Transglutaminase activity is decreased in large arteries from hypertensive rats, compared to normotensive controls

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Abstract:

Transglutaminases (TGs) catalyze the formation of covalent cross-links between glutamine residues and amine groups. This cross-linking activity has been implicated in arterial remodeling. Because hypertension is characterized by arterial remodeling we hypothesized that TG activity, expression, and functionality would be increased in aorta, but not vena cava (which does not undergo remodeling), from hypertensive rats relative to normotensive rats. Spontaneously hypertensive stroke-prone rats (SHRSP) and deoxycorticosterone acetate (DOCA)-salt rats, and their respective normotensive Wistar-Kyoto or Sprague-Dawley counterparts were utilized. Immunohistochemistry and western blot analysis measured the presence and expression of TGs 1 and 2, in situ activity assays quantified the active TGs, and isometric contractility was used to measure TG functionality. Contrary to our hypothesis, the activity (52% DOCA-salt vs control, 56% SHRSP vs control; p<0.05), expression (TG1: 54% DOCA-salt vs control p>0.05, TG2: 77% DOCA-salt vs control p<0.05), and functionality of TGs 1 and 2 were decreased in aorta, but not vena cava, from hypertensive rats. Mass spectrometry identified proteins uniquely amidated by TGs in the aorta that play roles in cytoskeletal regulation, redox regulation, and DNA/RNA/protein synthesis and regulation, and in the vena cava that play roles in cytoskeletal regulation, coagulation regulation, and cell metabolism. Consistent with the idea that growing cells lose TG2 expression, vascular smooth muscle cells placed in culture lose TG2 expression. We conclude that the expression, activity, and functionality of TGs 1 and 2 are decreased in aorta, but not vena cava, from hypertensive rats compared to controls.

Key words: Transglutaminase, hypertension, aorta, vena cava, arterial remodeling.
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Unusual terms and their abbreviations:

5-HT – 5-hydroxytryptamine (serotonin)

BAP – pentylamine-biotin

DAPI – 4',6-diamidino-2-phenylindole

DOCA – deoxycorticosterone

FXIII – Factor XIII

PE – phenylephrine

RA – rat thoracic aorta

RVC – rat thoracic vena cava

SHRSP – spontaneously hypertensive rat, stroke prone

TG1 – transglutaminase 1

TG2 – transglutaminase 2

WKY – Wistar-Kyoto rat
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Introduction:

Transglutaminases (TGs) are a family of calcium-dependent enzymes that are best known for catalyzing the formation of a covalent bond between an amine group and the \( \gamma \)-carboxamide group of a glutamine residue (8). The amine can come from a variety of sources including polyamines (7), monoamines such as serotonin (19), or peptide- or protein-bound lysines (14). Transglutaminases 1, 2, 4, and Factor XIII have been identified in the vasculature, but only TGs 1, 2 and 4 are expressed by vascular smooth muscle cells (4, 10). The cross-linking activity of TGs is of particular interest in blood vessels because such activity has the ability to stabilize cytoskeletal and extracellular matrix proteins (4), processes involved in vessel remodeling. TG1 helps maintain endothelial barrier function (4), and small artery inward remodeling in rat in response to low blood flow is dependent on the activity of TG2 (2). Additionally, TG activity is required for multiple steps in the pathogenesis of atherosclerosis (endothelial cell response, monocyte attachment, vascular remodeling, plaque stability; 4) and for the small artery remodeling seen in mice in response to L-NAME-induced hypertension (15). These studies suggest that TGs play a functional role in the progression of the arterial remodeling observed in these disease states.

Essential hypertension is a cardiovascular disease characterized by a chronic increase in systolic (>140 mmHg) or diastolic (>90 mmHg) blood pressure.

Hypertension is associated with vascular remodeling of both small and large arteries.

Small artery remodeling is often eutrophic and characterized by decreased inner and outer diameter without hypertrophy, while large artery remodeling is associated with increased collagen and fibronectin deposition, elastin fragmentation, and increases in
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arterial wall thickness and stiffness (5). Small artery remodeling is dependent on TG2 activity (2), but TG function has not been investigated in the remodeling of large vessels in response to hypertension. Because large arteries, such as the aorta, experience the highest blood pressures in the body, their adaptation to a chronic increase in blood pressure is critical.

The thoracic aorta and the inferior vena cava were investigated because each has a contrasting structure, function, and remodeling response to hypertension. The aorta is composed of seven layers of smooth muscle, is a conduit vessel, and undergoes extensive remodeling, while the vena cava is composed of one layer of smooth muscle, is a capacitance vessel, and undergoes very little remodeling in response to hypertension, at least in deoxycorticosterone acetate (DOCA)-salt hypertension (20). In this way, the vena cava served as a comparison vessel because it is of similar size and proximity to the aorta.

We hypothesized that the activity of vascular TGs is increased in the aorta, but not the vena cava, from hypertensive rats when compared to their normotensive counterparts. Two models of hypertension were used: the DOCA-salt rat model and the spontaneously hypertensive rat, stroke prone (SHRSP) model. A multidisciplinary approach was adopted: immunohistochemistry to visualize TG protein, western blot analysis to measure TG protein expression, a novel in situ activity assay to measure isozyme-specific TG activity, an in situ pentyamine-biotin (BAP) incorporation assay to measure general TG activity, isometric contractility to examine TG function, cell culture to investigate TG expression in proliferating vascular smooth muscle cells, and mass spectrometry to identify proteins uniquely amidated by TGs in the aorta and vena cava.
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Contrary to our hypothesis the expression, activity, and functionality of TGs 1 and 2 were decreased in the aorta, but not the vena cava, from hypertensive rats compared to their normotensive counterparts. Consistent with this idea, TG substrates that were aorta-specific were identified, and we found that cultured vascular smooth muscle cells, like the growing vascular smooth muscle cells in the artery from hypertensive rats, also lost TG2 expression.

Materials and methods:

Animal use/ethics:
All protocols involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) and followed guidelines put forth by Michigan State University. Rats were euthanized by intraperitoneal injection of 60-80 mg/kg pentobarbital; thoracic aorta and vena cava were removed by dissection for each of the following protocols. Rat systolic blood pressures were measured by standard tail cuff technique, as described previously (20).

