# Attenuation of Renal Vasopressin $V_2$ Receptor Upregulation by Bosentan, an ETA/ETB Receptor Antagonist

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**Circulating endothlein (ET) levels are elevated in heart failure and positively correlated with severity of heart failure. Recent studies demonstrated arginine vasopressin (AVP) $V_2$ mRNA expression was upregulated in the inner medullary collecting duct (IMCD) of cardiomyopathic hamsters (CM). The goal of the present studies was to determine if ET-1 is involved in upregulating the expression of AVP $V_2$ mRNA in the IMCD of CM by using a mixed ETA/ETB receptor antagonist bosentan. Our results showed plasma ET-1 levels increased in CM hamsters and related with the severity of heart failure. The competitive reverse-transcriptase polymerase chain reaction (RT-PCR) method was used to quantify the expression of AVP $V_2$ and aquaporin 2 (AQP2) mRNA in the IMCD. AVP $V_2$ mRNA expression was elevated in placebo-treated CM hamsters and decreased significantly with 14 days of bosentan treatment. Similar results were seen with AQP2 mRNA. The effect of bosentan in normalizing the expression of AVP $V_2$ and AQP2 mRNA in the IMCD of CM was confirmed by in situ hybridization studies. Bosentan treatments reduced the intensitites of the signals in the IMCD of CM hamsters to that seen in normal hamsters. This study demonstrated that AVP $V_2$ and AQP2 mRNA are upregulated in CM hamsters and these upregulations are attenuated by bosentan treatment, suggesting that ET-1 plays a role in upregulating the expression of AVP $V_2$ mRNA in CM hamsters.**



CIRCULATING ENDOTHELIN (ET) levels are elevated in experimental and human congestive heart failure (CHF)[1-5] and positively correlated with the severity of heart failure. It has been suggested that plasma level of ET in CHF is a predictor of mortality after myocardial infarction.[6] Studies in human volunteers have shown that ET-1 induces a decrease in glomerular filtration rate, renal plasma flow, and urinary excretion of sodium and water.[7] Thus, the potent vasoconstrictor action of ET can play a role in reducing renal function associated with CHF. Supporting this notion are experiments on heart failure dogs that demonstrated an increase in plasma ET-1 levels and an attenuated renal response to exogenous ET.[8] Similar results were seen in CHF rats where the renal hemodynamic and the natriuretic effects of big ET were severely attenuated.[9] These studies suggested an important role for ET in the pathogenesis of abnormal renal function in CHF.

Recent studies in our laboratory showed that enalapril, an angiotensin-converting enzyme inhibitor, restored the natriuretic and diuretic response to exogenous atrial natriuretic factor (ANF) in the kidney of cardiomyopathic hamsters (CM).[10] Administration of enalapril reduced the upregulation of vasopressin $V_2$ receptors in the inner medullary collecting duct (IMCD) of CM to normal levels.[11] These results suggested that angiotensin II plays a role in inducing upregulation of vasopressin $V_2$ receptors. Angiotensin II stimulates the expression of ET-1 mRNA in endothelial cells,[12] smooth muscle cells,[13] cardiomyocytes,[14] and renal mesangial cells.[15] Hence, changes in expression of vasopressin $V_2$ induced by angiotensin II can be in part mediated by ET-1. Herizi et al demonstrated that a mixed ETA/ETB receptor antagonist bosentan prevented albuminuria and reduction in renal blood flow caused by chronic infusion of angiotensin II.[16] This report suggested that ET-1 contributes to the renal effects of angiotensin II and can be involved in upregulating the expression of vasopressin $V_2$ in the IMCD of CM. The goal of the present studies was to determine if ET-1 is involved in upregulating the expression of vasopressin $V_2$ mRNA in the IMCD of CM by using a mixed ETA/ETB receptor antagonist, bosentan.

