Effects of chronic renal failure on caveolin-1, guanylate cyclase and AKT protein expression

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Abstract

Chronic renal failure (CRF) has been documented to cause oxidative stress and alter nitric oxide (NO) metabolism. However, the effect of CRF on proteins related to NO bioactivity has not been investigated. The present study was designed to test the hypothesis that CRF would induce changes in caveolin-1 (Cav-1), soluble guanylate cyclase (sGC) and Akt, three proteins important in regulating NO synthase (NOS) functionality. Male Sprague–Dawley rats were randomized to CRF via 5/6 nephrectomy or sham-operated control groups. After 6 weeks, body weight, blood pressure, creatinine clearance, plasma creatinine, urinary cyclic guanosine monophosphate (cGMP) and immunodetectable levels of Cav-1, sGC and Akt were determined in the renal, aorta, heart and liver tissues from both groups. CRF resulted in marked decreases in body weight and creatinine clearance, and elevation of blood pressure and plasma creatinine. An apparent upregulation of sGC protein abundance in renal tissue was noted, with no change in aorta, heart and liver tissues. This was accompanied by a reduction in urinary cGMP levels, indicative of sGC dysfunction. Cav-1 protein abundance was increased in aortic, liver and renal tissues. In contrast, CRF depressed Akt abundance in aorta, heart and liver tissues. These data document that CRF is characterized by alteration in the abundance of proteins regulating NO function in hepatic, vascular, cardiac and renal tissues, and a decrease in cGMP, which contributes to hypertension and changes in NO bioactivity previously noted in this model.

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1. Introduction

In the last few years, considerable progress has been made regarding the pathogenesis of hypertension in chronic renal failure (CRF). Recent evidence suggests that oxidative stress and altered nitric oxide (NO) bioactivity play critical roles in this process. CRF has been documented to decrease NO synthase (NOS) mRNA, protein and activity in the thoracic aorta and the remnant kidney [1,2], while oxidative stress in CRF promotes NO inactivation by reactive oxygen species (ROS) leading to functional NO deficiency and hypertension.

Three proteins intimately involved in the regulation of NO and NOS activity are caveolin-1 (Cav-1), Akt and guanylate cyclase (sGC). Cav-1 is the main structural component of caveolae, flask-shaped invaginations of the plasma membrane and is a predominant location of endothelial NO synthase (eNOS) in endothelial cells. Cav-1 coimmunoprecipitates with eNOS and its interaction with eNOS results in the inhibition of NOS activity [3]. Akt, also known as protein kinase B, is a serine threonine kinase that mediates the activation of eNOS via phosphorylation, leading to increased NO production [4,5]. Akt-induced phosphorylation of eNOS is known to reverse inhibitory conformation of eNOS when associated with Cav-1 [6].
Additionally, NO is the physiological activator that binds to the prosthetic heme group of the enzyme sGC to increase the conversion of guanosine triphosphate to cyclic guanosine monophosphate (cGMP), leading to the activation of several effector molecules [7]. To the best of our knowledge, no studies have investigated the effect of CRF on the abundance of these proteins.

In the present study, we achieved renal mass reduction by surgical resection of the upper and lower thirds of one kidney, followed by contralateral nephrectomy, which produces CRF with mild hypertension. We tested the hypothesis that CRF would cause alterations in Cav-1, sGC and Akt, key regulatory proteins associated with NO bioactivity, as well as reduce urinary cGMP.

2. Methods

2.1. Study groups

Male Sprague–Dawley rats with an average body weight of 270 g (Harlan Sprague–Dawley, Inc., Indianapolis, IN, USA) were used in this study. Animals were housed in a climate-controlled vivarium with 12-h day and night cycles and were fed a standard laboratory diet (Purina Rat Chow 5001; Purina Mills, Brentwood, MO) and water ad libitum. The animals were randomly assigned to the CRF and sham-operated control groups. The CRF group underwent 5/6 nephrectomy by surgical resection of the upper and lower thirds of left kidney, followed by right nephrectomy 4 days later. The control group underwent sham operations. The procedures were carried out under general anesthesia (pentobarbital sodium 50 mg/kg, IP) using strict hemostasis and aseptic techniques. The nephrectomy procedures were accomplished via dorsal incisions as described in our earlier studies [1]. The experimental protocol employed in the study was approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Animals were monitored for 6 weeks after nephrectomy and sham operation. The 6-week period was chosen because at this time point, the CRF animals exhibit a mild but significant elevation of their arterial pressure [1]. The utility of this model as opposed to that produced by renal artery branch ligation has been detailed earlier [1]. Body weights were determined weekly. At the conclusion of the six-week observation period, animals were placed in individual metabolic cages for a timed urine collection. Plasma creatinine and creatinine clearance were determined using standard laboratory procedures.

