Notice of Award

RESEARCH
Department of Health and Human Services
National Institutes of Health

Federal Award Date: 04/10/2019

NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

Grant Number: 7R01HL132953-04
FAIN: R01HL132953

Principal Investigator(s):
KUMUDA C DAS, PHD

Project Title: Amelioration and Reversal of Hypertension by Thioredoxin

Barnes, Victoria
Office of Sponsored Programs
Texas Tech University Health Sciences Center
3601 4th Street, MS 6271
Lubbock, TX 794306271

Award e-mailed to: SponsoredPrograms@TTUHSC.EDU

Period Of Performance:
Budget Period: 04/10/2019 – 01/31/2020
Project Period: 06/01/2016 – 01/31/2020

Dear Business Official:

The National Institutes of Health hereby awards a grant in the amount of $463,333 (see "Award Calculation" in Section I and "Terms and Conditions" in Section III) to TEXAS TECH UNIVERSITY HEALTH SCIS CENTER in support of the above referenced project. This award is pursuant to the authority of 42 USC 241 42 CFR 52 and is subject to the requirements of this statute and regulation and of other referenced, incorporated or attached terms and conditions.

Acceptance of this award including the "Terms and Conditions" is acknowledged by the grantee when funds are drawn down or otherwise obtained from the grant payment system.

Each publication, press release, or other document about research supported by an NIH award must include an acknowledgment of NIH award support and a disclaimer such as "Research reported in this publication was supported by the National Heart, Lung, And Blood Institute of the National Institutes of Health under Award Number R01HL132953. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health." Prior to issuing a press release concerning the outcome of this research, please notify the NIH awarding IC in advance to allow for coordination.

Award recipients must promote objectivity in research by establishing standards that provide a reasonable expectation that the design, conduct and reporting of research funded under NIH awards will be free from bias resulting from an Investigator's Financial Conflict of Interest (FCOI), in accordance with the 2011 revised regulation at 42 CFR Part 50 Subpart F. The Institution shall submit all FCOI reports to the NIH through the eRA Commons FCOI Module. The regulation does not apply to Phase I Small Business Innovative Research (SBIR) and Small Business Technology Transfer (STTR) awards. Consult the NIH website http://grants.nih.gov/grants/policy/coi/ for a link to the regulation and additional important information.

If you have any questions about this award, please contact the individual(s) referenced in Section IV.

Sincerely yours,

John Diggs
Grants Management Officer
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

Additional information follows

SECTION I – AWARD DATA – 7R01HL132953-04

Award Calculation (U.S. Dollars)

Salaries and Wages	$129,616
Fringe Benefits	$46,216
Personnel Costs (Subtotal)	$175,832
Materials & Supplies	$70,000
Travel	$8,000
Other	$45,000
Publication Costs	$4,000
Federal Direct Costs	$302,832
Federal F&A Costs	$160,501
Approved Budget	$463,333
Total Amount of Federal Funds Obligated (Federal Share)	$463,333
TOTAL FEDERAL AWARD AMOUNT	$463,333
AMOUNT OF THIS ACTION (FEDERAL SHARE)	$463,333

SUMMARY TOTALS FOR ALL YEARS		
YR	**THIS AWARD**	**CUMULATIVE TOTALS**
4	$463,333	$463,333

Fiscal Information:

CFDA Name:	Cardiovascular Diseases Research
CFDA Number:	93.837
EIN:	1752668014A1
Document Number:	RHL132953C
PMS Account Type:	P (Subaccount)
Fiscal Year:	2019

IC	CAN	2019
HL	8475146	$463,333

NIH Administrative Data:
PCC: eRA Commons / **OC:** 414E / **Released:** eRA Commons 04/08/2019
Award Processed: 04/10/2019 12:09:51 AM

SECTION II – PAYMENT/HOTLINE INFORMATION – 7R01HL132953-04

For payment and HHS Office of Inspector General Hotline information, see the NIH Home Page at http://grants.nih.gov/grants/policy/awardconditions.htm

SECTION III – TERMS AND CONDITIONS – 7R01HL132953-04

This award is based on the application submitted to, and as approved by, NIH on the above-titled project and is subject to the terms and conditions incorporated either directly or by reference in the following:

a. The grant program legislation and program regulation cited in this Notice of Award.
b. Conditions on activities and expenditure of funds in other statutory requirements, such as those included in appropriations acts.
c. 45 CFR Part 75.
d. National Policy Requirements and all other requirements described in the NIH Grants Policy Statement, including addenda in effect as of the beginning date of the budget period.
e. Federal Award Performance Goals: As required by the periodic report in the RPPR or in the final progress report when applicable.
f. This award notice, INCLUDING THE TERMS AND CONDITIONS CITED BELOW.

(See NIH Home Page at http://grants.nih.gov/grants/policy/awardconditions.htm for certain references cited above.)

Research and Development (R&D): All awards issued by the National Institutes of Health (NIH) meet the definition of "Research and Development" at 45 CFR Part§ 75.2. As such, auditees should identify NIH awards as part of the R&D cluster on the Schedule of Expenditures of Federal Awards (SEFA). The auditor should test NIH awards for compliance as instructed in Part V, Clusters of Programs. NIH recognizes that some awards may have another classification for purposes of indirect costs. The auditor is not required to report the disconnect (i.e., the award is classified as R&D for Federal Audit Requirement purposes but non-research for indirect cost rate purposes), unless the auditee is charging indirect costs at a rate other than the rate(s) specified in the award document(s).

An unobligated balance may be carried over into the next budget period without Grants Management Officer prior approval.

This grant is subject to Streamlined Noncompeting Award Procedures (SNAP).

This award is subject to the requirements of 2 CFR Part 25 for institutions to receive a Dun & Bradstreet Universal Numbering System (DUNS) number and maintain an active registration in the System for Award Management (SAM). Should a consortium/subaward be issued under this award, a DUNS requirement must be included. See http://grants.nih.gov/grants/policy/awardconditions.htm for the full NIH award term implementing this requirement and other additional information.

This award has been assigned the Federal Award Identification Number (FAIN) R01HL132953. Recipients must document the assigned FAIN on each consortium/subaward issued under this award.

Based on the project period start date of this project, this award is likely subject to the Transparency Act subaward and executive compensation reporting requirement of 2 CFR Part 170. There are conditions that may exclude this award; see http://grants.nih.gov/grants/policy/awardconditions.htm for additional award applicability information.

In accordance with P.L. 110-161, compliance with the NIH Public Access Policy is now mandatory. For more information, see NOT-OD-08-033 and the Public Access website: http://publicaccess.nih.gov/.

This award represents the final year of the competitive segment for this grant. See the NIH Grants Policy Statement Section 8.6 Closeout for complete closeout requirements at: http://grants.nih.gov/grants/policy/policy.htm#gps.

A final expenditure Federal Financial Report (FFR) (SF 425) must be submitted through the eRA Commons (Commons) within 120 days of the period of performance end date; see the NIH Grants Policy Statement Section 8.6.1 Financial Reports, http://grants.nih.gov/grants/policy/policy.htm#gps, for additional information on this submission requirement. The final FFR must indicate the exact balance of unobligated funds and may not reflect any unliquidated obligations. There must be no discrepancies between the final FFR expenditure data and the Payment Management System's (PMS) quarterly cash transaction data. A final quarterly federal cash transaction report is not required for awards in PMS B subaccounts (i.e., awards to foreign entities and to Federal agencies). NIH will close the awards using the last recorded cash drawdown level in PMS for awards that do not require a final FFR on expenditures or quarterly federal cash transaction reporting. It is important to note that for financial closeout, if a grantee fails to submit a required final expenditure FFR, NIH will close the grant using the last recorded cash drawdown level. If the grantee submits a final expenditure FFR but does not reconcile any discrepancies between expenditures reported on the final expenditure FFR and the last cash report to PMS, NIH will close the award at the lower amount. This could be considered a debt or result in disallowed costs.

A Final Invention Statement and Certification form (HHS 568), (not applicable to training, construction, conference or cancer education grants) must be submitted within 120 days of the expiration date. The HHS 568 form may be downloaded at: http://grants.nih.gov/grants/forms.htm. This paragraph does not apply to Training grants, Fellowships, and certain other programs—i.e., activity codes C06, D42, D43, D71, DP7, G07, G08, G11, K12, K16, K30, P09, P40, P41, P51, R13, R25, R28, R30, R90, RL5, RL9, S10, S14, S15, U13, U14, U41, U42, U45, UC6, UC7, UR2, X01, X02.

Unless an application for competitive renewal is submitted, a Final Research Performance Progress Report (Final RPPR) must also be submitted within 120 days of the period of performance end date. If a competitive renewal application is submitted prior to that date, then an Interim RPPR must be submitted by that date as well. Instructions for preparing an Interim or Final RPPR are at: https://grants.nih.gov/grants/rppr/rppr_instruction_guide.pdf. Any other specific requirements set forth in the terms and conditions of the award must also be addressed in the Interim or Final RPPR. *Note that data reported within Section I of the Interim and Final RPPR forms will be made public and should be written for a lay person audience.*

NIH strongly encourages electronic submission of the final invention statement through the Closeout feature in the Commons, but will accept an email or hard copy submission as indicated below.

Email: The final invention statement may be e-mailed as PDF attachments to: NIHCloseoutCenter@mail.nih.gov.

Hard copy: Paper submissions of the final invention statement may be faxed to the NIH Division of Central Grants Processing, Grants Closeout Center, at 301-480-2304, or mailed to:

National Institutes of Health
Office of Extramural Research
Division of Central Grants Processing
Grants Closeout Center
6705 Rockledge Drive
Suite 5016, MSC 7986
Bethesda, MD 20892-7986 (for regular or U.S. Postal Service Express mail)
Bethesda, MD 20817 (for other courier/express deliveries only)

NOTE: If this is the final year of a competitive segment due to the transfer of the grant to another institution, then a Final RPPR is not required. However, a final expenditure FFR is required and should be submitted electronically as noted above. If not already submitted, the Final Invention Statement is required and should be sent directly to the assigned Grants Management Specialist.

In accordance with the regulatory requirements provided at 45 CFR 75.113 and Appendix XII to 45 CFR Part 75, recipients that have currently active Federal grants, cooperative agreements, and procurement contracts with cumulative total value greater than $10,000,000 must report and maintain information in the System for Award Management (SAM) about civil, criminal, and administrative proceedings in connection with the award or performance of a Federal award that reached final disposition within the most recent five-year period. The recipient must also make semiannual disclosures regarding such proceedings. Proceedings information will be made publicly available in the designated integrity and performance system (currently the Federal Awardee Performance and Integrity Information System (FAPIIS)). Full reporting requirements and procedures are found in Appendix XII to 45 CFR Part 75. This term does not apply to NIH fellowships.

Treatment of Program Income:
Additional Costs

SECTION IV – HL Special Terms and Conditions – 7R01HL132953-04

Clinical Trial Indicator: No
This award does not support any NIH-defined Clinical Trials. See the NIH Grants Policy Statement Section 1.2 for NIH definition of Clinical Trial.

CHANGE OF RECIPIENT
Award reflects a Change of Recipient Institution. If the unobligated balance from the prior institution has been overestimated, it may be necessary to reduce the amount of this award.

NHLBI FUNDING GUIDELINES
This award is being issued in accordance with the NHLBI FY 2019 Operating Guidelines which can be found at: https://www.nhlbi.nih.gov/node-general/fy-2019-funding-and-operating-guidelines

STAFF CONTACTS

The Grants Management Specialist is responsible for the negotiation, award and administration of this project and for interpretation of Grants Administration policies and provisions. The Program Official is responsible for the scientific, programmatic and technical aspects of this project. These individuals work together in overall project administration. Prior approval requests (signed by an Authorized Organizational Representative) should be submitted in writing to the Grants Management Specialist. Requests may be made via e-mail.

Grants Management Specialist: Tyrone A Smith
Email: smithty@mail.nih.gov **Phone**: 301.827.8053 **Fax**: 301-451-4562

Program Official: Youngsuk Oh
Email: yoh@mail.nih.gov **Phone**: (301) 435-0560 **Fax**: (301) 480-2858

SPREADSHEET SUMMARY
GRANT NUMBER: 7R01HL132953-04

INSTITUTION: TEXAS TECH UNIVERSITY HEALTH SCIS CENTER

Budget	Year 4
Salaries and Wages	$129,616
Fringe Benefits	$46,216
Personnel Costs (Subtotal)	$175,832
Materials & Supplies	$70,000
Travel	$8,000
Other	$45,000
Publication Costs	$4,000
TOTAL FEDERAL DC	$302,832
TOTAL FEDERAL F&A	$160,501
TOTAL COST	$463,333

Facilities and Administrative Costs	Year 4
F&A Cost Rate 1	53%
F&A Cost Base 1	$302,832
F&A Costs 1	$160,501

PI: **DAS, KUMUDA C**	Title: Amelioration and Reversal of Hypertension by Thioredoxin	
Received: 12/28/2018	FOA: PA18-590 Clinical Trial:Optional	Council: 00/2019
Competition ID: FORMS-E-TYPE7-RESEARCH	FOA Title: Change of Grantee Organization (Type 7 Parent Clinical Trial Optional)	
7 R01 HL132953-04	Dual: AG	Accession Number: 4250704
IPF: 8285902	Organization: TEXAS TECH UNIVERSITY HEALTH SCIS CENTER	
Former Number: 5R01HL132953-03	Department: Internal Medicine	
IRG/SRG: HM	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A)	Animals: Y Humans: N Clinical Trial: N Current HS Code: Evaluat HESC: N	New Investigator: Early Stage Investigator:
Senior/Key Personnel:	*Organization:*	*Role Category:*
Kumuda Das Ph.D.	Texas Tech University Health Sciences Center	PD/PI
Venkatesh Kundumani-Sridharan Ph.D.	Texas Tech University Health Sciences Center	Faculty

OMB Number: 4040-0001
Expiration Date: 10/31/2019

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE	State Application Identifier

1. TYPE OF SUBMISSION*

❍ Pre-application ● Application ❍ Changed/Corrected Application

4.a. Federal Identifier
R01HL132953

b. Agency Routing Number

2. DATE SUBMITTED	Application Identifier	c. Previous Grants.gov Tracking Number

5. APPLICANT INFORMATION **Organizational DUNS*:** 6099807270000

Legal Name*: Texas Tech University Health Sciences Center
Department:
Division:
Street1*: 3601 4th Street
Street2: MS 6271
City*: Lubbock
County: Lubbock
State*: TX: Texas
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 79430-6271

Person to be contacted on matters involving this application
Prefix: First Name*: Victoria Middle Name: Last Name*: Barnes Suffix:
Position/Title: Senior analyst-Office of Sponsored Programs
Street1*: 3601 4th Street
Street2: MS 6271
City*: Lubbock
County: Lubbock
State*: TX: Texas
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 79430-6271
Phone Number*: 806-743-4565 Fax Number: Email: sponsoredprograms@ttuhsc.edu

6. EMPLOYER IDENTIFICATION NUMBER *(EIN) or (TIN)** 75-2668014

7. TYPE OF APPLICANT* H: Public/State Controlled Institution of Higher Education

Other (Specify):
Small Business Organization Type ❍ Women Owned ❍ Socially and Economically Disadvantaged

8. TYPE OF APPLICATION*
❍ New ❍ Resubmission
❍ Renewal ❍ Continuation ● Revision

If Revision, mark appropriate box(es).
❍ A. Increase Award ❍ B. Decrease Award ❍ C. Increase Duration
❍ D. Decrease Duration ● E. Other *(specify)* : Change of Grantee Organizatyion

Is this application being submitted to other agencies?* ❍Yes ●No What other Agencies?

9. NAME OF FEDERAL AGENCY*
National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER
TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*
Amelioration and Reversal of Hypertension by Thioredoxin

12. PROPOSED PROJECT		13. CONGRESSIONAL DISTRICTS OF APPLICANT
Start Date*	Ending Date*	TX-019
01/15/2019	01/14/2020	

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: Dr. First Name*: Kumuda Middle Name: C Last Name*: Das Suffix: Ph.D.

Position/Title: Professor and Director of Cardiopulmonary Res
Organization Name*: Texas Tech University Health Sciences Center
Department: Internal Medicine
Division: School of Medicine
Street1*: 3601 4th Street
Street2: MS 6598
City*: Lubbock
County: Lubbock
State*: TX: Texas
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 79430-6598
Phone Number*: 806-743-6747 Fax Number: Email*: kumuda.das@ttuhsc.edu

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested*	$555,169.00
b. Total Non-Federal Funds*	$0.00
c. Total Federal & Non-Federal Funds*	$555,169.00
d. Estimated Program Income*	$0.00

16.IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?*

a. YES ○ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR

○ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● I agree*

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION File Name:

19. AUTHORIZED REPRESENTATIVE

Prefix: Ms. First Name*: Erin Middle Name: Last Name*: Woods Suffix:

Position/Title*: Assistant vice president-sponsored programs
Organization Name*: Texas Tech University Health Sciences Center
Department:
Division:
Street1*: 3601 4th Street
Street2: MS 6271
City*: Lubbock
County: Lubbock
State*: TX: Texas
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 79430-6271
Phone Number*: 806-743-4569 Fax Number: Email*: sponsoredprograms@ttuhsc.edu

Signature of Authorized Representative*
Erin Woods

Date Signed*
12/28/2018

20. PRE-APPLICATION File Name:

21. COVER LETTER ATTACHMENT File Name:

424 R&R and PHS-398 Specific
Table Of Contents

OMB Number: 4040-0010
Expiration Date: 10/31/2019

Project/Performance Site Location(s)

Project/Performance Site Primary Location

❍ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name:	Texas Tech University Health Sciences Center
Duns Number:	6099807270000
Street1*:	3601 4th Street
Street2:	MS 6598
City*:	Lubbock
County:	Lubbock
State*:	TX: Texas
Province:	
Country*:	USA: UNITED STATES
Zip/ Postal Code*:	79430-6598
Project/Performance Site Congressional District*:	TX-019

Additional Location(s) File Name:

Funding Opportunity Number: PA-18-590. Received Date: 2018-12-28T13:38:59.000-05:00

OMB Number: 4040-0001
Expiration Date: 10/31/2019

RESEARCH & RELATED Other Project Information

1. Are Human Subjects Involved?* ○ **Yes** ● **No**

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ○ Yes ○ No

If YES, check appropriate exemption number: _ 1 _ 2 _ 3 _ 4 _ 5 _ 6 _ 7 _ 8

If NO, is the IRB review Pending? ○ **Yes** ○ **No**

IRB Approval Date:

Human Subject Assurance Number

2. Are Vertebrate Animals Used?* ● **Yes** ○ **No**

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ● Yes ○ No

IACUC Approval Date:

Animal Welfare Assurance Number A3056-1

3. Is proprietary/privileged information included in the application?* ● **Yes** ○ **No**

4.a. Does this project have an actual or potential impact - positive or negative - on the environment?* ○ **Yes** ● **No**

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ○ Yes ○ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place?* ○ **Yes** ● **No**

5.a. If yes, please explain:

6. Does this project involve activities outside the United States or partnership with international collaborators?* ○ **Yes** ● **No**

6.a. If yes, identify countries:

6.b. Optional Explanation:

	Filename
7. Project Summary/Abstract*	1234-Abstract-DAS.pdf
8. Project Narrative*	1235-Narrative-DAS.pdf
9. Bibliography & References Cited	1236-LiteratureCited.pdf
10.Facilities & Other Resources	1237-Resources_Das.pdf
11.Equipment	1238-major equipment-Das -TTUHSC.pdf

Abstract

Hypertension is a major risk factor for cardiovascular diseases, and especially poses health problems for people with advancing age. However, the pathogenesis of hypertension and the basic mechanism of blood pressure responses are incompletely understood. Thioredoxin is a multifunctional redox regulatory protein with powerful antioxidant properties that is essential for life as thioredoxin knockout mice die *in utero*. We recently developed a transgenic mouse line that is deficient in functional thioredoxin (dnTrx-Tg), and a complementary line that overexpresses the human protein (Trx-Tg). Unexpectedly, we observed that older (>2 years) dnTrx-Tg and wild-type mice showed markedly decreased arterial relaxation and high blood pressure, while aged-Trx-Tg mice continued to function normally with normal blood pressure. This hypertensive phenotype of dnTrx-Tg mice and anti-hypertensive phenotype of Trx-Tg mice prompted us to further evaluate these genotypes. Based on our preliminary data we hypothesize that Trx prevents age-dependent high blood pressure by maintaining arterial relaxation via increased eNOS expression and activation, and by upregulating the AT_2R receptor. In Aim 1 we will evaluate the role of vascular redox state of in control of hypertension in the three genotypes, in Aim 2 we will determine the effect of Trx on eNOS expression and function, and in Aim 3 we will evaluate the mechanism of AT_2R-dependent endothelium cell dysfunction in aged mice and how the receptor is regulated by Trx. We will also use an aged baboon model for validation our mice data. These studies will provide insight into blood pressure control in the elderly population, and will lay the groundwork for therapeutic development of thioredoxin.

