

Small Artery Remodeling and Erythrocyte Deformability in L-NAME-Induced Hypertension: Role of Transglutaminases

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Key Words

Vascular remodeling · Transglutaminase · Coagulation factor XIII · Resistance arteries · Hypertension · Nitric oxide · Knockout mice · Erythrocyte deformability

Abstract

Background: Hypertension is associated with inward remodeling of small arteries and decreased erythrocyte deformability, both impairing proper tissue perfusion. We hypothesized that these alterations depend on transglutaminases, cross-linking enzymes present in the vascular wall, monocytes/macrophages and erythrocytes. **Methods and Results:** Wild-type (WT) mice and tissue-type transglutaminase (tTG) knockout (KO) mice received the nitric oxide inhibitor N ω -nitro-L-arginine methyl ester hydrochloride (L-NAME) to induce hypertension. After 1 week, mesenteric arteries from hypertensive WT mice showed a smaller lumen diameter ($-6.9 \pm 2.0\%$, $p = 0.024$) and a larger wall-to-lumen ratio ($11.8 \pm 3.5\%$, $p = 0.012$) than controls, whereas inward remodeling was absent in hypertensive tTG KO mice. After 3 weeks, the wall-to-lumen ratio was increased in WT ($20.8 \pm 4.8\%$, $p = 0.005$) but less so in tTG KO mice ($11.7 \pm 4.6\%$, $p = 0.026$), and wall stress was normalized in WT but not in tTG KO mice. L-NAME did not influence expression of tTG or an alternative transglutaminase, coagulation factor XIII (FXIII). Suppression of FXIII by macrophage depletion was

associated with increased tTG in the presence of L-NAME. L-NAME treatment decreased erythrocyte deformability in the WT mice (-15.3% at 30 dynes/cm², $p = 0.014$) but not in the tTG KO mice. **Conclusion:** Transglutaminases are involved in small artery inward remodeling and erythrocyte stiffening associated with nitric oxide inhibition-related hypertension.

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Introduction

Hypertension is associated with a structural reduction in lumen diameter of small arteries [1–3], resulting in reduced maximal capacity for flow. Pressure-induced inward remodeling may thus generate a vicious circle that maintains high levels of arterial pressure [4]. Only a small decrease in lumen size is needed to induce a rather large loss in capacity for tissue perfusion, because flow is proportional to the 4th power of diameter. Indeed, inward resistance vessel remodeling is associated with an increased risk for cardiovascular ischemic events [5]. A further factor that could hamper local tissue perfusion in hypertension is erythrocyte stiffening [6], since stiff red blood cells may less easily pass through the microcirculation [7].

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Transglutaminases (Tgases) are calcium-activated enzymes that covalently cross-link structural proteins [8]. In hypertension, higher than normal calcium concentrations are found in both smooth muscle cells [9] and erythrocytes [10], potentially increasing Tgase activity. In circulating monocytes of hypertensive subjects, Tgase activity is markedly increased [11]. We previously found that matrix reorganization and inward remodeling of small arteries *in vitro* depend on Tgase activity [12]. Thus, we showed that small porcine coronary arteries and rat skeletal muscle arteries remodel inwardly *in vitro* upon exposure to exogenous guinea pig liver Tgase. Furthermore, manipulation of endogenous Tgase activity and expression modulated the remodeling of small arteries kept in organoid culture. In another *in vitro* study, we observed that flow prevented inward remodeling in healthy coronary small arteries in a nitric oxide (NO)-dependent way [13]. This observation is consistent with an NO-dependent inhibition of Tgase cross-linking activity as shown by others [14]. In a recent study, we have shown that tissue-type transglutaminase (tTG) knockout (KO) mice have delayed low-flow-induced inward remodeling [15].

Based on these findings, we hypothesized that tTG plays a key role in the inward remodeling of small arteries and loss of erythrocyte deformability following reduction of the NO availability. We therefore exposed wild-type (WT) and tTG KO mice to hypertension induced by the NO synthase inhibitor N ω -nitro-L-arginine methyl ester hydrochloride (L-NAME), and then assessed remodeling in mesenteric small arteries (after 1 and 3 weeks) and erythrocyte deformability (after 3 weeks). Monocytes/macrophages are known to accumulate in the vascular wall as a consequence of L-NAME treatment [16], possibly forming a source of both tTG and an alternative Tgase, coagulation factor XIII (FXIII) [17]. Therefore, we also aimed to assess the effect of macrophage depletion on tTG and FXIII expression and remodeling after 1 week of exposure to L-NAME in WT mice.

Methods

Mice

Mice deficient in tTG (tTG KO) and WT littermates, having a mixed Svj129/C57Bl6 background, were originally obtained from G. Melino, University of Rome Tor Vergata, Rome, Italy, and were then bred at the local animal facility. PCR analysis confirmed the genotype of tTG KO and WT animals. Mice were fed *ad libitum* and had free access to drinking water. Male and female mice were used for experiments at the age of 4–6 months. All experiments were approved by the local committee for animal experiments.

