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ORIGINAL RESEARCH

Adenosine A_{2A} Receptor Regulates microRNA-181b Expression in Aorta: Therapeutic Implications for Large-Artery Stiffness

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BACKGROUND: The identification of large-artery stiffness as a major, independent risk factor for cardiovascular disease–associated morbidity and death has focused attention on identifying therapeutic strategies to combat this disorder. Genetic manipulations that delete or inactivate the translin/trax microRNA-degrading enzyme confer protection against aortic stiffness induced by chronic ingestion of high-salt water (4%NaCl in drinking water for 3 weeks) or associated with aging. Therefore, there is heightened interest in identifying interventions capable of inhibiting translin/trax RNase activity, as these may have therapeutic efficacy in large-artery stiffness.

METHODS AND RESULTS: Activation of neuronal adenosine A_{2A} receptors (A_{2A}Rs) triggers dissociation of trax from its C-terminus. As A_{2A}Rs are expressed by vascular smooth muscle cells (VSMCs), we investigated whether stimulation of A_{2A}R on vascular smooth muscle cells promotes the association of translin with trax and, thereby increases translin/trax complex activity. We found that treatment of A7r5 cells with the A_{2A}R agonist CGS21680 leads to increased association of trax with translin. Furthermore, this treatment decreases levels of pre-microRNA-181b, a target of translin/trax, and those of its downstream product, mature microRNA-181b. To check whether A_{2A}R activation might contribute to high-salt water–induced aortic stiffening, we assessed the impact of daily treatment with the selective A_{2A}R antagonist SCH58261 in this paradigm. We found that this treatment blocked aortic stiffening induced by high-salt water. Further, we confirmed that the age-associated decline in aortic pre-microRNA-181b/microRNA-181b levels observed in mice also occurs in humans.

CONCLUSIONS: These findings suggest that further studies are warranted to evaluate whether blockade of A_{2A}Rs may have therapeutic potential in treating large-artery stiffness.

Key Words: adenosine A_{2A} receptor ■ microRNA ■ microRNA-181b ■ microRNA degradation ■ translin/trax ■ vascular stiffness

Large-artery stiffness (LAS) has emerged as a major cause of cardiovascular disease–related morbidity.[1–3] Increased aortic stiffness causes isolated systolic hypertension,[4] which reduces coronary artery flow and increases cardiac afterload.[5] These adverse effects lead to cardiac remodeling, dysfunction, and heart failure. Also, LAS mediates increased central arterial pressure and flow pulsatility, which has deleterious effects on target organs with low-resistance vascular beds, such as kidney and brain.[6] Therefore, LAS is an independent risk factor of cardiovascular disease, such as hypertension, myocardial infarction, heart failure, and stroke.[2,3,7–9] As

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RESEARCH PERSPECTIVE

What Is New?

- Stimulation of adenosine A_{2A} receptors on vascular smooth muscle cells activates the translin/trax microRNA-degrading enzyme, which plays a key role in eliciting age-associated aortic stiffness.
- Blockade of adenosine A_{2A} receptors in mice confers protection from aortic stiffness induced by ingestion of high-salt water.

What Question Should Be Addressed Next?

- Does adenosine A_{2A} receptor blockade have therapeutic benefit in patients with large artery stiffness, a major cause of cardiovascular morbidity?

Nonstandard Abbreviations and Acronyms

$A_{2A}R$	adenosine A_{2A} receptor
LAS	large-artery stiffness
pre-miR	premature microRNA
PWV	pulse wave velocity
TGF-β	transforming growth factor-beta
VSMC	vascular smooth muscle cell
WT	wild-type

the incidence of LAS increases markedly in individuals >60 years of age,[1,2] the epidemiological challenge posed by the anticipated growth in this segment of the population underscores the need to develop novel therapeutic options for combatting LAS.

Several microRNAs have been identified as candidates implicated in the pathophysiology of LAS.[10] In our previous studies, we demonstrated that microRNA-181b decreases with aging and deletion of the microRNA-181a/b-1 locus leads to premature onset of aortic stiffness.[11] Also, we showed that microRNA-181b overexpression blocks vasopressin-induced stiffening of vascular smooth muscle cells (VSMCs).[12] These findings suggest that strategies aimed at blocking the decrease in microRNA-181b levels associated with increased stiffness of aorta or VSMCs may have therapeutic potential in LAS.

As the translin/trax microRNA-degrading complex, an RNase that targets a small subpopulation of premature microRNAs (pre-miRs), selectively decreases levels of both pre-miR-181b and, its product, mature microRNA-181b in VSMCs,[13–15] we reasoned that interventions that inhibit translin/trax activity may confer protection from aortic stiffening. Consistent with this hypothesis, we found that deletion of *Tsn*, the gene encoding translin,[15] or inactivation of the translin/trax RNase by insertion of a point mutation, E126A, in *Tsnax*, the gene encoding trax, reverses the decline in both pre-miR-181b and microRNA-181b, as well as aortic stiffening, induced by high-salt water ingestion.[12] Furthermore, the *Tsnax* (E126A) mutation confers protection against aortic stiffening associated with aging.[12] Taken together, these findings indicate that manipulations that inhibit the activity of the translin/trax complex may provide a therapeutic approach to combat LAS by increasing microRNA-181b expression in aorta.

Recent studies conducted in neurons have demonstrated that TX is bound to the C-terminus of the unstimulated adenosine A_{2A} receptor ($A_{2A}R$) and that activation of this receptor triggers dissociation of trax.[16,17] As $A_{2A}R$ is also expressed on VSMCs and regulates vascular tone,[18,19] we hypothesized that VSMC A_{2A}Rs may provide a convenient way to regulate translin/trax formation and activity. Activation of A_{2A}Rs might trigger dissociation of trax from $A_{2A}R$ and promote formation of active translin/trax RNase complexes, whereas, conversely, blockade of $A_{2A}R$ would have the opposite effect. To test this hypothesis, we investigated (1) the effect of the $A_{2A}R$ agonist CGS21680 stimulation on microRNA-181b level in VSMCs in vitro and (2) the impact of $A_{2A}R$ antagonist SCH58261 treatment on the decline in pre-miR-181b/microRNA-181b and aortic stiffening induced by high-salt water (HSW) ingestion in vivo.

METHODS

The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

Animals

All experimental procedures were performed in accordance with the National Institute of Health's *Guide for the Care and Use of Laboratory Animals* and approved by the Johns Hopkins Animal Care and Use Committee. Male mice between 16 and 18 weeks of age were used for these experiments. In addition to wild-type (WT) C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), we also used mice with a floxed allele of *Tsn*, a mouse line that had been generated previously on a C57BL/6J background by using the easi-CRISPR protocol.[20] UBC-CreERT2 mice were obtained from JAX (Strain #007001). Conditional deletion of *Tsn* was induced in UBC-CreERT2 X $Tsn^{fl/fl}$ mice with the following tamoxifen treatment regimen.[20] Tamoxifen (MilliporeSigma, Burlington, MA) solution (10

mg/mL in corn oil) was administered intraperitoneally at a dose of 100 mg/kg daily for 6 consecutive days.

Cell Culture and CGS21680 Treatment

A commercially available rat vascular smooth muscle cell line (A7r5, Sigma-Aldrich, St. Louis, MO) was used for the in vitro experiments. Upon reaching 80% to 90% confluence, A7r5 cells were incubated with either 0.001% DMSO or 10 μM of CGS21680 (MilliporeSigma, Burlington, MA) at 37 °C for 24 hours.

Aortic rings were harvested from 10-week-old translin$^{-/-}$ mice and littermate WT mice following a protocol described by Hori et al[11] with minor modifications. In brief, whole mouse aortas from 3 to 4 mice of the same genotype were minced and digested with a collagenase type I (1750 units/mL) and papain (9.5 units/mL) solution at 37 °C for 18 to 24 hours or until the solution was cloudy. The resulting cell suspension was filtered using a 40-μm mesh and added to a 30-mm cell culture dish containing DMEM supplemented with 20% serum. VSMCs were maintained for 7 to 10 days to reach confluence. At passage 2, media were washed out and replaced with serum-free DMEM. After 24 hours of culture, VSMCs were treated with either 0.001% DMSO or 10 μM of CGS21680 at 37 °C for additional 24 hours.

The purity of VSMC cultures was checked by confirming that western blots were positive for α-actin and negative for vascular endothelial cadherin (endothelial cell marker), as described earlier.[11]

Immunoprecipitation

After either DMSO (vehicle) or CGS21680 ($A_{2A}R$ agonist) treatment, A7r5 cells were lysed with RIPA buffer (ThermoFisher Scientific, Waltham, MA) under nonreducing conditions. Protein content was measured using a Bradford assay. Magnetic beads (Dynabeads, catalog no. 10003D; ThermoFisher Scientific) were coated with translin antibody in 1:30 dilution. The A7r5 lysate (250 μg) was incubated with translin antibody-coated beads for 10 hours at 4 °C. The beads' bound fraction was then separated by electrophoresis on an SDS-PAGE gel under reducing conditions.

HSW Stress

Adult male Tsn$^{fl/fl}$ and UBC-CreERT2 X Tsn$^{fl/fl}$ mice were randomly selected and switched from their normal drinking water to 4% sodium chloride water (HSW) for 3 weeks.[15] Noninvasive pulse wave velocity (PWV) was measured at baseline before switching to HSW and then at weekly intervals for 3 weeks.

For in vivo $A_{2A}R$ antagonist treatment, 16- to 18-week-old male C57BL6 mice were randomly selected and placed into 4 groups: the mice were injected intraperitoneally once a day for 21 consecutive days with either 1% DMSO or SCH58261 (2 mg/kg; MilliporeSigma, Burlington, MA) under both normal drinking water and HSW.

