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Blunted cGMP response to agonists and enhanced glomerular cyclic 3′,5′-nucleotide phosphodiesterase activities in experimental congestive heart failure

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Blunted cGMP response to agonists and enhanced glomerular cyclic 3′,5′-nucleotide phosphodiesterase activities in experimental congestive heart failure. The natriuretic peptide (NP) and nitric oxide (NO) systems are activated in congestive heart failure (CHF), resulting in increased synthesis of cGMP, which serves as a second messenger for both humoral systems. These two regulatory systems play functional roles in the preservation of glomerular filtration rate (GFR) and sodium excretion in both acute and chronic CHF. A progressive decline in glomerular responsiveness to atrial natriuretic peptide (ANP) characterizes the terminal stage of chronic CHF despite elevation of plasma ANP. Phosphodiesterase isozymes (PDEs) are integral factors in determining cellular content and accumulation of cGMP, and up-regulation of PDE activity could participate in the glomerular resistance to ANP in severe CHF. To date, characterization of possible alteration of glomerular PDE isozyme activities in CHF is unknown, as is the *in vitro* glomerular response to the nitric oxide-soluble guanylyl cyclase pathway. We, therefore, first determined cGMP generation in response to particulate and soluble guanylyl cyclase activation by ANP and sodium nitroprusside (SNP) in isolated glomeruli from normal ($N = 6$) and CHF dogs ($N = 5$) in which CHF was induced by rapid ventricular pacing for 18 to 28 days. Secondly, we explored the presence of major PDE isozymes in glomeruli isolated from the control and CHF dogs. When ANP or SNP (10^{-10} to 10^{-4} M) were incubated with the suspension of isolated glomeruli, cGMP accumulation was lower by −72 to −96% with ANP and −42 to −77% with SNP in all glomerular medias obtained from CHF compared to controls. PDE hydrolyzing activity of both cAMP and cGMP were higher in the glomerular homogenates obtained from the kidneys of the CHF group ($N = 5$) compared to those of the control group ($N = 5$). We conclude that in severe chronic experimental CHF, glomerular cGMP accumulation decreases in response to both ANP and SNP, and CHF is characterized by enhanced cAMP- and cGMP-PDE activities that may participate in glomerular maladaptation to this cardiovascular syndrome.

Activation of the natriuretic peptide system (NPS), which includes atrial (ANP), brain (BNP) natriuretic peptides of myocardial cell origin [1, 2], and C-type (CNP) natriuretic peptide of endothelial [3] and renal cell origin [4], is a hallmark of chronic congestive heart failure (CHF) [5]. In addition to the NPS, the nitric oxide (NO) system is also activated in chronic CHF [6, 7]. Both humoral systems function through particulate and soluble guanylyl cyclases respectively resulting in an increase in cGMP generation. While the NPS and NO play important roles in the preservation of renal hemodynamics and sodium excretion in both acute and chronic heart failure [5–12], the terminal stage of CHF is characterized by decreases in glomerular filtration rate (GFR) and sodium excretion in addition to reduced glomerular and natriuretic responsiveness to exogenous ANP [13] and BNP [14]. While the mechanisms which mediate this glomerular hyporesponsiveness to NPS stimulation in CHF remain poorly defined, they may be multifactorial and include decreased number or reduced affinity of biological receptors or post-receptor events that may lead to reduced stimulation of guanylyl cyclases and/or an increased cGMP degradation.

Phosphodiesterase enzymes (PDEs) mediate hydrolysis of both cAMP and cGMP [15]. To date, seven major types of PDEs have been isolated and characterized. The first five include PDE-I (calcium-calmodulin dependent), PDE-II (cGMP-stimulated), and PDE-III (cGMP inhibited), hydrolyse both cAMP and cGMP while PDE-IV (cAMP-specific) hydrolyses only cAMP and PDE-V hydrolyses only cGMP. While five types of PDEs are present in rat glomeruli [16], their activities in CHF remain undefined.

Therefore, the current investigation was designed to address two objectives. First, we determined the ability of ANP and SNP to enhance cGMP accumulation in isolated glomeruli from normal and CHF canine kidneys. Second, we determined the activities of five types of PDEs in glomeruli from normal and CHF dogs. We hypothesized that in overt CHF, total PDE activity in isolated glomeruli would be increased and glomerular cGMP accumulation to ANP and SNP would be reduced. To test this hypothesis, glomeruli were isolated from canine kidneys in the presence and absence of overt CHF produced by three weeks of rapid ventricular pacing. This model is characterized by avid sodium retention in association with altered renal hemodynamic function, marked ventricular dysfunction, systemic vasoconstriction, and neurohumoral activation thus mimicking severe human CHF [10, 11, 17–20].

Methods

Animal model

Studies were conducted in two groups of male mongrel dogs (weight, 18 to 22 kg) in accordance to the Animal Welfare Act.

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The first group served as normal controls (Control group, $N = 6$) and the second group (CHF group, $N = 5$) served as the CHF group in which severe CHF was produced by chronic rapid ventricular pacing at a pacing rate of 245 beats/min for 21 days. Hemodynamics, renal function and neurohumoral function were assessed twice in the CHF group before pacing and after the appearance of signs and symptoms of CHF.

