The Range of Adaptation by Collateral Vessels After Femoral Artery Occlusion


Abstract—Natural adaptation to femoral artery occlusion in animals by collateral artery growth restores only ≈35% of adenosine-recruitable maximal conductance (C_{max}) probably because initially elevated fluid shear stress (FSS) quickly normalizes. We tested the hypothesis whether this deficit can be mended by artificially increasing FSS or whether anatomical restraints prevent complete restitution. We chronically increased FSS by draining the collateral flow directly into the venous system by a side-to-side anastomosis between the distal stump of the occluded femoral artery and the accompanying vein. After reclosure of the shunt collateral flow was measured at maximal vasodilatation. C_{max} reached 100% already at day 7 and had, after 4 weeks, surpassed (2-fold) the C_{max} of the normal vasculature before occlusion. Expression profiling showed upregulation of members of the Rho-pathway (RhoA, coflin, focal adhesion kinase, vimentin) and the Rho-antagonist Fasudil markedly inhibited arteriogenesis. The activities of Ras and ERK-1,-2 were markedly increased in collateral vessels of the shunt experiment, and infusions of L-NAME and L-NNA strongly inhibited MAPK activity as well as shunt-induced arteriogenesis. Infusions of the peroxinitrite donor Sin-1 inhibited arteriogenesis. The radical scavengers urate, ebselen, SOD, and catalase had no effect. We conclude that increased FSS can overcome the anatomical restrictions of collateral arteries and is potentially able to completely restore maximal collateral conductance. Increased FSS activates the Ras-ERK-, the Rho-, and the NO- (but not the Akt-) pathway enabling collateral artery growth. (Circ Res. 2006;99:656-662.)

Key Words: arteriogenesis ■ fluid shear stress ■ shunt ■ growth factors ■ microarrays

The restoration of maximal conductance (C_{max}) in animals after arterial occlusion remains defective (35% in the canine coronary circulation\(^1\) and 40% in the rabbit hind limb\(^2\)) in spite of the fact that normal resting blood flow is reached early. As a consequence exercise testing in experimental animals reveals defects similar to those in human patients.\(^3\) It was not known until now whether collateral vessels are potentially able to restore the full dilatory reserve of a normal vascular bed. Many observations would predict that this is not the case: the multitude of small vessels that replace an occluded artery is inefficient according to Poiseuille’s Law, and the tortuosity of collateral vessels offers finite resistance because of curvature flow and increased collateral length.\(^4\) One reason for the defective adaptation may lie in the fact that fluid shear stress normalizes prematurely: FSS falls by the third power of the growing radius. We tested the hypothesis whether a sustained increase of FSS is able to prolong the growth process and to restore normal maximal conductance. The method to achieve this was the creation of a shunt between the distal stump of the occluded femoral artery and the accompanying vein.\(^5\)

The novelty of the present findings is the demonstration that C_{max} can be reached and even surpassed by a drastic increase of fluid shear stress. Signaling pathways involved in the adaptation to fluid shear stress were interrogated, and it could be shown that three pathways have to converge to induce arterial growth, ie, the Ras-ERK pathway, the Rho-, and the NO-pathway.

Materials and Methods

An expanded Materials and Methods section can be found in the supplement available at http://circres.ahajournals.org.

We subjected rabbits to femoral artery occlusion and studied the effects of an arteriovenous shunt on the development of the collateral circulation (Figure 1). We measured collateral blood flow by ultrasonic flow probes and by MRI. The morphology of the vessels was studied in vivo by CT-scanning and post mortem by arteriography following pressure-controlled contrast medium injection. Col-
lateral artery tissue was prepared for RNA extraction, which was fashioned for microarray studies and for Western blot analysis. Animals were treated with a variety of tool drugs to interrogate the NO, Rho-, and Ras pathways. Smooth muscle cells in culture were treated with several agents used in the pharmacological in vivo studies.

Results

No animals were lost because of the surgical procedure or postoperatively. Clinical signs of peripheral ischemia were transiently present in a few animals but did not lead to ulceration or amputation. In two thirds of the treated animals, the A-V shunt was still open at termination of the experiment. Only animals with a functional shunt were included in the study.