Narrative

Hypertension is a high risk factor for the onset of many cardiovascular diseases including heart failure, cardiac hypertrophy, stroke and sudden death, and this risk further increases with advancing age. We have found that high levels of thioredoxin, one of our own proteins can decrease hypertension in the elderly and this age-related hypertension could be reversed by treatment with thioredoxin. Our project seeks to understand the mechanisms associated with protection and reversal of age-related hypertension by thioredoxin that will help in the development new therapeutics for treatment of hypertension in the elderly.

Resources

Laboratory

Dr. Das has a total of about 2000 sq ft of lab space in the 5th floor of the Bldg 1000 in the Texas Tech University Health Sciences Center. Cell culture facilities equipped with dual laminar flow hood, incubators, Biorad Cell system for cell counting and viability studies; and bright field/fluorescent inverted microscope, gel electrophoresis equipment, gel drier, speed vac concentrator, table-top microcentrifuges, incubators, -80° C and –20° C freezers, and refrigerators, liquid nitrogen storage facility, Berthhold luminometer, and BioTek microplate reader, Beckman DU800 spectrophotometer, Beckman DU700 spectrophotometer, liquid scintillation and gamma counters, STORM phosphoimager and gel documentation system, X-ray film processors. The PI has also access to core Flow Cytometry and fluorescent microplate readers, northern blotting and other types of imaging of a varieties of gels. There are incubators capable of regulating the oxygen environment using oxygen sensors and regulators. The lab has several computers for use by the post-doctoral fellows and research assistant and graduate students.

Animal surgery and hyperoxia exposure systems: Carl-Zeiss Stereo Microscope for mouse surgery, Ventillators, Thermal pads, Isoflurane automatic Anesthesia delivery system, Langendorff system, PowerLab 4 and PowerLab8 systems with high capacity computers for data recordings. Biospherix mouse exposure chambers for hyperoxia exposure system with Proox 110 oxygen monitoring system is available in the PI's Lab. Digital color camera for photomicrograph is also available in the PI's Lab.

Computers:

Dr. Das' office has a Macintosh computer with Intel processors with 1TB hard disk drive and 6 GB RAM, which is attached to a laser printer. His lab also contains several PC computers and Mac computers, printers, scanners. Additional computer resources are located throughout the campus. The university IT department is available for computer consultations and troubleshooting computer problems. Hard drives and network spaces are available for data storage. Several printers including laser color printers are located in the experimental pathology division and are available to the PI.

Animals:

The Department of Laboratory Animal medicine maintains an AAALAC accredited animal facility withsurgery sites on the ground floor of the Texas Tech University Health Sciences Center. The housing facilities include small and large rodents, and other animal. SCID mice are housed in an isolated room with filter cages. There is a full time veterinarian who supervises the operation of the facility, train investigators in animal handling techniques, and look after the health of the animals. Consultation with the veterinarian is available free of charge. Technical staff with the lab animal department maintains animals. State of the art animal surgery equipments are housed in the animal facility.

Office
Dr. Das has about 170 square feet of office space in his 5C168 room in the Bldg 1000 of TTUHSC, Lubbock, near his lab. The office space is with common facilities such asoffice equipment, fax and copy machine and other services.

TTUHSC CORE FACILITIES

TTUHSC Image Analysis Core Facility (Lubbock Campus) - The newly created Imaging Facility (1,200 square feet) has recently purchased the IVIS Lumina XR from Caliper Life Sciences for real time tracking of cells expressing bioluminescent or fluorescent proteins simultaneously with X-ray digital imaging technology for living mice. We also have in that facility two fully equipped laser scanning confocal microscopes. One of these confocal microscopes is a newly acquired Nikon Ti-E microscope with A1 confocal and STORM superresolution. The other is a Zeiss LSM 510/Meta confocal system attached to both inverted and up-right microscopes. The Nikon system is equipped to perform spectral imaging experiments, TIRF and FRAP. A high temporal and spatial resolution EMCCD camera is attached to this system. The microscope is also equipped with a stage-top environmental chamber in which precision temperature incubations can be performed. In addition, we also have up-right and inverted fluorescence microscopes attached to a CCD camera for routine observation of stained (fluorescence) or unstained (Differential Interference Contrast, DIC) specimens.

TTUHSC Molecular Biology Core Facility - Mission: The TTUHSC Molecular Biology Core Facility provides state-of-the-art instrumentation to support experimental demands in molecular biology, ranging from routine bacterial cell growth, monitoring of mammalian cell growth, advanced approaches for cell transfection, monitoring protein and gene expression, RT-PCR, flow cytometer and cell sorting capabilities, exon amplification and single cell separation for whole genomic analysis. Expertise and advice is available for the design of experiments using these approaches and technologies.

Services and Available Equipment: The Molecular Biology Core Facility has a Meso Scale Discover Sector Imager that uses multi-array approaches enabling the detection of bio-markers in single and multiplex formats. For quantification and analysis of DNA, RNA and proteins we have the ImageQuant LAS 4000 digital imager and the Typhoon FLA 9000 Biomolecular Imager for quantitative imaging of chemiluminescence, fluorescence, and gel documentation. The 2100 Bioanalyzer (Agilent) it uses microfluidics to run DNA, RNA and proteins samples and replaces agarose gels and SDS-PAGE. The Infinite M1000 PRO quadruple monochomator microplate reader (Tecan) is used to measure UV, VIS absorption, and a variety of fluorescence-based approaches with high spectral resolution. For transfection of cells with DNA or RNA, we have the 4D-Nucleofector Core X/Y (Lonza). For cell growth (mammalian cells in suspension, bacteria and yeast) we have the Biolector MP2 that allows continuous monitoring of growth mass, fluorescence, acid production and oxygen consumption in multi-well format. For analysis of gene expression, micro RNA profiling and non-coding RNA analysis we have the Quant Studio 12K real time PCR (Life Technologies). High throughput target enrichment (exons) and amplicon tagging is performed with Access Array (Fluidigm). The C1 Single Cell auto preparation system (Fluidigm) allows us to study in single cells gene expression and mRNA analysis in a 96 well format. A very simple image cytometric analysis to study apoptosis, cell cycle analysis, cell proliferation, viability assays and cell counting is performed using the Cellometer from Vision Image Cytometry. High resolution microscopy in

flow (ImageStream MarKK II, Amnis) is used to evaluate microscopically the localization of subcellular proteins with a high throughput approach using fluorescently labeled samples. The BD FACSJazz cell sorter and the BD Accuri C6 flow cytometer allows to perform cell sorting and study many cellular functions including apoptosis, cell cycle, cell proliferation, cell phenotyping.

Center for Biotechnology and Genomics (TTU General Academic Lubbock Campus)- Provides instrumentation, support and training in various aspects of modern biotechnology including spectrophotometry, protein identification, protein purification molecular interaction, DNA applications an functional genomics.

Membrane Protein Core Facility (Lubbock HSC Campus) - The Membrane Protein Laboratory Core (MPLC), is a shared facility located in the Center for Membrane Protein Research (CMPR), houses equipment that is primarily accessible to the CMPR faculty and their laboratory personnel, but is also available to Faculty throughout TTUHSC and TTU. The MPLC is equipped for the overexpression, purification and characterization of membrane proteins. Some of the equipment available includes digital gel-imaging systems (UV, visible and infrared), microscopes, spectrophotometers, a fluorescence/absorbance microplate reader, a microplate washer, a luminometer, a spectrofluorometer, refrigerated shaker incubators, a probe sonicator, small and large volume microfluidizers, FPLC systems, phosphorescence lifetime spectrometers for luminescence resonance energy transfer measurements, rapid-mixing stop-flow system for absorbance, fluorescence and light-scattering determinations, ATR/FTIR spectrometer for secondary structure determination, a picosecond lifetime system, static, and dynamic light scattering instruments, centrifuges and ultracentrifuges, crystallization incubators and mosquito robot, automatic UV microscopy system for crystal analysis, Rigaku Screen Machine for X-ray crystal screening and data collection, one- and two-photon confocal microscopy systems, high-throughput automatic frog-oocyte injector, liquid scintillation and gamma counters and an electron paramagnetic resonance spectrometer.

Neuroimaging Core (TTU General Academic Lubbock Campus) - The Texas Tech Neuroimaging Institute (TTNI) is a multi-user neuroimaging facility that promotes cutting-edge interdisciplinary research among Texas Tech University and Texas Tech University Health Sciences Center faculty and graduate students. The TTNI provides researchers with brain and body imaging technologies including structural (MRI), functional magnetic resonance imaging (fMRI), magnetic resonance spectroscopy, diffusion tensor imaging and techniques, including multimodal data fusion of EEG, fMRI, and DTI data.

Digestive Disease and Molecular Medicine Core (TTUHSC El Paso Campus) - Various clinical investigative testings are available including: Electrogastrogram (EGG); *SmartPill*, i.e. Wireless GI Motility Testing System; Interrogation Programming for gastric neurostimulation therapy (Enterra Device); autonomic testing and Transcutaneous Electrical Acupuncture System all of which are highly applicable for diagnostic and therapeutic intervention in GI tract motility disorders. The core is also capable of testing for genes and gene expression, gene SNP in health & disease, analyzing for cytokines and growth factors in serum as well as all collectible secretions and excretions with emphasis on promotion of translational research that may potentially lead to discovery of new biomarkers or therapeutic remedies for internal medicine. Laboratory Instrumentation available includes: Real-Time PCR System ViiA 7; Ultracentrifuge; Cone/Plate Viscometer; ELISA Plate Reader; Cell Culture CO_2 Incubator; UV/VIS Spectrophotometer; and Biological Safety Level 2 Laminar Flow Cabinets.

Department of Medical Photography and Electron Microscopy supports all faculty, staff and students of Texas Tech University Health Sciences Center. We specialize in producing a wide array of imaging services for a variety of needs including instructional use, research, student assignments or official business. We are well known for our award winning photography, high quality research posters, graphic design and specialized transmission and scanning electron microscopic work. We are located in Room BC200 of the Health Sciences Center. Please call (806) 743-1366 for additional information.

TTU Imaging Center -The Center is located in the Biology Department at the Lubbock TTU campus. They can prepare samples for both light and electron microscopy.

Cancer Center Cores: The TTUHSC SOM Cancer Center has several core laboratories that are also available to non-cancer faculty. It is located on the TTUHSC Lubbock Campus. Cell Culture Core, which serves as the Cell Culture and Xenograft Repository for the Children's Oncology Group (www.COGcell.org) and as the Texas Cancer Cell Repository (www.TXCCR.org). Cell Identification Core, which carries out short tandem repeat analyses of cell lines and compares them to a > 3000 cell line database to verify identity. Flow Cytometry Core, which has a BD LSR-II analyzer and a BD FACSaria cell sorter. Immunocompromised Mouse Core, which is part of the TTUHSC LARC and has laminar flow caging systems, animal irradiator, and anesthesia. Molecular Biology Core, which has a Taqman quantitative RT-PCR (TLDA-capable), and Agilent bioanalyzer, and an ABI sequencer optimized for STR analysis.

Major Equipment in the Laboratory of PI

The following are the major instruments available in the laboratory of the PI and placed in suitable location in the 2000 sq. ft. laboratory space.

Seahorse XF24 Analyzer: The PI's lab has recently acquired this instrument for mitochondrial function analysis. The PI is trained at Seahorse facility for using this instrument. The instrument is used for mitochondrial biogenetics assays using monolayer cells and isolated mitochondria from tissues. Glycolysis and oxidative phosphorylation assays are also performed in this equipment. We have recently published three papers using this flux analyzer.

Beckman DU 800 UV/VIS spectrophotometer: This is advanced equipment that is specifically important for our project. This spectrophotometer has kinetics analysis software with temperature control up to 45^0C. It can accommodate micro-quartz cuvettes capable of 50 ml volume. This *instrument is critical for activity assays of mitochondrial enzymes*. We routinely perform thioredoxin, thioredoxin peroxidase, thioredoxin reductase and many other assays of the mitochondrial enzymes such as NADH dehydrogenase, succinate dehydrogenase and a-Keto glutarate dehydrogenase. The PI has been using this equipment for a considerably long time and many current papers have published work with data generated by this advanced spectrophotometer.

Shimadzu UVPC Spectrophotometer: The spectrometer is very similar to DU800, but it is a simultaneous dual beam spectrophotometer with six cells for holding micro quartz cuvettes and is extremely suitable for enzyme kinetics, and endpoint measurement assays.

Carl-Zeiss Axiovert 200 and Carl-Zeiss Axiovision Fluorescent Microscopes: These are advanced fluorescent motorized microscope capable of Z-Stack function. The Axiovision software is capable of fluorescent intensity readout in the blue, red and green zones. The equipment is attached with advanced high-resolution camera for accurate image analysis. This microscope is used for live imaging of mitochondrial using live stains and has a 37^0C platform for culturing cells and visualizing fluorescence in real-time manner. DNA damage analysis using comet assay is performed in this microscope with special KS software.

Carl-Zeiss Dissecting microscope with color camera:
The laboratory of PI houses a state of the art Zeiss Discovery V12 dissecting microscope with two different types of base for illumination for microsurgery fitted with polarizing filters. The microscope facilitates animal surgery and microsurgery for removal of vessels and other difficult to see organs and tissues. Other dissecting microscopes such as Leica microsystems are used for placement of telemetry transmitters such as XD-11 or PA-10 catheters into carotid arteries.

Biotek Microplate Reader: This instrument can read visible and UV light, fluorescence and luminescence. Therefore we can perform fluorescent dye based assays such as caspase activity assay. In addition, we can determine the level of luciferase or b-gal luminescence.

LI-COR-ODYSSEY –Fc-Imaging System:
This is advanced imaging station capable of analyzing fluorescent-based western analysis and various gel imaging requirements. Fluorescent secondary and HPR-conjugated secondary antibodies can be used in western analysis using this machine.

Bruker EMX nano X-band EPR spectrometer:
The Bruker EMX nano is a full function EPR spectrometer suitable for spin trapping of free radicals as well as detection of nitric oxide radical. The PI has considerable expertise himself in handling samples for measurement and kinetics of free radical accumulation and other functional aspects related to pulmonary and vascular biology.

Quant Studio 7 Flex Real-Time PCR System A high throughput real-time PCR system capable of running many samples and utilizes premade primers from Thermo Electron.

Vevo 3100 High Frequency Ultrasound System:
This is new generation of high frequency ultrasound with several high frequency transducers. Mx250 transducer is used for non-linear contrast perfusion studies using micro-bubble. Mx400 transducer is used for routine mouse cardiac function analysis and Mx550S transducer is used for mouse vascular measurements. Ventricular function, micro-bubble perfusion measurements and other live imaging applications such as vascular flow dynamics using mesenteric, coronary or carotid artery are routinely performed in our lab using this instrument.

Data System International Mouse Telemetry System:
DST mouse telemetry system for continuous measurement of blood pressure in mice is available in PI's lab. The system contains 4 receivers with 4 HD-X11 and 4 PA-C10 transmitters. There is a separate animal housing for the telemetry system. Ponemah software for 4 animals with telemetry for Blood Pressure Analysis and ECG Analysis modules are available.

DMT 4 channel multi wire myograph System (620M):
This wire myography is routinely used in my lab for vascular reactivity studies of coronary, mesenteric and carotid arteries.

Pulsatile pressure myograph system (110P): This specialty line of Myograph is mostly used to study small vessel function under near physiological conditions of pressure and flow by digitally tracking diameter and flow in real-time. It includes single artery systems, for diameter measurement under user-controlled pressure and flow conditions. It comes with Zeiss microscope and software.

Universtiy Core Facilities: Besides these instruments the PIs lab has access to institutional flow cytometry core, two-photon microscopy core and pathology core laboratories of Texas Tech University Health Sciences Center.

OMB Number: 4040-0001
Expiration Date: 10/31/2019

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: Dr. First Name*: Kumuda Middle Name C Last Name*: Das Suffix: Ph.D.

Position/Title*: Professor and Director of Cardiopulmonary Res
Organization Name*: Texas Tech University Health Sciences Center
Department: Internal Medicine
Division: School of Medicine
Street1*: 3601 4th Street
Street2: MS 6598
City*: Lubbock
County: Lubbock
State*: TX: Texas
Province:
Country*: USA: UNITED STATES
Zip / Postal Code*: 79430-6598

Phone Number*: 806-743-6747 Fax Number:

E-Mail*: kumuda.das@ttuhsc.edu

Credential, e.g., agency login: eRA Commons User Name

Project Role*: PD/PI Other Project Role Category:

Degree Type: PhD Degree Year: 1992

Attach Biographical Sketch*: File Name: 1245-Biosketch_DAS-TTUHSC.pdf

Attach Current & Pending Support: File Name:

Funding Opportunity Number: PA-18-590 . Received Date: 2018-12-28T13:38:59.000-05:00

PROFILE - Senior/Key Person

Prefix: Dr. First Name*: Venkatesh Middle Name Last Name*: Kundumani-Sridharan Suffix: Ph.D.