In a sterile surgical suite, adult male mongrel dogs were anesthetized utilizing pentobarbital sodium anesthesia at a dose of 30 mg/kg i.v. for induction and repeated dosing as needed for maintenance of anesthesia. Supplemental oxygenation at 5 liter/min was provided via endotracheal tube utilizing a Harvard respirator, (Harvard Apparatus, Millis, MA, USA). A programmable cardiac pacemaker (Medtronic, Minneapolis, MN, USA) was implanted via a left thoracotomy with a 1 to 2 cm pericardiotomy. Following the pericardiotomy, the heart was exposed and a screw-in epicardial pacemaker lead was implanted into the right ventricle. The pacemaker lead was connected to a pulse generator which was then implanted subcutaneously in the chest wall. Pacing capture was verified intraoperatively prior to closing the chest cavity. The pericardium was then sutured close with great care not to distort the anatomy of the pericardium. The chest cavity and incisions were then closed in layers.

A chronic femoral artery catheter (Model GPV Vascular-Access Port, Access Technologies, Skokie, IL, USA) for mean arterial pressure monitoring and arterial plasma sampling was placed in each dog during the sterile surgical procedure. The catheter was implanted into the left femoral artery with the self-sealing silicone rubber septum port tunneled subcutaneously to the left upper hind limb. Prophylactic antibiotic treatment with 225 mg clindamycin subcutaneously and 400,000 U procaine penicillin G plus 500 mg dihydrostreptomycin intramuscularly (Combiotic; Pfizer, Inc., New York, NY, USA) were administered preoperatively and on the first two post-operative days.

Following a 14 day post-operative recovery period, rapid right ventricular pacing was initiated at 245 bpm. Each dog was then continuously paced at 245 bpm through the 21 day protocol. Pacemaker capture was verified by surface electrocardiogram two times each week.

The following acute protocol was undertaken in each of the individual dogs twice: first at baseline, before the onset of ventricular pacing, and second, after 21 days of ventricular pacing at 245 bpm. Briefly, for each of the two studies, on the night prior to the experiment, each dog was fasted, but allowed free access to water. On the day of the acute experiment, each dog was anesthetized with thiopental sodium (15 mg/kg, i.v.) to allow sterile percutaneous placement of a flow-directed balloon tip pulmonary artery catheter (model 93131-7F; American Edwards Laboratories, AHS del Caribe, Anasco, PR) via an external jugular vein. The femoral artery catheter was connected to a pressure monitor for on-line measurement of aortic pressure and for blood sampling. A second balloon-tip catheter was inserted in the urinary bladder for urine collection.

After recovery from anesthesia, each dog was allowed to stand freely in a minimally restricting sling. After administration of a loading dose of inulin and PAH, an infusion of an inulin/PAH solution was infused at a rate of 1 ml/min via central access to achieve a stable plasma inulin concentration of approximately 50 mg/dl. Each dog was allowed to stabilize for a 60-minute equilibration period prior to measuring any parameters. The study period consisted of a 30-minute clearance period with hemodynamics and blood sampling occurring at the midpoint of the clearance.

During the clearance, the following hemodynamic data were collected: mean arterial pressure (MAP), right atrial pressure (RAP), pulmonary artery capillary pressure (PCWP), and cardiac output (CO). Cardiac output was measured by thermodilution (American Edwards Cardiac Output Computer model 9510-A). For each clearance, cardiac output was measured four times and then averaged. Systemic vascular resistance (SVR) was calculated as [(mean arterial pressure-right atrial pressure)/cardiac output]. Pulmonary vascular resistance (PVR) was calculated as [(pulmonary artery pressure-pulmonary capillary wedge pressure)/cardiac output]. Renal vascular resistance (RVR) was calculated as [(mean arterial pressure − right atrial pressure)/renal blood flow]. Mean arterial pressure was assessed via direct measurement from the chronic arterial port. Glomerular filtration rate (GFR) was measured by inulin clearance. Renal blood flow (RBF) was calculated from estimated renal plasma flow (PAH clearance) and hematocrit. Urine was collected on ice during the clearance period for assessment of urine volume, inulin, PAH, endothelin, and cGMP.

Glomerular isolation and cGMP generation

Dogs were anesthetized with sodium pentobarbital (30 mg/kg) and kidneys were harvested. The control group underwent euthanasia by harvesting of the kidneys as did the CHF group utilizing a similar protocol. The control group did not undergo pacemaker insertion. We have previously reported that neurohumoral function is not different between a normal control group (as was utilized in the current study) and a sham operated group that underwent thoracotomy and cardiac manipulation [21].

Glomeruli were isolated using a minor modification of the technique of Chaumet-Riffaud et al [22]. Briefly, after the kidneys were removed, renal cortex was isolated, sliced, minced then squeezed through sieve with pore sizes of 250 and 212 μm, respectively. After each sieving, the suspension was centrifuged at 160 g for three minutes and the supernatant was discarded. The final pellet was resuspended and was buttered through a 60 μm sieve; the glomeruli which were retained on the sieve were resuspended, recentrifuged and the supernatant was discarded. The pellet was resuspended in ice-cold Kreb's buffer, pH 7.4, containing 135 mM NaCl, 4.7 mM KCl, 25 mM Na bicarbonate, 1.2 mM, K_2HPO_4, 2.5 mM $CaCl_2$, 0.026 mM Ca Versanate, and 10 mM glucose, equilibrated with 95% O_2 and 5% CO_2. Aliquot of the final centrifugation uniformly yielded material containing > 90% glomeruli and less than 5% tubular contamination when examined by light microscopy.