Hemodynamics

Immediately after occlusion of the femoral artery blood pressure in the peripheral stump of the occluded artery fell to low values of 31±4.2% of the aortic pressure. Peripheral pressure rose to 50±8% after 7 days and remained at that level for several weeks. In contrast, in animals with femoral artery ligature and an additional A-V shunt the peripheral arterial pressure dropped to 6±1% of the arterial pressure (immediately after the operation), increased within 7 days to 17±3%, and reached 29±3% after 4 weeks. When the shunt was occluded—routinely done during the terminal experiment—7 days after femoral artery occlusion, peripheral pressure increased to 60±3% of the aortic pressure and to 73±1% when measured 4 weeks after femoral artery occlusion.

Collateral blood flow ratio (shunt versus control side) measured by MRI was 5.8±0.8 at day 7 and 13.7±3.7 at day 28. One week after shunt treatment calculated $C_{max}$ values under optimal vasodilatation were 88±4% of $C_{max}$ of nonoccluded legs, representing a 2-fold increase in comparison to $C_{max}$ of control ligated legs (44±7%). After 4 weeks, shunt treated legs showed a $C_{max}$ of 199±19% of nonligated legs. In contrast, legs with control ligatures did not show any further improvement after 4 weeks when compared with the results after 1 week (Figure 2A).

Effects of Pharmacological Inhibitors

Fasudil, a Rho-pathway inhibitor, and L-NAME and L-NNA, both inhibitors of endothelial NO synthetase (eNOS), almost completely blocked the positive effects of high shear stress on collateral artery growth (shunt 306±15 versus Fasudil 176±4 versus L-NAME 163±8 versus control 155±27 mL/min/100 mm Hg) (Figure 2B).

Moreover, the hypothesis that shear stress leads to an uncoupling of eNOS resulting in increased production of oxygen- and nitrogen-based radicals was investigated. To demonstrate such an effect, 3-Morpholinosydnonimine (Sin-1), which acts as a NO- and superoxide anion radical donor (both react to produce peroxynitrite), was infused but arteriogenesis was inhibited in a dose-dependent manner. In contrast, infusion of the scavengers Ebselen, urate, catalase, and superoxide dismutase showed no effect (data not shown).

Angiography and Computerized Tomography

Postmortem angiograms (Figure 3A and 3B) showed marked increases in the number of enlarged collaterals on the shunt side (day 7: 42±2.5 versus 18±0.95 [control]; day 28: 48±2.3 versus 12±2.0 [control]), which corresponded with the number and size of collaterals detected in vivo by CT (Figure 4A and 4B). Treatment with Fasudil or L-NAME resulted in a significant decrease in collateral count (fasudil 31±0.9 versus L-NAME 23.8±1.2 versus shunt 42±2.5). In contrast to the natural course of collateral growth, which leads to the elimination of most vessels that had initially participated in the growth transformation in favor of a few large ones ("pruning"), no pruning had occurred on the shunt side without further treatment: all preexistent arteriolar connections had enlarged and stayed that way.

High Shear Stress Activates the Ras/ERK Pathway and Increases H-Ras Protein Levels

Using antibodies that react specifically with dual (Thr202/Tyr204) phosphorylated extracellular signal-regulated kinases (ERK), an activation of ERK-1 and ERK-2 in fluid shear stress induced collaterals was detected in Western blot analysis. Quantitative analysis showed that levels of phosphorylated (activated) ERK-1 and ERK-2 were increased in both cytosolic (2.5-fold) and particulate fractions (1.5-fold) obtained from collateral arteries of the shunted side when compared with collaterals of the control side. This shear stress–induced ERK activation was abrogated by L-NAME. Total ERK-1 and ERK-2 protein levels showed no difference between control and shunted side collaterals with or without L-NAME treatment (Figure 5).