Western Blot Analysis

A7r5 cells and mouse aortic tissue were lysed with RIPA buffer containing protease and phosphatase inhibitors under reducing condition. Protein content was measured with a Bradford assay. Protein samples and molecular weight standards were separated by 1-dimensional gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes, which were blocked for 2 hours in 5% nonfat dry milk dissolved in Tris-buffered saline (pH 7.4) with 1% TWEEN 20. Membranes were then incubated in 5% nonfat dry milk for 2 hours at room temperature with antibodies to translin and trax, which were generated in our lab,[21] phosphorylated-SMAD2/3 (catalog no. 8828) and total-SMAD2/3 (catalog no. 8685) from Cell Signaling Technologies (Danvers, MA); Hsp90 (catalog no. 610419, BD Transduction Laboratories, Franklin Lakes, NJ). Membranes were incubated with secondary antibody with the appropriate horseradish peroxidase–conjugated IgG in Tris-buffered saline with 1% TWEEN 20 with 5% nonfat dry milk for 1 hour at room temperature. Immunoreactive proteins were visualized using an enhanced chemiluminescence reagent kit (catalog no. 34577, ThermoFisher Scientific). The signals emitted for chemiluminescence were detected with the iBright FL100 Imaging system (ThermoFisher Scientific), and band density was analyzed with ImageJ.

RNA Isolation and Quantitative Polymerase Chain Reaction

The microRNA-enriched total RNA fractions were isolated from the A7r5 lysate and from aortic tissue using miRNeasy kit (Qiagen, Venlo, Netherlands), per the company's instructions. To avoid genomic DNA contamination, DNase digestion was performed using an RNase-free DNase kit (Qiagen), per the company's instructions.

To characterize the integrity of the isolated RNA, spectrophotometric evaluation was performed using Nanodrop (Thermo Scientific). Only RNA samples with an Ab_{260} (absorbance at 260 nm) value >0.15 were used for further experiments. The ratio of the readings at 260 nm and 280 nm (Ab_{260}/Ab_{280}) was also measured in order to check the purity of the isolated RNA. Only high-quality RNA samples, as defined by Ab_{260}/Ab_{280} ≈2.00 were used for the experiments.

For the samples that passed the RNA quality control, the isolated RNA was reverse transcribed using the miScript Reverse Transcription Kit (Qiagen).

Quantitative polymerase chain reaction was performed using a miScript SYBR green polymerase chain reaction kit (Qiagen) and detected with a QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific). All reactions were performed in triplicates. The expressions of microRNA-181b, let-7a, microRNA-126-3p, and pre-miR-181b were analyzed using commercially available primers (miScript assay, Qiagen).

Noninvasive PWV Measurements

PWV measurements were made using a high-frequency, high-resolution Doppler spectrum analyzer (DSPW; Indus Instruments, Webster, TX) on mice anesthetized with 1.5% to 2% isoflurane.[22,23]

Tensile Testing

Thoracic aortas were harvested and cut into 2-mm rings. The aortic ring and a 0.5 mm segment proximal to each ring were imaged at ×10 magnification to measure inner diameters, thickness, and the vessel length. The diameter and the length were measured with ImageJ software. The aortic rings were mounted onto the pins of an electromechanical puller (DMT560; Danish Myo Technology A/S, Aarhus, Denmark). After calibration and alignment, the pins were slowly moved apart using an electromotor at a rate of 20 μm/s to apply radial stress on the specimen until breakage. Displacement and force were recorded continuously. Engineering stress was calculated by normalizing force to the initial stress-free area of the specimen (engineering stress = force/2t × L; where t=thickness and L=length of the sample). Engineering strain (λ) was calculated as the ratio of displacement to the initial stress-free diameter. The stress–strain relationship was represented by the equation stress=α exp (βλ), where α and β are constants. α and β were determined by nonlinear regression for each sample using Excel (Microsoft, Redmond, WA).[24]

The RNA In Situ Hybridization (ISH) Assay

Human aorta samples from deidentified male subjects were obtained from the Autopsy Pathology Program at the Johns Hopkins School of Medicine. The average post-mortem delay for these samples is ≈7 to 10 hours from the time of death. Only subjects without any history of cardiovascular illness were selected. The samples were separated into 2 groups: (1) <40 years of age and (2) >60 years of age. The samples were cleaned with sterile PBS and fixed in formalin for 12 to 16 hours. Then, paraffin blocks were prepared from the aortic tissues, and sections were placed on 5 mm plus-slides. The average age of group 1 was 37.7 ± 1.9, and group 2 was 73.3 ± 7.7 years. To evaluate RNA quality in these samples, sections were stained with RNU6. After passing the RNA quality control, the same samples were then stained for pre-miR-181b or microRNA-181b, using BaseScope and miRNAscope, respectively. The BaseScope Detection Reagent Kit v2– RED (BaseScope, catalog no. 323910, Advanced Cell Diagnostics, Newark, CA) and microRNANAscope HD Detection Reagent Kit –RED (miRNAscope: catalog no. 324510, Advanced Cell Diagnostics) were used, per the company's instructions. Both the custom hsa-pre-miR-181b probe and hsa-pre-miR-181b primers were validated by the company for specificity in a human cell line before being used to stain the human aorta slides. The validated probe was hybridized and amplified for each kit, and either pre-miR-181b or microRNA-181b was detected using a mixture of each Fast-RED solution A and B (1:60) included in each kit.

Digital Image Analysis

The Masson-Trichrome, pre-miR-181b and microRNA-181b–stained slides were digitized with a SeBaCam Digital Microscope Camera (Laxco, Bothell, WA). Using ImageJ software, the area of staining was measured. That area was divided by the total area of the vascular layer or the vascular media in the same aortic ring section.

Statistical Analysis

The results are presented as mean and standard error of the mean (mean ± SEM). For multiple comparisons, 1-way or 2-way ANOVA and the Tukey or Bonferroni post hoc tests were used. $P<0.05$ was considered statistically significant. All analyses were performed using Prism 9 (GraphPad Software Inc., La Jolla, CA).

RESULTS

Activation of $A_{2A}R$ Increases Translin-Trax Association

To test our hypothesis that $A_{2A}R$ stimulation would increase the association of trax with translin, we stimulated A7r5 cells with the $A_{2A}R$-specific agonist CGS21680 and then monitored translin-trax association by immunoprecipitation with translin antibody. We first confirmed that treatment of A7r5 cells with CGS21680 does not affect the expression of translin or trax in total lysates (Figure 1A through 1C). Then, we checked whether $A_{2A}R$ stimulation increased the immunoprecipitation of trax by translin antibodies (Figure 1D). We found that CGS21680 treatment increased the amount of trax immunoprecipitated with translin antibody (Figure 1E and 1F), consistent with our hypothesis that $A_{2A}R$ stimulation increases the association of translin with trax.

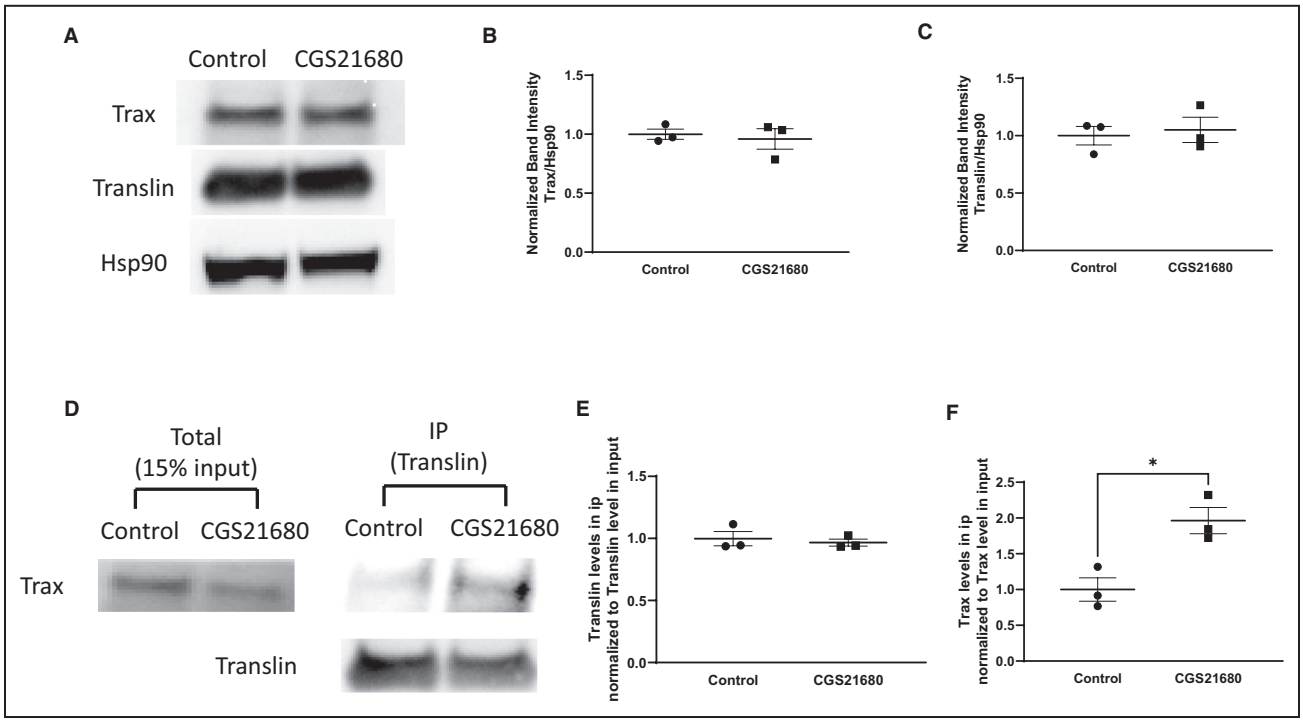

Figure 1. Adenosine A_{2A} receptor activation facilitates translin/trax association.
A. Western blot for trax (upper band) and translin (middle band) was performed on A7r5 cell lysates harvested after 24 hours of treatment with either vehicle control (0.001% DMSO) or CGS21680 (adenosine A_{2A} receptor–specific agonist). Hsp90 (lower band) was used as a loading control. Band intensity data presented in trax (**B**) and translin (**C**) were analyzed by *t*-test (n=3/group). **D,** Following treatment of A7r5 cells with either vehicle control or CGS21680, lysates were harvested and then used for immunoprecipitation with translin antibodies. The pellets were immunoblotted for trax (upper band) and translin (lower band), shown under the IP Translin label. Before adding translin antibodies to the lysate, an aliquot was removed and processed for western blotting for trax, shown under the Total label. The intensities of the immunoprecipitated translin (**E**) or trax (**F**) bands were analyzed following normalization to the input translin or trax band intensity for that condition. Data were analyzed by *t*-test (n=3/group). *P<0.05 vs control. IP indicates immunoprecipitation.

