Smooth muscle cell function and organization of the resistance artery wall

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Chapter 5

Smooth muscle contractile plasticity in rat mesenteric small arteries: sensitivity to specific vasoconstrictors, distension and inflammatory cytokines

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Abstract

Small artery remodeling may involve a shift in the diameter-dependent force generating capacity of SMC. We tested to what extent and under which conditions such contractile plasticity occurs. Rat mesenteric arteries were mounted on isometric myographs. Active diameter-tension relations were determined after application of several stimuli for 16 or 40 hours at 40% or 110% of the passive diameter at 100mmHg. At 40%, 16 hour incubation with endothelin-1 (ET-1) but not U46619 shifted force capacity towards smaller diameters. Inflammatory cytokines (TNF-α,IL-1β,IFN-γ), TGFβ or serum neither induced such shift nor augmented the effect of ET-1. The ET-1 mediated change was not affected by superoxide dismutase and catalase. Inward matrix remodeling in the presence of ET-1 was slower, occurring after 40 hours. Arteries maintained at 110% showed a shift of force capacity to larger diameters, which was prevented by ET-1 but not by U46619. In the active but not the passive state, SMC had altered nuclear lengths after incubation at 40%. These data demonstrate contractile plasticity in small arteries, where chronic strain is an outward drive and specifically ET-1 an inward drive, acting through mechanisms that do not seem to relate to oxidative stress, inflammatory pathways or major reorganization of the SMC.

Keywords: Vascular Smooth Muscle Cells, Mesenteric Artery, Vasoconstriction, Vascular Remodeling, Inflammation
Introduction

Adequate local tissue perfusion and perfusion reserve depend on proper diameter regulation in vessels forming the resistance vasculature. Such regulation is a continuous process, ranging from tone adaptation in seconds to remodeling of matrix and cellular organization in the course of days to months [1, 2]. The process is impaired in a range of cardiovascular and metabolic pathologies. As an example, inward small artery remodeling occurs in many hypertensive disorders. This strongly reduces vascular reserve and is believed to contribute to end-organ damage in hypertension [3].

The caliber and contractile properties of blood vessels depend not only on the amount of matrix elements and vascular cells, but more crucially so on the way these elements are organized. Remodeling of small arteries towards a new caliber is frequently dominated by a reorganization of existing elements rather than proliferation or apoptosis of cells and synthesis or degradation of matrix element. Indeed, in essential hypertension, inward remodeling has been demonstrated to be eutrophic in many organs [4]. Passive biomechanical properties of small arteries, i.e. radius-tension and pressure-diameter relations, have been used to characterize the remodeling and to infer its consequences for organ resistance [5-7]. Yet, the role of smooth muscle cell (SMC) reorganization during small artery remodeling has received very little attention. The organization of SMC in the wall is however crucial for vascular contractile function and maintenance of a state of vasoconstriction (tone). The capacity for active tension development is known to increase with distension up to a certain optimal diameter. Further distension then causes a fall in tension generating capacity, simultaneously with rapidly increasing passive tension. The shape of this active radius-tension relation is of importance for at least three reasons. Firstly, it contributes to diameter stability in pressurized arteries, where according to the Laplace relationship more tension is needed at higher distension. Secondly, while the maximum diameter of blood vessels is dictated by the matrix organization, its minimum diameter, obtained at full activation, very strongly depends on this active relation, and a shift toward lower diameters allows development and maintenance of vascular spasm. Thirdly, peak active tension occurs at around 90% of the maximum diameter of blood vessels for many branching orders [8, 9], suggesting the existence of mechanisms that tune the architecture of SMC and matrix during development and remodeling. The nature of such mechanisms and their possible role in vascular diseases however are elusive.

Contractile plasticity, defined as a shift in the active strain-tension relation, has been clearly identified in non-vascular SMC [10, 11]. Moreover, contractile plasticity depends on mechanical load and activation, and such dependence has been
inferred in airway and bladder hyperresponsiveness [11, 12]. The purpose of this study is to test to what extent and under which conditions such plasticity occurs in small arteries. In particular we tested the hypotheses that maintained strong activation (tone) in combination with low distension induces a shift of capacity for active force development towards smaller diameters, and that this process is promoted by inflammatory cytokines and depending on oxidative stress. For this purpose, we kept isolated small artery segments mounted on isometric myographs for 1 or 2 days, and determined shifts in active diameter-tension relations after application of a range of stimuli at varying distension. The results depict endothelin-1 as a strong stimulator of shifts of the active curve towards smaller diameters.