We next sought to analyze expression levels of H-Ras in collaterals from the shunted and control side. H-ras is a member of GTP-binding proteins of small molecular weight, known to act as biological switches for various cellular processes. As shown in Figure 6A, Western blot analyses showed a 2.3-fold increase in expression of H-Ras on the shunted side. This effect was reduced by 50% if shunt collaterals were additionally treated with L-NAME (Figure 6C).
Fluid Shear Stress and L-NAME Treatment Did Not Influence the Activation of SEK/JNK and Akt Kinase Pathways

Western blot analysis revealed that neither shear stress nor inhibition of NO synthesis by L-NAME influence the activation of SEK/MKK4, an upstream activator of JNK, and Akt kinase (Figure 6D). No differences in P-SEK1/MKK4 levels were observed when comparing collaterals of control and shunted side. Furthermore, no differences in P-SEK1/MKK4 levels were detectable when collaterals of the shunted side (without L-NAME) were compared with collaterals of shunted side with L-NAME. Moreover, FSS and L-NAME treatment did not change the levels and specific phosphorylation (activation) of Akt kinase (Figure 6F).

Microarray Expression Profiling and Pathway Analysis

For details see supplemental methods section.

SNAP and L-NAME (But Not L-NNA) Inhibited Cell Proliferation in MVSMC

For details see supplemental methods section.

Discussion

That flow determines arterial size, ie, that form follows function, was known for a long time. Thoma, Schretzmayr, Rodbard, Langille, Holtz, Tronc, and Ben Driss made the observation that arterial size depends on flow during development, that adult arteries respond with structural changes to changes in blood flow, and that the lumen is controlled by “an immediate physiological adjustment in vascular tone induced by the change in flow, and a delayed anatomical change that occurs when the change in flow persists”. Thereafter many studies identified a host of molecules (vasoactive, growth regulatory, inflammatory, adhesive) whose endothelial production is also mediated by fluid shear stress. Recent studies by the laboratories of...
Tedgui,16–18 Busse,19 Dejana,20 and from our own group21 have shed light on the mechanisms of transduction of the mechanical stimulus into a growth response.

The most important present new findings are that markedly increased fluid shear stress, created by high shunt flows, overcomes the anatomical restrictions of collateral vessels and leads to complete normalization and overcompensation of maximal conductance. The pathways involved are the Ras-Raf-MEK-ERK- and the Rho-pathway. NO plays an important but complex role.

An often-repeated observation is that the spontaneous growth of collateral arteries remains defective in that only ≈35% to 40% of the maximal physiological resistance is restored after arterial occlusion.1,22 This defect in the natural restoration of maximal conductance was often tried to ameliorate with the application of angiogenic growth factors, but even in the rigorous studies by Unger23,24 high doses of FGF-2 increased Cmax from only 40% to 50% of Cmax. One explanation for the limited usefulness of growth factors could be that because of anatomical restrictions collateral vessels had reached their limit and even if further stimulated do not respond with a functional improvement. The tortuosity of collateral vessels, leading to energy-wasting curvature flow, their increase in length, and the relatively large number of small vessels would make them inefficient from the viewpoint of Poissielle’s Law.4 However, if this defective natural restoration is caused by premature normalization of FSS by the initial increases in collateral diameter, (which reduces FSS by the third power of the radius), an increase in FSS should lead to further growth. In our distal-stump arteriovenous shunt experiments we show for the first time that collateral vessels are not growth-limited and that their mentioned shortcomings do not restrict the restoration of maximal collateral blood flow. In fact, their growth is finally only limited by the size of the surgical Anastomosis and by the increased flow-load on the heart. Four weeks after installment of the shunt, the collateral vessels are able to conduct twice as much maximal blood flow compared with the normal femoral artery bed (see Figure 2a). It could be argued that even under the conditions of the shunt, FSS should fall with the radius in 3rd power caused by the enlargement of the collateral vessels. However because the venous system acts like a flow sink, Poissielle’s law with the radius in the 4th power applies in the absence of a flow limiting peripheral resistance, creating a positive feedback loop.

