 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(ω)-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 transamination reaction.4,5 The role of TG2 in regulating endothelial barrier function,4,12 small
artery remodeling,7 induction of vascular calcification program,10 and atherosclerosis9,13 is emerging.

Crosslinking activity of TG2 has been shown to be inhibited in vitro by NO through protein S-nitrosylation14,15 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 modulating crosslinking activity, NO is shown to influence TG2 crosslinking activity and location in endothelial cells. Decreased endothelial-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 ~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(amide)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-Nitrosoylation 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-nitrosoylation 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 1-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.
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 ± 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 μmol/L, 1 hour), reducing agent (dithiothreitol [DTT]; 200 μmol/L, 1 hour), or NOS inhibitor (L-NAME; 200 μmol/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 μmol/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). 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. DTI 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 μmol/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). Cholinergic stimulation of HAEcs with acetylcholine (ACh) (1 μmol/L, 30 minutes) leads to increased TG2 S-nitrosylation (n=3) and decreased TG activity (n=4). The data did not reach statistical significance.
NO/SNO Regulates TG2 Subcellular Distribution

We next determined whether NO/SNO regulates TG2 location in HAECS. Cells were treated with GSNO (200 μmol/L), GSNO + L-cysteine (200 μmol/L each), DTT (200 μmol/L), or L-NAME (20 or 200 μmol/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 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. Cy3 and Cy5 corresponded to Cy3 fluorescence. DTT and L-NAME (200 μmol/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).

Figure 2. NO regulates TG2 subcellular distribution in HAECS. A, HAECS were treated with DTT (200 μmol/L), NOS inhibitor L-NAME (20 or 200 μmol/L), GSNO (200 μmol/L), or GSNO + L-cysteine (200 μmol/L each), or 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. Cy3 and Cy5 corresponded to Cy3 fluorescence. DTT and L-NAME (200 μmol/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).

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 TG2−/− 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 TG2−/− mice (Figure 3A and 3B). Finally, L-NAME treatment led to increased central aortic stiffness, as measured by PWV (Figure 3C). Consistent with existing
literature, 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 TG2/−/− 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...
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
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. Previous studies have shown that TG2 is expressed in all layers of the blood vessel. 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-nitrosylation and is involved in small-artery remodeling in an NO-dependent manner. 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. This highlights the importance of nitrosoredox-dependent posttranslational modifications in determining function and their role in human disease and stress 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. Examples include delayed inward remodeling of resistance arteries in response to surgical reduction of blood flow and L-NAME–induced hypertension. 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. 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−/− mice 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 e-γ-glutamyllysine 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. 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. 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, activating RhoA, and enhanced growth factor receptor signaling, 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 collagen in an NO-dependent manner. 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. 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.

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Disclosures
None.

References


**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.
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Correction

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In the article that appears on page 117 of the July 9, 2010, issue, all L-NAME treatments in cells and tissue were performed for 24 hours and not 2 hours as originally reported in the manuscript. Each instance of an incorrect treatment time has been corrected throughout.

The authors regret this error. This error has been noted and corrected in the online version of the article, which is available at http://circres.ahajournals.org/cgi/content/full/107/1/117

Reference


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Detailed Methods:

Animals: The animal protocols used in this paper have been approved by the Institutional Animal Care and Use Committee at Johns Hopkins University School of Medicine. Fisher 344 rats were utilized for this study and were supplied by the National Institute of Aging. Young animals were approximately 3 months of age while old animals were between 22-24 months of age. Male TG2−/− mice (a kind gift from Robert Graham, Victor Chang Cardiovascular Institute NSW Australia) were used (3 – 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 (HAEC) were purchased from Cascade Biologics, cultured using ECM Media (ScienCell labs) and used between passage 7 and 10. Intact cells were treated as indicated and used to determine TG2 activity, expression, and S-nitrosylation. NIH3T3 cells stably transfected with full length TG2 construct were cultured and used as described in literature1, 2.

Shear stress: HAEC were grown to ~80% confluence and subjected to a laminar shear stress of 20 dynes/cm² for 20 h using a cone-and-plate viscometer3-5. BPA and Ca²⁺ were added (see below for activity assay) and plates sheared for an additional 4 h. Alternately, cells were sheared at 20 dynes/cm² for 24 h, and used for S-nitrosylation assays.

