Sympathetic blockade prevents the decrease in cardiac VEGF expression and capillary supply in experimental renal failure

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Amann K, Odoni G, Benz K, Campean V, Jacobi J, Hilgers KF, Hartner A, Veelken R, Orth SR. Sympathetic blockade prevents the decrease in cardiac VEGF expression and capillary supply in experimental renal failure. Am J Physiol Renal Physiol 300: F105–F112, 2011. First published October 20, 2010; doi:10.1152/ajprenal.00363.2010.—Uremic cardiomyopathy of men and rodents is characterized by lower myocardial capillary supply that in rats could be prevented by central and peripheral blockade of the sympathetic nervous system. The underlying pathomechanisms remain largely unknown. We investigated whether alterations of cardiac vascular endothelial growth factor (VEGF) gene and protein expression were involved. In our long-term experiment, we analyzed whether VEGF gene and protein expression was altered in the heart of male Sprague-Dawley rats with either sham operation (sham, n = 10) or subtotal nephrectomy (SNX, n = 10). In our short-term experiment (17 sham, 24 SNX), the effect of a putative downregulation of sympathetic nervous activity by surgical renal denervation (interruption of renal afferent pathways) on cardiac gene expression of VEGF, flt-1, and flk-1 and on myocardial capillary supply was analyzed. In the long-term study, cardiac capillary supply and vascular endothelial growth factor gene and protein expression were significantly lower in SNX than in sham. In the short-term experiment, cardiac VEGF mRNA expression was significantly lower in untreated SNX (4,258 ± 2.078 units) than in both sham groups (11,709 ± 4,169 and 8,998 ± 4,823 units); this decrease was significantly prevented by renal denervation (8,190 ± 3,889, P < 0.05). We conclude that cardiac VEGF gene and protein expression is reduced in experimental renal failure, and this may be considered as one potential reason for impaired myocardial adaptation under the condition of cardiac hypertrophy. The beneficial effect of sympathetic downregulation on cardiac structure and function in renal failure may be at least in part explained by increased cardiac VEGF gene expression.

uremic cardiomyopathy; capillary supply; vascular endothelial growth factor; capillary/myocyte mismatch; sympathetic nervous system; renal denervation; chronic renal failure

LEFT VENTRICULAR HYPERTROPHY (LVH) develops early in patients with chronic renal failure (CRF) and has important prognostic value in terms of mortality (22, 36). LVH is invariably associated with structural remodeling of the myocardium that has been extensively characterized in CRF patients as well as in experimental models of CRF such as the subtotal nephrectomy (SNX) model (9). In particular, a specific blood pressure-independent reduction of capillary supply has been documented that leads to capillary/myocyte mismatch and may be the main cause of ischemic injury to the myocardium (1, 10, 12). This capillary reduction is unique to the heart in SNX rats and does not occur in other skeletal muscles (8).

It has been shown, however, that angiogenesis is also impaired in a hindlimb ischemia model of SNX rats (27), most presumably because of decreased vascular endothelial growth factor (VEGF) and VEGF receptor gene and protein expression. Recently, lower VEGF levels in parallel with increased soluble VEGF receptor flt-1 levels have been shown in CKD patients (19), indicating some VEGF resistance under the condition of CRF.

At present, the pathogenesis of inadequate capillary adaptation in LVH is unknown. In CRF patients, partial regression of LVH has been documented after correction of anemia (35) or optimized heart failure therapy (24). Changes of myocardial structure, however, have not been addressed in these studies. In experimental studies, blockade of the endothelin system (7, 33) as well as central or peripheral blockade of the sympathetic nervous system were effective in preventing the development of capillary rarefication in SNX rats, even when nonhypotensive doses were used (4, 7, 40). In contrast, other interventions like angiotensin-converting enzyme inhibition, administration of calcium channel blockers, or correction of anemia with erythropoietin were not successful (2, 40).

Sympathetic overactivity is a known feature of CRF and has been documented both in experimental (16) and in clinical (18, 28–30) studies.

The mechanisms underlying increased sympathetic nerve activity in uremia are not yet fully understood. Besides uremic toxins (38), an altered activity of renal afferent nerve fibers that are intrarenally colocalized with sympathetic nerve fibers may play a role (39). It is known for quite some time that specific afferent renal denervation will delay and alleviate the development of high blood pressure in almost all models of experimental hypertension (11); this is interpreted as an indirect hint that increases of central sympathetic outflow are inhibited during development and maintenance of experimental hypertension. In experimental models of CRF, afferent nerve fibers were involved in the development of hypertension and sympathetic activity (16). Afferent nerve fibers like the ones found in the kidney are complexly involved in inflammatory processes in a variety of organs (21, 37).