Experiment Design

Hypertension was induced by inhibition of NO production. L-NAME (Sigma, Steinheim, Germany) was dissolved in the drinking water (1 mg/ml) and given for a period of 1 or 3 weeks. A subset of WT mice received liposome-encapsulated clodronate (clodr-lip) to suppress the peritoneal monocyte/macrophage population. In order to cover the 1 week of L-NAME exposure, clodronate-containing liposomes were injected in 3 doses of 0.2 ml (2 days before the onset of L-NAME treatment, at the onset and 3 days after starting L-NAME treatment). Clodronate-containing liposomes were prepared as previously described [18]. Clodronate was a gift of Roche Diagnostics GmbH, Mannheim, Germany. For each intervention group, a matched control group was built, consisting of littermates of the treated animals.

At the end of the L-NAME treatment, mice were anesthetized with a mixture of ketamine 1.25 mg/kg (Nimatek; Eurovet, Blandel, The Netherlands), medetomidine 0.2 mg/kg (Domitor; Orion, Espoo, Finland) and atropine sulfate 0.5 mg/kg (Pharmachemie, Haarlem, The Netherlands). The carotid artery was cannulated with a polyethylene tube filled with phosphate-buffered physiological saline solution containing heparin 20 U/ml (Leo Pharma, Breda, The Netherlands), connected to a pressure transducer. Mean arterial pressure, heart rate and respiratory rate were determined. The mesentery was then excised and placed in cold calcium-free physiological solution buffered with 3-(N-morpholino)propane-sulfonic acid (MOPS; Sigma). Isolated mesenteric arteries (first order) were mounted in a pressure myograph as described previously [19] and a pressure-diameter relationship was determined in calcium-free MOPS buffer in the presence of papaverine 0.1 mM (Sigma). From each mouse, two arteries were isolated and data were averaged. Wall-to-lumen ratio and wall cross-sectional area were calculated from the measured values for the inner and outer diameter at 80 mm Hg. Fully dilated arteries of control animals had an inner diameter which ranged from 192 to 339 μ m and the wall-to-lumen ratio ranged from 0.084 to 0.125 at 80 mm Hg.

Immunohistochemistry

After measuring dimensions in the pressure myograph, arteries were fixed in 4% formalin. Following membrane permeabilization using pure cold acetone and antigen retrieval with trypsin (Gibco, Paisley, UK), vessels were incubated overnight with 33 μ g/ml FITC-coupled rat anti-mouse CD68 antibody (MCA1957F, Serotec, Oxford, UK). Nuclei were stained with ethidium bromide. Vessels were afterwards washed with phosphate-buffered saline, embedded in Tissue-Tek (Sakura, Zoeterwoude, The Netherlands) on microscope glass and scanned over their length using a confocal microscope (Leica, Heidelberg, Germany) to count monocytes/macrophages. Cells were counted by three experienced observers who were blinded to the intervention and results were averaged.

Real-Time Polymerase Chain Reaction

Real-time PCR was performed on small mesenteric arteries isolated using previously described protocol, primers and amplification cycles [15]. Briefly, mesenteric small arteries were dissected in MOPS buffer at 4°C and kept in 1 ml Trizol (Gibco) for RNA extraction. cDNAs were subsequently synthesized using an Omniscript reverse transcriptase kit (Qiagen, Venlo, The Netherlands). Real-time PCR was performed in iQTM SYBR[®] Green Supermix buffer (Invitrogen, Breda, The Netherlands) using a MyCycler (Bio-Rad, Hercules, Calif., USA) thermal cycler.

Table 1. Mean arterial blood pressure, heart rate and hematocrit in the various groups

Treatment	Mean arterial pressure, mm Hg		Heart rate, beats/min		Hematocrit, %	
	WT	KO	WT	KO	WT	KO
Control	115.1 ± 3.2 (18)	116.6 ± 3.0 (14)	452 ± 34 (10)	515 ± 26 (13)	44.7 ± 1.9 (14)	44.2 ± 1.4 (10)
1 week L-NAME	141.9 ± 3.7** (15)	137.2 ± 5.1* (6)	546 ± 21 (7)	468 ± 37 (6)	-	-
1 week L-NAME + clodr-lip	143.3 ± 3.3** (7)	-	-	-	-	-
3 weeks L-NAME	139.6 ± 4.8** (8)	147.4 ± 5.6** (7)	533 ± 50 (6)	566 ± 40 (7)	44.7 ± 1.6 (9)	42.2 ± 0.9 (9)

* $p = 0.01$; ** $p < 0.001$ versus control [ANOVA, Dunnett (2-sided) correction]. There was no significant difference between WT and tTG KO mice in any of the study groups (unpaired Student's t test). Figures in parentheses indicate number of animals per group.

