Blunted transcriptional response to skeletal muscle ischemia in rats with chronic kidney disease: potential role for impaired ischemia-induced angiogenesis

Rafael U. Heiss,[1,2] Fabian B. Fahlbusch,[3] Johannes Jacobi,[1] Christoph Daniel,[4] Arif B. Ekici,[5] Nada Cordasic,[1] Kerstin Amann,[4] Andrea Hartner,[3]* and Karl F. Hilgers[1]*

[1]*Department of Nephrology and Hypertension, Friedrich-Alexander University, Erlangen-Nuremberg (FAU), Germany;* [2]*Department of Radiology, Friedrich-Alexander University, Erlangen-Nuremberg (FAU), Germany;* [3]*Department of Pediatrics and Adolescent Medicine, Friedrich-Alexander University, Erlangen-Nuremberg (FAU), Germany;* [4]*Institute for Nephropathology, Friedrich-Alexander University, Erlangen-Nuremberg (FAU), Germany; and* [5]*Institute of Human Genetics, Friedrich-Alexander University, Erlangen-Nuremberg (FAU), Germany*

Heiss RU, Fahlbusch FB, Jacobi J, Daniel C, Ekici AB, Cordasic N, Amann K, Hartner A, Hilgers KF. Blunted transcriptional response to skeletal muscle ischemia in rats with chronic kidney disease: potential role for impaired ischemia-induced angiogenesis. *Physiol Genomics* 49: 230–237, 2017. First published February 17, 2017; doi:10.1152/physiolgenomics.00124.2016.—Chronic kidney disease (CKD) is associated with increased cardiovascular morbidity and mortality. Previous studies indicated an impairment of ischemia-induced angiogenesis in skeletal muscle of rats with CKD. We performed a systematic comparison of early gene expression in response to ischemia in rats with or without CKD to identify potential molecular mechanisms underlying impaired angiogenesis in CKD. CKD was induced in male rats by 5/6 nephrectomy (SNX); control rats were sham operated (sham). Eight weeks later, ischemia of the right limb was induced by ligation and resection of the femoral artery. Rats were killed 24 h after the onset of ischemia, and RNA was extracted from the musculus soleus of the ischemic and the nonischemic hindlimb. To identify differentially expressed transcripts, we analyzed RNA with Affymetrix GeneChip Rat Genome 230 2.0 Arrays. RT-PCR analysis of selected genes was performed to validate observed changes. Hindlimb ischemia upregulated 239 genes in CKD and 299 genes in control rats (66% overlap), whereas only a few genes were downregulated (14 in CKD and 34 in controls) compared with the nonischemic limb of the same animals. Comparison between the ischemic limbs of CKD and controls revealed downregulation of 65 genes in CKD; 37 of these genes were also among the ischemia-induced genes in controls. Analysis of functional groups (other than angiogenesis) pointed to genes involved in leukocyte recruitment and fatty acid metabolism. Transcript expression profiling points to a relatively small number of differentially expressed genes that may underlie the impaired postischemic angiogenesis in CKD.

chronic kidney disease; angiogenesis; microarray; ischemia; skeletal muscle

PATIENTS WITH chronic kidney disease (CKD) are at a higher risk to develop cardiovascular disease compared with patients with normal kidney function. The relation between increased cardiovascular complications and CKD was described by Lindner et al. (21a) in a small cohort of hemodialysed patients. In patients with CKD cardiovascular diseases not only occur more frequently but also tend to be more progressive despite recent therapeutic advances. This also applies to the outcome of interventional therapy in acute coronary syndrome and peripheral occlusive disease (4, 7, 22, 25, 29). The outcome of peripheral vascular disease in CKD patients may be adversely affected by an impaired capability for compensatory capillary sprouting because of defective angiogenesis in these individuals (8).

Animal experiments with subtotally nephrectomized rats, which develop CKD, as well as autopsy studies of patients with CKD provide evidence of a decreased capillary density in the myocardium (1–3). Contradictory data exist for the capillary supply of skeletal muscle in CKD. In an animal study with subtotally nephrectomized rats capillary density of the psoas muscle was not significantly reduced in response to chronic renal insufficiency (2). On the other hand, Flisiński et al. (13) detected a reduction in the capillary vessels of the gastrocnemius muscle in a comparable experimental setup in a later study. This group also showed decreased mRNA levels of Hif1a and Vegfa, both key proteins of angiogenesis, in the gastrocnemius muscle of rats with CKD (12, 19). Angiogenesis is defined as capillary growth through sprouting and enlargement from preexisting vessels (27, 38). This process is primarily induced by hypoxia or low oxygen tension (27) and contributes to restoring the oxygen supply of tissues after ischemia. Jacobi et al. (19) were able to demonstrate that renal insufficiency impairs ischemia-induced adaptive angiogenesis and reperfusion in CKD rats with hindlimb ischemia, an observation confirmed by recent reports (18, 32). While the underlying mechanisms are yet poorly understood, an impaired transcriptional response to ischemia in skeletal muscle tissue could obviously contribute. The aim of the present study was to search for potential molecular mechanisms underlying the impaired angiogenesis in CKD by systematic comparison of early transcript expression in response to hindlimb ischemia in rats with or without CKD by using a DNA microarray approach.

* A. Hartner and K. F. Hilgers contributed equally to this work.

Address for reprint requests and other correspondence: K. F. Hilgers, Dept. of Nephrology and Hypertension, Univ. Hospital Erlangen, Ulmenweg 19, 91054 Erlangen, Germany (e-mail: Karl.Hilgers@uk-erlangen.de).