2.2. Measurement of arterial pressure

Arterial pressure was determined by tail plethysmography (Harvard Apparatus, Natick, MA, USA) as described previously [8]. Briefly, the conscious animal was placed in a restrainer and permitted to rest for 10–15 min. The cuff was then placed on the tail and was inflated and released several times to condition the animal to the procedure. After stabilization, blood pressure was measured three times, and the average of these values was used.

2.3. Tissue preparation and immunoblotting of Cav-1, sGC and Akt

The animals were anesthetized (pentobarbital 50 mg/kg, IP) and exsanguinated by cardiac puncture. Remnant kidney, thoracic aorta, heart and liver tissues were immediately harvested, cleaned with PBS, and snap-frozen in liquid nitrogen. Plasma and frozen tissues were then stored at −70 °C until processed.

Homogenates (25% w/vol) of kidney, heart, liver and thoracic aorta were prepared in 10 mmol/l HEPES buffer, pH 7.4, 1 mmol/l ethylenediaminetetraacetic acid (EDTA), 1 mmol/l dithiothreitol (DTT), 10 μg/ml leupeptin, 2 μg/ml apro tinin and 1 mmol/l phenylmethylsulfonyl fluoride (PMSP) at 0–4 °C with a Polytron homogenizer set on an output of 5. Homogenates were centrifuged at 9000×g for 10 min at 4 °C to remove nuclear fragments and tissue debris without precipitating plasma membrane fragments. A portion of the supernatant was used for the determination of total protein concentration by using a Bio-Rad kit (Hercules, CA, USA).

Total cellular protein (20 μg each) was electrophoresed in 4–20% Tris-glycine sodium dodecyl sulfate (SDS) polyacrylamide gels (Novex, San Diego, CA, USA). Proteins were transferred onto polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA, USA), blocked in 5% dry milk in T-TBS and incubated with the following primary antibodies for 3 h at room temperature: anti-sGC-β subunit (Calbiochem, San Diego, CA, 1:1000, Cat. #371712), anti-Cav-1 (Affinity Bioreagents, Inc., Golden, CO, 1:1000, Cat. #PAI-064), and anti-AKT (BD Biosciences, 1:2000, Cat. #610861). After washing five times with T-TBS, the blots were incubated with secondary antibodies (anti-rabbit, 1:2000 for sGC and Cav-1, Cat. #NA 934V) and anti-mouse, 1:2000 for AKT (Amersham Biosciences, Piscataway, NJ, Cat. #931V) conjugated with horseradish peroxidase at room temperature for 2 h. After washing five times with T-TBS, the blots were incubated with secondary antibodies (anti-rabbit, 1:2000 for sGC and Cav-1, Cat. #NA 934V and anti-mouse, 1:2000 for AKT (Amersham Biosciences, Piscataway, NJ, Cat. #931V) conjugated with horseradish peroxidase at room temperature for 2 h. After washing five times with T-TBS, the membranes were developed using enhanced chemiluminescent (ECL) reagent (Amersham Life Science, Inc., Arlington Heights, IL, USA) and subjected to autoradiography for 1–5 min. The autoradiographs were scanned with a laser densitometer (Model PD 1211; Molecular Dynamics, Sunnyvale, CA, USA) to determine the relative optical densities of the bands. Normalization to β-actin or glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used to verify the uniformity of protein load and transfer efficiency across the test samples in liver, heart and aorta. However, β-actin increased in renal tissue, as expected, most likely from the
surgical procedures, which may result in the alteration of expression of structural proteins. Consequently, GAPDH (anti-GAPDH, 1:2500, Chemicon International, Temecula, CA, Cat. #MAB 374) was used to normalize protein expression in this tissue (Fig. 4).

2.4. Measurement of urinary cGMP

cGMP levels in urine were determined using an enzyme immunoassay kit from Sigma (sGC-201) with intra and inter assay coefficient of variations of 4.0% and 3.5%, respectively, and a sensitivity of 0.37 pmol/ml.

2.5. Data analysis

Student’s t-test were used in statistical evaluation of the data that are presented as mean±S.E. P values <0.05 were considered significant.

3. Results

3.1. General data (Table 1)

Body weights at the conclusion of the study were significantly lower in the CRF group than in the control group (P<0.01), suggesting a significant growth retardation in the CRF animals (Table 1). The CRF group exhibited a marked increase in arterial blood pressure (P<0.02), whereas the sham-operated control group showed no significant change in blood pressure over the 6-week study. As anticipated, the CRF group exhibited a significant reduction in creatinine clearance compared with the sham-operated controls and significantly higher plasma creatinine (P<0.001).

