A decrease in total renal blood flow (RBF) associated with an increase in renal vascular resistance is a common finding in patients and in experimental animals with congestive heart failure (CHF) (8, 10). However, analysis of intrarenal blood flow measurements reveals that although cortical blood flow (CBF) is markedly diminished, medullary blood flow (MBF) appears to be preserved (3, 16). Such a conservation of medullary perfusion is apparently associated with an adaptive decrease in the resistance of the medullary vascular bed, since renal perfusion pressure tends to be decreased in CHF. This implies that, in CHF, medullary vasodilation may occur in the face of severe cortical vasoconstriction. The mechanisms responsible for such a differential regulation of intrarenal blood flow in CHF have not been fully elucidated.

Experimental evidence has accumulated in recent years suggesting that locally produced vasoactive substances, such as nitric oxide (NO) and endothelin (ET), play an important role in the regulation of vascular tone in various tissues including the kidney (18, 27). These paracrine agents are generated in large concentrations in the kidney, particularly in the renal medulla (17, 39), and have been documented to be potent modulators of renal vascular tone (18, 25). Recently, we demonstrated that systemic infusion of ET-1 to normal rats resulted in a marked and sustained decrease in CBF but, at the same time, increased medullary perfusion (13). The latter vasodilatory response in the renal medulla was mediated by stimulation of the ETB receptor subtype coupled to activation of the NO system (13). The possibility that such an interaction between the two paracrine systems may be involved in the regulation of intrarenal blood flow and the maintenance of medullary perfusion in CHF, although appealing, has not been previously evaluated.

Several studies have indeed suggested that the ET system is activated in CHF (21, 34). This assumption is based on the demonstration that plasma levels of ET-1 and its precursor big ET-1 are elevated in patients and in animals with experimental CHF (6, 20, 22). Likewise, evidence for altered activity of, and responsiveness to, endothelium-dependent vasodilators such as acetylcholine has been reported in heart failure (9, 19, 34). However, it is not clear whether and to what extent these alterations are involved in the adaptations of intrarenal hemodynamics in CHF.

Recently, we reported that administration of bosentan, a mixed ET1/ETB receptor antagonist, significantly improved cortical perfusion in rats with severe decompensated CHF induced by aortocaval fistula (12). This finding indicates that ET may be involved in the altered renal hemodynamics and the pathogenesis of cortical vasoconstriction in CHF.

The present study was therefore undertaken to further evaluate the contribution of the ET and NO systems to the pathophysiological adjustments in renal regional blood flow in rats with experimental CHF. Specifically, this study was done to test whether altered responsiveness to, or the expression of, various components of these two paracrine systems may be involved in the pathogenesis of renal cortical vasoconstriction and medullary vasodilation in rats with aortocaval fistula. Previous studies from our laboratory have demonstrated that this experimental model mimics the
characteristic renal and neurohumoral manifestations found in patients with CHF (35, 36). These include a decrease in RBF and glomerular filtration rate with a tendency to salt and water retention, as well as marked activation of compensatory systems like the renin-angiotensin and atrial natriuretic peptide systems.

METHODS

Studies were conducted on a local strain of male Wistar rats weighing 280–350 g. The animals were kept in individual metabolic cages in a controlled-temperature room and were fed standard rat chow containing 0.5% NaCl and tap water ad libitum.

Experimental Model

An aortocaval fistula was surgically created between the abdominal aorta and inferior vena cava according to the method originally described by Stumpe et al. (31) and adapted in our laboratory (1, 36). Briefly, the rats were anesthetized with a mixture of halothane (Fluothane, ICI Pharmaceuticals) and oxygen. A midabdominal incision was performed to expose the vena cava and abdominal aorta distal to the origin of the renal arteries. Miniature surgical clamps were placed around both vessels, 10–15 mm apart, and a longitudinal incision was performed in the outer wall of the vena cava. The common wall between the aorta and the vena cava was grasped through incision under binocular magnification, and a fistula (1.0–1.2 mm OD) was created between the two vessels. The opening of the outer wall of the vena cava was then closed with a continuous suture (7-0 prolene nonabsorbable suture, Ethicon). After the surgical procedure, the animals were allowed to recover and then returned to the metabolic cages for daily monitoring of urine output and sodium excretion. A matched group of normal rats served as controls. Five to seven days after operation, rats with aortocaval fistulae were divided into two subgroups (36) according to their daily absolute rate of sodium excretion (UNaV): rats with compensated CHF (UNaV < 1,200 µeq/24 h) and rats with decompensated CHF (UNaV > 1,200 µeq/24 h). Previously, we reported that the subgroup of rats with decompensated CHF (UNaV > 1,200 µeq/24 h) displayed marked congestion of the lung and liver with edema and ascites formation, and the animals usually die within 7–10 days from symptoms of pulmonary edema (36). In contrast, in rats with compensated CHF, UNaV returned to normal levels after 1 wk, and survival was extended. However, plasma atrial natriuretic peptide levels remain elevated in this subgroup, while RBF and cortical perfusion are diminished (12, 36).

