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Original article

Calcium-dependent signalling is essential during collateral growth in the pig hind limb-ischemia model

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ABSTRACT

We investigated the effect of pharmacological activation of the Ca^{2+}-channel *transient receptor potential cation channel, subfamily V, member 4* (TRPV4) on collateral growth in a pig hind limb-ischemia model thereby identifying subcellular mechanisms. Domestic pigs received femoral artery ligature and were randomly assigned to one of the following groups (each $n=6$): (1) 4α-phorbol 12,13-didecanoate (4αPDD) treatment; (2) treatment with an arterio-venous shunt (AV-shunt) distal to the occlusion; or (3) implantation of NaCl-filled minipump. Six sham-operated pigs acted as controls. Aortic and peripheral mean arterial pressure (MAP) measurements were performed to assess the collateral flow index (CFI). Tissue was isolated from M. quadriceps for immunohistochemistry and from isolated collateral arteries for quantitative real time PCR (qRT-PCR). Shortly after ligature the CFI dropped from 0.96 ± 0.02 to 0.21 ± 0.02 in all ligature-treated groups. In ligature-only-treated pigs CFI increased to 0.56 ± 0.03 after 7 days. Treatment with 4αPDD led to an enhancement of CFI compared with ligature alone (0.73 ± 0.03). CD31-staining showed improved arteriolar density. Increased Ki67 staining in collaterals indicated proliferation. qRT-PCR and Western blot analysis showed upregulation or modulation of Ca^{2+}-dependent transcription factors *nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1* (NFATc1), *Kv channel interacting protein 3, calsenilin* (KCNIP3/CSEN/DREAM), and *myocyte enhancer factor 2C* (MEF2C) in 4αPDD- and AV-shunt-treated pigs compared with controls. Improved CFI after 4αPDD treatment identifies TRPV4 as an initial fluid shear-stress sensor and collateral remodelling and growth trigger. Subcellularly, modulation of Ca^{2+}-dependent transcription factors indicates a pivotal role for Ca^{2+}-signalling during arteriogenesis.
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1. Introduction

After occlusion of a large artery the vascular system has the capacity for autonomous healing, although imperfect, through the development and growth of a collateral circulation. Elucidation of the molecular pathways leading to a functional "natural bypass" would be the basis for stimulating the non-optimal processes pharmacologically. The goal is to produce full restoration of arterial function.

One approach is to stimulate collateral growth using angiogenic growth factors. However, these agents did not live up to expectations in controlled clinical trials despite previous anecdotal evidence [1].

Adhering monocytes were shown to play an important role in the transformation of pre-existing small arterioles into significant conduits of blood flow, taking over the role of an occluded artery [2–4]. However, overtransfusion of monocytes increased the restoration of bloodflow after femoral artery ligation (FAL) in mice but the result was significantly below normal flow. We demonstrated that other or additional factors are necessary to develop a functional artery, suggesting the presence of an arteriogenic pathway, which exists in addition to the paracrine action of monocytes. In a hind limb-ischemia model in rabbits and rats with an arterio-venous anastomosis (AV-shunt) leading to chronically-elevated fluid shear stress (FSS) in the collateral system, we showed that this physical force markedly triggered collateral growth [5,6]. Furthermore, it was possible to completely compensate blood flow deficits. One of the genes significantly upregulated during these processes is the *transient receptor potential cation channel, subfamily V, member 4* (TRPV4) [7].

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Since TRPV4 is known for its role in modifying intracellular Ca^{2+}-concentrations [8,9], we hypothesized that calcium-dependent transcriptional regulation is an important molecular mechanism during arteriogenesis.

The aim of the present study was to investigate whether drug-induced activation of TRPV4 leads to an improved perfusion distal to an occluded major artery in a clinically relevant pig model of hind limb ischemia, and to elucidate the underlying molecular mechanism.

2. Methods

2.1. Animal models

The present study was performed according to section 8 of the *German Law for the Protection of Animals*, which conforms to the US National Institutes of Health (NIH) guidelines. Twenty four juvenile, male, crossbred pigs with a body weight (BW) of 38.5 ± 4.7 kg were included in this study. Eighteen pigs were subjected to left femoral artery occlusion and 6 each were randomly assigned to the following groups: (1) NaCl-filled osmotic minipumps (termed ligature-only); (2) 4α-phorbol-12,13-didecanoate (4αPDD) treatment (18 µg/kg BW/day) using osmotic minipumps (termed 4αPDD); or (3) AV-shunt. Six sham-operated pigs served as controls (termed sham) (Table 1, Figs. 1(A) and (B)).

Animals were premedicated with an intramuscular injection of ketamine (20 mg/kg BW), azaperone (4 mg/kg BW), and atropine sulfate (0.02 mg/kg BW). Anesthesia was performed by intravenously injected propofol given as a 1.5 mg/kg BW bolus followed by a continuous infusion of 5 mg/kg BW/h. Pigs were intubated and ventilated with 40% oxygen using an Oxylog 3000 (Draeger medical, Lübeck, Germany). Analgesia was maintained with piritramide (0.3 mg/kg BW i.v.). In twelve pigs (ligature-only and 4αPDD group) the left femoral artery was dissected and the catheter of a minipump (ALZET® 2ML1, osmotic pumps Company, Cupertino, USA) was retrogradely inserted with the tip placed distal to the branching of the A. profunda femoris, which was then occluded. Simultaneously, the catheter was fixed at the site of occlusion. Also, the A. circumflexa femoris lateralis was ligated to prevent the direct bridging of the collateral arteries. The pigs in the AV-shunt-group received an arterio-venous fistula between the distal part of the femoral artery and the accompanying vein by side-to-side anastomosis. All animals received an antibiotic (2 mg/kg KG cefquinom i.m.) and analgesic (25 µg/h fentanyl via a transdermal patch) treatment during the first 3 post-operative days.