Animal models:
Animals were housed according to Michigan State University IACUC standards with a 12-hour light/dark cycle and free access to standard rat chow and drinking water.

DOCA-salt rats:
Male Sprague-Dawley rats (250 to 300 g; Charles River Laboratories Inc, Portage, MI) were randomly assigned to a DOCA or sham group. During a
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uninephrectomy, while under isoflurane anesthesia, a DOCA- (150 mg sc; Sigma-
Aldrich, St. Louis, MO, USA) impregnated silicone elastomer (Silastic, Dow Corning,
Midland, MI, USA) was implanted subcutaneously in the DOCA group. Rats assigned to
the sham group also underwent uninephrectomy but did not receive the DOCA implant.
Postoperatively, DOCA-salt rats were given a solution of 1% NaCl and 0.2% KCl
(wt/vol) to drink; sham rats drank normal tap water. Rats were on this regimen for 3-4
weeks prior to experimentation. DOCA-salt rats had an average systolic blood pressure
of 212 ± 4 mmHg while sham rats had an average systolic blood pressure of 115 ± 7
mmHg (p<0.05 t-test). Rats were 13-15 weeks old when sacrificed.

SHRSP rats:

Male SHRSP rats were obtained from the breeding colony at the Department of
Pharmacology and Toxicology at Michigan State University in East Lansing, MI, USA
maintained by Dr. Anne M. Dorrance. Age-matched normotensive Wistar-Kyoto (WKY)
rats were obtained from Charles River, Wilmington, MA, USA. SHRSP rats had an
average blood pressure of 202 ± 9 mmHg while the WKY rats had an average blood
pressure of 117 ± 1 mmHg. Rats were 12 weeks old when sacrificed.

Tissues were taken from animals for one of the following protocols:

Brightfield immunohistochemistry:

Immunohistochemistry was performed as previously described (20). Following
harvesting and cleaning, rat thoracic aorta and vena cava were formalin-fixed, paraffin-
embedded, and sectioned (8 µm). Sections were dewaxed in Histochoice Clearing
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Agent (Cat #H103-4L; Amresco, Solon, OH, USA), unmasked with Antigen Unmasking Solution (Cat #H-3300; Vector Laboratories, Burlingame, CA, USA), and incubated in 0.3% \( \text{H}_2\text{O}_2 \) in Dulbecco’s phosphate buffered saline (PBS; Cat #D8537; Sigma-Aldrich) for 30 minutes at room temperature to eliminate endogenous peroxidase activity.

Sections were blocked for 60 minutes at room temperature in 1.5% blocking serum in PBS, incubated in primary antibody [mouse anti-TG1 (1:100; Cat #sc-166467; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-TG2 (1:100; Cat #ab421; Abcam, Cambridge, MA, USA)], and equilibrated at 4 °C overnight. Primary antibody was not included in negative control sections. The next day, sections were incubated in secondary antibody (1:200 secondary and 1.5% blocking serum in PBS) for 30 minutes at room temperature. The secondary antibody was visualized using Vectastain ABC Elite Reagent (Cat #PK-61000; Vector Laboratories) and ImmPACT NovaRED (Cat #SK-4805; Vector Laboratories), according to manufacturer instructions. Vectastain Hematoxylin QS (Cat #H-3404; Vector Laboratories) was used as a counterstain.

Coverslips were mounted and sealed with Vecta Mount (Cat #H-5000; Vector Laboratories). Brightfield imaging was performed using a Nikon Eclipse inverted microscope (Nikon Group, Otowara, Japan) and images captured using MMI Cell Tools (Molecular Machines & Industries, Zurich, Switzerland). Human Skin (Cat #12-701-XA1; ProSci, Loveland, CO, USA) was used as a positive control for TG1 and Human Breast Ductal Carcinoma Tumor (Cat #10-010-XA1; ProSci) for TG2.

In situ TG activity assay:
In situ TG1 and TG2 activity was detected using fluorescently labeled isozyme-specific glutamine donor peptides as described previously (9, 10). In addition to active peptides, negative control peptides composed of the same amino acid sequence with the reactive glutamine (Q) replaced with a non-reactive asparagine (N) were utilized (QN peptides). Following harvesting and cleaning, rat thoracic aorta and vena cava were fresh frozen, 8 μm sections mounted on slides, and stored at -80 °C until use. The sections were equilibrated to room temperature and blocked for 30 minutes in PBS with 150 mM NaCl and 1% bovine serum albumin (Cat #A7906-10G; Sigma-Aldrich). Next, the sections were incubated in substrate reaction solution (5 mM CaCl₂, 100 mM Tris-HCl pH 8.0, 1 mM DTT) with 0.1 μM FITC labeled peptide (K5 [YEQHKLPSSWF] or K5QN for TG1 activity and T26 [HQSYVDPWMLDH] or T26QN for TG2 activity) at 37 °C for 90 minutes. The samples were then incubated in stop solution (25 mM EDTA in PBS) for 5 minutes at room temperature, washed in PBS (3 x 5 minutes at room temperature), coverslips applied using ProLong Gold antifade reagent with DAPI (Cat #P36931; Invitrogen, Eugene, OR, USA), and sealed with clear nail polish once dry. Fluorescent imaging was performed using a Nikon Eclipse inverted microscope (Nikon Group) and images captured using NIS Elements Br 3.0 (Nikon Group).

In situ BAP assay and BAP/RECA-1 colocalization assay:

Following harvesting and cleaning, rat thoracic aorta and vena cava were fresh frozen, 8 μm sections mounted on slides, and stored at -80 °C until use. Sections were equilibrated to room temperature and blocked for 30 minutes in PBS with 1% bovine serum albumin (Cat #A7906-10G; Sigma-Aldrich). Sections incubated with substrate
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reaction solution (100 mM Tris-HCl pH 8.0, 1 mM DTT) with or without the amine donor substrate 5-biotinamidopentylamine (4 mM, BAP; Cat #21345; Pierce Biotechnology, Rockford, IL, USA) at 37 °C for 90 minutes. Stop solution (25 mM EDTA in PBS) was added for 5 minutes at room temperature. Sections were washed in PBS (3 x 5 minutes at room temperature), incubated in Alexa Fluor 555-conjugated streptavidin (1:1000 in PBS; Cat #S32355; Invitrogen) at 37 °C for one hour, washed in PBS again (3 x 5 minutes at room temperature), coverslips mounted using ProLong Gold antifade reagent with DAPI (Cat #P36931; Invitrogen), and sealed with clear nail polish once dry. Fluorescent imaging was performed using a Nikon Eclipse inverted microscope (Nikon Group) and images captured using NIS Elements BR 3.0 (Nikon Group). The colocalization assay was performed in the same manner, but with the following additions: 1.5% goat serum in the blocking solution and reaction solution, RECA-1 primary antibody (1:50; Cat #ab22492; Abcam) in the reaction solution, and Alexa Fluor 488 goat anti-mouse secondary antibody (1:1000; Cat #A11029; Invitrogen) in the secondary antibody solution.

