Activation of Resistance Arteries with Endothelin-1: From Vasoconstriction to Functional Adaptation and Remodeling

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Key Words

Collagen · Endothelin-1 · Integrins · Microcirculation · Organ culture · Resistance arteries · Vasoconstriction

Abstract

Remodeling of resistance arteries is a key feature in hypertension. We studied the transition of vasoconstriction to remodeling in isolated rat skeletal muscle arterioles. Arterioles activated with 10 nM endothelin-1 showed functional adaptation when kept at low distension in a wire myograph setup, where contractile properties shifted towards a smaller lumen diameter after 1 day. Pressurized arteries kept in organoid culture showed physical inward remodeling after 3-day activation with 10 nM endothelin-1, characterized by a reduction in relaxed diameter without a change in the wall cross-sectional area (eutrophic remodeling). The relaxed lumen diameter (at 60 mm Hg) decreased from 169 ± 5 (day 0) to 155 ± 4 μ m (day 3). An antibody directed to the β_3 -integrin subunit (but not one directed to the β_1 -integrin subunit) enhanced remodeling, from a reduction in relaxed diameter at 60 mm Hg of 15 ± 2.4 to 22 ± 1.8 μ m (both on day 3). Collagen gel contraction experiments showed that the antibody directed to the β_3 -integrin subunit enhanced the compaction of collagen by smooth

muscle cells, from 83 ± 1.5 to $68 \pm 1.5\%$ of the initial gel diameter. In conclusion, these data show that inward eutrophic remodeling is a response to sustained contraction, which may involve collagen reorganization through β_3 -integrins.

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Introduction

Hypertension is associated with a structurally determined increase in the wall-to-lumen ratio of the vasculature. In the conduit arteries, this remodeling is characterized by either no change or an increase in the lumen diameter, the increased wall-to-lumen ratio thus being due to hypertrophy, an increase in wall material. In the resistance arteries, the lumen diameter is decreased, and the amount of wall material is either increased (hypertrophy) or unchanged, being due to a rearrangement of the wall material around the smaller lumen (eutrophy), depending on the type or model of hypertension [1]. Thus the rat angiotensin-II infusion model [2, 3] of hypertension results in hypertrophic remodeling of the small arteries, a finding which has also been reported for the spontaneously hypertensive rat [4], although in that model the results are variable. In human renovascular hypertension, hyper-

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trophic remodeling of small arteries has been reported [5], but in essential hypertension, the form of remodeling appears to be eutrophic [6–10]. An understanding of the mechanisms involved in eutrophic remodeling is therefore needed if the vascular changes seen in essential hypertension are to be clarified.

Until recently, mainly *in vivo* models have been used for studying the altered structure of small arteries in hypertension. However, recently a number of groups have used isolated vessels, cannulated and held under pressure for various periods of time. This has enabled the investigation of the effects of pressure and mitogens on mechanisms (e.g. extracellular signal-related kinase, ERK1/2) known from cell culture experiments to be involved in growth processes [11–13]. Such experiments may be expected to be of relevance to situations involving hypertrophic remodeling, although the experiments so far reported have not been long enough to detect changes in wall material. Other experiments by some of us have involved organ culture [14, 15]. These experiments have shown that when cultured for 3 days under pressure and during continuous activation either by fetal calf serum or by endothelin-1, physical remodeling can be detected; that is, when examined under relaxed conditions and a given pressure, the diameter of the vessel is reduced following the culture. Moreover, this physical remodeling is not associated with an increase in wall material: it is due to eutrophic remodeling. Thus an *in vitro* model has now been developed which can mimic the type of remodeling which is seen in human essential hypertension.

The aim of the present study was to delineate the transition of vasoconstriction to physical remodeling. The questions which have been addressed are: (1) which signal transduction pathways are involved in remodeling; (2) how is activation linked to rearrangement of the extracellular matrix, and (3) does activation result in functional adaptation, i.e. changes in the contractile properties, prior to physical remodeling. Physical remodeling was studied using isolated vessels in organoid culture, and the interaction of smooth muscle cells with the extracellular matrix was further explored using a collagen contraction assay. The possibilities of the wire myograph setup were exploited to study functional adaptation. In most cases, endothelin-1 was used to induce activation, and the effects of inhibitors of ERK1/2 activation and tyrosine kinase were used to study possible signal transduction pathways. Antibodies against β_1 - and β_3 -integrin subunits were used to study possible mechanisms of the transmission of cell signals to the extracellular matrix.

