

Decreased S-Nitrosylation of Tissue Transglutaminase Contributes to Age-Related Increases in Vascular Stiffness

Lakshmi Santhanam,* Eric C. Tuday,* Alanah K. Webb,* Phillip Dowzicky, Jae Hyung Kim, Young Jun Oh, Gautam Sikka, Maggie Kuo, Marc K. Halushka, Anne M. Macgregor, Jessilyn Dunn, Sarah Gutbrod, David Yin, Artin Shoukas, Daniel Nyhan, Nicholas A. Flavahan, Alexey M. Belkin, Dan E. Berkowitz

Rationale: Although an age-related decrease in NO bioavailability contributes to vascular stiffness, the underlying molecular mechanisms remain incompletely understood. We hypothesize that NO constrains the activity of the matrix crosslinking enzyme tissue transglutaminase (TG2) via S-nitrosylation in young vessels, a process that is reversed in aging.

Objective: We sought to determine whether endothelium-dependent NO regulates TG2 activity by S-nitrosylation and whether this contributes to age-related vascular stiffness.

Methods and Results: We first demonstrate that NO suppresses activity and increases S-nitrosylation of TG2 in cellular models. Next, we show that nitric oxide synthase (NOS) inhibition leads to increased surface and extracellular matrix-associated TG2. We then demonstrate that endothelium-derived bioactive NO primarily mediates its effects through TG2, using TG2^{-/-} mice chronically treated with the NOS inhibitor L-N^G-nitroarginine methyl ester (L-NAME). We confirm that TG2 activity is modulated by endothelium-derived bioactive NO in young rat aorta. In aging rat aorta, although TG2 expression remains unaltered, its activity increases and S-nitrosylation decreases. Furthermore, TG2 inhibition decreases vascular stiffness in aging rats. Finally, TG2 activity and matrix crosslinks are augmented with age in human aorta, whereas abundance remains unchanged.

Conclusions: Decreased S-nitrosylation of TG2 and increased TG activity lead to enhanced matrix crosslinking and contribute to vascular stiffening in aging. TG2 appears to be the member of the transglutaminase family primarily contributing to this phenotype. Inhibition of TG2 could thus represent a therapeutic target for age-associated vascular stiffness and isolated systolic hypertension. (*Circ Res.* 2010;107:117-125.)

Key Words: tissue transglutaminase ■ S-nitrosylation ■ S-nitrosation ■ aging ■ vascular stiffness

Aging is associated with alterations in the properties of all elements of the vascular wall including endothelium, vascular smooth muscle, and matrix.¹ These changes result in increased vascular stiffness and isolated systolic hypertension. In addition, increased vascular stiffness promotes atherosclerosis at various sites in the vascular tree, such as the carotid artery.^{2,3} Both dynamic changes (alterations in endothelial function and effects on vascular smooth muscle contractility), as well as structural alterations (eg, fracturing of elastin, increased collagen content, and accumulation of advanced glycation end products) have been described in aging. Vessel structure can additionally be regulated by alterations in matrix crosslinking.¹

Transglutaminases (TGs) are enzymes that catalyze a transamidation reaction, leading to the crosslinking of

proteins through the formation of the stable N-ε-(γ-glutamyl)lysine isopeptide bonds.^{4,5} At least 3 of the 9 members of the TG superfamily are expressed in vascular systems. Tissue transglutaminase (TG2) in particular is ubiquitously expressed in vasculature, including in endothelial cells, smooth muscle cells, fibroblasts, and monocytes/macrophages.⁴⁻¹¹ TG2 is confined mainly to the cytosol, and a portion of it is associated with the cell membrane and secreted out of the cell to the extracellular matrix (ECM) through an as yet unidentified mechanism.⁴ The reaction catalyzed by TG2 is dependent on its location: cytosolic TG2 acts mainly as a GTPase and extracellular TG2 catalyzes the transamidation reaction.^{4,5} The role of TG2 in regulating endothelial barrier function,^{4,12} small

Original received December 17, 2009; revision received May 10, 2010; accepted May 11, 2010.

From the Johns Hopkins University School of Medicine (L.S., E.C.T., A.K.W., P.D., J.H.K., Y.J.O., G.S., M.K., M.K.H., A.M.M., J.D., S.G., D.Y., A.S., D.N., N.A.F., D.E.B.), Baltimore, Md; Anesthesiology and Pain Medicine (Y.J.O.), Anesthesia and Pain Research Institute, Yonsei University College of Medicine, Seoul, Korea; and Center for Vascular Inflammatory Diseases (A.M.B.), University of Maryland School of Medicine.

*These authors contributed equally to this work.

Correspondence to Dan E. Berkowitz, Johns Hopkins University School of Medicine, 720 Rutland Ave, Traylor 621, Baltimore MD 21205. Phone: 410-614-1517; Fax: 410-614-0019. E-mail dberkow1@jhmi.edu

© 2010 American Heart Association, Inc.

Circulation Research is available at <http://circres.ahajournals.org>

DOI: 10.1161/CIRCRESAHA.109.215228

Non-standard Abbreviations and Acronyms

DTT	dithiothreitol
ECM	extracellular matrix
GSNO	S-nitrosoglutathione
HAEC	human aortic endothelial cell
L-NAME	L-N ^G -nitroarginine methyl ester
NOS	nitric oxide synthase
PWV	pulse wave velocity
TG	transglutaminase
TG2	tissue transglutaminase
WT	wild type

artery remodeling,⁷ induction of vascular calcification program,¹⁰ and atherosclerosis^{4,13} is emerging.

Crosslinking activity of TG2 has been shown to be inhibited *in vitro* by NO through protein S-nitrosylation^{14,15} of key cysteine residues. Furthermore, NO reverses small artery remodeling by TG2 in mice.^{7,10} In addition to directly modulating crosslinking activity, NO is shown to influence TG2 subcellular distribution in fibroblasts,¹⁶ wherein the NO donor SNAP was shown to decrease the deposition of TG2 to the ECM over 72 hours. It is well established that endothelial NO bioavailability diminishes with aging.^{17,18} We therefore tested the hypothesis that decreased NO bioavailability contributes to an increase in TG activity in aging vessels. In this study, we demonstrate that endothelial nitric oxide synthase (NOS)-dependent NO regulates TG2 crosslinking activity and location in endothelial cells. Decreased endothelium-dependent NO synthesis in the aging vasculature leads to reduced TG2 S-nitrosylation and, thus, enhanced transamidation activity. This, in turn, results in increased crosslinking of matrix proteins and, consequently, to decreased compliance and increased stiffness of aging conduit blood vessels.

Methods

An expanded Methods section is available in the Online Data Supplement at <http://circres.ahajournals.org>.

Animals

The animal protocols used in this report have been approved by the Johns Hopkins University School of Medicine Institutional Animal Care and Use Committee. Fisher 344 rats were used for this study and were supplied by the National Institute of Aging. Young animals were \approx 3 months of age, whereas old animals were between 22 to 24 months of age. Male TG2^{-/-} mice (a kind gift from Robert Graham, Victor Chang Cardiovascular Institute, New South Wales, Australia) were used (3 to 5 months of age) in the study with BL6129S as background. All animals were fed *ad libitum* and had free access to drinking water.