Arteriogenic Pathways

Our present studies provide evidence for 3 signaling pathways in addition to the already described cell–cell interaction largely based on bone marrow–derived cells adhering to activated endothelium.25,26 The 3 signaling pathways are the Ras-ERK pathway, the Rho-pathway, and the NO-pathway. For the RAS-ERK pathway we could show a marked increase of phosphorylation in Western blots of collateral vessel tissue. Whereas the protein amount of ERK did not change, that of Ras had increased. The RAS-ERK pathway plays a prominent role in the transmission of growth factor–initiated signals leading to cell proliferation.27 It is therefore not
surprising to find members of that signaling chain in high activity in growing collateral vessels. However, within the constraints of the present experiment, we cannot identify with any certainty the growth factor(s) that had caused this. Our observation that classical growth factors did not show changes of expression in the microarray screen makes their role uncertain.

A new finding is the prominent role of the Rho-pathway in arteriogenesis, which is based on the observed upregulation of RhoA itself, and members of the Rho signaling chain like...
cofilin (CFL1), vimentin and the increased protein expression, and increased degree of phosphorylation of focal adhesion kinase (FAK). Furthermore, the Rho inhibitor Fasudil inhibited arteriogenesis. Although the Rho-pathway is essential for cell motility, necessary for collateral remodeling, a role in smooth muscle cell proliferation was also discussed in a recent review. Its main role lies in the modification of the actin cytoskeleton via cofilin and dextrin as we could recently show and confirm with our present microarray experiments. The dissolution of the contractile phenotype of the SMCs is a prerequisite for their mobility during remodeling and is paralleled by the transcriptional downregulation of alpha smooth muscle actin. Cofilin exists in 2 forms: cofilin1, which is expressed in endothelial cells, and cofilin 2, which is expressed in smooth muscle cells. The cofilins are activated by effectors of Rho GTPases, involving LIM kinases, and the phosphatase slingshot. The fact that focal adhesion kinase is also increased in SMCs indicates that this flow sensing system is probably also a general stress sensor in SMCs that are under increased circumferential wall stress because of the vasodilating influence of NO. The high pressure– and stretch-related forces must now be borne by the SMCs alone because the elastin skeleton had dissolved under the influence of the monocyte elastase. High fluid shear stress is one of the activators of eNOS. It is therefore not surprising to discover that NO plays a role in arteriogenesis which is highlighted by our previous finding of its strong expression in growing collateral vessels and by the almost complete inhibition of the shunt effect by treatment with L-NAME which also caused a shutdown of the RAS-ERK pathway. The role of eNOS may be more complex than the clear-cut L-NAME effect at first suggests, because NO is a known antimitogen for SMCs and it prevents leukocyte adhesion which we also confirmed in SM cell culture studies. Reports on the negative effects of L-NAME on positive remodeling of large conductance arteries under high fluid shear stress may therefore allow for a different interpretation. However, L-NAME is not the most specific antagonist of eNOS, and we tried therefore the more specific antagonist L-NNA in our shunt studies and found that it exerted also a marked antiangiogenic effect without exhibiting an antiproliferative effect in cultured smooth muscles cells. This strengthened the NO-hypothesis beyond reasonable doubt but did not eliminate the complexity of the action of NO because of the strong antiproliferative action of NO itself, because NO donor infusion did not increase \( C_{\text{max}} \), and because eNOS overexpression in transgenic mice does not stimulate arteriogenesis. We hypothesize therefore that NO, produced by high shear stress, most probably induces a Rho-activating factor. Both pathways, the NO as well as the Rho-pathway, assume equal rank because the inhibition of either one inhibits arteriogenesis. The final common pathway for both Rho- and NO is most probably the Ras/ERK signaling chain.

We explored yet another hypothesis: an uncoupling of eNOS may have happened under the influence of the intense and long lasting shear stress and under the influence of oxygen based radical production. NO plus superoxide might have given rise to the formation of peroxinitrite, a radical recently described as being involved in vascular remodeling. Radical scavengers like Ebselen and urate had no effect on \( C_{\text{max}} \) and the peroxi radical donor SIN-1 markedly and significantly decreased collateral conductance thereby refuting this hypothesis.

Our own present and previous findings would favor the view that stress-induced deformation of the endothelial cell and the change in cytoskeletal tension and arrangement may initiate signals that lead to a variety of responses that act in concert: chemokine expression, adhesion molecule production, marked upregulation of integrins, Rho-activation (present report), and NO-production.