**Materials and Methods**

**Animal handling**

Male Wistar rats (Charles River), 8-12 weeks old, were anaesthetized with an i.p. injection of pentobarbital (100 mg/kg) and decapitated. All experiments were approved by the local committee for animal experiments.

**Isolation and mounting of vessels**

Second order mesenteric small arteries were dissected immediately in cold MOPS-buffered physiological solution (composition (mM): 145.0 NaCl, 4.7 KCl, 1.17 MgSO<sub>4</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 2 CaCl<sub>2</sub>, 5.0 glucose, and 2.0 pyruvate; pH 7.35) and segments approximately 2 mm in length were transferred to the organ baths of a four-channel wire myograph (Danish Myo Technology, Arhus, Denmark) filled with physiological salt solution (PSS, composition (mM): NaCl 119,NaHCO<sub>3</sub> 25, KCl 4.7, KH<sub>2</sub>PO<sub>4</sub> 1.18, MgSO<sub>4</sub> 1.17, CaCl<sub>2</sub> 1.6, EDTA 0.027, glucose 5.5) at 37 °C and gassed with 95% air and 5% CO<sub>2</sub>.

**Experimental protocol**

Using standard procedures for wire myography [13]; each segment was first normalized to determine the passive diameter at an equivalent pressure of 100 mmHg (D<sub>100</sub>). Segments were then set at 90% of D<sub>100</sub>. After a 15 min equilibration period, segments were challenged twice with 10 µM noradrenaline and 125 mM K<sup>+</sup>, with equimolar reduction of the Na<sup>+</sup> concentration (KPSS), to test for reactivity.

Diameter-tension relations were determined at 0.4 to 1.2 D<sub>100</sub> in steps of 0.1 D<sub>100</sub> and starting at the lowest distension. For each distension, passive tension was recorded after 5 minutes of relaxation in PSS when tension nearly reached steady state. The vessel was then stimulated with KPSS for 5 minutes and total tension (i.e. sum of active and passive tension) was recorded, followed by washout and 5 minutes of relaxation. The vessel was then stretched and the stimulation was repeated. This
protocol was applied to freshly isolated vessels and was repeated after incubation of the vessels for 16 or 40 hours (see below). High potassium concentrations may result in neurotransmitter release, affecting tension indirectly. To test for this possibility, in a subset of experiments this protocol was repeated after 15 minutes incubation at 0.9 D_{100} with 1 µM capsaicin or 300 µg/ml 6-hydroxydopamine, or in the presence of 0.1 µM prazosin. Repeated KPSS stimulation may result in very early alterations in force capacity that could interfere with the recording of the active diameter-tension relation. In separate time control experiments, the vessels were set to 0.6 D_{100} and repeatedly stimulated with KPSS for 5 min, followed by two washes with PSS and a 10 min relaxation period, for up to 4 hours. This was repeated after preincubation of 6-hydroxydopamine, capsaicin or in the presence of prazosin.

For overnight incubation, vessels were maintained in Leibovitz culture medium containing 1% of a mix of antibiotic-antimycotic solution on the wire myograph at low (0.4 D_{100}) or high (1.1 D_{100}) distension. After finishing the day 0 diameter-tension curve, PSS was replaced by Leibovitz medium. We chose this medium because it is a phosphate-buffered medium that does not require gassing by CO_2. In initial experiments using other media we noted that continuous gassing during overnight incubation resulted in instable tension recordings. Incubation was maintained for 16 hours, or in some cases 40 hours (including media refreshment), after which the buffer was switched back to PSS and a new diameter-tension curve was constructed. ‘In supplemental experiments using sodium nitroprusside or calcium-free solution, we established that these mesenteric vessels in the presence of PSS remain passive during construction of the passive diameter-tension relation before or after culture (Fig. S1, S2). We applied a series of incubation conditions, aimed at studying the role of contractile activation and distension on the changes in passive and active tension. In a first set of experiments, we tested the effect of 10 nM endothelin-1 (ET-1) and 1 µM of the thromboxane mimetic 9,11-dideoxy-1α,9α-epoxy methanoprostaglandin (U46619) as vasoconstrictors, for vessels maintained at 0.4 D100 and 1.1 D100. Next, we investigated the mechanisms involved in ET-1-induced contractile plasticity, by repeating the ET-1 experiments in the presence of pro-inflammatory cytokines (combination of TNF-α, IL-1β and IFN-γ, 10 ng/ml), transforming growth factor beta (TGF-β, 5 ng/ml), superoxidase dismutase (SOD), 100 U/ml combined with catalase (cat), 1000 U/ml), 200 U/ml pegylated superoxide dismutase (PEG-SOD) together with 1000 U/ml pegylated catalase (PEG-cat) or 10% heat-inactivated fetal calf serum (FCS).