For measurement of ANP-stimulated or NO-stimulated cGMP accumulation, we used a method employed in our previous studies on rat kidney glomeruli with a few minor modifications [22, 24]. In short, aliquots of fresh glomeruli were suspended in Kreb's buffer and preincubated for 10 minutes at 37°C and incubation was started by adding synthetic ANP at a concentration of 10^{-10} to 10^{-4} M or sodium nitroprusside (SNP) at similar range of concentrations. This was terminated after 10 minutes by adding ice-cold trichloroacetic acid (TCA), final concentration 6.6%, and cooling to 4°C then recentrifuged, and the pellet was dissolved in 1 N NaOH and assayed for protein content by the method of Lowry et al [25] using bovine serum albumin as the standard. The

supernatant fluid, containing cGMP from glomeruli and the incubation media, was extracted five times with four volumes of water-saturated ethyl ether to remove the TCA before being evaporated to dryness under a stream of air and was stored at −70°C until assayed for cGMP content. For the cGMP assay, samples were dissolved in 50 mM sodium acetate buffer, pH was adjusted to 6.2 mixed thoroughly, and aliquot acetylated according to the manufacturer's instructions (Dupont, Boston, MA, USA). Averaged results of triplicate determinations were expressed as fmol cGMP accumulated per 10 minutes of incubation per mg protein. The supernatant fluid was used for the assay of cGMP with a method slightly modified from that described by Imura et al [26].

Phosphodiesterase (PDE) assay

Homogenization of isolated glomeruli was obtained and extracted for PDE assays in buffer containing 0.1% Triton X-100 as mentioned previously [27, 28]. The activity of PDE was measured by incubating the glomerular homogenate in a reaction mixture (final volume 100 μl) of the following composition (final concentrations): 10 mM $MgSO_4$, 2 mM EGTA, 0.1% bovine serum albumin, 15 mM Tris-HCl adjusted to pH 7.4, and either 0.5 μM [^{3}H]-cAMP, or 0.5 μM [^{3}H]-cGMP as substrate [27, 29]. In experiments examining the activity of Ca^{2+}-calmodulin (CaM)-dependent PDEs, the reaction mixture also contained 2.01 mM $CaCl_2$, to obtain 10 μM Ca^{2+}, and 10 μg/ml of calmodulin (CaM). The PDE activity in aliquot samples incubated without Ca^{2+} and CaM (but containing 2 mM EGTA, *vide supra*) was subtracted as "basal activity" [27, 29]. Activities of isozymes PDE III and PDE-IV were determined as cAMP-PDE inhibitable by 3 μM cilostamide or 3 μM rolipram, respectively [28]. At these concentrations (3 μM) both rolipram and cilostamide cause maximal inhibition of corresponding PDEs in rat glomeruli [16]. The PDE II-activity was determined as the difference in cAMP-PDE assayed without or with 5 μM cGMP [28] and PDE-V activity was demonstrated as the difference in cGMP-PDE without or with 10 μM zaprinast. This concentration of zaprinast was slightly higher than that described by Chini et al [16]. In experiments examining the effect of PDE inhibitors, stock solutions of all inhibitors of PDE were made in 100% dimethylsulfoxide (DMSO), and the incubation media (including controls) contained, after final dilution, 0.1% DMSO. Hydrolysis of c-3′,5′-nucleotides was less than 20% of the substrate and was linearly proportional to incubation time and enzyme protein concentration [27–29]. Protein content was measured by the Bradford's method [30].

Hormone analysis

Arterial blood was collected at the midpoint of the clearance for analysis of atrial natriuretic peptide (ANP), 3′5′ guanosine monophosphate (cGMP), endothelin (ET), plasma renin activity (PRA), electrolytes and hematocrit. Plasma ANP was measured by a specific radioimmunoassay (RIA) as previously described [8]. Briefly, blood for hormone analysis was collected in an ethylenediamine-tetraacetic acid (EDTA) tube and immediately placed on ice. After centrifugation of 2500 rpm at 4°C, plasma was separated and stored at −20°C until assay. ANP was extracted by use of C^{18} Bond Elut with recovery of 86%. ANP was measured by a radioimmunoassay utilizing a specific antibody to human ANP. Interassay variation was 9%, intra-assay variation was 6%, and cross reactivity was 100% with canine ANP. Cross reactivity to BNP or CNP was less than 0.1%.

Plasma endothelin was determined by ET-1,2 [^{125}I] assay system from Amersham (Amersham, UK). Before the radioimmunoassay, plasma was acidified with 0.5% trifluoroacetic acid (TFA). C8 Bond Elut cartridges were washed with 4 ml of methanol and 4 ml of water to extract the plasma. After the plasma was applied, cartridges were washed with 2 ml of normal saline and 6 ml of water. ET was eluded from the cartridges with 2 ml of 90% methanol in 1% TFA, then dried and reconstituted for the radioimmunoassay. The recovery of the extraction procedure was 81% as determined by the addition of synthetic ET to plasma; interassay and intra-assay variations were 9% and 5%, respectively. The minimal level of detection was 0.5 pg per tube. The cross reactivity of endothelin-2 and -3 and proendothelin in this assay were < 5%, < 3%, and < 37%, respectively.