Methods

In accordance with institutional guidelines, male Wistar rats (Møllegaard, Denmark), 10–12 weeks of age, were stunned by a blow on the head and decapitated. Cremaster muscles were excised under sterile conditions and placed in cold, sterile Krebs-Henseleit buffer (Sigma), pH 7.35. Segments of the first-order arteriole of 2–4 mm length were dissected from the muscle and used in an organ culture setup (Danish Myo Technology) or a wire myograph setup (Danish Myo Technology).

Wire Myograph

The bath of the wire myograph setup was filled with physiological saline solution of the following composition (in mM): NaCl 119, NaHCO₃ 25, KCl 4.7, KH₂PO₄ 1.18, MgSO₄ 1.17, CaCl₂ 2.5, EDTA 0.027 and glucose 5.5. The solution was gassed with 5% CO₂/95% air, pH 7.4. Segments were mounted using 20- μ m wires and normalized according to a standard procedure [16] in the presence of papaverine to prevent active force development. A distension versus active tension relationship was established using 5-HT (10^{-6} M). Active force induced by 5-HT at each level of distension was calculated from the maximal force minus the passive force. Then, vessels were set at either 0.3 L₁₀₀ (low distension) or 0.9 L₁₀₀ (normal distension) and activated with 10^{-8} M endothelin-1 overnight. After this period, endothelin-1 was washed out for 1 h and a second distension versus active tension relationship was established.

Organoid Culture

Organoid culture was performed as described previously [14]. Briefly, vessels were tied to glass cannulas on both ends and pressurized to 75 mm Hg. The organ bath was mounted on top of a microscope, equipped with a digital camera that was connected to a computer. Diameter of the vessels was measured using Vediview software and recorded continuously. The vessels were equilibrated to 34°C and developed a spontaneous tone within 1 h after equilibration. Medium, agonists and inhibitors were substituted daily.

The first series of culture experiments was done using DMEM (Gibco), which was recirculated from a reservoir of 50 ml that was gassed with 5% CO₂ and 95% air. Vessels were activated with 10^{-8} M endothelin-1 for 3 days, with or without inhibitors, either genistein (10^{-5} M; Sigma) to inhibit tyrosine kinases or PD98059 (10^{-5} M; New England Laboratories) to block ERK1/2 activation. This concentration of genistein does not affect tone in these vessels [17]. PD98059 effectively blocks ERK1/2 activation at this concentration [11, 13]. In a separate group, segments were stimulated with 35 mM potassium medium, which comprised DMEM with additional potassium chloride.

In the second series of culture experiments, Leibovitz (Gibco) medium was used to reduce the amount of medium and inhibitors. This medium does not require CO₂ to maintain pH. The medium was not recirculated, thereby reducing the volume of medium to ~1.5 ml. Monoclonal antibodies (mAb) directed against the β_1 -integrin subunit (clone Ha2/5; hamster IgM, Pharmingen) and the β_3 -integrin subunit (clone F11; mouse IgG₁, Pharmingen) were used at a concentration of 50 μ m/ml. Both antibodies react with rat tissue.

In both series, contractile responses to 5-HT (10^{-7} M) were established on day 3 after washout of endothelin-1, potassium, and inhibitors. Passive pressure-diameter relationships were established at the start and end of the experimental protocol. For this purpose, vessels were incubated with 10^{-4} M papaverine on day 0 and incubated with

Ca²⁺-free physiological saline solution containing 10⁻⁴ mol/l papaverine on day 3. Pressure-diameter relationships were made after a 30-min incubation with the dilatory medium.

Immunostaining

Immunostaining for integrins containing the β_3 -subunit was performed on sections of the cremaster muscle. Specimens were embedded in Tissue-tek medium and frozen in liquid nitrogen. Sections were fixed in cold acetone. Sections were incubated overnight at 4°C with a dilution of 1:200 of the primary antibody (F11; Pharmingen). A kit for mouse antibody on rat tissue (DAKO) was used to detect the primary antibody and diaminobenzidine was used to reveal positive staining. Negative control sections were processed without the primary antibody.

