



Knockdown of transglutaminase-2 prevents early age-induced vascular changes in mice¹

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Abstract

Purpose: To determine whether the absence of transglutaminase 2 enzyme (TG2) in TG2 knockout mice (TG2^{-/-}) protect them against early age-related functional and histological arterial changes.

Methods: Pulse wave velocity (PWV) was measured using non-invasive Doppler and mean arterial pressure (MAP) was measured in awake mice using tail-cuff system. Thoracic aortas were excised for evaluation of endothelial dependent vasodilation (EDV) by wire myography, as well as histological analyses.

Results: PWV and MAP were similar in TG2^{-/-} mice to age-matched wild type (WT) control mice. Old WT mice exhibited a markedly attenuated EDV as compared to young WT animals. The TG2^{-/-} young and old mice had enhanced EDV responses (p<0.01) as compared to WT mice. There was a significant increase in TG2 crosslinks by IHC in WT old group compared to Young, with no stain in the TG2^{-/-} animals. Optical microscopy examination of Old WT mice aorta showed thinning and fragmentation of elastic laminae. Young WT mice, old and young TG2^{-/-} mice presented regularly arranged and parallel elastic laminae of the tunica media.

Conclusion: The genetic suppression of TG2 delays the age-induced endothelial dysfunction and histological modifications.

Key words: Aging. Transglutaminases. Endothelium-Dependent Relaxing Factors. Mice.

■ Introduction

Age is the most important predictor of cardiovascular disease, even in the absence of modifiable risk factors¹. Vascular aging is associated with changes in arterial wall structure and function. The arterial ageing process results from alterations in the properties of all elements of the vascular wall, including endothelium, vascular smooth muscle, and extra-cellular matrix (ECM)^{2,3}. These alterations contribute to the development of vascular stiffness and to an additional impairment of the endothelial function, both considered as independent risk factors for cardiovascular morbidity and mortality⁴⁻⁶.

TG2 is the most abundantly expressed member of the transglutaminase family of enzymes in the vasculature and catalyzes a transamidation reaction, producing the crosslinking of proteins through the formation of *N*- ϵ -(γ -glutamyl)lysine isopeptide bonds⁷. Importantly, TG2 crosslinking activity as well as TG2 subcellular distribution relates to endothelial NO bioavailability in endothelial cells^{8,9}, fibroblasts¹⁰ and smooth muscle cells (SMC)¹¹. More recently, TG2 expression has been involved in multiple vascular pathophysiological processes, including vascular remodeling¹²⁻¹⁴, atherosclerosis¹⁵, vascular calcification¹⁶ and age-dependent aortic stiffening^{8,17}.

We conducted an experimental rodent model using a genetic approach to assess early age-related functional and microstructural arterial changes mediated by TG2. We therefore, hope to provide insights on future therapeutic targets to prevent subclinical vascular aging deleterious processes.

■ Methods

All protocols used in this study were approved by the Institutional Animal Care and Use Committee at Johns Hopkins University

School of Medicine and ratified by the Committee of Ethics in Experimental Research of the Universidade Federal de São Paulo (protocol n° 3516260515).

Male TG2^{-/-} mice were used in the study with their wild-type background BL6/129S as control. For the experimental model, 20 young mice (10 TG2^{-/-} and 10 WT) and 20 old mice (10 TG2^{-/-} and 10 WT) were used. To assess early age-related changes, we used young animals at 3 months of age and old animals at 8 months of age. All animals were fed and watered *ad libitum*. Thereafter, the animals were sacrificed and the thoracic aortae were removed for assessing endothelial function, morphological and immunohistochemical (IHC) studies.

Noninvasive mean arterial pressure and pulse-wave velocity

Blood pressure was measured concurrently in unanesthetized mice using an XBP1000 non-invasive tail-cuff blood pressure system (Kent Scientific Corporation). For analytic purposes, we computed noninvasive mean arterial pressure (MAP).

Noninvasive pulse-wave velocity (PWV) was assessed as a measure of the central aortic stiffness index. For this purpose, we used a high frequency, high resolution Doppler spectrum analyzer (DSPW), a real-time signal acquisition and spectrum analyzer system (Indus Instruments, Houston, Texas) as previously described¹⁸. Mice were lightly anesthetized with 1.5% isoflurane, blood pressures and heart rates were allowed to stabilize into the physiologic range prior to study. 10 and 20 MHz probes were used to measure the descending aortic and abdominal aortic flow velocity.