TG2 expression: Expression was determined by western blotting using CUB7402 antibody (Neomarkers) for HAEC and rat tissue (1:5000 dilution in 3 % milk), and rabbit polyclonal antibody (Ab-4, Neomarkers, 1:1000) for mouse aortic tissue. Blots were analyzed by densitometry using the ImageJ software.

Transglutaminase Activity Assay: A dot blot assay was used to determine Transglutaminase activity as described6 with minor modifications; we used HRP-conjugated streptavidin and chemiluminescent detection instead of infrared fluorophore. The assay is based on the incorporation of the TG substrate 5-(biotinamido)pentylamine (BPA) (Pierce, USA) into proteins7, 8. In brief, intact
cells/tissues were incubated with 1 mM BPA and 2.5 mM Ca\textsuperscript{2+} at 37 °C for 4 h in culture media and then rinsed free of unreacted BPA using PBS. Following this, samples were homogenized/lysed to recover proteins. 1 – 2 ug total proteins were loaded onto nitrocellulose membrane (BioRad Dot Blot apparatus). The membrane was rinsed and blocked in 3 % BSA overnight and probed with HRP-conjugated streptavidin (Amersham Bioscience; 1:10,000 dilution in 1% BSA) to determine BPA incorporation. Blots were then stripped using Restore Plus stripping buffer (Pierce) and reprobed with GAPDH to determine protein loading. BPA incorporation and GAPDH levels were determined by densitometry analysis using the NIH Image J software. For each sample, activity was calculated as a ratio of BPA/GAPDH. In one blot, we had 2 or more biological replicates of each group; therefore, the average value of the BPA/GAPDH ratio from the baseline group was set to 100% activity. A time course of BPA incorporation in HAEC is shown in Fig S1A and a comparison to the standard western blotting approach is provided (Fig S1B).

**S-nitrosylation assay:** TG2 S-nitrosylation was determined using the biotin switch\textsuperscript{9, 10} assay in cell lysates/tissue homogenates. As 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. 100 µg samples were blocked using methylmethane thiosulfonate (MMTS, 50 mM in Tris-HCl pH 7.5 (50 mM), neocuproine (0.01mM), SDS (1 % w/v), pH 7.5)) at 50 °C accompanied by vigorous shaking. Proteins were recovered by cold acetone precipitation, resuspended in labeling buffer (Tris HCl (50 mM) pH 7.5, EDTA (1 mM)) and labeled with HPDP Biotin (4 mM final concentration) for 1 h at room temperature in the presence or absence of the reducing agent ascorbate (5 mM). After the labeling step, proteins were recovered by acetone precipitation, and biotinylated proteins enriched using streptavidin coated agarose. Samples were resolved by SDS PAGE and probed with TG2 antibody to determine TG2 S-nitrosylation levels. Densitometry analysis was performed using ImageJ software.

**Isolation of Cell surface proteins:** Surface proteins were enriched using sulfo-NHS-LC biotin (Pierce). Briefly, HAECs were cultured to confluence in 100 mm dishes and treated with indicated drugs/reagents, Plates were then rinsed in cold PBS and surface proteins were biotinylated using sulfo-NHS-LC-biotin (Pierce) following manufacturer’s protocol.
At the end of labeling, cells were trypsinized, recovered in PBS, and counted. 1.5 x 10^6 cells from each sample were pelleted and lysed in 200 µl 1x RIPA buffer. Biotinylated proteins were enriched using streptavidin-coated agarose. TG2 was determined by western blotting.

**Recovery of ECM proteins:** HAEC were grown to confluence and treated with indicated reagents. ECM was recovered following the protocol described by Soucy et al. In brief, cells were rinsed twice in PBS. Cellular and nuclear materials were extracted by incubation with cell removal solution (0.05% Triton X-100 (Fisher Scientific, Pittsburgh, PA) and 50 mM NH4OH (Sigma, St. Louis, MO) in PBS) until the cells were floating. The matrix was then briefly washed once with 50 mM NH4OH (in PBS) and then three times with phosphate buffered saline (PBS) (Quality Biological Inc, Gaithersburg, MD). ECM was scraped into 1x RIPA buffer and TG2 was determined by western blotting.