Conclusion

Sustained high fluid shear stress caused by a surgically created shunt between the distal end of an occluded femoral artery and its accompanying vein leads to a much prolonged growth process of collateral arteries which completely restores full maximal conductance, even surpassing normal maximal conductance of a normal arterial bed by a factor of 2. At least 3 signaling pathways converge, ie, the RAS-ERK-, the Rho-, and the NO-pathway, which enable the substantial and sustained growth of collateral arteries and allow for the great range of adaptation potential.

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Disclosures

None.

References

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Detailed description of all methods and of the Micro-array results

**Methods**

The present study was performed according to Section 8 of the *German Law for the Protection of Animals*, which confirms to the US National Institutes of Health (NIH) guidelines.

**Anesthesia and Surgery**

Male rabbits (New Zealand White) with an average body weight of 3.0 kg were anesthetized with i.m. injections of 40 mg/kg of ketamine hydrochloride and 4.0 mg/kg xylazine. This produced a sufficiently deep anesthesia for the ligature of both femoral arteries and for the side-to-side anastomosis operation of the distal stump of the left femoral artery with the accompanying vein. The wound was closed in layers and the animals received an antibiotic (enrofloxacine) and were outfitted for 2 days with a collar to prevent self-mutilation. For pain relief each animal received 0.15 mg s.c. buprenorphine postoperatively. This was repeated on the first postoperative day or as needed. For the terminal experiment the animals were premedicated with 5 mg midazolam and 40 mg/kg ketamine hydrochloride i.m. followed by 50 mg/kg intravenous sodium pentobarbital and fentanyl, 0.06 mg/kg.

**Design of the Experiment**

**Arterio-venous shunt experiments.** Both femoral arteries were occluded by ligature distally to the origin of the deep femoral artery. Additionally, the distal stump of the left femoral artery was then side-to-side anastomosed with the accompanying vein (shunt) (figure 1). The AV-shunt animals (n=12) were studied with magnetic resonance imaging (MR) and with Computerized Roentgen Tomography (CRT) at 7 days after the operation and 4 weeks with the shunt still open. For the hemodynamic experiments (n=14) the shunts were surgically closed and pressures and flows were measured under the influence of adenosine. After the terminal hemodynamic experiment animals were either selected for histology and angiography or for RNA- and protein extraction of the collateral vessels. Collaterals of the non-shunted side served as controls.

**Drug administration.** Pharmacological agents were either applied via Alzet minipumps or orally. Groups of rabbits (n = 40) were outfitted with subcutaneously
implanted Alzet minipumps connected to a catheter, which was introduced into the proximal stump of the occluded femoral artery with the tip located in such a way that it did not hinder inflow into the deep femoral artery, the feeder artery for most collateral vessels. Minipumps (2.5 ml) with a duty cycle of 7 days were loaded with Sin-1 (0.4 mg/kg, 0.6 mg/kg, 1.0 mg/kg), fasudil (10 mg/kg), urate (saturated solution), Catalase (1300U/kg) or SOD (3500U/kg). L-NAME (60 mg/kg), L-NNA (40mg/kg) or ebselen (10 mg/kg) were administered per os.

Terminal experiments including determination of collateral conductances were done seven days after femoral artery ligature.

**Magnetic Resonance Imaging.** All examinations were performed on a 1.5 T Scanner (Magnetom Sonata, Siemens Medical Systems, Erlangen, Germany). The anesthetized rabbits were placed in supine position on the scanner table and a six segment phased array coil was placed over the examination area.

A Flash 2D phase contrast sequence was used for flow measurements with the following parameters: ECG-gated, repetition time (TR) 27 ms, echo time (TE) 8.4 ms, 6 acquisitions. Slice thickness: 2.5 mm, Field of view (FOV): 100 mm x 65 mm, matrix size: 192 x 128. Temporal resolution was 31 ms, and spatial resolution amounted to 0.5 mm x 0.5 x 2.5 mm. Velocity encoding was set at 70 cm/s.

Flow was measured at a transverse plane 1cm distal to the aortic bifurcation. Four separate regions of interest (ROI) for each common iliac artery and common iliac vein covered each vessel entirely. Twelve to 18 velocity measurements per heart cycle were acquired for the heart rate range observed (125-180/min).