Erythrocyte Deformability

Erythrocytes deform under the influence of externally applied shear stresses, acquiring shapes that can be fitted to ellipses [20]. The length (L) and the width (W) of the fitted ellipse yield the elongation index (EI), calculated as: $EI = (L - W)/(L + W)$. The effect of shear stress on erythrocyte shape was assessed using a laser-assisted optical rotational cell analyzer (LORCA; Mechatronics, Hoorn, The Netherlands). Erythrocyte deformability was measured in arterial blood from mice (WT and tTG KO) treated for 3 weeks with L-NAME and their controls. Blood was harvested on anticoagulant (K₂EDTA, BD Microtainer, New Jersey, N.J., USA) and further diluted (based on measured hematocrit) in a highly viscous medium (31 mPa · s), consisting of 0.14 mM of polyvinylpyrrolidone in phosphate-buffered saline, to obtain a final volume fraction of erythrocytes of 2×10^{-3} l/l. One milliliter of this suspension was injected in the gap between the fixed inner cylinder and the rotating, concentric, outer cylinder of the automated LORCA ektacytometer and exposed to shear stress values between 0 and 30 Pa. For each shear stress step, data from 50 erythrocytes were used for calculating an average elongation index, using the LORCA 2.1 software.

Data Analysis

Results on vascular structure and real-time PCR are expressed as percentage change relative to the average value measured in untreated littermates. For statistics, we normalized the values to the littermate controls, and applied a one-way ANOVA and Dunnett post hoc test on three groups: the controls (100% but with a within-group variation), the 1-week and 3-week L-NAME groups. Results are presented as mean ± SEM; error bars are SEM. Statistical tests were performed using SPSS 12.0.2 for Windows.

Results

L-NAME Effect on Blood Pressure

Mean arterial pressure was determined in 75 animals (table 1). Treatment with L-NAME induced a significant increase in blood pressure within 1 week in both WT and

KO mice. The L-NAME effect on blood pressure was persistent after 3 weeks. Treatment with clodr-lip had no influence on the hypertensive effect of L-NAME. Heart rate was not significantly influenced by L-NAME treatment in WT and tTG KO mice. There were no significant differences in blood pressure or heart rate between WT and tTG KO mice in any of the groups.

Hypertension and Remodeling

Structural effects were assessed as relative changes of the inner diameter and wall-to-lumen ratio in the intervention groups as compared to the corresponding controls. Hypertension induced faster and larger structural changes in the WT than in the tTG KO vessels. Thus, after 1 week of L-NAME treatment, mesenteric arteries of WT mice showed a significant decrease in the lumen diameter (fig. 1a). The wall-to-lumen ratio was significantly increased by $11.8 \pm 3.5\%$ (fig. 1b). However, this was not accompanied by a significant change in wall cross-sectional area (fig. 1c). Thus, in WT mice, hypertension for 1 week was associated with eutrophic inward remodeling of small mesenteric arteries. In contrast, no change in lumen diameter was observed after 1 week in arteries from tTG KO mice (fig. 1a). In these mice, L-NAME did not induce significant changes in the wall-to-lumen ratio: $8.8 \pm 4.3\%$ (fig. 1b). The wall cross-sectional area was not significantly different in control versus L-NAME-treated tTG KO mice. After 3 weeks of L-NAME exposure, there was a trend of inner diameter decrease in both WT and tTG KO mice, but not enough to reach statistical significance (fig. 1a). Wall-to-lumen ratio was significantly increased in both WT (by $20.8 \pm 4.8\%$) and tTG KO mice (by $11.7 \pm 4.6\%$), without significant changes in wall cross-sectional area (fig. 1c).