Measurements of Intrarenal Hemodynamics

Intrarenal blood flow in control and CHF rats was analyzed by laser-Doppler flowmetry, as previously described (13). On the day of the experiment, rats were anesthetized with thiobutabarbital sodium salt (Inactin, RBI, Natick, MA; 110 mg/kg ip) and prepared for hemodynamic studies. The rats were placed on a temperature-regulated table (37°C), and the kidneys were removed and immediately placed in liquid nitrogen. Total RNA was extracted from the renal cortex and medulla, as described by Chomczynski and Sacchi (7), with the use of a commercial solution (RNAzol B, Tel-Test) and quantified by spectrophotometry. Reverse transcription (RT) followed by quantitative polymerase chain reaction (RT-PCR) was then applied.

RT-PCR. The effects of CHF induction on mRNA levels of ET-1, ETα, ETβ, and endothelial NOS (eNOS) in the renal cortex and medulla were examined. cDNA for these mRNAs was synthesized from 2 µg of total RNA with the use of specific primers (Table 1). Avian myeloblastosis virus reverse transcriptase (16 U/reaction; Promega) was used for RT, along with the reaction mixture recommended by the enzyme manufacturer, in a volume of 20 µl, using 1.25 µM down-
Table 1. Oligonucleotide primers for PCR used in the study

<table>
<thead>
<tr>
<th>Primer Sequences</th>
<th>Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ET-1</strong></td>
<td></td>
</tr>
<tr>
<td>Upstream</td>
<td>5' ATGGATATTATTTTCGCTGAT 3' (231)</td>
</tr>
<tr>
<td>Downstream</td>
<td>5' GGGAGTTGAGCCWGATGA 3' (212-231)</td>
</tr>
<tr>
<td><strong>ETα</strong></td>
<td></td>
</tr>
<tr>
<td>Upstream</td>
<td>5' ATGATGCTGCTGAATCTCAGG 3' (217)</td>
</tr>
<tr>
<td>Downstream</td>
<td>5' ACCAGTCTCCACGCTGG 3' (727-753)</td>
</tr>
<tr>
<td><strong>eNOS</strong></td>
<td></td>
</tr>
<tr>
<td>Upstream</td>
<td>5' TTTGCTCTGGTGGTCC 3' (991-1010)</td>
</tr>
<tr>
<td>Downstream</td>
<td>5' GTATTTTATGCTGAGGCGG 3' (1296-1315)</td>
</tr>
<tr>
<td><strong>β-actin</strong></td>
<td></td>
</tr>
<tr>
<td>Upstream</td>
<td>5' CCNGAGTGCTACAGGCAGCTGATCCATGGAA 3' (2456-2487)</td>
</tr>
<tr>
<td>Downstream</td>
<td>5' CACCTCAGGCAGGTGCACCCGATG 3' (3036-3069)</td>
</tr>
</tbody>
</table>

ET, endothelin; eNOS, endothelial nitric oxide synthase.