Cultured VSMC assay:

Aortic and vena cava smooth muscle cells were derived from explants of the thoracic aorta or inferior vena cava and fed with DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamate. Cells were verified as smooth muscle cells by staining with a smooth muscle cell-specific alpha-actin antibody. Both aortic and vena cava cells were used between passages 1 and 2. Cells were then prepared for western analysis, as described below.
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Western blots:
Protein isolation, protein concentration determination, and western analysis were performed as previously described (20). Briefly, 50 μg of homogenized total protein from rat aorta and vena cava or smooth muscle cells cultured from these vessels was separated by gel electrophoresis, blotted onto a polyvinylidene fluoride (PVDF) membrane (TG1) or a nitrocellulose membrane (TG2), blocked for 3 hours at 4 °C in 4% wt/vol chick egg ovalbumin, and incubated overnight in primary antibody [mouse anti-TG1 (1:100; Cat #sc-166467; Santa Cruz Biotechnology) or rat anti-TG2 (1:2,000; Hybridoma Facility, University of Alabama at Birmingham, AL, USA)] diluted in blocker. Samples were incubated in secondary antibody [HRP-linked anti-mouse IgG (TG1; Cat #NA931V; GE Healthcare, Little Chalfont, Buckinghamshire, UK) or HRP-linked anti-rat (TG2, Abcam)] for one hour at 4 °C. The positive control for TG1 was rat skin lysate (Cat #1480; ProSci). Blots were developed with ECL western blotting detection reagents (Cat #RPN2209; GE Healthcare) on film (Cat #F-9023-8X10; GeneMate, BioExpress, Kaysville, UT, USA) using a Kodak X-OMAT film developer (Eastman Kodak Company, Rochester, NY, USA). ImageJ (U. S. National Institutes of Health, Bethesda, MD, USA) was used for densitometric analysis and quantification.

Isometric contraction:
Cleaned rings (~ 5 mm length) of endothelial cell-intact thoracic aorta from sham and DOCA-salt rats were mounted in tissue baths for isometric tension recordings using Grass transducers and PowerLab data acquisition system (Chart 7.0; Colorado Springs,
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Tissues were placed under optimum resting tension (4000 milligrams) and equilibrated for one hour, with washing, before exposure to compounds. Tissue baths contained warmed (37 °C), aerated (95% O₂/CO₂) physiological salt solution [PSS: 103 mM NaCl, 4.7 mM KC, 1.18 mM KH₂PO₄, 1.17 mM MgSO₄-7H₂O, 1.6 mM CaCl₂-2H₂O, 14.9 mM NaHCO₃, 5.5 mM dextrose, and 0.03 mM CaNa₂ EDTA]. Administration of an initial concentration of 10 μM phenylephrine (PE) was used to test tissue viability. The status of the endothelial cell was examined by testing the ability of the cholinergic muscarinic agonist acetylcholine (Ach) to relax aortic rings contracted half-maximally to PE. Tissues were again washed and were incubated for one hour with vehicle (water) or cystamine (1 mM) prior to cumulative addition of 5-HT (10⁻⁹ – 10⁻⁴ M). Data are reported as the percentage of the initial contraction to PE.

**TG substrate identification:**
To have sufficient protein for analysis, two to three aorta or 5 to 6 vena cava from normal Sprague-Dawley rats were pooled to make protein homogenates. Pooled isolation was performed a total of three times. Tissues were taken fresh from animals, cleaned of perivascular fat and clotted blood, frozen using liquid nitrogen, and pulverized into a powder using a mortar and pestle. Lysis buffer (10 mM Tris, 0.5 mM EDTA, 0.5 mM β-mercaptoethanol, and 0.1% Triton X-100; pH 8.0) plus protease inhibitors (1 mM PMSF, 2 mg/ml aprotinin and leupeptin, 1 mM sodium orthovanadate) was added (250 μl per rat aorta, 50 μl per rat vena cava), tissues were ground for an additional minute in buffer, and then lysates were collected in centrifuge tubes. Tubes were briefly sonicated (6 x 1 minute), centrifuged at 18,000 g for 10 minutes, and
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supernatant was removed and placed in a fresh tube while the remaining pellet was
discarded. Protein concentration of the supernatant was immediately determined using
a BCA protein assay. Samples were then frozen at -80°C until the reaction was
performed.

BAP incorporation was performed in lysis buffer in the presence of 5 mM CaCl$_2$
and 1 mM dithiothreitol (DTT), plus protease inhibitors (same as above). Rat aorta and
vena cava protein was diluted to 1 μg/μl (a total of 400 μg protein was used per
substrate condition), and 4 mM BAP or control (lysis buffer) was added. Samples were
incubated at 37°C for one hour, at which point samples were removed and an aliquot of
protein (20 μg) was collected. This aliquot was separated by electrophoresis on an
SDS-polyacrylamide gel (SDS-PAGE) to visually examine differences between aorta
and vena cava TG substrates. This allowed us to determine if there were TG protein
substrates specific to each vessel.