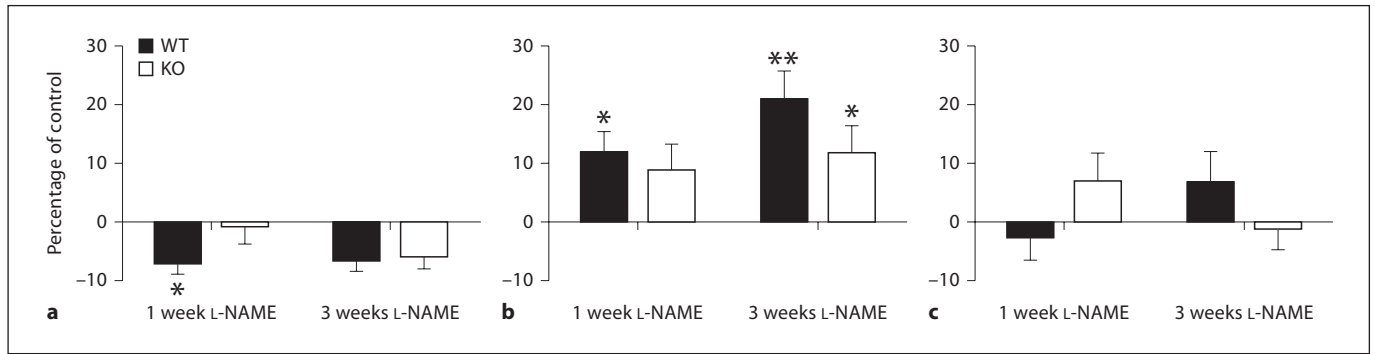


Fig. 1. Relative change of structural parameters [inner diameter (a), wall-to-lumen ratio (b) and wall cross-sectional area (c)], after 1 or 3 weeks of L-NAME treatment. * $p < 0.05$; ** $p < 0.01$ versus control (one-way ANOVA, Dunnett correction applied).

Wall Stress during Remodeling

Estimates were made of *in vivo* wall stress, on the basis of the *in vivo* systemic pressure and the *in vitro* determined passive diameter and wall thickness at this pressure (fig. 2). After 1 week of L-NAME treatment, wall stress was significantly increased in both WT (by $121.3 \pm 3.6\%$ of control littermates; $p < 0.01$) and tTG KO mice (by $15.5 \pm 5.3\%$; $p < 0.05$). After 3 weeks, wall stress was completely normalized in WT mice ($2.6 \pm 6.7\%$ of controls, $p = \text{NS}$), but remained elevated in tTG KO mice (by $20.6 \pm 5.6\%$ of controls, $p < 0.01$, all tests one-way ANOVA with Dunnett correction).

Role of Macrophages

Macrophages may be a source of tTG and FXIII. Therefore, we aimed to test whether the inward remodeling could be affected by macrophage depletion using the clodr-lip treatment in WT mice exposed to L-NAME for 1 week. In the presence of clodr-lip and L-NAME, the inner diameter and wall-to-lumen ratio did not change (by 0.3 ± 2.2 and $3.5 \pm 3.0\%$ of control littermates, $p = \text{NS}$). There was no significant difference in the wall-to-lumen ratio between L-NAME alone and L-NAME in the presence of clodr-lip. Monocytes/macrophages often appeared in small groups in the adventitial layer of the vessel wall and thus showed a relatively high variation in counts per length unit. CD68-positive cell counts were not significantly changed (L-NAME: by $20.1 \pm 30.6\%$ of controls; clodr-lip + L-NAME: by $-50.2 \pm 37.8\%$ of controls; fig. 3).

tTG and FXIII Expression

Real-time PCR was performed on small mesenteric arteries of WT and tTG KO mice exposed to L-NAME treat-

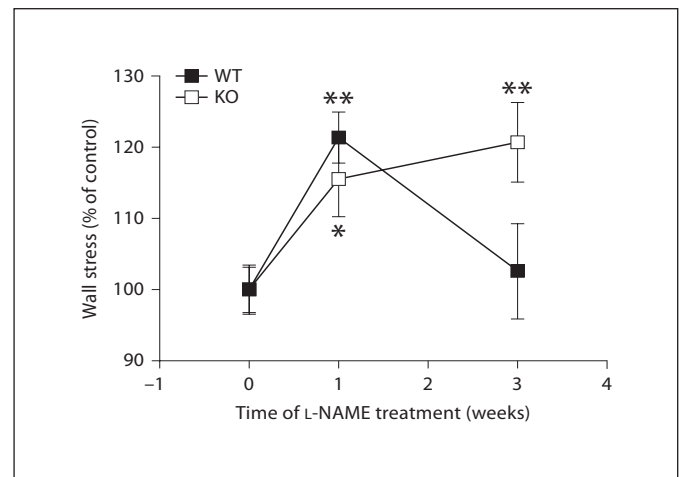


Fig. 2. Estimation of *in vivo* wall stress, based on individual measurements of blood pressure and *in vitro* dimensions at this pressure. * $p < 0.05$; ** $p < 0.01$ versus control (one-way ANOVA, Dunnett correction applied).

ment for 3 weeks and their controls. Additionally, expression of tTG and FXIII was assessed in the subgroup of WT mice receiving 1 week of L-NAME treatment, in the presence or absence of clodr-lip, and the corresponding controls. tTG and FXIII expression were not significantly influenced by L-NAME treatment alone in both WT and tTG KO mice (the latter not shown). Treatment with clodr-lip in the WT mice was associated with significantly decreased FXIII expression (by $-49 \pm 13\%$ of controls, $p = 0.04$), confirming its origin in the macrophages. Interestingly, the low level of FXIII expression was complemented by a significant increase (by $82 \pm 30\%$ of controls, $p = 0.03$) in tTG expression (fig. 4).