MATERIALS AND METHODS

Animals. All experiments were performed in male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) kept in a temperature-controlled animal facility on a 12 h light/dark cycle, with tap water and rodent chow ad libitum. The study protocol was approved by the animal research ethics committee of the local government authorities (Bezirksregierung Mittelfranken, AZ 54-2532.1-18/11).

Subtotal (5/6) nephrectomy. At 8 wk of age, animals underwent left subcapsular nephrectomy through a flank incision under 2.5% isoflurane anesthesia (Forene 100%; Abbott, Wiesbaden, Germany). One week later, the upper and lower thirds of the right kidney were resected and weighed to achieve subtotal (5/6) nephrectomy (SNX) ($n = 5$). Control animals were sham operated ($n = 4$). Renal mass reduction was performed by surgical resection, rather than ligation of arterial branches to avoid significant elevations in blood pressure (15).

Hindlimb ischemia. Eight weeks after renal mass reduction or sham surgery, all animals underwent unilateral (right limb) induction of hindlimb ischemia. Briefly, under aseptic conditions, the common femoral artery was ligated at the level of the inguinal ligament and excised down to the branching of the deep femoral artery, which was also ligated using 5-0 silk sutures (Ethicon, Somerville, NJ). A sham procedure (preparation of arteries without ligation) was performed on the contralateral leg. Postsurgery animals received subcutaneous buprenorphine (0.03 mg/kg twice daily) for pain relief. The animals were killed 24 h after surgery. Blood samples were taken, and soleus muscles of both legs were collected, weighed, and snap-frozen in liquid nitrogen for RNA isolation (19).

Metabolic cages. For urine collection, animals were housed in metabolic cages over 24 h once before nephrectomy and once before hindlimb ischemia. Body weight, food intake, water consumption, and urine output were monitored. Proteinuria was measured and expressed as urinary albumin excretion per gram creatinine.

RNA isolation. RNA was isolated from muscle tissue samples (200 mg) after homogenization with a disperser (T10 basic Ultra-Turrax; IKA, Staufen, Germany) using the RNeasy Fibrous Tissue Midi Kit (Qiagen, Hilden, Germany). In this procedure Proteinase K was used as well as DNase to remove genomic DNA. The entire process was carried out according to the manufacturer's instructions.

In all cases purity and quantity were assessed by Spectrophotometer Nano Drop 1000 (peqlab Biotechnologie, Erlangen, Germany) with relative absorbance at 260 vs. 280 nm. To avoid distortions caused by excessive protein levels, only RNA eluates were used at a ratio of RNA to protein content in the range of 1.6–2.0.

Microarray analysis. Isolated RNA samples were subjected to quality control using the Agilent 2100 Bioanalyzer equipped with an Agilent RNA 6000 Nano kit and related software (Agilent, Santa Clara, CA). RNA integrity number (RIN) values of ≥ 5 were deemed suitable for analysis (11). The RIN values of our samples ranged from 8.8 to 9.5.

Preparation of cDNA, cRNA, hybridization, and scanning of microarrays were performed by the Chip Facility Unit, Institute of Human Genetics, Friedrich-Alexander University, Erlangen-Nürnberg, according to established methods (16), following the manufacturer's instructions (Affymetrix, Santa Clara, CA). To identify differentially expressed transcripts in our analysis the GeneChip Rat Genome 230 2.0 was employed (Affymetrix). The data sets are accessible through the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database repository (GSE88925).

We used the step-up method to control the false discovery rate according to Benjamini and Hochberg (6) as implemented in the Partek Genomics Suite and filtered for corrected *P* values accordingly. The statistical data analysis of the recorded results was performed using the Partek Genomics Suite software package (Partek, St. Louis, MO) by calculating a two-way ANOVA for each gene and an F-test for group comparisons. The following groups were analyzed: ischemic vs. nonischemic hindlimb, within the SNX group as well as within the sham group and the ischemic hindlimbs of each group (SNX vs. Sham).

IPA functional pathway analysis. For further functional pathway analysis of biological and molecular networks underlying muscular ischemia under CKD we employed the web-based Ingenuity Pathways Analysis tool (IPA; Ingenuity Systems, Redwood City, CA), as described in detail earlier by us (5) and others (37). Using IPA we compared our filtered microarray data with the current Ingenuity Pathways Knowledge Base. Based on our list of significantly altered target genes, IPA computed sets for the comparative analysis of generic networks. No fold-change cut-off was applied to the normalized output data ($P < 0.05$). In addition to the *P* value, the IPA software additionally attributes a Z-score based on an internal algorithm. In each comparison between the groups all functions with a $P < 0.05$ and a Z-score of > 2 (increase) or < -2 (decrease) were rendered statistically significant. Comparative network analysis was carried out using the web-based software BioVenn by Hulsen et al. (17). For functional pathway analysis the confidence level was set to "experimentally observed" including direct and indirect relationships, with cut-off settings of 2.0-fold (up and down) and $P < 0.05$.

Real-time reverse transcription-polymerase chain reaction. The extracted RNA was utilized to synthesize first-strand cDNA by using TaqMan Reverse Transcription Reagent with random hexamers (Applied Biosystems, Darmstadt, Germany).

Polymerase chain reaction was performed with the thermocycler StepOnePlus Real-Time PCR System (Applied Biosystems) and SYBR green reagents (Applied Biosystems) according to the manufacturer's instructions. All samples were run in duplicate. The relative amount of the specific mRNA of interest was normalized to 18S rRNA, which proved to be most suitable after screening of the literature and after testing of other potential housekeepers (beta actin, GAPDH) (35). Dissociation curves were performed to confirm the specificity of the polymerase chain reaction. Primer sequences are depicted in Supplement 1. (The online version of this article contains supplemental material.) The analysis of the obtained data was performed with the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis. Statistical group comparison of animal data and of the real-time PCR results was carried out using PASW Statistics 18 software (SPSS, Chicago, IL) employing one-way analysis of variance (ANOVA) followed by LSD (least significant difference) post hoc multiple comparison testing. A *P* value of <0.05 was considered statistically significant.