To the remaining TG reaction solution, 1% SDS was added [to prevent non-
specific protein binding to Dynabeads MyOne streptavidin-coated C1 beads (Cat #650-
02; Invitrogen)] and samples were placed on ice while dialysis units were prepped.
Slide-A-Lyzer® Dialysis cassettes (Cat #66330; Thermo Scientific, Rockford, IL, USA)
were used according to the manufacturer’s instructions. Cassettes were hydrated by
incubating in PBS for at least 2 minutes prior to use. Samples were diluted to 2 mL with
PBS plus protease inhibitors (Complete Mini Protease Inhibitor Cocktail Tablets, Cat
#11836153001; Roche Applied Science, Indianapolis, IN, USA; 1 tablet/10 mL of PBS)
prior to loading sample into dialysis cassette. Samples were allowed to dialyze with 500
mL of PBS for 2 x 2 hours, then overnight, at 4°C. Samples were then removed from
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dialysis unit, added to streptavidin-coated Dynabeads (600 µl of beads per sample
prepared by washing 3x in PBS, per the manufacturer’s instructions, and resuspended
in 300 µl of PBS plus Roche Inhibitor Cocktail and 0.1% SDS), and allowed to tumble
for 2 hours at 4°C. This volume of beads was determined to be necessary by
preliminary experiments. Beads were captured using a magnet (Dynal MPC®-M, Cat
#120.09; Invitrogen). Supernatant was removed, beads were washed 3x with PBS plus
protease inhibitors, placed on ice, and taken to the proteomics facility at Michigan State
University to be analyzed by tandem mass-spectrometry to identify proteins captured.

SDS-PAGE and In-Gel Digestion for Mass Spectrometric Analysis:

Bead-bound protein samples were incubated with 40 µl of 2x SDS-PAGE sample
buffer at 60°C for 10 minutes in an Eppendorf ThermoMixer R (Eppendorf, Hauppauge,
NY, USA). The solutions were then cooled to room temperature and spun at 21,000 x g
to pellet particulates. The supernatant was loaded onto a Criterion 12.5% Tris-HCl
precast gel (Bio-Rad, Hercules, CA, USA) and electrophoresed at 50V constant for ~15
minutes or until the dye front migrated 2-3mm below the well. Electrophoresis was then
stopped; the gel fixed in 40% Methanol/20% Acetic Acid for at least 2 hours followed by
overnight staining with Colloidal Coomassie Blue stain. After de-staining, visualized gel
bands were individually cut from the gel and subjected to in-gel tryptic digestion
according to Shevchenko et al. (16), with modifications. Briefly, gel bands were
dehydrated using 100% acetonitrile and incubated with 10 mM dithiothreitol in 100 mM
ammonium bicarbonate, pH ~8, at 56°C for 45 minutes, dehydrated again, and
incubated in the dark with 50 mM iodoacetamide in 100 mM ammonium bicarbonate for
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Gel bands were then washed with ammonium bicarbonate and dehydrated again. Sequencing grade modified trypsin was prepared to 0.01 \( \mu \text{g/\mu l} \) in 50mM ammonium bicarbonate and ~50 \( \mu \text{l} \) of this was added to each gel band so that the gel was completely submerged. Bands were then incubated at 37°C overnight. Peptides were extracted from the gel by water bath sonication in a solution of 60% acetonitrile/1% trichloroacetic acid and vacuum dried to ~2 \( \mu \text{l} \).

Mass Spectrometric Analysis:

Peptides were re-suspended in 2% acetonitrile/0.1% trifluoroacetic acid to 20 \( \mu \text{l} \) and from this 10 \( \mu \text{l} \) were automatically injected by a nanoAcquity Sample Manager (Waters Corporation, Milford, MA, USA) and loaded for 5 minutes onto a Waters Symmetry C18 peptide trap (5 \( \mu \text{m} \), 180 \( \mu \text{m} \) x 20 mm) at 4 \( \mu \text{l/minute} \) in 2% acetonitrile/0.1% formic acid. The bound peptides were then eluted using a Waters nanoAcquity UPLC (Buffer A = 99.9% Water/0.1% Formic Acid, Buffer B = 99.9% Acetonitrile/0.1% Formic Acid) onto a Waters BH130 C18 column (1.7\( \mu \text{m} \), 100\( \mu \text{m} \) x 100 mm) and eluted over 60 minutes with a gradient of 5% B to 30% B over 47 minutes, ramped to 90%B at the 49th minute, held at 90%B for 1 minute and dropped back to 5%B at the 50.1 minute, all at a constant flow rate of 0.8 \( \mu \text{l/minute} \). Eluted peptides were sprayed into either a LTQ-FT Ultra mass spectrometer (ThermoFisher, Waltham, MA, USA) or a ThermoFisher LTQ Linear Ion-Trap mass spectrometer, both using a Michrom ADVANCE nanospray source. For LTQ-FT analysis, survey scans were taken in the FT (25000 resolution determined at m/z 400) and the top five ions in each survey scan were then subjected in a data-dependent fashion to automatic low energy collision
Decreased TG 1 and 2 in aorta from hypertensive rats induced dissociation (CID) in the LTQ. For the LTQ analysis, the top five ions in each survey scan were then subjected to data-dependent zoom scans followed by CID in the ion trap.

Data analysis:
The brightness and contrast of images were adjusted for clarity equally for all IHC, in situ activity assay, and in situ BAP assay image sets. Additionally, the exposure time and look-up tables (LUTs) were set equally for all sections in an experimental set. The in situ BAP assay images were taken using the TRITC (red) channel fluorescence and were converted to green fluorescence for clarity using ImageJ (NIH). All images of western blots are unaltered for either brightness or contrast.

The “Measure, Length” command of NIS Elements BR 3.0 (Nikon Group) was used with brightfield images at 4x magnification to measure aorta lumen diameter and wall thickness. For each condition (DOCA-salt, sham, SHRSP, WKY), measurements from 4 animals were pooled. Statistical significance between conditions was established using one-tailed, unpaired t-tests (p=0.05).

Quantification of fluorescent images was performed using ImageJ (NIH). The FITC (green fluorescent) channel of the image was converted to grayscale. For aorta, the entire artery was selected and the area and mean gray value were measured. For vena cava, the luminal smooth muscle layer was selected and the area and mean gray value were measured. Density of fluorescence was then calculated by dividing the mean gray value by the area. Statistical analysis was performed using a 2-way ANOVA.
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with a level of significance set at p<0.05. Data were graphed using Graph Pad Prism
(GraphPad Software, San Diego, CA, USA).

