Liposomal Hsp90 cDNA induces neovascularization via nitric oxide in chronic ischemia

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Abstract

Objective: Induction of angiogenesis has been reported subsequent to eNOS overexpression or activation, the latter involving Hsp90 as a chaperone protein. Here, we investigated the potential of regional Hsp90 overexpression to induce therapeutic neovascularization in vivo in a chronic rabbit hindlimb ischemia model.

Methods: In rabbits (n=7 per group), the external femoral artery was excised at day 0 (d0). At d7, liposomes containing eGFP (control group) or Hsp90 were retroinfused into the anterior tibial vein. At day 7 and day 35, angiographies were obtained and analyzed for collateral formation and perfusion velocity (frame count score (% of d7 values). Capillary/muscle fiber (C/MF) ratio was calculated from five muscle areas of the ischemic limb. l-NAME and Geldanamycin were co-applied, where indicated.

Results: Compared to mock-treated controls, Hsp90 transfected increased C/MF ratio at day 35 (1.78±0.15 vs. 1.19±0.13, p<0.05), an effect blunted by l-NAME (1.39±0.11). Hsp90 transfection increased collateral formation (157±11% vs. 110±13%) and frame count score (174±18% vs. 117±10%), both sensitive to inhibition by l-NAME co-application (135±17% and 134±14%, respectively). Of note, C/MF ratio was found elevated 3 days after Hsp90 transfection (1.61±0.16 at d10), at a time point when collateral formation was unchanged (106±6%), and tended to remain elevated in the presence of l-NAME applied thereafter (1.64±0.35 at d35), though l-NAME blocked subsequent changes in collateral growth or increase in perfusion at d35.

Conclusions: We conclude that Hsp90 is capable of inducing angiogenesis and arteriogenesis via nitric oxide (NO) in a rabbit model of chronic ischemia. Our findings describe the capillary level as an initial site of Hsp90-cDNA-induced neovascularization, followed by growth of larger conductance vessels, resulting in an improved hindlimb perfusion.

Keywords: Angiogenesis; Nitric oxide; Rabbit hindlimb ischemia; Gene therapy; Retroinfusion

1. Introduction

Patients with hypoperfusion of the ileo-femoro-popliteal arteries are prone to develop ischemic leg syndromes including claudication, inability to move, ischemic ulcers and gangrene, if the arterial stenosis is not amenable to surgical or percutaneous treatment. For a growing number of these no-option patients, therapeutic neovascularization has been established as novel concept in a body of experimental studies. Therapeutic neovascularization requires angiogenesis [1], defined as capillary growth by sprouting from enlarged venules, and arteriogenesis [2,3], e.g., enlargement of preexisting collaterals. In addition, several recent studies found a contribution of vasculogenesis [4,5], depending on circulating progenitor cells, to either angiogenesis [5] or arteriogenesis [6] in the adult organism.

Of note, growth factors of the VEGF [7] or IGF family [8] as well as pharmacologic agents like statins [9], all
2. Materials and methods

New Zealand rabbits (3–3.5 kg, female) were purchased at Charles River (Sulzfeld, Germany), rompun was obtained from Bayer (Leverkusen, Germany) and ketamine from Ratiopharm (Ulm, Germany). The contrast agent Solutrast (Ratiopharm, Ulm, Germany). The human Hsp90 cDNA was obtained from W.C. Sessa [15]. All procedures concerning use of animals and cells conformed with the Declaration of Helsinki.

2.1. Endothelial cells

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics and used at early passages. When indicated, cells were exposed to an hypoxic environment by flushing a mixture of 95%N\textsubscript{2}/5%CO\textsubscript{2} in a home-made sealed chamber leading to a measured pO\textsubscript{2} of ~10 mm Hg. In some experiments, cells were transfected in EBM medium (Clonetics) with a mixture of the Hsp90 plasmid formulated in Lipofectamine (Invitrogen) according to the manufacturer’s protocol; the empty plasmid vector was used as a control to maintain identical amounts of DNA in each transfection. In some experiments, cells were exposed to 20 \textmu M radicicol or 5 mM \textit{L}-NAME before harvesting.