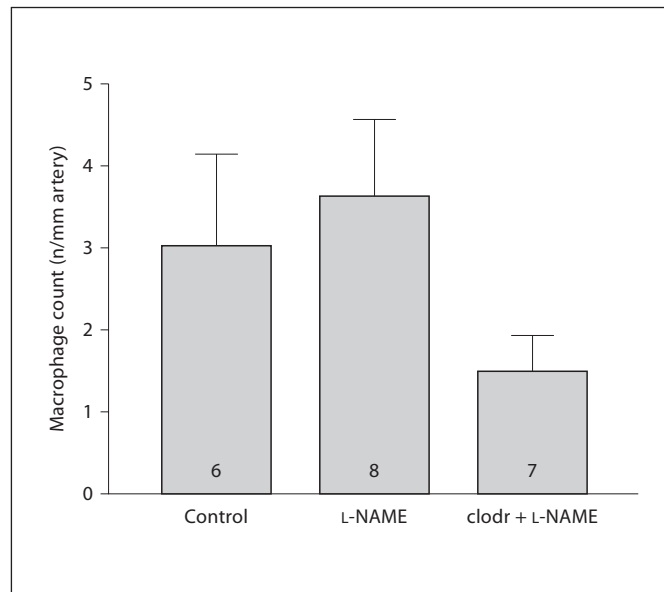
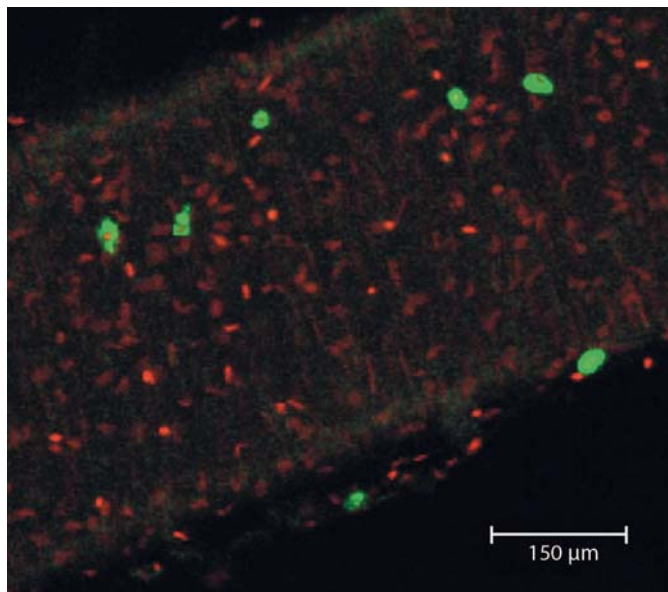


Fig. 3. Confocal microscopy. Macrophages (stained green with an FITC-bound anti-CD68 antibody) were counted to assess the effect of clodronate-containing liposomes and/or L-NAME treatment. Nuclei are stained red with ethidium bromide. Within the bars, the numbers of animals per group are given.

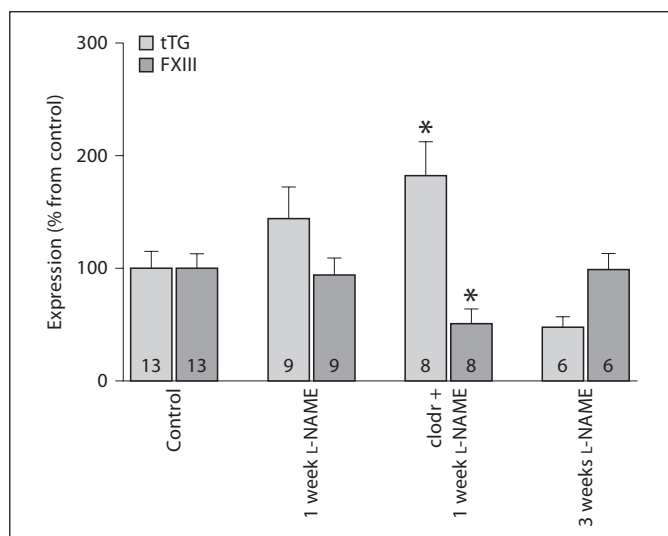


Fig. 4. Real-time PCR. tTG and FXIII expression in small mesenteric arteries of WT mice, given as percentage of the matched control group. * $p < 0.05$ versus control.

Erythrocyte Deformability

Hematocrit was identical in WT and tTG KO and did not change following 3 weeks of L-NAME treatment (table 1). Erythrocyte deformability was identical in untreated WT and KO mice: elongation indices at a shear

rate of 30 dynes/cm² were 0.383 ± 0.017 in WT versus 0.386 ± 0.011 in tTG KO mice ($p = 0.91$). After 3 weeks of L-NAME treatment, erythrocytes of WT mice were significantly stiffer ($15.3 \pm 3.6\%$ at a shear rate of 30 dynes/cm², $p = 0.03$ vs. control), whereas little effect ($5.1 \pm 2.8\%$) was observed in the erythrocytes of tTG KO mice ($p = 0.02$ vs. treated WT) (fig 5a, b). The importance of such stiffening can be assessed by comparing the shear stress needed to bend an erythrocyte to half of the maximum deformation [21, 22] (fig. 5c, d). This shear stress value is not different in nontreated WT (18.8 ± 2.5 dynes/cm²) versus tTG KO mice (16.1 ± 1.3 dynes/cm²). After L-NAME treatment, a significant increase is observed in the WT mice ($50.2 \pm 16.5\%$, $p = 0.04$) but not in the tTG KO mice ($13.7 \pm 8.8\%$, $p = 0.27$).