Densitometric analysis of TG1 and TG2 was done using ImageJ (NIH). Protein
levels of the TGs were normalized to β-actin or α-actin by dividing the densitometric
data obtained for the protein of interest with the densitometric data obtained for the actin
control. β-actin was used for normalization when comparing cultured smooth muscle
cells with whole tissue to take into account the contribution of other cell types within
whole tissue, and because α-actin levels can be profoundly different between aorta and
vena cava smooth muscle cells in culture. When comparing tissues from hypertensive
rats with control rats α-actin was used because TG expression in the smooth muscle
was then the focus. Statistical significance between groups was established using two-
tailed, unpaired t-tests (p=0.05).

Contractility data were recorded in milligrams, normalized to an initial maximal
response to 10 μM phenylephrine (PE), and reported as a percentage of the initial
maximal response. Statistical significance between maximums was established using
two-tailed, unpaired t-tests (p=0.05).

For analysis of the mass spectrometry data, the resulting MS/MS spectra were
converted to peak lists using BioWorks Browser v3.3.1 (ThermoFisher) using the default
FT or LTQ parameters and searched against all rat protein sequences available from
the SwissProt protein database (downloaded from UniProt [www.uniprot.org] on
8/06/2012) using the Mascot searching algorithm, v 2.4.1. The Mascot output was then
analyzed using Scaffold, v3.6.4 (www.proteomesoftware.com) to probabilistically
validate protein identifications using the ProteinProphet (13) computer algorithm.
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Assignments validated above the Scaffold 95% confidence filter were considered true. Proteins that were present in the treatment group at a level greater than two times the control group were considered a “hit”. Proteins were considered “aorta-specific” if they registered 3 hits in the rat aorta and 0 or 1 hit in the rat vena cava. Proteins were considered “vena cava-specific” if they registered 3 hits in the rat vena cava and 0 or 1 hit in the rat aorta. Protein levels were compared using the “Qualitative Value” option in Scaffold.

Results:

Decrease in TG1 and TG2 immunohistochemical staining in aorta from DOCA-salt rats:

An increase in wall-to-lumen ratio of the aorta, measured by brightfield microscopy, was used as an indicator of smooth muscle hypertrophy/hyperplasia and confirmed that arterial remodeling had occurred (Table 1). Immunohistochemical detection of TG1 (Figure 1A) and TG2 (Figure 1B) protein in aorta and vena cava from DOCA-salt and sham rats was performed, with human skin (TG1) and human breast tumor (TG2) as positive controls. Staining was absent in sections lacking the primary antibody (insert in corner of images). For both TG1 and TG2, staining was significant in the smooth muscle layers of both vessels, although it was not exclusive to those areas. This was particularly apparent in the vena cava, which contain only one layer of luminal smooth muscle. A qualitative decrease in both TG1 and TG2 staining was observed in aorta from DOCA-salt rats, compared to aorta from sham rats. This decrease was not observed in the vena cava.

Decreased TG activity in aorta from both DOCA-salt and SHRSP rats:
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In addition to aorta and vena cava from DOCA-salt and sham rats we investigated aorta and vena cava from SHRSP rats and their WKY controls using the \textit{in situ} TG activity assay and the \textit{in situ} BAP assay. \textit{In situ} incorporation of a fluorescently labeled isozyme specific glutamine donor peptide (K5 for TG1, T26 for TG2) was used to measure \textit{in situ} TG1 (Figure 2A, 3A) and TG2 activity (Figure 2B, 3B). Negative control peptides (K5QN or T26QN; insert in corner of images), which have the reactive glutamine replaced by a non-reactive asparagine, were utilized in parallel with active peptides. The wavy lines seen between smooth muscle layers in the aorta are caused by elastin autofluorescence. The right hand panels quantify fluorescence for each set of tissues. \textit{In situ} glutamine donor peptide incorporation supports a significant decrease in TG1 and TG2 activity in aorta from DOCA-salt rats when compared to aorta from sham (Figure 2; p<0.05). No significant difference in TG1 or TG2 activity was observed in vena cava. Similarly, \textit{in situ} glutamine donor peptide incorporation showed a significant decrease in TG1 and TG2 activity in aorta but not vena cava from SHRSP rats when compared to aorta from WKY rats (Figure 3; p<0.05).

\textit{In situ} incorporation and fluorescent detection of an amine donor substrate (BAP assay) was used to measure general \textit{in situ} TG activity (Figure 4). As a negative control, parallel sections were included without the addition of BAP in the reaction solution (insert in corner of images). Signal was not present in negative control sections. The right hand panels quantify fluorescence of images. Aorta from DOCA-salt rats had decreased incorporation of BAP compared to aorta from sham rats (Figure 4A; p<0.05). Similarly, aorta from SHRSP rats had decreased incorporation of BAP compared to aorta from WKY rats (Figure 4B; p<0.05). The incorporation of BAP was not reduced in
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the vena cava of either DOCA-salt or SHRSP rats compared to controls. We performed
an endothelial marker (RECA-1)/BAP colocalization to verify that transglutaminase
activity in the vena cava is present in the luminal smooth muscle layer, as well as the
endothelial layer (Figure 5). We found that TG activity was present in both the
endothelial layer and the luminal smooth muscle layer. This is consistent with IHC
staining for TG protein.

Decreased TG1 and TG2 protein expression in aorta from DOCA-salt rats:

Western analysis was used to measure TG1 and TG2 protein expression in aorta
and vena cava from DOCA-salt and sham rats, with protein levels normalized to α-actin
(TG1 - Figure 6A; TG2 - Figure 6B). When measuring TG1 protein expression bands of
generally lower intensity were seen in aorta from DOCA-salt rats compared to aorta
from sham rats, but this difference was not statistically significant. TG2 protein
expression was decreased in aorta from DOCA-salt rats when compared to aorta from
sham rats (p<0.05). No significant difference in either TG1 or TG2 protein expression
was seen in vena cava from DOCA-salt rats compared to control.

Decreased TG expression in cultured VSMC:

Western analysis of fresh whole tissue and cultured aorta and vena cava VSMCs
from normal Sprague-Dawley rats was performed, with protein levels normalized to β-
actin. Cultured rat aorta VSMCs showed a complete loss of TG2 protein expression
compared to whole rat aorta, but TG1 was not decreased (Figure 7A). Additionally,
cultured rat vena cava VSMCs showed a decrease in TG1 and a loss of TG2 protein
Decreased TG 1 and 2 in aorta from hypertensive rats expression compared to fresh rat vena cava (Figure 7B). These data are quantified in Figure 6C. The 76 kDa is the generally accepted molecular weight for TG2. The 52 kDa band may be due to alternate posttranslational modifications and is not indicative of TG2 expression. These data suggest that actively proliferating VSMCs undergo a phenotypic change associated with a decrease in TG1 and, in particular, TG2 expression.