Collagen Gel Contraction

Vascular smooth muscle cells were isolated from rat aorta using 4 mg/ml collagenase, 1 mg/ml soybean trypsin inhibitor and 0.4 mg/ml elastase. After enzymatic digestion of the aorta at 37°C for 75 min, cells were centrifuged and washed with Leibovitz medium. Cells were grown till confluence in culture flasks containing Leibovitz medium supplemented with 10% heat-inactivated FCS and antibiotics. For collagen gel contraction, cells were used at passage 3. Collagen gels were formed using rat tail collagen type I (Fluka). Collagen was dissolved in Leibovitz medium supplemented with 10% heat-inactivated FCS, using 0.2 N HAc at 4°C. The pH of the solution was then adjusted to pH 7.4 at room temperature. Collagen was allowed to polymerize in a 12-well culture plate at 37°C for 1 h, using 1 ml of collagen solution per well. Cells were released from the culture flask with trypsin-EDTA (Gibco), counted, and 10⁵ cells were gently placed on top of each gel. Antibodies were added to the same concentration as in the organoid culture experiments and the gels were incubated at 37°C for 4 days.

Statistics

Data are expressed as means $\pm$ SEM, with n indicating the number of experiments. Mean data were compared by t test or ANOVA, followed by post-hoc analysis with Bonferroni correction or Dunnett's t test as appropriate. Data were considered significant when $p < 0.05$.

Results

Functional Adaptation

The wire myograph setup was used to study functional adaptation. Contraction was induced with 5-HT, which induced maximal tension within 3 min after addition of the agonist. Subsequent steps in distension revealed a typical lumen distension versus tension relationship (fig. 1). Segments that were activated overnight at 0.3 L₁₀₀ showed a marked shift in contractile responses. Thus, contraction was enhanced at low distension, and impaired at high distension on day 1 as compared to day 0 (fig. 1a). The segments that were kept at 0.9 L₁₀₀ did not show a change in the active tension-distension relationship (fig. 1b).

Fig. 1. Distension versus active tension relationship of cremaster muscle arterioles in a wire myograph setup. Active tension induced by 10⁻⁶ M 5-HT was determined after successive steps in distension. Relationships were determined before and after a 1-day activation period with 10⁻⁸ M endothelin at 0.3 L₁₀₀ (**a**; n = 6) and 0.9 L₁₀₀ (**b**; n = 6). Vessels activated at low distension showed responses that were significantly increased at 0.3 L₁₀₀ and 0.5 L₁₀₀, and decreased at 1.0 L₁₀₀ and 1.1 L₁₀₀ as compared to day 0. * $p < 0.05$, ** $p < 0.01$.

Physical Remodeling

Physical remodeling was studied using the organ culture approach. In the first series of culture experiments, vessels were studied in DMEM. In this medium, segments developed spontaneous tone, resulting in a ~30% reduction in inner diameter. Activation with endothelin-1 further enhanced vasoconstriction. While endothelin-1 was refreshed daily, some decrease in contractile response during the 3-day experimental period was noted. Addition of neither PD98059 nor genistein affected constriction induced by endothelin-1 (fig. 2). Stimulation with 35 mM potassium induced a remarkably stable level of

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Fig. 2. Diameter of arterioles during organoid culture in DMEM. The maximal diameter at the start of the experiment was determined after incubation with 10^{-4} M papaverine. Arteriolar segments developed spontaneous tone after washout of papaverine, and were subsequently activated with 10^{-8} M endothelin-1 or 35 mM potassium ($n = 6$) during a 3-day culture period. Arterioles were incubated with endothelin-1 only ($n = 7$; control), or with endothelin-1 in combination with either 10^{-5} M PD98059 ($n = 4$) or 10^{-5} M genistein ($n = 4$). Endothelin-1 enhanced constriction throughout the culture period, as compared to spontaneous tone ($p < 0.01$ for days 0–3 vs. spontaneous tone).