Position/Title*:	Research Assistant Professor
Organization Name*:	Texas Tech University Health Sciences Center
Department:	Internal Medicine
Division:	School of Medicine
Street1*:	3601 4th Street
Street2:	MS 6598
City*:	Lubbock
County:	Lubbock
State*:	TX: Texas
Province:	
Country*:	USA: UNITED STATES
Zip / Postal Code*:	79430-6598

Phone Number*: 806-743-9212 Fax Number:

E-Mail*: Venkatesh.Kundumani-Sridharan@ttuhsc.edu

Credential, e.g., agency login: eRA Commons User Name

Project Role*: Faculty Other Project Role Category:

Degree Type: PhD Degree Year: 2007

Attach Biographical Sketch*: File Name: 1246-Biosketch_new VKS.pdf

Attach Current & Pending Support: File Name:

OMB No. 0925-0001 and 0925-0002 (Rev. 10/15 Approved Through 10/31/2018)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Kumuda C Das

eRA COMMONS USER NAME (credential, e.g., agency login): [eRA Commons User Name]

POSITION TITLE: Professor & Director of Cardiopulmonary Research

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Utkal University, Bhubaneswar, India	B.Sc. (Honors)	1975	Zoology
Gujarat University, Ahmedabad, India	M.Sc.	1978	Cell Biology (Zoology)
Virginia Polytechnic Institute & State University, Blacksburg, VA.	Ph.D.	1992	Biochemistry/Toxicology Respiratory Sciences
National Jewish Medical & Research Center, Denver, Colorado.	Post-Doc.	1996	Respiratory Molecular Biology

A. Personal Statement

I have the expertise, training, experience and motivation to successfully carry out the proposed research project. I have broad background in free radical biochemistry, molecular biology and endothelial biology in cardiovascular diseases. My research includes understanding the complex interaction of redox systems in the pathophysiology of cardiovascular diseases underlying the important contribution of endothelial redox homeostasis in the initiation and propagation various cardiovascular diseases. My research is specifically focused on mechanistic as well as therapeutic aspects of intervention of human cardiovascular and pulmonary diseases. As a PI and Co-PI in several university, private foundation and NIH research projects I established the importance of redox systems in gene expression, alteration of protein function and initiation of cardiovascular and pulmonary diseases. My earlier studies with lung ischemia-reperfusion models have provided a basis for the free radical mechanisms in the onset of lung injury due to reperfusion of ischemic lung where novel class of antiarrhythmic ages provided protection as free radical scavenger. I have advanced these fundamental principles to myocardial ischemia reperfusion and more recently to remote ischemic preconditioning. After a number of years of studies I have embraced the idea that endothelium is the critical player in onset or propagation of diseases of heart and blood vessels. In the current project we have proposed to uncover an endothelial mechanism that operate in remote ischemic preconditioning resulting in protection against myocardial infarction in ischemia-reperfusion injury. I have assembled a team of outstanding junior scientist to carryout the proposed research that has significant translational potential.

1. **Das, K.C**., Venkatesh Sridharan K. and Subramani, J. **2018**. INVITED REVIEW: Role of Thioredoxin in Age-related Hypertension. (Oparil, S and Carey, R Edited) **Current Hypertension Reports**
2. Hilgers, R. H., Kundumani-Venkatesh, V., Subramani, Chen, **L**., Rusch, N.J., and **Das, K.C. 2017**. Thioredoxin reverses age-related hypertension by chronically improving vascular redox and restoring eNOS function. **Sci. Transl. Med. 9 eaaf6094**
3. Subramani, J, Kundumani-Sridharan, V, Hilgers, R. H., Owens, C and **Das, K. C**. 2016. Thioredoxin protects against coronary endothelial dysfunction in ischemia-reperfusion injury by deglutathionylation of eNOS. **J Biol Chem 291 23374**

4. Kundumani-Sridharan, V., Swaminathan J and **Das, K. C**. 2015. Redox control of MKK4 activation and MnSOD gene expression via Cys246 and Cys266 in Human Microvascular Endothelial Cells. **J Biol Chem. 290 17505-19**

B. Positions and Honors

Positions and Employment

1996-1998 Instructor of Pediatrics, National Jewish Medical and Research Center, Denver CO
1998-2002 Assistant Professor of Molecular Biology, University of Texas Health Center at Tyler, TX
2002-2004 Associate Professor of Molecular Biology, University of Texas Health Center at Tyler, TX
2004-2012 Associate Professor of Pathology, University of Arkansas for Medical Sciences, Little Rock AR
2012- Professor and Director of Research, Department of Anesthesiology, Texas Tech University Health Sciences Center at Lubbock, TX.
2012- Professor of Pharmacology and Neuroscience, TTUHSC, Lubbock, TX
2012- Professor of Cell Physiology and Biophysics, TTUHSC, Lubbock, TX
2014- Professor, Internal Medicine
2014- Director, Center of Excellence for Cardiovascular Research, TTUHSC, Lubbock
2016/09 Professor and Chairman, Department of Translational and Vascular Biology, UTHSCT, Tyler, TX

Other Experience and Professional memberships

Member, American Heart Association, Member, American Thoracic Society, Member, European Society for Cardiology, European society of Hypertension, American Association for Advancement of Science

Grant Review Panels: Hypertension and Microcirculation Study Section, 2016,NIH, Special Emphasis Panel, Vascular Hematology, 2016,NIH, Vascular Cell Molecular Biology Study section, 2016, NIH, Lung Injury, Repair and Remodeling study section, 2016,NIH Special Emphasis Panel, Vascular Hematology 2015, NIH Hypertension and Microcirculation Study Section 2015, NIH Special Emphasis Panel, Vascular Hematology, 2015,NIH Special Emphasis Panel, Vascular Biology, 2013, NIH Special Emphasis Panel, Vascular Hematology, June 2012,NIH Atherosclerosis and inflammation (AICS) Study Section, 2012, NIH Special Emphasis Panel, Vascular Hematology, October 2011, NIH Special Emphasis Panel, Microcirculation & Vascular Biology, April 2011, NIH Special Emphasis Panel, Toxicology, March 2011,NIH Special Emphasis Panel, Countermeasures against Radiation Injury, April 2010, NIH Special Emphasis Panel, Radiation-induced Lung Injury, June 2008, NIH Lung Cell Molecular and Immunobiology Study Section, June 2004, Department of Pathology Grant Review, 2008 , Center for Clinical and Translational Research (CCTR) UAMS, 2009, Wellcome Trust Foundation, London, 2002 -2003, Cancer Institute, London. 2006

Honors

American Lung Association, Post-Doctoral Fellowship Award 95-97
AHA, Esther Ludwig Memorial Research Award 1997-1998, Colorado
AHA, Scientist Development Award, 1998-2002
University of Texas Presidents Research Council Award 2003
The Wolf Benevolent Trust Award, University Texas Health Center, Tyler 2004
Chancellor's Distinguished Research Award, 2015, Texas Tech University System

1. During my graduate studies I first discovered that local anesthetics, which are also antiarrhythmic agents are potent scavengers of hydroxyl radicals and quenchers of singlet oxygen. Employing an *ex vivo* lung ischemia-reperfusion injury we provided evidence that these agents ameliorate lung injury due to hyperoxia or ischemia-reperfusion. Further, we demonstrated that hyperoxia-mediated lung injury (ex vivo) is ameliorated by these anesthetics. Thus, we provided the mechanism of prevention of oxidative lung injury by local anesthetics. This is my first contribution to science in using anesthetics for oxygen-mediated lung injury.
   a. **Das, K. C**. and H.P. Misra. 1992. Antiarrhythmic agents: scavengers of hydroxyl radical and inhibitors of NADPH-dependent lipid peroxidation in bovine lung microsomes. *J. Biol. Chem. 267:19172-19178.*
   b. **Das, K. C**. and H.P. Misra.1992 Lidocaine, a hydroxyl radical scavenger and singlet oxygen quencher. *Mol. Cell. Biochem. 115:179-185.*

c. **Das, K. C**. and H.P. Misra. 1994. Impairment of mouse macrophage cell line RAW 264.7 function by antiarrhythmic drugs. *Mol. Cell. Biochem., 132:151-162*
d. **Das, K. C**. and H.P. Misra 1994. Amelioration of reperfusion injury by antiarrhythmic agents in isolated rat lung. *Environ. Health Perspec. 102(Suppl.10):117-122*

2. My early publications demonstrated that superoxide dismutase-2 (mitochondrial) is induced not by oxidants, but by reductants. This discovery was counter-intuitive at that time, because only oxidants were believed to induce the expression of antioxidant genes. We also published that there is close association between NFκB activation and MnSOD expression induced by reductants. Later, we demonstrated that Thioredoxin, a protein dithiol induces MnSOD. Many investigators later confirmed these discoveries in many other systems. For this discovery I have been awarded a patent for use of thioredoxin in lung diseases. I believe that Thioredoxin will be a therapeutic agent in future as it could be injected and internalized by cells.

US Patent: **5,985,261** - Use of thioredoxin like molecules for induction of MnSOD to treat oxidative lung damage. Licensed to OrPro Therapeutics, San Diego, CA

a. **Das, K. C**., Yvette Lewis-Mollock, and C. W. White. 1995. Activation of NF-κB and elevation of MnSOD gene expression by thiol reducing agents in lung adenocarcinoma (A549) cells. *Am. J. Physiol, 269 (Lung Cell. Mol. Physiol. 13):L588-L602.*
b. **Das, K. C**., Yvette Lewis-Mollock, and C. W. White. 1997. Elevation of MnSOD by thioredoxin in A549 cells *Am. J. Resp. Cell. Mol. Biol. 17:713-726.*
c. **Das, K. C**. 2001. c-Jun N-terminal kinase-mediated redox-dependent degradation of IkB: Role of thioredoxin redox in activation of NF-kB. *J. Biol. Chem. 276:4662-4670.*
d. **Das, K. C**., Xiao-ling, G. and C.W. White. 1999 Induction of thioredoxin and thioredoxin reductase gene expression in lungs of newborn primates by oxygen. *Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L6L*

3. While working with mechanism of activation of NFκB I discovered that anticancer agents such as taxol, vincristine, vinblastine and doxorubicin induce NFκB. This was first discovery of activation of NFkB by anticancer agents, which was cited by many investigators in the filed of cancer biology. We also discovered for the first time that MnSOD is induced by anticancer agents via activation of PKCδ and NFkB. Following this work I discovered that Thioredoxin increases the cytotoxic effect of anthracyclines, but not other anticancer drugs. The mechanism was found to be enhancing the redox cycling of quinone moiety by donating electrons to many reductases such as cytochrome P450 system.

a. **Das, K. C** and C. W. White. 1997. Induction of NF-kB by anti neoplastic agents: Role of protein kinase C. *J. Biol. Chem. 272:14914-14920*
b. **Das, K. C**., Xio-ling, G. and C. W. White. 1998. Protein kinase Cδ dependent induction of manganese superoxide dismutase gene expression by antineoplastic agents. *J. Biol. Chem. 273:34639-34645*
c. Dashanamoorthy, R. and **Das, K.C**. 2005. Thioredoxin increases anthracycline redox cycling and sensitizes breast cancer cells to p53-dependent apoptosis. *J Biol Chem. 280, 40084-40096*
d. Dashnamoorthy R. and **K. C. Das**. 2004. Redox-cycling of anthracyclines by thioredoxin system: Increased superoxide generation and DNA damage. *Canc. Chemother. Pharmacol. 54:449-458*

My contribution to Thioredoxin biology started during my early career. It is important to emphasize that with continued research in Thioredoxin I have acquired several skills for many difficult assays and endpoint measurements. Using these skills I created a new transgenic mice that express mutant protein (in a dominant-negative manner) with greatly reduced thiol reducing capacity. Since Thioredoxin knockout mice die *in utero,* this is the only alternative available to study the exact role of this protein in role of *in vivo* redox in endothelial dysfunction in aged mice. Using these mouse models we found that preservation of vascular redox is important for regulation of hypertension. I have also found that overexpression of Thioredoxin in mice maintains the vascular redox state during aging and also preserves normal endothelial functioning in aged mice. For this discovery I have obtained an international patent. The novel idea that age-related hypertension could be reversed by thioredoxin is not only

exciting, but also may renew the vascular bed with regenerated proteins that will allow the elderly people to defy age-related hypertension.

Recent Patent: PATENT PENDING

PATENT PENDING patent filing, reported to NIH via eEdision

a. Subramani J, Kundumani-Sridharan, V, Hilgers, R.P and **Das**, **K. C.** 2016. Thioredoxin uses a GSH-independent route to deglutathonylate endothelial nitric oxide synthase and protect against myocardial infarction. *J Biol Chem. 291:23374-89*
b. **Das, K.C**. 2015. Thioredoxin-deficient mice, a novel phenotype sensitive to ambient air and hypersensitive to hyperoxia-induced lung injury. *Am J Physiol Lung Cell Mol Physiol. 308:L429-4.*
c. Kundumani-Sridharan, V., Swaminath J and **Das, K. C. 2015**. Redox control of MKK4 activation and MnSOD gene expression via Cys246 and Cys266 in Human Microvascular Endothelial Cells. *J Biol Chem. 290:17505-19*

D. Research Support:
Ongoing Research Support

7R01 HL 130061-02 DAS(PI) 9/1/2016 - 3/31/2020

Amelioration of Mitochondrial dysfunction by Thioredoxin in hyperoxia

The major goal of this proposal is to determine the mechanisms of action of thioredoxin in protection against mitochondrial dysfunction in hyperoxia in the lung

R01 HL132953-01 DAS(PI) 6/1/16 - 3/31/2020

Amelioration and Reversal of Hypertension by Thioredoxin

The major goal of this proposal is to determine the antihypertensive role of Thioredoxin in age-related hypertension. Gentically modified mice overexpressing thioredoxin or expressing the mutant redox inactive thioredoxin will be used to evaluated in vivo effect of thioredoxin in age-related hypertension.

Completed Research Support

OMB No. 0925-0001 and 0925-0002 (Rev. 10/15 Approved Through 10/31/2018)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Kundumani Sridharan, Venkatesh

eRA COMMONS USER NAME (credential, e.g., agency login): eRA Commons User Name

POSITION TITLE: Research Assistant Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Madurai Kamaraj University, Madurai, India	BS	06/1998	Industrial Microbiology
Central Food Technological Research Institute, Mysore, India	MS	08/2000	Food Technology
Central Food Technological Research Institute, Mysore, India	PhD	06/2007	Biotechnology

A. Personal Statement

My primary research goals are directed toward understanding the role of ErbB2 in cardiac endothelial cell function and survival during ischemia reperfusion injury. I also study the role of ErbB2 and its interaction with Nrg1 in remote ischemia preconditioning (RIPC) and subsequent protection of heart from ischemic injury. During my postdoctoral training and as junior faculty, I worked extensively in angiogenesis and atherosclerosis. My research work made several striking advancements in understanding of pathological retinal angiogenesis, eicosanoids' role in pathological deep tissue angiogenesis, restenosis and atherosclerosis and resulted in 20 publications in well-respected journal in the field of cardiovascular biology. My current research focuses on studying the redox control of function of ErbB family of receptors in ischemic cardiac diseases. My recent studies show that ErbB2 receptor loose its stability and function during ischemia-reperfusion injury in heart which leads to cell death. I am currently developing an interventional method through which ErbB2 can be protected using thioredoxin, a redox damage repair protein. In the recent past, I received a grant award from the Private Source to study function of endothelial ErbB2 in the survival of endothelial cells during myocardial ischemia reperfusion injury. Along with my colleagues in Dr. Das laboratory, I demonstrated the reversal of age-related hypertension by administering human recombinant thioredoxin to aged mice. This work was published in Science Translational Medicine. In summary, I have a proven record of accomplishments and productivity in cardiovascular research field and my expertise and experience have prepared me to take important role in the proposed project.

B. Positions and Honors

Positions and Employment:

2000-2001 Research Officer, Unilever Research India, Bangalore, India
2001-2003 Junior Research Fellow, Central Food Technological Research Institute, Mysore, India
2003-2005 Senior Research Fellow, Central Food Technological Research Institute, Mysore, India
2005-2007 Postdoctoral Trainee, Department of Physiology, University of Tennessee Health Science Center, Memphis, TN, USA
2007-2008 Postdoctoral Researcher, Department of Microbiology (MIP) & Stanley S. Scott Cancer Center, LSU Health Sciences Center, 1901 New Orleans, LA 70112 USA
2008-2012 Assistant Professor, Department of Physiology, University of Tennessee Health Science Center, Memphis, TN, USA

2012-2016 Research Assistant Professor, Department of Anesthesiology, Texas Tech University Health Science Center, Lubbock, TX, USA
2016-2018 Research Assistant Professor, Department of Translational & Vascular Biology, University of Texas Health Sciences Center at Tyler, Tyler, TX, USA
2018- Research Assistant Professor, Department of Internal Medicine, Texas Tech University Health Science Center, Lubbock, TX, USA

Honors:

1) Junior Research Fellowship and Lectureship, Council of Scientific and Industrial Research & University Grants Commission, India, 2001-2003
2) Senior Research Fellowship, Council of Scientific and Industrial Research & University Grants Commission, India, 2003-2005

Memberships in Scientific Societies:

1). American Society for Microbiology
2). American Heart Association
3). American Society for Biochemistry and Molecular Biology
4). International Anesthesia Research Society
5). American Association for the Advancement of Science

C. Contributions to Science

My recent studies show that ErbB2 receptor loose its stability and function during ischemia-reperfusion injury in heart, which leads to cell death. Further, my study demonstrated that hioredoxin is a deglutathionylating agent for the first time and it can reactivate glutathionylated proteins by deglutathionylation.