## MATERIALS AND METHODS

Studies were performed in male CM and age-matched controls purchased from Biobreeders (Watertown, MA). Hamsters used for these experiments weighed between 125 and 175 g. The hamsters were divided into 4 groups: groups 1 and 2 were normal hamsters and CM that were not treated with bosentan; groups 3 and 4 were normal hamsters and CM that were treated with bosentan (100 mg/kg/d, orally) for 14 days before experiments were conducted.

Hamsters were anaesthetized with phenobarbital (50 mg/kg, intraperitoneally) and the renal papillary tissues were dissected out for isolation of the IMCD, methodology described in our previous publication.[17] The renal papillary tissues were minced and digested in RPMI-1640 medium (Sigma-Aldrich Canada, Oakville, Canada) containing collagenase (1.5 g/mL) (United States Biochemical, Cleveland, OH) for 30 minutes at 37°C. The digestion was terminated by the addition of equal volume of RPMI-1640 medium containing 10% fetal calf serum. The IMCD were separated with percoll (specific gravity, 1.07). The cells were tested by histological and biochemical methods to confirm them to be IMCD.[17]

The competitive reverse-transcriptase polymerase chain reaction (RT-PCR) method was used to quantify the expression of arginine vasopressin (AVP) $V_2$ and aquaporin 2 (AQP2) mRNA in the IMCD.[11] Known amounts of competitor DNA molecules were added to each PCR and the amount of target cDNA present in the sample was determined from the added competitor, which gave an equimolar amount of PCR products as the target cDNA. A single set of primers to amplify both target cDNA and added competitor of known concentration were used. PCR was done with a GeneAMP PCR System 2400 (Perkin-Elmer, Norwalk, CT) in a total volume of 50 $\mu$L containing 2

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$\mu$L of cDNA, 2 $\mu$L of competitor, 20 pmol of each primer, 100 $\mu$mol/L dNTPs, 10 mmol/L Tris-HCl, 0.75 mmol/L $MgCl_2$, and 1.25 U of *Taq* DNA polymerase (Gibco BRL, Grand Island, NY). After completion of PCR, an aliquot of the reaction mixture was electropheresed on 1.5% agarose followed by staining with ethidium bromide. The appropriate bands were scanned and analyzed by computer densitometry (Alphalmager, 1200 Alph Inotech Corp, San Leandro, CA).

The kidneys of normal hamsters and CM were removed and fixed overnight in 4°C paraformadldehyde–phosphate-buffered saline (PBS). The tissues were dehydrated through successive baths of ethanol (75%, 95%, and 100% at 10 minutes each) and toluene (2 × 30 minutes each), and embedded in 3 successive baths of Paraplast (Oxford Labware, St Louis, MO) at 58°C. After paraffin solidification at room temperature, the tissue were kept at 4°C until used. Paraffin sections were cut (8 $\mu$m thick), mounted on poly-L-lysinated slides, air-dried overnight, and stored at 4°C until used for in situ hybridization. In situ hybridization was done according to Braissant and Wahli.[18] The paraffinized tissue sections were rehydrated through successive baths of toluene (2 × 30 minutes), ethanol (100%, 95%, and 70% at 10 minutes each), and diethlyprocarbonate (DEPC)-treated water for 15 minutes. The sections will then be postfixed for 10 minutes in 4% paraformaldehyde-PBS. Following postfixation, sections were incubated for 30 minutes in PBS containing 1% active DEPC, and equilibrated for 15 minutes in a 5X saline sodium citrate buffer (SSC). Each section was then be prehybridized for 2 hours at 58°C in 500 $\mu$L of hybridization mix (50% formamide, 5X SSC, salmon sperm DNA 40 $\mu$g/mL). The probes were denatured for 5 minutes at 80°C and added to 20 $\mu$L of hybridization mix. The hybridization reaction was performed overnight at 42°C. After incubation, the sections were washed for 15 minutes in 2X SSC at room temperature, 30 minutes in 2X SSC at 65°C, 30 minutes in 0.1X SSC at 65°C, and equilibrated for 10 minutes in Buffer 1 (Tris-HCl 100 mmol/L and NaCl 150 mmol/L, pH 7.5). The sections were incubated for 2 hours at room temperature with an alkaline phosphatase–coupled anti-digoxigenin antibody (Roche Diagnostic, Laval, Canada). Excess antibodies were removed by two 15-minute washes in Buffer 1, and the sections were equilibrated for 10 minutes in Buffer 2 (Tris-HCl 100 mmol/L, NaCl 100 mmol/L, and $MgCl_2$ 50 mmol/L, pH 9.5). Color development was done overnight at room temperature in Buffer 2 containing substrates NBT and BCIP (Roche Diagnostic). Staining was terminated by successive rinses with water and 70% ethanol, and nonspecific staining was removed by overnight wash in 95% ethanol. The sense strands were used as the probe for negative control.