Fig. 3. a Pressure vs. relaxed lumen diameter relation before (day 0) and after the culture period (day 3) in DMEM. Arterioles were activated during the culture period with 10^{-8} M endothelin-1 ($n = 7$). * $p < 0.05$, ** $p < 0.01$ for day 0 vs. day 3. **b** Inward remodeling, shown as reduction in relaxed lumen diameter at 60 mm Hg. Arterial segments were activated during organoid culture with endothelin-1 ($n = 7$), endothelin-1 in combination with PD98059 ($n = 4$), endothelin-1 in combination with genistein ($n = 4$) or with 35 mM potassium in the absence of endothelin-1 ($n = 6$).

Fig. 4. Diameter of arterioles during organoid culture in Leibovitz medium. The maximal diameter at the start of the experiment was determined after incubation with 10^{-4} M papaverine. Arteriolar segments developed spontaneous tone after washout of papaverine, and were subsequently activated with 10^{-8} M endothelin-1. Segments were incubated with endothelin-1 only (control; $n = 7$), or with endothelin-1 and mAb directed against either the β_3 -integrin subunit (F11; $n = 7$) or with endothelin-1 and mAb against the β_1 -integrin subunit (Ha2/5; $n = 5$).

Fig. 5. a Pressure versus relaxed lumen diameter before (day 0) and after the culture period (day 3) in Leibovitz medium. Arterioles were activated during the culture period with endothelin-1 (n = 7). **b** Pressure versus relaxed lumen diameter of arterioles incubated with endothelin-1 and mAb F11, directed against either the β_3 -integrin subunit (n = 7). **c** Pressure versus relaxed lumen diameter of arteri-

oles incubated with endothelin-1 and the mAb Ha2/5, directed against the β_1 -integrin subunit (n = 5). **d** Inward remodeling, shown as a reduction in relaxed lumen diameter at 60 mm Hg. Remodeling was significantly increased in segments treated with the mAb directed against the β_3 -integrin subunit as compared to control. * p < 0.05; ** p < 0.01.

tone throughout the experiment, to a level similar to that obtained with endothelin-1.

The passive pressure-diameter relationship showed a marked change after a 3-day activation period with endothelin-1. A significant decrease in diameter at higher pressure levels was found, when day 3 was compared to day 0 (fig. 3a). Segments that were incubated with PD98059 or genistein showed a similar pattern and extent of remodeling. Activation with 35 mM potassium mimicked the outcome of the experiments with endothelin-1 and resulted in a similar degree of remodeling (fig. 3b).

In the second series of culture experiments Leibovitz medium was used. In this medium, vessels developed a similar level of spontaneous tone and endothelin-1-

induced constriction as compared to DMEM. Addition of antibodies against β_1 - or β_3 -integrin subunits (Ha2/5 and F11) did not alter vasoconstriction induced by endothelin-1 (fig. 4). However, inward remodeling of vessels incubated with the anti- β_3 -antibody (F11) was enhanced as compared to endothelin-1 alone (fig. 5a, b). Incubation with the β_1 -antibody induced a similar degree of remodeling as compared to endothelin-1 alone (fig. 5c). At 60 mm Hg (fig. 5d), the relaxed lumen diameter of arterioles incubated with a combination of endothelin-1 and mAb F11 showed a reduction in maximal diameter of 22 ± 1.8 vs. 15 ± 2.4 μ m (F11 vs. control, p < 0.05). To exclude any interference of differences in vessel size on day 0, the reduction in passive diameter was also calculated as percentage.

Fig. 6. Responses to 10^{-7} M 5-HT, recorded on day 3 in DMEM (left 4 bars) and Leibovitz medium (right 3 bars). Responses were recorded during spontaneous tone, after washout of endothelin-1, inhibitors and 35 mM potassium. A significant increase in contractile response was observed in segments activated with potassium versus endothelin-1. * $p < 0.01$.

This showed a similar result, with a reduction of 13 ± 1.1 vs. $8.6 \pm 1.2\%$ (F11 vs. control, $p < 0.05$), indicating a 47% increase in remodeling with the antibody F11. The result of another approach to interfere with the $\alpha_v\beta_3$ -integrin, using a cyclic Arg-Gly-Asp (RGD) peptide (XJ735) at a concentration of 10^{-5} M, was negative as neither remodeling nor vascular tone were affected (data not shown).