For confocal imaging mesenteric small arteries were dissected in MOPS buffer at 4 °C and incubated for 1h with 10^{-5} M of the vital nuclear dye Hoechst 33342. Vessels were mounted on an isometric wire myograph suitable for confocal imaging (Danish Myo Technology, Denmark), and imaged with a Leica TCS SPII confocal microscope. Vessels were acclimatized for 30 minutes, normalized, set to 0.9D_{100} and
tested with KPSS twice. Confocal optical sections were acquired with a dry objective (x40) by averaging 8 frames at z steps of 1 µm. Hoechst 33342 was excited at 405 nm, and the emission was recorded at 410-510 nm. These 3D data on nuclear location in the vessel wall were obtained at distensions of 0.4, 0.6, 0.8 and 1.1D₁₀₀ in relaxed vessels and at 0.4D₁₀₀ in KPSS activated vessels. After finishing the set for day 0, MOPS buffer was replaced by Leibovitz culture medium including 10 nM ET-1 or 1 µM U46619 as vasoconstrictors. Vessels were then incubated on the wire myograph at 0.4 D₁₀₀ distension for 12 hours, making sure that the wire position on the microscope was maintained, and the protocol was repeated for day 1.

**Chemicals**

ET-1 was purchased from Bachem, the cytokines were obtained via Tebu-bio. Leibovitz medium was obtained from GIBCO Invitrogen (Breda, The Netherlands). FCS was obtained from BioWhitaker (Verviers, Belgium). All other chemicals were purchased from Sigma. Capsaicin was dissolved in chloroform. Prazosin was dissolved in dimethyl sulfoxide (DMSO) and the other chemicals were dissolved in deionized water.

**Data analysis**

Active tension was calculated by subtracting the passive tension from the total tension. For the active diameter-tension relations, the response to KPSS was determined at two points: Ta₁ refers to the peak tension during the 5 minutes stimulation, while Ta₂ reflects the steady state tension taken at 5 minutes (Fig 5.1).

During incubation, a small and slow drift in the force recording occurred. Active tension during the overnight incubation was therefore determined by subtracting an interpolated passive component from the recorded total tension. This interpolation was based on the passive tension recorded in PSS just before and after application of the overnight incubation medium (Fig 5.2). Active tension was averaged per 8 hours during the 16 or 40 hour culture period and was normalized to the maximum as determined at 0.9 D₁₀₀ on day 0.
Active diameter-tension relations were compared at day 1 or 2 versus day 0 in a paired fashion. In addition, for all determined relations, the diameter corresponding to maximum tension development (D\text{opt}) was calculated using parabolic curve fitting of the 5 highest points in the active diameter-tension relation. Furthermore, the ratio of tension generating capacity at 0.4 D\text{100} and 1.0 D\text{100} (T_{0.4/1.0}) was calculated to characterize shifts in active diameter-tension relations.

Two blinded observers measured the lengths of the SMC nuclei in 3D, using dedicated software in Matlab. The program allowed navigation through the volumetric image stack and selection of the two most distant points in 3D on each nucleus. Nuclear length was then determined from the Euclidian distance between these points. The observers selected all clearly identifiable smooth muscle nuclei in the image stacks. These data were processed by calculating the average lengths and by comparing length and position of individual nuclei at the start and end of the experiment.

Figure 5.1: Example of changes in contraction dynamics after overnight incubation with ET-1 at low distension (0.4 D\text{100}), for a vessel stimulated with 125 mM K+ at 0.7 D\text{100}. Shown are transient (T\text{a}_1) and steady state (T\text{a}_2) responses to KPSS at the start (a) and end (b) of 16 hours incubation. The transient (T\text{a}_1) response disappeared after 16-hour incubation, while a large increase in steady state tension development (T\text{a}_2) occurred for this incubation condition. Active tension was calculated by subtracting the passive tension from the total tension, as illustrated by the arrows. The subsequent figures indicate this active tension.
Figure 5.2: Examples of vascular tension recorded during 16 hours incubation at low and high distension in the presence of 10 nM ET-1 or 1 μM U46619. The recorded tension is the sum of active and passive tension; the dotted lines indicate the passive component as determined from interpolation of the values recorded before and after incubation.
**Statistics**

Data are expressed as mean ± SEM and are compared by one-way or two-way analysis of variance (ANOVA) with Bonferroni post-hoc tests or paired t-tests as appropriate, using SPSS Statistics version 18.0. Differences were considered statistically significant at p < 0.05; n values represent the number of rats used in the studies.