Plasma ET was determined by radioimmunoassay using a commercial assay kit (Peninsula Laboratory, Belmont, CA) after extraction with $C_{18}$ Sep-Pak (Water Associates, Milford, MA). .

Data were shown as mean ± SEM. Correlation coefficients were obtained by linear regression analysis. Student's *t* tests were used to determine statistical significance for intergroup comparisons. When multiple groups were involved, 1-way analysis of variance (ANOVA) was used. A *P* value less than .05 was considered significant.

## RESULTS

The mean plasma ET-1 level, body weight, and heart weight to body weight ratio in the 4 groups of hamsters are summarized in Table 1. Heart weight increased significantly in CM compared to the corresponding age-matched control. Bosentan treatment did not have an effect on cardiac hypertrophy in CM. Heart weight to body weight ratios were significantly elevated in placebo-treated and bosentan-treated CM and were unaffected by treatment. There was no significant difference between heart weight to body weight ratios between placebo-treated and bosentan-treated CM. Plasma ET-1 levels increased in CM compared to normal hamsters. Bosentan did not attenuate the increased plasma ET-1 in CM. Figure 1 shows the relationship between plasma endothelin levels and heart weight to body weight ratios. It can be seen that ET-1 correlates with heart weight to body weight ratio for both untreated ($y = 2054.2x - 0.5444$, $r = 0.57$, $P < .05$) and bosentan-treated hamsters ($y = 2107.8x - 0.1599$, $r = 0.67$, $P < .01$). These results suggested that increased in plasma ET-1 levels in CM are related to the severity of the disease.

The RT-PCR results are shown in Table 2. In the placebo-treated CM, AVP $V_2$ mRNA expression was increased compared with its age-matched control. Bosentan treatment for 14 days significantly decreased AVP $V_2$ mRNA expression in the CM to a level similar to that of normal hamsters. A positive correlation was observed between AVP $V_2$ mRNA and heart weight to body weight ratio in the placebo-treated CM ($y = 178.94x - 0.1385$, $r = 0.51$, $P < .05$) (Fig 2). These results suggested that AVP $V_2$ mRNA expression increases with the progression of heart failure. This correlation was not seen after bosentan treatment ($y = 21.77x + 0.49$, $r = 0.12$, difference not significant [NS]). When AVP $V_2$ mRNA was plotted against plasma ET-1 levels (Fig 3), a correlation between these 2 parameters was found ($y = 0.0667x + 0.1041$, $r = 0.63$, $P < .025$). This relationship suggests that ET-1 may play a part in upregulating the expression of AVP $V_2$ mRNA in the IMCD. Similar results were seen with AQP2 mRNA. AQP2 mRNA expression was higher in placebo-treated CM when compared to the control group. A relationship between AQP2 mRNA and heart weight to body weight ratio was detected and is illustrated in Fig 4 ($y = 10.629x - 0.0219$, $r = 0.70$, $P < .01$). Further analysis of the results showed a significant correlation between AVP $V_2$ and AQP2 mRNA ($y = 0.0141x + 0.0175$, $r = 0.68$, $P < .05$) in the IMCD of placebo-treated CM.