Activation of A_{2A}R Degrades Pre-miR-181b

Previous studies indicate that the translin/trax RNase selectively degrades pre-miR-181b in the VSMCs and, thereby, blocks its conversion to microRNA-181b by Dicer.[12,15] Therefore, to assess whether the increased association of translin with trax leads to enhanced RNase activity of the translin/trax complex, we examined the effect of CGS21680 treatment on levels of both pre-miR-181b and microRNA-181b. We confirmed that activation of A_{2A}R, by treating with CGS21680, decreased levels of both pre-miR-181b (Figure 2A), and microRNA-181b (Figure 2B). Furthermore, these results indicate that CGS21680 affects microRNA-181b selectively, as levels of microRNA-126-3p (Figure 2C) or let-7a (Figure 2D) microRNAs that are abundant in the aorta, were not significantly different following this treatment.

To test our hypothesis that the ability of CGS21680 to decrease levels of pre-miR-181b and microRNA-181b expression is mediated by its activation of the translin/trax RNase activity, we assessed its effect on primary aorta VSMCs that were isolated from translin$^{-/-}$ mouse[15] and their littermate WT controls. To this end, we measured the levels of pre-miR-181b (Figure 2E) and microRNA-181b (Figure 2F) after the primary VSMCs were incubated with either DMSO or CGS21680 for 24 hours. Consistent with our previous findings in A7r5 cells, CGS21680 treatment decreased the levels of both pre-miR-181b and microRNA-181b in the VSMC cells isolated from WT aorta. However, CGS21680 treatment did not alter pre-miR-181b (Figure 2E) or microRNA-181b (Figure 2F) expression in cells isolated from translin knockout mice, consistent with our hypothesis that the ability of A_{2A}R activation to markedly decrease levels of both pre-miR-181b and microRNA-181b is mediated by its activation of translin/trax RNase.

Activation of A_{2A}R Stimulates Transforming Growth Factor-Beta Pathway Signaling

Our previous studies indicate that downregulation of microRNA-181b leads to aortic stiffening by augmenting transforming growth factor-beta (TGF-β) signaling.[11,12] To evaluate whether CGS21680 also elicits an enhanced

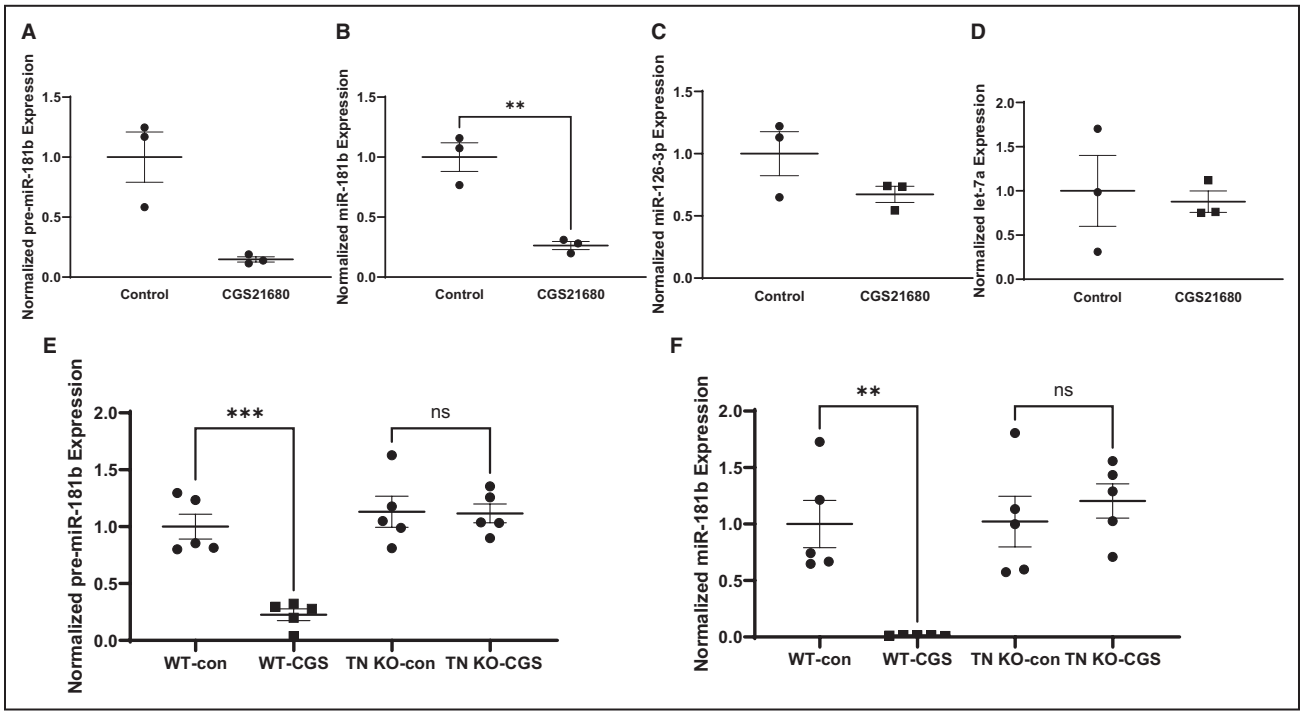

Figure 2. Adenosine A_{2A} receptor activation decreases levels of pre-miR-181b and microRNA-181b via activation of translin/trax RNase.

The bar graphs present quantitative PCR data examining the effect of CGS21680 on levels of pre-miR-181b (**A**); microRNA-181b (**B**); microRNA-126a-3p (**C**); and let-7a (**D**) in total RNA in A7r5 cell lysates harvested after 24 hours of treatment with either vehicle control (0.001% DMSO) or CGS21680 (adenosine A_{2A} receptor–specific agonist). n=3/group. Pre- or mature microRNA-181b, microRNA-126a-3p, and let-7a expression were normalized to RNU6. Data were analyzed by *t*-test. **P*<0.05, ***P*<0.01 vs control. Pre-miR-181b (**E**) and microRNA-181b (**F**) in total RNA in primary aortic VSMCs from WT-control mice and translin$^{-/-}$ mice harvested after 24 hours of treatment with either vehicle control (0.001% DMSO) or CGS21680. n=5/group. Pre- or mature microRNA-181b expressions were normalized to RNU6. Data were analyzed by *t*-test. ***P*<0.01, ****P*<0.001 vs control. KO indicates knockout; pre-miR, premature microRNA; VSMCs, vascular smooth muscle cells; TN, translin; and WT, wild-type.

TGF-β signaling pathway in A7r5 cells, we checked the effect of CGS21680 on the levels of SMAD 2/3 phosphorylation, downstream targets of TGF-β. Western blotting for p-SMAD2/3 showed statistically significant higher levels of pSMAD2/3 following CGS21680 treatment (Figure 3A and 3B). Taken together, these findings indicate that translin/trax activation elicited by A_{2A}R stimulation leads to reduced levels of microRNA-181b, which in turn enhances TGF-β signaling.

A_{2A}R Antagonist SCH58261 Protects From HSW-Induced Aortic Stiffening

Since the A_{2A}R receptor agonist CGS21680 mimics the biochemical changes that have been implicated in mediating HSW-induced aortic stiffening, that is, reduction of pre-miR-181b/microRNA-181b levels and enhanced TGF-β signaling pathway,[11] we hypothesized that A_{2A}R activation might play a key role in mediating HSW-induced aortic stiffening.

Furthermore, this line of reasoning implies that A_{2A}R inhibition might exert the opposite effect, that is, block the HSW-induced decrease in pre-miR-181b/microRNA-181b levels and protect from HSW-induced aortic stiffening. However, before examining the possibility that this pharmacological treatment might be able to confer protection from aortic stiffening in the HSW paradigm, we first wanted to check if conditional deletion of translin in adulthood is able to confer protection in this paradigm.

We opted to use the HSW paradigm because it provides a convenient way to mimic the pathophysiology of LAS in a relatively short period of time (3 weeks). In contrast, C57BL/6J mice develop age-associated aortic stiffening at ≈15 to 18 months.[12] Furthermore, HSW-induced aortic stiffening displays similar pathological features as observed in age-associated aortic stiffening, including increased aortic diameter/perimeter, increased thickening of the media, and decreased levels of microRNA-181b.