RESULTS

Animal characteristics and physiological parameters. Eight weeks after induction of SNX, ischemia was induced and animals were killed 24 h later. At this time, no differences were observed between SNX and control rats regarding body weight (Table 1). The specific total kidney weight [body weight at sacrifice/whole kidney weight at sacrifice)*100] was significantly reduced in SNX rats (Table 1). In SNX animals albuminuria was detected with a significantly elevated albumin-to-creatinine ratio (Table 1). Serum creatinine and serum urea were markedly elevated in SNX rats (Table 1).

Table 1. *Biometric and biochemical parameters*

	Sham	SNX	*t*-Test
Body weights of rats at death, g	510.9 ± 33.0	486.4 ± 36.9	n.s.
Specific kidney weight at death, %	0.57 ± 0.05	0.40 ± 0.10	$P < 0.05$
Albuminuria 8 wk after SNX, alb/crea ratio	0.07 ± 0.05	11.05 ± 5.25	$P < 0.01$
Serum creatinine at death, mg/dl	0.27 ± 0.04	0.64 ± 0.31	$P < 0.05$
Serum urea at death, mg/dl	35.3 ± 4.3	99.9 ± 41.7	$P < 0.05$

SNX, 5/6 nephrectomy; n.s., not significant.

DNA microarray analysis (all genes). Analysis of transcripts points to an ischemia induced upregulation of 239 genes in CKD and 299 genes in control rats (66% overlap), whereas only a few genes were downregulated (14 in CKD and 34 in control rats with an overlap of 36%) compared with the respective nonischemic side. Comparison between the ischemic sides of CKD and control animals revealed a downregulation of 65 genes in CKD without any intersection of downregulated genes of the comparison of the ischemic and nonischemic hindlimb in CKD or within the sham group. In contrast, 37 (Table 2, Fig. 1*A*) of these 65 in CKD downregulated genes were also among the ischemia-induced genes in controls. In the same comparison 11 genes were slightly upregulated (Supplement 2). Not one of these 11 genes could be arranged to the intersection of induced genes of the comparisons of ischemic and nonischemic hindlimb in the sham or within the 5/6 SNX group. (Fig. 1)

Functional analysis (all genes). Ischemia upregulated 132 biological functions in CKD and 138 functions in control rats (62% overlap), whereas only few functions were downregulated (4 in CKD and 16 in control rats) compared with the respective nonischemic side. Comparison between the ischemic sides of CKD and control animals revealed downregulation of 14 biological functions in CKD; 10 of these were also among the ischemia-induced functions in controls (Table 3).

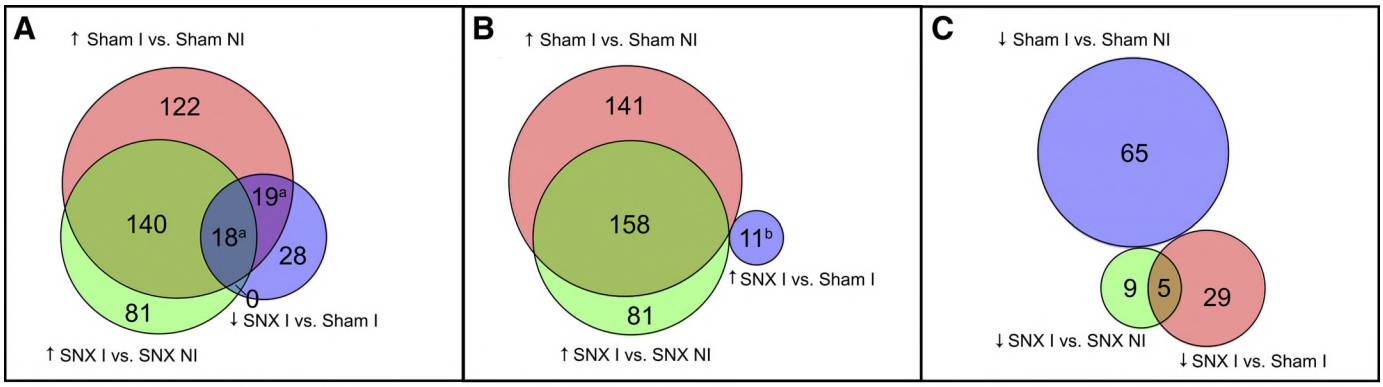
Differentially regulated genes involved in angiogenesis, leukocyte recruitment, and fatty acid metabolism. Real-time PCR was used to confirm the results of the DNA-microarray (Supplement 3) and to investigate additional genes involved in angiogenesis, leukocyte recruitment, and fatty acid metabolism.