Decreased TG function in aorta from DOCA-salt rats:
Cystamine, a non-isozyme selective TG inhibitor, was used in an isometric contraction assay to measure relative TG function. The purpose of this experiment was to assess TG function in remodeled aorta from hypertensive rats. 5-HT was used as an agonist given its recognized dependence on TG function for contraction (19).

Contraction to 5-HT is reported as % of initial contraction to PE (Sham RA: 1273.4 ± 71.8 mg; sham RA + cystamine: 1018 ± 163mg; DOCA RA: 1366 ± 33.96mg; DOCA RA + cystamine: 1300 ± 133mg). ACh-induced relaxation was 37.8 ± 6.24% [EC$_{50}$ PE contraction in sham RA, average of vehicle and cystamine] and 82.47 ± 13% [EC$_{50}$ PE contraction in DOCA-salt RA, average of vehicle and cystamine]. The ability of cystamine to reduce 5-HT-induced maximal contraction was significantly diminished in aorta from DOCA-salt rats compared to aorta from sham rats (Figure 8). These data suggest that TG function may be decreased in aorta from DOCA-salt rats.

Aorta- and vena cava-specific TG substrates:
Decreased TG 1 and 2 in aorta from hypertensive rats

Because aortic TG activity was selectively decreased in hypertension when compared to vena cava TG activity, we examined the targets of TG activity that are aorta- and vena cava-specific using BAP to capture proteins. Seven TG substrates were identified that are specific to the aorta and six TG substrates were identified that are specific to the vena cava (Table 2). Proteins that had 3 hits in the aorta and 0 or 1 hit in the vena cava or 3 hits in the vena cava and 0 or 1 hit in the aorta are shown on the left and the associated full name and function of each protein is shown on the right. These proteins play roles in cytoskeletal organization, redox regulation, DNA/RNA/protein synthesis and regulation, coagulation regulation, and cell metabolism.

Discussion:
Small artery inward remodeling in response to low blood flow is dependent on TG2 (2), and work in our lab has shown the presence of TG1 and TG2 in the aorta and vena cava (10). Additionally, TG activity is required for multiple steps in the pathogenesis of atherosclerosis (4) and the small artery remodeling seen in mice in response to L-NAME-induced hypertension (15). Despite this, no research has investigated the role of TGs in the remodeling of large arteries and veins in response to high blood pressure. Large arteries experience the greatest pressures in hypertension, so they may be the most challenged. We investigated the expression, activity, and function of TGs 1 and 2 in the aorta and vena cava from hypertensive rats and their normotensive counterparts. We hypothesized that protein expression, activity, and functionality of TGs 1 and 2 would be increased in the aorta, but not the vena cava. Contrary to our hypothesis, we found that protein expression, activity, and function of TGs 1 and 2 were decreased in the aorta from hypertensive rats compared to
Decreased TG 1 and 2 in aorta from hypertensive rats normotensive rats, while no difference was seen in the vena cava between the two groups.

A two-model, two-vessel approach:

We used two rat models of hypertension to test our hypothesis. This was done to determine whether the results were specific to a specific model of hypertension.

The DOCA-salt model is an experimental model based on hyperaldosteronism and is characterized by increased vasoconstrictor efficacy (21), reduced vasodilator efficacy, endothelial dysfunction, and centrally mediated increases in vascular smooth muscle tone (17). DOCA-salt hypertensive rats exhibit increased aortic superoxide production and renal inflammation (12) and undergo vascular remodeling in hypertension (20). The SHRSP model is a genetic model characterized by increased sympathetic activity (18), elevated renin levels, increased VSMC growth and proliferation (17), and increased vascular release of superoxide (12). Importantly, the SHRSP model exhibits pathophysiological changes similar to those seen in human essential hypertension (18) and has been used to investigate the role of TG2 in small artery remodeling (6).

The thoracic aorta and inferior vena cava each have fundamentally different roles in cardiovascular physiology. The aorta is an elastic conduit vessel that, due to the cushioning function of large arteries, experiences high blood pressures, contributes to systolic blood pressure (17), and undergoes significant remodeling in response to hypertension (20). In contrast, the vena cava carries a high blood volume, is highly
Decreased TG 1 and 2 in aorta from hypertensive rats

compliant, contributes greatly to vascular capacitance, is a low-pressure vessel (17), and undergoes little remodeling in response to hypertension (20).

A five-method approach:

The multiple approaches taken – immunohistochemistry, *in situ* activity assay using a glutamine donor, *in situ* BAP assay using an amine donor, western analysis, and contractility – are internally consistent. However, the outcome refutes our hypothesis. In direct contrast to research in small arteries, the protein expression, isozyme specific activity, general activity, and functionality of TGs were decreased in aorta from hypertensive rats, but not from the vena cava of these rats, when compared to normal rats. These changes in TG activity are meaningful to aortic function because contraction to 5-HT, a TG-dependent process (19), was inhibited to a lesser degree in aorta from DOCA-salt rats compared to sham. This suggests that TG function is decreased in these vessels.

Arteries versus veins:

TG activity in the vena cava of hypertensive rats, unlike in the aorta, was not reduced compared to the normotensive controls. The aorta experiences higher pressures, and typically remodels to a greater extent than the vena cava (20). If remodeling were associated with decreased TG activity, this would explain the decrease in TG activity seen in the aorta but not in the vena cava. The aorta has 7 layers of smooth muscle, while the vena cava only has one layer of smooth muscle. If remodeling were related to phenotypic changes in vascular smooth muscle cells, characterized by a
Decreased TG 1 and 2 in aorta from hypertensive rats

decrease in TG activity, the difference in amount of vascular smooth muscle would
explain the significant decrease in TG activity seen in the aorta. In support of this idea,
cultured VSMCs from both aorta and vena cava lost TG expression. Vena cava smooth
muscle cells have the potential to lose TG expression and enter a proliferative state
similar to aortic smooth muscle cells, and do so in culture, but do not do so in vivo in
response to hypertension.