Even though our previous studies demonstrated that two lines of mice with constitutive loss of translin/trax activity display protection against HSW-induced aortic stiffening,[12,15] it remains unclear whether

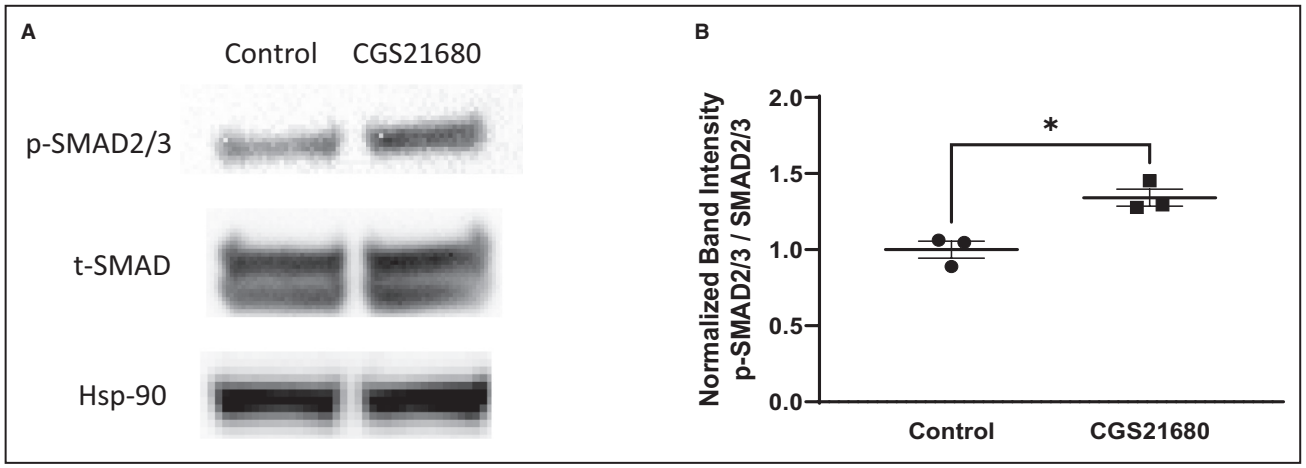

Figure 3. Adenosine A_{2A} receptor activation enhances TGF-β signaling.
A, Western blot was performed on A7r5 cell lysates harvested after 24 hours of treatment with either vehicle control or CGS21680 to detect phospho-SMAD2/3 (p-SMAD2/3; upper band) and total SMAD2/3 (t-SMAD; middle band). Hsp-90 (lower band) was used as a loading control to normalize the intensity of phospho- and total SMAD2/3 bands. **B**, Band net intensity data were determined by calculating the ratio of p-SMAD2/3 and t-SMAD2/3 band intensities and analyzed by *t*-test (n=3/group). **P*<0.05 vs control.

pharmacological strategies aimed at suppressing translin/trax activity in adulthood would be able to mimic these protective effects. A major limitation of those studies is that both lines of mice display robust adiposity.[20] This phenotype is due to loss of translin/trax during development, as conditional deletion of translin/trax in adulthood does not elicit this phenotype.[20] Therefore, it is unclear whether the protection observed in mice with constitutive loss of translin/trax activity is due to indirect, compensatory effects elicited by loss of translin/trax during development or is dependent on the absence of translin/trax during adulthood when aortic stiffening occurs. This distinction is important, as it has direct implications for whether pharmacologic strategies aimed at suppressing translin/trax activity in adulthood might be able to confer protection. Therefore, before testing the impact of SCH58261 in the HSW paradigm, we first assessed whether conditional deletion of TN during adulthood is able to block aortic stiffness induced by exposure to HSW.

To this end, we treated both *Tsn*$^{fl/fl}$ (7 mice) and UBC-CreERT2 X *Tsn*$^{fl/fl}$ mice (8 mice) with the tamoxifen regimen found to be effective at producing conditional deletion of *Tsn* in previous studies.[20] Then, we compared the impact of switching mice from normal drinking water to HSW for 3 weeks on aortic stiffening in these groups of mice.[15] We found that conditional deletion of *Tsn* prevented development of HSW-induced aortic stiffening as measured by PWV in vivo (Figure 4A), and tensile testing of aortic rings ex vivo (Figure 4B) (n=4 animals with 3 aortic rings per animal). In addition, we found, as expected, that conditional deletion of *Tsn* (n=8) increased levels of both pre-miR-181b (Figure 4C), and mature microRNA-181b in aorta (Figure 4D) compared with the corresponding control mice (n=7).

Based on these results, we proceeded to examine whether the $A_{2A}R$ antagonist SCH58261 in vivo confers protection against development of HSW-induced aortic stiffening. As predicted by this hypothesis, 6 mice treated with SCH58261 displayed protection from HSW-induced aortic stiffening on the basis of monitoring PWV during the HSW regimen (Figure 5A) and tensile testing at the end of HSW (Figure 5B) (n=4 animals with 3 aortic rings per animal). SCH58261 did not have any effect on aortic stiffness under normal drinking water (Figure 5A). In addition, we found that 4 or 5 mice treated with SCH58261 did not display the reduction in both pre-miR-181b and mature microRNA-181b induced by HSW treatment (Figure 5C and 5D). HSW-induced aortic stiffening is associated with increased collagen staining. Consistent with the ability of SCH58261 treatment to prevent aortic stiffening, we found that it also blocks HSW-induced increase in collagen deposition (Figure 6A and 6B). Furthermore, SCH58261 significantly attenuated the increases of aortic diameter, thickness, and perimeter induced by HSW (Figure 6C, 6D and 6E). The aortic sections were isolated from the descending aorta of 3 mice in each group.

Decrease in Pre-miR-181b and MicroRNA-181b in Human Aorta With Aging

Since $A_{2A}R$ blockade prevents the decrease in pre-miR-181b/microRNA-181b induced by the HSW paradigm, it is tempting to speculate that this approach might have therapeutic potential in LAS. However, even though we have found that microRNA-181b levels

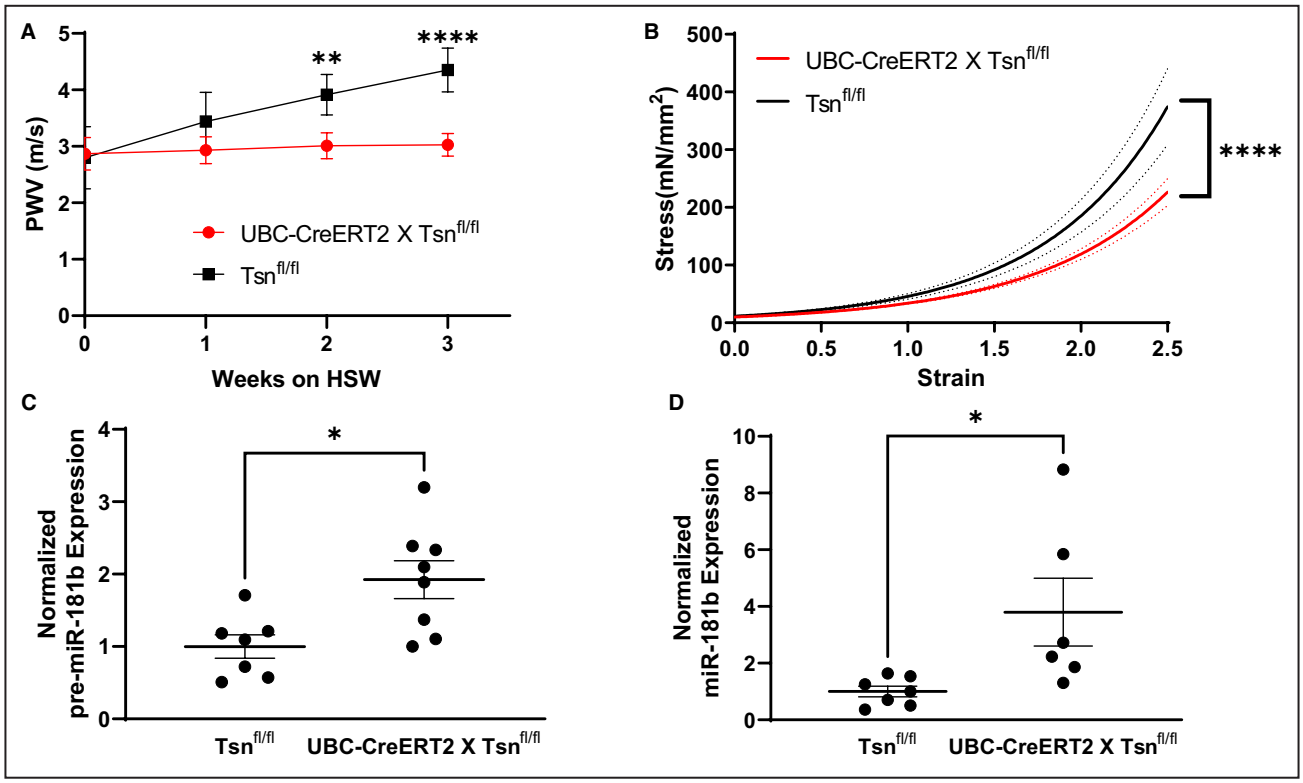

Figure 4. Conditional deletion of translin (Tsn) protects from increased vascular stiffness and decreased microRNA-181b expression produced by high-salt water (HSW) stress.

A. Weekly pulse wave velocity (PWV) measurements comparing responses of UBC-CreERT2 X Tsn$^{fl/fl}$ and littermate control (Tsn$^{fl/fl}$) (n=5) mice treated with HSW. Data were analyzed by 2-way ANOVA with repeated measures, which showed significant effects of genotype (P<0.0001) and time (P<0.0001), as well as a significant interaction between these variables (P<0.01). **B.** Tensile testing was performed on the thoracic aorta from UBC-CreERT2 X Tsn$^{fl/fl}$ and littermate Tsn$^{fl/fl}$ mice treated with HSW (n=3 animals with 4 aortic rings per animal). Data were analyzed by 2-way ANOVA with repeated measures, which showed significant effects of genotype (P<0.0001) and strain (P<0.0001), as well as a significant interaction between these variables (P<0.0001). Post hoc testing by t-test at a strain of 2.5 showed P<0.0001. Quantitative polymerase chain reaction data examining pre-miR-181b (**C**) and microRNA-181b (**D**) expression in total RNA from aortas of UBC-CreERT2 X Tsn$^{fl/fl}$ and littermate Tsn$^{fl/fl}$ mice treated with HSW (n=7, 8). Pre- or mature microRNA-181b expression was normalized to SNORD61. Data were analyzed by t-test. *P<0.05, **P<0.01, ****P<0.00001. pre-miR indicates premature microRNA.

drop in mouse aorta with HSW[15] and aging[12] and that translin/trax deletion or inactivation, as well as $A_{2A}R$ blockade, increases microRNA-181b levels in mouse aorta, it is unclear if humans display a similar decline in microRNA-181b with aging. To demonstrate the impact of aging on microRNA-181b expression in the middle layer (media) of human descending aorta, we implemented RNA in situ hybridization staining to monitor pre-miR-181b and mature microRNA-181b expression. The quantitative staining for RNU6 was the same in all the samples in both groups, suggesting the RNA quality in the fixed slides were the same (Figure S1). BaseScope staining showed a statistically significant downregulation of pre-miR-181b levels in the 4 subjects of the aged group compared with the 3 subjects of the young group (Figure 7). Further, miRNAscope staining showed statistically significant downregulation of mature microRNA-181b in the aged group compared with the young group (Figure 8).