The effect of bosentan in normalizing the expression of AVP V*2* and AQP2 mRNA in the IMCD of CM was confirmed by in

**Table 1. Hematocrit, Body Weight, Heart Weight, and Hormonal Activity for All Groups**

| | Normal, Untreated | CM, Untreated | Normal, Bosentan-Treated | CM, Bosentan-Treated |
|---|---|---|---|---|
| n | 7 | 7 | 8 | 8 |
| Hematocrit (%) | 49 ± 0.9 | 42 ± 0.5† | 47 ± 1.2 | 44 ± 1.2* |
| Body weight (g) | 160 ± 3.7 | 131 ± 5.4† | 162 ± 5.1 | 131 ± 2.8† |
| Heart weight (g) | 0.66 ± 0.02 | 0.80 ± 0.05* | 0.62 ± 0.02 | 0.71 ± 0.03* |
| Heart weight body weight ratio | 0.00424 ± 0.0001 | 0.0065 ± 0.0003† | 0.0039 ± 0.0001 | 0.0055 ± 0.0003† |
| Plasma endothelin (pg/mL) | 7.8 ± 0.8 | 12.4 ± 1.4* | 8.1 ± 0.6 | 11.0 ± 1.2* |

*$P < 0.05$,

†$P < 0.01$ compared to corresponding normal hamster

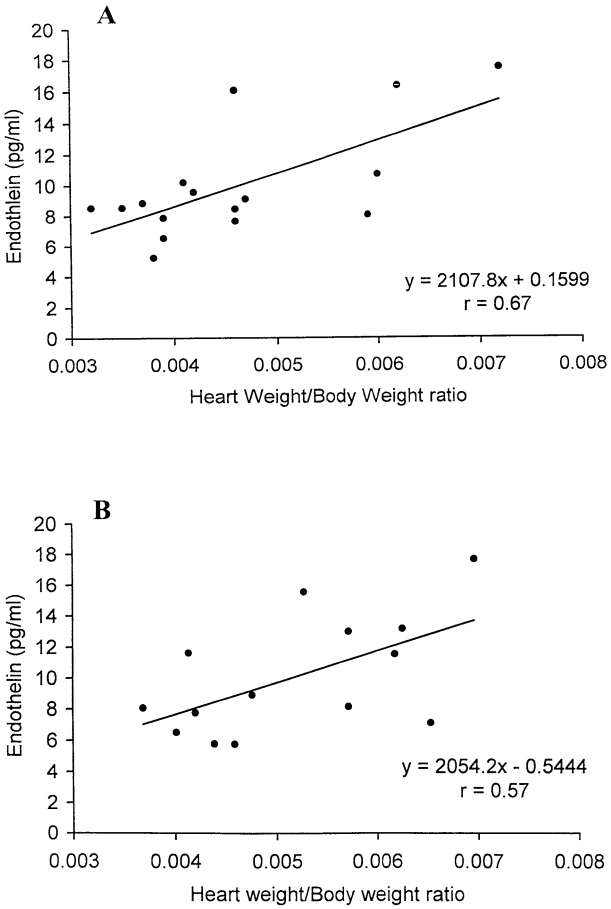


**Fig 1. Relationship between plasma ET levels and heart weight to body weight ratio for (A) bosentan-treated and (B) untreated hamsters.**

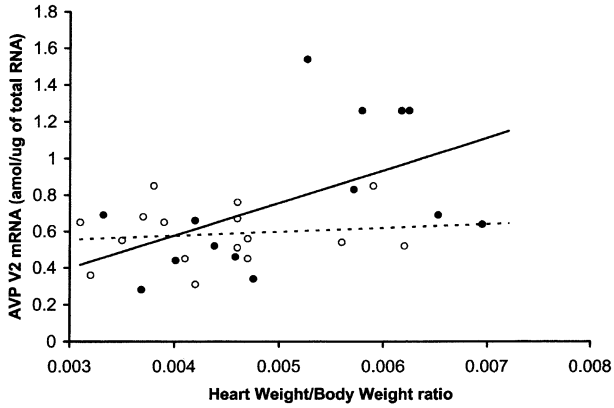


**Fig 2. A positive correlation was observed between AVP $V_2$ mRNA expression in the IMCD and heart weight to body weight ratio in the placebo-treated hamsters (●) (y = 178.94x − 0.1385, $_r$ = 0.51, $P < .05$), but was not seen in the bosentan-treated hamsters (○) (y = 21.77x + 0.4892, $r = 0.12$, NS).**