2.2. NO determination

The determination of nitric oxide (NO) level, e.g., the accumulation of NO derivatives (NO\textsubscript{x}) in the (serum-deprived) cell-bathing medium, was carried out using the Nitric Oxide Colorimetric Assay (Roche Diagnostics, Mannheim, Germany). We used 150-mm dishes with confluent HUVEC bathing in a minimal amount of medium in order to minimize the dilution of NO\textsubscript{x} and fresh medium was used for the last hour of incubation (e.g., when adding VEGF and the indicated inhibitors) to reduce background.

2.3. Western blotting

Immunoblottings and immunoprecipititation were carried out as previously described [12] with eNOS and Hsp90 antibodies from Beckton Dickinson (Lexington, KY, USA).

2.4. Animal protocol

All animal experiments conformed with the German and NIH animal legislation guidelines and were approved by the Bavarian Animal Care and Use Committee (AZ 2531-82/02). At day 0, after i.v. anesthesia induction with rompun (2 mg/kg) and ketamine (50 mg/kg), the complete femoral artery was excised [16]. The wound was closed and animals were brought back to the animal facility with free access to food and water.

At day 7, animals were anesthetized as described above, and a baseline angiography was performed after positioning a 4F catheter (Cordis, Haan, Germany) via the right carotid artery into the common iliac artery. Contrast agent was injected into the ischemic limb with an automatic injector (2 ml/s, 4 ml total) and fluoroscopy was performed with a Ziehm system (Nurnberg, Germany). Twenty-five angiographic pictures (=frames) per second were acquired and stored for later analysis.

2.5. Pressure-controlled retroinfusion of the ischemic hind limb

The anterior tibial vein was surgically prepared and a polyethylene catheter (0.80 mm outer diameter, 0.40 mm inner diameter) was inserted. A tourniquet was placed proximal to the femoral vein around the ischemic limb to prevent venous outflow for the therapeutic interval. Then, retroinfusion of 5 ml heparinized sodium chloride 0.9% with 500 \textmu g of either eGFP- (mock) or Hsp90-cDNA formulated in liposomes (QIAGEN, Hilden, Germany), was performed over 20 min. Pressure was continuously monitored and maintained at 80 mm Hg, providing the entire limb with the therapeutic agent (data not shown, cf. Ref. [17]). No venous rupture observed throughout the experimental series.

2.6. Application of \textit{L}-NAME and Geldanamycin

One series of animals (n=7) received the NOS inhibitor \textit{L}-NAME orally from d7 until d34 (500 \textmu mol/l). In order to dissect angionic from arteriogenic effects, minipumps (Alzet, Cupertino, CA, USA) were inserted subcutaneously,
delivering 50 nmol L-NAME per hour. Insertion was performed either d7 (concomittantly with Hsp90 transfection, n=5) or d10, i.e., after the onset of capillary formation (n=4). An additional experimental group received Geldanamycin, a specific inhibitor of Hsp90 protein interactions [18], via minipump (2 nmol/h, n=4).

2.7. Capillary density

At the end of the experiment, tissue samples from the thigh (m. adductor, m. vastus medialis) and calf muscles (m. gastrocnemius, m. tibialis anterior, m. fibularis) were obtained for quantification of capillary density. Transverse 10-μm tissue sections were cut from each muscle using a cryostat (Leitz, Wetzlar, Germany). Sections were routinely stained for alkaline phosphatase, an endothelial cell marker [16], or for confirmation identified with a CD31-antibody (Santa Cruz SC1506) and a FITC-labeled secondary antibody [19]. Capillaries and myofibers were counted in a blinded fashion in three microscopic fields (40x magnification/muscle, capillary density being expressed as capillary/myofiber ratio as described earlier [20].