Cell Cultures

Human aortic endothelial cells (HAECs) were purchased from Cascade Biologics, cultured using ECM Media (ScienCell Labs), and used between passages 7 and 10. Intact cells were treated as indicated and used to determine TG2 activity, expression, and S-nitrosylation.

Shear Stress

HAECs were sheared at 20 dyne/cm² (400 rpm) using a cone-and-plate viscometer as described elsewhere.^{19–21}

TG2 Expression

Expression was determined by Western blotting.

TG Activity Assay

A dot blot assay was used to determine TG activity as described²² with minor modifications (see the Online Data Supplement for details). A time course of biotin(amido)pentylamine incorporation in HAECs is shown in Online Figure I (A) and a comparison to the standard Western blotting approach is provided (Online Figure I, B).

S-Nitrosylation Assay

TG2 S-nitrosylation was determined using the biotin switch assay²³ in cell lysates/tissue homogenates. Because the activity assay also relies on biotinylation, the S-nitrosylation assays were performed on a separate set of samples in parallel with the activity assays.

Isolation of Cell Surface Proteins and ECM

Cell surface proteins were enriched using sulfo-NHS-LC biotin (Pierce) following the protocol of the manufacturer. ECM fraction was recovered by removing cells and nuclear material following the protocol of Soucy and Romer.²⁴

Immunofluorescent Staining of TG2

HAECs were grown on fibronectin-coated coverslips and treated as indicated. Extracellular/ECM associated TG2 was first stained in live cells by incubating with TG2 primary followed by Cy3-conjugated secondary antibody. Cells were then fixed, permeabilized, and intracellular TG2 labeled by incubating with TG2 primary followed by Cy5-conjugated secondary antibody. Samples were then mounted, sealed, and imaged on a Nikon Eclipse 80i equipped with a photometrics CoolSnap HQ2 camera (Cy3, green; Cy5, red). The entire labeling procedure was performed in the dark and at 4°C (see the Online Data Supplement for details).

In Vivo NOS Inhibition in Mice

Wild-type (WT) (BL6129S) and TG2^{-/-} mice were used. Animals were randomized into 2 groups and implanted with an osmotic pump (Alzet) filled with a 4-week dose of either L-N^G-nitroarginine methyl ester (L-NAME) (20 mg/kg per day) or vehicle control.

In Vivo Inhibition of TG in Aging Rats

Eighteen- to 19-month-old rats were randomized to 2 groups and implanted with an osmotic pump, filled with a 4-week dose of either cystamine (40 mg/kg per day) or vehicle control. Pumps were exchanged every 4 weeks for 3 months.

Pulse Wave Velocity Measurement

Aortic pulse wave velocity (PWV) was measured using high-frequency Doppler with a Doppler Signal Processing Workstation (Indus Instruments) as previously described.^{25,26} Blood pressure was measured concurrently (see the Online Data Supplement for details).

Carotid Artery Compliance

The carotid artery was dissected and cannulated in a perfusion chamber. The artery was perfused with oxygenated calcium-free Krebs buffer using a peristaltic pump (Cole-Parmer Instrument Co), which also continuously monitored perfusion pressure. Pressure was incrementally increased from 0 to 100 mm Hg in steps of 10 mm Hg, each for 30 second intervals. Vessel outer diameter was simultaneously recorded using microscopic imaging

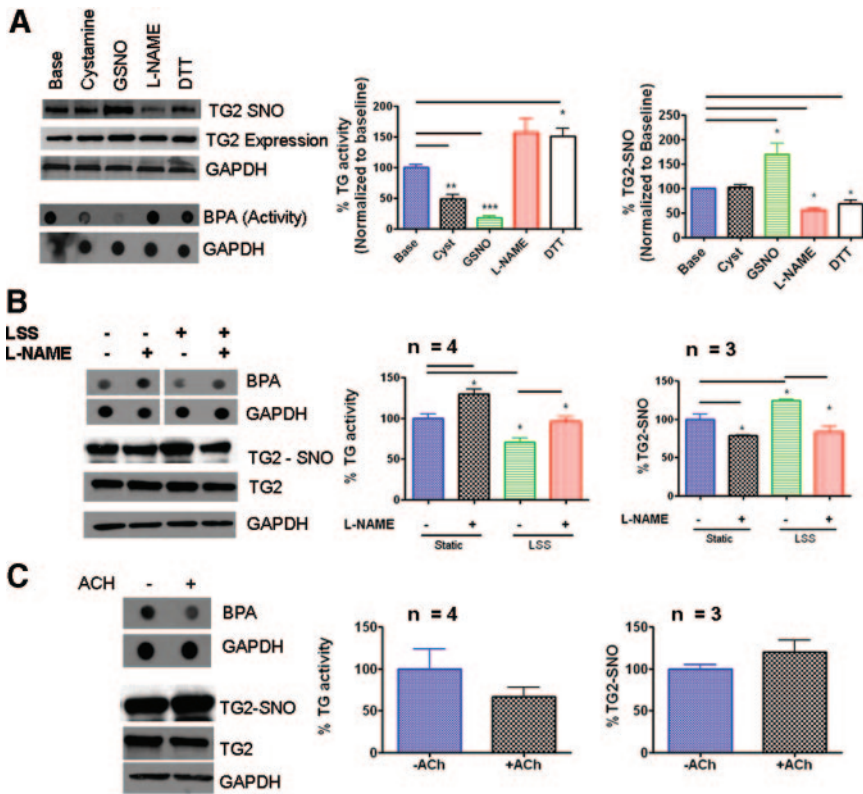


Figure 1. TG2 is regulated by protein S-nitrosylation in HAECs. **A**, NOS inhibition (L-NAME; 200 $\mu\text{mol/L}$, 2 hours) and reducing agent (DTT; 200 $\mu\text{mol/L}$, 1 hour) lead to increased activity and decreased TG2 S-nitrosylation in HAECs, but increased NO (GSNO; 200 $\mu\text{mol/L}$, 1 hour) leads to decreased TG activity and increased TG2 S-nitrosylation in HAECs. TG2 expression is constant in all experiments ($*P<0.05$, $**P<0.01$, $***P<0.001$, 1-way ANOVA with Tukey post test). **B**, Lamina shear stress (LSS) (20 dyne/cm² for 24 hours) decreases TG2 activity in HAECs compared to unsheared controls; L-NAME (200 $\mu\text{mol/L}$, 24 hours) increases TG2 activity toward unsheared controls (n=4 for each group), the reverse is true for S-nitrosylation levels (n=3) ($*P<0.05$, 1-way ANOVA with Bonferroni post test). **C**, Cholinergic stimulation of HAECs with acetylcholine (ACh) (1 $\mu\text{mol/L}$; 30 minutes) leads to increased TG2 S-nitrosylation (n=3) and decreased TG activity (n=4). The data did not reach statistical significance.

and video dimension analysis (Analog Digital Instruments). Compliance (ability to stretch and hold volume) and distensibility (ability to stretch; elastic property of vessel) were calculated from these data. It is important to note that pressure was measured at the pump proximal to the vessel, and, thus, actual vessel pressure may be different depending on the resistances exerted by components of the flow circuit. This, however, remains constant for all experimental groups.