Plasma and urinary cGMP was measured by radioimmunoassay using previously described methods [31–33]. Plasma renin activity was measured by radioimmunoassay as previously described [34]. Glomerular filtration rate was determined by the clearance of inulin. Urinary and plasma inulin concentration was measured by the anthrone method. Urinary and plasma electrolytes were determined by flame emission spectrophotometry (model 357; Instrumentation Laboratory, Wilmington, MA, USA).

Materials

Synthetic h α-ANP was purchased from Phoenix Laboratories (Mountview, CA, USA), sodium nitroprusside was from Sigma Chemical Co. (St. Louis, MO, USA), The cGMP Kit was purchased from Dupont Co. Inc. (Boston, MA, USA). The cAMP and cGMP were from Sigma Chemical Co.; [^{3}H]-cAMP and [^{3}H]-cGMP (ammonium salt 30 to 50 Ci/mmol) were from Amersham Co. (Arlington Heights, IL, USA). Cilostamide was a gift from Parke-Davis Pharm. (Warner-Lambert Co., Ann Arber, MI, USA). Rolipram was purchased from Biomol. Zaprinast was purchased from Sigma Chemical Co. All other reagents and chemicals were of the highest purified grade and were purchased from standard suppliers. [^{3}H]-cAMP and [^{3}H]-cGMP were, prior to use as substrates in PDE assays, purified according to a method of Kincaid and Manganiello [35].

Statistical analysis

Data were expressed as mean ± SEM. All data from each experimental group were compared and analyzed by ANOVA for repeated measures with Fisher's exact for the least significant post hoc test applied when appropriate. The comparison between groups was analyzed by ANOVA and unpaired *t*-test when appropriate. Statistical significance was accepted for $P < 0.05$.

Results

Cardiovascular, renal and hormonal function

Table 1 reports cardiovascular, renal, hormonal function before and after the development of overt CHF. Right atrial pressure (RAP) and pulmonary capillary wedge pressure (PCWP) increased while mean arterial pressure (MAP), cardiac output (CO), and urinary sodium excretion (UNaV) decreased during CHF. Glomerular filtration rate (GFR) tended to decrease in association with a marked decrease in renal blood flow (RBF). Plasma ANP, cGMP and ET increased during CHF while PRA increased but not significantly.

Table 1. Hemodynamics, Renal Function and Sodium Excretion, and Hormonal Function from CHF Group Before and During Symptomatic CHF

		Baseline	CHF
MAP	mmHg	122 ± 3	109 ± 2*
RA	mmHg	5.6 ± 0.6	15.4 ± 1.7*
PCWP	mmHg	9.8 ± 0.4	33 ± 1.9*
CO	L/min	5.3 ± 0.3	2.2 ± 0.1*
SVR	R.U.	22 ± 2	42 ± 1*
GFR	ml/min	119 ± 15	94 ± 30
RBF	ml/min	456 ± 83	217 ± 41*
UNaV	mEq/min	171.0 ± 41.2	25.7 ± 5.9*
P-ANP	pG/ml	38 ± 8	970 ± 118*
PRA	ng/ml/min	0.5 ± 0.2	1.6 ± 0.8
P-ET	pG/ml	3.7 ± 1.4	11 ± 1.8*
P-cGMP	pM/ml	1.9 ± 0.9	7.2 ± 1.2*

Data in Mean ± SEM; * denotes $p < 0.05$ compared to baseline: MAP, mean arterial pressure; RA, right atrial pressure; PCWP, pulmonary capillary wedge pressure; CO, cardiac output; SVR, systemic vascular resistance; GFR, glomerular filtration rate; RBF, renal blood flow; UNaV, urinary sodium excretion; P-ANP, plasma atrial natriuretic peptide; PRA, plasma renin activity; P-cGMP, plasma cGMP; P-ET, plasma endothelin1

cGMP accumulation in isolated glomeruli

Accumulation of cGMP generated in incubated glomeruli increased dose-dependently in response to added ANP (Fig. 1A) reaching plateau at 10^{-6} to 10^{-4} M ANP. Incubation with SNP also resulted in a dose dependent increment of accumulated cGMP (Fig. 1B), however, the total amount of accumulated cGMP was many-fold less than with ANP ($P < 0.05$). In CHF, less glomerular cGMP accumulation was observed compared to normal control at all concentrations of ANP ($P < 0.05$) or SNP at 10^{-4} M of SNP ($P = 0.059$). Table 2 demonstrates 72 to 96% less cGMP accumulation in the incubation medias of CHF glomeruli compared to control after incubation with different concentrations of ANP, and 42 to 77% less cGMP accumulation after incubation with different concentrations of SNP.

Phosphodiesterase activity in isolated glomeruli

PDE isozyme activities, defined by cAMP, cGMP hydrolysis and the type specific criterias as mentioned earlier in the **Methods** section, are depicted in Figures 2, 3 and 4. Figure 2 A and B report activities of each type of cAMP- and cGMP-PDE hydrolyzing activities, respectively, from normal controls without (basal) and with stimulation or inhibition by type-specific agents. Figure 2A illustrates that calcium-calmodulin did not enhance cAMP hydrolysis but cGMP increased the cAMP hydrolysis from the basal activity. The cAMP-hydrolyzing PDE activities were specifically inhibited by cilostamide and rolipram. Figure 2B reports the cGMP hydrolyzing PDE activities without (basal), and with calcium-calmodulin, or with zaprinast in glomeruli from normal controls. Calcium/calmodulin enhanced cGMP-hydrolyzing PDE activity significantly and zaprinast tended to attenuate the cGMP-hydrolyzing PDE activity from the basal activity.