1. Kundumani-Sridharan V, Dyukova E, Hansen DE 3rd, Rao GN. (2013). 12/15-Lipoxygenase mediates high-fat diet-induced endothelial tight junction disruption and monocyte transmigration: a new role for 15(S)-hydroxyeicosatetraenoic acid in endothelial cell dysfunction. **J Biol Chem**. 288(22):15830-15842. PMCID: PMC3668740.
2. Kundumani-Sridharan V, Subramani J, Das KC. 2015. Thioredoxin activates MKK4-NFκB pathway in a redox dependent manner to control manganese superoxide dismutase gene expression in endothelial cells. **J Biol Chem**. 290:17505–17519. PMCID: PMC4498085
3. Subramani J, Kundumani-Sridharan V, Hilgers RH, Owens C, Das KC. 2016. Thioredoxin uses a GSH-independent route to deglutathionylate endothelial nitric-oxide Synthase and protect against myocardial infarction. **J Biol Chem**. 291: 23374-23389. PMCID: PMC5095395.
4. Hilgers RH*, Kundumani-Sridharan V*, Subramani J, Chen LC, Cuello LG, Rusch NJ, Das KC. (2017) Thioredoxin reverses age-related hypertension by chronically improving vascular redox and restoring eNOS function. **Sci Transl Med**. 9(376): eaaf6094. PubMed PMID: 28179506. *** Equal first authors.**

OMB Number: 4040-0001
Expiration Date: 10/31/2019

RESEARCH & RELATED BUDGET - SECTION A & B, Budget Period 1

ORGANIZATIONAL DUNS*: 6099807270000

Budget Type*: ● Project ○ Subaward/Consortium

Enter name of Organization: Texas Tech University Health Sciences Center

Start Date*: 01-15-2019 **End Date*:** 01-14-2020 **Budget Period:** 1

A. Senior/Key Person

	Prefix	First Name*	Middle Name	Last Name*	Suffix	Project Role*	Base Salary ($)	Calendar Months	Academic Months	Summer Months	Requested Salary ($)*	Fringe Benefits ($)*	Funds Requested ($)*
1.	Dr.	Kumuda	C	Das	Ph.D.	PD/PI	Institutional Base Salary	EFFORT			47,400.00	10,769.00	58,169.00
2.	Dr.	Venkatesh		Kundumani-Sridharan	Ph.D.	Faculty					30,000.00	10,847.00	40,847.00

Total Funds Requested for all Senior Key Persons in the attached file

Additional Senior Key Persons: File Name:

Total Senior/Key Person **99,016.00**

B. Other Personnel

Number of Personnel*	Project Role*	Calendar Months	Academic Months	Summer Months	Requested Salary ($)*	Fringe Benefits*	Funds Requested ($)*
1	Post Doctoral Associates	EFFORT			52,000.00	23,250.00	75,250.00
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						
1	Research Assistant	12.00			48,000.00	22,590.00	70,590.00
2	**Total Number Other Personnel**					**Total Other Personnel**	**145,840.00**
						Total Salary, Wages and Fringe Benefits (A+B)	**244,856.00**

RESEARCH & RELATED Budget (A-B) (Funds Requested)

Funding Opportunity Number: PA-18-590 . Received Date: 2018-12-28T13:38:59.000-05:00

RESEARCH & RELATED BUDGET - SECTION C, D, & E, Budget Period 1

ORGANIZATIONAL DUNS*: 6099807270000
Budget Type*: ● Project ○ Subaward/Consortium
Organization: Texas Tech University Health Sciences Center

Start Date*: 01-15-2019 **End Date*:** 01-14-2020 **Budget Period: 1**

C. Equipment Description		
List items and dollar amount for each item exceeding $5,000		
Equipment Item		**Funds Requested ($)***
Total funds requested for all equipment listed in the attached file		
	Total Equipment	
Additional Equipment: File Name:		

D. Travel		Funds Requested ($)*
1. Domestic Travel Costs (Incl. Canada, Mexico, and U.S. Possessions)		4,000.00
2. Foreign Travel Costs		4,000.00
	Total Travel Cost	**8,000.00**

E. Participant/Trainee Support Costs		Funds Requested ($)*
1. Tuition/Fees/Health Insurance		0.00
2. Stipends		
3. Travel		
4. Subsistence		
5. Other:		
Number of Participants/Trainees	**Total Participant Trainee Support Costs**	**0.00**

RESEARCH & RELATED Budget {C-E} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTIONS F-K, Budget Period 1

ORGANIZATIONAL DUNS*: 6099807270000
Budget Type*: ● Project ○ Subaward/Consortium
Organization: Texas Tech University Health Sciences Center

Start Date*: 01-15-2019 **End Date*:** 01-14-2020 **Budget Period:** 1

F. Other Direct Costs	Funds Requested ($)*
1. Materials and Supplies	60,000.00
2. Publication Costs	5,000.00
3. Consultant Services	
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	
6. Equipment or Facility Rental/User Fees	
7. Alterations and Renovations	
8 . Animal Charges	45,000.00
Total Other Direct Costs	**110,000.00**

G. Direct Costs	Funds Requested ($)*
Total Direct Costs (A thru F)	**362,856.00**

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)*
1 . Total direct cost	53.00	362,856.00	192,313.00
		Total Indirect Costs	**192,313.00**

Cognizant Federal Agency (Agency Name, POC Name, and POC Phone Number): DHHS, Matthew Dito, 214-767-3261

I. Total Direct and Indirect Costs	Funds Requested ($)*
Total Direct and Indirect Institutional Costs (G + H)	**555,169.00**

J. Fee	Funds Requested ($)*

K. Total Costs and Fee	Funds Requested ($)*
	555,169.00

L. Budget Justification*: File Name: 1247-Budget justification-HY-Das.pdf
(Only attach one file.)

RESEARCH & RELATED Budget {F-K} (Funds Requested)

BUDGET JUSTIFICATION

Kumuda C. Das, Ph.D., Principal Investigator [EFFORT] effort, [EFFORT] months). Dr. Das is Professor and Director of Cardiopulmonary Research in the department of internal medicine. Dr. Das has been working on biological role of Thioredoxin in vascular and pulmonary systems. Dr. Das will be responsible for experimental design, direction of laboratory staff, including postdoctoral fellows and technicians, data acquisition and analysis, budget management, and adherence to rules and regulations regarding the use of hazardous substances and animals. Dr. Das is also trained as a veterinary medical graduate and is expert in laboratory animal handling and experiments. Therefore, he will assist postdoctoral fellows, students and laboratory technicians in animal procedures. Dr. Das will be responsible for preparation and submission of all manuscripts and reports derived from the project.

Venkatesh Sridharan, PhD, ([EFFORT] months). Salary requested for all years. Dr. Sridharan is a research assistant professor in Dr. Das's lab. He has about six years of experience in various molecular biological techniques and mouse models. He will perform biochemical and molecular biological assays in age-related hypertension using transgenic and wildtype mice. He will specifically perform eNOS functional assays and all the microscopy and signal transduction studies. He will also assay the redox state of Trx after various experimental manipulations of mesenteric artery and carotid artery. He will perform immunohistochemistry and immunofluorescence of markers of endothelial dysfunction. He will generate data, interpret data and will produce publication quality figures. He will analyze data and prepare manuscripts. [EFFORT] of his salary is requested.

Post-Doctoral Fellow (TBA): ([EFFORT] months) A post doctoral fellow will be recruited to replace Dr. Hilgers, who is no longer working in PI's Lab. He/She will perform vascular reactivity, and blood pressure measurement in mice. He/She will also perform all studies related to AT-1R and AT2-R studies proposed in the application.

Research Assistant/Associate (BS or MS): (100%, 12 Calendar months salary is requested): The research assistant will maintain mouse colony, perform genotyping and maintain accurate mice log book. Because we anticipate several strains of mice and several breeding cages to be taken care, dedicated personnel would be best to handle these needs. Thus, the need for a full time research assistant is almost essential for these studies. He will process the tissue for redox assays and other enzymatic assays and western blotting. He will also work in coordination with Dr. Sridharan for assays and other experiments.

Mice

Mice breeding, generation of strains and aging: ($45,000): We will also generate about six new strains (NOS3$^{-/-}$-Trx, NOS3$^{-/-}$-dnTrx, NOS3$^{-/-}$; AT2R$^{-/-}$-Trx, and AT2R$^{-/-}$-dnTrx and AT2R with breeding of mice. Breeding of these mice will be done in our TTUHSC animal facility. Thus, total charges for mice include our breeding, per diem charges and holding these mice strains for about 1.8 to 2 years for aging studies. This budget for mice is the minimum requirement based on our experience in maintaining an aging colony for 3 strains for last three years at TTUHSC. Large number of animals is necessary for 6-10 replicates for vascular reactivity, telemetry and other enzymatic assay studies.

The AT2R strain will cost €2400 and will be purchased from European Union. NOS3-/- strain will be purchased from Jackson Laboratory and will cost about $250/ pair. These mice will be obtained and cross-bred in the first year with our Trx-Tg or dnTrx-Tg mice.

We expect to hold 200-300 mice at a time for allowing them to age in addition to our young mice which will be continuously produced at a constant rate by breeding. Animals are

timings.
We expect first year animal cost to be lower, but it will sharply increase over next budget periods, as we will have several hundred cages to manage.

Supplies: ($60,000) Regular lab supplies for various biochemical assays, animal related small surgical instruments and replacement telemetry transmitters are included in this category. Refurbishing of XD11 with new batteries cost approximately $800/transmitter for 30 battery days. Antibodies, siRNA, acrylamide and other plastic and cell isolation materials are included in this category.

Travel: ($8000) Travel is budgeted for national and international meetings. Additionally, travel money is budgeted for travel to Oklahoma City for baboon experiments by our research assistant professors and post-doctoral fellows. In addition, the PI will also visit the baboon resources center and will have discussions with [Redacted by] as required.

Publications ($5000): We have experienced significant increase in page charges for our publications. Thus, depending upon the journal these charges could be very high. However, a modest amount of $5000 is budgeted for the grant period.

RESEARCH & RELATED BUDGET - Cumulative Budget

	Totals($)	
Section A, Senior/Key Person		99,016.00
Section B, Other Personnel		145,840.00
Total Number Other Personnel	2	
Total Salary, Wages and Fringe Benefits (A+B)		244,856.00
Section C, Equipment		
Section D, Travel		8,000.00
1. Domestic	4,000.00	
2. Foreign	4,000.00	
Section E, Participant/Trainee Support Costs		0.00
1. Tuition/Fees/Health Insurance	0.00	
2. Stipends		
3. Travel		
4. Subsistence		
5. Other		
6. Number of Participants/Trainees		
Section F, Other Direct Costs		110,000.00
1. Materials and Supplies	60,000.00	
2. Publication Costs	5,000.00	
3. Consultant Services		
4. ADP/Computer Services		
5. Subawards/Consortium/Contractual Costs		
6. Equipment or Facility Rental/User Fees		
7. Alterations and Renovations		
8. Other 1	45,000.00	
9. Other 2		
10. Other 3		
Section G, Direct Costs (A thru F)		362,856.00
Section H, Indirect Costs		192,313.00
Section I, Total Direct and Indirect Costs (G + H)		555,169.00
Section J, Fee		
Section K, Total Costs and Fee (I + J)		555,169.00

PHS 398 Cover Page Supplement

OMB Number: 0925-0001
Expiration Date: 03/31/2020

1. Vertebrate Animals Section

Are vertebrate animals euthanized? ● Yes ○ No

If "Yes" to euthanasia

Is the method consistent with American Veterinary Medical Association (AVMA) guidelines?

● Yes ○ No

If "No" to AVMA guidelines, describe method and provide scientific justification

2. *Program Income Section

*Is program income anticipated during the periods for which the grant support is requested?

○ Yes ● No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period	*Anticipated Amount ($)	*Source(s)

PHS 398 Cover Page Supplement

3. Human Embryonic Stem Cells Section

*Does the proposed project involve human embryonic stem cells? ○ Yes ● No

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://grants.nih.gov/stem_cells/registry/current.htm. Or, if a specific stem cell line cannot be referenced at this time, check the box indicating that one from the registry will be used:

Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Cell Line(s) (Example: 0004):

4. Inventions and Patents Section (Renewal applications)

*Inventions and Patents: ○ Yes ○ No

If the answer is "Yes" then please answer the following:

*Previously Reported: ○ Yes ○ No

5. Change of Investigator/Change of Institution Section

❑ Change of Project Director/Principal Investigator

Name of former Project Director/Principal Investigator

Prefix:

*First Name:

Middle Name:

*Last Name:

Suffix:

☑ Change of Grantee Institution

*Name of former institution:

University of Texas Health Sciences Center at Tyler

PHS 398 Research Plan

OMB Number: 0925-0001
Expiration Date: 03/31/2020

Introduction	
1. Introduction to Application (for Resubmission and Revision applications)	
Research Plan Section	
2. Specific Aims	1239-Specific Aims.pdf
3. Research Strategy*	1240-Research-Strategy.pdf
4. Progress Report Publication List	1241-Progress report publication list-HY-Das.pdf
Other Research Plan Section	
5. Vertebrate Animals	1242-VertebrateAnimals-DAS.pdf
6. Select Agent Research	
7. Multiple PD/PI Leadership Plan	
8. Consortium/Contractual Arrangements	
9. Letters of Support	
10. Resource Sharing Plan(s)	1243-Resource sharing Plan-DAS .pdf
11. Authentication of Key Biological and/or Chemical Resources	1244-Authentication_-Das copy.pdf
Appendix	
12. Appendix	

Specific Aims:

Hypertension is a major risk factor for cardiovascular diseases, and especially poses health problems for the aged people. In fact, people who are non-hypertensive at 55 years of age have a 90% lifetime risk of eventually developing the disease. High blood pressure predisposes individuals to the development of left ventricular hypertrophy, heart failure, stroke, aneurysm and other vascular and cardiac pathophysiology. Obviously, identifying age-specific factors is necessary to pursue therapies that are specific for treatment of elderly for this problem. However, animal models that specifically address age-linked causative factors of hypertension are rare.

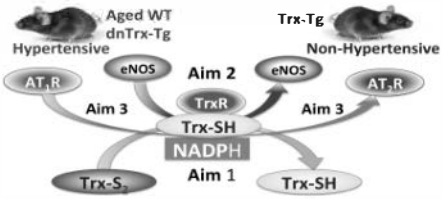

FIG 1. Hypothesis: High levels of hTrx attenuate hypertension in aged Trx-Tg mice. Aim 1. Improved vascular redox state; Aim 2. Functional eNOS; Aim3, Increased AT_2R and decreased AT_1R

In this proposal we will study novel mechanisms associated with prevention and reversal of age-related hypertension using our newly created mice models that express high levels of Thioredoxin (Trx; Trx-Tg) and another strain that is deficient in functional Trx (dnTrx-Tg). Our proposed study is based on strong preliminary data that aged Trx-Tg mice: **1)** are non-hypertensive compared to aged WT or dnTrx-Tg mice; **2)** have greater endothelium-dependent relaxation; **3)** generate increased levels of nitric oxide (NO), and decreased levels of superoxide in the arteries; **4)** show increased eNOS expression and phosphorylation; **5)** have decreased AT_1R expression; and **6)** increased AT_2R expression. All of these findings are opposite in aged WT or dnTrx-Tg mice. Additionally, injection of recombinant human Trx (rhTrx) to aged WT mice brought down the blood pressure to the level of young WT mice, and the normal blood pressure (BP) was maintained for at least for 12 days. Further, impairment of mesenteric artery relaxation of aged WT mice was reversed in rhTrx-injected mice. Collectively, these exciting preliminary data show for the first time that high levels of Trx not only protect against the development of age-related hypertension and endothelial dysfunction, but also reverse both. Conceptually, this finding is very different than the conventional antioxidant-mediated protection, as Trx reverses hypertensive phenotype to normotensive with the effect lasting for several days.

We propose to establish that Trx confers protection against age-dependent hypertension by performing a multi-faceted analysis of murine arteries from transgenic and knockout mice and to identify endothelial proteins that are targets of Trx-mediated amelioration. Given the fact that Trx is able to reverse the hypertensive phenotype to a normotensive one due to chronic administration of rhTrx, it is exciting to consider a significant benefit of rhTrx as an anti-hypertensive agent to lower and reverse age-related hypertension in the elderly. Toward this goal, we will pursue additional studies to demonstrate the efficacy of rhTrx as an antihypertensive agent in a non-human primate model as a pre-clinical study. We strongly believe that this research will lay the foundation for clinical use of rhTrx as a BP lowering therapy, and is truly a translational bench-to-bedside approach for novel treatment of hypertension.

Central Hypothesis:

Based on our extensive and strong preliminary studies we hypothesize that **high levels of Trx attenuate and reverse age-related hypertension due to preservation of vascular redox state resulting in improved endothelial function via eNOS functionality, and increased AT_2R and decreased AT_1R expression.** The hypothesis will be tested using the following three interrelated specific aims as depicted in Fig 1.

Aim 1. **Establish that high levels of Trx attenuate and reverse hypertension by protecting against vascular endothelial dysfunction** We will establish that high levels of hTrx protect against endothelial dysfunction by maintaining a functional Trx system in Trx-Tg mice. Further, we will also show that high blood pressure in aged WT mice and aged baboons is decreased with rhTrx injection concomitant with decreased endothelial dysfunction for several weeks.

Aim 2. **Determine that high levels of Trx improve endothelial function and decrease hypertension by restoring eNOS expression and function.** The mechanisms of restoration of eNOS function by Trx will be determined by studying the effect of Trx on eNOS uncoupling, eNOS glutathionylation and BH_4 oxidation, using $NOS3^{-/-}$-Trx-Tg, $NOS3^{-/-}$ and dnTrx-$NOS3^{-/-}$-Tg mice; and aged WT mice injected with rhTrx.

Aim 3. Proprietary Info

B. RESEARCH STRATEGY

I. Significance

Hypertension is a high risk factor for the onset of many cardiovascular diseases (including heart failure, cardiac hypertrophy, stroke, aneurysm and sudden death) and this risk is significantly increases with advancing age. Progressive increase in the life expectancy has created challenges for simultaneously combating the health problems that arise from aging and hypertension (6, 7). Recent (2015 September) NIH SPRINT (Systolic Blood Pressure Intervention Trial) with 9,361 hypertensive patients above age of 50 showed that a more aggressive targeted systolic blood pressure of 120 mmHg reduced the risk of cardiovascular diseases by 25-30% (AHA & NBC) underscoring the severity and adverse impact of hypertension. It is recommended that two drugs could simultaneously be used to achieve the targeted BP of 120 mmHg. However, this recommendation could further increase the "polypharmacy', which is already a critical issue for the elderly (8). Although a great deal of research has been devoted to discovering causative factors and treatments for hypertension, few studies have coordinately examined the effects of age and this disease, so basic mechanisms of age-dependent hypertension have not been established (9-11). In this proposal, we will show the mechanisms that underlie Trx –mediated protection against development of hypertension, and the mechanisms by which Trx reverse this process using aged mouse models.