2.8. Collateral score

Angiographies were analyzed in a blinded fashion for collaterals by counting the number of vessels in the femoral region (from the inguinal ligament to the knee joint) intersecting an overlaying grid, as described previously [21]. The score obtained at d35 was normalized to baseline score obtained at d7.

2.9. Frame count cinedensitometry

Quantitative measurement of blood flow by assessing the time contrast agent requires for passage between two defined anatomical landmarks has been established in femoral, iliac and coronary arteries [22–24]. In the present study, we used cine angiograms obtained as described above, defining the landmarks as the proximal and distal end of the excised femoral artery, i.e., the inguinal ligament and the knee joint. Peak densitometry at the proximal and distal landmark was determined, and the number of frames required for peak densitometry to pass from the proximal to the distal landmark counted [19]. Since perfusion velocity of contrast agent is indirectly proportional to frame count, and frame count in the ischemic limb (i.l.) was normalized to the non-ischemic contralateral limb (n.l.), we assessed blood according to the ratio of frame count (non-ischemic limb)/frame count (ischemic limb), termed frame count score. The values at d35 are given in % of d7 levels. This flow assessment correlated well with the established perfusion measurements, e.g., microsphere analysis, which was conducted for the experimental groups receiving Hsp90 retroinfusion with or without oral L-NAME coapplication (data not shown).

2.10. Statistical analysis

Data are given as mean±S.E.M. Differences between several groups were tested using ANOVA and Student–Newman–Keul’s post hoc analysis. p<0.05 was considered statistically significant.

3. Results

3.1. eNOS activity under hypoxic conditions: relevance of Hsp90 interaction

The first observation obtained in the present study was a fading eNOS activity under hypoxic conditions in vitro. Following an initial increase of NO production (at 3 h, Fig. 1A), which was almost entirely sensitive to treatment with the Hsp90 inhibitor radicicol, a progressive decrease below control levels was obtained at 24 and 48 h of hypoxia. Therefore, we investigated the ability of increased Hsp90 abundance to overcome the fading eNOS activity under hypoxia. Indeed, transfection of Hsp90 led to stable eNOS activity between 3 and 24 h of hypoxia (Fig. 1B). This finding was consistent with co-immunoprecipitation experiments displaying similar levels of eNOS/Hsp90 interaction at 3 and 24 h of hypoxia only after Hsp90 transfection (Fig. 1C). Of note, eNOS expression was not significantly altered according to the time of exposure to hypoxia.

3.2. Decreased Hsp90 expression in ischemic tissue

Guided by the in vitro observations, we next investigated the levels of Hsp90 expression in ischemic musculature in vivo. Indeed, Hsp90 expression at d10 after femoral artery excision was profoundly decreased in ischemic hindlimbs, when intraindividually compared to the contralateral, non-ischemic limbs (Fig. 2). Liposomal Hsp90 transfection (500 μg) via retroinfusion at d7 was able to restore Hsp90 protein to the level of the non-ischemic limb at the same timepoint.

3.3. Hsp 90 induces therapeutic neovascularization

Subsequently, we analyzed the effect of increased Hsp90 abundance on vessel growth of the ischemic limb. Our analysis of Hsp90 on vessel growth included the capillary level (i.e., angiogenesis) and the collateral vessel level (i.e., arteriogenesis). Fig. 3 revealed a significant increase in the capillary/muscle fiber (C/MF) ratio at 4 weeks after Hsp90 transfection, as depicted in examples of untreated and Hps90 transfected muscle sections (Fig. 3A,B) and in a quantitative analysis (Fig. 3C, 1.78±0.15% versus 1.21±0.07). This effect was blunted by oral application of L-NAME (C/MF ratio 1.39±0.11) or local infusion of the Hsp90 inhibitor Geldanamycin (C/MF ratio 1.48±0.21).