The wall cross-sectional area, as determined in vessels activated with endothelin-1 in the absence of inhibitors and antibodies, did not change with remodeling: $8,636 \pm 720 \mu m^2$ on day 0 vs. $8,275 \pm 652 \mu m^2$ on day 3 ($n = 14$). The reduction in the cross-sectional area was $5 \pm 7\%$ on day 3 vs. day 0.

On day 3 of the culture period, all segments constricted in response to 5-HT (10^{-7} M). Contractile responses were similar in all groups after endothelin-1 activation, but enhanced in vessels after activation with potassium as compared to endothelin-1-activated vessels (fig. 6).

Immunostaining

Positive staining for the β_3 -integrin subunit was found in the media of the arteriole (fig. 7a). Note that staining was absent in the first-order vein. Control sections where the primary antibody was absent were negative (fig. 7b).

Collagen Gel Contraction

Control gels were contracted to $83 \pm 1.5\%$ of the initial diameter after 4 days. The antibody directed against the β_3 -integrin subunit augmented contraction to $68 \pm 1.5\%$ of the initial diameter, whereas the antibody directed to the β_1 -integrin subunit completely inhibited collagen gel contraction (fig. 8).

Discussion

Both experimental and clinical evidence support an emerging concept of physical remodeling as a result of persistent functional responses. Thus, Langille et al. [18] showed that, after conduit artery ligation, inward remodeling followed persistent vasoconstriction, with similar results being obtained in rat mesenteric small arteries [19]. Using an in vitro approach, we provided direct evidence that active tone induces inward eutrophic remodeling of resistance arteries [15]. The possible clinical significance of the finding has been supported by analysis of the available literature on hypertensive treatment, which suggests that vasodilation, but not hypotension, is effective in the correction of vascular structure [20].

Functional Adaptation

We hypothesized that chronic activation would not only lead to rearrangement of the extracellular matrix, as evidenced by inward remodeling, but also of cellular elements. Such reorganization, which could involve the cytoskeleton or the active filaments, would be evidenced by changes in the contractile properties. We exploited the possibilities of the isometric wire myograph, to specifically test the role of distension in the response to endothelin-1. The distension was set at $0.3 L_{100}$, equivalent to the constriction induced by endothelin-1 in cannulated vessels, or $0.9 L_{100}$. Contractile properties were tested after 1-day activation with endothelin-1, when physical remodeling is not yet present [15]. The results show that activation at low distension induces a change in the contractile properties. Contractile force increased at lower levels of distension, at the expense of force development at higher distension. Such changes were not observed when vessels were activated at $0.9 L_{100}$, suggesting that the changes are related to the small diameter associated with normal vasoconstriction. Whether these changes depend on the concentration or duration of activation with endothelin-1 remains to be determined. In addition, further research is needed to elucidate whether these changes relate to remodeling of the contractile apparatus within the cell, or if it is due to cell rearrangement.

Fig. 7. a Immunostaining of cremaster muscle sections with the mAb directed against the β_3 -integrin subunit (F11). Positive staining was observed in the media of the arteriole. No staining was observed in the vein or the surrounding tissue. **b** Control sections without the primary antibody were negative. The micrographs are typical of results from 2 specimen.

Fig. 8. Effect of antibodies on collagen gel contraction. The mAb F11, directed against the β_3 -integrin subunit ($n = 5$), significantly enhanced contraction. The mAb Ha2/5, directed against the β_1 -integrin subunit ($n = 5$), significantly inhibited contraction, as compared to control ($n = 8$). * $p < 0.001$.