stream primer. PCR was then applied with 2 µl of the resulting cDNA using the upstream and downstream primers at 1.25 µM each. Each PCR mixture contained 1.5 U of the enzyme Taq polymerase (Takara Ex Taq, 5 U/µl) and 60 µM dNTP. When possible, primers were chosen to span introns that would distinguish by size PCR products derived from cDNA from those derived from genomic DNA contaminants. In a preliminary study we found that 35 PCR cycles for ET-1, ETα, and cDNA from those derived from genomic DNA contaminants. Ten microliters of the product was in proportion to the amount of cDNA used. After an initial denaturation step at 94°C for 2 min, cycles of annealing at 56°C for 45 s, elongation at 72°C for 1.5 min, and denaturation at 94°C for 45 s were performed with 10% of the cDNA (2 µl) described above. The RT-PCR product of the gene encoding β-actin served as a quantity marker, and control, and 23 cycles were required to obtain a visible product. Six microliters of PCR products were electrophoresed on agarose Tris-acetate-EDTA buffer gel. The resulting gel was stained with ethidium bromide (0.5 µg/ml) for 15 min until clear bands were visible in ultraviolet light. The relative levels of mRNA encoding the above products were quantified by densitometric analysis of the resulting X-ray film exposed to the gel. These experiments yielded results very similar to those obtained with the conventional nonradioactive technique.

Determination of eNOS Immunoreactive Levels by Western Blot Analysis

Renal cortex and medulla of control, compensated CHF, and decompensated CHF rats were removed as described in RT-PCR, and tissues were homogenized with a Polytron homogenizer (M. Zipperer) in 2.5 ml of 10 mM sodium phosphate buffer, pH 7.4, containing 1 mM MgCl2, 30 mM NaCl, 0.02% sodium azide, and the following set of protease inhibitors: antipain-HCl, bestatin, chymostatin, E-64, leupeptin, pepstatin, phosphoramidon, Pefabloc, EDTA, pepstatin, and aprotonin. The homogenates were stored at –70°C until assayed. Kaleidoscope prestained standard molecular markers (Bio-Rad) were used for determination of the molecular weight of the immunoreactive products. Ten microliters of the tissue homogenates (~120 µg of protein) were treated with 20 µl of sample buffer (10% SDS, 50% glycerol, 1 M Tris, 0.1% bromophenol blue, and 1 M dithiothreitol, pH 6.8) and placed in a boiling water bath for 5 min. Samples were then eletrophoresed on polyacrylamide Tris-glycine gels (4–20%, Bio-Rad) and transferred electrophotically to a nitrocellulose membrane (300 mA for 90 min). The blots were blocked in 3% (wt/vol) dried milk powder in Tris-buffered saline (TBS) and 0.1% Tween 20 overnight. The nitrocellulose membranes were incubated with 1,000-fold-diluted eNOS monoclonal antibodies (Transduction Laboratories, Lexington, KY) for 120 min, then washed three times with TBS for 5 min each. After they were washed, the blots were incubated with 5,000-fold-diluted peroxidase-conjugated rabbit anti-mouse IgG (Sigma Chemical) in TBS containing 0.1% Tween 20 for 60 min. Immunoreactive bands were visualized by the chemiluminescence detection system (Sigma Chemical).

Statistical Analysis

ANOVA for repeated measures followed by Dunnett’s test was utilized to evaluate the difference between the time points and baseline value in each group. Two-way ANOVA was used to compare data of control and CHF groups. Data obtained in the in vitro studies in control, compensated CHF, and decompensated CHF were evaluated by one-way ANOVA. P < 0.05 was considered statistically significant. Values are means ± SE.

RESULTS

Figure 1 depicts the basal values, in perfusion units, of CBF and MBF in control and CHF rats. In rats with CHF there was a significant decrease in CBF compared with control animals (by –58%), whereas MBF remained unaltered. Also, the MBF-to-CBF ratio was significantly higher in CHF rats than in controls (0.45 ± 0.03 vs. 0.31 ± 0.02, P < 0.001), indicating that a redistribution of intrarenal blood flow was present in this experimental model of CHF.

The effects of administration of ET-1 on CBF and MBF in rats with CHF and in control animals are shown in Fig. 2. Commensurate with our previous report in normal animals (13), ET-1 induced a significantly higher increase in CBF in control animals than in controls (0.45 ± 0.03 vs. 0.31 ± 0.02, P < 0.001), indicating that a redistribution of intrarenal blood flow was present in this experimental model of CHF.
with values different from baseline throughout the experimental period (Fig. 2A).