Interestingly, Hsp90 also induced collateral growth at d35, as depicted in an angiographic example (Fig. 4A, right
Fig. 1. Role of endogenous and recombinant Hsp90 in VEGF-induced eNOS activation during hypoxia. ECs were placed in an hypoxic chamber (10 mm Hg) for the indicated amounts of time. The accumulation of NOx (nitrates+nitrites) in the extracellular medium was measured for the last 60-min period in the presence of 25 ng/ml VEGF and either L-NAME or radicicol. (A) Time course of NOx production in the absence (total) or in the presence of L-NAME or radicicol. (B) Effect of Hsp90 transfection on the L-NAME sensitive production of NOx after 24 h of hypoxia; for the comparison, the extents of NOx production after 0, 3 and 24 h are presented in the absence and in the presence of radicicol (*p<0.05; **p<0.01 versus time 0, §p<0.05; §§p<0.01 versus corresponding total condition; n=3–4). (C) Immunoblot of Hsp90 (upper panel), eNOS (middle panel) and Hsp90 after eNOS immunoprecipitation (eNOS IP, lower panel).

Panel) in comparison to a control experiment (Fig. 4A, left panel). Quantitative analysis (Fig. 4B) revealed an increase in collateral score after Hsp90 transfection (157±12% of d7 level), but not in mock-transfected animals (112±7%, p<0.05). Similar to the capillary density, collateral growth induction by Hsp90 was sensitive to L-NAME application.
Consistent with both, capillary and collateral vessel growth, an increased perfusion score was found elevated at d35 in Hsp90-transfected hindlimbs (177 ± 19% of d7 level, compared to 111 ± 17% in mock-transfected controls, Fig. 5). The inhibitory effect of L-NAME (134 ± 14%) indicated a mediating role of nitric oxide, which appeared to rely on Hsp90, since Hsp90 transfection was ineffective in the presence of the Hsp90 inhibitor Geldanamycin (108 ± 28%). Thus, we were able to demonstrate a coherent improvement at both the microvascularity and conductance vessel level, resulting in an improvement of perfusion velocity.

3.4. Hsp90 affects capillary density before collateral growth

To gain further insight into the cascade of events leading to functionally relevant neovascularization after Hsp90 transfection, i.e., by dissection of microvascular and macrovascular vessel growth, we terminated experiments at d10, 3 days after Hsp90 transfection. At this time point, C/MF ratio at day 10 was already increased (1.61 ± 0.16, Fig. 6A), whereas the collateral number was virtually identical to the d7 level (99 ± 2.5%, Fig. 6B). Blockade of the intermediate Hsp90 effects with an L-NAME-loaded minipump at d10 did
not affect the capillary response to Hsp90 transfection in the three monitored calf muscle areas, all of which still displayed significant increases in C/FM ratio (data not shown). However, a lack of capillary response in the thigh muscles prevented a significant gain of C/FM ratio compared to mock-transfected animals (Fig. 6A). Importantly, local L-NAME application from d10 abrogated collateral growth induction (Fig. 6B), with perfusion velocity (115±19%) remaining at control levels (Fig. 6C). In contrast, implantation of the L-NAME minipump at d7 concomittant with Hsp90 transfection yielded no changes in C/MF ratio, collateral growth or perfusion score (Fig. 6A–C), similar to the oral L-NAME application (Figs. 3–5). Thus, the Hsp90-induced neovascularatory response is observed first at the capillary level, whereas the conductance vessels growth occurs subsequently (between d10 and d35). Capillary growth alone, obtained by blocking collateral growth via L-NAME minipump from d10, however, did not suffice to increase perfusion of the ischemic hindlimb (Fig. 6C), pointing to the role of conductance vessel growth and of nitric oxide as a central mediator therein.

4. Discussion

In the present study, we were able to overcome hypoxia-induced loss of eNOS activity in vitro by Hsp90
transfection into endothelial cells. Moreover, in vivo, we induced neovascularization of an ischemic rabbit hindlimb by Hsp90 overexpression, an effect abolished by co-application of either L-NAME or the specific Hsp90 antagonist Geldanamycin.