**Results**

**Characteristics of active diameter-tension relations**

Repeated stimulation with KPSS at 0.6 $D_{100}$ revealed that transient tension development ($Ta_1$) increased significantly between first and second stimulus, remaining constant for another 12 stimuli (n=3). Steady state tension ($Ta_2$) did not significantly increase during the repeated stimulations (Fig 5.3). These control experiments thus indicate that SMC plasticity in the course of constructing a diameter-tension relation is very limited. We tested to what extent the evolution of $Ta_1$ for multiple stimulations depends on release of neurotransmitters. However, the patterns of force development did not change after pretreatment of the vessels with 300 µM 6-hydroxydopamine (n=3) or 1 µM capsaicin (n=9), or during incubation with 0.1 µM prazosin (n=4), arguing against contributions of peripheral adrenergic and sensory nerves in the difference between $Ta_1$ and $Ta_2$ (data not shown). The difference between $Ta_1$ and $Ta_2$ depended strongly on the distension (Fig 4.4A). This difference disappeared almost completely after overnight incubation under all tested conditions in the study, as demonstrated in Fig 5.4B-C for vessels cultured under control conditions. Therefore, below we only consider the steady state active diameter-tension curves ($Ta_2$). We tested whether $Ta_2$ changes after maintenance of the vessels on the wire myograph at low (0.4 $D_{100}$) or high distension (1.1 $D_{100}$). Fig. 3D-E depicts these data for 16 hours. As can be seen, vessels incubated at low distension showed a minor increase in tension at low distensions (P<0.05 for 0.4, 0.5 and 0.7 $D_{100}$), while the vessels incubated at high distension lost some force capacity at 0.4 $D_{100}$. Indeed, $D_{opt}$ did not significantly change for the vessels incubated at 0.4 $D_{100}$ but shifted significantly outward for the vessels incubated at high distension (Table 5.1). Overall, $Ta_2$ did not greatly change overnight.

**ET-1 but not U46619 induces inward SMC reorganization**

Tension development was monitored during the entire incubation period. Figure 5.2 depicts examples of tension at low and high distension in the presence of contractile stimuli and control. These examples indicate that vessels stimulated at
Figure 5.3: Transient and steady-state responses to repeated stimulation with KPSS. Ta₂ increased slightly and not significantly during the repeated stimulations, i.e. covering the time needed for constructing the diameter-tension relations.

Figure 5.4: Comparison of Ta₁ and Ta₂ responses to KPSS at the start of a 16-hour incubation period (a; n = 38) and after maintenance of the vessel for 16 h at 0.4 D₁₀₀ (b; n = 19) and 1.1 D₁₀₀ (c; n = 19). In order to compare Ta₂ before and after culture, part of these data are replotted in d (0.4 D₁₀₀, n = 19) and e (1.1 D₁₀₀, n = 19). * p < 0.05 [paired t tests: Ta₁ vs. Ta₂ (a–c); day 0 vs. day 1 (d, e)].
1.1D$_{100}$ with either ET-1 or U46619 develop and maintain activity, characterized as more or less regular rhythmicity. Slight rhythmic active tension was also seen in some of the control vessels at 1.1 D$_{100}$. In contrast, at the start of the incubation, vessels at 0.4 D$_{100}$ did not reveal active tension. While the control and U46619-incubated vessel remained passive, small oscillations started to develop in the ET-1 incubated vessel at this distension. Fig. 5.5 summarizes these data. Despite the oscillations in the presence of ET-1 at 0.4D$_{100}$, average active tension remained very low in this group. No active tension occurred in U46619-stimulated vessels at this distension. For incubation at 1.1 D$_{100}$, vessels stimulated with U46619 developed significantly more active tension than either the control or ET-1 stimulated vessels (one-way ANOVA with Bonferroni post-hoc tests, p<0.05). Peak tension during the oscillations was also higher for U46619 as compared to ET-1 (data not shown). Despite the common observation that ET-1 induced tension does not readily fall back after wash-out in acute experiments, we found that wash-out of ET-1 after overnight exposure resulted in full relaxation within seconds (data not shown).