Comparison of transcripts between ischemic and nonischemic hindlimbs within the sham group indicated a significant induction of genes involved in angiogenesis, leukocyte recruitment and fatty acid metabolism, e.g., Vegfa, Il6, Timp1, and the transcription factor E2f8 (Fig. 2), but also of Ccl2, Hsp70 and Mt1a. All of these genes within the sham group were also significantly upregulated in comparison with the ischemic hindlimb of the SNX group. Similar to the findings of the microarray, comparison between nonischemic sides showed a

Table 2. *Intersection of all upregulated genes of Sham I vs. Sham NI (FC > 2, P < 0.05) and all downregulated genes of SNX I vs. Sham I (FC < −2, P < 0.05) ordered by the FC of Sham I vs. Sham NI*

			24 h Sham I vs. Sham NI		24 h SNX I vs. Sham I	
Symbol	Gene Name	Probe Set ID	*P* Value	FC	*P* Value	FC
Ccl2*	chemokine (C-C motif) ligand 2*	1367973_at	0.000	53.130	0.032	−3.280
Mt1a /// Ttr	metallothionein 1a /// transthyretin	1371237_a_at	0.000	40.580	0.037	−4.610
Egr1	early growth response 1	1368321_at	0.000	17.600	0.027	−2.950
Junb*	jun B proto-oncogene*	1387788_at	0.000	11.110	0.009	−2.170
Serpine1*	serpin peptidase inhibitor. clade E (nexin. plasminogen activator inhibitor type 1)*	1368519_at	0.000	10.440	0.029	−3.290
Fosb	FBJ osteosarcoma oncogene B	1373759_at	0.005	8.720	0.022	−5.010
Csrnp1	cysteine-serine-rich nuclear protein 1	1389402_at	0.001	8.570	0.002	−6.940
Tnfaip6	tumor necrosis factor alpha induced protein 6	1371194_at	0.000	6.750	0.001	−3.890
Maff	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	1380229_at	0.001	6.660	0.009	−3.620
Myc*	myelocytomatosis oncogene*	1368308_at	0.001	6.390	0.015	−3.180
Egr2	early growth response 2	1387306_a_at	0.003	6.260	0.007	−4.510
E2f8	E2F transcription factor 8	1377410_at	0.004	5.590	0.010	−4.040
Serpina3n	serine (or cysteine) peptidase inhibitor clade A member 3N	1368224_at	0.000	5.260	0.000	−6.620
Fgl2	fibrinogen-like 2	1370623_at	0.001	4.300	0.007	−2.210
Adamts4	ADAM metallopeptidase with thrombospondin type 1 motif 4	1393730_at	0.000	4.130	0.001	−3.070
Inhbb	inhibin beta-B	1377163_at	0.000	4.050	0.000	−3.840
Efna1*	ephrin A1*	1398273_at	0.000	3.920	0.004	−2.050
Gadd45b	growth arrest and DNA-damage-inducible beta	1372016_at	0.001	3.890	0.015	−2.250
Fnip2	folliculin interacting protein 2	1391315_at	0.007	3.870	0.034	−2.600
Srxn1	sulfiredoxin 1	1372510_at	0.014	3.730	0.030	−2.940
Adm*	adrenomedullin*	1387219_at	0.004	3.570	0.041	−2.200
Stc1	stanniocalcin 1	1377404_at	0.006	3.360	0.023	−2.490
Eno2	enolase 2. gamma. neuronal	1370341_at	0.002	3.300	0.013	−2.280
Il6*	interleukin 6*	1369191_at	0.028	2.880	0.025	−2.790
Depdc7	DEP domain containing 7	1384971_at	0.003	2.830	0.010	−2.290
Pgf	placental growth factor	1368919_at	0.011	2.760	0.004	−3.030
Ier5	immediate early response 5	1389355_at	0.049	2.750	0.013	−3.530
Pmepa1	prostate transmembrane protein androgen induced 1	1389809_at	0.017	2.610	0.021	−2.380
Btg2	BTG family member 2	1386995_at	0.002	2.560	0.005	−2.250
Kcne4	Potassium voltage-gated channel. Isk-related family member 4	1390969_at	0.020	2.560	0.042	−2.460
Cxcl9*	chemokine (C-X-C motif) ligand 9*	1373544_at	0.047	2.550	0.019	−2.950
Irak3	interleukin-1 receptor-associated kinase 3	1380336_at	0.005	2.530	0.011	−2.170
Slc1a1	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter system	m1370367_at	0.002	2.440	0.004	−2.080
Tes	testis derived transcript	1383401_at	0.047	2.420	0.042	−2.360
Gch1	GTP cyclohydrolase 1	1387221_at	0.020	2.400	0.011	−2.530
Hsd11b1	hydroxysteroid 11-beta dehydrogenase 1	1386953_at	0.008	2.150	0.004	−2.260
Man1a1	mannosidase. alpha. class 1A member 1	1383574_at	0.011	2.020	0.004	−2.170

I, ischemic; NI, nonischemic; FC, fold change. Also see Fig. 1*A*. *Labeled genes are detected by the IPA software as angiogenesis related.



[a] for a complete list of genes see Table 2
[b] for a complete list of genes see Supplement 2

Fig. 1. Venn diagrams based on all genes of the DNA microarray. I, ischemic; NI, nonischemic; SNX, 5/6 nephrectomy. ↑, upregulated [fold change (FC) > 2, $P < 0.05$]; ↓, downregulated (FC > 2, respectively, FC < −2, $P < 0.05$).

trend toward reduction of gene expression on the nonischemic side of the SNX group as well (Table 4).