The cumulative flow (ROI area x mean velocity) per heart cycle, given in ml, was calculated to access shunt effects. Results for each, the shunted as well as the non-shunted, side were compared to the venous flow on each side to check measurement accuracy.

Time of flight (TOF) angiograms were produced using transversal slabs of a Flash 3D sequence with the following parameters: TR 28 ms, TE 4.3 ms, four slabs with 24 partitions each, matrix size 208 x 256 FOV 14.7 x 12 cm, slab thickness 0.8mm, four averages, acquisition time 16 min.

Maximum intensity projection images (MPI) visualized the vessels.
**Computerized Roentgen Tomography (CRT)**

All examinations were performed on a 16 row CT scanner (Somatom Sensation Cardiac, Siemens Medical Systems). A contrast-enhanced angiogram was acquired using 1.7 ml/kg body weight of iodinated contrast medium with 400 mg iodine/ml (Imeron 400, Altana Pharma, Konstanz, Germany). The contrast medium was injected by hand at 2 ml/s, followed by a 5 ml saline flush. The image acquisition started automatically four seconds after a predetermined threshold of 120 Houndsfield units (HU) in a ROI over the descending thoracic aorta was exceeded.

Parameters of the raw 16row-spiral images were: collimation 0.75 mm, 0.5 s rotation time, table feed 15 mm/rot, pitch 1.25, 120 kV, 100 mAs. An acquisition time of 9-11 sec sufficed to cover the distal abdominal aorta to the rabbit’s foot at the arterial enhancement phase.

From these raw images, transverse images were calculated with 0.75 mm slice thickness, and 0.5mm image spacing, with the reconstructed FOV measuring 150 mm and a 512 x 512 matrix size. Volume rendering (VR) images were constructed from these images for further evaluation.

**Hemodynamic measurements**

For the terminal experiment the animals were anesthetized again and both iliac arteries were exposed and outfitted with ultrasonic flow probes to measure blood flow in ml/min. These were positioned proximal to the deep femoral artery, i.e., the stem of most of the growing collateral vessels. A small PE 50 catheter was advanced through a side-branch into the abdominal aorta for the infusion of adenosine. A fluid filled catheter was introduced into the carotid artery for the measurement of systemic arterial pressure in mmHg. Both pedal arteries were prepared and catheters inserted for the measurement of the pressure gradient over the collateral network or along the intact femoral artery and its tributaries. Adenosine was infused at increasing concentrations until the central aortic pressure began to fall. This happened usually at a concentration of 600 micrograms/kg per minute. Pressure and flow signals were fed into an AD-converter and were digitally recorded on a MacLab computer. Maximal conductances ($C_{max}$) were calculated by dividing maximal adenosine-recruited flows by the pressure gradient across the collateral network (ml/min/mmHg).
Postmortem angiography

Angiographies were performed as previously described. Briefly, to obtain postmortem angiographies or to visualize collateral arteries for mRNA isolation or protein extraction, rabbit hind limbs were perfused with barium sulphate/gelatin contrast agent. Angiograms of each hindlimb were taken in a Balteau radiography apparatus (Machlett).

RNA isolation, amplification and microarray analysis

Per rabbit, two RNA samples were isolated (Rneasy Mini kit, Qiagen) from collateral wall tissue (one of each, ligature non-shunt control and AV-shunt) of four rabbits. After additional treatment with DNase-I, the eight RNA samples were linearly amplified for two cycles to average yields of 11 micrograms cRNA (average I 260/280 ratio 1.96) and subsequently labeled with Cy3- and Cy5 dyes (Amino Allyl MessageAmp™ aRNA Kit, cat#1752, Ambion). Each sample pair of AV-shunt and ligature control collaterals (1 microgram cRNA per Cy-dye) was hybridized in duplicates according to the dye-swap design for 16h at 40°C (25% de-ionized formamid; 20 mg yeast tRNA; Hyb buffer, Amersham).