A third factor that could account for the difference in TG 1 and 2 activity between
the aorta and vena cava of hypertensive rats is the difference in TG substrates in each
vessel. Three functional groups of aorta-specific TG protein substrates were identified:
cytoskeletal proteins (destrin, profilin 1, myosin heavy chain 10), a redox regulation
protein (glutathione s-transferase mu5), and DNA/RNA/protein synthesis and regulation
proteins (cysteine and glycine-rich protein 1, GTP-binding nuclear protein RAN, protein
niban). The vena cava does not lose TG activity and does not undergo remodeling
during hypertension, so vena cava-specific TG substrates may account for the
stabilization and lack of remodeling in this vessel. Three functional groups of vena cava-
specific TG protein substrates were identified: cytoskeletal proteins (vimentin,
dihydropyrimidinase-related protein 2, tubulin beta-2A chain), coagulation regulation
proteins (fibrinogen gamma chain, alpha-1-macroglobulin), and a cell metabolism
protein (guanine deaminase). Literature searches indicate that no research has been
performed investigating how TG-mediated modifications affect the activity or function of
these proteins, so the discussion of these proteins’ roles in arterial remodeling is
speculation. Our findings raise the question of how TG-driven modification of these
Decreased TG 1 and 2 in aorta from hypertensive rats proteins through TG-mediated reactions changes their activity such that the loss of TG activity in the aorta is associated with remodeling.

Differences between large and small artery TGs in hypertension:

Our results are in direct contrast with previous work that has shown the inward remodeling of small arteries is TG2-dependent (2). Bakker et al found that both the inward remodeling of rat mesenteric arteries induced by low blood flow, and the inward remodeling of rat skeletal muscle arteries induced by endothelin-1, were decreased by the addition of the TG inhibitor cystamine (2). Additionally, inward remodeling of rat mesenteric arteries in response to both L-NAME-induced hypertension and low blood flow is delayed in TG2 KO mice (15, 3). Our results may be different because the vessels our lab and other labs used have fundamentally different physiological roles.

Small arteries contribute to total peripheral resistance. Large arteries, on the other hand, are highly elastic conduit vessels. It is not unreasonable to suggest that remodeling of these vessels plays different roles in the regulation of blood pressure and that transglutaminases have different roles in this remodeling.

Limitations:

The results and conclusions of this research are limited to vessels of similar size and caliber as those used – the aorta and vena cava. Indeed, our research is in direct contrast to similar research using smaller vessels. The use of sections of vessels and standardized conditions to test for TG activity may remove or modify regulators of TG activity, such as oxidation-reduction balance and intracellular Ca^{2+} levels, which may
Decreased TG 1 and 2 in aorta from hypertensive rats

play a different role in vivo in hypertensive animals compared to normotensive. Despite this, the results of IHC and western analysis are consistent with the measurements of TG activity, supporting the idea that TG expression and activity are decreased. Although the use of exogenous Ca\(^{2+}\)/DTT may artificially induce TG activity, it normalizes experimental conditions and reflects physiological conditions in which intracellular TG is activated. While it has become a general view in the field that intracellular TG2 is not active, there is evidence that suggests the reducing milieu in cells, increases in intracellular Ca\(^{2+}\) levels, and decreases in intracellular GTP levels can lead to sensitization and activation of TG2, as outlined in (11). The inhibitor cystamine was used because it is not isoform specific and therefore was ideal for our research, as opposed to other inhibitors such as the TG2-specific L682.777. That said, the use of cystamine in the contractility experiments might have resulted in off-target effects. The use of \(\alpha\)-actin, which is expressed in smooth muscle cells, to normalize TG protein expression when comparing tissues from hypertensive rats and control rats did not take the endothelial tissue into account. We normalized TG expression using \(\alpha\)-actin because we are primarily interested in TG activity within the vascular smooth muscle cells. That said, when comparing cultured smooth muscle cells with whole tissue (Figure 7) the protein expression was normalized to \(\beta\)-actin, which does take other cell types into account. While the presence of endothelial cells could contribute to the difference in TG expression observed between tissue and cultured smooth muscle cells, endothelial cells account for only a small percentage of total tissue, at least in the aorta. The similarity of TG expression in whole aorta (Figure 6) and cultured cells lends confidence to the conclusion that TG expression is decreased in these vascular smooth muscle
Decreased TG 1 and 2 in aorta from hypertensive rats

cells. We also cannot assume that these results extend beyond the DOCA-salt and
SHRSP rat models of hypertension, however, internal consistency supports that these
findings are general to hypertension. Additionally, the results are limited to the activity
and function of TG1 and TG2. TG4 and macrophage-derived FXIII (4, 10) were not
investigated. Lastly, we investigated rats with established hypertension. TGs may play
contrasting roles in the development of hypertension compared to its maintenance.

Conclusion:

A multi-faceted approach allowed determination that there was a decrease in TG
protein expression, activity, and function in the aorta but not vena cava of hypertensive
rats compared to normotensive rats. Additionally, we identified aorta- and vena cava-
specific TG substrates and discovered that cultured vascular smooth muscle cells
undergo a phenotypic change characterized by increased proliferation and loss of TG2
expression. These findings are in direct contrast with previous research investigating
TG2 in small artery remodeling, indicating that the role of TGs in arterial remodeling
may differ in large and small arteries.

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Decreased TG 1 and 2 in aorta from hypertensive rats

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Conflict of interest statement:

The authors declare that the research was performed in the absence of any financial or commercial relationships that could be construed as a potential conflict of interest.
Decreased TG 1 and 2 in aorta from hypertensive rats

References


Decreased TG 1 and 2 in aorta from hypertensive rats


Decreased TG 1 and 2 in aorta from hypertensive rats


Decreased TG 1 and 2 in aorta from hypertensive rats

**Figure 1.** Immunohistochemistry for TG1 (A) and TG2 (B) in sham and DOCA-salt rat aorta and vena cava. Negative controls are included in the corner of images. Human skin was used as a positive control for TG1 and human breast tumor was used as a positive control for TG2. A capital L indicates the luminal side and arrows indicate areas of interest. Images are representative of n=4 for all tissues.