We recently developed a transgenic mouse line (Trx-Tg) that expresses high levels of human Trx (hTrx), and a complementary line (dnTrx-Tg) expressing an inactive hTrx mutant (in which the catalytic Cys32 and Cys35 residues are replaced by serine) in a dominant-negative manner (12, 13). Unexpectedly, we found that aged (>2 yrs.) Trx-Tg mice maintain a significantly lower basal blood pressure than comparable WT mice. Thus, phenotypically these mice are *non-hypertensive*. In contrast, aged dnTrx-Tg mice are phenotypically hypertensive. This intriguing phenotypic variations due to gain or loss of function of Trx points to a critical role of Trx in the regulation of hypertension. To gain further insight into the mechanisms of actions of Trx, we performed further analysis on the vascular factors relevant in hypertension and found that, like aged WT animals, older dnTrx mice do not release NO in response to acetylcholine, but aged Trx-Tg mice do. Thus, in terms of hypertension, the aged Trx-Tg mice resemble younger WT animals. Additionally, we found that mesenteric arteries from aged WT mice failed to relax *ex vivo* using myograph studies. Consistent with Trx-Tg mice, aged WT mice injected with rhTrx demonstrated normal BP for at least 12-days after the rhTrx injection, demonstrating that impairment of vascular relaxation is corrected or reversed by using pharmacologically active hTrx. This fact not only provides a novel treatment method to reverse hypertension in aged individuals, but also conceptually advances the idea that age-related hypertension is a reversible process. Undoubtedly, the treatment with Trx could reduce polypharmacy and improve the quality of life of the elderly. These novel and never before interesting concept and findings will be critically evaluated in our research proposal, and significant novel information is expected to be forthcoming that will further shed light into the mechanisms of age-related hypertension. The significance of our proposal is further enhanced as we propose to evaluate anti-hypertensive effect of rhTrx *in a baboon model of age-related hypertension based on our very exciting studies with mice*.

II. Innovation

Although age-related hypertension is an established phenomenon the treatments are only palliative, but not curative. Current treatment recommendations from various agencies include calcium channel blockers, diuretics or angiotensin II receptor blockers or other(8) (14). Our proposed research will show that Trx would restore our vessel proteins that have been oxidatively inactivated during the aging process resulting improved endothelial function and amelioration of hypertension. This reversal of hypertension is a novel conceptual advance in hypertension research and is a shift in existing paradigm of treatment of hypertension. For these reasons, we have included an aged baboon model in addition to our mouse studies. The proof-of-concept is shown by our data that aged WT mice injected with rhTrx show normal BP and improved vascular relaxation, and decreased arterial stiffness. Since I have first discovered the anti-hypertensive properties of Trx, I have obtained an international patent (*# PCT/US2015/037131 "Thioredoxin and thioredoxin derivatives or peptides for treatment of High blood pressure")* for my discovery, which demonstrates the novelty and innovativeness of antihypertensive role of hTrx.

One of the unique functions of Trx in comparison with a classical "antioxidant" is its ability to regenerate a protein after it is oxidatively inactivated, whereas an antioxidant intercepts a free radical or other ROS at the site of generation, and thereby prevents the oxidation process to occur. This property makes Trx an ideal system to regenerate proteins oxidized during the life span of an organism restoring their function. There have been no studies yet that utilized redox-altered mouse models to study the role of Trx *in vivo* in age-related hypertension. Thus, for the very first time we will examine the effect of Trx in endothelial dysfunction and hypertension in mice with gain-of-function and loss-of-function of Trx. We will use an aged baboon hypertension model (See attached letter) and determine whether therapeutic levels of recombinant-human Trx could reverse age-related hypertension in non-human primates. Thus, our proposal will have a lasting impact on hypertension research with significant conceptual advance.

Approach:

BACKGROUND:

Age-related Hypertension: The incidence of hypertension is strikingly high with advancing age, and is an independent prognostic factor for the onset or progression of a variety of cardiovascular disorders. For example, prevalence of hypertension increased from 7.3% to 23.6%to 66.6% among individuals aged 18-39, 40-59 and equal to or greater than 60 years. An increase of as low as 1-5 mmHg higher over 140 makes us hypertensive and over 120 makes us pre-hypertensive(15). A 4-year incidence of increased hypertension with a normal BP of 120/80 in 35-64 years age is about 6%, which doubles to about 18% in the 65-94 years of age (15). The molecular pathophysiology of age-related hypertension is largely attributed to endothelial dysfunction and arterial stiffness due to loss of balance between vasodilators and vasoconstrictors(16, 17). Decreased availability of NO is a major reason for impairment of vascular relaxation (16, 17). Studies have shown that endothelium-independent relaxations are unaffected by aging, suggesting that endothelial dysfunction is primarily involved in age-related hypertension (16-18).

Thioredoxin in age-related hypertension: Trx is a multifunctional protein that maintains cellular redox state and is an electron donor for ribonucleotide reductase (RNR). The Trx system, which includes thioredoxin reductase (TrxR) in addition to Trx, taps NADPH as the source of reducing equivalents to perform protein-disulfide reduction. The *Trp-Cys-Gly-Pro-Cys* active site of Trx is highly conserved across species. The mammalian Trx has 5 cysteine residues at positions 32, 35, 62, 69 and 73. Whereas Cys32 and Cys35 perform the direct transfer of electrons to a disulfide, Cys62, 69 and 73 perform regulatory functions. For example, nitrosylation of Cys73 results in increased activity of hTrx (19-21) and oxidation of 62-69 decreases the disulfide reductase activity of hTrx (22). Although Trx does not scavenge superoxide anion ($O_2^{\cdot-}$) it scavenges hydroxyl radicals and quenches singlet oxygen (23). Further, Trx induces mitochondrial manganese superoxide dismutase (MnSOD) (2, 24). Additionally, Trx induces the expression of peroxiredoxin (Prx) and also donates electrons to Prx for its peroxidase activity (25). Thus, Trx is a powerful antioxidant that can neutralize major ROS (directly scavenging $^{\cdot}OH$ & $^1O_2$, and indirectly $O_2^{\cdot-}$ via MnSOD induction, and H_2O_2 via Prx) in the vascular cells. It has been shown that mice treated with rhTrx (intra-peritoneal injection) show decreased myocardial infarction due to ischemia-reperfusion injury(26), indicating the efficacy of injectable rhTrx. Given the fact that vascular proteins do undergo oxidative modification during aging and Trx regenerates oxidatively modified proteins, Trx may have important role in the control of hypertension during aging by restoring age-dependent oxidation of critical vascular proteins such as eNOS, resulting in improved function. However, the **role Trx in hypertension is unknown.** Our proposal addresses this critical gap in the knowledge in hypertension.

Role of AT_1R and AT_2R in age-related hypertension: Although AT_1R has been intensely studied; the role of AT_2R in age-related hypertension is yet to be fully established. AT_2R is developmentally regulated and has vasorelaxing effects when activated (27). However, an intriguing study showed that in old rats AT_2R blockade improved flow-mediated dilation, NO-mediated vasodilation and increased eNOS in resistance arteries, suggesting vasoconstrictor action of AT_2R in older rats (28, 29). Further, in old rats AT_2R expression primarily occurs in VSMC in contrast to young rats where AT_2R primarily occurs in ECs (28, 29). We found that the expression of AT_2R, which functions in an opposite manner to AT_1R is increased in aged Trx-Tg mice, but not in dnTrx-Tg mice (Fig 15). Because AT_2R is beneficial in control of high BP, our data show a likely reciprocal control of AT_1R and AT_2R by Trx, resulting in balance and control of hypertension. Additionally, in contrast to WT or dnTrx-Tg mice, expression of AT_1R occurs at lower levels in aged Trx-Tg mice (Fig 15 & 17). We speculate that increased appearance of AT_2R in VSMC is prevented in aged Trx-Tg mice due to preservation of Trx redox state and increased AT_2R occurs in the endothelial cells of resistance arteries in aged Trx-Tg mice, but not in WT or dnTrx-Tg mice. We propose to delineate whether Trx prevents the switching of AT_2R from ECs to VSMC in aged Trx-Tg mice, and thereby maintaining high levels of AT_2R in ECs as reported in young WT rats (29).

EXPERIMENTAL STRATEGY:

We will pursue 3 inter-related specific aims to elucidate the mechanism by which high levels of Trx not only protect against age-related hypertension, but also reverse an aged hypertensive phenotype to a normotensive phenotype. In aim 1 we will establish that high levels of Trx is anti-hypertensive in Trx-Tg mice but not in dnTrx-Tg mice or mice where Trx is knocked out in a vessel-specific manner (*using V-Cadherin-Cre mice and breeding with Trx-floxed mice that we have successfully developed (please see letter in appendix); Cdh5-CreERT2:$Trx^{fl/fl}$*). We will further confirm whether chronic rhTrx treatment of aged WT mice or aged baboons would show decreased hypertension and endothelial dysfunction. In Aim 2 we will determine the mechanism of eNOS dysregulation and how exactly high levels of Trx restores its function; and in Aim 3 we will determine the mechanisms of Trx-mediated induction of AT_2R in the endothelium in aged Trx-Tg mice in contrast to AT_2R expression in VSMC in aged WT mice. We specifically will focus on vascular mechanisms in aging, as studies have shown that endothelial-independent relaxations are unaffected by aging, suggesting that endothelial dysfunction is primarily involved in age-related hypertension. Conclusive evidence will be provided by

genetically altered mice such as our newly created Trx-Tg and dnTrx-Tg mice in addition to vessel-specific Trx-conditional-KO mice(see attached letter). We will also pursue a pre-clinical study in **non-human primate model** where rhTrx will be injected to aged baboons and its effect on blood pressure levels and endothelial dysfunction will be evaluated.

Specific Aim 1: Establish that high levels of Trx attenuate and reverse hypertension by protecting against vascular endothelial dysfunction.

Rationale: Trx function is severely decreased in aged arteries and Trx is required for endothelial function (Figs 2 and 7). Thus it is important to understand the mechanisms by which Trx become dysfunctional and whether increased levels of Trx would protect against its own oxidation and would improve endothelial function in the arteries resulting in normal BP in aged Trx-Tg mice. Further, we will determine whether injecting aged WT mice with rhTrx would result in maintenance of normal BP for prolonged period of time. These studies not only will establish that high level of Trx in Trx-Tg mice is anti-hypertensive, but also will show that treatment with rhTrx could be a realistic therapy for treatment of hypertension in the elderly. We further will test the efficacy of rhTrx as an antihypertensive agent in a non-human primate model.

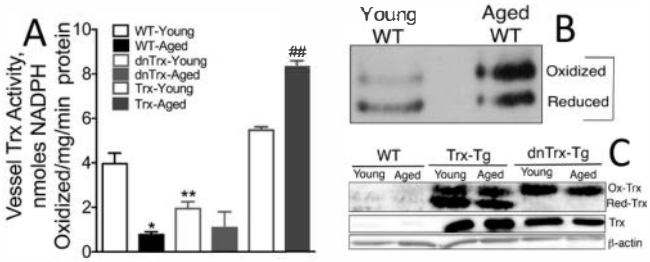

FIG 2. Carotid arteries from young or aged WT, Trx-Tg and dnTrx-Tg mice (n=4-5 each) were isolated and lysates were assayed for: (A) Trx activity (1, 2); (B) Level of oxidized and reduced Trx in young and aged WT mice; (C) Redox state of Trx in young and aged mice (1, 2). *Significantly lower than young WT; **Significantly lower than young WT; ##Significantly higher than WT or dnTrx-Tg mice.

PRELIMINARY STUDIES FOR SPECIFIC AIM 1:

Vascular redox state is predominantly oxidized in aged WT or dn-Trx-Tg, but remains reduced in Trx-Tg mice: To evaluate the effect of Trx on hypertension, we generated mice overexpressing human Trx (Trx-Tg) and another strain expressing mutant (C32S, C35S) human Trx (13, 30). The overexpression of mutant Trx decreased the level of active Trx in a dominant-negative manner by competing with TrxR (31). We created this mice as Trx knockout mice die *in utero* (32). We measured Trx activity using pooled samples of carotid artery (CA). The activity of Trx was significantly decreased in CA from young dnTrx-Tg mice compared to WT or Trx-Tg mice (Fig 2A). As expected, CA of young Trx-Tg mice showed higher Trx activity than similar WT mice. Aging was associated with a marked loss of Trx activity in CA of WT mice and persistently low Trx activity in CA of aged dnTrx-Tg mice. In contrast, the activity of Trx in CA from aged Trx-Tg mice was enhanced compared to all CA preparations. We compared the redox state of Trx between CA of young and aged WT, Trx-Tg and dnTrx-Tg mice. Trx remained in a reduced state in young WT mice, but most of Trx was oxidized in aged WT mice (Fig 2B). The redox state of Trx in CA of young and aged Trx-Tg mice revealed similar high levels of reduced Trx. In contrast, CA of young and aged Trx-deficient mice showed high levels of oxidized Trx and sparse reduced Trx (Fig 2C). These data show that aging is associated with a marked shift from an reduced to oxidized vascular redox state, and reveal that increased Trx levels during aging maintain a vascular redox state similar to younger mice. From a redox point, vessels of aged Trx-Tg mice appeared similar to young Trx-Tg or young WT mice, but not young dnTrx-Tg mice.

Aged WT and dnTrx-Tg mice are hypertensive, but aged Trx-Tg mice are normotensive:

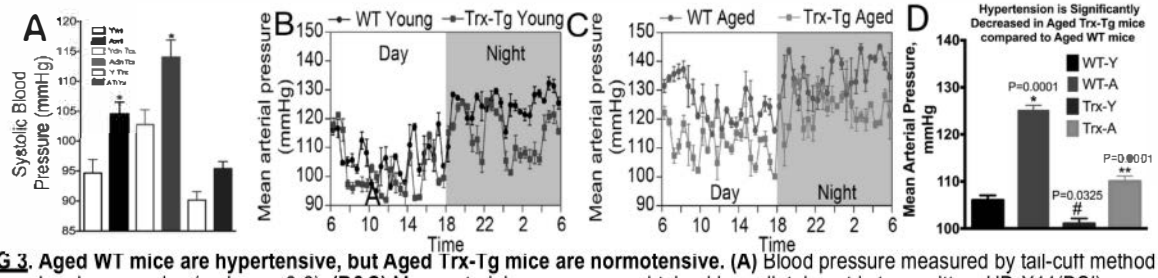

FIG 3. Aged WT mice are hypertensive, but Aged Trx-Tg mice are normotensive. (A) Blood pressure measured by tail-cuff method in aged and young mice (males, n=6-8); **(B&C)** Mean arterial pressure was obtained by radiotelemetric transmitters HD-X11(DSI) young (3-6 mo) and aged (18-24 mo) male WT and male Trx-Tg mice (n=4-5 each). **(D).** 12h time averaged MAP from each group is shown as bar graph (Mean± SEM). *Significantly higher than WT Young. **Significantly lower than WT aged and #Significantly lower than WT young.

We evaluated whether increased Trx expression alters systolic blood pressure (SBP) measured by tail cuff plethysmography in young and aged mice. SBP was lower in young Trx-Tg mice compared to young dnTrx-Tg or WT. However, SBP of aged dnTrx-Tg mice was significantly higher than aged WT or Trx-Tg mice (Fig 3A). Interestingly, SBP levels of aged Trx-Tg mice were not significantly different than young WT mice (P = NS). To further verify that expression of human Trx prevents age-related hypertension, we compared mean arterial pressures (MAP) between young and aged WT and Trx-Tg mice using radio-biotelemetry. The 24 -hr. recordings (Figs 3B-C) and averaged values (Fig 3D), indicate that MAPs of young and aged Trx-Tg mice were lower than age-matched WT. Additionally, whereas aged WT mice exhibited age-dependent hypertension, aged Trx-Tg mice exhibited a 15 mm Hg reduction in MAP compared to young Trx-Tg mice and no significant difference from the average MAP of 106 of young WT mice. Thus, aged Trx-Tg mice exhibiting overexpression of human Trx failed to develop age-related hypertension. We could not obtain

corresponding blood pressure measurements in aged dnTrx-Tg mice using telemetry due to lack of aged dnTrx-Tg mice. Thus, high level of Trx is able to lower blood pressure in aged mice.

Reversal of age-related hypertension in WT mice injected with rhTrx:

Since Trx-Tg mice have high levels of Trx at birth, lower MAP in these animals suggests protection against development of high BP during aging, but does not show whether it is effective in aged mice who already have high BP. To determine whether recombinant human Trx (rhTrx) would be effective in decreasing age-related hypertension, rhTrx was injected via tail vein to aged male WT mice (2.5 mg/Kg) at 24 hours intervals for 3-days. After the 3rd injection blood pressure was recorded after 3 and 12 days using radio-telemetry. As shown in Fig 4A-B, the MAP of aged WT mice was significantly decreased to the level seen in young WT mice. For the daytime a drop of 17 mmHg was noted and for nighttime a drop of 13 mmHg was noted. Intriguingly, the blood pressure remained lowered for at least 12-consecutive days after the rhTrx injection, demonstrating chronic changes in the arterial relaxing factors that maintained lower MAP in these animals. High level of injected rhTrx was detected in the heart (Fig 4C), and in the blood plasma 24h after the 3rd injection. The injected rhTrx was mostly found to be in reduced state in the plasma (Fig 4D).

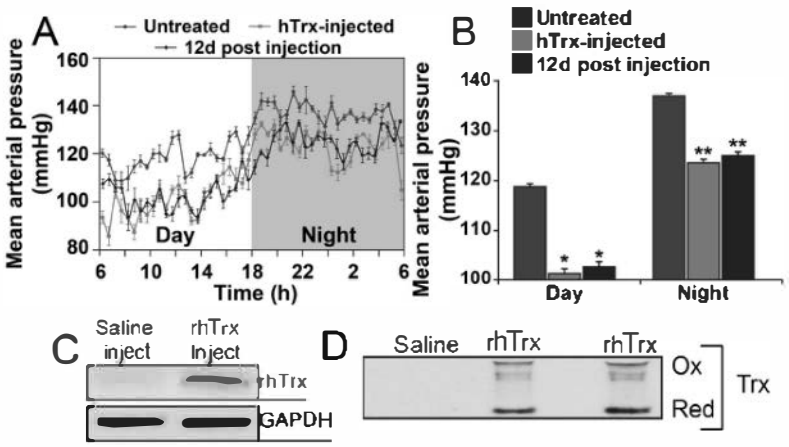

FIG 4. Age-related hypertension is alleviated by chronic rhTrx treatment: (A) Aged WT male mice (obtained from NIA) were either untreated or treated consecutively with 3 dosages of recombinant hTrx (2.5 mg/kg, Sigma) via tail vein (n=4-5 for each group). After the third dose radiotelemetric readings (using PAC-10 transmitter) collected for 24h period. (MAP) values are plotted for 3d and 12d post Trx injection (n=3). **(B)** Day and night (12h period) time averaged mice. * p<0.0001, ** p<0.0001; (C) injected rhTrx detected in the heart of aged WT mice; **(D)** Redox state of injected rhTrx in the plasma of aged WT mice after 3-days.