DISCUSSION

Although the translin/trax RNase has been implicated in mediating synaptic plasticity,[25–27] adipogenesis,[20] and aortic stiffening,[10,12,15] the cellular signaling pathways that regulate translin/trax RNase activity in these paradigms are poorly understood. Previous studies have suggested that treatments that enhance dissociation of trax from phospholipase C-β may promote formation and activation of the translin/trax RNase[28,29]; however, it is unclear if this mechanism operates in VSMCs. In this study, we examined whether $A_{2A}R$ activation, which has been shown to trigger dissociation of trax from its C-terminus in neuronal cells, elicits a similar response in VSMCs and if that mechanism could enhance association of trax with translin. In addition to confirming this prediction, we also demonstrated that $A_{2A}R$ activation elicits a decrease in pre-miR-181b, a substrate of the translin/trax RNase. Furthermore, we

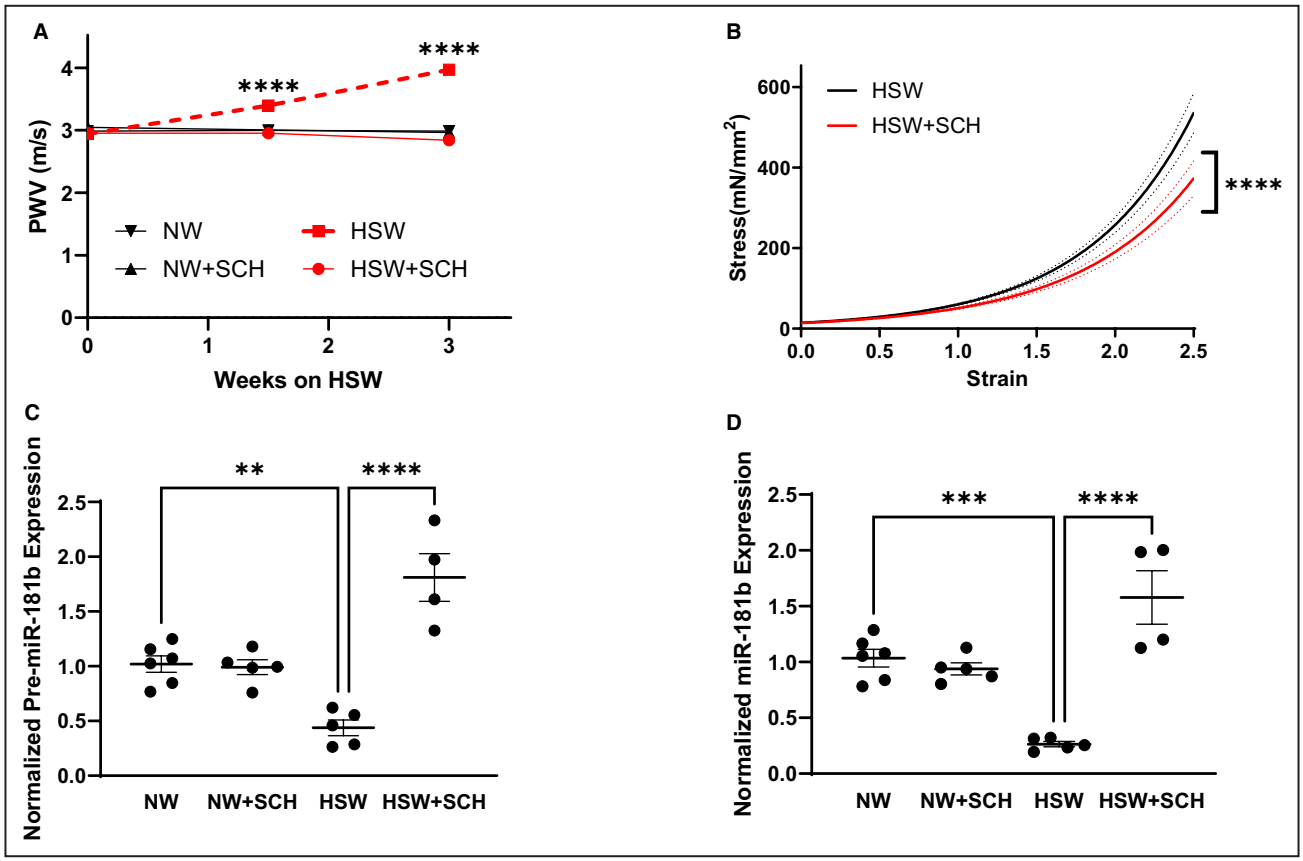

Figure 5. Adenosine A_{2A} receptor–specific antagonist (SCH58261) protects the aorta from the degradation of pre-microRNA-181b/microRNA-181b and increased vascular stiffness induced by high-salt water (HSW) stress.
A, Pulse wave velocity (PWV) was monitored before the onset of HSW treatment and twice during the 3 weeks of HSW regimen. The graph compares PWV values in mice treated daily with SCH58261 or vehicle control (n=6) during HSW and also with normal drinking water (NW). Data were analyzed by 2-way ANOVA with repeated measures, which showed significant effects of treatment (P<0.0001) and time (P<0.0001), as well as a significant interaction between these variables (P<0.0001). **B**, Tensile test was performed on the thoracic aorta from SCH58261-treated and PBS-injected mice treated with HSW (n=3 animals with 4 aortic rings per animal). Data were analyzed by 2-way ANOVA with repeated measures, which showed significant effects of treatment (P<0.0001) and strain (P<0.0001), as well as a significant interaction between these variables (P<0.0001). Post hoc testing by t-test at a strain of 2.5 showed P<0.0001. Quantitative polymerase chain reaction was performed for pre-miR-181b (**C**), and microRNA-181b (**D**) in total RNA from the aortic tissue of mice that were subjected to NW or HSW ingestion and treated daily with either with vehicle control or SCH58261 (SCH). Data presented in **C** and **D** were analyzed by Tukey post hoc analysis after intergroup differences were found by 1-way ANOVA. *P<0.05, **P<0.01, ****P<0.0001 by 2-sample t-test. pre-miR indicates premature microRNA.

confirmed that this effect is absent in aortic smooth cells isolated from translin knockout mice, indicating that the decreases in pre-miR-181b and microRNA-181b are mediated by translin/trax. Thus, these findings indicate that the increased association of trax with translin also increases translin/trax activity in VSMCs. Consistent with previous studies demonstrating that decreased microRNA-181b levels activate the intracellular TGF-β signaling cascade,[11] we also found that A_{2A}R activation elicits increased phosphorylation of SMAD 2/3, downstream targets of TGF-β receptor activation (Figure 9).

Based on these findings, we explored the possibility that A_{2A}R activation may mediate the increase in aortic stiffening induced by HSW ingestion, which is associated with a decline in both pre-miR-181b and mature microRNA-181b levels.[15] Our results strongly support this hypothesis, as treatment with the A_{2A}R-specific antagonist SCH58261 was highly effective in blocking both HSW-induced aortic stiffening and decreases in pre-miR-181b/microRNA-181b. As adenosine is released from all cells and acts in an autocrine or paracrine fashion, these findings suggest that HSW ingestion elevates extracellular adenosine levels in aorta that elicit aortic stiffening via activation of VSMC A_{2A}Rs. This scenario seems plausible since high-salt stress increases adenosine production in renal cells.[30,31] However, it will be important, in future studies, to directly assess the impact of HSW treatment on adenosine disposition in VSMCs.