Figure 3 A and B illustrates the higher basal PDE activities in the CHF group compared to the control group for both hydrolysis of cAMP and cGMP, respectively. Figure 4 A and B compares activities of each type of cAMP- and cGMP-hydrolyzing PDEs between the control and CHF groups, and without (basal) and with stimulation or inhibition with type-specific agents. In contrast to the control group, both parts of Figure 4 illustrate higher PDE activities in the CHF group by stimulation or inhibition with the type specific agents.

Figure 4A indicates that cAMP hydrolyzing activity of the glomeruli from the CHF group was enhanced (29%) by the calcium-calmodulin, while a slight enhancement of the activity occurred in the control group (5%). Enhanced cAMP hydrolyzing PDE activity was observed by 38% in both the CHF and the control groups by cGMP. Attenuation of cAMP-hydrolysis in CHF group were observed by cilostamide (−25%) and rolipram (−32%), and these observations were similar to the control group (−35% by cilostamide and −38% by rolipram, respectively), but higher PDE activities after the inhibition were observed in the CHF group.

In the CHF group, Figure 4B illustrates calcium-calmodulin enhanced cGMP-PDE hydrolysis (58%) from the basal activity, while zaprinast attenuated the cGMP-PDE hydrolysis (31%) below the basal activity. The PDE activities in the presence of calcium-calmodulin and zaprinast of the CHF group tended to be higher than of the control group.

Discussion

The current studies were designed to provide further insight into the glomerular hyporesponsiveness to atrial natriuretic peptide and sodium nitroprusside in a canine model of overt CHF. The current studies demonstrate that these two agonists to particulate and soluble guanylyl cyclases are characterized by a reduced ability to accumulate cGMP in isolated glomeruli from dogs with overt CHF. Second, these studies provide new insight into alterations in glomerular PDEs and demonstrate for the first time enhanced cAMP and cGMP hydrolysis in CHF. These studies therefore extend previous investigations and provide a possible mechanism by which glomerular responses to humoral factors that are linked to the cyclic nucleotides, cAMP and cGMP, occur in CHF.

Rapid ventricular pacing induced heart failure has been widely accepted as a model which results in the hemodynamic, renal, and neurohumoral adaptations which mimic those of human heart failure [10, 11, 18–20]. Recently, we have reported in preliminary studies that in acute heart failure (AHF), despite decreased CO and MAP, GFR and urinary sodium excretion are well maintained in the presence of activation of the NPS with enhanced cGMP generation [12]. Such a compensatory renal response to AHF occurs in the absence of activation of the renin-angiotensin-aldosterone system similar to the early phase of chronic heart failure observed early in human and experimental CHF [10, 11, 17–20]. In this preliminary report, we also observed that the NO system functions to maintain renal blood flow in AHF [12].

In contrast to AHF, overt CHF is characterized by a glomerular and tubular resistance to exogenous ANP [13]. Such a renal resistance is associated with a reduced cGMP response to exogenous ANP [36] with a blunted increase in GFR [37] and an attenuated cGMP response to ANP in isolated glomeruli [38]. In the present studies, we explored mechanisms of this glomerular resistance to ANP and to an agonist of the soluble guanylyl cyclase pathway. Specifically, we utilized isolated glomeruli for studying cGMP accumulation and PDE activities based upon the knowledge that the NPS and NO system induce marked generation of cGMP in glomeruli, particularly in epithelial cells (podocytes) and mesangial cells [39, 40]. Although a reduced cGMP response in glomeruli from CHF kidneys has been reported with ANP,