Figure 5.6A-D shows Ta$_2$ after 16 hours incubation. For vessels incubated at low distension, a clear leftward shift of Ta$_2$ was found for ET-1, but not in vessels incubated with U46619. This shift was associated with significant lower D$_{opt}$ and larger T$_{0.4/1.0}$ (Table 5.1). In vessels incubated at high distension, D$_{opt}$ did not change. However, U46619-incubated vessels showed significant reduction of T$_{0.4/1.0}$ while ET-1 incubation prevented this shift (Table 5.1). These overnight shifts in individual groups

![Graphs showing mean active tension during incubation](attachment:graphs.png)
resulted in significant dependence of day 1 $D_{\text{opt}}$ and $T_{0.4/1.0}$ on type of stimulus (control day 1, ET-1, U46619, $p<0.05$) and incubation distension (0.4, 1.1$D_{100}$, $p<0.05$), without a significant interaction between these effects for both parameters (two-way ANOVA test). Also in this overall test including both distensions, ET-1 induced a significant leftward shift as compared to either the control or U46619 (two-way ANOVA with Bonferroni post-hoc tests, $p<0.05$).

Figure 5.6: $T_{a2}$ diameter-tension relation before and after 16 h of incubation with 10 nM ET-1 (a, b), 16 h of incubation with 1 μM U46619 (c, d) and 40 h of incubation with ET-1 (e, f) at 0.4 and 1.1 $D_{100}$. *$p < 0.05$, day 0 vs. day 1 or day 2, paired t tests.
ET-1 induced SMC reorganization is a robust response

The above results indicate that shifts in $T_{a_2}$ do not only depend on the distension and activation during incubation, but also on the nature of the vasoconstrictor, with ET-1 causing less average overnight tension but far more contractile plasticity than U46619. ET-1 is known to have a range of effects on SMC in addition to contraction, including generation of oxidative stress. However, 100 U/ml superoxide dismutase (SOD) together with 1000 U/ml catalase did not prevent the leftward shift of $T_{a_2}$ (Table 5.1). In particular, we tested whether the added intracellular effects would better prevent the ET-1 induced plasticity. However, 200 U/ml pegylated superoxide dismutase (PEG-SOD) together with 1000 U/ml pegylated catalase (PEG-cat) did not prevent the leftward shift of $T_{a_2}$ (on-line supplement Fig. S3). Thus, $T_{0.4/1.0}$ still increased significantly in the presence of the radical scavengers (Table 5.1).

Growth factors and pro-inflammatory cytokines may also induce plasticity [14, 15]. We tested whether either TGF-β or the combination of TNF-α, IL-1β and IFN-γ could mimic the effect of ET-1 at 0.4 $D_{100}$. However, incubation with these cytokines did not mimic the ET-1 response. The combined inflammatory cytokines did not augment the response to ET-1. In a third experiment, we incubated vessels with FCS, based on the previous observation of FCS-induced tone and inward remodeling in cannulated small mesenteric arteries [6]. However, also this incubation condition did not induce SMC contractile plasticity. No active tension occurred in TGF-β or the combination of TNF-α, IL-1β and IFN-γ or FCS-stimulated vessels at 0.4 $D_{100}$ during 16 hours incubation (data not shown). We finally tested whether the ET-1 induced shift in $T_{a_2}$ is maintained or augmented during longer incubation. After 40 hours of incubation, peak active tension development induced by KPSS was unchanged, underlining the long-term viability of these wire-mounted vessels. Moreover, the inward shift of $T_{a_2}$ became more dominant (Fig 5.6E), with a significantly higher $T_{0.4/1.0}$ after 40 hours as compared to 16 hours incubation. In contrast, after such stimulation for 40 hours at 1.1 $D_{100}$, the $T_{a_2}$ relation remained unchanged (Fig. 5.6F).

Characteristics of passive diameter-tension relations

Vascular matrix remodeling has been shown to occur in cultured vessels, and is characterized by a change in the passive diameter-tension relation [6, 7]. In the current study, in most of the groups a small (~2-3%) though consistent and significant outward remodeling occurred (Fig 5.7, Table 5.1). Vessels incubated with ET-1 did not show this outward remodeling (Fig 5.7C-D). After 40 hours incubation in the presence of ET-1 at low distension, significant inward matrix remodeling occurred, reducing $D_{100}$ to 0.93±0.02 of its original value. This normalized passive diameter at the end of culture was significantly different from that in all other groups maintained at low distension (one way ANOVA with Bonferroni post-hoc tests, p<0.05).
Figure 5.7: Passive diameter-tension relation before and after 16 h of incubation (control; a, b), 16 h of incubation with 10 nM ET-1 (c, d) and 16 h of incubation with 1 μM U46619 (e, f) at 0.4 and 1.1 \( D_{100} \). *p < 0.05, day 0 vs. day 1 or day 2, paired t tests.
ET-1 and U46619 induces nuclei lengthening