situ hybridization studies. In CM, prominent hybridization signals of AVP $V_2$ and AQP2 mRNA were localized to the paranuclear region of the IMCD cells (Figs 5A and 6A). In contrast, these signals were weaker in the IMCD of normal hamsters (Figs 5C and 6C). When CM were treated with bosentan, the strong signals noted in the IMCD of CM were not seen (Figs 5B and Fig 6B) and the intensities of the signals were comparable to that seen in normal hamsters. There were no signals detected when the probe was omitted or used in the presence of ribonuclease A, suggesting the probes were highly specific.

## DISCUSSION

In this study, we evaluated the effect of bosentan (an ETA/ETB mixed receptor antagonist) on the pathology of heart failure in CM. The increase in heart weight, and heart weight to body weight ratios in CM, were not attenuated by bosentan treatment. These parameters remained elevated following bosentan treatment. These findings were not surprising because cardiac hypertrophy was developed over a long time period and these hamsters were treated with bosentan for only 14 days before they were studied. This corroborates with the report by Willette et al,[19] which showed that SB 2177242, an ETA/ETB receptor antagonist, had no effect on the development of cardiac hypertrophy in an inter-renal aortic banding rat model. On the other hand, studies in rats with coronary artery ligation and were treated with bosentan for more than 2 months showed that ET antagonist reduced hypertrophy.[20] These extended treatment with bosentan of heart failure subjects could reduce cardiac hypertrophy.

Numerous studies have shown that circulating ET-1 is increased in patients with CHF and correlated to severity of heart failure.[1-6] In the present study, the plasma ET-1 levels were significantly elevated in CM and the increase in circulating ET-1 was not attenuated by bosentan treatment. This is in contrast to the acute effect of bosentan on plasma ET-1 in dogs with CHF. Bolus injection of bosentan caused a massive increase in circulating ET-1 in 15 minutes, possibly due to ET-1

**Table 2. AVP $V_2$ and AQP2 mRNA Expression in the Inner Medullary Collecting Ducts of Untreated and Bosentan-Treated Hamsters**

| | Normal, Untreated | CM, Untreated | Normal Bosentan-Treated | CM, Bosentan-Treated |
|---|---|---|---|---|
| n | 7 | 7 | 8 | 8 |
| AVP $V_2$ mRNA, amol/$\mu$g of total RNA | 0.48 ± 0.05 | 1.26 ± 0.3* | 0.60 ± 0.05 | 0.56 ± 0.06† |
| AQP2 mRNA amol/$\mu$g of total RNA | 0.018 ± 0.002 | 0.035 ± 0.007* | 0.019 ± 0.002 | 0.018 ± 0.002† |

*$P < .05$ compared to the corresponding normal control.
†$P < .05$ compared to untreated cm.