Aorta preparation

Aorta was used to assess vascular elasticity and endothelial dysfunction. Mice were anesthetized in a closed chamber with

isoflurane. Anesthesia was maintained by mask ventilation of 1.5% isoflurane with a coupled charcoal scavenging system. Animals were positioned supine with limbs taped to the surgical table. Midline laparotomy was performed towards caudal-cranial, with extension into the chest after opening the diaphragm. The animals were administered an intracardiac injection of sodium heparin (1,00 IU), 1 min prior the aorta dissection. The mice were euthanized and a maximum length of aorta was isolated and removed. The dissected vessel was immediately placed in a culture dish with iced Krebs buffer (containing [mM] 118 NaCl, 4.7 KCl, 25.0 NaHCO₃, 1.16 MgSO₄, 1.18 KH₂PO₄, 11.1 D-(+)- glucose, 3.24 CaCl₂). The aorta was cleaned of connective tissue *ex-vivo*. The aorta was segmented in 2 mm rings at the level of descending thoracic aorta for assessment of vasoreactivity as well as histological analyzes.

Endothelial function assessment

We characterized endothelial function *ex vivo* assessing endothelial dependent vasodilation (EDV) using vascular tension experiments. In brief, aortic rings were suspended from strain gauges in 25-mL organ chambers filled with oxygenated (95/5% O₂/ CO₂) Krebs buffer maintained at pH 7.4 and 37°C. The rings were stretched at 10-min intervals in 100-mg increments to reach optimal tone (500mg). Indomethacin (10⁻⁵ M) was added to the aortic rings to prevent any generation of endothelium-derived contracting prostanoids for NO-dependent relaxation responses. Vessels were precontracted with phenylephrine (PE, 10⁻⁶ M) (Sigma) for 15 min. Cumulative dose-responses to acetylcholine (ACh, 10⁻⁹–10⁻⁵ M) (Sigma) were obtained to determine relaxation activity. Furthermore, cumulative dose-responses to sodium nitroprusside (SNP, 10⁻⁹–10⁻⁵ M) (Sigma) were obtained to characterize endothelial

independent vasorelaxation. Vasorelaxation is expressed as percent relaxation, as calculated by the percent decrease in tension from the PE-induced precontraction.

Tissue preparation and staining techniques

Mice aortic tissue segments were fixed for 24 hours in 10% neutral formaldehyde. The tissue segments were embedded in paraffin, and 5-7mm transverse sections were cut using a Reichert-Jung 2030 biocut rotary microtome. The tissue sections were mounted on positively charged aminopropyl- triethoxysilane-coated glass slides and deparaffinized using successive washes with xylene/ethanol/water. Briefly, after deparaffinization and rehydration, slides were incubated with 3% H₂O₂ to block endogenous peroxidase activity. Antigen retrieval was performed using 0.01 mol/L of sodium citrate buffer in a microwave oven for 10 minutes. Before IHC staining, the sections were blocked with 10% goat serum for 30 minutes. To localize the TG2-generated cross-links, the sections were incubated with 10 µg/mL of mAb 81D4 specific

for Nε-(γ -glutamyl)-lysine isodipeptide cross-links (Covalab) for 18 hours at 4°C. The slides were washed and incubated with anti-mouse IgM (for cross-links) conjugated with HRP for 30 minutes at room temperature. The slides were washed and incubated with secondary goat anti-mouse immunoglobulin (Ig)G conjugated with HRP for 30 minutes at room temperature. HRP activity was developed with 3,30-diaminobenzidine chromagenic substrate (Vector Laboratories, Burlingame, CA) for 5 minutes per the manufacturer's instructions. The sections were counterstained with Harris Hematoxylin and Eosin (H&E) for histological examination.

IHC scoring

Digital images were taken using an

Olympus DP20 camera system (Olympus, Tokyo, Japan) and white balanced using Photoshop CS2 (Adobe). An open-source image analysis software program (FrIDA) was used to score individual images. Regions of interest (ROIs) were separately created for the media and adventitia of each vessel. Brown pixels were identified using a color-picking algorithm that captured a user-defined region of hue, saturation, and luminosity, and the percent of brown pixels to total pixels within each ROI was determined. For TG2, the media ROI was evaluated and for the cross-links the adventitia ROI was used.