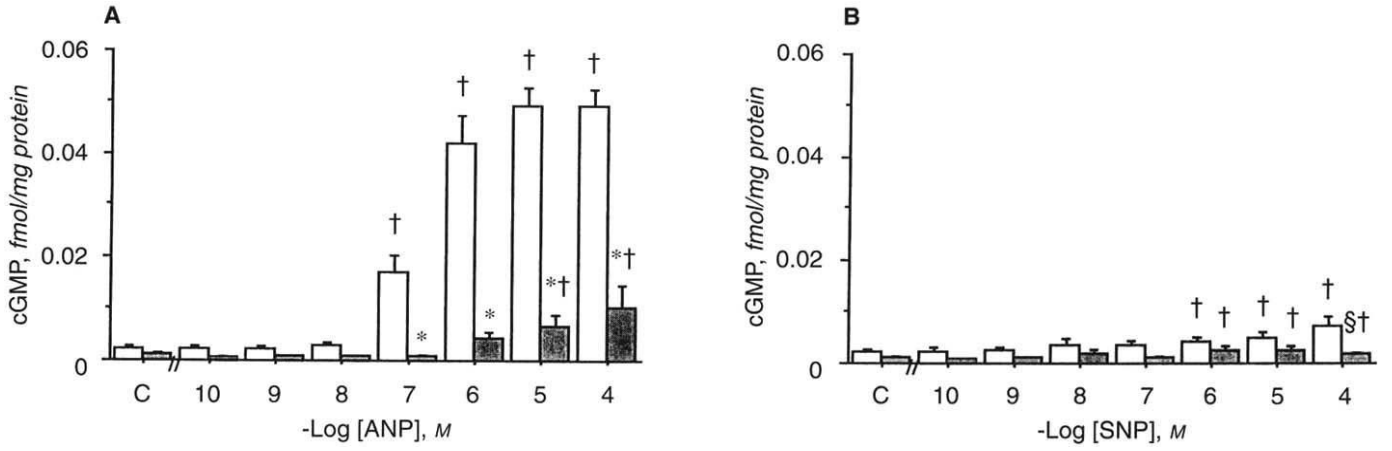

Fig. 1. *Accumulation of cGMP in the incubation media of glomerular suspension with incremental concentration from 10^{-10} to 10^{-4} M of ANP (atrial natriuretic peptide,* **A***) and SNP (sodium nitroprusside,* **B***), compared between control group and heart failure group.* Data are expressed in means ± SEM in fem to mole per mg of glomerular protein; C denotes 0 M concentration of ANP in (A) and SNP in (B), respectively; $^*P < 0.05$ compared to normal and $\S P = 0.059$ compared to normal; $\dagger P < 0.05$ compared to 0 M concentration of the stimulating agent used in the same group. Symbols are: (□) control; (▨) CHF.

Table 2. The relative differences of mean cGMP accumulation between CHF and control glomeruli in percent of control glomeruli after 10 minutes of incubation with ANP or SNP at concentration of 10^{-10} to 10^{-4} M respectively

Concentration of ANP or SNP (M)	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
ΔcGMP accumulation after incubation with ANP (%)	−76	−72	−77	−96*	−90*	−87*	−79*
ΔcGMP accumulation after incubation with SNP (%)	−67	−61	−52	−67	−42	−49	−77§

ANP, atrial natriuretic peptides; SNP, sodium nitroprusside

ΔcGMP accumulation = [(cGMP accumulation from CHF glomeruli-cGMP accumulation from control glomeruli)/cGMP accumulation from control glomeruli] × 100; *Denotes $p < 0.05$ compared cGMP accumulation between the CHF and the control glomeruli, and § denotes $p = 0.059$ compared cGMP accumulation between the CHF and the control glomeruli after stimulation by SNP

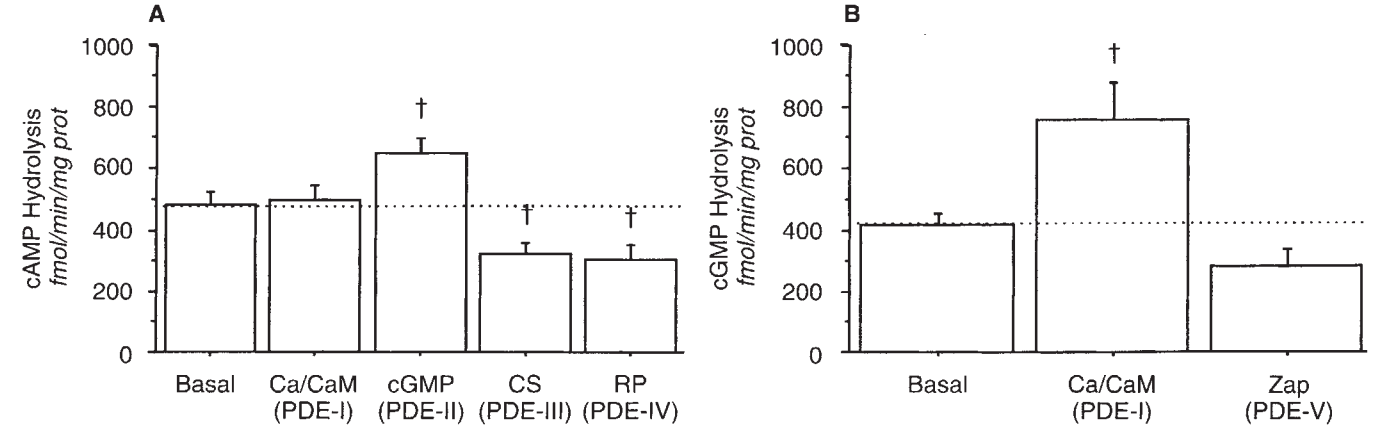

Fig. 2. *Glomerular phosphodiesterase (PDE) normal dogs express per minute per mg of glomerular homogenizing protein in basal condition (Basal), calcium calmodulin stimulated c AMP or cGMP hydrolyzing PDE-1 (Ca-CaM), cGMP stimulated cAMP PDE-II (cGMP), cilostamide inhibited cAMP hydrolyzing PDE-III (Cs), rolipram inhibited PDE-IV (Rp), and zaprinast inhibited hydrolyzing PDE-V (Zap).* Doted line represents the level of basal PDE hydrolyzing activity. Data are expressed as means ± SEM; $\dagger P < 0.05$ compared to basal activity.

controversy continues to persist [38, 41–43]. Levin et al reported that cGMP generation after stimulation with ANP in glomeruli from cardiomyopathic hamsters with CHF was lower than in normal hamsters [38]. In contrast, Luk et al reported glomerular cGMP responses to ANP or BNP in hamster cardiomyopathy and CHF was higher than control [41]. In CHF induced by aortocaval shunt in rat, Abassi et al found cGMP accumulation from glomeruli to be higher in CHF [42] while Garcia, Bonhomme and Schiffrin reported lower generation of glomerular cGMP in the same model of CHF in the presence of elevation of PRA compared to normal control rats [43]. In the current study, the finding of a reduced glomerular response to ANP is consistent