However, as shown in Fig. 2B, a different pattern of cortical vasoconstrictor response was observed when CHF rats were analyzed according to the severity of cardiac dysfunction. Namely, in rats with compensated CHF the ET-1-induced cortical vasoconstriction was similar to that observed in control animals, whereas in rats with decompensated CHF the vasoconstrictor effect of ET-1 was markedly blunted, with percent change values not different from zero throughout the experiment. In contrast, the vasodilator effect of the peptide in the medulla was significantly prolonged in both subgroups of animals with CHF (Fig. 2B).

To test whether this sustained effect of ET-1 in the medulla may be achieved through activation of the ETB receptor subtype, we evaluated the response to IRL-1620, a specific agonist of the ETB receptor (38). The results of this set of experiments are shown in Fig. 3. Because ET-1 produced an extended vasodilatory response in the medulla in rats with compensated and decompensated CHF, animals with CHF were not divided into two subgroups in this experimental protocol. Infusion of IRL-1620 into rats with experimental CHF also resulted in a prolonged vasodilatory response that was of a significantly longer duration than that observed in control animals. Infusion of the same dose of the agonist had no effect on CBF in control animals or in rats with CHF.

The effects of ET-1 on MBF in control and CHF rats in the presence of NOS blockade are shown in Fig. 4. Animals with CHF, both compensated and decompensated, were grouped together in this protocol. As reported previously in normal animals, the transient medullary vasodilatory response after ET-1 administration was completely abolished and, in fact, was replaced by a vasoconstrictor response (13). In CHF rats pretreated with L-NAME, the medullary vasodilatory response to ET-1 administration was markedly blunted yet differed considerably from the response observed in
normal rats with NO blockade. This might suggest that L-NAME administration in rats with CHF was not as effective in blocking the enhanced medullary NO production in heart failure. Yet, because chronic NOS inhibition had an intense renal vasoconstrictor effect and changed baseline perfusion values in control and CHF rats, other mechanisms not related directly to NO blockade cannot be completely ruled out.

In Vitro Studies

RT-PCR data. Figure 5 shows PCR products of representative agarose gels for ET-1, ETA, ETB, eNOS, and β-actin in the renal cortex and medulla of control rats and animals with compensated and decompensated CHF. The size of the RT-PCR products was as predicted by means of genomic maps (Table 1). When the PCR was performed in the absence of RT or RNA, these products were not observed, indicating that this reaction is highly specific and that these products derived from the mRNA and not the genomic DNA. The optimum number of amplification cycles used in the present study was chosen on the basis of preliminary experiments which showed that the PCR products obtained after these cycles were within the linear phase of amplification. Also the amount of cDNA was within the linear range of cDNA amplification.

Figure 6 summarizes the results obtained by scanning the gel photographs (n = 5–6 for each parameter) with computerized densitometry. The values are expressed as the relative amount of ET-1, ETA, ETB, and eNOS mRNA normalized to β-actin mRNA.

After 35 PCR cycles, ET-1 mRNA levels were in general significantly higher (4-fold) in the renal medulla than in the renal cortex. Induction of CHF provoked a significant increase in the cortical expres-
sion of the ET-1 gene in proportion to the severity of CHF. In contrast, the high ET-1 mRNA levels in the renal medulla were not affected and remained unchanged in CHF rats. ETA and ETB receptor subtypes were abundantly expressed in the renal cortex and medulla. Similar to ET-1 mRNA, the mRNA levels of ETA and ETB receptors were significantly higher in the medullary tissue (2-fold) than in the cortex. However, no significant differences were found between control rats and animals with heart failure in cortical and medullary ETA mRNA levels. Likewise, cortical and medullary mRNA levels encoding ETB were comparable in control animals and animals with CHF (Fig. 6).

Compared with the currently examined components of the ET system, only 28 PCR cycles of amplification were sufficient to observe eNOS mRNA products within the linear range of amplification, suggesting a higher expression of the latter. Similar to the components of the ET system, the expression of eNOS mRNA was significantly higher (4.4-fold) in the medulla than in the cortex. Interestingly, cortical and medullary abundance of eNOS mRNA increased significantly after the induction of CHF. The cortical expression of eNOS was significantly enhanced by 144% in decompensated animals (P < 0.05), and the medullary concentrations of eNOS mRNA increased in rats with decompensated CHF (+67%, P < 0.01).