Aging-induced endothelial dysfunction is prevented in Trx-Tg mice, but not WT or dnTrx-Tg mice:

Vascular endothelium plays a crucial role in maintaining vasomotor tone, which is a major contributing factor for control of blood pressure. We determined whether endothelium – dependent relaxation is preserved in aged Trx-Tg mice due to maintenance of vessel redox state of Trx. Endothelium-dependent acetylcholine (ACh)-mediated relaxations were decreased in superior mesenteric artery (SMA) derived from young dnTrx-Tg mice compared to Trx-Tg mice (Fig 5A). Aging resulted in a statistically significant rightward shift in the concentration-response curves to ACh in all three mice groups (Fig 5B). However, this shift was lower in Trx-Tg mice compared to WT and dnTrx-Tg mice, as evidenced by a significantly greater pEC_{50} value in aged Trx-Tg mice (6.74 ± 0.06) compared to WT mice (6.35 ± 0.09). Sensitivity for ACh in SMA from aged dnTrx-Tg mice could not be determined, since relaxations did not reach more than 50% in most SMA. Strikingly, E_{max} values to ACh were not different in SMA between young and aged Trx-Tg mice (98 ± 1 % versus 94 ± 2%). This E_{max} for aged Trx-Tg mice was significantly higher compared to aged dnTrx-Tg mice (47 ± 11 %), but not to aged WT mice (85 ± 2 %; Fig 5B). Fig 5C summarizes the relaxing responses from young and aged mice by plotting the calculated "area above the curve" values. The aging-induced changes were specific to the endothelium, since relaxing responses to the endothelium-independent NO donor sodium nitroprusside were comparable in SMA from young (Fig 5D) and aged (Fig 5E) mice of all three groups. These data suggest that high levels of hTrx protect endothelium-dependent relaxations in aged Trx-Tg mice.

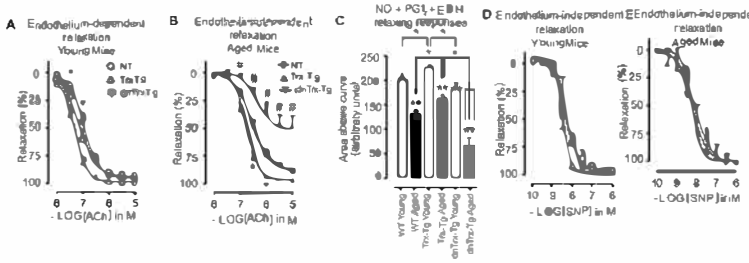

FIG 5. Aging-induced endothelial dysfunction is suppressed in Trx-Tg mice: (A-C) Endothelium-dependent relaxations in SMA derived from young **(A)** and aged **(B)** mice were contracted with a submaximal concentration of PHE before assessing relaxing responses to cumulative concentrations of ACh (0.01 – 10 mM). **(C)** Calculated area above the curve expressed as arbitrary units from all individual response curves. **(D&E)** Endothelium-independent relaxations in SMA derived from young **(D)** and aged **(E)** mice were treated for 30 min with L-NAME (100 µM) and indomethacin (10 µM), contracted with a submaximal concentration of PHE, and assessed with cumulative concentrations of SNP (0.0001 – 10 µM). Values are means ± SEM (n=8-10 mice). * $P < 0.05$ versus Trx-Tg, # $P < 0.05$ dnTrx-Tg versus WT and Trx-Tg, ** $P < 0.05$ versus young mice.

Treatment with rhTrx increases SMA inner diameter with decreased SMA stiffening: We determine if high levels of rhTrx injected into aged WT mice would show decreased arterial stiffness, as stiffness is one of the indices for age-related hypertension (33). We determined pressure-diameter relationship in saline-injected and rhTrx-injected (2.5mg/Kg rhTrx/daily for 3 days via tail vein) aged WT mice (same mice used for BP

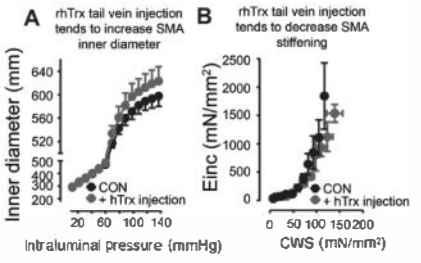

FIG 6. rhTrx treatment enhances SMA lumen diameter and elasticity: Aged WT mice treated with rhTrx as done in Fig 4. **(A)** Intraluminal pressure versus diameter relationships for pressurized segments of the SMA; (B) Average incremental elastic modulus versus circumferential wall stress relationships.

measurement, Fig 4). SMA lumen diameters tended to be higher in rhTrx-injected aged WT mice compared to saline-injected littermates (Fig 6A). From wall thickness and diameter values we calculated circumferential wall stress (CWS) and incremental elastic modulus (E_{inc}) for each pressure step. Fig 6B shows that E_{inc} values tended to be higher for comparable CWS values in SMA from saline-infused WT aged mice compared to Trx-injected aged littermates, indicating a decreased arterial stiffness in arteries of rhTrx injected mice.

rhTrx treatment improves endothelial function in aged wt mice: Since rhTrx treatment decreased hypertension in aged WT mice, we determined whether endothelial dysfunction is also ameliorated in these mice. As shown in Fig 7A, rhTrx treatment significantly improved overall endothelium-dependent ACh-mediated relaxing responses. pEC_{50} was statistically significantly reduced in saline-injected WT mice (6.83 ± 0.26) compared to Trx-injected WT littermates (7.07 ± 0.06). NO-dependent relaxations were assessed under conditions in which vasodilator prostaglandins and endothelium-dependent hyperpolarization (EDH) were pharmacologically blocked. In SMA derived from rhTrx-injected mice, NO-mediated relaxing responses were statistically significantly larger compared to its saline-injected littermates (E_{max} 72 ± 4% versus 49 ± 8%, Fig 7B). Collectively, these data establish that injected rhTrx is therapeutically effective to lower MAP of aged mice via promoting endothelium-dependent relaxation.

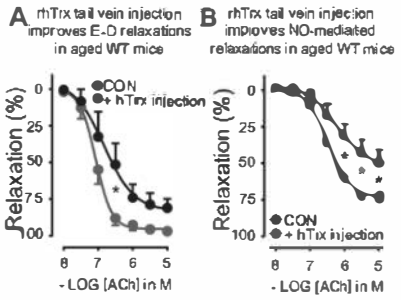

FIG 7. Endothelium-dependent relaxing responses to cumulative concentrations of ACh (0.01 – 10 mM) without any inhibitors. NO-mediated ACh-induced relaxations in SMA treated for 30 min in the combined presence of INDO (10 mM), TRAM-34 (10 mM) and UCL 1684 (10 mM) (A) untreated, (B) rhTrx treated. Values are means ± SEM (n=6 mice). * $P < 0.05$ versus CON.

Is Trx required for vascular relaxation? We determined whether vascular Trx is required for relaxation of arteries. As shown in Fig 8A, the expression of Trx was effectively depleted in mesenteric artery by siRNA specific to Trx (34) using *ex vivo* transfection. As shown in Fig 8B, endothelium-dependent relaxations due to ACh was decreased in Trx depleted SMA but not in NT siRNA treated SMA. This data suggest that vascular Trx is required for relaxations in SMA isolated form WT mice. Further, this experimental technique also established that we could down-regulate vascular genes in *ex vivo* vessels to study its effect on vascular reactivity. This novel data show for the first time that arterial Trx is an important regulator of endothelium-dependent relaxation.

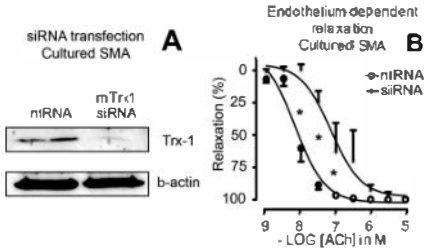

FIG 8. Trx is required for endothelium-dependent relaxation of SMA: One segment of SMA was transfected with Trx siRNA using lipofectamine for 6 hrs. Then, the the segment was incubated for an additional 16 hrs and the segment was mounted on the wire-myograph.

Experimental Design for Aim 1:
The experiments proposed in this aim are designed to elucidate the mechanisms by which arterial Trx is oxidized in aging and establish whether maintenance of arterial redox state by high levels of Trx decreases endothelial dysfunction and reverses hypertension in mice or non-human primates injected with rhTrx.

1.a. Whether increased oxidation or post-translational modifications of Trx occurs due to loss of enzymatic function of TrxR and Prx: Reduced Trx is regenerated by TrxR, which transfers reducing equivalents from NADPH(2). We will determine if a decrease in TrxR activity in aged mice accounts for the vascular accumulation of oxidized Trx, by measuring it in mesenteric arteries of young and old WT, Trx-Tg, and dnTrx-Tg mice using our previously published methods (13, 30, 35). We will also use methods we have described (13, 25) to measure the activity of Trx-dependent peroxiredoxin (Prx), to evaluate if age-dependent decreases in the activity of this enzyme may contribute to higher Trx oxidation levels. We will also determine whether Trx it self under goes oxidation and nitrosylation that reduces its enzymatic activity (22). We expect that decreased activities of TrxR and Prx would occur in aged WT mice vessels, but Trx-Tg would show undiminished or higher activities of these redox enzymes. Further, we will downregulate TrxR or Prx using RNAi in SMA/CA and determine its effect on artery Trx redox state *ex vivo*, and also in HCAEC. Conversely, we will increase intracellular Trx concentration by incubating arteries or cells with rhTrx and determine its effect on arteries previously oxidized with reversible oxidizer diamide. These determinations will reveal fundamental molecular contributions to the changes in redox balance that occur with aging, and by involving our transgenic mice in these experiments.

1.b. Does increased glutathionylation of Trx or TrxR inactivates these proteins during aging? Protein modifications can cause dysfunction of key enzymes involved in redox maintenance (36). For example, when the exposed thiol groups of Trx are glutathionylated, its disulfide-thiol exchange reaction becomes compromised. Likewise, modification of catalytic thiols in TrxR renders it inactive, causing redox dysfunction. Accordingly, we will determine if Trx or TrxR becomes glutathionylated in older mice, as Trx has been shown to be glutathionylated (37). In addition to catalytic Cys32 and Cys35 (which we mutated to create dnTrx), Trx has three other cysteines, which themselves control the activities of the catalytic thiol. Therefore, we will examine potential, age-linked glutathionylation of Cys62, Cys69, and Cys72 in the human Trx that is expressed in Trx-Tg mice (38-40). The catalytic cysteines are mutated in the dnTrx-Tg line, so we can also determine if their loss promotes glutathionylation of Cys62, 69, and 72 in mice at the age when they begin to develop increased

blood pressure. We will perform the assay as shown in our preliminary studies (Fig 14). Next, we will show whether loss of redox balance would cause endothelial dysfunction in aged mice. These experiments will show whether S-glutathionylation of Trx is a major cause of its inactivation in aging. We also will perform control experiments using high levels of GSSG or using BCNU to inactivate glutathione reductase to induce S-glutathionylation and determine the role of Trx-TrxR in deglutathionylation.

1.c. Determine if compromised in vivo Trx redox homeostasis in aged mice causes endothelial dysfunction: Our preliminary studies with WT mice depleted of Trx (Fig 8) demonstrate that Trx is required for vascular relaxation. Studies with dnTrx-Tg mice vessel suggest that active Trx is required for proper endothelium-dependent relaxation (Fig 5). We propose that vessel specific knockout of Trx (Cdh5-CreERT2:$Trx^{fl/fl}$) would impair endothelial-dependent relaxations and show age-dependent hypertension. We will determine endothelium-dependent relaxation in young and aged *Cdh5-CreERT2:*$Trx^{fl/fl}$ mice in a myograph as described in our preliminary studies (Fig 5). If Trx is absolutely required for relaxation, as shown in Fig 5, we will find that vessels from either young or aged Cdh5-CreERT2:$Trx^{fl/fl}$ mice would show dysfunction. For comparison, we will also use Cdh5-Trx (endothelial-specific overexpression of Trx) vessels for relaxation studies. Aged Cdh5-Trx mice are expected to show unimpaired endothelial dysfunction compared to young Cdh5-Trx mice. Further, we should be able to restore endothelial function in Cdh5-CreERT2:$Trx^{fl/fl}$ vessels by injection of rhTrx via tail vein as shown in our preliminary data. Both of these strains will be aged and the blood pressure will be measured via telemetry to correlate endothelial function (or dysfunction) with blood pressure. Collectively, our experiments will show if compromised redox perturbations affect age-related vascular endothelial dysfunction.

1.d. Establish that rhTrx injection protects against hypertension for a prolonged period in aged mice: To determine whether Trx would be effective in lowering the BP for a prolonged time period we will inject rhTrx (2.5 mg/Kg/mouse/day, Sigma Chemicals) into aged WT mice for 3 consecutive days and monitor blood pressure for 1-3 months using radio telemetry. If required we will inject additional rhTrx dosages and continue the BP measurement. We will also perform similar experiments using dnTrx-Tg mice; we expect that injection of Trx will initially lower the blood pressure of dnTrx-Tg mice, but the BP is expected to increase after 1-3 days because the injected active Trx will fail to be reduced because of dominant-negative effect of mutant Trx on TrxR of these mice. We will have a floxed Trx-KO mice (*the mouse is already produced and currently undergoing F1 breeding; please see attached letter*) soon, and will generate an endothelium specific Trx-KO by breeding it with pCadherin-Cre (Dr. Luisa Iruela-Arispe, UCLA, Please see the letter in appendix). We will monitor BP of aged endothelial-Trx-KO mice to determine whether these mice would show high BP compared to WT mice. If it does develop hypertension, we will treat these mice with rhTrx, and determine whether rhTrx treatment would reverse the hypertensive phenotype to normotensive one. Collectively, these in vivo studies with novel mouse models would show specificity of anti-hypertensive effects of Trx during aging.

1.e. Does pharmacological levels of hTrx reverse age-related hypertension in a baboon model of aging? Based on our strong and unequivocal preliminary studies demonstrating the efficacy of rhTrx in lowering age-related hypertension in mice, we propose to further test whether our mice studies could be extended to non-human primates. Thus, the data obtained from this funding period would be clinically relevant to undertake clinical trials for the efficacy of rhTrx in lowering hypertension in human patients. We strongly believe that our non-human primate studies proposed here is truly a bench to bedside approach, which will be beneficial to millions of hypertensive patients in future.

In collaboration with Redacted by agreement (see attached letter, a collaborator in this application) we first will measure the blood pressure of young (2-5 yrs.) and aged (>25 years equivalent to 75 yr. humans) baboons repeatedly for 1-2 months. We expect to detect high blood pressure in aged baboons (~>15 mmHg) (28, 41). We will inject rhTrx to aged baboons (cleared of any endotoxin contamination using endotoxin column as done previously (2)) at an initial dose of 2.5 mg/Kg b.w consecutively for 3 days. Following which blood pressure will be recorded for 1-3 months. The blood pressure in baboons will be measured by cuff-method as mentioned in the vertebrate animal section. However, we will use radio-telemetry if required to accurately measure blood pressure using DSI personnel to implant the sensors. For these experiments we will use male baboons. Based on the blood pressure differences we will further set up experiments to assay EC dysfunction in arterial biopsies.

1.f. Anticipated results, potential pitfalls and alternative approaches: We anticipate that increased levels of Trx will decrease age-related hypertension, enhance vascular relaxation and prevent endothelial dysfunction in aged mice. We also anticipate that aged mice or baboons treated with rhTrx will show normal BP compared to high BP seen in aged WT mice or baboons. Since Trx-Tg mice express the protein in all organs including the vascular tissue, it is possible that non-vascular functions of Trx may indirectly influence blood pressure. To avoid this complication, we will utilize our proposed endothelium-specific expression of Trx or endothelial-specific KO for Trx, and determine if they produce similar data. In fact, we are already in the process of generating these mice using a cadherin promoter, and expect them to be available in next 6-8 months. As an alternate approach, we may either infuse (mice with mini-osmotic pumps) or baboons (injections) with angiotensin II (dose relevant to slow pressure models such as 500 ng/Kg for mice) to increase blood pressure in aged animals and evaluate the effect of pharmacological levels of rhTrx on blood pressure using telemetry for mouse and cuff method for baboons. We will also use higher lower rhTrx dose as needed to lower BP in

baboons. We have already shown that Trx-Tg mice are resistant to hypertension induced by angiotensin infusion (13). In case, we determine that the baboon blood pressure measurement is not sensitive in cuff method we will consider use of telemetry for our baboon colony. Transmitters are available for baboons and DSI personnel could implant these transmitters for our studies.

Specific Aim 2: Determine that high levels of Trx improve endothelial function and decrease hypertension by restoring eNOS expression and function.

Rationale: Clinical studies have shown that endothelium-independent relaxations to sodium nitroprusside is unaffected by aging. Further, a decline in flow-mediated vasodilator capacity owing to decreased EC-derived NO has been implicated in aging (14, 16, 18). Decreased NO availability is also related to mechanical and inflammatory injury of aging arteries (17). Therefore, we will focus on endothelial mechanisms that may operate in the protection of endothelial dysfunction in aged Trx-Tg mice.

PRELIMINARY STUDIES FOR SPECIFIC AIM 2:

NO-mediated relaxation response is preserved in aged Trx-Tg mice, but not in WT or dnTrx-Tg:

Since NO is the major endothelium-derived vasorelaxing factor in SMA, we determined whether NO contributed to ACh-mediated relaxations in Trx-Tg mice. To rule out vasorelaxing factors derived from cyclooxygenases, SMA were continuously treated with the non-selective cyclooxygenase inhibitor indomethacin (INDO; 10 μM). In addition, SMA were incubated with TRAM-34 (1 μM) and UCL 1684 (1 μM) to inhibit both endothelial calcium-activated potassium channels IK1 and SK3, respectively, to prevent endothelium-dependent hyperpolarization (EDH). Comparable NO-mediated relaxations were observed in SMA from young mice (Fig 9A). In SMA of Trx-Tg mice, NO-mediated relaxations were unaffected by aging (Fig 9B&C). However, as shown in Fig 9B&C, WT and dnTrx-Tg mice showed markedly reduced NO-mediated relaxations with aging. We next determined whether high levels of Trx directly impacts eNOS function.