DNA microarray analysis of angiogenesis-related genes. A considerable portion of angiogenesis-related genes were among the most evident differentially regulated transcripts in our model. Therefore, a Venn analysis was performed for angiogenesis-related genes only. Ischemia upregulated 35 angiogenesis-related genes in CKD (Supplement 4) and 38 genes in control rats (Supplement 5) (74% overlap), whereas only one single gene in the CKD group was downregulated (Cxcl12, FC = 2.03, $P = 0.009$, Supplement 4) compared with the respective nonischemic side. Comparison between the ischemic sides of CKD and control animals revealed a downregulation of 13 angiogenesis-related genes in CKD (Supplement 6) without any intersection of downregulated genes of the comparison of the ischemic and nonischemic hindlimb in CKD or within the sham group. In contrast 8 (Table 2, gray-marked genes) of these 13 in CKD downregulated genes were also among the ischemia-induced genes in controls. In the same comparison not one angiogenesis-related gene was upregulated (Fig. 3). These 13 (Supplement 6) downregulated angiogenesis-related genes showed strong connection in the network analysis. Among the eight known angiogenesis-related genes that were induced by ischemia but reduced in CKD were interleukin-6, adrenomedullin, c-myc, and the chemokines Ccl2 and Cxcl9 (Table 2, *-labeled genes). Thus, differential expression of transcripts caused by ischemia primarily points to increased gene expression on the ischemic side compared with the nonischemic side in both groups, which overlapped by the majority of upregulated genes. This applies to the analysis of all transcripts as well as for angiogenesis-related transcripts only. However, none of these transcripts were upregulated in the ischemic hindlimb of 5/6 SNX in the comparison of the ischemic sides of SNX and controls, but they were significantly downregulated in general.

Network analysis. From our network analysis, Il6 in particular appeared as a central gene (Fig. 4). In conjunction with Vegfa, which was found to be regulated in a similar way and is a central gene in the regulation of angiogenesis, a network of angiogenesis-related genes that are differentially regulated in ischemic control tissue vs. ischemic SNX tissue can be established (Fig. 4).

Table 3. *All biological functions of the intersection Sham I vs. Sham NI (Z-score > 2, $P < 0.05$) and SNX I vs. Sham I (Z-score < −2, $P < 0.05$)*

Function Annotation	*P* Value	Z-score	Predicted Activation State	Molecules
Size of body	0.001	−3.36	decreased	15
Fatty acid metabolism	0.001	−2.71	decreased	11
Cell viability	0.001	−2.71	decreased	18
Vasculogenesis	0.000	−2.70	decreased	16
Development of cardiovascular system	0.000	−2.64	decreased	17
Synthesis of fatty acid	0.000	−2.48	decreased	9
Angiogenesis	0.000	−2.35	decreased	15
Recruitment of leukocytes	0.002	−2.27	decreased	7
Vascularization	0.000	−2.21	decreased	9
Synthesis of lipid	0.000	−2.10	decreased	17

Molecules, number of regulated molecules per function.

DISCUSSION

CKD patients suffer from an increased risk for cardiovascular morbidity and mortality (14, 31). This seems at least in part be due to a reduced angiogenetic potential, which limits reperfusion of ischemic tissue (23, 34). Previous studies confirmed that angiogenesis after limb ischemia is severely reduced in the presence of impaired kidney function (18, 19, 32). The underlying molecular mechanisms, however, are poorly understood. The findings of our study suggest that this impairment of angiogenesis is accompanied by a reduced expression of a limited set of genes in skeletal muscle tissue in response to ischemia. Most of these genes play a role in angiogenesis by regulating vascular development, inflammation, or metabolism. In a former study Jacobi et al. (19) detected an impairment of ischemia-induced angiogenesis in skeletal muscle tissue of rats with CKD. These results were confirmed by Schellinger et al. (32) and by Hung et al. (18) in current studies. Both groups observed a higher capillary density in the ischemic hindlimb of sham animals 2 wk/4 wk after induction of hindlimb ischemia compared with the ischemic hindlimb of SNX animals (18, 32).

In our study we used the experimental setting of Jacobi et al. (19) and Schellinger et al. (32) to further investigate potential molecular mechanisms underlying this phenomenon by a sys-