Micro array analysis was performed essentially as described. In brief, spotted microarrays (MAD, SILS, The Netherlands) were used, containing 60-mer oligonucleotide sequences encoding for 18650 human genes (Sigma/Compugen library). Images were acquired using the Agilent-II scanner and processed by ArrayVision8.0 software. Background-subtracted intensities were Loess-normalized for non-linearity (limma package, www.bioconductor.org), and imported into the Rosetta Resolver database and analysis software (version 5.0, Rosetta Biosoftware).

Baysian statistical analysis was performed using a paired Cyber-T test and false discovery rate (FDR)-correction of the resultant P-values was recalculated using the Benjamini-Hochberg correction for multiple testing. Micro array intensity differences were considered significant when absolute intensity was greater than 50 and q<0.01, i.e. a Baysian FDR-corrected p-value that implies 1% false positives in selected genes. All genes with a Locuslink ID (16.234) were imported into Pathway analysis software for analysis (Mappfinder 2.0, www.GenMapp.org).
**Preparation of soluble and particulate protein fractions**

Tissue samples (shunt-collaterals and control-collaterals) were suspended in ice-cold buffer A containing (in mmol/L): 20 Tris-HCl, 250 sucrose, 1.0 EDTA, 1.0 EGTA, 1.0 DTT, 0.1 sodium orthovanadate, 10 NaF and 0.5 PMSF, pH 7.40 and homogenized with a Teflon homogenizer. The homogenates were centrifuged at 14,000xg for 30 min at 4 °C. The supernatants represented the soluble (cytosolic) fractions. For particulate fractions, the pellets after the first centrifugation were resuspended in buffer A containing 0.2% Triton-X100 and centrifuged at 10,000xg for 10 min at 4°C. The Triton X-100 soluble supernatants represented the particulate fraction. The protein concentrations were estimated by the method of Bradford.

**Electrophoresis and immunochemical Western blot analysis**

Samples of soluble or particulate fractions containing equivalent amounts of proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). For Western blot assays, proteins after electrophoretic separation were transferred to nitrocellulose membranes. Total contents or activation of proteins were determined using specific antibodies. For primary immunodetection the specific antibodies against ERK, H-Ras, Akt kinase (all from Santa Cruz Biotechnology), phospho-ERK (Thr202/Tyr204), phospho-SEK1 (Thr223), phospho-Akt kinase (Ser473) were used (all from Cell Signaling Technology). Peroxidase-labelled anti-rabbit or anti-mouse immunoglobulins (Amersham Biosciences) were used as secondary antibodies. Bound antibodies were detected by the ECL detection method. Quantitative gel analysis was performed using Phosphorimager SF (Molecular Dynamics, Krefeld, Germany).

**Cell Culture**

Mouse vascular smooth muscle cells (MVSMC) were isolated as described earlier and were cultivated in Iscove’s modified medium containing 10 % (vol/vol) heat-inactivated fetal calf serum (FCS), 10 units/ml penicillin, 10 g/ml streptomycin, and 2 mM L-glutamine.
Cell proliferation assay

Cell proliferation was determined by a colorimetric immunoassay, which based on the uptake of BrdU during DNA synthesis and the quantitative binding of a monoclonal anti-BrdU-antibody (Roche Diagnostics, Mannheim, Germany). MVSMC were cultivated for 1 day in a 96-well microtiter plate. Subsequently, cells were cultivated in serum-free medium for 24 h. Thereafter, test agents were added in serum-free medium in a total volume of 100 µl/well. After incubating three days BrdU-incorporation was determined.