The differential effect of ET-1 and U46619 on Ta₂ may well relate to re-organization of the SMC architecture. However, a quantification of such changes is far from trivial for the intact vessel. As a first approach for addressing this question, we considered that alterations in the SMC cytoskeletal arrangements are likely to affect the nuclear length and its dependence on vascular distension. Confocal imaging of wire-mounted vessels provided excellent visualization of smooth muscle cell nuclei (Fig. 5.8). Nuclear length in relaxed vessels was found to depend on vascular distension. However, this relation was only modest, with a 175% increase in vascular diameter (from 0.4 D₁₀₀ to 1.1 D₁₀₀) resulting in a ~30% increase in nuclear length. Moreover, overnight incubation with neither U46619 nor ET-1 changed this relation (Fig. 5.9A, B, paired t-tests, p=N.S.). Slack length of mesenteric vessels is around 0.5 D₁₀₀ [16]. Therefore, activation at 0.4 D₁₀₀ would not be completely isometric, providing room for changes in nucleus length. For that reason we also determined the nuclear length after stimulation with KPSS at

Figure 5.8: Positions of nuclei at 0.8 D₁₀₀ before (green) and after (red) 12 h of incubation with 10 nM ET-1 (a) and 1 µM U46619 (b). Local movements of nuclei were observed for either constrictor. The two arrows in each image indicate opposite movement of two nuclei, indicating that such movement is a local event rather than a global shift or limited registration of the images before and after culture.
this distension. In this activated state, nuclear length at 0.4 was substantially and significantly larger at day 1 as compared to day 0 for both ET-1 and U46619-incubated vessels (one way ANOVA with Bonferroni post-hoc tests, p<0.05).

It has been suggested that repositioning of individual SMC within the wall is an early event in remodeling after maintained strong activation [17]. The repositioning of individual nuclei would provide a strong indication for such mechanisms as basis for the shift in $T_{a2}$. We found that the majority of nuclei maintained their position after overnight incubation, although a few clearly moving SMC were observed for either constrictor (Fig. 5.8A, B). While we could not quantify these local movements, there was no obvious difference in this respect between both constrictors.

**Figure 5.9:** Passive nuclear length-vascular diameter relation and nuclear length during stimulation with KPSS at 0.4 $D_{100}$ before and after 12 h of incubation with 10 nM ET-1 (a) and 1 $\mu$M U46619 (b) at 0.4 $D_{100}$ (n = 5).

**Table 5.1:** Characterization of the steady-state active diameter-tension relation.
Discussion

Main results
We showed in this study that the capacity for active tension generation in rat mesenteric small arteries is malleable. Maintained stretch shifted the active diameter-tension relation to larger diameters while this was prevented by endothelin-1 (ET-1) but not U46619. At low distension, prolonged activation by ET-1 but not U46619 shifted this relation to smaller diameters, possibly forming an early event in inward remodeling. The ET-1 mediated change in tension generation was not related to oxidative stress. In addition, TGF-β, combined inflammatory cytokines (TNF-α, IL-1β and IFN-γ) or presence of serum failed to induce similar SMC adaptation. These data together suggest that ET-1 shifts the capacity for active tension development towards smaller diameters through mechanisms that are not clearly related to oxidative stress, inflammatory pathways or major reorganization of the SMC.

Mechanisms of contractile plasticity
Some initial observations on contractile plasticity induced by ET-1 were made by Bakker et al. [18]. This inspired us to test a range of different conditions, including a comparison of vasoconstrictors. We found significant effects of both type of stimulus (control day 1, ET-1, U46619) and distension (0.4, 1.1 D_{100}) on both D_{opt} and T_{0.4/1.0} without significant interaction. Thus, active tension-diameter relations shifted relatively more leftward for all the stimuli after incubation at 0.4 D_{100} as compared to 1.1 D_{100} (Fig. 5.3D-E and Fig. 5.7). In addition, ET-1 induced more leftward shift than the other stimuli at both distensions, and also this can be appreciated from the figure 5.7. These data suggest that maintained distension may in general be a drive for adaptation of the active radius-tension relation towards larger diameters, while vasoconstrictor-specific mechanisms drive the inward shift of this relation. Future work needs to be done to test such concepts for other stimuli and other vascular beds.” The current observation that this behavior is induced by specifically ET-1 raises the question which mechanisms can be involved. Two sequential processes are relevant here: the intracellular signaling events, and their effects on structural changes in the SMC.