**Figure 2.** In situ activity assay for TG1 (A) and TG2 (B) activity in sham and DOCA-salt rat aorta and vena cava. Sham rat images are representative of n=4 and DOCA-salt rat images are representative of n=5. Quantification results are presented to the right of each image set. Negative controls are included in the corner of images. A capital L indicates the luminal side and arrows indicate areas of interest. * = significantly different (p<0.05) from sham. ** = significantly different (p<0.05) from active peptide.

**Figure 3.** In situ activity assay for TG1 (A) and TG2 (B) activity in WKY and SHRSP rat aorta and vena cava. All images are representative of n=4. Quantification results are presented to the right of each image set. Negative controls are included in the corner of images. A capital L indicates the luminal side and arrows indicate areas of interest. * = significantly different (p<0.05) from WKY. ** = significantly different (p<0.05) from active peptide.

**Figure 4.** BAP assay for general TG activity in sham and DOCA-salt rat aorta and vena cava (A) and WKY and SHRSP rat aorta and vena cava (B). All images are representative of n=4. Quantification results are presented to the right of each image.
Decreased TG 1 and 2 in aorta from hypertensive rats

set. Negative controls are included in the corner of images. A capital L indicates the luminal side and arrows indicate areas of interest. * = significantly different (p<0.05) from sham/WKY. ** = significantly different (p<0.05) from BAP.

Figure 5. BAP assay/RECA-1 colocalization in SHRSP rat vena cava. BAP (red), RECA-1 (green), colocalization (yellow). Negative controls are included on the corner of images. All images are representative of n=4. A capital L indicates the luminal side and arrows indicate areas of interest.

Figure 6. Western blot and densitometric analysis of TG1 (A) and TG2 (B) protein expression in sham and DOCA-salt rat aorta (n=4) and vena cava (n=4). Densitometric analysis of TG1 and TG2 protein expression was performed in both sham and DOCA-salt aorta and vena cava, corrected to α-actin. * = significantly different (p<0.05) from sham.

Figure 7. Western blots and densitometric analysis of TG1 and TG2 protein expression in cultured VSMCs (n=4) and fresh tissue (n=4). Aorta (A), vena cava (B). + = Rat skin lysate; MW of TG1 = 67kDa (proteolytically activated); MW of TG2 = 77kDa. Densitometric analysis (C) is corrected to β-actin. * = significantly different (p<0.05) from whole tissue.

Figure 8. Contractility to 5-HT with and without the addition of cystamine in sham and DOCA-salt thoracic aorta. Points are means ± SEM for number of animals in
Decreased TG 1 and 2 in aorta from hypertensive rats parenthesis. Brackets indicate the difference in reduction caused by cystamine in sham vs DOCA-salt aorta. * = significantly different (p<0.05) from 1 mM cystamine.
Table 1. Morphological measurements of aorta from hypertensive and control rats.

<table>
<thead>
<tr>
<th></th>
<th>Wall thickness (μm)</th>
<th>Lumen diameter (μm)</th>
<th>Wall-to-lumen ratio</th>
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<tr>
<td>DOCA-salt</td>
<td>180.9 ± 13.57*</td>
<td>1862 ± 46.26</td>
<td>0.0971 ± 0.0065*</td>
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<tr>
<td>Sham</td>
<td>127.5 ± 7.764</td>
<td>1814 ± 22.77</td>
<td>0.0702 ± 0.0039</td>
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<td>SHRSP</td>
<td>158.2 ± 22.16*</td>
<td>1863 ± 70.65*</td>
<td>0.0841 ± 0.0085*</td>
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<tr>
<td>WKY</td>
<td>91.47 ± 5.120</td>
<td>1569 ± 24.29</td>
<td>0.0582 ± 0.0027</td>
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</table>

Values are means ± SEM of n=4 for all groups. * = significantly different (p<0.05) from sham/WKY.
Figure 3

(A) WKY Aorta vs. SHRSP Aorta

(B) WKY Vena Cava vs. SHRSP Vena Cava
Figure 8  Rat Thoracic Aorta +E

![Graph showing percentage PE (10^-5 M) contraction versus log 5-HT [M] for different conditions: Sham - Vehicle, Sham - 1 mM Cystamine, DOCA-salt - Vehicle, DOCA-salt - 1 mM Cystamine. The graph indicates a dose-response relationship with increasing 5-HT concentrations leading to higher contractions.](image-url)
Table 2. Identification of rat aorta-specific and vena cava-specific TG substrates using BAP as a non isozyme-specific substrate linker to endogenous proteins.

<table>
<thead>
<tr>
<th>Hits:</th>
<th>Abbrv:</th>
<th>Protein:</th>
<th>Function:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta-specific</td>
<td>3 Hits RA</td>
<td>NIBAN</td>
<td>Protein Niban</td>
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<tr>
<td></td>
<td>1 Hit RVC</td>
<td>PROF1</td>
<td>Profilin-1</td>
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<tr>
<td></td>
<td>0 Hits RVC</td>
<td>CSRP1</td>
<td>Cysteine and glycine-rich protein 1</td>
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<tr>
<td></td>
<td></td>
<td>GSTM5</td>
<td>Glutathione S-transferase Mu 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEST</td>
<td>Destrin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MYH10</td>
<td>Myosin-10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RAN</td>
<td>GTP-binding nuclear protein RAN</td>
</tr>
<tr>
<td>Vena cava-specific</td>
<td>3 Hits RVC</td>
<td>VIME</td>
<td>Vimentin</td>
</tr>
<tr>
<td></td>
<td>1 Hit RA</td>
<td>DPYL2</td>
<td>Dihydropyrimidinase-related protein 2</td>
</tr>
<tr>
<td></td>
<td>0 Hits RA</td>
<td>TBB2A</td>
<td>Tubulin beta-2A chain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FIBG</td>
<td>Fibrinogen gamma chain</td>
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<tr>
<td></td>
<td></td>
<td>A1M</td>
<td>Alpha-1-macroglobulin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GUAD</td>
<td>Guanine deaminase</td>
</tr>
</tbody>
</table>

Proteins that were present in the treatment group at a level greater than two times the control group were considered a “hit”. Proteins were considered “aorta-specific” if they registered 3 hits in the rat aorta and 0 or 1 hit in the rat vena cava. Proteins were considered “vena cava-specific” if they registered 3 hits in the rat vena cava and 0 or 1 hit in the rat aorta.