Because the sympathetic nervous system is known to modulate capillary growth via direct and indirect effects (38, 39), a possible role of the sympathetic nervous system in the pathogenesis of cardiac capillary-myocyte mismatch in CKD seems plausible (11).

In previous studies, we had already shown that central and peripheral sympathetic blockade could prevent the decrease in...
myocardial capillary supply (5, 40). In the present study, we now investigated 1) whether expression of the major angio-
genic factor VEGF or other major angiogenic factors is altered in the heart of SNX with lower cardiac capillary supply and 2) whether cardiac VEGF expression could be modified by a downregulation of sympathetic activity interrupting afferent renal pathways.

MATERIALS AND METHODS

Long-Term Experiment

In a long-term study, 20 male Sprague-Dawley (SD) rats (8 wk of age) were either sham operated (sham, n = 10) or SNX (n = 10) and followed for 8 wk. One-half of the animals was then perfused fixed via the abdominal aorta with ice-cold 0.9% NaCl for mRNA and protein analysis. The other one-half was perfusion fixed with glutar-
aldehyde for quantitative morphological investigations as described in detail (6).

Short-Term Experiment

In a short-term study, we investigated the effect of complete surgical blockade of the renal sympathetic nervous system by selective renal denervation on myocardial capillary supply and particularly cardiac gene expression of VEGF and its receptors flt-1 and flk-1 in sham and SNX rats. Sham operation was performed in 17 SNX and in 24 SD rats as described in detail (12). Briefly, SNX was performed in a two-step procedure. First, the left kidney was carefully taken out and weighed. Next, the animals recovered for 1 wk followed by a SNX of the right kidney by standardized renal ablation, i.e., two-thirds of the weight of the initially removed left kidney was surgically removed from the cortical tissue. Control animals were sham-operated, i.e., first, a left sham uninephrectomy was performed. Later (1 wk), a sham SNX of the right kidney was performed by decapsulating the kidney.

After SNX (5 days), one-half of the animals of each group was left untreated, the other one-half underwent sympathetic blockade by selective renal denervation as described earlier (34). Briefly, blockade of the sympathetic nervous system was achieved by removing all nerve fibers and fibrous tissue surrounding the renal arteries followed by application of phenol as described earlier (for further details, see Ref. 34). The effectivity of renal denervation was tested in sham and SNX animals by uptake and fractional release of the sympathetic transmitter [3H]norepinephrine from cortical slices that were nearly completely prevented by renal denervation.

The following groups were investigated: untreated sham (n = 8), sham + denervation (n = 9), untreated SNX (n = 12), and SNX + denervation (n = 12).

After 10 days, blood pressure (tail cuff plethysmography) was measured in conscious rats, and blood was taken to determine serum

Statistics

Nonradioactive in situ hybridization. In addition, cardiac expres-
sion of VEGF mRNA was analyzed using nonradioactive in situ hybridization as described before (13).

TaqMan PCR. Gene expression of VEGF and its receptors flk-1 and flt-1 was quantitated by TaqMan PCR using the same primers and protocol that was described in detail elsewhere (13, 23).

Quantitative morphological investigations. After perfusion fixa-
tion, the heart of each animal was resected, and the total heart weight as well as the left ventricular weight were determined. Tissue samples and sections were obtained and stained according to the orientator method (4, 32). Briefly, uniform random sampling of the myocardium was achieved by preparing a set of equidistant slices of the left ventricle and the interventricular septum with a random start. Two slices were selected by area-weighted sampling and processed accordingly. Eight pieces of the left ventricular muscle, including the septum, were prepared and afterwards embedded in Epon-Araldit. Semithin sections (0.8 μm) were stained with methylene blue and basic fuchsin and examined by light microscopy with oil immersion and phase contrast at a magnification of 1:1,000.

All animal work has been conducted according to relevant national and international guidelines. Formal approval was given by the local authorities (Regierungspräsidium Karlsruhe, Germany). All investigations were performed in a blinded manner, i.e., the observer was unaware of the study group to which the animal belonged. Stereological analysis was performed on eight random samples of differently oriented sections of the left ventricular myocardium per animal according to the orientator method (4). This technique was developed to determine the capillary number per area and to allow a simple estimation of capillary length density (by multiplying capillary density by 2) as a three-dimensional parameter of myocardial capillarization (32). Thereby, myocardial structures can be easily identified and counted on semithin sections using standard criteria. Thus length density (Lv) was determined using the equation Lv = 2Qv/X (where Qv is area density, i.e., the no. of capillary transsects/area of myocardial reference tissue). Reference volume was the total myocardial tissue (exclusive of noncapillary vessels, i.e., arterioles and veins and tissue clefts). Total length of capillaries per heart (Lwp) was calculated, using the formula: Lwp = Lwp × V with V = m/6 and b = 1.04 g/cm³ (40). Intercapillary distance (ICD) was calculated according to a modification of the formula of Henquell and Hornig (26) as ICD = [(3/4) × Qv × X × V 2].