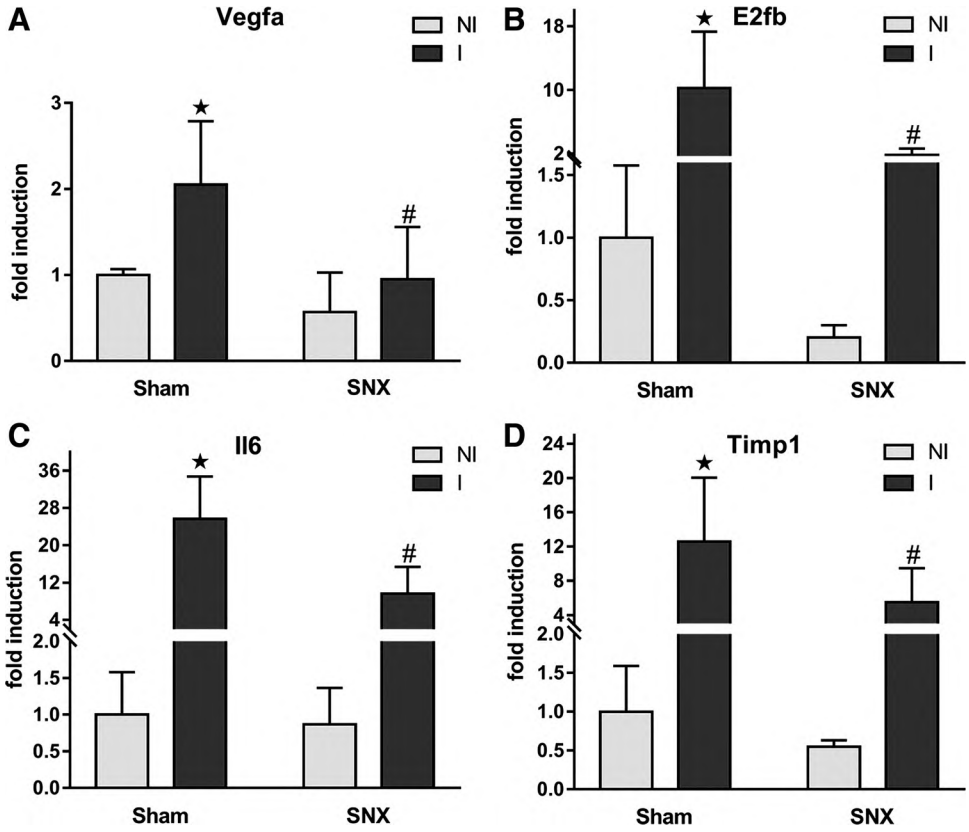


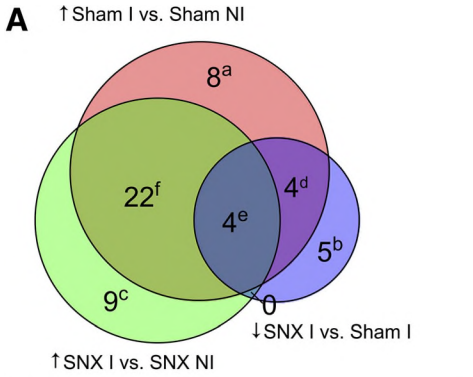
Fig. 2. Expression analysis of Vegfa (*A*), E2fb (*B*), Il6 (*C*), and Timp1 (*D*). Means ± standard deviation. I, ischemic; NI, nonischemic. *$P < 0.05$ for Sham I vs. Sham NI; #$P < 0.05$ for SNX I vs. Sham I.

tematic comparison of gene expression in response to ischemia in rats with or without CKD. A DNA microarray analysis was performed 24 h after induction of ischemia. This time point was chosen based on previous work of Lee et al. (21) and Paoni et al. (28), who examined ischemia-related expressional changes in the hindlimb model of mice without the additional influence of CKD. Using a microarray technique, both found that ischemia triggered a significant increase in gene expres-

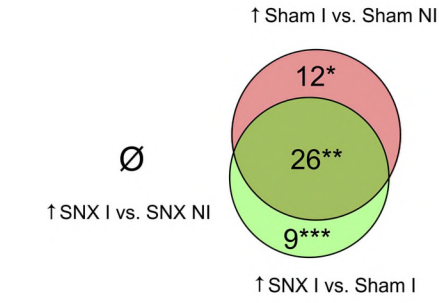
Table 4. *Results of real-time PCR from soleus muscle*

	24 h				24 h		
	Sham		SNX		*P* Value		
Gene Symbol	Nonischemic	Ischemic	Nonischemic	Ischemic	Sham I/NI	SNX I/NI	Sham I/SNX I
Vegfa	1.00 ± 0.07	2.05 ± 0.73	0.57 ± 0.46	0.95 ± 0.61	$P = 0.024$	$P = 0.283$	$P = 0.011$
Flt1	1.00 ± 0.25	1.50 ± 0.69	0.78 ± 0.61	1.02 ± 0.64	$P = 0.298$	$P = 0.545$	$P = 0.272$
Kdr	1.00 ± 0.19	2.04 ± 0.37	0.67 ± 0.53	1.31 ± 0.77	$P = 0.027$	$P = 0.086$	$P = 0.083$
Angpt1	1.00 ± 0.07	1.04 ± 0.33	1.05 ± 0.24	1.05 ± 0.36	$P = 0.886$	$P = 0.978$	$P = 0.955$
Angpt2	1.00 ± 0.25	2.81 ± 0.82	0.73 ± 0.28	2.12 ± 0.92	$P = 0.003$	$P = 0.005$	$P = 0.165$
Ccl2	1.00 ± 0.20	68.80 ± 67.90	0.37 ± 0.26	11.31 ± 11.34	$P = 0.014$	$P = 0.592$	$P = 0.021$
Ccl7	1.00 ± 0.42	28.36 ± 19.95	0.50 ± 0.26	9.40 ± 5.38	$P = 0.016$	$P = 0.417$	$P = 0.070$
Hsp70	1.00 ± 0.56	3.93 ± 1.20	0.39 ± 0.33	1.65 ± 1.37	$P = 0.002$	$P = 0.063$	$P = 0.005$
Il6	1.00 ± 0.58	25.64 ± 9.11	0.87 ± 0.50	9.61 ± 5.76	$P = 0.000$	$P = 0.018$	$P = 0.001$
Mt1a \\\ Ttr	1.00 ± 0.43	15.39 ± 10.16	0.54 ± 0.14	4.01 ± 2.21	$P = 0.003$	$P = 0.299$	$P = 0.009$
Timp1	1.00 ± 0.59	12.59 ± 7.46	0.55 ± 0.08	5.52 ± 3.96	$P = 0.002$	$P = 0.087$	$P = 0.033$
E2f8	1.00 ± 0.58	10.24 ± 7.08	0.20 ± 0.10	1.84 ± 0.80	$P = 0.004$	$P = 0.511$	$P = 0.006$
Pgf	1.00 ± 0.35	1.67 ± 0.56	0.90 ± 0.62	0.98 ± 0.53	$P = 0.138$	$P = 0.812$	$P = 0.099$
Tgfb1	1.00 ± 0.63	3.56 ± 2.65	0.93 ± 0.61	2.83 ± 3.47	$P = 0.175$	$P = 0.215$	$P = 0.661$
Thbs1	1.00 ± 0.49	6.29 ± 4.09	0.66 ± 0.26	6.29 ± 4.66	$P = 0.053$	$P = 0.120$	$P = 0.344$
Thbs2	1.00 ± 0.44	1.67 ± 0.84	0.52 ± 0.18	1.1 ± 0.37	$P = 0.093$	$P = 0.080$	$P = 0.118$
Spp1	1.00 ± 0.77	2.46 ± 0.83	0.24 ± 0.23	2.08 ± 1.08	$P = 0.046$	$P = 0.004$	$P = 0.526$
Gstm2	1.00 ± 0.23	1.51 ± 1.35	2.31 ± 1.49	1.89 ± 1.12	$P = 0.598$	$P = 0.593$	$P = 0.660$
Prkag3	1.00 ± 0.68	1.11 ± 1.04	1.44 ± 0.51	1.26 ± 0.54	$P = 0.855$	$P = 0.720$	$P = 0.783$
Csrnp1	1.00 ± 0.53	8.54 ± 5.78	0.45 ± 0.18	1.43 ± 0.64	$P = 0.003$	$P = 0.599$	$P = 0.003$
Gprc5a	1.00 ± 0.43	10.13 ± 0.88	0.46 ± 0.10	6.07 ± 5.12	$P = 0.001$	$P = 0.013$	$P = 0.072$
Hoxd10	1.00 ± 0.68	1.03 ± 0.62	1.27 ± 1.07	1.91 ± 1.68	$P = 0.974$	$P = 0.402$	$P = 0.307$