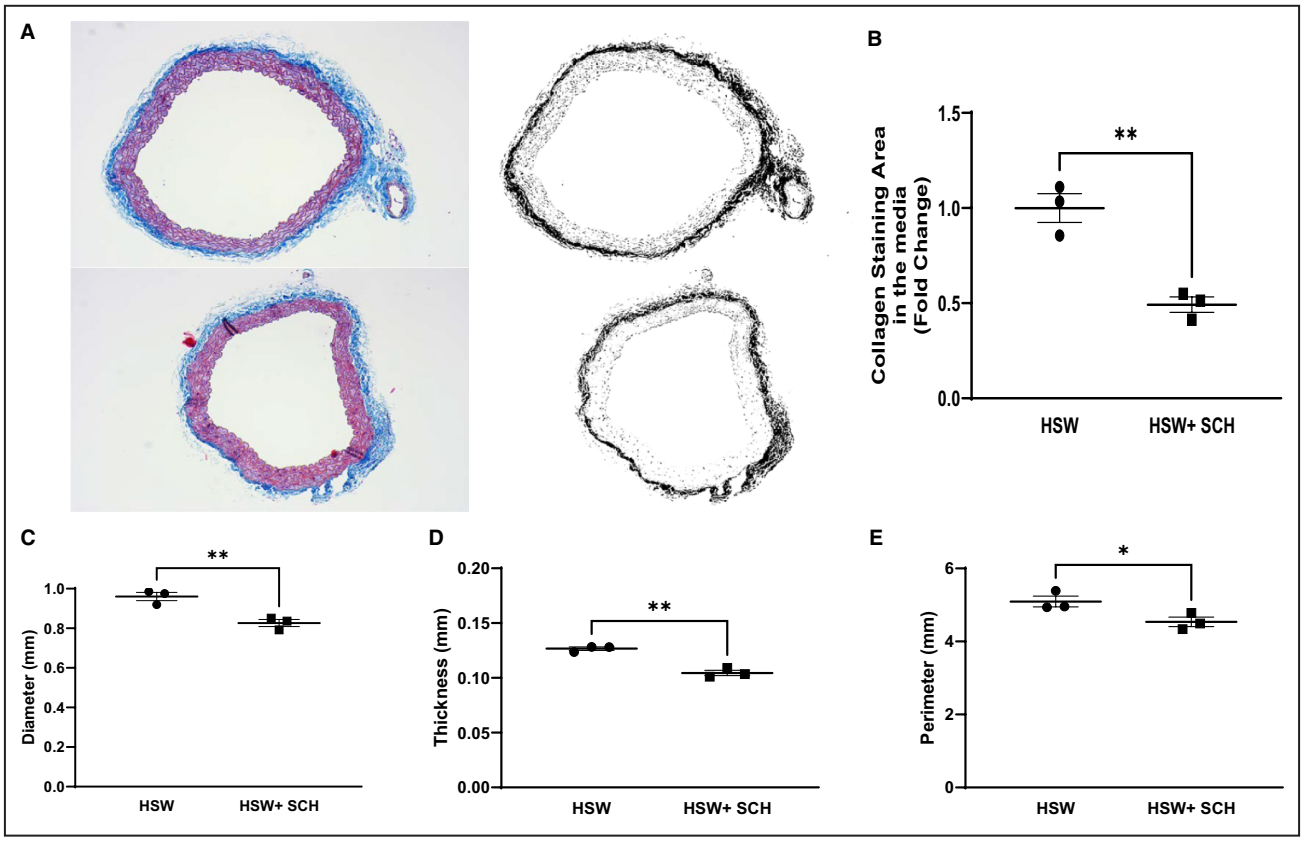

Figure 6. Treatment with adenosine A_{2A} receptor–specific antagonist (SCH58261) prevents histological alterations associated with high-salt water (HSW)-induced aortic stiffening.

A, Left panels show Pre-miR staining of aortic rings from vehicle control- and SCH58216 (SCH)-injected mice that were treated with HSW for 3 weeks. The intensity of the blue staining, which corresponds to collagen, is shown in the right panel. **B**, The areas stained only in the media for collagen were quantified and are presented in the bar graph at right, which shows reduced collagen staining in aorta sections from mice that underwent daily SCH58216 treatment. Data were analyzed by *t*-test (n=3/group). ***P*<0.01 vs PBS-injected. Mice that underwent daily SCH58216 treatment have reduced aortic diameter (**C**), aortic wall thickness (**D**), and aortic perimeter (**E**) compared with control PBS-injected mice. Data were analyzed by *t*-test (n=3/group). **P*<0.05, ***P*<0.01 vs PBS-injected.

The possibility that increased adenosine may mediate HSW-induced aortic stiffening fits well with several previous studies implicating adenosine in eliciting increased collagen deposition and TGF-β signaling, key aspects of the pathophysiology of aortic stiffening.[11] For example, in hepatocytes, adenosine activates collagen 1 and 3 depositions via extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase signaling pathways.[32] Further, adenosine promotes extracellular TGF-β in hepatic stellate cells.[32] Additionally, adenosine acts in an autocrine fashion, via A_{2B}R activation, to stimulate TGF-β secretion from corpus cavernosum fibroblasts and increase collagen deposition.[33] Previously, we found that microRNA-181b knockout mice have increased aortic stiffness that is accompanied by augmented TGF-β signaling and increased deposition of extracellular collagen in the aorta.[11] In that study, we also demonstrated that microRNA-181b directly binds to the 3' segment of the TGF-βi transcript. In fact, we have also observed a markedly higher level of plasma TGF-β level in a microRNA-181b knockout mouse compared with its littermate control. Taken together, these findings support our model that blockade of A_{2A}R activation may confer protection from aortic stiffening by dampening TGF-β signaling elicited by reduced levels of microRNA-181b.

In this study, we focused on the role of changes in microRNA-181b levels in VSMCs, rather than vascular endothelial cells, in mediating or protecting from aortic stiffness. Our focus on VSMCs stems from our previous study in which we identified activation of the TGF-β signaling pathway with microRNA-181b deficiency only in VSMCs.[11] In contrast, there were no changes in TGF-β signaling in vascular endothelial cells. These findings were obtained in primary cell culture experiments, in which the thoracic aorta was excised from 12-week-old mice from both microRNA-181a/b$^{-/-}$ and corresponding WT mice. VSMCs and vascular endothelial cells were cultured in 2 different plates from the

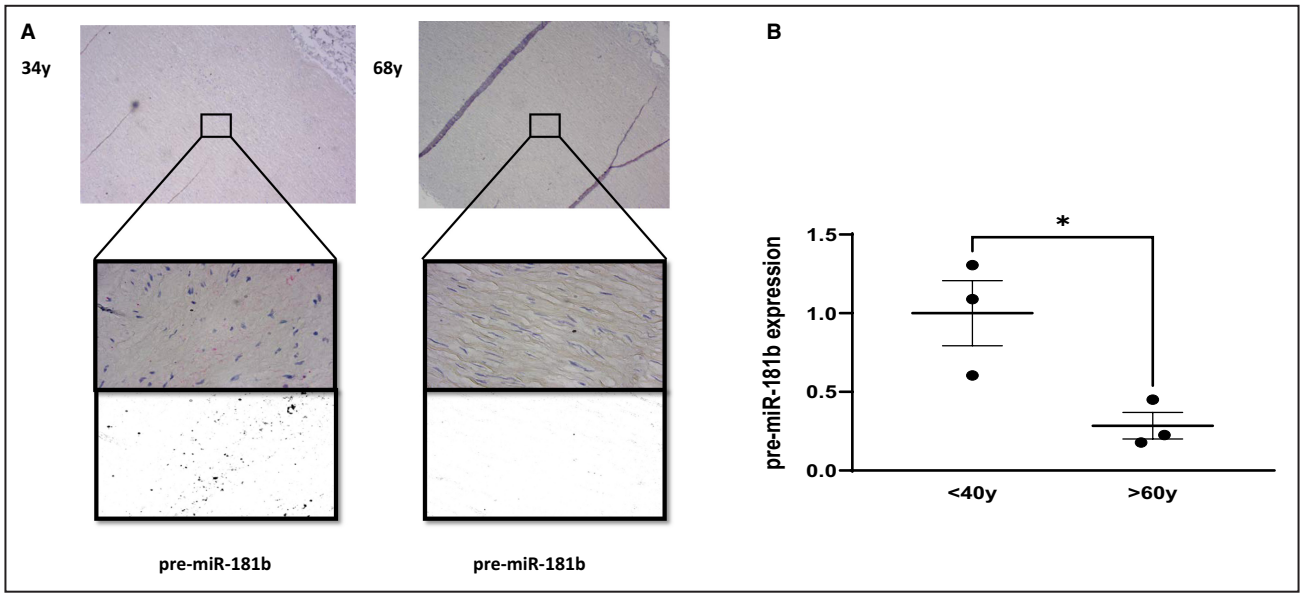

Figure 7. Loss of pre-miR-181b expression in media of human aorta with aging.
A, Representative ×4 staining picture of pre-miR-181b in full thickness sections of human aorta of 34- and 68-year-old men (top) and in higher magnification (×40) views of the middle layer (middle). The bottom panel presents a digitally processed version of these images performed with ImageJ software to display the density of pre-miR-181b staining. **B**, Digitalized slides were analyzed for pre-miR-181b stained area using ImageJ software. n=3–4/group. Data were analyzed by *t*-test. *P<0.05 vs <40 years group. pre-miR indicates premature microRNA.

same aorta.[11] Conversely, primary VSMCs isolated from the aortas of transgenic mice with a mutation in the gene coding for trax, *Tsnax* (E126A), that inactivates translin/trax RNase activity display lower TGF-β release compared with that observed in VSMCs isolated from aortas of WT mice.[12] However, even though these culture-based studies have led us to focus on the role of microRNA-181b in regulating TGF-β in VSMCs, it is certainly possible that the ability of translin deletion and translin/trax inactivation to confer protection from aortic stiffening may also be due to the impact of these interventions on vascular endothelial cells.