Discussion

The main findings of this study were: (1) inward remodeling of small arteries following L-NAME treatment was delayed in tTG KO mice; (2) neither tTG nor FXIII expression was stimulated by L-NAME-induced hypertension; (3) macrophage depletion by clodronate reduced FXIII expression but increased tTG expression, and (4) erythrocyte stiffening following L-NAME treatment occurred in WT but not tTG KO mice.

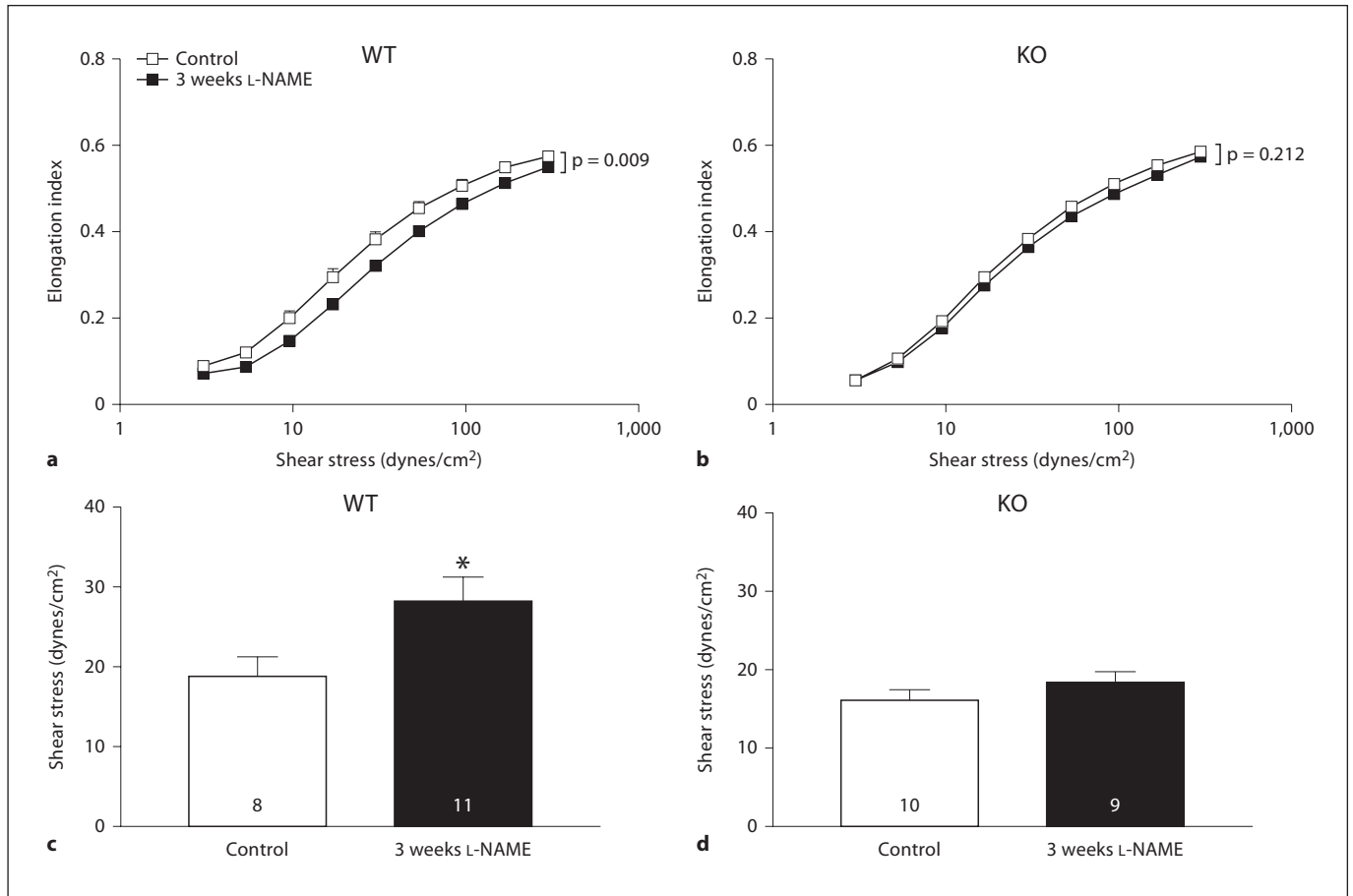


Fig. 5. a, b Elongation index of erythrocytes subjected to increasing shear stress. Significance was tested using the general linear model for repeated measurements. **c, d** Shear stress needed for half-maximum deformation of erythrocytes. * $p < 0.05$ versus control.