Results

Microarray expression profiling and pathway analysis

The aim was to find candidate genes and pathways that would be responsible for the strong stimulation of collateral artery growth by increased FSS. Therefore, a genome-wide expression profiling was performed directly comparing in 4 individual rabbits the collaterals from shunt versus ligated side that were harvested 7 days after the initial surgery. Expression profiling was performed using a dye-swap design, directly comparing shunt versus ligated samples from individual rabbits on duplicate arrays. A total of 10778 genes (58%) could be reliably detected in our samples using a microarray background corrected intensity cut-off of 20. Reliable differences in expression were determined by Bayesian statistical analysis, including correction for multiple testing, which yield a total of 366 differentially expressed genes (Bayesian FDR-corrected q<0.01, I>50) (Online Figure 1A). Further analysis by hierarchical clustering showed a good correlation in gene expression profiles between the 4 individual rabbits (Online Figure 1B). Next, biological significance was determined by ranking the genes by ontology term using GenMAPP/MAPPfinder pathway analysis software. No significant change in expression levels of major vascular growth factors or their cognate receptor could be detected. Specifically, intensities for fibroblast growth factors-2 (FGF-2) and platelet derived growth factor-D (PDGF-D) were not changed, whereas expression of FGF-4, PDGF-A and –C, monocyte chemoattractant protein-1 (MCP-1) as well as vascular endothelial growth factor-B (VEGF-B) and –C was not detected. Still a complex panel of diverse genes showed consistent differential
expression in our model, as shown by the line graphs (Online Figure 1C), indicating that a number of diverse signaling pathways is operational during sustained remodeling in the shunted side, like RhoA, coflin and vimentin that were up-regulated more than 5-fold.

**L-NAME (but not L-NNA) inhibited cell proliferation in MVSMC**

To confirm the in vivo results of the inhibitory effect of L-NAME on collateral artery growth, the effect of L-NAME and L-NNA was analyzed in vitro. Influence of L-NAME on FCS- and fibroblast-growth-factor-2 (FGF-2)-induced cell proliferation was determined by measurements of DNA synthesis in MVSMC. FCS or FGF increased DNA synthesis 18 and 1.7 fold, which both were significantly inhibited by L-NAME but not by L-NNA. L-NAME did not change DNA synthesis of unstimulated cells. The inhibitory action of L-NAME started at concentrations of 250 micromolar. It is of note that the NO donor SNAP exerted also a potent anti-proliferative effect.

**References**

Online Fig. 1:

A) Genes with differential intensities between ligature controls and AV-shunt grown collateral arteries. An M vs. A plot of the micro-array experiment is shown here. The genes with differential intensities between shunted collaterals and ligature controls (int>50; p<0.01) are shown as coloured dots: (●) red, 262 genes up in AV-shunt; (●) green, 104 genes down in AV-shunt; (+) blue, no criteria met.

B) Inter-rabbit comparison of genes with differential micro-array intensities between AV-shunt and ligature-grown collaterals. The 2log-transformed ratios of 366 genes with differential intensities (Bayesian p < 0.01; micro-array intensity>100) were used for the hierarchical clustering. The clustering columns represent genes within n = 4 individual rabbits. Colour information of the micro-array intensity ratios (shunt/ligature): Red; up in shunt; Green; down in shunt. Clustering method: weighted average; similarity measure: Euclidean distance; ordering function: average value.

C) Within-rabbit differences between micro-array intensities of AV-shunt and ligature grown collaterals. The line graphs show the percentile within-rabbit differences between the ligature control collaterals (L), and the collaterals derived from the AV-shunts (S), for each gene in four rabbits. Ligature controls were set to 100%.
Figure 1

A. Scatter plot showing the log intensity on the x-axis and log ratio on the y-axis. Different symbols represent different rabbit samples.

B. Heatmap showing gene expression levels with downregulated (104 genes) and upregulated (262 genes) genes highlighted.

C. Line graphs for specific genes:
- CUL1: int. 22270 (+1.6-fold; p=3.8×10^-10)
- E2F2: int. 10328 (+2.0-fold; p=4.8×10^-7)
- IGF2R: int. 206 (+1.3-fold; p=4.0×10^-5)
- IGF2: int. 503 (+1.3-fold; p=1.3×10^-4)
- CFL1: int. 216 (+1.3-fold; p=5.5×10^-4)
- MEFL2C: int. 15590 (+1.7-fold; p=2.7×10^-3)
- OS-9: int. 193 (-1.1-fold; p=7.2×10^-37)
- GPC1: int. 116 (-12.2-fold; p=7.8×10^-30)
- MIA: int. 60 (-3.4-fold; p=9.9×10^-23)
- RGS5: int. 52 (-2.4-fold; p=1.3×10^-12)
- FGF20: int. 59467 (-1.5-fold; p=6.3×10^-9)
- GPR24: int. 141 (-1.3-fold; p=1.2×10^-4)

Online Figure 1