Human Tissue

Thoracic descending aortic segments for immunohistochemistry and TG activity were taken from 16 subjects (8 young [33 to 49 years old] and 8 old [62 to 101 year old]) undergoing autopsy as part of a wider study of vascular tissues as described.²⁷ The collection of all tissues was approved by the institutional review board of The Johns Hopkins Hospital.

Data Analysis

All Western blots and dot blots were analyzed by densitometry using the ImageJ software (NIH). Results are expressed as a percentage change relative to the average value measured in the baseline group. Statistics were performed using GraphPad Prism software. One-way ANOVA with Bonferroni or Tukey correction were used to compare 3 or more groups; unpaired *t* test was used to compare 2 groups. All data are represented as means \pm SEM.

Results

TG2 Is Regulated by S-Nitrosylation in Cellular Models

We first established the dependence of TG crosslinking activity on bioactive NO in 2 cellular models: NIH3T3 cells overexpressing myc-tagged TG2 (Online Figure II) and HAECs (Figure 1A). Intact cells were pretreated with

NO donor (*S*-nitrosoglutathione [GSNO]; 200 $\mu\text{mol/L}$, 1 hour), reducing agent (dithiothreitol [DTT]; 200 $\mu\text{mol/L}$, 1 hour), or NOS inhibitor (L-NAME; 200 $\mu\text{mol/L}$, 2 hours). Cystamine (1 mmol/L, 1 hour) was used to inhibit TG crosslinking activity. Activity was measured in intact cells using the biotin(amido)pentylamine (1 mmol/L, 4 hours) incorporation assay. TG2 S-nitrosylation was measured in cell lysates using the biotin switch assay. NO/SNO inhibits TG activity: GSNO decreased and L-NAME increased TG activity in HAECs. Conversely, TG2 S-nitrosylation was increased with GSNO and decreased with L-NAME. DTT treatment led to increased activity and decreased S-nitrosylation, suggesting NO likely exerts its effect through a reversible modification of protein thiols. TG2 expression levels remained constant in all these studies. In addition, endogenous NO production was modulated using shear stress (20 dyne/cm², 24 hour, using cone-and-plate viscometer) in the presence and absence of NOS inhibitor L-NAME (200 $\mu\text{mol/L}$), and TG2 activity and S-nitrosylation were measured. Effect of shear was confirmed by inspecting cell alignment, increased NOS3 phosphorylation,¹⁹ and increased KLF-2 mRNA levels (Online Figure III, A through C).²⁸ Shear stress decreased TG2 activity and increased S-nitrosylation compared to static conditions. L-NAME reversed the effect of shear on TG2 activity and S-nitrosylation (Figure 1B). In addition, HAECs were treated with acetylcholine (1 $\mu\text{mol/L}$, 30 minutes) to induce NO production. This resulted in decreased TG2 activity (Figure 1C) and increased TG2 S-nitrosylation.