Statistics

Statistical analysis was performed with SPSS 13. Data are given as means ± SD. Student’s t-test or ANOVA was used for comparison of means followed by appropriate post hoc tests. If distributional assumptions were in doubt, the nonparametric Kruskal-Wallis test was chosen. The zero hypothesis was rejected at P < 0.05.
RESULTS

Long-Term Experiment

Animal data and cardiac capillary supply. SNX led to LVH, which was paralleled by lower myocardial capillary supply. After 8 wk, body weight and systolic blood pressure were comparable between sham and SNX. Left ventricular weight (after perfusion) was significantly higher in SNX than in sham, indicating LVH ($P < 0.05$). Serum urea as one parameter of renal function was also significantly increased in SNX compared with sham ($P < 0.01$), indicating moderate stable renal failure. In parallel with the development of LVH, length density and total length per volume of myocardial capillaries as three-dimensional parameters of cardiac capillary supply decreased in SNX compared with sham-operated control animals (−22.1%, $P < 0.01$). This was accompanied by a significant increase in ICD, i.e., the distance between the center of a cardiomyocyte and a surrounding capillary (26.4 ± 1.43 vs. 23.5 ± 1.68 μm, $P < 0.01$) (Table 1).

Analysis of expression of angiogenic factors in the hearts of sham and SNX. Cardiac gene and protein expression of VEGF, but not of other major angiogenic factors, is reduced in SNX compared with sham. As shown in Fig. 1, VEGF mRNA (A and B) and protein (C and D) was expressed in myocyte nuclei, and capillary endothelial cells, HIF-1α, angiopoietin1 and -2, and Tie-2 were localized predominantly in the cardiac interstitium, but even stronger in the subendocardium as well as in myocyte nuclei and in capillary endothelial cells (Fig. 2). Total cardiac gene expression of these angiogenic factors was not significantly different between SNX and sham (Figs. 1 and 2).

In contrast, cardiac VEGF gene (Fig. 1, A and B) and protein (Fig. 1, C and D) expression was significantly lower in the SNX group compared with sham. The same was true when mRNA expression of nonradioactive in situ hybridization (Fig. 1E) and protein (Fig. 1F) expression was quantitated. Moreover, analysis of quantitative cardiac VEGF mRNA expression related to glyceraldehydes-3-phosphate dehydrogenase by TaqMan PCR revealed significantly lower values in SNX animals compared with sham-operated controls (Fig. 1G). In contrast, after 8 wk of moderate CRF, cardiac gene expression of flk-1 and flt-1 was not significantly different between SNX and sham (Fig. 1G).

Short-Term Experiment

Animal data. Sympathectomy by selective renal denervation improves renal function independent of blood pressure. After 10 days, there was no significant difference in body weight and heart weight between the four groups. Systolic blood pressure was slightly higher in both SNX groups; the difference, however, was not statistically significant. S-creatinine as a parameter of kidney function was comparable in both sham groups, whereas values were significantly higher in both SNX groups, indicating mild renal failure ($P < 0.001$). Of note, S-creatinine was significantly lower in the SNX + denervation group than in untreated SNX ($P < 0.01$), pointing to a renoprotective effect of sympathetic blockade by selective renal denervation (Table 2).

Morphology of the heart. Sympathectomy by selective renal denervation prevents the decrease in myocardial capillarization and normalizes cardiac VEGF expression. Analysis of myocardial capillarization revealed a significantly lower number of capillary transsects per area myocardium in SNX vs. sham (244.7 ± 83.9 vs. 323.5 ± 95.3/mm² of myocardium, $P < 0.05$). This early decrease in myocardial capillary supply in the heart of SNX was completely prevented by surgical denervation (396.7 ± 91.6), whereas, in sham-treated animals, sympathectomy did not affect capillary density (336 ± 102.3).