Means ± SD. I, ischemic; NI, nonischemic.



a	Itgb2, S1pr3, Ptafr, Nab2, Cxcr4, Tnfrsf1a, Ptgs2, Fgr
b	Gem, Ctgf, Nr4a1, Cxcl14, Ptgs1
c	Fn1, Alcam, Tnc, Ancxa2, Col4a1, Sema3b, Vim, Hspb1, Rgcc
d	Efna1, Adm, Il6, Cxcl9
e	Ccl2, Junb, Serpine1, Myc
f	Ccl7, Timp1, Spp1, Hmox1, Runx1, Col8a1, Tnfrsf12a, Lgals3, Cd44, Apln, Klf5, F2r, Igfbp3, Stat3, Gadd45a, Angpt2, Ednrb, Icam1, Ehd4, Ctsb, G6pd, Ccnd1



*	Itgb2, Efna1, Adm, S1pr3m, Il6, Ptafr, Cxcl9, Nab2, Cxcr4, Tnfrsf1a, Ptgs2, Fgr
**	Ccl2, Ccl7, Timp1, Spp1, Junb, Serpine1, Hmox1, Myc, Runx1, Col8a1, Tnfrsf12a, Lgals3, Cd44, Apln, Klf5, F2r, Igfbp3, Stat3, Gadd45a, Angpt2, Ednrb, Icam1, Ehd4, Ctsb, G6pd, Ccnd1
***	Fn1, Alcam, Tnc, Anxa2, Col4a1, Sema3b, Vim, Hspb1, Rgcc

Fig. 3. Venn diagrams based on angiogenesis-related genes of the DNA microarray. I, ischemic; NI, nonischemic; ↑, upregulated; ↓, downregulated (FC > 2, respectively, FC < −2, $P < 0.05$).

sion within the first 24 h, especially of angiogenesis-related genes.

Underlining the efficacy of the ischemic stimulus in our model, the comparison of transcript expression profiles between the ischemic and nonischemic hindlimb of our 24 h sham control group showed similar results as found by Lee et al. (21) and Paoni et al. (28). In line with our data, both studies detected an increase of general gene expression to a comparable extent. In CKD animals, ischemia also induced a prominent increase of gene expression compared with the rats' nonischemic hindlimbs. We found that many of these differentially regulated transcripts in CKD animals were identical to the ones found in sham animals. However, the absolute number of upregulated transcripts was reduced when compared with the sham group. None of these ischemia-induced transcripts showed a higher expression in the ischemic muscle of CKD animals in comparison with the ischemic side of the sham group. This relation also applied to angiogenesis-related genes. Thus, chronic renal insufficiency not only restricts the absolute number of regulated transcripts but also causes a quantitatively reduced expression of ischemia-induced transcripts. This is in accordance with data from Flisiński et al. (12), who described reduced gene expression of Hif1a, Vegfa, and Vegf receptors on the nonischemic gastrocnemius muscle of 24 h 5/6 SNX rats compared with muscle of sham controls. Among the known angiogenesis-related genes that were induced by ischemia but reduced in CKD were Vegfa, c-Myc, E2f8, Timp1, Adm, Il6, and the chemokines Ccl2, and Cxcl9. These downregulated