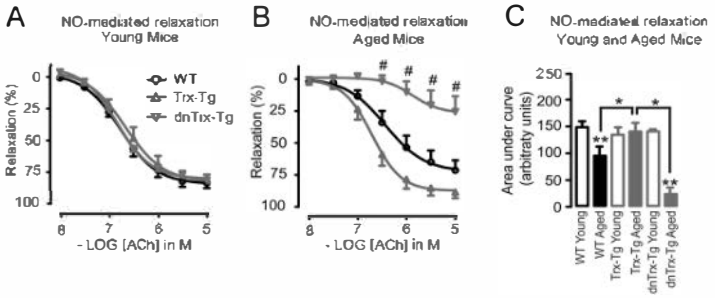

FIG 9. Preserved NO-mediated relaxing responses in aged Trx-Tg mice, but not in WT or dnTrx-Tg mice. (**A - C**) SMA derived from young (**A**) and aged (**B**), SMA relaxation was measured as mentioned in Fig 5, (C) Calculated area above the curve expressed as arbitrary units from all individual response curves shown in Fig 9A&B. Values are means ± SEM (n=6-8 mice). * $P < 0.05$ versus Trx-Tg, # $P < 0.05$ dnTrx-Tg versus WT and Trx-Tg, ** $P < 0.05$ versus young mice.

eNOS expression and phosphorylation is increased in arteries of aged Trx-Tg mice:

eNOS is known to be inactivated in aging mice (11) , we explored if high levels of Trx could protect eNOS activity. As shown in Fig. 10A, phosphorylation of eNOS Ser1179 was significantly increased in the aorta of young Trx-Tg mice compared to wt or dnTrx animals. Such phosphorylation was significantly higher in aged Trx-Tg mice compared to wt or dnTrx-Tg mice, in which almost no phosphorylation of this residue occurs. Unexpectedly, the expression level of eNOS was also increased in aged Trx-Tg mice, in contrast to aged dnTrx-Tg mice (Fig. 10A). Thus, both eNOS activation and expression are increased in aging animals by the influence of Trx. Additionally, when we injected rhTrx to aged WT mice, eNOS expression and phosphorylation at S1179 were increased in the mesenteric arteries (Fig. 10B), indicating that chronic administration of rhTrx protects against age-related eNOS dysfunction. eNOS expression is normally controlled by the activation of Rac1(42), so we propose to determine if such a process occurs in Trx-Tg mice in this aim.

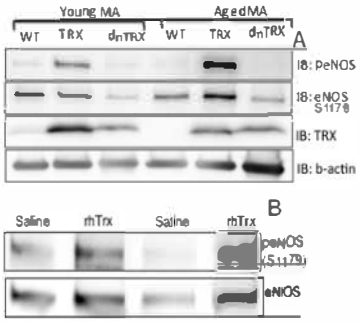

FIG. 10. (A) eNOS phosphorylation is increased in aged Trx-Tg mice, but not in wt or dnTrx-Tg. SMA from aged mice were homogenized and western analysis for eNOS (S1179), eNOS, Trx and β-actin was performed.**(B) Treatment of aged mice with rhTrx increases eNOS expression and phosphorylation.** Aged WT mice were injected with rhTrx as described for Fig 4D-E, peNOS and eNOS detected by western blotting in the mesenteric arteries.

Effects of Trx on NO release in *ex vivo* carotid arteries:

We used the fluorescent NO indicator DAF-FM to measure release of NO in carotid arteries isolated from young or aged wt, Trx-Tg, or dnTrx-Tg mice. We did not see statistically significant differences in basal NO release in arteries derived from young mice (Fig. 11), and incubation with ACh caused a rapid increase in fluorescence from all three genotypes (they tended to be more prominent in Trx-Tg mice). Inhibition of NO synthesis with L-NAME prevented this increase in signal. Aging did not

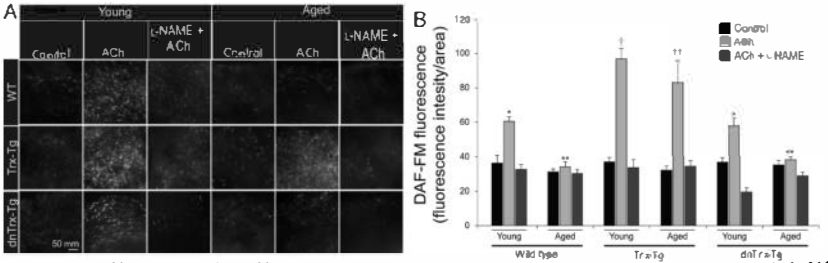

FIG. 11. eNOS-mediated NO release in young and aged arteries in Trx-Tg. (**A**) NO release by endothelium in longitudinally opened arterial segments was visualized by loading with 5 μM DAF-FM and treating with 10 μM ACh for 5 seconds. Images were captured using a Zeiss Axio Imager Z2 fluorescent microscope . Some arteries were first incubated with 100 μM L-NAME for 30 min at 37°C before loading with DAF-FM and treatment with ACh. All incubations and treatments were carried out in oxygenated Krebs-Henseleit buffer. (**B**). Quantitation of fluorescence (n=5) *, $p < 0.01$ versus wt young control; ** or †, $p < 0.01$ versus wt young + ACh, ††, $p < 0.01$ versus wt aged + Ach

alter basal levels of NO release in all of the genotypes. However, although ACh stimulation did not evoke a significant signal in arteries from aged wt and dnTrx mice, ***it clearly did so in arteries from aged Trx-Tg mice*** (Fig. 11), and this was ***inhibited with L-NAME***. These data suggest that NO release in aged WT mice is impaired due to oxidation and high levels of Trx restore NO release in aged Trx-Tg arteries. Next, we determined whether eNOS is the major source of $O_2^{\cdot-}$ in aged arteries.

eNOS is a major source of $O_2^{\cdot-}$ in aged vessels of WT mice: Given that NO release is maintained in arteries of aged Trx-Tg mice, we determined whether dysfunctional eNOS in aged WT or dnTrx-Tg mice is a source of $O_2^{\cdot-}$. If eNOS were the source of $O_2^{\cdot-}$, then we would expect a decrease in $O_2^{\cdot-}$ production after treatment of aorta with L-NAME. Alternatively, if NADPH oxidase (Nox) is the source of $O_2^{\cdot-}$ in the aged WT or dnTrx-Tg mice, $O_2^{\cdot-}$ release should be reduced after VAS2870, a non-selective NADPH oxidase inhibitor (43). There was no difference in $O_2^{\cdot-}$ generation between aortae of young WT, Trx-Tg or dnTrx-Tg mice (Fig 12A). However, aortae of aged WT mice showed higher levels of nuclear DHE staining suggestive of increased $O_2^{\cdot-}$ generation; this signal was decreased by L-NAME. In contrast, VAS2870 did not decrease DHE staining. These data suggest that eNOS underlies $O_2^{\cdot-}$ generation in aortae of aged animals. Surprisingly, DHE staining in aortae of aged dnTrx-Tg mice was decreased when the vessels were treated with either VAS2870 or L-NAME, indicating that both Nox and eNOS in arteries of these mice release O2.-. To conclusively establish the $O_2^{\cdot-}$ release by eNOS in aged arteries, we utilized EPR spin-trapping to specifically detect superoxide anion. As shown in Fig 12B-G, we detected strong BMPO-OH adduct (signifies $O_2^{\cdot-}$) in the aged arteries of WT or dnTrx-Tg mice, but this adduct was markedly absent in the vessels of Trx-Tg mice. Further, we detected BMPO-OOH adduct in the presence of VAS2870 in aged arteries (Fig 12H), but the BMPO-OOH adduct was undetectable in the presence of L-NAME in aged WT arteries (Fig 12I), indicating that eNOS uncoupling, but not NADPH oxidase is a major source of $O_2^{\cdot-}$ in aged arteries of WT mice.

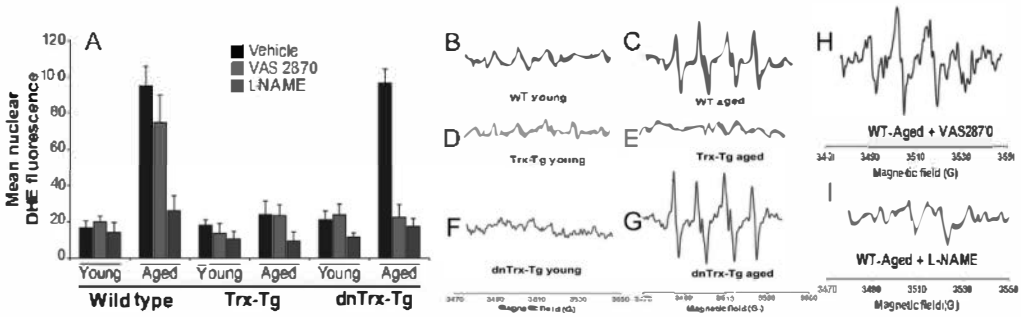

FIG 12. (A)eNOS is a major source of $O_2^{\cdot-}$ in aged WT mice: Aortae were treated with or without 1 μM VAS 2870 or 100 μM L-NAME for 1 hour and incubated with DHE (10 mM) for an additional 3 hours. $O_2^{\cdot-}$ were measured by quantitating mean red fluorescence intensity localized in nucleus and represented by the bar graph.* $P < 0.05$ level (ANOVA, Tukey's post test), ** $P < 0.05$ versus L-NAME. (B) EPR spin-trappping of $O_2^{\cdot-}$ detected using 5-*tert*-Butoxycarbonyl-5-methyl-1-pyrroline-N-oxide (BMPO), as BMPO-OOH adduct in the carotid artery (3-5); spectra for WT young, (C) aged WT, (D) young Trx-Tg, (E) Aged Trx-Tg, (F)Young dnTrx-Tg, (G) Aged dnTrx-Tg, (H) Aged WT with L-NAME, (I) Aged WT with VAS2870

Endothelium-dependent $O_2^{\cdot-}$ generation is decreased in the presence of increased levels of rhTrx:
To further determine whether O_2.- is produced in in aged arteries, we used EPR spin trapping experiment using BMPO as the O_2.- spin trap (3-5). As shown in Fig 13C denudation of EC from the aged vessels significantly decreased the generation of O_2.- . In addition, injecting rhTrx via tail vein resulted in decreased levels of $O_2^{\cdot-}$ as shown by a decrease in BMPO-OOH signal height (Fig 13B). Collectively, these data demonstrate that $O_2^{\cdot-}$ is derived from the endothelium, and the EC-dependent $O_2^{\cdot-}$ was decreased in aged WT mice treated with rhTrx.

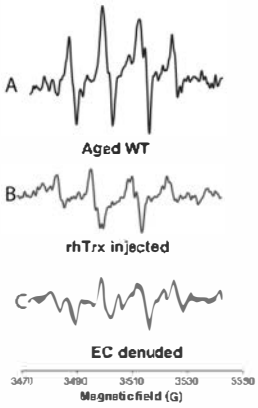

FIG 13. EPR spin-trapping of $O_2^{\cdot-}$ using BMPO as spin-trap. (A) BMPO-OOH adduct in carotid artery of aged WT mice; (B) Aged WT carotid artery from rhTrx injected mice; (C) Aged WT artery denuded of the endothelium

Depletion of Trx induces eNOS glutathionylation in endothelial cells:
S-glutathionylation occurs in increased oxidative environment due to redox balance favoring increased accumulation of GSSG. The GSSG can spontaneously conjugate with protein thiols (Pr-SSG) resulting in inactivation of protein function (36, 44). Since high levels of Trx protected eNOS activity, we determined whether depletion of Trx would promote eNOS glutathionylation. As shown in Fig 14A, Trx was effectively downregulated due to siRNA treatment of human coronary artery endothelial cells (HCAECs). This depletion of endogenous Trx promoted significant increase in eNOS glutathionylation and decreased enzymatic activity (Fig. 14 B&C). These results indicate that physiological level of Trx is required to prevent glutathionylation and maintain eNOS function. However, loss of Trx could also shift cellular redox balance to a more oxidizing state that would result in increased accumulation of GSSG, inducing

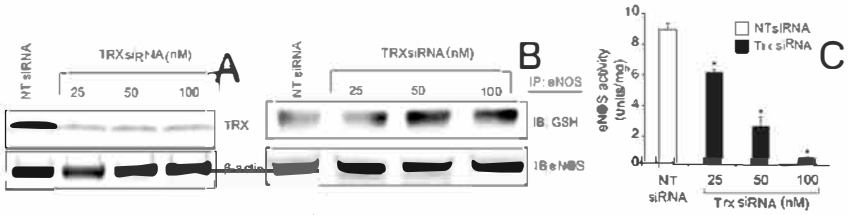

FIG 14: Trx deficiency promotes eNOS S-glutathionylation and reduces its enzymatic activity. (A) HCAEC were transfected with Non-Target (NT) or Trx siRNA and analyzed for level of Trx and β-actin by western blotting. (B) Lysate in A was Immunoprecipitated using anti- eNOS, followed by anti-GSH antibody to detect eNOS-SG and eNOS. (C) eNOS enzymatic activity by monitoring conversion of radiolabeled L-arginine to L-citrulline.

eNOS glutathionylation. We will determine whether S-glutathionylation of eNOS occurs in aged WT mice and whether increased levels of rhTrx would deglutathionylate eNOS and improve its function.

Experimental Design for Aim 2:

2.a. Determine if high levels of Trx protects against age-related BH_4 oxidation: Tetrahydrobiopterin is an important cofactor of eNOS (45, 46), and oxidation of BH_4 to BH_2 extinguishes the NO-generating activity of eNOS (47). This process (which is referred to as eNOS uncoupling) is characteristic of the aging process (48). Loss of BH_4 not only prevents NO formation, but also increases $O_2^{\cdot-}$ generation by activating the oxygenase function of eNOS (45). BH_2 is reduced back to BH_4 by dihydrofolate reductase (DHFR) in a reaction that uses folate as substrate, and reducing power provided by NADPH (45). We **hypothesize that Trx can regenerate BH_4, through its thiol reductase capability** (using reducing equivalents transferred from NADPH via TrxR), and/or by activating DHFR; both of which are potential mechanisms by which Trx may reduce eNOS uncoupling during aging. In these experiments, we will first measure BH_4 and eNOS activity using the L-[^{14}C] arginine-to-L[^{14}C]citrulline conversion assay (49) in mesenteric arteries from young or aged wt, dnTrx-Tg, and Trx-Tg mice. Next, we will evaluate its metabolites (biopterin, 7,8-dihydrobiopterin, pterin, and dihydroxanthopterin) in the same preparations, assaying them by HPLC using electrochemical detection (49). These studies will provide basic information on whether high levels of Trx confer protection from endothelial dysfunction by decreasing BH_4 oxidation and thereby promoting NO production, and whether the converse situation occurs when Trx is deficient. Using vessel-specific overexpression of Trx and vessel specific knockout of Trx mice we will delineate the role of Trx in *in vivo* BH_4 oxidation in aging.

2.b. Does aging induce eNOS glutathionylation in WT resistance arteries, but not in arteries from Trx-Tg? Various forms of protein modification are expected to occur due to cumulative oxidative process over the life span of an organism. We speculate that eNOS, being a redox-sensitive protein could be glutathionylated due to increased GSSG formation during the aging process, and increased availability of Trx in Trx-Tg mice would prevent or reverse such glutathionylation. Our preliminary data show that depletion of Trx by RNAi increases glutathionylation of HCAEC (Fig 14). We will determine whether eNOS is glutathionylated in aged WT mice and high levels of Trx in Trx-Tg mice would regenerate free eNOS by reversal of glutathionylation. We will first perform glutathionylation assay as shown in our preliminary studies using aged and young mesenteric artery lysates of WT, Trx-Tg or dnTrx-Tg mice. We will also determine eNOS activity in these lysates as shown in our preliminary studies for HCAEC. These data will show whether Trx would regenerate eNOS in aged mice inactivated by glutathionylation. These studies will also be repeated in aged WT mice injected with rhTrx.

2.c. Determine if eNOS activity is regulated by Trx via differential phosphorylation of Ser1179 and Thr497: Although phosphorylation of serine 1179 is required for eNOS activation, this modification by itself is not sufficient, nor is it the sole indicator of eNOS activity (50). For example, phosphorylation of threonine 497 prevents induction of eNOS by signaling molecules such as calmodulin (51). Activation by phosphorylation of S1179 occurs via various enzymes, including Akt, AMPK, PKA, CamKII and PKG (52, 53), whereas inactivation by phosphorylation of T497 or S116 is mediated by ERK (52, 53). We have recently shown that increased expression of Trx abrogates ERK expression in response to oxidative stress (12), and we suspect that by doing so, Trx reduces the phosphorylation of T497 and S116 in aged mice, thereby permitting the activation of eNOS via phosphorylation of S1179 by other kinases. To examine this possibility, we will measure T497 and S116 eNOS phosphorylation using phospho-specific antibodies in western analyses (as described above; Fig. 10) of arterial lysates from aged wt, Trx-Tg and dnTrx-Tg mice. We will also evaluate vascular ERK phosphorylation in these animals. We expect to observe decreased ERK activation in aged Trx-Tg mice concomitant with decreased T497 and S116 phosphorylation, and the converse in aged dnTrx-Tg mice. We will also use an eNOS construct (AddGene, on hand) to overexpress the enzyme in microvascular endothelial cells, and compare it with cells co-transfected with eNOS and phCMV-Trx (on hand) to determine if Trx specifically modulates eNOS via phosphorylation (as reflected by modification of S1197, T497 and S116). Further, we will alter these sites by site-directed mutagenesis, then determine if Trx changes eNOS activity and by measuring NO release using fluorescent probes as described in Fig 11. These experiments will reveal the specific role each phosphorylation site has in Trx-mediated regulation of hypertension. Complementary studies with NOS3$^{-/-}$-Trx and NOS3$^{-/-}$-dnTrx-Tg mice will also be performed.

2.d. Determine if increased arterial expression of VEGF in aged Trx-Tg mice induces eNOS. We now know that eNOS expression is increased in aged Trx-Tg compared to wt and dnTrx-Tg mice (Fig. 10), but it is unclear how Trx brings this about. Overexpression of Trx induces vascular endothelial growth factor (VEGF) in cultured ECs (54, 55), and considering that VEGF can stimulate eNOS expression (56), we speculate that **Trx increases eNOS synthesis via VEGF**. Here, we propose to explore Trx-associated VEGF and eNOS upregulation by comparing their occurrence in mesenteric arteries of aged mice, and establish if VEGF is required for increased eNOS expression. Two sets of experiments are planned as follows: 1) We will compare arterial VEGF and eNOS mRNA and protein (using real-time PCR and western analysis) from young and aged wt, dnTrx-Tg, and Trx-Tg mice; and 2) We will determine if Trx-stimulation of eNOS decreases concomitantly with VEGF that has been down-regulated using RNAi, in low-passage mouse EC cultures (isolated from aged mesenteric arteries per published methods; (57, 58) and/or human microvascular EC cultures (that take up Trx and respond by expressing VEGF). These experiments should establish the role of VEGF in Trx-mediated

eNOS upregulation and lowering of blood pressure. We will further evaluate the role of VEGF in eNOS induction using *Trx-EC-KO* mice and *Cdh5-EC-Trx* mice and injecting aged WT mice with rhTrx.