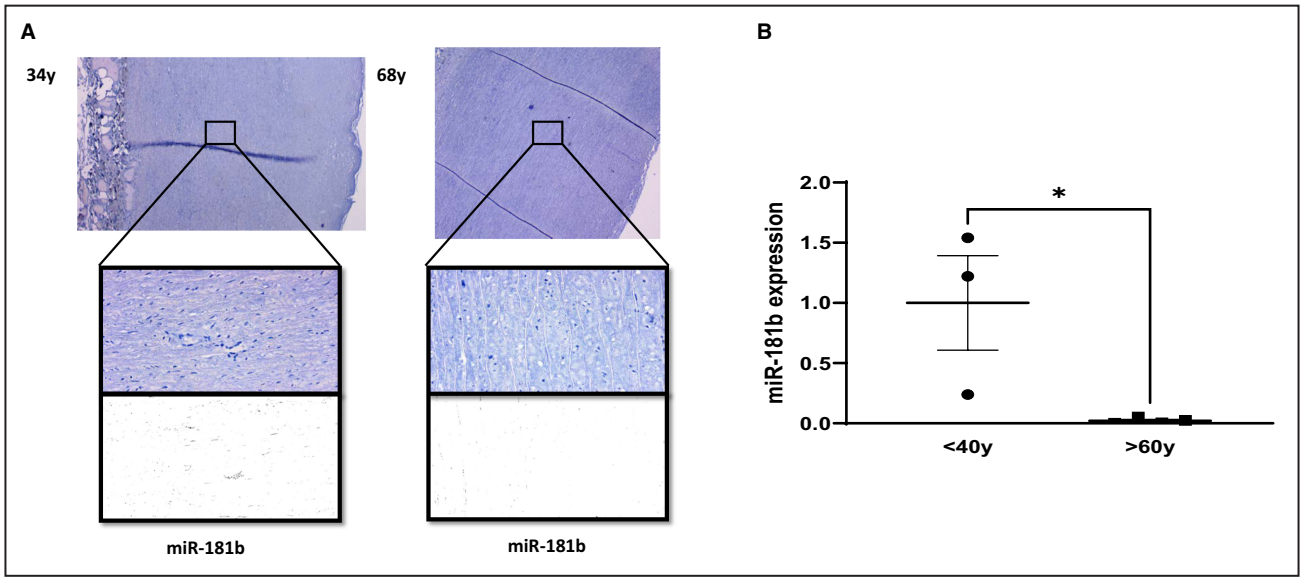

Figure 8. Loss of microRNA-181b expression in VSMCs of human aorta with aging.
A, Representative ×4 staining picture of microRNA-181b in full thickness sections of human aorta from same samples presented in Figure 5 (top). Higher magnification (×40) view of middle layer taken from area indicated on top panel (middle). Digitally processed image showing pre-miR-181b staining (bottom). **B**, Digitalized slides were analyzed for pre-miR-181b stained area using ImageJ software. n=3-4/group. Data were analyzed by *t*-test. *P<0.05 vs <40 years group. miR indicates microRNA; and pre-miR, premature microRNA.

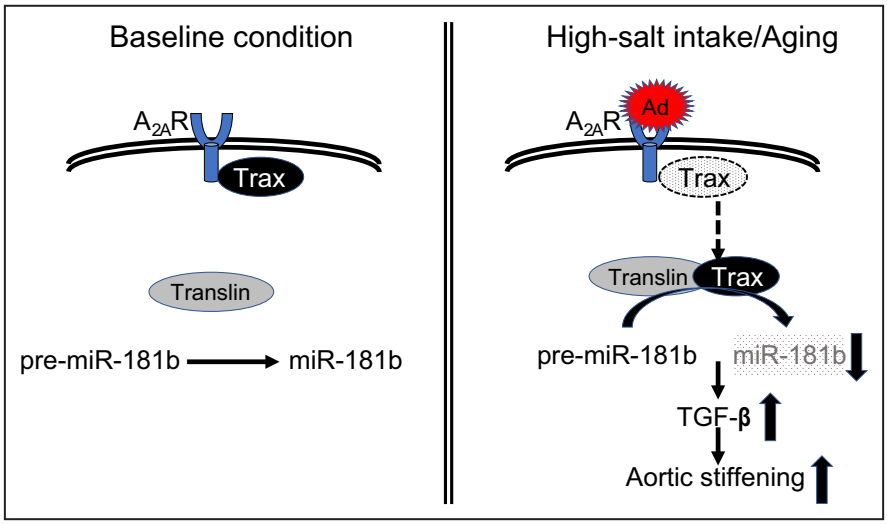

Figure 9. Role of adenosine A_{2A} receptor ($A_{2A}R$) activation in mediating increased aortic stiffness elicited by high-salt intake or aging.
This schematic diagram illustrates key steps in the signaling pathway linking $A_{2A}R$ activation with increased aortic stiffness. According to this model, high-salt intake or aging increases extracellular levels of adenosine which activate $A_{2A}Rs$ on VSMCs. Stimulation of $A_{2A}Rs$ triggers dissociation of trax from its C-terminus enabling trax to bind to translin to form active translin/trax complexes. Enhancing translin/trax RNase activity triggers degradation of its substrate, pre-miR-181b, and prevents its conversion into mature microRNA-181b by Dicer. Decreased silencing of mRNAs targeted by microRNA-181b leads to increased extracellular levels of TGF-β, which, in turn, drives aortic stiffening. miR indicates microRNA; pre-miR, premature microRNA; and VSMCs, vascular smooth muscle cells.

Our prior studies have also provided additional evidence implicating enhanced TGF-β signaling in mediating aortic stiffness since interventions that suppress TGF-β signaling, such as losartan treatment of microRNA-181b knockout mice[11] or adding TGF-β neutralizing antibody to the culture media of VSMCs,[12] mitigates the increase in VSMC stiffness induced by downregulation of microRNA-181b. In the present study, we have shown that blocking the $A_{2A}R$, which stimulates formation of the translin/trax complex and activation of its RNase activity prevents the degradation of microRNA-181b in the VSMCs, as well as aortic stiffening induced by HSW treatment. Therefore, this pharmacological strategy should be investigated further as a potential intervention to prevent or reverse LAS in humans.

In previous studies, we have found several similarities between the pathophysiology of aortic stiffening induced by HSW and that which is associated with aging.[11,15] In particular, aortic stiffening in both these paradigms is associated with selective decreases in levels of both pre-miR-181b and mature microRNA-181b, indicating that these paradigms elicit heightened activity of translin/trax. Furthermore, we have demonstrated that mice with a point mutation in *Tsnax* (E126A), which inactivates translin/trax RNase activity, display dramatic protection from developing aortic stiffening in both these paradigms. In addition, in both paradigms aortic stiffening is characterized by increased collagen deposition, aortic diameter, and aortic wall thickness. Since we have found that pre-miR-181b and microRNA-181b also decline in human aorta with aging, as found previously in mice, it is tempting to speculate that $A_{2A}R$ blockade may also confer protection from aging-associated aortic stiffening and have therapeutic potential in treating LAS, which is strongly associated with aging.[34,35] To pursue this hypothesis, it may be valuable to expand our analysis of microRNA-181b expression in human post-mortem aorta samples to test the prediction that the decline in microRNA-181b expression in aorta inversely correlates with PWV in humans.

Our studies implicating translin/trax and microRNA-181b in the pathophysiology of LAS have, to date, been limited to studies conducted on male mice. However, since the incidence of LAS is higher in postmenopausal women[36,37] and female C57BL/6 mice also display aortic stiffening with aging,[38,39] it will be important in future studies to assess if translin/trax and microRNA-181b play a similar role in the pathophysiology of LAS in aging women. To our knowledge, this study is the first to demonstrate age-associated pre-miRNA (pre-miR-181b) and microRNA (microRNA-181b) downregulation in human aorta. However, there is a lack of additional information about the post-mortem subjects of younger and older groups. While aging itself may

indeed be responsible for loss of pre-miR-181b and microRNA-181b expression, there could also be other characteristics of the 2 groups of samples that underlie the differences. Finally, as an $A_{2A}R$ antagonist, istradefylline (Nourianz; Kyowa Kirin, Tokyo, Japan) has been approved for treatment of patients with Parkinson disease, it may be interesting to assess the impact of this treatment on aortic stiffening in this population.

CONCLUSIONS

In this study, we demonstrated that activation of $A_{2A}R$ facilitates the formation and activity of the translin/trax RNase complex in VSMCs. We have also found that treatment with an $A_{2A}R$ antagonist blocks the ability of HSW treatment to elicit degradation of microRNA-181b in the aorta, as well as aortic stiffening.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Figure S1