**Immunofluorescent staining of TG2:** HAECs were grown on fibronectin coated coverslips and treated as indicated. The entire labeling procedure was performed in the dark. Extracellular/ ECM associated TG2 was stained first in live cells as follows: samples were rinsed in cold PBS, blocked (PBS with 0.1 % BSA) for 15 min, and treated with TG2 antibody (CUB7402, 1:100) at 4 °C for 45 min. Samples were then rinsed 3 times in cold PBS, and treated with Cy3-conjugated goat-anti-mouse IgG (1:100, Jackson Immuno) at 4 °C for 45 min. Then, intracellular TG2 was labeled: samples were rinsed in cold PBS, fixed (3 % paraformaldehyde 45 min) and permeabilized (PBS containing 0.1% BSA and 2% w/v saponin; 15 min). Next, samples were blocked using Goat Serum Dilution Buffer (GSDB; 10 % Goat serum v/v, 2 % saponin w/v, 10 mM Glycine; 30 min), treated with primary antibody again (1:100 in GSDB; 45 min), followed by rinsing and labeling with Cy5-conjugated goat-anti-mouse IgG (Jackson Immuno, 1:100 in GSDB; 45 min). Samples were then mounted using FluorSave reagent containing DAPI (Calbiochem). Samples were sealed with clear nail polish and imaged on a Nikon Eclipse 80i equipped with a photometrics CoolSnap HQ2 camera. Images were acquired at 10x magnification with the exposure time kept constant for each channel across all samples. The object count function was used to determine live cells by counting nuclei. Cy3 and Cy5 intensities were determined and (Cy3/Cy5) ratios were calculated and divided by number of cells in the field to yield extracellular/intracellular TG2 per cell. In addition,
representative images were captured at 40x magnification, again, keeping exposure times constant for all samples.

**In vivo NOS inhibition in mice:** WT (BL6129S) and TG2−/− mice were treated with the NOS inhibitor L-NAME for a period of 4 weeks. Animals were randomized into two groups and implanted with an osmotic pump (Alzet) filled with a four week dose of either L-NAME (20 mg/kg/day) or vehicle control. Pulse wave velocity was measured as described below. At the end of the study, the aortas were excised and TG2 activity and expression were determined.

**In vivo inhibition of transglutaminase in aging rats:** 18-19 month old rats were treated with the TG inhibitor cystamine for a period of 12 weeks to assess the role of TG in mediating vascular stiffness. Animals were randomized into two groups and implanted with an osmotic pump (Durect Corporation) filled with a 4 week dose of either cystamine (40 mg/kg/day) or vehicle control. Pumps were exchanged every 4 weeks for 3 months. Pulse wave velocity was measured as described below. At the end of the study, rats were euthanized, and aorta excised for biochemical assays.

**Pulse wave velocity (PWV) measurements:** To assess vascular stiffness, the aortic PWV was measured at the beginning and end of the study period using high frequency Doppler with a Doppler Signal Processing Workstation (Indus Instruments) as described previously. In mice, blood pressure was measured concurrently using an XBP1000 non-invasive tail blood pressure system (Kent Scientific Corporation) at the beginning and end of treatment period. For rats, BP was measured noninvasively at the beginning of the treatment period. At the end of the treatment period, intraventricular pressure was measured in anesthetized rats using a combined conductance micromanometric catheter as previously described.

**Carotid artery compliance:** To investigate passive vascular properties, 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 120 mmHg in steps of 10 mmHg, each for 30 second intervals. Vessel outer diameter was simultaneously recorded using microscopic
imaging and video dimension analysis (Analog Digital Instruments). Compliance can be described as the ability to stretch and hold volume (V). Since the aortic section is cannulated at fixed length, the relative compliance can be calculated from the relationship between luminal pressure (P) and cross-sectional area (A), or diameter (d), assuming a circular lumen.

\[
Compliance = \frac{\Delta V}{\Delta P} \approx \frac{\Delta A}{\Delta P} \approx \frac{\Delta d^2}{\Delta p}
\]

To minimize the influence of aortic geometry, the vascular distensibility can be calculated by normalizing the stressed condition (V, A, or d^2) to the unstressed condition (Vo, Ao, or d_0^2):

\[
Distensibility = \frac{\Delta V}{V_o \Delta P} \approx \frac{\Delta A}{A_o \Delta P} \approx \frac{\Delta d^2}{d_0^2 \Delta P}
\]