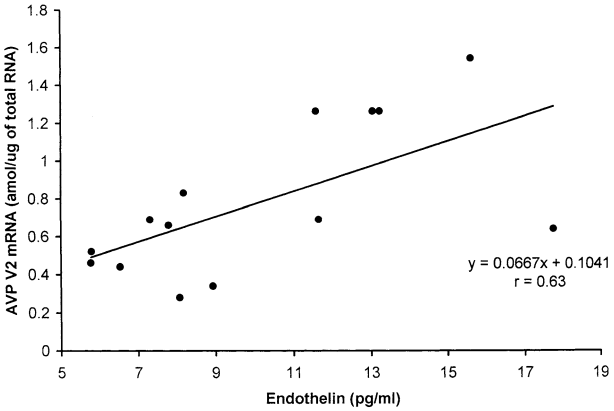


**Fig 3. Correlation between AVP $V_2$ mRNA expression in the IMCD and plasma ET-1 levels.**

receptor blockade.[21] Increase in circulating ET-1 levels correlate with increase in heart weight to body weight ratios that reflect the severity of heart failure in these hamsters. Our results are in agreement with those seen in humans, where elevated plasma ET-1 levels in heart failure correlates with the severity of the condition. The source of elevated circulating ET-1 levels can be from increase production of ET-1 in the hypertrophic heart,[22,23] caused by hemodynamic pressure overload. Neurohumoral systems activated in CHF, such as angiotensin II and vasopressin, may also stimulate ET-1 production.[24] In addition, ETB receptors, which are responsible for the clearance of ET-1, are downregulated in the lung[25] and kidney[10] of heart failure animals, and may play a role in increase circulating ET-1 levels.

AVP $V_2$ mRNA levels were significantly elevated in the CM compared to its corresponding control. This upregulation of AVP $V_2$ mRNA was shown by RT-PCR measurement and confirmed by in situ hybridization. The increase in AVP $V_2$ mRNA in the IMCD correlates with the increase in heart weight to body weight ratio, suggesting that upregulation of AVP $V_2$ mRNA expression is related to the severity of heart failure. These observations suggest that upregulation of the AVP $V_2$ receptors in the IMCD of the kidney of CHF animals contributes to the excessive reabsorption of water by the renal tubules. The importance of AVP $V_2$ receptors in enhancing water reabsorption in heart failure is affirmed by studies that showed $V_2$ receptor antagonists corrected the impaired water excretion by the kidneys of dogs[26] and rats[27] with induced cardiac failure. $V_2$ receptor antagonists also restored the kidney of CM to ANP-induced natriuresis and diuresis.[10] The upregulation of AVP $V_2$ mRNA can be due to neurohumoral disturbances associated with heart failure. Recent studies in CM suggested that the increase in circulating angiotensin II levels in heart failure is responsible for promoting the increase in AVP $V_2$ receptors in the IMCD.[11] Angiotensin II has been found to stimulate ET-1 production in endothelial cells, smooth muscle cells, cardiomyocytes, and renal mesangial cells,[12-15] whereas angiotensin-converting enzyme inhibitors lower circulating ET-1 levels.[28] This suggests that angiotensin II–induced upregulation of AVP $V_2$ mRNA is mediated in part by ET-1. In the present report we showed that AVP $V_2$ mRNA correlates with plasma ET-1, indicating that ET-1 might be responsible for increased expression of AVP $V_2$ mRNA in the IMCD. This notion is further strengthened by the consideration that when CM were treated with bosentan, a mixed ETA/ETB receptor antagonist, the upregulation of AVP $V_2$ mRNA in the IMCD was attenuated. These data suggested that ET-1 might play a role in the upregulation of AVP $V_2$ mRNA expression in the IMCD of CM. This action of ET-1 is most likely mediated by ETB receptors, because mRNA and binding studies indicated that renal tubule mainly express ETB receptors; moreover, functional studies also imply that ETB receptors play a role in mediating most of ET's effects on the renal tubules.[29] Preliminary in vitro studies in our laboratory showed that incubating IMCD with ET-1 led to upregulation of AVP $V_2$ mRNA and AVP $V_2$ protein, and this effect was probably mediated by ETB receptors. These results suggest that ET-1 has a direct effect on upregulating the expression of vasopressin $V_2$ receptors in the IMCD.