Data analysis

All statistical analyses were performed using Prism 5 for Mac by GraphPad Software Inc. and Microsoft Excel version 14.1.3 statistical analysis software. The results were expressed as mean and standard error (mean \pm SEM). One-way analysis of ANOVA and the Bonferroni post hoc test for multiple-comparison were used for comparing all groups and pairs of groups respectively. A value of $p < 0.05$ was considered statistically significant.

Results

PWV and MAP of TG2^{-/-} mice were similar to those of age-matched WT mice

In Figure 1A, PWV did not have a statistically significant difference in TG2^{-/-} mice compared to that of WT age-matched controls (Old TG2^{-/-} 4.44 \pm 0.20m/s vs. Old WT 3.85 \pm 0.2m/s, $n = 10$, $p = 0.06$). There was also no statistically significant difference between young and old animals within the same groups (Young TG2^{-/-} 4.39 \pm 0.22m/s vs. Old TG2^{-/-} 4.44 \pm 0.20m/s; $n = 10$, $p = 0.88$ / Young WT 3.71 \pm 0.18m/s vs. Old WT 3.85 \pm 0.21m/s; $n = 10$, $p = 0.62$).

Also shown in Figure 1B, MAP was not statistically different between age-matched WT and TG2^{-/-} mice (Old TG2^{-/-} 78.23 \pm 4.77mmHg vs. old WT 83.69 \pm 5.37mmHg; $p = 0.45$; $n = 10$ / young TG2^{-/-} 85.99 \pm 3.45mmHg vs. young WT 87.90 \pm 3.7mmHg; $n = 10$, $p = 0.71$). There was no statistically significant difference between young and old animals within the same groups (young TG2^{-/-} 85.99 \pm 3.45mmHg vs. old TG2^{-/-} 78.23 \pm 4.7mmHg; $n = 10$, $p = 0.20$ / young WT 87.90 \pm 3.79mmHg vs. old WT 83.69 \pm 5.37mmHg; $n = 10$, $p = 0.53$).

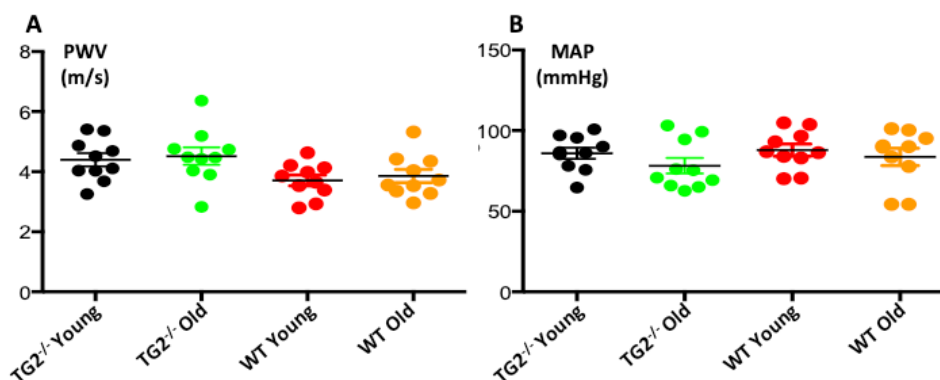


Figure 1 - Comparisons for pulse-wave velocity (PWV; **A**) and mean arterial pressure (MAP; **B**) within study groups ($n = 10$; all p values between groups > 0.05). TG2^{-/-} - mice knockout to Transglutaminase 2 enzyme; WT - wild type mice.

Endothelial nitric oxide-dependent aortic relaxation was increased in TG2 knockout mice.

The EDV responses to acetylcholine in aged mice were significantly attenuated compared to those from the young animals (old WT 54.48±5% vs. young WT 72.11±2%,

$p < 0.001$, $n = 10$; old TG2^{-/-} 70.55±4% vs. young TG2^{-/-} 92.87±6%, $p = 0.001$, $n = 10$). The young and old TG2^{-/-} mice had enhanced EDV responses as compared to control mice, as shown in Figure 2. The response to the endothelium-independent vasodilator SNP was not significantly different in any of the groups.