Experimental Design

We chose to use L-NAME treatment as a model for hypertension. It leaves little doubt that the initial mode of action of L-NAME is by vasoconstriction [23–25], amongst others related to impaired shear stress sensing. The raised resistance vessel tone increases peripheral resistance and, through central regulation, the blood pressure. Whether it is the lack of sensed shear, the raised blood pressure, or the associated increased tone that drives the inward remodeling cannot be derived from our experiments. The literature provides evidence that each of these mechanisms is active in resistance vessels. Thus, in mouse small arteries, eutrophic inward remodeling following shear reduction was shown by us [15] and several other groups [26, 27]. On the other hand, many NO-independent protocols for experimental hypertension exist that also induce inward remodeling of the mes-

enteric bed [28, 29]. We previously demonstrated a causal relation between increased tone and inward remodeling in primarily in vitro settings [30] and suggest that tone is also the drive in the current study. Tone may have increased equally well in WT and tTG KO animals. The difference between the groups would be that in the WT but less so in the tTG KO mice, the continuous vasoconstriction induces inward remodeling through cross-linking by Tgases. Yet, this interpretation is complicated due to the presence of the various stimuli. Adding to the complexity, the remodeling response affects pressure, shear stress, and the relation between smooth muscle cell activation and actual vasoconstriction. Monitoring cardiac output would help unravel the sequence of causes and effects in this model of experimental hypertension. Yet, irrespective of the drive, a vicious circle of inward resistance vessel remodeling and increased pressure

could develop that resembles essential hypertension. Indeed, stopping L-NAME treatment in mice does not lead to full normalization of blood pressure at 10 weeks [31]. It is unknown whether the inward remodeling is also maintained here.

We aimed to allow sufficient time to have a clear remodeling response. In pilot experiments, we observed that inward remodeling in WT mice occurred already after 1 week of L-NAME treatment. We therefore chose this as our first time point. We considered that the tTG KO mice may have delayed rather than suppressed inward remodeling and therefore decided to also study the mice at 3 weeks. Such remodeling was indeed found. However, it remains to be established whether remodeling has reached a steady state in both genotypes after 3 weeks.

We did not test the effect of macrophage depletion by clodr-lip in control vessels without L-NAME in this study. However, we previously found that in the absence of a remodeling stimulus, clodr-lip is without effect. Thus, in a study addressing flow-dependent remodeling [15], clodr-lip treatment for 7 days did not affect the inner diameter or wall-to-lumen ratio in control vessels (i.e. in the absence of L-NAME and under normal flow). This was the case for both WT and tTG KO mice.

While the current data point at the involvement of tTG in L-NAME-induced remodeling, we cannot conclude that macrophage-derived FXIII is involved. The observed inward remodeling after 1 week of L-NAME treatment was modest. We attempted to test whether this remodeling was suppressed by clodr-lip. However, in this specific subgroup, the remodeling in littermates treated with L-NAME only was too limited to draw meaningful conclusions about a structural effect of clodr-lip. It does not seem feasible to extend the clodronate treatment to 3 weeks, since this would require many intraperitoneal injections. Therefore, a more robust model for hypertension-induced inward remodeling and an alternative approach for macrophage depletion is needed to test whether macrophage-derived FXIII is involved in hypertensive inward remodeling.

Hypertension and Small Artery Inward Remodeling

We have recently shown that tTG and FXIII are involved in the eutrophic inward remodeling following reduction of local flow in small mesenteric arteries [15]. Thus, tTG KO mice have no obvious cardiovascular phenotype when unchallenged, but show slower and smaller inward remodeling when exposed to a flow-modifying protocol. We attributed the remaining inward remodeling to FXIII, and suggested that remodeling occurred

through cross-linking of chronically folded matrix fibers in the vascular wall resulting in shortened structural elements [32]. The current work demonstrates that Tgases are also involved in inward remodeling in the L-NAME model of hypertension. We therefore speculate that Tgase activity forms a very general modus for inward microvascular remodeling.

Inward remodeling of small arteries in hypertension can be regarded as an adaptation mechanism leading to normalization of wall stress [33]. Based on the Laplace law, this requires increasing the wall-to-lumen ratio. This can be achieved by wall hypertrophy (i.e. smooth muscle cell growth and replication, matrix deposition), by eutrophic inward remodeling (rearranging the existing material around a smaller lumen), or by a combination of both. Since wall cross-sectional area following L-NAME treatment was not changed in any of the groups, the current remodeling is clearly eutrophic, in accordance with the view that the remodeling is mediated by matrix cross-linking through Tgases. The estimations of in vivo wall stress based on the Laplace law suggest impairment of wall stress adaptation by such eutrophic remodeling in the tTG KO group, since estimated wall stress remained elevated after 3 weeks of L-NAME treatment. Some care should be taken in interpreting these data since local pressure in the vessels would be slightly below systemic and since in vivo these mesenteric vessels have tone [34].