**Human tissue:** Thoracic descending aortic segments were taken from 16 subjects (8 young (33-49 yr old) and 8 old (62-101 yr old) undergoing autopsy as part of a wider study of vascular tissues as described\(^\text{16}\). These tissues were processed to be ~1 x 3 cm, frozen and kept at -80 °C until use. Ten iliac arteries from 5 young (33-49 yr old) and 5 old (62-101 yr old) individuals were also used from the same collection. Unstained slides, for immunohistochemistry (IHC), were created from the formalin fixed paraffin-embedded iliac artery tissues. The collection of all tissues was approved by the institutional review board (IRB) of The Johns Hopkins Hospital. The previously detailed assays (TG2 activity, Western Blot expression) were also carried out using human tissue collected post mortem.

**Immunohistochemistry in aortic samples**

**TG2 expression/ cross links:** All IHC staining was performed using a mouse antihuman TG2 antibody for expression (1:100, Covalab; France) or monoclonal antibody 81D4 (1:100 dilution, Covalab, France) for cross-links. Staining was performed using the Lab Vision UltraVision Detection System, per the manufacturer’s instructions (Lab Vision, Fremont, CA). Briefly, following deparaffinization and rehydration, slides were incubated with 3% H2O2 to block endogenous peroxidase activity. Antigen retrieval was done using
0.01M sodium citrate buffer in a microwave oven for 10 minutes. Slides were then blocked with Ultra V Block and incubated with the mouse anti-human TG2 antibody (1:100 dilution) at room temperature for 90 minutes. Slides were incubated with biotinylated anti-mouse secondary antibody for 10 minutes at room temperature, and subsequently incubated with streptavidin peroxidase. Slides were then incubated with DAB chromagen and counterstained with hematoxylin before being coverslipped.

**Scoring of IHC staining intensity**

IHC staining intensity was evaluated in the tunica media and was scored based on the strongest staining of the vessel using a 0-3 (0 = none, 1 = mild, 2 = moderate, 3 = strong) scale. Scoring was performed independently and blindly by three authors (ET, AMM and MKH) and the average score for each tissue was used.

**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. For statistics, 1-way ANOVA and Tukey post test were used to compare three or more groups; unpaired t-test was used to compare two groups using GraphPad Prism software. All data are represented as Mean±SEM.
Online Figure I: A dot blot approach was developed to measure TGase activity. HAEC were incubated with BPA for indicated time. Proteins were recovered and BPA incorporation, TG2 expression, and GAPDH (loading control) were measured. A) 1 μg protein from each sample was loaded onto nitrocellulose membrane using the BioDot dot blot apparatus (Bio Rad), blocked overnight in 3% BSA, followed by detection of biotin incorporation using streptavidin-conjugated HRP (1:10,000 in 1% BSA; Amersham Bioscience); B) Biotin incorporation was determined by western blotting using 10 μg protein from each sample; TG2 expression blot is also shown; C) densitometry analysis of Dot blot and western blot approaches shows excellent correlation. The dot blot approach is a good representation of TG activity. It is sensitive and faster and requires less protein (1 – 2 μg) than the western blot approach typically used; and D) densitometry analysis of TG2 expression. All densitometry analyses were performed using ImageJ software (NIH). Data are representative of 4 independent experiments.
Online Figure II: NO regulates TG2 activity. NIH3T3 cells stably transfected with mifepristone-inducible plasmid encoding full-length TG2 were stimulated for 8 h and used. GSNO (200 μM, 1 h) increased TG2 S-nitrosylation while expression was not altered (top); TG2 activity decreased with GSNO treatment (bottom).
Online Figure III: Effect of shear on HAEC. HAEC were grown to 80% confluence and subjected to 20 dynes/cm² shear stress (= 400 rpm). A) Cell alignment in the direction of shear was confirmed by brightfield microscopy; B) KLF-2 mRNA was determined by RT-PCR, and C) phospho-eNOS levels were determined by western blotting.
Online Figure IV: Ex vivo pressure-dimension relationship of carotid artery. 
A) pressure-diameter correlation in WT and TG2−/− mice with and without L-NAME treatment; B) Pressure vs. Normalized diameter; and C) Distensibility (= slope of normalized graph B).
Supplemental References:


15. Soucy KG, Lim HK, Attarzadeh DO, Santhanam L, Kim JH, Bhunia AK, Sevinc B,