After 7 days, the pigs were anesthetized for catheter-based angiography and hemodynamic measurements. After euthanasia with a thiopenthal bolus and 60 mmol potassium chloride, the left hind limbs were perfused with warm (39 °C) gelatine-bromphenol blue-solution and immediately covered with ice. Collateral arteries were dissected free, cryo-preserved, and stored at −80 °C. Additionally, tissue from the aorta, femoral artery proximal and distal to the ligature, both saphenous arteries, and the M. quadriceps were obtained.

Table 1
Six pigs were randomly assigned to one of three experimental groups.

Experimental groups	Sham	Ligature-only	4αPDD	AV-shunt
Surgical treatment	Sham operation	Occlusion of the A. femoralis including NaCl-filled osmotic minipumps	Occlusion of the A. femoralis including 4αPDD-filled osmotic minipumps	Occlusion of the A. femoralis combined with an arterio-venous shunt distal to the ligature
n	6	6	6	6

2.2. Hemodynamic measurements; collateral flow index

Catheter-based hemodynamic measurements were performed just before and immediately after ligature on day 0 and during terminal surgery on day 7. Shortly before pressure measurement the pig received a single bolus of 0.3 mg Adenosin (Adrekar®, Sanofi Aventis, Frankfurt a. M., Germany) by i.a. injection (proximal part of the left A. femoralis) to achieve maximal vasodilatation (Supplementary Fig. 3) as previously described [10]. The systemic mean arterial pressure (sMAP) was measured in the right carotid artery. The peripheral mean arterial pressure (pMAP) was measured in the left saphenous artery and the central venous pressure (CVP) in the right external jugular vein. In AV-shunt-treated pigs, the shunt was acutely ligated for assessment of the hemodynamic parameters (Fig. 1(A)). The pressure-derived collateral flow index (CFI) was estimated with the following formula: CFI = (sMAP-CVP)/(pMAP-CVP) [11,12].

2.3. In vivo angiography

On days 0 and 7, a catheter (7F) was placed in the abdominal aorta under fluoroscopic guidance (Siremobil, Siemens AG, Munich, Germany). Angiographies were performed with the iodine-based contrast medium, Iomeprol (Imeron, Bracco Imaging GmbH, Konstanz, Germany) in order to visualize collateral growth. Contrast medium was infused via catheter into the abdominal aorta and the collaterals were visualized and photographed using fluoroscopic guidance (Siremobil Compact, Siemens AG, München, Germany).

2.4. Immunohistochemistry

Frozen sections (6 µm) were fixed in 4% paraformaldehyde for 5 min and incubated with primary antibodies against TRPV4 (Alomone Labs Ltd., Jerusalem, Israel), Ki67 (Dako, Glostrup, Denmark), *nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent* 1 (NFATc1; Santa Cruz Biotechnology, Santa Cruz, CA, USA), *Jun activation domain binding protein* (C-JUN/AP1; Calbiochem® Merck, Darmstadt, Germany), *calcineurin* (CaN, Sigma, München, Germany), *cAMP responsive element binding protein 1* (CREB; Sigma, München, Germany), *platelet/endothelial cell adhesion molecule* (CD31 or PECAM1; Antigenix America Inc., New York, USA), *actin, alpha, vascular smooth muscle* (αSMA; Sigma, München, Germany) or CD45 (PTPRC, *protein tyrosine phosphatase, receptor type, C*; AK online, Aachen, Germany). Cryosections were treated with the secondary antibodies anti-mouse-cy3 or anti-rabbit-cy3 (Chemicon, Hofheim, Germany). In case of treatment with a biotinylated secondary antibody, a third incubation was carried out with carbocyanin-linked streptavidin (Biomol, Hamburg, Germany). Nuclei were stained with DAPI (Invitrogen, Groningen, Netherlands) or Draq5 (Axxora, Lörrach, Germany). Sections were analyzed using a Leica confocal microscope (TCS SP, Leica, Wetzlar, Germany). Incubation with PBS instead of the first antibody was used as negative control. Arteriole density was assessed by counting CD31-positive stained vessels in all four experimental groups in representative images (40×; 24 images with $n = 4$ for each pig). Results were extrapolated and presented as arterioles/mm^2. The proliferative activity of vascular cells was determined by staining cryosections for Ki67. Proportion of leukocytes were elucidated by counting CD45-postive cells in the vascular wall and the adjacent perivascular space. Ki67- and CD45-positive cells were counted using 40× magnifications (24 images with $n = 4$ for each pig) and normalized to all nuclei in the vascular wall. Perivascular cells were included in total cell count in case of CD45

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