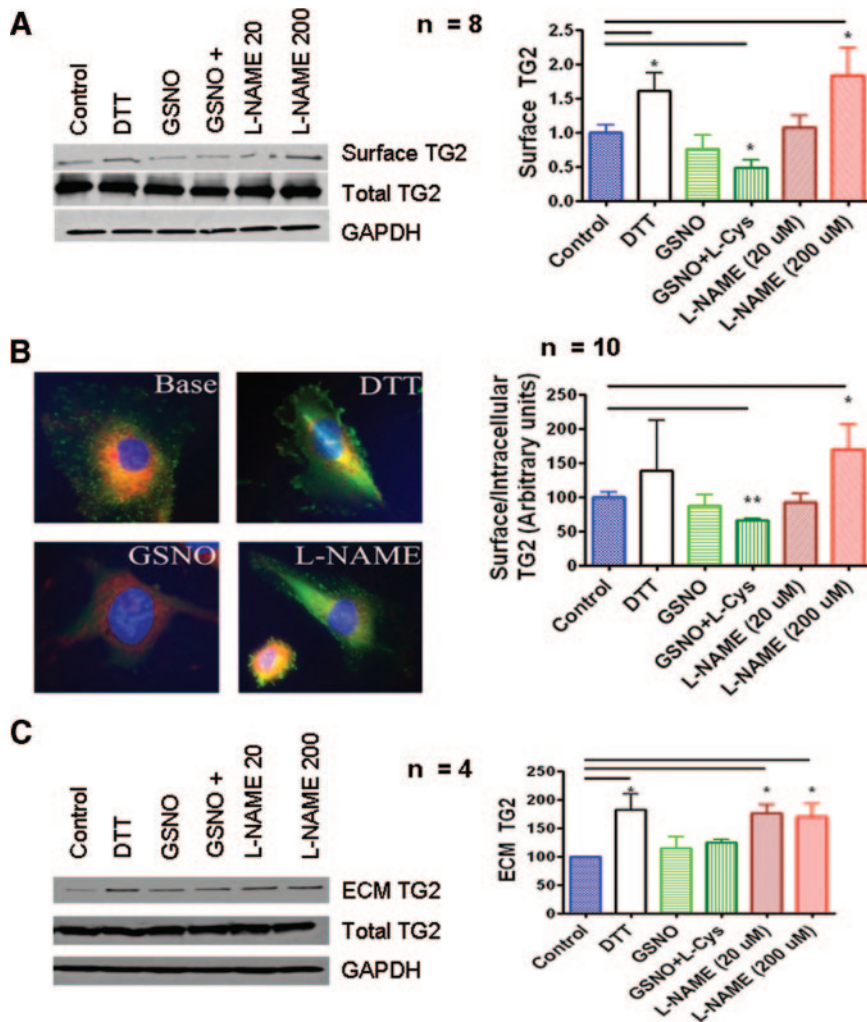


Figure 2. NO regulates TG2 subcellular distribution in HAECs. **A**, HAECs were treated with DTT (200 $\mu\text{mol/L}$), NOS inhibitor L-NAME (20 or 200 $\mu\text{mol/L}$), GSNO (200 $\mu\text{mol/L}$), or GSNO+L-cysteine (200 $\mu\text{mol/L}$ each), and controls were left untreated. Surface/ECM and cytosolic TG2 levels were determined by enrichment of surface proteins (as described in Methods); DTT and L-NAME led to increased levels of cell surface TG2, whereas GSNO and GSNO+L-cysteine treatments led to decreased surface TG2; total (whole cell lysate) TG2 was unaltered ($n=8$; $*P<0.05$, $**P<0.01$). **B**, Immunofluorescence. In this case, HAECs were grown on fibronectin-coated coverslips and treated as indicated. Extracellular TG2 was labeled by treating live cells with TG2 antibody followed by Cy3-conjugated secondary antibody followed by fixing, permeabilizing, and labeling of intracellular TG2 by treating samples with TG2 antibody followed by Cy5-conjugated secondary antibody. Samples were analyzed by fluorescence microscopy, with green corresponding to Cy3 and red corresponding to Cy5 fluorescence. DTT and L-NAME (200 $\mu\text{mol/L}$) treatment led to increased surface TG2 (higher Cy3 signal than baseline), whereas GSNO+L-Cys led to decreased surface TG2 (lower Cy3 signal than baseline). Cy3/Cy5 ratios for the **bar graph** were obtained as described in the Online Data Supplement ($n=12$; $*P<0.05$; $**P<0.01$). **C**, HAECs were grown to confluence and treated as indicated; ECM fraction was recovered as described in Methods. DTT and L-NAME led to increased deposition of TG2 in the ECM, whereas GSNO treatment did not alter ECM-associated TG2 compared to untreated cells ($*P<0.05$, $**P<0.01$).

NO/SNO Regulates TG2 Subcellular Distribution

We next determined whether NO/SNO regulates TG2 location in HAECs. Cells were treated with GSNO (200 $\mu\text{mol/L}$), GSNO+L-cysteine (200 $\mu\text{mol/L}$ each), DTT (200 $\mu\text{mol/L}$), or L-NAME (20 or 200 $\mu\text{mol/L}$) for 2 hours. We first determined surface TG2 by labeling cell surface proteins with sulfo-NHS-LC biotin followed by enrichment using streptavidin-coated agarose. TG2 was determined in the biotinylated (Surface TG2) fraction and in whole cell lysates (Total TG2) by Western blotting (Figure 2A). Increased NO led to decreased surface associated TG2. L-Cysteine is shown to increase S-nitrosylation of intracellular proteins,²⁹ and, indeed, corresponded to increased intracellular TG2. DTT and L-NAME led to increased surface associated TG2. Next, HAECs cultured on fibronectin-coated coverslips were subjected to the same treatments. In this case, we used immunofluorescence to label surface TG2 with Cy3- and cytosolic TG2 with Cy5-conjugated secondary antibodies (Figure 2B; see Methods for details) followed by analysis using fluorescence microscopy (Cy3, green; Cy5, red). There is an increase in the Cy3-labeled (surface/ECM) TG2 in DTT and L-NAME (200 $\mu\text{mol/L}$) conditions and decreased Cy3-labeled TG2 in the GSNO+L-cysteine-treated cells compared to untreated (baseline) cells. Cy3/Cy5 ratios were calculated as a measure of surface/intracellular TG2. Finally, ECM fractions of

HAECs treated as indicated (Figure 2C) were isolated, and TG2 in the ECM was determined by Western blotting. Whereas short-term GSNO (2 hours) treatment had no effect on ECM associated TG2, inhibiting NOS led to increased ECM deposition of TG2, as did DTT. Together, these results demonstrate that decreased NO leads to increased TG2 externalization.

TG2 Is the Predominant TG That Regulates Vascular Stiffness

At least 3 TGs are expressed in vasculature.^{10,11} To determine the role of TG2 specifically, we used a TG2^{-/-} mouse model. WT and TG2^{-/-} mice were treated with the NOS inhibitor L-NAME using osmotic infusion pumps (40 mg/kg per day for 4 weeks) to assess the role of bioactive NO in mediating TG activity in vivo. Controls were treated with vehicle alone. Control WT mice showed much higher TG activity compared to control TG^{-/-} mice (Figure 3A and 3B). TG activity was increased in L-NAME-treated WT mice (compared to control WT) but not in TG2^{-/-} mice (Figure 3A and 3B). TG2 expression was unaltered in WT mice with L-NAME treatment and was undetected in TG^{-/-} mice (Figure 3A and 3B). Finally, L-NAME treatment led to increased central aortic stiffness, as measured by PWV (Figure 3C). Consistent with existing