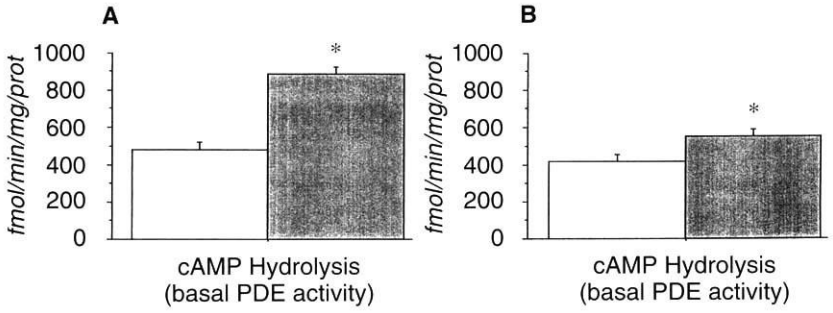

Fig. 3. *Basal glomerular phosphodiesterase (Basal) hydrolyzing activities of cAMP (A) and cGMP (B) after five minutes of incubation per mg of glomerular homogenizing protein, compared between the control group (□) and the heart failure group (▨; N = 5 in each group).* Data are expressed as means ± SEM; $^*P < 0.05$ compared between heart failure group and control.

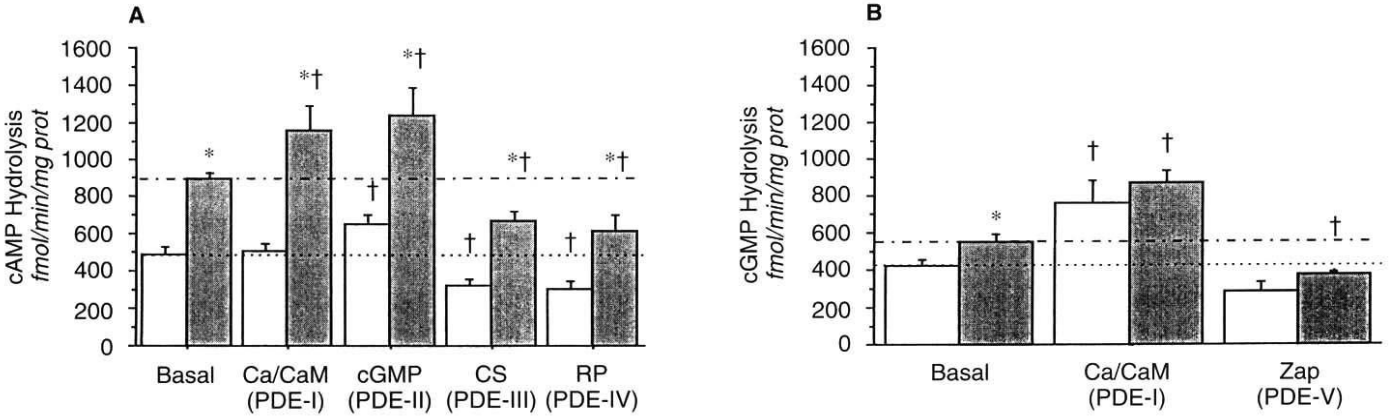

Fig. 4. *Glomerular phosphodiesterase (PDE) hydrolyzing activities of cAMP (A) and cGMP (B) express per minute per mg of glomerular homogenizing protein in basal condition (Basal), calcium calmodulin stimulated cAMP or cGMP hydrolyzing PDE-1 (Ca-CaM), cGMP stimulated cAMP PDE-II (cGMP), cilostamide inhibited cAMP hydrolyzing PDE-III (CS), rolipram inhibited PDE-IV (RP), and zaprinast inhibited hydrolyzing PDE-V (Zap), compared between control group and heart failure group (N = 5 in each group).* Data are expressed as means ± SEM; $^*P < 0.05$ compared between the heart failure group and controls, $\dagger P < 0.05$ compared to basal activities of each group. Doted lines (. . . . . or . . . . .) indicate the levels of baseline cAMP (A) or cGMP (B) hydrolysis PDE activities obtained from the control and CHF groups, respectively.

with Levin et al and Garcia et al. Moreover, we extended these observations and also established that the soluble guanylyl cyclase pathway is also involved as demonstrated by the attenuated response to SNP in CHF. However, we are aware that species difference in renal responsiveness between rat and dog might exist and this needs to be investigated further.

The glomerular cGMP response to ANP was higher than the response to SNP, in isolated glomeruli from dogs either with or without CHF. In the present study, glomeruli were incubated with agonists as described and, at the end of incubation, TCA was added to inactivate all enzymes and disrupt glomeruli. As in our previous studies [23, 24, 44], we measured total accumulation of cGMP which includes intracellular cGMP in glomerular cells and cGMP effused into medium. The amount of cGMP accumulation is related to protein from glomeruli. This established method is well suited to detect differences in capacity of ANP-sensitive and NO-sensitive systems in glomeruli from CHF dogs and controls. The amount of cGMP that effused into the media escapes hydrolytic action of PDEs that are endocellular enzymes. It was also shown that ANP-guanylyl cyclase catalyzes cGMP formation close to plasma membrane [45]. The ANP-stimulated cGMP likely escapes to extracellular media more readily than cGMP pool synthetized by cytoplasmic guanylyl cyclase that is stimulated by NO. This interpretation is consistent with localization of cGMP generated in response to ANP or SNP analyzed by immunocytologic methodology [45]. Also, our findings are consistent with investigation on aortic strips [46] that show that ANP increased cGMP synthesis and extrusion into the incubation media whereas SNP elevated only intracellular content of cGMP. The observation that cGMP accumulation in CHF was lowered to the same Δ% relative degree in ANP-stimulated or SNP-stimulated glomeruli is consistent with the notion that increased hydrolysis of cGMP-PDE in CHF was an underlying cause of the difference.