2.e. Determine if Trx increases eNOS expression in aged arteries through Rac1 activation; Rac1 is a redox-controlled factor (42) that increases eNOS expression, but the role it has in blood pressure regulation and the development of hypertension is not clear. Rac1 induces eNOS gene transcription through PAK1, and promotes activity of the enzyme by increasing L-arginine uptake (42), so we will determine if Trx-mediated eNOS upregulation involves these actions, and if consequent eNOS phosphorylation is mediated by PI3K. First, we will measure Rac1 activities in the lysates of aged arteries using a pull-down kinase assay; we expect Rac1 activation will occur in Trx-Tg, but not dnTrx-Tg vessels. Next, we will simultaneously upregulate Trx and downregulate Rac1 by co-transfecting ECs with phCMV-Trx and Rac1 siRNA, and measure eNOS expression using RT-PCR and western analysis; the data will establish if downregulating Rac1 prevents the induction of eNOS expression by Trx. Finally, we will downregulate PAK1 and use western blots to evaluate its effect on Trx-stimulated eNOS expression. Together, these studies will establish if Rac1 is involved in Trx-mediated increases in eNOS expression.

2.f. Age-related changes in eNOS function in baboon's resistance arteries and effect of Trx: We will repeat experiments described in 2.a -2.e with baboon femoral artery branch in control and Trx-injected baboons.

2.g. Anticipated results, pitfalls and alternate approaches: We anticipate that the dysfunctional eNOS in aged WT mice will be reversed in WT mice SMA treated with Trx. eNOS function is also expected to be protected and will show similarity to WT young mouse. Thus, studies outlined in this aim will establish the mechanism by which Trx increases eNOS expression and its activation. Like Specific Aim 1, we will perform some of these experiments in mice that have endothelium-specific expression of Trx to confirm these findings. If baboons will show significant effects in response to Trx, we will sacrifice baboons for detail analysis similar to proposed mice studies.

Specific Aim 3: Determine that Trx-mediated increased AT_2R expression and decreased AT_1R expression ameliorates endothelial dysfunction Trx-Tg mice

Rationale: We have observed increased expression of AT_2R in the aorta of Trx-Tg mice, but not in WT mice or dnTrx-Tg mice. Additionally, The expression of AT_1R is increased in aged WT and dnTrx-Tg mice, but not in Trx-Tg mice. Further, injection of rhTrx into aged WT mice increased AT_2R, but decreased AT_1R expression. These *in vivo* data point to a previously unrecognized redox-based mechanism regulating these receptor expressions. Here, we will determine if up regulation of AT_2R plays a role in Trx-mediated regulation of blood pressure as AT_2R functions in an opposite manner to that of AT_1R, and brings about lower blood pressure and improved relaxation (9, 27).

PRELIMINARY STUDIES FOR SPECIFIC AIM 3

Proprietary Info

Proprietary Info

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Literature Cited:

Progress report publication list

1. **Patents:** PATENT PENDING (Filed) PATENT PENDING
   PATENT PENDING
2. **Das, K.C**., Venkatesh Sridharan K. and Subramani, J. **2018**. INVITED REVIEW: Role of Thioredoxin in Age-related Hypertension. ***Curr Hypertens Rep 20:6.*** PMID 29445879
3. Shannon M. Dunn and **Das. K.C**. **2017**. Decreased EDHF relaxation is a major mechanism in endothelial dysfunction in resistance arteries of aged mice on prolonged high fat sucrose diet. ***Physiol. Rep. 5. 13502*** PMC5727270
4. Hilgers, R. H., Kundumani-Venkatesh, V., Subramani, Chen, L., Rusch, N.J., and **Das, K.C. 2017**. Thioredoxin reverses age-related hypertension by chronically improving vascular redox and restoring eNOS function. ***Sci. Transl. Med. 9 eaaf6094.*** PMC5808940
5. Subramani, J, Kundumani-Sridharan, V, Hilgers, R. H., Owens, C and **Das, K.C**. **2016**. Thioredoxin protects against coronary endothelial dysfunction in ischemia-reperfusion injury by deglutathionylation of eNOS. ***J Biol Chem 291 23374.*** PMC5095395

PHS Human Subjects and Clinical Trials Information

OMB Number: 0925-0001 and 0925-0002

Expiration Date: 03/31/2020

Are Human Subjects Involved	○ Yes ● No
Is the Project Exempt from Federal regulations?	○ Yes ○ No
Exemption Number	❑ 1 ❑ 2 ❑ 3 ❑ 4 ❑ 5 ❑ 6 ❑ 7 ❑ 8
Does the proposed research involve human specimens and/or data	○ Yes ● No
Other Requested information	

F. Vertebrate

1. Use of animals:

Mice: A total of 1144 male mice will be used in the proposed experiments including 18-24 week old, C57BL6 wildtype, C57BL/6-Trx, C57BL/6-dnTrx Further we will generate transgenic mice specifically overexpressing Trx in the EC (Cdh5-Trx-Tg). We are also developing a conditional Trx knockout mouse in the vessels using our Trx floxed mouse and breeding it with EC-specific Cre mouse. The purpose of these studies is to examine the role of thioredoxin in age-related hypertension. All of our transgenic mice will be allowed to age in house in our animal facility. We have already producing aged mice (>2 years) in our facility without much difficulty or mortality and will continue to do the same. We will specifically use male mice for our studies of age-related hypertension, as we have observed that male aged mice are more hypertensive than the females. However, the beneficial role of Trx in males may be equally applicable to female age-related hypertension.

Baboons: A total of 16 baboons aged 3-30 will be used in this study. The purpose is to take non-invasive blood pressure measurements in aged and young baboon control animals. Human Thioredoxin will be injected (0.5 mg/Kg) as a single dose followed by repeated blood pressure measurement for a period of 6-8 months.

New strains of mouse that are already being developed:

1. Trx-conditional Knockout (Cdh5-CreERT2:$Trx^{fl/fl}$): This strain will be developed by breeding a V-Cadherin-Cre with with Trx floxed mice. We already have Trxfl/fl mice produced by Genoway (please see attached letter). This mice will be shipped to Jackson Laboratories in December for breeding. We have filed MTA for obtaining Cdh5-CreERT2 from Dr. Luisa Iruela-Arispe, UCLA (Please see the letter in appendix).

Using the conditional knock out of Trx in the vessels we will be able to precisely delineate the role of vascular Trx in protection against age-related hypertension for the first time.

New strains of mice that will be generated by breeding mice:

3. NOS3-KO-Trx-Tg: This mouse strain will be created by crossing NOS3-KO mice with Trx-Tg mice. The strain will be identified by PCR analysis of SMA for detection of NOS3 and Trx. NOS3-KO mice will be purchased from Jackson Laboratories (B6.129P2-*Nos3*^{tm1Unc}/J; 002684).

4. NOS3-KO-dnTrx-Tg: This strain will be created by breeding NOS3-KO mice with dnTrx-Tg mice. The strain will be identified by PCR analysis of SMA for detection of NOS3 and Trx. Trx activity assay will be performed in these mice to determine the level of Trx activity. NOS3-KO mice will be purchased from Jackson Laboratories (B6.129P2-*Nos3*^{tm1Unc}/J; 002684).

5. AT_2R-KO-Trx-Tg: This mouse strain will be developed by breeding AT2R-KO mice with Trx-Tg mice, and the transgenic strain will be identified by qPCR/Western analysis for AT2R and Trx in the SMA lysates. The strain, "B6Brd; B6N-Tyr-Agtr2-tm1a(EUCOMM)Wts/WtsiCnbc" is available from EUCOMM for 2400 euros and a MTA.

6. AT_2R-KO-dnTrx-Tg: This mouse strain will be developed by breeding AT2R-KO mice with dnTrx-Tg mice, and the transgenic strain will be identified by qPCR/Western analysis for AT2R and Trx in the SMA lysates, and assay of Trx activity in the SMA. The strain, "B6Brd; B6N-Tyr-Agtr2-tm1a(EUCOMM)Wts/WtsiCnbc" is available from EUCOMM for 2400 euros and a MTA.

Baboons: (*Papio cynocephalus*)
A total of 16 baboons will be used in this study. Eight aged baboons (25-30 years age, human equivalent age ~75 years) and eight young baboons (3-5 years age) will be used. The blood pressure of all baboons will be measured once a week (for six months) in unanesthetized animals with low dose of ketamine (1.5 mg/Kg, i.m.), an agent commonly used to immobilize non-human primates, does not alter the blood pressure, but relaxes the animal for the blood pressure measurement. We expect that aged baboons will have significantly high blood pressure compared to young adults. Redacted by agreement will be in charge of these experiments (a sub-awardee collaborator). Redacted by agreement has significant experience in maintenance of baboon colony in University of Oklahoma Medical Center.

2. Quantitation of expression of Trx. We estimated that protein expression would require 18 animals per group to identify differences in the expression of Trx (µg/mg protein) between groups by activity assays as well as by western analysis. This will be a single factor two level analysis of variance. The assumed value detectable difference is about 8 (µg/mg protein) with assumed standard deviation of 0.8 (µg/mg protein). This number is based statistical analysis for ANOVA at a = 0.05 level, and a power of 1.

Isolation of mesenteric artery and aorta for vascular reactivity studies and NO release studies to assess blood pressure measurements and endothelial dysfunction, and biochemical and molecular biological assays:

We will determine the expression of several proteins in the mesenteric artery of aged and young wildtype, Trx-Tg or dnTrx-Tg in aged untreated and aged angiotensin II treated groups. Further the tension studies will also be performed in isolated mesenteric vessels. This will be a single factor two level analysis of variance. The assumed value detectable difference is about 3-20 (µmoles or other units/mg protein) with assumed standard deviation of 0.8 (µmoles or other units/mg protein). We estimate the sample size to be 6 for each experimental group based power analysis for contrasts and comparisons (power of 0.999 at a=0.05). These studies will also measure more than six endpoints. Therefore, we have included three replicates.

Extra animals generated in breeding: The breeding process will be controlled for obtaining optimal number of animals. However any excess animals will be euthanized by CO_2 from a tank source.

Rationale Hypertension is a major contributor to many cardiovascular disorders, including ventricular dysfunction, coronary artery disease, and heart failure. Aging is an independent factor for the onset of high blood pressure; in fact, people who are non-hypertensive at 55 years of age have a 90% lifetime risk of eventually developing the disease. Although a

number of biochemical processes (*e.g.*, oxidative stress, reduced nitric oxide, high renin-angiotensin activity, and endothelial dysfunction) have been identified as contributors to hypertension, the fundamental mechanisms involved in blood pressure control are not completely understood. Obviously, identifying age-specific factors is necessary to pursue therapies for this problem, but animal models that specifically address age-linked causative factors of hypertension are rare.

Baboon aged model for hypertension:
Our mice studies with injectable recombinant human thioredoxin were very successful in lowering the mean arterial pressure of hypertensive mice as reported in our preliminary studies. Therefore, we propose to use a lower non-human primates model of age-related hypertension to evaluate the efficacy of thioredoxin in lowering the blood pressure before we take it to humans. Since, results of experiments with baboons may directly be useful in understanding human hypertension in aging we propose to use this model for confirming our findings with mechanistic data obtained using mice studies with recombinant human thiroedoxin. Further, we propose to inject human Thioredoxin to baboons as it will provide a pre-clinical evidence of lowering or reversal of age-related hypertension relevant to humans. Since mouse is a very lower form of vertebrate, baboon studies, although expensive will be worthwhile for getting confirmatory data on role of thioredoxin on possible use in human thioredoxin. In addition, using injection of human thioredoxin in to baboons is expected to induce minimal immunological reactions as these genes are highly conserved and the antibody recognizes both human and monkeys with equal affinity.

3. Justification of use of animals, choice of species and numbers to be used: The goal of the proposed research is to understand whether thioredoxin overexpression would protect against hypertension in aged mice. Our preliminary studies strongly suggest that thioredoxin could effectively protect against hypertension due to amelioration of endothelial dysfunction, promotion of NO release and decreased expression of NADPH oxiase. We will determine the expression levels of various proteins including Trx in the mesenteric artery or aorta of aged wildtype, Trx-Tg or dnTrx-Tg mice in untreated and angiotensin treatment groups. The groups are sham treatment, and angiotensin II treatment (400-600 ng/Kg bw). However, after initial experiment we will choose an optimal dose on our derived data. Thus, there will be three groups with 3 strains males+female a total of 9 groups (young, aged aged+angiotensin II). 6 mice for each groups and one replicate makes a total of 108 mice for one set of experiments. Thus the total number is 432. Further, for our mesenteric artery experiments we will pool arteries from about 4 mice making 24 mice for each group. That comes to a total of 432 with one replicate. Our experiment with new transgenic mice that will be created (TrxEc-Tg) would require 108 animals. For maintenance of 5 transgenic colonies we need 100 mice on hand (20/Tg). Thus, our requirement will be a total of 1144 mice.

Additional justification for repeating experiments with very high power: Biological experiments even with very high power is required to be repeated at least 2-3 times independently. Thus, all of our cellular and animal experiments will be repeated at least 2 times. Moreover, if one person has performed a specific experiment we may get the same experiment performed again by another person to verify the results. It is important to point out here that we measure several endpoints in the same animal experiment maximizing the animal use.

4. The Texas Tech University Health Sciences Center (TTUHSC) complies with the Public Health Service (PHS) policy on Care and Use of Laboratory Animals, the Animal Welfare Act, and the NIH Guide for the Care and Use of Laboratory Animals, (NIH Publication No. 85-23). The TTUHSC is fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and is committed to maintaining AAALAC accreditation.

5. We anticipate that none of the described experiments will result in excessive discomfort, distress or pain to animals as most dissections will be terminal under appropriate anesthesia, and the animal would die as a result of removal of vessels and the heart.

Justification for use of baboon and number of baboons:
The goal of any experiments pertaining to therapeutic efficacy of a protein or reagent in humans must be tested in a species with close homology to humans. In this respect non-human primates have been used in several drug trial studies. However, the efficacy should be tested first in animals of lower phylogeny. In this regard, we have performed extensive studies using overexpression of human thioredoxin in mice and have obtained strong data that these mice are non-hypertensive. Further, as a therapeutic approach, we injected human recombinant thioredoxin to mice and observed that age-related hypertension and endothelial dysfunction is significantly lower in these mice (preliminary data). Thus, strong protective action of Trx as an injectable protein provided justification to use this safe and beneficial protein in baboons to find whether age-related hypertension could be decreased in these animals. Experimental success in non-human primates would pave the pathway for further testing in humans. As the ultimate goal of this research is to use therapeutic efficacy of hTrx in lowering age-related hypertension, we believe that our proposed baboon studies are well justified after the success of injectable thioredoxin in lowering the blood pressure in our mice model.

We propose to use initially **N=4** for young and aged baboons and young and aged baboons that will be injected with a single does of human Thioredoxin (0.5 mg/Kg). This determination comes from examining studies that have measured blood pressure in monkeys including baboons using effectively **N** = 3 to 5. Thus, we believe **N** = 4 is an effective size for blood pressure measurement propose in our studies.

5. **Euthanasia** will be performed using light anesthesia of mice with isoflurane followed by cervical dislocation in accordance with the recommendations of the panel on euthanasia of the American Veterinary Medical Association.

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Resource Sharing Plan

We have generated two lines of transgenic mice: (1) expressing human thioredoxin (Trx-Tg) and (2) expressing human mutant thioredoxin in a dominant-negative manner (dnTrx-Tg). To our best knowledge Trx-Tg mice is generated for the first time in this country; Trx-Tg mice was generated in Japan previously. However, we have generated the dnTrx mouse for the first time in the world. This line of transgenic mice is particularly useful as a redox-inactive control for oxidant-related research.
We also plan to generate the following mouse strains:

1. Trx-conditional Knockout
2. Cdh5-Trx-Tg
3. NOS3-KO-Trx-Tg
4. NOS3-KO-dnTrx-Tg
5. AT2R-KO-Trx-Tg
6. AT2R-KO-dnTrx-Tg:

Following the characterization and peer-reviewed publication of the transgenic mouse strain generated, mice will be freely distributed to investigators at academic institutions wanting mice for non-commercial research. Individual requests for shipment of mice generated by this program project funding to AAALAC (Association for Asses s ment and Accreditation of Laboratory Animal Care International) accredited institutions will be honored. The recipient investigators would provide written assurance and evidence that the animals will be used solely in accord with their local IACAC review and that animals will not be further distributed by the recipient without the consent of the Principal Investigator and the Institutional official. The animals should not be distributed for use for commercial purposes. Requests for mice from for-profit corporations to use the mice commercially will be negotiated by our institution's technology transfer office. All licensing shall be subject to distribution pursuant to my institution's policies and procedures on royalty income. The technology transfer office will report any invention disclosure submitted to them to the appropriate Federal Agency.

To facilitate sharing and distribution of the mice strains and associated resources developed under this grant will be maintained in a specific pathogen free facility. This facility will maintain the mice free of the following micro-organisms and pathogens (e.g., pinworms, mouse hepatitis virus (MHV), Sendai virus, mycoplasma, mites, etc.). Should our mice strains become infected with any of these micro-organisms, the mice will be re-derived through embryo transfer at the Charles River Laboratories.

"Other Research Resources" generated with funds from this grant will include DNA constructs, etc. These resources, as available, would also be freely distributed upon request to qualified academic investigators for non-commercial research.

My institution and I will adhere to the NIH Grants Policy on Sharing of Unique Research Resources including the "Sharing of Biomedical Research Resources: Principles and Guidelines for Recipients of NIH Grants and Contracts" iss ued in December, 1999. Specifically, material transfers would be made with no more restrictive terms than in the Simple Letter Agreement or the UBMTA and without reach through requirements. Should any intellectual property arise which requires a patent, we would ensure that the technology remains widely available to the research community in accordance with the NIH Principles and Guidelines document.

Authentication of key biological and chemical resources:

1. All antibodies will be authenticated by matching it with the literature, and in case of new antibodies, the testing will be performed using purified protein antigens to authenticate and validate antibodies for specific western analysis or immunological staining purposes. For new antibodies or antibodies that may show non-specific reactivity, we will use peptide blocking of proteins using blocking peptides.

2. All strains of mice will be screened for expression or deletion of genes by PCR or southern blotting techniques. The expression of cell-specific overexpression or deletion will be confirmed with isolation of cells from the specific organ and quantified via western analysis.

3. Cell type specific markers will be used for confirmation of origin of cells. For example, isolectin or cadherin will be always used as markers of endothelial cells and alpha-actinin as the marker for cardiomyocytes. These markers will be used during isolation of endothelial or myocytes from heart tissue and subsequent growth.

4. For cultured cell studies, Cells will be purchased from ATCC or other vendors, but will not be obtained from any other laboratory to maintain strict authenticity of the primary cells or cell lines.