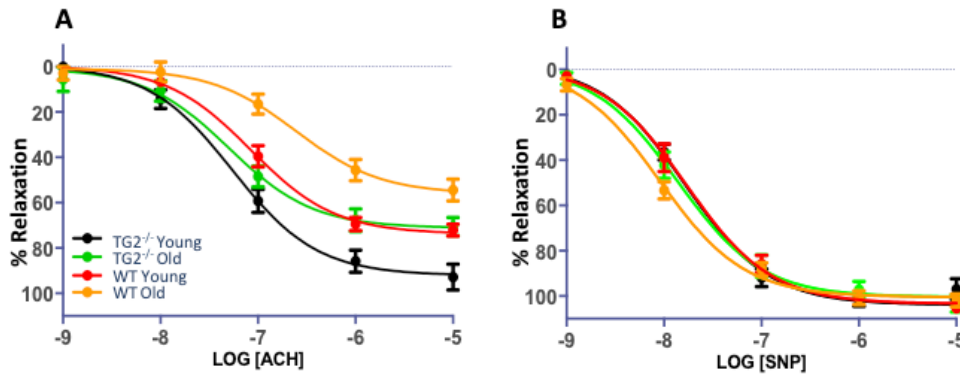


Figure 2 – (A) Old WT mice exhibited a markedly attenuated vascular relaxation as compared to Young WT animals. The TG2^{-/-} young and old mice had enhanced vasorelaxation responses (** $p < 0.001$) as compared to control mice. **(B)** SNP dose response curve demonstrates no significant differences in endothelial independent responses to SNP. $p < 0.001$ WT young vs. WT old; TG2^{-/-} young vs. TG2^{-/-} old; WT old vs. TG2^{-/-} old and WT young vs. TG2^{-/-} young. Abbreviations as in Figure 1.

TG2-generated cross-links is increased in old WT mice compared to young ones.

TG2-generated cross-links was determined

by IHC (Figure 3). There was a significant increase in old WT compared to young WT (3.66 ± 0.33 vs. 1.33 ± 0.33 , $p = 0.007$, $n = 3$), with no stain in the TG2^{-/-} animals.

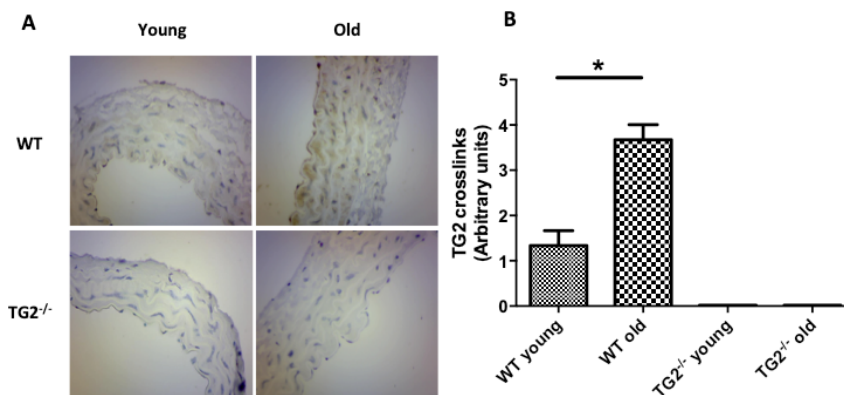


Figure 3 - A. Immunohistochemical analysis of TG2 presence (immune staining with anti-TG2 monoclonal antibody, x400) in wildtype young and old groups compared to TG2^{-/-} young and old groups. **B.** IHC scores are represented in the bar graphs ($n = 3$ in each group). There was no statistically significant difference between the WT young vs. TG2^{-/-} young and TG2^{-/-} young vs. old TG2^{-/-} groups. * represents values of $p < 0.05$. Abbreviations as in Figure 1.

Aorta arteries of old WT mice present ageing histological modifications, not observed in the old TG2^{-/-} mice.

Examination of old WT mice aortas with optical microscopy (H&E, x400) showed reduced elastic laminae, with areas of fragmentation compared to old TG2^{-/-} mice. Also, old TG2^{-/-} mice maintain tissue structure similar to young groups (Figure 4). In addition, cellular infiltrate in media tunica was found in old WT mice.