3.2. cGMP

The CRF animals exhibited a significant reduction in urinary cGMP (142.8±23.9 vs. 243.3±28.5 pmol/18 h urine, P<0.05, Table 1).

3.3. CAV-1, sGC and Akt protein expression

The CRF group exhibited a significant upregulation of immunodetectable sGC protein in kidney tissue (Fig. 1C, P<0.01) as compared with values found in the normal control group. However, there was no change in sGC protein abundance in the thoracic aorta (Fig. 1A) heart (Fig. 1B) or liver (Fig. 1D). Additionally, the CRF group showed a significant upregulation in Cav-1 protein abundance in aorta (Fig. 2A, P<0.01) kidney (Fig. 2C, P<0.01) and liver

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Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Body weight (g)</th>
<th>Plasma Cr (mg/dl)</th>
<th>Ccr (ml/min)</th>
<th>Arterial pressure (week 0)</th>
<th>Arterial pressure (week 6)</th>
<th>cGMP (pmol/18 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRF</td>
<td>8</td>
<td>358±8*</td>
<td>0.77±0.05*</td>
<td>1.28±0.07*</td>
<td>119±3</td>
<td>164±4*</td>
<td>142±23.9*</td>
</tr>
<tr>
<td>CON</td>
<td>6</td>
<td>404±5</td>
<td>0.47±0.01</td>
<td>2.32±0.12</td>
<td>119±3</td>
<td>121±3</td>
<td>243±28.5</td>
</tr>
</tbody>
</table>

Values are mean±S.E. Cr, creatinine concentration; Ccr, creatinine clearance; CRF, chronic renal failure; CON, control.

* P<0.05 vs. other groups.

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Fig. 1. Representative Western blots of aortic (A), cardiac (B), renal (C), and hepatic (D) sGC (β subunit) in four Control and four CRF animals. Group data is shown below, illustrating relative optical densities of sGC protein bands in the study animals. N=6 in each group. *P<0.01 relative to other groups.
In contrast, AKT protein abundance in aorta (Fig. 3A, \(P<0.01\)), heart (Fig. 3B, \(P<0.01\)) and liver (Fig. 3D, \(P<0.01\)) was decreased in the CRF animals compared to that found in the control group, with no change in kidney tissue (Fig. 3C).

4. Discussion

The pathogenesis of CRF appears to intimately involve alterations in NO bioactivity and signaling. Previous studies have documented that oxidative stress plays a role in the decrease in NO bioactivity in CRF, as noted by the reported elevation of malondialdehyde, tissue nitrotyrosine accumulation [9] and superoxide radical levels [10]. Additionally, elevated NAD(P)H oxidase protein expression and depressed SOD enzyme abundance have been documented in renal insufficiency [9,11,12]. Recent evidence suggests that NADPH oxidase is a major source of \(O_2^-/CO\) production in cardiovascular systems [13,14]. Therefore, observed marked upregulation of NADPH oxidase and decreased SOD protein abundance in the kidneys of CRF animals compared to sham-operated, strongly suggests the presence of oxidative stress in CRF [9]. Mitigation of these events occurred after administration of the SOD-mimetic drug,

![Fig. 2. Representative Western blots of aortic (A), cardiac (B), renal (C), and hepatic (D) Cav-1 in four Control and four CRF animals. Group data is shown below, illustrating relative optical densities of Cav-1 protein bands in the study animals. N=6 in each group. *\(P<0.01\) relative to other groups.](image)

![Fig. 3. Representative Western blots of aortic (A), cardiac (B), renal (C), and hepatic (D) Akt in four Control and four CRF animals. Group data is shown below, illustrating relative optical densities of Akt protein bands in the study animals. N=6 in each group. *\(P<0.01\) relative to other groups.](image)
tempol [9]. CRF has also been documented to decrease aortic and renal tissue NOx levels, eNOS mRNA, protein and activity [2,11,15], although some investigators have documented an increase in vascular NOS expression [15], which is most likely attributed to ligation of the upper and lower renal artery branches of one kidney followed by contralateral nephrectomy, which produces a dramatic hypertensive effect. Amelioration of oxidative stress by high-dose of vitamin E enhances NO availability, improves hypertension, lowers protein nitration products, and increases NOS expression and vascular NO production in CRF animals [11]. These data clearly document altered NO bioactivity in the pathogenesis of CRF. Nevertheless, the role of key regulatory proteins that affect NO bioactivity, such as Cav-1, sGC and Akt, has not been investigated in CRF.