eNOS immunoreactive levels. In parallel to our findings with RT-PCR, a protein fraction of 140 kDa was clearly recognized in the renal medulla and, to a lesser extent, in the renal cortex, after incubation of cortical and medullary homogenates with anti-eNOS antibodies under denaturing and reducing conditions (Fig. 7). The immunoreactive levels of eNOS increased in the medulla of rats with CHF in proportion to the severity of the disease. Also, despite its low abundance, eNOS levels in the cortical tissue were enhanced in CHF rats in proportion to cardiac dysfunction (Fig. 7). These data suggest that the elevated levels of eNOS mRNA in the renal tissue are accompanied by a similar increase in protein immunoreactivity.
DISCUSSION

The findings of the present study provide important and novel information on the regulation of renal regional blood flow in rats with experimental CHF. Our data clearly demonstrate that experimental CHF is associated with alterations in the expression and activity of at least two important paracrine systems in the kidney, NO and ET. Moreover, these adjustments are associated with altered responsiveness of the cortical and medullary vascular beds to the vasoactive effects of ET-1. Specifically, infusion of ET-1 into rats with CHF results in an attenuated cortical vasoconstriction and a prolonged vasodilatory response in the medulla compared with control rats. Finally, our results suggest that these local modifications in the paracrine systems within the kidney may play a role in the phenomenon of redistribution of intrarenal blood flow in CHF. Namely, these alterations may be a part of the adaptive processes in CHF that produce a sustained cortical vasoconstriction but, at the same time, preserve medullary perfusion.

Earlier studies in patients and in experimental models of CHF have documented that this disorder is associated with a redistribution of intrarenal blood flow and diversion of CBF to subcortical areas (3, 16). Our study indicates that similar intrarenal hemodynamic adjustments are present in rats with aortocaval fistula, an experimental model that mimics the renal and neurohumoral manifestations of CHF (31, 35, 36). As pointed out earlier, because of the decrease in mean arterial pressure in this model, preservation of MBF must be associated with active medullary vasodilation, which occurs in the face of a marked cortical vasoconstriction. The findings of the present study provide evidence for the involvement of the NO and ET systems in mediating this differential regulation of intrarenal blood flow. Specifically, our data indicate that CHF is associated with enhanced expression of the prepro-ET-1 mRNA, particularly in the renal cortex, and of eNOS mRNA in the renal cortex and medulla. Moreover, these alterations were in proportion to the severity of the disease, as reflected in the two subgroups of rats with CHF. The observations on the altered responsiveness of the cortical and medullary vascular beds to ET-1 administration are also in line with the above findings. Thus our data demonstrate that the cortical vasoconstrictor effect of ET-1 is blunted in rats with CHF. However, careful examination of the data indicates that this attenuated response was noted primarily in rats with decompensated CHF, a group in which the ET system is highly stimulated. Such an activation of the ET system could conceivably lead to increased occupation of the vasoconstrictor ET<sub>A</sub> receptors, thereby explaining the decrease in baseline CBF and the blunted vasoconstrictor response to exogenous infusion of the peptide. An ET<sub>A</sub> receptor-mediated cortical vasoconstriction may likewise explain the findings in our previous report (12), which demonstrated that bosentan, a mixed ET<sub>A/B</sub> receptor antagonist, significantly improved cortical perfusion, but only in rats with decompensated CHF. In addition, activation of the cortical NO system, as reflected by the increased eNOS expression in rats with severe heart failure, could be an important mechanism to counterbalance the cortical vasoconstrictor effect of ET-1. Thus increased receptor occupancy by excessive ET-1 generation as well as augmented NO production in the cortex could contribute to the blunted vasoconstrictor action of the peptide in rats with decompensated CHF.