As concerns signaling, to the best of our knowledge no information is available on regulation of vascular smooth muscle contractile plasticity by ET-1. However, the current SMC contractile plasticity (affecting the diameter for optimal active tension) may be an early process in vascular inward matrix remodeling (reducing the passive diameter). Some of the identified processes in matrix remodeling may relate to the plasticity. Inward remodeling of small arteries is associated with
combined vascular constriction and inflammation in various pathological events, including hypertension and altered flow [14, 19]. Cytokines are powerful regulators of inflammatory responses. Some of the cytokines that have found to be up-regulated in constrictive remodeling include TNF-α, IL-1β and IFN-γ [14, 20]. Such cytokines have also been implicated in ET-1 signaling through both upstream and downstream effects [21]. Therefore, we tested whether these combined pro-inflammatory cytokines could mimic ET-1 in inducing contractile plasticity, but found no evidence for such induction nor for augmentation of plasticity response to ET-1.

It is generally accepted that TGF-β plays a role in inward arterial remodeling, inducing matrix hypertrophy and a shift of the phenotype of vascular cells towards contractile SMC [15, 22]. However, in the current experiments this growth factor induced neither clear matrix remodeling nor induction or augmentation of SMC contractile plasticity.

We next considered oxidative stress (ROS) as another possible mediator in smooth muscle contractile plasticity, based on the observation that ET-1 induces oxidative stress [23]. Indeed, Amiri et al. [24] have shown that overexpression of ET-1 increases ROS and causes structural remodeling and endothelial dysfunction of resistance arteries in mice. ET-1 effects on matrix remodeling may occur through combined prolonged tone and formation of ROS [25]. Superoxidase dismutase and catalase reduce ROS production and constrictive remodeling of iliac arteries [26]. However, in the current study, incubation with these antioxidant enzymes did not prevent the inward shift of active tension capacity by ET-1.

Both U46619 and ET-1 regulate contraction of smooth muscles through G-protein coupled receptors. Thrombrane receptors and ET-1 receptors show much overlap in signaling, raising the question why only ET-1 induced the plasticity. Possibly, this could be the result of divergences downstream of receptor signaling. Thus, Gaq/11 is dominating for ET-1 and Ga12/13 for U46619, leading to activation of different pathways [27]. The differences in activity transients during the overnight incubation may be reminiscent of this. Whether these differences are indeed relevant for the leftward shift in contractile capacity remains to be determined.

**Cell organization during contractile plasticity**

While we were not able to identify the specific ET-1 induced signaling events leading to contractile plasticity, we believe that such plasticity is based on reorganization of the SMC structure or of their embedding in the vascular wall. Similar processes, involving reshaping of the contractile apparatus and cytoskeleton, have been identified in airway and bladder SMC [28, 29]. Based on evidence from airway SMC, Bosse et al. [10] proposed that additional contractile units in series are formed during maintained lengthening of the cell, maintaining optimized overlap between actin and myosin filaments and causing Dopt to increase towards the new habitual cell length. In analogy,
the inward shift of $D_{opt}$ in the current vessels incubated with ET-1 at 0.4D$_{100}$ could be related to a reduction of the number of contractile units in series. Data from Martinez-Lemus et al. [17] indicate increased overlap and relengthening of SMCs in rat cremaster muscle arterioles subjected to deep constriction by noradrenaline for 4 hours.

A full characterization of changes in SMC architecture and length is experimentally difficult and was beyond the scope of the current work. However, we argued that large alterations in ultrastructure and especially relengthening of SMC should lead to an altered relation between nuclear length and vascular diameter. We used nuclear staining because this is straightforward and provides 3D images with excellent contrast. We found that under passive conditions this relation remained unchanged (Fig. 5.9). The very limited dependence of nuclear length on vascular distension indicates large local differences in cytoskeletal deformation and reorganization of peri-nuclear and distal cell locations during stretch. Activation at 0.4 D$_{100}$ caused a large reduction of nuclear length at day 0. While the vessel may not have been fully isometric at this low stretch, a comparison with the nuclear length under passive conditions reveals that the nuclear shortening cannot be attributed to possible tightening of the vessel around the wires. Therefore, also during activation there seem local differences in cytoskeletal deformation and reorganization. After overnight culture at low distension, such nuclear length reduction upon stimulation with KPSS was virtually abolished (Fig. 5.9). Speculatively, a possible explanation of these findings is that the cytoskeletal architecture did not change overnight, reflected by the unchanged passive vascular diameter-nuclear length relation, but that extra tension-generating capacity has developed in series with the nuclei, keeping them relatively extended during activation. In contrast to the active diameter-tension relations, these effects on nuclear length were not different between the two agonists. Therefore, while these data provide some indication of cellular rearrangement, it is clear that much more work will be needed to correlate these structural and functional data.