We found no increase in tTG and FXIII expression following L-NAME treatment. Yet, several mechanisms have been identified for the regulation of activity. Thus, activation of both tTG and FXIII needs calcium [35], providing a possible link between tone and activity. Also, NO is believed to suppress activation of tTG and FXIII by direct nitrosylation [36], giving a rationale for L-NAME-induced activation. Indeed, we previously found that extrinsic FXIII induced inward remodeling of coronary vessels kept in culture at low pressure in the absence of tone, and this effect was inhibited by an NO donor [12]. The mechanisms that regulate activity and transport to the interstitial space are far from clear. Assessing extracellular activity is not trivial. Immunostaining against cross-links shows that these are already present in large amounts in any normal vessel, overwhelming the effects of interventions. Immunostaining against tTG and the Tgase-specific cross-link has previously been performed in other protocols [12, 15], but provided little information since both are present in control. A quantitative assessment of expression at the protein level by western blotting against the Tgases would have required much more tissue

and would still leave questions with respect to localization and activity. Therefore, a true assessment of extracellular activity awaits the design of new assays for this purpose.

We previously showed in a ligation model that monocytes/macrophages could form the source of FXIII causing the inward remodeling in the tTG KO mice [15]. These cells accumulate in the vascular wall after L-NAME treatment [16] and are able to synthesize both tTG and FXIII [17]. FXIII is known as a marker of the alternative activation of macrophages [37], associated with a fibrotic, reparatory phenotype and induced by cross-talk with type 2 T helper lymphocytes [38]. Interestingly, this immunologic profile has been associated with fibrotic ventricular remodeling of L-NAME-treated mice [39]. In the current study, the suppression of FXIII expression by clodrilip treatment in combination with the reduced CD68-positive cell count shows that the macrophages were the source of FXIII. The significant increase in tTG expression following destruction of these cells demonstrates that the main source of tTG in the wall of small arteries is not the macrophage, but more likely the endothelium or the smooth muscle cells. These data indicate that the absence of FXIII leads to local upregulation of tTG, if an inward remodeling stimulus such as L-NAME is present. The reverse does not appear to be true, since the tTG KO mice had no upregulation of FXIII. The substrates for Tgases are not only the structural proteins but also a wealth of other extracellular and intracellular proteins, including elements of signaling cascades [3, 36]. It is thus conceivable that tTG expression is negatively regulated by cross-links in any of these proteins, irrespective of whether these are formed by tTG or FXIII.

Erythrocyte Deformability

Erythrocyte deformability is decreased in untreated patients with essential hypertension [40]. Moreover, the free calcium concentration was found to be increased in the erythrocytes of hypertensive patients [10]. Erythrocyte stiffening and cytosolic calcium increase were also shown to be correlated in such patients [41]. The causal link between high cytosolic calcium and erythrocyte stiffening could be tTG activity, which is known to depend on a rise in cytosolic calcium [42]. Indeed, we observed that erythrocytes permeabilized with a calcium ionophore became stiffer when exposed to increasing concentrations of calcium and this phenomenon could be prevented by adding a Tgase-specific blocker, cystamine (data not shown). The current data show a large increase in stiffness in the WT mice following L-NAME treatment

(50% increase in shear for half-maximal deformation). The lack of such stiffening in the tTG KO mice indicates that this enzyme can modulate erythrocyte stiffness by cross-linking of structural proteins. Indeed, actin, spectrin [3], and band 3 protein [43] have the lysine and glutamine residues on which tTG can act.

While we suggest Tgases as a common pathway for erythrocyte stiffening and inward remodeling, we do not believe that the two processes are causally linked. For erythrocyte stiffening, lack of NO is far more likely to be the stimulus than is the raised pressure, because red blood cells cannot sense the blood pressure: unlike the vascular wall where the transmural pressure gradient generates a wall tension, the erythrocytes are fully embedded in the pressurized blood and hence miss a transmural gradient. We therefore attribute the stiffening to chronically increased tTG activation following NO inhibition. The raised erythrocyte stiffness and the vascular inward remodeling both increase the resistance to perfusion. Stiff red blood cells could furthermore add an ischemic component to hypertension by reducing the functional capillary density [44] and providing an additional mechanism to induce structural capillary rarefaction [45].

In conclusion, we show involvement of Tgases in inward microvascular remodeling and erythrocyte stiffening in an L-NAME model for hypertension. These enzymes may provide a possible therapeutic target in the treatment of hypertensive and ischemic disorders.

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