In the present studies, we assayed both cAMP and cGMP hydrolyzing PDE activities since both intracellular signaling systems are essential and may adapt differently in CHF. In glomeruli from normal dogs, cAMP hydrolysis by PDE was elevated by cGMP and depressed by cilostamide and rolipram, but remained unchanged after calcium-calmodulin. This suggests that PDE-II, -III, and -IV activities exist and respond promptly to their stimulation in the control of cAMP in canine glomeruli. Additionally, our data that calcium-calmodulin induced elevation of PDE hydrolysis of cGMP also underscores the preference of cGMP as the substrate of hydrolysis of PDE-I. In addition, zaprinast, a specific PDE-V inhibitor depressed cGMP-hydrolysis activity slightly without a statistical significant difference, suggesting that the PDE-V present in this tissue may possibly have low sensitivity to this antagonist.

In CHF, we observed that cAMP hydrolysis by PDE in glomeruli was elevated by calcium-calmodulin and by cGMP. Additionally, cilostamide and rolipram depressed cAMP hydrolysis. For

cGMP as a substrate, calcium-calmodulin increased while zaprinast depressed hydrolysis. These findings indicate the presence of all five types of PDE in canine glomeruli, and that all types play active roles in prompt hydrolysis of cAMP and cGMP in CHF. Both basal cAMP and cGMP hydrolytic activities of PDE from glomeruli of CHF were higher than those obtained from normal glomeruli, suggesting that the environment of CHF had activated all or some PDE types, particularly PDE-I and PDE-V. The current study supports a need to further assess cGMP accumulation in isolation from degradation. This, however, should be done with specific isozyme PDE antagonists for PDE-I, PDE-IV, and PDE-V.

Studies by Haneda et al may provide insight into mechanisms which activate PDEs in CHF [47]. These investigators demonstrated that angiotensin II and other calcium stimulating peptides inhibited ANP induced cGMP accumulation in cultured rat glomerular mesangial cells. By employing a specific calmodulin inhibitor, phosphodiesterase inhibitor, protein kinase C inhibitor, calcium inophore, and an activator of protein kinase C, two mechanisms were advanced. The first one involved the activation of calcium-dependent, calmodulin-stimulated nucleotide phosphodiesterase, and the second involved the inhibition of guanylyl cyclase resulting from protein kinase C activation. As ET-1 is known to increase intracellular calcium, calcium-calmodulin dependent-PDE could be activated. Thus, increases in plasma ET-1 as observed in the present studies and in other studies [48–50], could play a role in PDE activation in overt CHF.

In the current study, the marked reduction in cGMP accumulation in isolated glomeruli to ANP occurred in association with a modest but significant increase in basal cGMP hydrolyzing PDE. Such alteration in dynamics of cyclic nucleotide in disease is not without precedent. For example, Homma et al [51] investigated the role of PDEs in pathogenesis of murine nephrogenic diapedes insipidus (NDI) and reported that a doubling of basal PDE activity in inner medullary collecting duct cells (IMCD) of NDI (control vs. NDI, 31.1 ± 2.8 vs. 75.9 ± 9.6 fmol cAMP · min^{-1} · mm tubule ^{1}) was associated with greater than a 40 times decrease in cAMP accumulation in IMCD of NDI in the response to 10^{-6} M vasopressin (control vs. NDI, 45.8 ± 15.6 vs. 1.2 ± 0.2 fmol cAMP · mm $tubule^{-1}$). Thus, previous studies and other studies in IMCD have demonstrated that a modest change in PDE activity may have marked and profound effects upon reducing the generation of cyclic nucleotides to humoral stimulation [51–54]. The current studies suggest that the marked alteration in cGMP accumulation with less degree of enhancement of cGMP-PDE hydrolysis are consistent with this concept.

While the current studies were not designed to provide therapeutic insights into glomerular alterations in CHF, the current findings, however, suggest a role and mechanism of action of PDE inhibition in augmenting glomerular function in response to ANP in CHF as suggested *in vivo* by Margulies et al [55]. In this recent study, a specific PDE-V inhibitor, zaprinast, improved renal function. The glomerular response to PDE-V inhibitor alone or with ANP improved GFR and sodium excretion. Further studies are required to pursue this therapeutic potential.

In summary, in overt CHF, we demonstrate in isolated canine glomeruli reduced cGMP accumulation in CHF to ANP and SNP. For the first time, we demonstrate increased glomerular PDE activities in CHF with alterations in five subclasses of PDE. These findings provide new insight into alterations in glomerular function in CHF that may result in a reduced glomerular responsiveness to endogenous humoral ligands linked to cyclic nucleotides in CHF.

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