Perhaps of more potential importance are the findings on the adaptive hemodynamic changes in the renal medulla in rats with experimental heart failure. In this respect, several findings deserve consideration. First, basal expression of ET-1 mRNA and eNOS mRNA is significantly higher (by at least 4-fold) in the medulla than in the cortex. This finding is in agreement with the observations that ET immunoreactive level in the renal medulla is the highest in the body (17), as well as with the recently reported direct measurements of NO production in the medulla (39). Second, CHF is associated with increased expression of the eNOS isoform in the renal medulla in proportion to the severity of the disease. Third, infusion of ET-1 into CHF rats induces a prolonged medullary vasodilatory response in the compensated and decompensated subgroups. Similar to our observation in normal animals (13), this prolonged vasodilatory response could be mimicked by administration of a specific ET<sub>B</sub> agonist and was dependent on an intact NO system. Previously, we speculated that the NO-dependent medullary vasodilation induced by ET-1 could serve as an important homeostatic mechanism in the maintenance of medullary blood supply, particularly under pathophysiological conditions (13). It is possible that the sustained vasodilatory response observed in rats with experimental CHF in the present study could represent such an adaptive process aimed at preserving medullary perfusion. The importance of maintaining adequate blood supply to the renal medulla is underscored by the finding of low PO<sub>2</sub> levels, even under physiological conditions, suggesting that this region of the kidney is on the verge of hypoxia (4). Thus any further diminution in MBF due to cardiac failure could result in severe anoxic cellular damage.

Our data clearly indicate that the expression of eNOS, but not of ET-1 or the ET<sub>B</sub> receptor, was increased in the medulla of rats with CHF. This suggests that the regulation occurred at the final step of the ET-ET<sub>B</sub> receptor-eNOS axis. In that respect, regulation of the final step of the signaling cascade might be advantageous, since the eNOS may serve as a final common pathway to the action of several vasodilatory systems. Taken together, it is tempting to suggest that the NO system in the medulla is an obligatory determinant of the vasodilatory action of ET and that the prolongation of this effect in CHF rats is due to overexpression of eNOS. The significant elevation of cortical eNOS mRNA and immunoreactivity in rats with decompensated CHF, which still falls far behind its abundance in the medulla, was perhaps sufficient to abolish the ET-1-induced cortical vasoconstriction but
not adequate to induce vasodilation similar to that observed in the medulla.

The exact mechanisms underlying the enhanced expression of ET-1 mRNA in the renal cortex of rats with CHF are unclear. Several potential stimuli are known to induce ET expression in the endothelium of various tissues, including angiotensin II, tumor necrosis factor-α, transforming growth factor-β, interleukin, and hypoxia (32). Most of these agents increase ET production via the activation of protein kinase C, AP-1 site, and other regulatory elements in the ET-1 promoter (32). It should be emphasized that the severity of CHF in patients and animals often correlates with the degree of activation of some of these substances (30, 33, 36). Taken together, our present observation that ET-1 is expressed in the renal cortex in positive correlation to cardiac dysfunction suggests that it may be a secondary response to the activation of these ET-promoting hormonal, neural, and cytokine systems.

The findings of the present study further support the notion that the two paracrine systems, ET and NO, are involved in the pathogenesis of CHF. The evidence for the involvement of the ET system in this cardiovascular disorder is based on several observations, including increased circulating levels of ET-1 in CHF (6, 21, 26) and increased expression of ET-1 and altered ET receptor density in myocardial tissues of animals with experimental CHF (5, 20). The data of the present report further extend these observations and attest to the importance of this system in modulating CBF in this cardiovascular disorder. Similarly, there is ample experimental evidence indicating that the NO system is altered in CHF (9, 34). Most notable is the finding that CHF is associated with a blunted vasodilatory response to endothelium-dependent vasodilators such as acetylcholine (15, 19). However, there is also evidence that CHF may be associated with increased generation of NO (14, 37). In that regard, our data demonstrate an increased expression of eNOS mRNA in the kidney of rats with CHF. This finding, however, cannot rule out the possibility that other isoforms of NO may also be activated in the renal medulla in CHF. It is noteworthy that the neural isoform (bNOS) in the renal medulla has been recently documented to be modulated by dietary salt intake and is thought to play an important role in the long-term control of blood pressure (24). Moreover, it is also possible that other systems, such as natriuretic peptides, prostaglandins, and adenosine (2, 23, 28, 29), as well as other agents acting through NO, i.e., bradykinin (11), may take part in modulating MBF in heart failure. Additional studies are required to elucidate the possible role of each of these agents.

In summary, the present study demonstrates that experimental CHF is associated with altered regulation of renal regional blood flow, most likely reflecting alterations in the expression and activity in the renal cortex and medulla of two important paracrine systems, ET and NO. Moreover, it is suggested that these changes may contribute to the preservation of intact medullary perfusion in the face of severe cortical vasoconstriction during the development of cardiac decompensation.

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