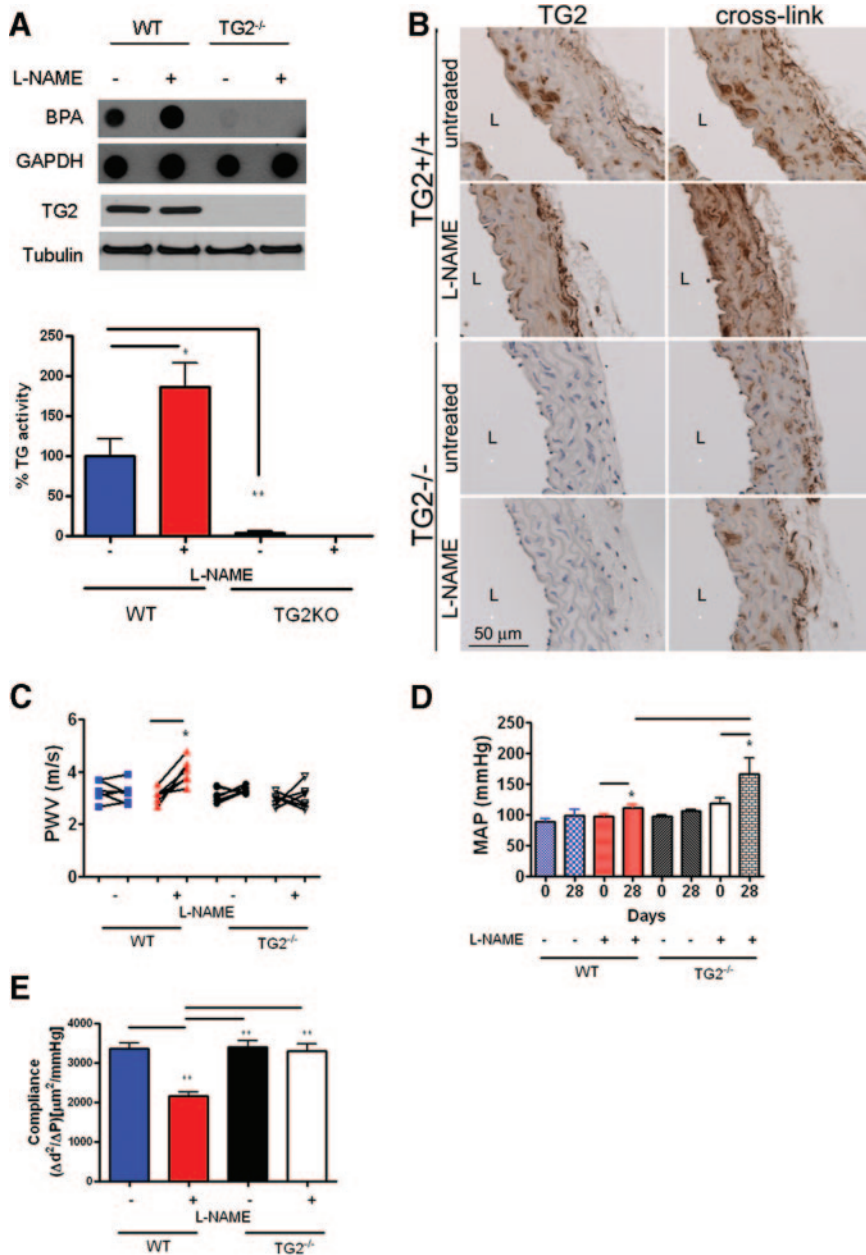


Figure 3. TG2 is the TG important in mediating vascular stiffness. NOS was inhibited in WT and TG2^{-/-} mice using L-NAME (20 mg/kg per day) administered through osmotic infusion pumps for 4 weeks. **A and B**, TG activity increases with L-NAME inhibition of NOS in WT mice but not in TG^{-/-} mice, whereas TG2 expression is unchanged. **C**, PWV increases in L-NAME-treated WT but not TG2^{-/-} mice compared to untreated controls. **D**, Mean arterial pressure (MAP) for mice were measured before and after treatment. **E**, Compliance of carotid artery measured ex vivo by pressure–dimension analysis decreases in L-NAME-treated WT but not TG2^{-/-} mice compared to untreated controls (n=8 each group; *P<0.05, **P<0.01; 1-way ANOVA followed by Bonferroni post test).

literature,³⁰ mean arterial pressures (Figure 3D) were comparable in the WT and TG2^{-/-} mice at baseline. L-NAME treatment led to increased blood pressure in both WT and TG2^{-/-} mice but a significantly higher level in TG2^{-/-} mice. Finally, carotid artery compliance measured ex vivo by pressure–dimension analysis (Figure 3E) decreased in WT but not in TG^{-/-} mice with L-NAME treatment. Pressure–dimension traces, normalized diameter, and distensibility of carotids are shown in Online Figure IV (A through C).

TG Crosslinking Activity Is Higher in Old Rat Aorta Because of Impaired TG2 S-Nitrosylation by Endothelium-Dependent Bioactive NO

We next addressed age-related alterations in TG activity in aorta of rats. Old rats have higher TG crosslinking activity,

as measured using the biotin(amido)pentylamine incorporation assay (Figure 4A) and immunohistochemistry (Figure 4B). TG2 abundance remained unchanged (Figure 4A and 4B). We probed the role of endothelium-dependent bioactive NO in regulating TG2 by inhibiting NOS using L-NAME (200 μmol/L, 2 hour; Figure 4A) and by comparing endothelium-denuded (E-) with intact (E+) aortic rings (Figure 4C). Both NOS inhibition and removal of endothelium (diminished NO availability) led to a marked increase in TG activity, lending support to the role of NOS in modulating TG activity. We also measured greater levels of S-nitrosylated TG2 in young compared to old rat aorta, suggesting a role for S-nitrosylation in modulating TG activity (Figure 4D). We next tested whether in vivo chronic TG inhibition using cystamine (40 mg/kg per day for 4 weeks) could improve vascular stiffness (as measured

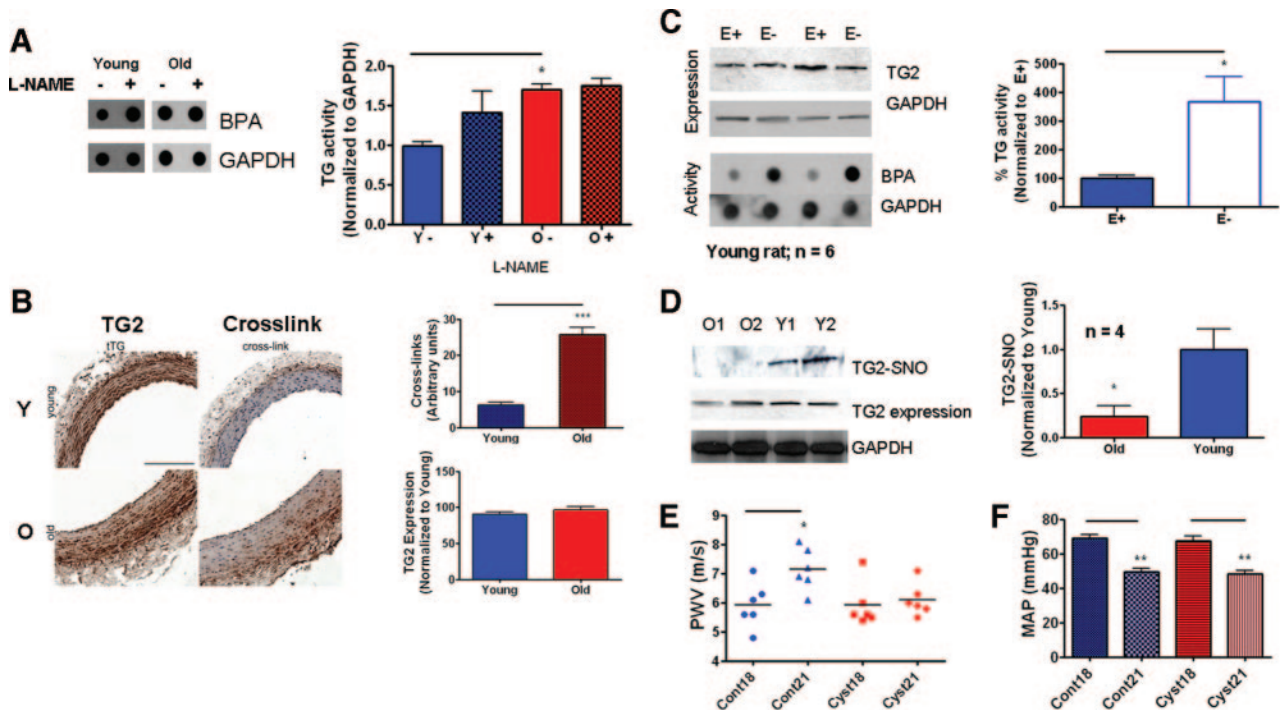


Figure 4. TG2 activity augmented and S-nitrosylation is diminished in aging rat aorta in an NOS3-dependent manner. **A**, TG activity is higher in old rats compared to young (n=6 each group; * $P < 0.05$ by Student's *t* test); ex vivo L-NAME treatment markedly enhances activity in young but not in old. **B**, TG-specific crosslinks determined by immunohistochemistry are higher in old compared to young rat aorta; expression is unchanged (right). **C**, TG activity is markedly enhanced in aortic segments of young rats lacking endothelium (E⁻) compared to intact endothelium (E⁺), suggesting endothelial NO is important in regulating TG2 from other cellular sources (n=6). **D**, S-Nitrosylation of TG2 diminishes with age, whereas expression is unchanged. **E**, Untreated rats show an increase in PWV over 12 weeks, whereas rats treated with TG inhibitor cystamine (40 mg/kg per day) administered via osmotic infusion pumps for 12 weeks show significantly lower PWV compared to untreated animals (n=8 each group). **F**, Mean arterial pressure (MAP) decreases with age in rats and is not significantly different in age-matched treated and untreated groups (n=8 each group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; 1-way ANOVA with Tukey post test).

by PWV) associated with aging. Cystamine-treated animals show significantly lower PWV at the end of the treatment period compared to controls (Figure 4E). Blood pressure decreased with age equally in both cystamine-treated and untreated rats (Figure 4F).