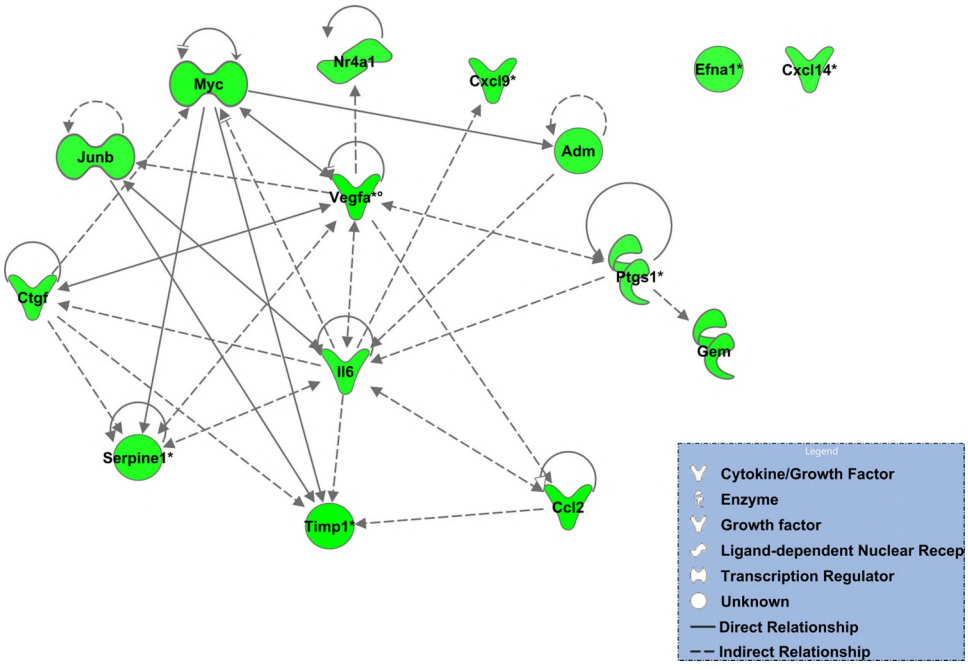


Fig. 4. Network analysis of all angiogenesis-related genes of 24 h SNX I vs. sham I. I, ischemic; NI, nonischemic. *Multiple identifiers: represented on the microarray several times; ° taken from PCR analysis (FC > 2, respectively, FC < −2, $P < 0.05$).

angiogenesis-related genes showed network connectivity with Vegfa and especially Il6 as central nexus genes. Il6 was found to be an angiogenic factor in tumors (24) and was recently described as a biomarker of cardiovascular disease in advanced CKD (33). The transcription factors c-myc and E2f8 are known to regulate angiogenetic processes at least in part via activation of Vegfa (26, 36). Timp1 has cytokine-like properties and can influence angiogenesis in tumors (30). Similarly, Adm contributes to tumor angiogenesis and is therefore discussed as a therapeutic target in angiogenesis (9). Chemokines not only recruit leukocytes to sites of inflammation but also play an important role in neovascularization (20). Therefore, it is conceivable that a reduced induction of these genes after ischemia in CKD will translate into a reduced capacity to form new blood vessels to ameliorate ischemia in skeletal muscle tissue. To some extent, some of these findings might possibly be applied to the known decreased capillary density and restricted angiogenesis in the myocardium in the context of CKD (1–3).

In the analysis of biological functions a large overlap of ischemia-induced functions in comparison to the corresponding nonischemic hindlimb within the sham and SNX group appeared. The categories inflammatory response, cellular growth and proliferation, and hematological system development and function belong to the functional categories with the most regulated transcripts. Comparison between the ischemic sides of CKD and control animals revealed a downregulation of 10 biological functions in CKD, in which ischemia-induced genes are involved. These genes are induced in muscle tissue of our sham group only. Our in silico analysis indicates that these biological functions are primarily related to vascular system development and lipid metabolism and could subsequently be limited in their ischemia-induced activity by chronic renal failure. This finding is in line with Jacobi et al. (19), showing impairment of ischemia-induced angiogenesis in renal failure, with reduced activity of several biological functions of vascular system development within the 24 h after ischemia-induction in 5/6 SNX animals.

Interestingly, Fang et al. (10) recently found a dependency of functional lipid metabolism on an intact regulation of angiogenesis. Inefficient Apoa1 binding protein-mediated cholesterol efflux from cells interferes with Vegf signaling and with neovascularization. Thus, the differential expression of genes involved in lipid metabolism in muscles of ischemic sham rats compared with ischemic SNX might be involved in an altered activity of genes responsible for angiogenesis.

Our data are limited to the early transcriptional response of skeletal muscle tissue to ischemia; we did not investigate whether transcriptional effects translated to corresponding changes of the abundance of the respective proteins. Nor did we perform targeted interventions to test the functional role of some transcripts. However, we do know that the reduced expression of several genes of the angiogenetic pathway is at least associated with a subsequent impairment process of restoring perfusion to the ischemic hindlimb in 5/6 SNX rats to the control group after 2 wk of ischemia (19, 32).

In summary, CKD significantly reduced ischemia-induced expression of transcripts in the hindlimbs of our ischemia model in rats. Besides the reduction of the absolute number of regulated genes, impaired renal function affected the expression of ischemia-induced transcripts quantitatively. As a consequence, the physiologically early induction of angiogenesis-related genes in the presence of ischemia is restricted. This may be the reason for the known impaired angiogenesis in the skeletal muscle of CKD animals. Thus, we conclude that the reduced capacity of CKD patients to compensate for ischemia and/or hypoxia may originate from an impaired angiogenic transcriptional response, resulting in a reduced capillary sprouting in ischemic tissue. Candidate genes that contribute to these alterations include factors regulating cardiovascular development, inflammation, and metabolism. All these genes might possibly have the potential to influence angiogenesis in CKD. Therefore, a therapeutic intervention to stimulate the transcriptional response to ischemia in CKD may hold promise to improve angiogenesis.

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The data sets generated and analyzed during the current study are accessible through the NCBI GEO database repository (GSE88925). All other data generated or analyzed during this study are included in this published article.

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DISCLOSURES

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

R.U.H. and N.C. performed experiments; R.U.H., F.F., A.B.E., and K.A. analyzed data; R.U.H., F.F., C.D., A.H., and K.F.H. interpreted results of experiments; R.U.H. drafted manuscript; R.U.H., K.A., A.H., and K.F.H. edited and revised manuscript; R.U.H., F.F., J.J., C.D., A.B.E., N.C., K.A., A.H., and K.F.H. approved final version of manuscript; F.F. prepared figures; J.J., C.D., and K.F.H. conceived and designed research.

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