In the present study, we accomplished renal mass reduction by surgical resection of the upper and lower thirds of one kidney, followed by contralateral nephrectomy, which produces CRF with moderate hypertension. As expected, the CRF animals exhibited a moderate, but significant rise in arterial blood pressure, coupled with a significant increase in plasma creatinine, a hallmark of renal insufficiency. The results showed that this surgical-resection model of CRF is associated with significant alteration in tissue sGC, Cav-1 and AKT protein expressions. Based on the results of the present study, we believe that CRF, per se, leads to an alteration in the abundance of these proteins and as such, contributes to the pathogenesis of hypertension in CRF. Furthermore, the reduction in urinary cGMP is indicative of depressed NO activity previously noted in this model.

By reacting with the heme moiety of sGC, NO activates this enzyme which catalyzes the formation of the second messenger, cGMP. The CRF animals employed in the present study exhibited a significant reduction of urinary cGMP excretion coupled with a significant rise in immunodetectable sGC abundance in the remnant kidney. This is in agreement with Schneider et al. [10], who documented a progressive increase in sGC-β in the first 2 weeks of acute renal failure. While on the surface, these findings appear to be contradictory, the apparent paradox can be readily reconciled by the following considerations. First, since generation of cGMP is NO dependent, previously demonstrated depressed NO availability occasioned by marked reductions of kidney and vascular tissue NOS expression [1] and ROS-mediated NO inactivation [9,11] can readily account for diminished urinary cGMP excretion despite elevated kidney tissue sGC abundance. Second, the magnitude of increase in remnant kidney sGC content was far less than the magnitude of renal mass reduction occasioned by 5/6 nephrectomy and the ensuing progression in the chronic phase. Thus, the estimated total kidney tissue sGC content would be far lower in the CRF animals compared to that in the control rats with two intact kidneys. Accordingly, to the extent that urinary cGMP may reflect its renal production, the combination of depressed NO availability and overall reduction in enzyme quantity can account for depressed urinary cGMP excretion observed in the CRF rats. It should also be pointed out that in recent years several laboratories have demonstrated NO-independent regulation of sGC (reviewed in Ref. [16]). Stasch et al. [17] have reported the presence of a NO-independent regulatory site on sGC. NO-independent downregulation of sGC has been demonstrated in PC12 cells exposed to nerve growth factor [18] and in rat cerebellar granule neurons exposed to aluminium [19] or high ammonium [20]. It is also of note that Koglin and Behrends [21] have shown biliverdin to be an endogenous inhibitor of sGC. Biliverdin is formed by heme oxygenases.
(HO), which convert heme to carbon monoxide and biliverdin. Earlier studies from this laboratory have shown a marked induction of hepatic HO-1 as well as HO-2 6 weeks after CRF [22].

One post-translational modification that can potentially regulate eNOS activity is phosphorylation [23]. Cotransfection of wild-type Akt and eNOS in COS cells increases eNOS phosphorylation in a wortmannin-sensitive manner and is inhibited by mutation of serine 1177/1179 to alanine [4]. Akt-phosphorylated eNOS is 15- to 20-fold more active than unphosphorylated eNOS [24]. Additionally, immuno-precipitation of eNOS results in coprecipitation of Akt [25].

In the present study, we demonstrated that CRF causes widespread downregulation of Akt, which can result in a decreased capacity to activate NOS. In addition to Akt deficiency, the CRF group exhibited significant upregulation of Cav-1 protein abundance in the kidney, thoracic aorta and liver tissues. Cav-1 is a structural component of caveolae and the principal binding site for eNOS in cells. Association of Cav-1 with eNOS results in inhibition of NOS activity (reviewed in Ref. [23]). Accordingly, NO production is negatively regulated by eNOS interactions with Cav-1 and that for NO release to occur, the inhibitory clamp by Cav-1 must be overcome, an event which is normally facilitated by calmodulin [26]. Only one study has investigated the effects of renal insufficiency on Cav-1. In a model of acute renal failure, Mahmoudi et al. [27] documented a marked increase in Cav-1 expression at the site of renal injury and was found to be localized within apoptotic cells.

Taken together, the present data indicate that CRF results in an alteration of cellular proteins regulating NO bioactivity. Given the pivotal contribution of these proteins in NO bioactivity, these changes may contribute to the genesis of the associated hypertension by compounding the effects of CRF-induced downregulation of renal, vascular, and cardiac NOS isoforms shown in earlier studies of this model [1,11]. NOS deficiency in CRF animals was confirmed by in vitro incubation experiments, which demonstrated a marked reduction in NO generation by isolated vascular tissues from CRF kidneys [10]. NOS deficiency, the CRF group exhibited significant upregulation of Cav-1 and downregulation of Akt. These changes compound the effects of previously noted downregulation of NOS isotypes and oxidative stress in lowering NO availability, contributing to hypertension and cardiovascular disease in CRF.

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References