Hsp90 overexpression restored eNOS activity after 24 h of hypoxia in vitro, which otherwise faded substantially, although eNOS protein levels were maintained (Fig. 1A–C). In a recent study analyzing myocardial response to Hsp90 transfection, we observed a robust phosphorylation of eNOS at serin residue 1177, which, of note, was accompanied by dephosphorylation of threonin 495, providing increased eNOS activation [12]. Co-immunoprecipitation of proteins provided evidence of AKT as the kinase and calcineurin as the phosphatase involved in Hsp90-dependent eNOS activation under normoxic conditions. In the present study, early hypoxia (3 h) increased eNOS activity in vitro, an effect almost entirely inhibited by radicicol, a specific Hsp90 blocker [25]. The subsequent loss of eNOS function (at 24 and 48 h) was responsive to Hsp90 transfection, indicating that this strategy might overcome an eNOS activation deficiency under chronic hypoxic conditions in vivo.

We tested this hypothesis in a model of complete femoral artery excision inducing chronic hindlimb ischemia, as indicated by, e.g., VEGF upregulation [16]. The occlusion procedure is different from femoral artery ligation [2], which fails to provide tissue ischemia and increasing VEGF expression, though inducing collateral growth in-between 1 week. In this scenario, arteriogenesis appears as a neovascularity entity of its own, and an exclusive therapeutic target of agents like MCP-1 [2], reflecting the relevance of the monocyte/macrophage compartment [26] for this process. Of note, exogenous VEGF application in high concentrations did not enhance collateral growth in this arteriogenesis model [2], despite an increased responsiveness of vascular smooth muscle cells towards VEGF under hypoxic conditions [27]. A potential difference between microvascular arterioles (~50 μm diameter), where VEGF increases vessel maturation [28], and larger conductance vessels visible in angiography, where no effect was observed after VEGF transfection [29], might explain this discrepancy.

In the femoral artery excision model used in our study, collateral growth is preceded by capillary growth, with a maximum within 5 days after the onset of ischemia [16]. Therefore, performing therapeutic interventions not earlier than 7 days after the onset of ischemia, physiologic responses at the microcirculatory and conductance level were allowed to take place, providing a stable ischemia model at the time of therapeutic intervention without significant changes in collateral score or perfusion over the following 4 weeks (cf. Figs. 4B and 5). The response towards Hsp90 transfection included both, capillary and collateral growth, compatible with the application mode via retroinfusion into the venous system, distributing the therapeutic agent in the calf and thigh tissue [19].

Interestingly, capillary/muscle fiber ratio was found above the control level as early as 3 days after Hsp90 cDNA retroinfusion, whereas collaterals remained unaltered at that point (Fig. 6A–B). Time selective inhibition of NO formation with a minipump releasing L-NAME revealed that collateral formation in response to Hsp90 could be prevented even after capillary formation took place (Fig. 6A–C). This observation points to nitric oxide as a central mediator of vascular remodeling leading to collateral growth. Although L-NAME represents an unselective NOS inhibitor, capable of eNOS or iNOS inhibition, the contribution of the iNOS to neovascularization of ischemic muscle tissue has been found to be minor in a chronic ischemia model [30], as opposed to tumor angiogenesis [31,32].

Of note, nitric oxide has been found to stimulate vasculogenesis [33], i.e., by bone marrow derived endothelial progenitor cells. The contribution of bone marrow derived
progenitor cells to the neovascularization observed was not investigated in this study. However, the local activation of eNOS via Hsp90 cDNA retroinfusion differs from the eNOS introduction via bone marrow transplantation into otherwise eNOS deficient mice. Moreover, systemic (oral) or local t-NAME application yielded similar inhibition of neovascularization, rendering an influence of Hsp90 overexpression on vasculogenesis unlikely.

In summary, we have demonstrated that under hypoxic conditions, eNOS activity is fading at d2, a loss of function responsive to overexpression of Hsp90. In vivo, overexpression of Hsp90 in ischemic hindlimb muscles increases capillary density in-between the first 3 days after transfection, to be followed by collateral growth and improved perfusion thereafter. These results point to the relevance of local eNOS activation as target of therapeutic neovascularization.

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References