Upregulation of AQP2 mRNA in the IMCD of CM was also observed. This increase in AQP2 mRNA expression correlated with heart weight to body weight ratios, suggesting that AQP2 upregulation was related with the seriousness of heart failure. This confirms the observation of Xu et al in CHF rats.[30] Studies have shown that acute elevation of plasma AVP upregulates AQP2 mRNA.[31] Thus, the increase in AQP2 in CHF can be due in part to elevation in plasma AVP associated with CHF.[11] It has also been shown that administration of $V_2$ agonist to normal[32] and Brattleboro rats[33] resulted in a significant increase in AQP2 protein. These results also indicate that AVP upregulates AQP2 mRNA and protein. The increase in AQP2 mRNA by AVP is mediated by a dual effect of the cyclic adenosine monophosphate (cAMP) signal on the CREB and AP1 site on the AQP2 promoter.[34] The increase in cAMP is from the stimulation of the AVP $V_2$ receptors by AVP that activates the G protein and adenylyl cyclase. Since both plasma AVP[11] and AVP $V_2$ receptors are upregualted in CM, cAMP formation is increased, leading to the upregulation of AQP2 mRNA seen in these studies. The upregulation of AQP2 mRNA was attenuated after bosentan treatment. This attenuation can be due to downregulaion of the AVP $V_2$ receptors with bosentan treatment and possibly a reduction in cAMP formation.

The upregulation of AVP $V_2$ receptors and AQP2 protein

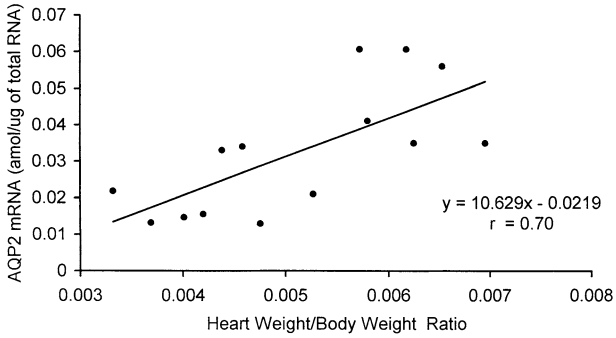


**Fig 4. Relationship between AQP2 mRNA expression in the IMCD and heart weight to body weight ratio.**

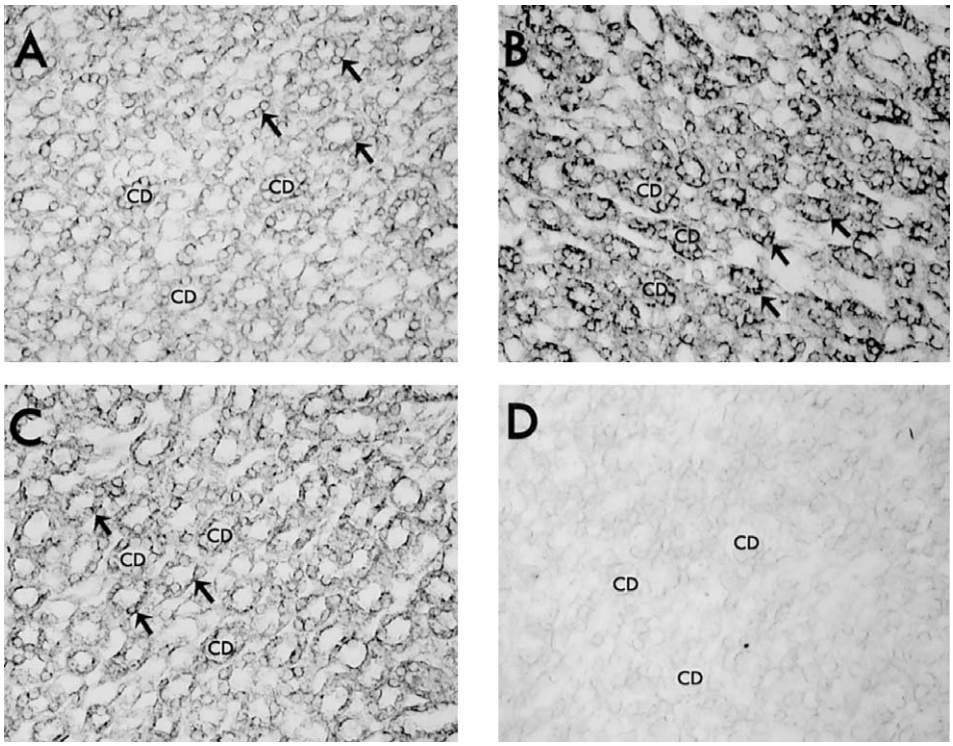


**Fig 5. In situ hybridization studies for the detection of AVP $V_2$ mRNA signals in the IMCD of (A) normal hamsters, (B) cardomyopathic CM, and (C) CM treated with bosentan. (D) Effect of RNAase treatment on detecting AVP $V_2$ mRNA signal.**