TG Activity Is Increased in Aging Humans

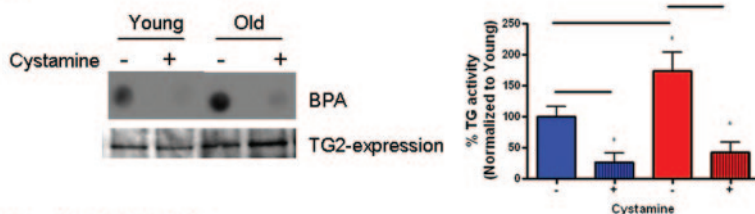
We compared the TG activity and expression in young and old human aorta obtained at autopsy. TG activity (Figure

5A) and crosslinks (Figure 5B) were higher in aged humans compared to young controls. This suggests that age-related increases in TG2 activity contribute to vascular changes in humans and that TG2 is a potential target for therapy in treating age-related vascular dysfunction.

Discussion

Aging is accompanied by increased remodeling of the vascular wall and altered endothelial function. Whereas

A Activity and Expression



B Cross-links

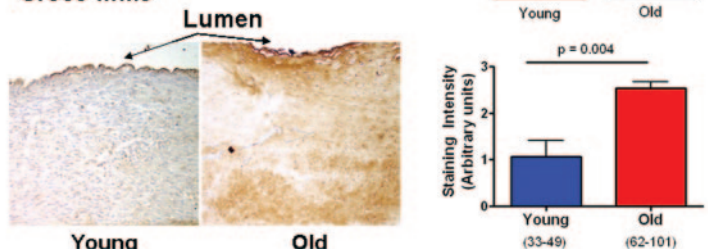


Figure 5. TG activity increases with age in human aorta. Samples were obtained during autopsy, and TG2 activity/expression was determined. **A**, TG activity is higher in old (ages 62 to 101 years) compared to young (ages 33 to 49 years) human aorta; expression levels are unaltered with age. **B**, TG-specific crosslinks increase with age (immunohistochemistry) (n=8; * $P < 0.05$, ** $P < 0.01$ by Student's *t* test).

elastin fracture, increased collagen content, and increased activity of MMP-2 are known to be important mediators of this phenomenon, the role of TG2 in vascular remodeling and atherosclerosis is emerging.^{4,30} Previous studies have shown that TG2 is expressed in all layers of the blood vessel.^{4,5} The *N*- ϵ (γ -glutamyl)lysine crosslinks formed by TG are mainly found in the adventitia and the (sub)endothelium. TG2 is thought to be inhibited by *S*-nitrosylation¹⁴ and is involved in small-artery remodeling in an NO-dependent manner.⁷ In this study, we demonstrate that TG activity increases with age in rat and human aorta whereas TG2 *S*-nitrosylation decreases. It is well accepted that aging is associated with diminished NO bioavailability. Together, these observations suggest that an NO-dependent posttranslational modification of TG2 likely mediates the increase in activity observed in aging. It is interesting to note that the abundance of TG2 remained unchanged in both aging human and rat aorta, an observation supported by a recent proteomic study identifying age-associated alterations in protein expression levels in rat aorta.³¹ This highlights the importance of nitroso-redox-dependent posttranslational modifications in determining function and their role in human disease³² and stresses the critical role of functional proteomics in target identification.

TG2^{-/-} mice do not show any overt cardiovascular phenotype when unchallenged, but have altered response(s) compared to WT littermates under several stresses.³³ Examples include delayed inward remodeling of resistance arteries in response to surgical reduction of blood flow³⁴ and L-NAME-induced hypertension.³⁰ The presence of remodeling in TG2^{-/-} mice is attributable to alternative mechanisms involving other members of the TG superfamily (eg, factor XIII and TG5). Additionally, in the case of L-NAME induced hypertension, TG2^{-/-} mice show enhanced blood pressure responses but smaller structural changes compared to WT littermates.³⁰ In this study, we used L-NAME inhibition of NOS in WT and TG2^{-/-} mice and show that TG2 is the predominant TG-mediating conduit artery (aortic) stiffness. WT mice show a significant increase in arterial stiffness (PWV) in response to NOS inhibition by L-NAME, whereas TG2^{-/-} mice do not. The distensibility of carotid arteries from TG2^{-/-} is significantly higher than WT at baseline (Online Figure IV, B); however, their compliance is comparable. Additionally, whereas TG2 expression was undetected in TG2^{-/-} mice, the aorta of these mice stained positively for the ϵ -(γ -glutamyl)lysine crosslinks, albeit at lower intensities than WT mice. We predict that the lower matrix crosslinking leads to increased distensibility, but there are other complementary/compensating mechanisms contributing toward maintaining compliance in the TG2^{-/-} mice, such as those observed in small arteries.^{10,30,34} This remains to be confirmed.

In HAECs, NOS-dependent NO regulates TG2 *S*-nitrosylation and activity, as demonstrated using NOS inhibitor L-NAME, shear stress, and acetylcholine. Furthermore, we show that decreased nitrosylation is accompanied by increased externalization of TG2. The specific

enzymatic reaction catalyzed by TG2 is dependent on its subcellular location. In the cytosol, with high GTP and low Ca²⁺ levels, the crosslinking activity of TG2 is held latent. Conversely, because extracellular TG2 encounters low GTP and high Ca²⁺ levels, it performs its transamidation reaction. Our study suggests that sustained decrease in NO bioavailability, such as those encountered in aging vasculature, can lead to decreased TG2 *S*-nitrosylation, increased externalization, and, thus, increased matrix crosslinking. Furthermore, our data suggest that endothelial NO regulates the activity of TG2 derived from other cell types (fibroblasts and SMCs) in the aorta, as denuding the endothelium (and thereby reducing bioavailable NO) leads to increased TG2 activity. Thus, the role of endothelium derived bioavailable NO in regulating *S*-nitrosylation of TG2 derived from other cell types of the vessel remains to be elucidated, for example, through coculture systems. In addition to reduced NO bioavailability, Ca²⁺ dysregulation in aging aorta may also contribute to TG2 activity because Ca²⁺ is essential for TG2 crosslinking activity and can also influence TG2 *S*-nitrosylation.¹⁴ Moreover, dysregulated denitrosylation pathways can also contribute to this phenomenon. The contribution(s) of these mechanisms to TG2 regulation in aging remains to be studied.