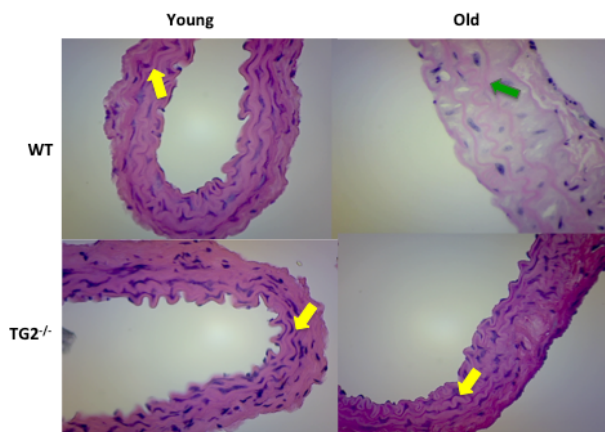


Figure 4 - Optical microscopy (H&E, x400) of aortic rings from wildtype old and young groups compared to TG2^{-/-} old and young groups. Aorta of old wildtype animals show reduced elastic laminae (*green arrow*) compared to old TG2^{-/-} animals (*yellow arrow*), which maintains tissue structure similar to young groups. Abbreviations as in Figure 1.

■ Discussion

We show that young WT mice had a similar endothelial function response when compared to 8-months old TG2^{-/-} mice. In our findings, old WT mice had the worse endothelial response among all groups. Furthermore, we show histological modification in aorta arteries media layers as related to the TG2-mediated early vascular ageing process.

Endothelial dysfunction appears to be one of the main markers of aging-related vascular changes, as a reduction in the endothelium-dependent vasodilatation is strongly associated to age-related endothelial dysfunction^{19,20}. In this regard, preventive efforts have been focusing on new therapeutic targets that may delay the vascular ageing process.

In our study, TG2 has an important role in early phases of vascular ageing, as young WT animals had similar degrees of endothelial response when compared to TG2^{-/-} old mice. In addition, we found early aortic histological abnormalities in old WT animals that are known to relate to arterial aging process^{21,22}. Interestingly, noninvasive measures of PWV and MAP were not significantly different among groups at this 8-month follow-up assessment.

Our results show TG2-mediated aortic microscopic changes as early as at 8 months of age. In accordance with our findings, previous studies on microscopic abnormalities in the aortic elastic laminae have shown relation to the vascular ageing process²¹⁻²³. Histological examination stained with H&E and IHC are validated methods to assess age-related microstructural early arterial changes^{8,21}.

TG2 has consistently shown an important role in age-dependent vascular stiffness^{8,9,11,24,25}. In particular, Santhanam *et al.*⁸ showed that PWV measures of TG2^{-/-} mice were similar to age-matched WT controls, but those TG2^{-/-} were protected from the increase in PWV resulting from NOS inhibition using L-NAME⁸. In addition, elevated arterial pressure is known to relate to older ages in clinical and experimental settings²⁶. Other aging studies, however, were performed on older animals when compared to our study, with ages ranging from 13 to 32 months²⁷⁻³⁰.

Our findings suggest that, even in mice ages as young as 8-months, TG2 relates to an early degree of endothelial dysfunction.

In the vascular ageing process, endothelial dysfunction and vascular histological abnormalities seem to precede changes detected by standard noninvasive methods widely used in clinical settings, such as PWV and MAP. As the animal ages, the endothelial dysfunction possibly progresses to a threshold point in which subclinical abnormalities may appear as clinically identifiable vascular diseases. Therefore, our study indicates that a comprehensive understanding of the role of TG2 in age-related vascular changes may help in the preventive efforts of the early subclinical vascular disease.

■ Conclusions

Genetic suppression of TG2 in mice delays early age-induced endothelial dysfunction and aortic microscopic abnormalities. Furthermore, TG2 inhibition augments vasorelaxation in an endothelial-dependent manner. Endothelial dysfunction and microstructural abnormalities were found as early age-related changes mediated by TG2, in the absence of significant alterations in noninvasive standard parameters such as pulse-wave velocity and blood pressure. These findings suggest that TG2 inhibition rises as a promising therapeutic target for future drug development aiming to prevent early subclinical age-related vascular abnormalities.

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