In parallel, the decrease of cardiac VEGF mRNA expression in SNX was prevented by selective renal denervation (Fig. 3A). VEGF mRNA expression as measured by TaqMan PCR was significantly lower in untreated SNX than in both sham groups ($P < 0.05$). It was significantly higher in SNX + denervation than in untreated SNX. In contrast, mRNA expression of both VEGF receptors, flk-1 and flt-1, was not significantly altered by either SNX or renal denervation, respectively (Fig. 3, B and C).

Activation of cardiac interstitial and endothelial cells as assessed by PCNA-positive cells per square millimeter area myocardium was significantly higher ($P < 0.05$) in untreated SNX (30.3 ± 15.4) compared with sham (2.91 ± 0.66) and was significantly lowered by selective surgical renal denervation (10.1 ± 5.99). In contrast, cardiac apoptosis as assessed by activated caspase-3 was not significantly different between the four groups (data not shown).

DISCUSSION

The salient feature of the present study is the finding that expression of the main proangiogenic factor VEGF, which is significantly lower in the heart of SNX animals with mild CRF compared with sham-operated controls, is normalized by interventions that ameliorate overactivity of the sympathetic system in CKD. Furthermore, the study strongly supports the assumption that, in CKD, altered renal afferent nerve activity contributes significantly to a general increase in central sympathetic outflow to the heart.

Studies in experimental models of CKD as well as in patients have clearly shown that myocardial capillary supply is

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<th>Table 1. Animal data and parameters of myocardial capillary supply of long-term experiment (8 wk of moderate stable CRF)</th>
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<td><strong>Parameter</strong></td>
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<td>Body Wt, g</td>
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<td>Left Ventricular Wt, g</td>
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<td>Total Capillary Length, mm</td>
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*Values are means ± SE; n, no. of rats. CRF, chronic renal failure; $L_{vcap}$, length density of capillaries/unit myocardium; SNX, subtotal nephrectomy; NS, not significant. ¹Weight after perfusion fixation.*

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This finding is specific for LVH in CKD, since it was not found in other models of cardiac hypertrophy due to hypertension, i.e., the SHR rat model of essential hypertension and the 1C-2K model of renovascular hypertension (1, 10, 12). The selective capillary deficit is unique to the heart and does not appear under physiological conditions in other skeletal muscle, i.e., the psoas muscle (8). Using the hindlimb model of ischemia in nonhypertensive 5/6 nephrectomized rats, however, Jacobi et al. (27) reported similar findings in the gastrocnemius and soleus muscles as we found in the myocardium. They also observed decreased capillary supply in parallel with lower VEGF mRNA expression. In contrast to our findings in the myocardium, in the peripheral muscle of SNX under the condition of ischemia, also both VEGF receptors were down-regulated.

In the heart, the selective reduction of capillary growth under the condition of LVH leads to so-called capillary-myocyte mismatch with increased diffusion distance for blood and oxygen and may thus provide at least one expla-
nation for impaired ischemia tolerance in CKD. This was elegantly shown in the myocardial infarction model where the infarcted area as well as the percentage of infarcted tissue were significantly higher in SNX than in sham animals (20). The above-mentioned lack of cardiac VEGF gene expression may provide an explanation for the failure of capillaries to adapt to hypertrophy of the left ventricle under the condition of renal failure. This decrease in cardiac VEGF mRNA expression seems to be specific for the SNX model or CKD, since a similar significant decrease in VEGF

Fig. 2. Long-term experiment. Cardiac protein expression (by immunohistochemistry) of angiopoietin1 (Ang1, A) and -2 (Ang2, B), their receptor Tie-2 (C), and of hypoxia inducible factor (HIF)-1α (D) in sham and SNX animals. After 8 wk of moderate stable renal failure, cardiac protein expression of major angiogenic factors other than VEGF is not different between sham and SNX animals. Quantification is given as positive nuclei per area myocardium (0.035 mm² for A, B, and D and μm² for C).
mRNA was not seen in the heart of 1C-2K rats with renovascular hypertension (data not shown).

In contrast to VEGF, the expression of other angiogenic factors like angioptiokin and -2 is not altered in the heart of SNX rats compared with controls. This emphasizes the diverse roles of the various angiogenic factors in the myocardium, with, i.e., FGF-2 being mostly involved in angiogenesis after ischemic injury like after myocardial infarction (3). Of note, protein expression of HIF-1α, the master regulator of oxygen sensing, was not altered in the hearts of rats with mild stable CRF of 8 wk duration. This may be due to methodological reasons and does not necessarily rule out a role of HIF-1α in hypoxia sensing and upregulation of VEGF under the conditions of CKD.