It is important to note that protein crosslinks catalyzed by TG2 are very stable and have a very low turnover. Thus, increased crosslinks observed in the aorta of aged rats and humans could be either attributable to increased TG2 activity or a result of accumulation of crosslinks at constant TG2 levels. In both cases, TG2 crosslinking activity contributes to the resultant vascular stiffening. Our study demonstrates that loss of NO bioavailability leads to increased TG2 transamidation activity and might accelerate the stiffening process. Thus, TG2 inhibition is a potential therapeutic route toward treating age-related vascular disease.

Finally, in addition to increased matrix crosslinking, there are a number of downstream mechanisms that might ultimately contribute to TG2-mediated increases in vascular stiffness. These include integrin signaling,³⁵ activating RhoA,³⁶ and enhanced growth factor receptor signaling,³⁷ which can contribute to vascular proliferation and fibrosis. In addition, TG2 is important for targeting of latent transforming growth factor β complex, which leads to enhanced synthesis and deposition of matrix proteins including collagen³⁸ in an NO-dependent manner.¹⁶ For example, a recent study demonstrated significantly lower plaque areas in TG2-ApoE double knockout mice on a Western-type diet compared to ApoE-null mice. The plaques in the double knockout also had lower collagen content and increased inflammation and, therefore, more unstable plaque. This was matched by decreased transforming growth factor β activity. Here again, factor XIII was shown to play a potential compensatory role in the double knockout mice.³⁹

In conclusion, we show that TG2 is the primary TG mediating age-associated stiffening of conduit arteries. TG2 is regulated by endothelium-derived bioavailable NO; TG2 *S*-nitrosylation decreases and crosslinking activity

increases with age and contributes to a decrease in vascular compliance. The cellular source of TG2 in the vasculature that contributes to this phenomenon remains to be identified.

Acknowledgments

We thank Dr Hanjoong Jo and Chih-Wen Ni (Emory University) for help with preparing the cone-and-plate shear stress viscometer. We thank Dr Norimichi Koitabashi, Dr Takahiro Higuchi, Dr Kenji Fukushima, Vanessa Pau, and Helena Cortes (Johns Hopkins University) for technical assistance.

Sources of Funding

This work was supported by the American Heart Association Beginning Grant in Aid 09BGIA2220181 (to L.S.) and American Diabetes Association Junior Faculty Award 1-05-JF-20 (to M.K.H.).

Disclosures

None.

References

- Greenwald SE. Ageing of the conduit arteries. *J Pathol*. 2007;211:157–172.
- van Popele NM, Grobbee DE, Bots ML, Asmar R, Topouchian J, Reneman RS, Hoeks AP, van der Kuip DA, Hofman A, Witteman JC. Association between arterial stiffness and atherosclerosis: the Rotterdam Study. *Stroke*. 2001;32:454–460.
- Wilkinson IB, Franklin SS, Cockcroft JR. Nitric oxide and the regulation of large artery stiffness: from physiology to pharmacology. *Hypertension*. 2004;44:112–116.
- Bakker EN, Pisteia A, Vanbavel E. Transglutaminases in vascular biology: relevance for vascular remodeling and atherosclerosis. *J Vasc Res*. 2008;45:271–278.
- Bergamini CM, Griffin M, Pansini FS. Transglutaminase and vascular biology: physiopathologic implications and perspectives for therapeutic interventions. *Curr Med Chem*. 2005;12:2357–2372.
- Auld GC, Ritchie H, Robbie LA, Booth NA. Thrombin upregulates tissue transglutaminase in endothelial cells: a potential role for tissue transglutaminase in stability of atherosclerotic plaque. *Arterioscler Thromb Vasc Biol*. 2001;21:1689–1694.
- Bakker EN, Buus CL, Spaan JA, Perree J, Ganga A, Rolf TM, Sorop O, Bramsen LH, Mulvany MJ, Vanbavel E. Small artery remodeling depends on tissue-type transglutaminase. *Circ Res*. 2005;96:119–126.
- Chowdhury ZA, Barsigian C, Chalupowicz GD, Bach TL, Garcia-Manero G, Martinez J. Colocalization of tissue transglutaminase and stress fibers in human vascular smooth muscle cells and human umbilical vein endothelial cells. *Exp Cell Res*. 1997;231:38–49.
- Faverman L, Mikhaylova L, Malmquist J, Nurminskaya M. Extracellular transglutaminase 2 activates beta-catenin signaling in calcifying vascular smooth muscle cells. *FEBS Lett*. 2008;582:1552–1557.
- Johnson KA, Polewski M, Terkeltaub RA. Transglutaminase 2 is central to induction of the arterial calcification program by smooth muscle cells. *Circ Res*. 2008;102:529–537.
- Vanbavel E, Bakker EN. A vascular bone collector: arterial calcification requires tissue-type transglutaminase. *Circ Res*. 2008;102:507–509.
- Baumgartner W, Golenhofen N, Weth A, Hiiragi T, Saint R, Griffin M, Drenckhahn D. Role of transglutaminase 1 in stabilisation of intercellular junctions of the vascular endothelium. *Histochem Cell Biol*. 2004;122:17–25.
- Cho BR, Kim MK, Suh DH, Hahn JH, Lee BG, Choi YC, Kwon TJ, Kim SY, Kim DJ. Increased tissue transglutaminase expression in human atherosclerotic coronary arteries. *Coron Artery Dis*. 2008;19:459–468.
- Lai TS, Hausladen A, Slaughter TF, Eu JP, Stamler JS, Greenberg CS. Calcium regulates S-nitrosylation, denitrosylation, and activity of tissue transglutaminase. *Biochemistry*. 2001;40:4904–4910.
- Melino G, Bernassola F, Knight RA, Corasaniti MT, Nistico G, Finazzi-Agro A. S-nitrosylation regulates apoptosis. *Nature*. 1997;388:432–433.
- Telci D, Collighan RJ, Basaga H, Griffin M. Increased TG2 expression can result in induction of transforming growth factor beta1, causing increased synthesis and deposition of matrix proteins, which can be regulated by nitric oxide. *J Biol Chem*. 2009;284:29547–29558.
- Cernadas MR, Sanchez de Miguel L, Garcia-Duran M, Gonzalez-Fernandez F, Millas I, Monton M, Rodrigo J, Rico L, Fernandez P, de Frutos T, Rodriguez-Feo JA, Guerra J, Caramelo C, Casado S, Lopez F. Expression of constitutive and inducible nitric oxide synthases in the vascular wall of young and aging rats. *Circ Res*. 1998;83:279–286.
- van der Loo B, Labugger R, Skepper JN, Bachschmid M, Kilo J, Powell JM, Palacios-Callender M, Erusalimsky JD, Quaschnig T, Malinski T, Gygi D, Ullrich V, Luscher TF. Enhanced peroxynitrite formation is associated with vascular aging. *J Exp Med*. 2000;192:1731–1744.
- Boo YC, Sorescu G, Boyd N, Shiojima I, Walsh K, Du J, Jo H. Shear stress stimulates phosphorylation of endothelial nitric-oxide synthase at Ser1179 by Akt-independent mechanisms: role of protein kinase A. *J Biol Chem*. 2002;277:3388–3396.
- Hwang J, Saha A, Boo YC, Sorescu GP, McNally JS, Holland SM, Dikalov S, Giddens DP, Griendling KK, Harrison DG, Jo H. Oscillatory shear stress stimulates endothelial production of O₂⁻ from p47phox-dependent NAD(P)H oxidases, leading to monocyte adhesion. *J Biol Chem*. 2003;278:47291–47298.
- Shin J, Jo H, Park H. Caveolin-1 is transiently dephosphorylated by shear stress-activated protein tyrosine phosphatase mu. *Biochem Biophys Res Commun*. 2006;339:737–741.
- McConoughey SJ, Niatsetskaya ZV, Pasternack R, Hills M, Ratan RR, Cooper AJ. A nonradioactive dot blot assay for transglutaminase activity. *Anal Biochem*. 2009;390:91–93.
- Jaffrey SR, Snyder SH. The biotin switch method for the detection of S-nitrosylated proteins. *Sci STKE*. 2001;2001:PL1.
- Soucy PA, Romer LH. Endothelial cell adhesion, signaling, and morphogenesis in fibroblast-derived matrix. *Matrix Biol*. 2009;28:273–283.
- Kim JH, Bugaj LJ, Oh YJ, Bivalacqua TJ, Ryoo S, Soucy KG, Santhanam L, Webb A, Camara A, Sikka G, Nyhan D, Shoukas AA, Ilies M, Christianson DW, Champion HC, Berkowitz DE. Arginase inhibition restores NOS coupling and reverses endothelial dysfunction and vascular stiffness in old rats. *J Appl Physiol*. 2009;107:1249–1257.
- Soucy KG, Ryoo S, Benjo A, Lim HK, Gupta G, Sohi JS, Elser J, Aon MA, Nyhan D, Shoukas AA, Berkowitz DE. Impaired shear stress-induced nitric oxide production through decreased NOS phosphorylation contributes to age-related vascular stiffness. *J Appl Physiol*. 2006;101:1751–1759.
- Halushka MK, Cornish TC, Lu J, Selvin S, Selvin E. Creation, validation, and quantitative analysis of protein expression in vascular tissue microarrays. *Cardiovasc Pathol*. 2010;19:136–146.
- Boon RA, Horrevoets AJ. Key transcriptional regulators of the vasoprotective effects of shear stress. *Hamostaseologie*. 2009;29:39–40, 41–33.
- Zhang Y, Hogg N. The mechanism of transmembrane S-nitrosothiol transport. *Proc Natl Acad Sci U S A*. 2004;101:7891–7896.
- Pisteia A, Bakker EN, Spaan JA, Hardeman MR, van Rooijen N, VanBavel E. Small artery remodeling and erythrocyte deformability in L-NAME-induced hypertension: role of transglutaminases. *J Vasc Res*. 2008;45:10–18.
- Fu Z, Wang M, Gucek M, Zhang J, Wu J, Jiang L, Monticone RE, Khazan B, Telljohann R, Mattison J, Sheng S, Cole RN, Spinetti G, Pintos G, Liu L, Kolodgie FD, Virmani R, Spurgeon H, Ingram DK, Everett AD, Lakatta EG, Van Eyk JE. Milk fat globule protein epidermal growth factor-8: a pivotal relay element within the angiotensin II and monocyte chemoattractant protein-1 signaling cascade mediating vascular smooth muscle cells invasion. *Circ Res*. 2009;104:1337–1346.
- Foster MW, Hess DT, Stamler JS. Protein S-nitrosylation in health and disease: a current perspective. *Trends Mol Med*. 2009;15:391–404.
- Iismaa SE, Mearns BM, Lorand L, Graham RM. Transglutaminases and disease: lessons from genetically engineered mouse models and inherited disorders. *Physiol Rev*. 2009;89:991–1023.
- Bakker EN, Pisteia A, Spaan JA, Rolf T, de Vries CJ, van Rooijen N, Candi E, VanBavel E. Flow-dependent remodeling of small arteries in mice deficient for tissue-type transglutaminase: possible compensation by macrophage-derived factor XIII. *Circ Res*. 2006;99:86–92.
- Akimov SS, Krylov D, Fleischman LF, Belkin AM. Tissue transglutaminase is an integrin-binding adhesion coreceptor for fibronectin. *J Cell Biol*. 2000;148:825–838.