Physical Remodeling

In the present study we used our previously established model of eutrophic remodeling [15] to investigate the mechanisms involved in activation-induced remodeling. Physical remodeling was observed after 3 days of strong vasoconstriction, induced by endothelin-1. The reduction in diameter is evident at high pressure levels only, resembling the characteristic changes seen in spontaneously hypertensive rats as compared to Wistar-Kyoto rats [21]. Work from our laboratory and that of others suggest that signal transduction pathways induced by vasoconstrictors, pressure, and growth factors converge at the level of ERK1/2. Thus, pressure and angiotensin II act synergistically to activate ERK1/2 in resistance arteries [11]. Pressure may also cross-activate the platelet-derived growth factor receptor and activate ERK1/2 [13]. Therefore, we hypothesized that a key role in the transition of constriction to physical remodeling may be played by ERK1/2. However, the data obtained in the culture experiments show that tyrosine kinases and ERK1/2 are not essential in remodeling, as induced by endothelin-1 in these vessels. In addition, the data with high potassium medium show that a relatively simple means of vasoconstriction is sufficient to induce remodeling. Thus, these results do not provide evidence for a role of signal transduction pathways initiated by ERK1/2 in this model, and alternative mechanisms need to be considered.

One such alternative, but less-defined mechanism of remodeling may involve integrins. Integrins are transmembrane proteins that serve many functions, including adhesion, migration, signal transduction and perhaps also fulfill a mechanosensory role [22]. The $\alpha_v\beta_3$ integrin is a receptor for vitronectin, fibronectin and osteopontin [23], proteins that may form a provisional matrix during growth and remodeling processes. Expression of the $\alpha_v\beta_3$ -integrin is enhanced specifically during low blood flow-related inward remodeling [24]. Thus, both the $\alpha_v\beta_3$ integrin and its ligands have been associated with inward remodeling. In addition, the $\alpha_v\beta_3$ -integrin is overexpressed in spontaneously hypertensive rats over Wistar-Kyoto rats, leading others to the suggestion that remodeling may relate to the anchoring function of integrins [25]. The present study now suggests an active role for integrins in remodeling, as the β_3 -integrin antibody enhanced remodeling induced by endothelin-1. The β_3 -integrin subunit was located by immunostaining in the media of the arteriole, in agreement with a smooth muscle-dependent process.

Collagen Contraction

To study the role of integrins in the relationship between two main players in the remodeling process, smooth muscle cells and collagen fibers, more directly, a collagen contraction assay was used [26]. This assay is used frequently as a model for wound healing and involves the reorganization of collagen fibers by fibroblasts or smooth muscle cells. It has recently also been used to study processes related to vascular remodeling [27]. We speculated that integrin-dependent collagen rearrangement plays a similar role in vascular remodeling. Thus, we hypothesized that the increase in remodeling as induced by the mAb F11 may result from an augmented capacity to rearrange collagen fibers in the vessel wall. The results of the collagen gel experiments substantiate this hypothesis, as collagen compaction was clearly enhanced by the β_3 -integrin subunit antibody. Such enhanced capacity to compact collagen gels in the presence of certain antibodies directed against the $\alpha_v\beta_3$ -integrin or its subunits has been observed by others, using either smooth muscle cells [28] or fibroblasts [29], and involves the increase in α -smooth muscle actin expression and its organization into stress fibers [28]. This effect is specifically related to some function-blocking antibodies, and not observed with RGD peptides [28]. This may explain the negative result with the cyclic RGD peptide XJ735 in the present study. Of relevance here may also be the observations that the antibody F11 inhibits smooth muscle migration [30] and proliferation [31]. Whether these effects are indirectly contributing to inward remodeling remains to be determined.

We also observed that the antibody directed against the β_1 -integrin collagen inhibited collagen contraction. This finding did not correspond to remodeling events in the cultured arterioles. The reason for this difference may be that the expression pattern of integrins or the accessibility of antibodies to their ligands differs between cultured cells and the intact vessel preparation. It can therefore not be ruled out that besides the $\alpha_v\beta_3$ integrin, other integrins play a role in remodeling.

Conclusions

In this study, several approaches were used to elucidate the relevant stimuli and signaling pathways that are involved in the processes of vascular remodeling. The data are consistent with both experimental and clinical evidence, which have also suggested that physical remodeling may occur as a result of persistent functional responses. The results show that conventional growth

mechanisms (which would be expected to involve tyrosine and mitogen-activated kinases) seem not to be involved in eutrophic remodeling. Rather, the process appears to require the transmission of information from activated smooth muscle cells via integrins to the extracellular matrix. Therefore, the present study reveals a novel mechanism for eutrophic inward remodeling, which involves the reorganization of the extracellular matrix by activated smooth muscle cells through integrins.

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