In patients with CKD, Di Marco et al. (19) recently found significantly higher plasma soluble Flt-1 levels together with anti-angiogenic serum activity compared with nonrenal control patients. Interestingly, higher soluble Flt-1 serum levels were also found in % nephrectomized rats compared with sham-operated animals (19). When Mallamaci et al. (31) investigated the relationship between VEGF, left ventricular function, and mortality in a prospective cohort study in 228 hemodialysis patients, they found that VEGF was inversely related with midwall fractional shortening (P = 0.002), and this predicted mortality (P = 0.02). Taken together, these two studies in patients with CRF may point to some VEGF resistance under the condition of renal failure.

Although it is known that, in CKD, sympathetic activity is increased, the exact mechanisms to explain this observation are as yet not fully understood. Partly, this may be interpreted by central effects of ANG II of baroreceptor activity (30). In the study by Hausberg et al. (25) in CKD patients, the results suggested that input from the diseased kidneys may increase central sympathetic outflow: even after renal transplantation, sympathetic activity in patients was only normalized if these patients had undergone prior nephrectomy. It was suggested that uremia-related toxins that accumulate in CKD and cannot be completely cleared by dialysis treatment may be responsible for afferent sympathetic nerve discharge in the kidneys (14).

On the other hand, several studies in animal models of CKD suggest that excitation of renal afferent nerves results in increased efferent sympathetic nerve discharge, with resulting increases in blood pressure. Blood pressure effects of increased sympathetic nerve activity have definitely to be taken into account but do not seem to fully explain the specific capillary deficit in CKD (27), (4, 7, 40) nor the significant decrease in sympathetic nerve activity have definitely to be taken into account but do not seem to fully explain the specific capillary deficit in CKD (27), (4, 7, 40) nor the significant decrease in cardiac VEGF gene expression observed in SNX rats. In the present study, blood pressure was measured by tail plethysmography, and we did not see significant blood pressure effects of increased sympathetic nerve discharge in the kidneys (14).

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It was observed that increased sympathetic nerve activity of central origin in an ablation model of CKD was prevented by dorsal rhizotomy, a procedure that selectively blocks subdiaphragmatic afferent nerves (15). Also, the development of
hypertension in the 5% nephrectomized rats could be prevented by dorsal rhizotomy (14). The same group showed that acute renal injury by intrarenal injection of phenol caused an immediate rise in blood pressure and in norepinephrine secretion from the posterior hypothalamus that could be prevented by renal denervation (42). Thus afferent impulses from the diseased kidney to central integrative structures in the brain may cause increased sympathetic nerve discharge and contribute to hypertension in CKD. We have to acknowledge, however, that we did not provide any direct measurement of myocardial sympathetic activity. These measurements are technically very difficult and are precluded by tissue fixation that was necessary for other analyses. By studying uptake and fractional release of the sympathetic transmitter [3H]norepinephrine from cortical slices, we were able to show, however, that selective renal denervation abolished renal sympathetic nerve activity in the kidney. It is therefore at least conceivable that also systemic sympathetic nerve activity (and presumably also cardiac sympathetic nerve activity) was lowered.

Our findings extend on the general concept that increased afferent renal nerve activity increases general central sympathetic outflow in that the increased sympathetic activity eventually triggered by intrarenal processes even induces pathological damage in the heart.

These findings in an experimental model of CKD are of potential clinical importance, since, in CKD patients, sympatholytic agents had shown beneficial effects on cardiovascular survival. In a recent study, Welten et al. (41) provided evidence for a potential benefit of β-blockers for the outcome of CKD patients after noncardiac vascular surgery. The authors followed 2,126 vascular surgery patients with and without kidney disease for a mean of 5.98 ± 3.68 yr; among those, 36% received β-blockers before surgery. As a result, the authors found that patients treated with β-blockers underwent less limb arterial revascularization procedure. Furthermore, the risk of all-cause mortality was reduced by perioperative β-blocker treatment in CKD patients. Thus these clinical data are consistent with a proangiogenic effect of β-blockade in CKD patients, although this aspect was not formally investigated in the study. In view of this and other clinical studies (17) documenting a beneficial effect of β-blockade in patients with CKD, the present study may shed some light on the underlying pathomechanisms and may thus lead to a better understanding of the pathogenesis and therapy of cardiovascular alterations in patients with CKD.

In summary, we conclude that decreased myocardial VEGF expression possibly due to overactivity of the sympathetic nervous system might be responsible for the deficit in capillary adaptation in response to myocardial hypertrophy in renal failure which is accompanied by negative and potentially lethal functional consequences.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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