36. Janiak A, Zemskov EA, Belkin AM. Cell surface transglutaminase promotes RhoA activation via integrin clustering and suppression of the Src-p190RhoGAP signaling pathway. *Mol Biol Cell*. 2006;17:1606–1619.
37. Zemskov EA, Loukinova E, Mikhailenko I, Coleman RA, Strickland DK, Belkin AM. Regulation of PDGF receptor function by integrin-associated cell surface transglutaminase. *J Biol Chem*. 2009;284:16693–16703.
38. Nunes I, Gleizes PE, Metz CN, Rifkin DB. Latent transforming growth factor-beta binding protein domains involved in activation and transglutaminase-dependent crosslinking of latent transforming growth factor-beta. *J Cell Biol*. 1997;136:1151–1163.
39. Van Herck JL, Schrijvers DM, De Meyer GR, Martinet W, Van Hove CE, Bult H, Vrints CJ, Herman AG. Transglutaminase 2 deficiency decreases plaque fibrosis and increases plaque inflammation in apolipoprotein-E-deficient mice. *J Vasc Res*. 2009;47:231–240.

Novelty and Significance

What Is Known?

- Diminished NO bioavailability in aging conduit arteries contributes to increased vascular stiffness.
- Tissue transglutaminase (TG2) is involved in small artery remodeling in response to various pathophysiologic stresses.
- TG2 activity is thought to be regulated by NO via S-nitrosylation.

What New Information Does This Article Contribute?

- TG2 crosslinking activity and externalization are regulated by NO in endothelial cells.
- Endothelium-derived NO constrains TG2 activity in rat aorta through S-nitrosylation.
- Decreased TG2 S-nitrosylation leads to increased crosslinking activity in old rat aorta.
- TG inhibition ameliorates age-associated increase in vascular stiffness in rats.

Age-associated increase in central vascular stiffness and resultant systolic hypertension are the hallmark of the aging vascular system and contribute to cardiovascular morbidity in the elderly. Therapy, however, remains elusive because there are few established protein targets. In this study, we established the role of TG2 in mediating vascular stiffness. We show that TG2 activity increases with age in the aorta of rats and humans. We demonstrate that decreased NO leads to decreased TG2 S-nitrosylation and increased TG2 externalization and crosslinking activity in endothelial cells. We further demonstrate that endothelium-derived NO regulates TG2 crosslinking activity and TG2 S-nitrosylation diminishes with age in rat aorta. Finally, TG inhibition in rats prevents age-associated increases in vascular stiffness. This is the first study demonstrating the role of decreased S-nitrosylation of TG2 by endothelium-derived NO in increased vascular stiffness with aging. This study provides a foundation to further study the therapeutic potential of TG2 in treated age-related vascular disease.