can play a significant role in water retention in CHF. Studies in heart failure rats showed that upregualtion of AQP2 protein led to a significant increase in urine osmolaltiy.[30] Clearance studies in CM have shown ANP-induced diuresis and natriuresis is impaired compared to enalapril-treated CM.[10] These untreated CM had a significant increase in AVP $V_2$ receptors and AQP2 mRNA,[11] whereas enalapril-treated CM had AVP $V_2$ receptors and AQP2 mRNA levels comparable to that seen in normal

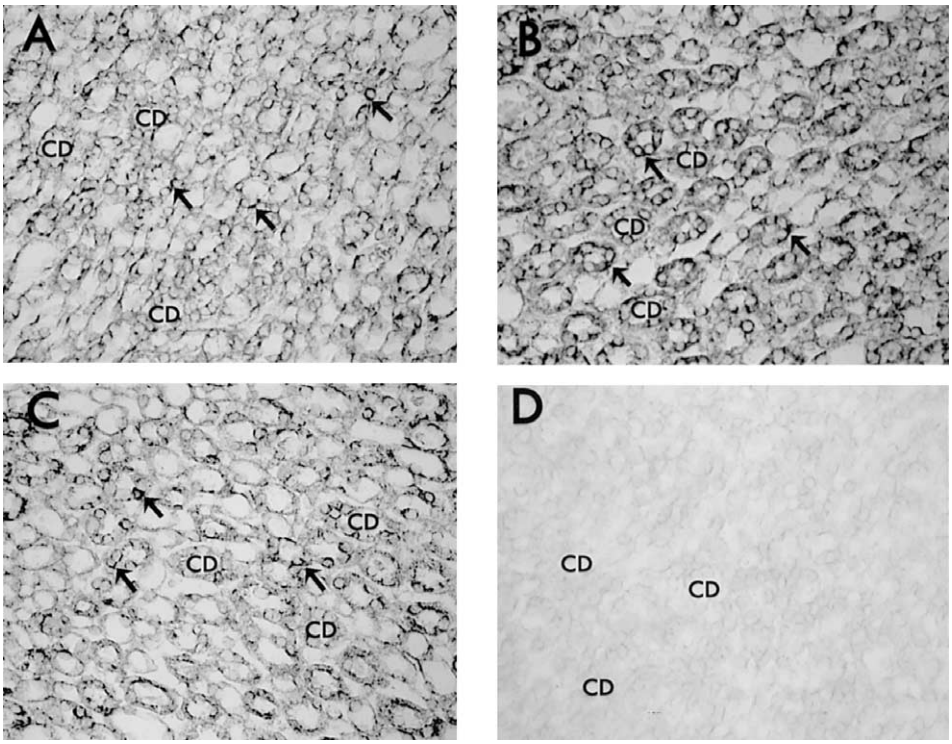


**Fig 6. In situ hybridization studies for the detection of AQP2 mRNA signals in the IMCD of (A) normal hamsters, (B) CM, and (C) CM treated with bosentan. (D) Signals are not detected in slides treated with RNAase.**

hamsters. These results suggested that upregulation of AVP $V_2$ receptors and AQP2 protein in CHF is associated with impairment in water excretion. The present report showed that AVP $V_2$ receptors and AQP2 mRNA upregulation in CM were attenuated by bosentan, suggesting that bosentan can have a beneficial effect on fluid retention; this remains to be examined.

The present study demonstrated that AVP $V_2$ and AQP2 mRNA are upregulated in CM and this upregulation is attenuated by bosentan treatment, suggesting that ET-1 plays a role in upregulating the expression of AVP $V_2$ mRNA in CM.

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