Critique on used methodology

We recorded the active diameter-tension relations by isometric testing at increasing diameters. Contractile plasticity might be fast enough to already allow adaptation during this process. Indeed, initial adaptation to length has been shown to occur already after only a few stimuli in the course of minutes in rabbit femoral and carotid arteries [30, 31]. We found such fast length adaptation between the first and second stimulation (Fig. 5.2). However, this was only the case for the phasic part of the response (Ta$_1$). Moreover, for the remaining hours the Ta$_1$ and Ta$_2$ responses remained unchanged, and a clear shift of the active diameter-tension relations was only found after overnight incubation. These data point towards the co-existence of adaptive
processes at completely different time scales, with possibly different underlying mechanism and physiological roles.

It is clear that recording nuclear lengths and their changes upon distension or activation is a very limited and indirect way for assessing changes in cellular organization, and these data can only serve to generate initial hypothesis for future work. Yet the data were collected by two blinded observers, supported by imaging software that acknowledged the 3D nature of the wall. Considering the heterogeneity in the vascular wall, additional automated software could help collecting more extensive datasets on distribution of lengths and positions of cellular structures.

Implications

The current contractile plasticity has several implications for vascular function. First, the minimum diameter that can be obtained by activated vessels is greatly influenced by the active diameter tension relation. As an example, based on the current data and assuming the Laplace relation, a vessel subjected to 100 mmHg would be able to constrict to $0.62 D_{100}$ in control, while this value reduces to $0.35 D_{100}$ after overnight incubation with ET-1 at low distension, and increases to $0.64 D_{100}$ after overnight maintenance at high distension without contractile activation. Second, in vivo, such changes in minimum attainable diameter could be self-enhancing: a deeply constricted vessel will induce more tension-generating capacity at low distension, allowing again deeper constriction, and so on. Third, this process would eventually also affect the matrix remodeling towards a smaller diameter, based on previous data showing that deep tone and small distension drive inward remodeling [5]. Therefore, changes or differences in contractile plasticity may be expected to affect the design and function of the resistance vasculature. Evidence for a role of SMC reorganization in vascular development and remodeling comes from the observation that SMC lengths are by and large equal for vessels ranging orders of magnitude in diameter [32]. While there is no information that contractile plasticity indeed plays a role in pathologies such as hypertension or cerebral artery spasm after sub-arachnoid hemorrhage, for airway smooth muscle a role in asthma has been clearly identified [29, 33], and the current data make clear that the plasticity can be modulated by agents such as ET-1 that have been indicated in cardiovascular pathologies.

Conclusions

In this study several conditions were tested to elucidate the mechanisms of contractile plasticity in small artery remodeling. We found that such plasticity indeed occurs in small arteries, albeit at a slower pace than seen in non-vascular smooth muscle, and specifically in the presence of ET-1. Oxidative stress, inflammatory pathways or major reorganization of the SMC seem not to be involved in contractile plasticity. Changes in
force-generating capacity preceded matrix remodeling. While many aspects of the causes and mechanisms of such adaptation remain to be addressed, we believe that contractile plasticity is an essential element of vascular remodeling, and possible changes in this process could be involved in pathological remodeling and affect vascular structure and integrity.

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References


Supplements

**Figure S1:** Passive diameter-tension relations in PSS or PSS+Sodium Nitroprusside (10 µM) at the end of 40 hours incubation with ET-1 at 0.4 and 1.1 D_{100}, n=3. These data show that vessels remain passive in PSS at the end of culture.

**Figure S2:** Passive diameter-tension relations in PSS or Ca-Free solution at day 0 and day 1, n=6.
Figure S3: Steady state ($T_{a2}$) diameter-tension relation before and after 16 hours incubation with 10 nM ET-1 or 10 nM ET-1 together with 200 U/ml pegylated superoxide dismutase (PEG-SOD) and 1000 U/ml pegylated catalase (PEG-cat), n=6. In addition to the statistics in Table 1 of the main manuscript, we tested for both $D_{opt}$ and $T_{0.4/1.0}$ whether the shifts after overnight incubation in ET-1 were significantly suppressed. This was not the case (ttests of ET-1 versus ET-1+PEGSOD/PEGCAT, ET-1 versus SOD/CAT, ET-1 versus pooled SOD/PEGSOD data: p=N.S. for either parameter). These data show that ET-1 induced plasticity is not suppressed by pegylated or non-pegylated SOD and catalase.