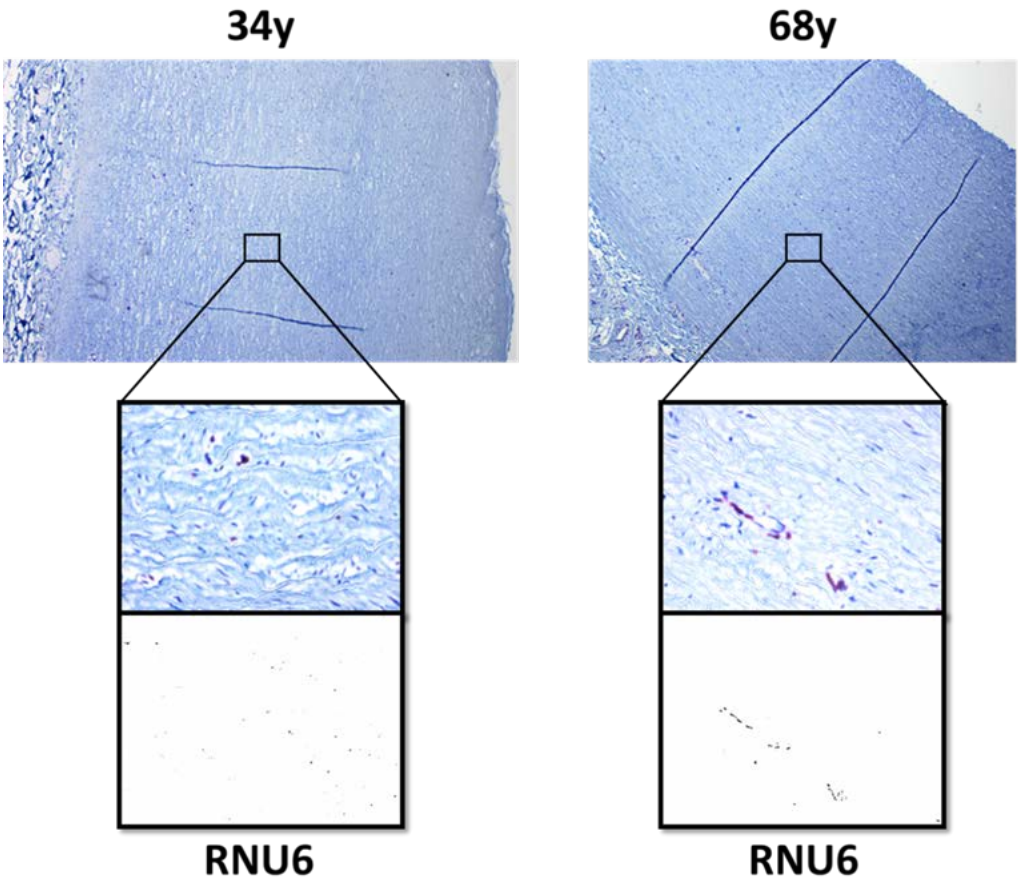
REFERENCES

1. Chirinos JA, Segers P, Hughes T, Townsend R. Large-artery stiffness in health and disease: JACC state-of-the-art review. *J Am Coll Cardiol*. 2019;74:1237–1263. doi: 10.1016/j.jacc.2019.07.012
2. Mitchell GF, Hwang SJ, Vasan RS, Larson MG, Pencina MJ, Hamburg NM, Vita JA, Levy D, Benjamin EJ. Arterial stiffness and cardiovascular events: the Framingham Heart Study. *Circulation*. 2010;121:505–511. doi: 10.1161/CIRCULATIONAHA.109.886655
3. Laurent S, Alivon M, Beaussier H, Boutouyrie P. Aortic stiffness as a tissue biomarker for predicting future cardiovascular events in asymptomatic hypertensive subjects. *Ann Med*. 2012;44(Suppl 1):S93–S97. doi: 10.3109/07853890.2011.653398
4. Kaess BM, Rong J, Larson MG, Hamburg NM, Vita JA, Levy D, Benjamin EJ, Vasan RS, Mitchell GF. Aortic stiffness, blood pressure progression, and incident hypertension. *JAMA*. 2012;308:875–881. doi: 10.1001/2012.jama.10503
5. Chirinos JA, Segers P. Noninvasive evaluation of left ventricular afterload: part 2: arterial pressure-flow and pressure-volume relations in humans. *Hypertension*. 2010;56:563–570. doi: 10.1161/HYPERTENSIONAHA.110.157339
6. Sedaghat S, Mattace-Raso FU, Hoorn EJ, Uitterlinden AG, Hofman A, Ikram MA, Franco OH, Dehghan A. Arterial stiffness and decline in kidney function. *Clin J Am Soc Nephrol*. 2015;10:2190–2197. doi: 10.2215/CJN.03000315
7. Mattace-Raso FU, van der Cammen TJ, Hofman A, van Popele NM, Bos ML, Schalekamp MA, Asmar R, Reneman RS, Hoeks AP, Breteler MM, et al. Arterial stiffness and risk of coronary heart disease and stroke: the Rotterdam study. *Circulation*. 2006;113:657–663. doi: 10.1161/CIRCULATIONAHA.105.555235
8. Laurent S, Boutouyrie P, Asmar R, Gautier I, Laloux B, Guize L, Ducimetiere P, Benetos A. Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. *Hypertension*. 2001;37:1236–1241. doi: 10.1161/01.HYP.37.5.1236
9. Aisu H, Saito M, Inaba S, Morofuji T, Takahashi K, Sumimoto T, Okura T, Higaki J. Association of worsening arterial stiffness with incident heart failure in asymptomatic patients with cardiovascular risk factors. *Hypertens Res*. 2017;40:173–180. doi: 10.1038/hr.2016.116
10. Baraban JM, Tuday E, Berkowitz DE, Das S. Deciphering the role of microRNAs in large-artery stiffness associated with aging: focus on miR-181b. *Front Physiol*. 2021;12:747789. doi: 10.3389/fphys.2021.747789
11. Hori D, Dunkerly-Eyring B, Nomura Y, Biswas D, Steppan J, Henao-Mejia J, Adachi H, Santhanam L, Berkowitz DE, Steenbergen C, et al. MiR-181b regulates vascular stiffness age dependently in part by regulating TGF-β signaling. *PLoS One*. 2017;12:e0174108. doi: 10.1371/journal.pone.0174108
12. Tuday E, Nakano M, Akiyoshi K, Fu X, Shah AP, Yamaguchi A, Steenbergen C, Santhanam L, An SS, Berkowitz D, et al. Degradation of premature-miR-181b by the translin/trax rnase increases vascular smooth muscle cell stiffness. *Hypertension*. 2021;78:831–839. doi: 10.1161/HYPERTENSIONAHA.120.16690
13. Asada K, Canestrari E, Fu X, Li Z, Makowski E, Wu YC, Mito JK, Kirsch DG, Baraban J, Paroo Z. Rescuing dicer defects via inhibition of an anti-dicing nuclease. *Cell Rep*. 2014;9:1471–1481. doi: 10.1016/j.celrep.2014.10.021
14. Baraban JM, Shah A, Fu X. Multiple pathways mediate microRNA degradation: focus on the translin/trax RNAse complex. *Adv Pharmacol*. 2018;82:1–20. doi: 10.1016/bs.apha.2017.08.003
15. Tuday E, Nomura Y, Ruhela D, Nakano M, Fu X, Shah A, Roman B, Yamaguchi A, An SS, Steenbergen C, et al. Deletion of the microRNA-degrading nuclease, translin/trax, prevents pathogenic vascular stiffness. *Am J Physiol Heart Circ Physiol*. 2019;317:H1116–H1124. doi: 10.1152/ajpheart.00153.2019
16. Sun CN, Cheng HC, Chou JL, Lee SY, Lin YW, Lai HL, Chen HM, Chern Y. Rescue of p53 blockage by the A(2A) adenosine receptor via a novel interacting protein, translin-associated protein x. *Mol Pharmacol*. 2006;70:454–466. doi: 10.1124/mol.105.021261
17. Chien T, Weng YT, Chang SY, Lai HL, Chiu FL, Kuo HC, Chuang DM, Chern Y. Gsk3β negatively regulates TRAX, a scaffold protein implicated in mental disorders, for NHEF-mediated DNA repair in neurons. *Mol Psychiatry*. 2018;23:2375–2390. doi: 10.1038/s41380-017-0007-z
18. Balwierczak JL, Sharif R, Krulan CM, Field FP, Weiss GB, Miller MJ. Comparative effects of a selective adenosine A2 receptor agonist, CGS 21680, and nitroprusside in vascular smooth muscle. *Eur J Pharmacol*. 1991;196:117–123. doi: 10.1016/0014-2999(91)90416-N
19. Conti A, Monopoli A, Gamba M, Borea PA, Ongini E. Effects of selective A1 and A2 adenosine receptor agonists on cardiovascular tissues. *Naunyn-Schmiedeberg's Archives of Pharmacology*. 1993;348:108–112. doi: 10.1007/BF00168545
20. Fu X, Shah AP, Li Z, Li M, Tamashiro KL, Baraban JM. Genetic inactivation of the translin/trax microRNA-degrading enzyme phenocopies the robust adiposity induced by Translin (Tsn) deletion. *Mol Metab*. 2020;40:101013. doi: 10.1016/j.molmet.2020.101013
21. Finkenstadt PM, Kang WS, Jeon M, Taira E, Tang W, Baraban JM. Somatodendritic localization of Translin, a component of the Translin/Trax RNA binding complex. *J Neurochem*. 2000;75:1754–1762. doi: 10.1046/j.1471-4159.2000.0751754.x
22. Jung SM, Jandu S, Steppan J, Belkin A, An SS, Pak A, Choi EY, Nyhan D, Butlin M, Viegas K, et al. Increased tissue transglutaminase activity

contributes to central vascular stiffness in eNOS knockout mice. *Am J Physiol Heart Circ Physiol*. 2013;305:H803–H810. doi: 10.1152/ajpheart.00103.2013
23. Sikka G, Pandey D, Bhuniya AK, Steppan J, Armstrong D, Santhanam L, Nyhan D, Berkowitz DE. Contribution of arginase activation to vascular dysfunction in cigarette smoking. *Atherosclerosis*. 2013;231:91–94. doi: 10.1016/j.atherosclerosis.2013.08.026
24. Steppan J, Sikka G, Jandu S, Barodka V, Halushka MK, Flavahan NA, Belkin AM, Nyhan D, Butlin M, Avolio A, et al. Exercise, vascular stiffness, and tissue transglutaminase. *J Am Heart Assoc*. 2014;3:e000599. doi: 10.1161/JAHA.113.000599
25. Park AJ, Havekes R, Fu X, Hansen R, Tudor JC, Peixoto L, Li Z, Wu YC, Poplawski SG, Baraban JM, et al. Learning induces the translin/trax RNAse complex to express activin receptors for persistent memory. *Elife*. 2017;6:6. doi: 10.7554/eLife.27872
26. Chern Y, Chien T, Fu X, Shah AP, Abel T, Baraban JM. Trax: a versatile signaling protein plays key roles in synaptic plasticity and DNA repair. *Neurobiol Learn Mem*. 2019;159:46–51. doi: 10.1016/j.nlm.2018.07.003
27. Park AJ, Shetty MS, Baraban JM, Abel T. Selective role of the translin/trax RNAse complex in hippocampal synaptic plasticity. *Mol Brain*. 2020;13:145. doi: 10.1186/s13041-020-00691-5
28. Aisiku OR, Runnels LW, Scarlata S. Identification of a novel binding partner of phospholipase cβ1: translin-associated factor x. *PLoS One*. 2010;5:e15001. doi: 10.1371/journal.pone.0015001
29. Philip F, Guo Y, Aisiku O, Scarlata S. Phospholipase Cβ1 is linked to RNA interference of specific genes through translin-associated factor X. *FASEB J*. 2012;26:4903–4913. doi: 10.1096/fj.12-213934
30. Siragy HM, Linden J. Sodium intake markedly alters renal interstitial fluid adenosine. *Hypertension*. 1996;27:404–407. doi: 10.1161/01.HYP.27.3.404
31. Carroll MA. Role of the adenosine(2A) receptor-epoxyeicosatrienoic acid pathway in the development of salt-sensitive hypertension. *Prostaglandins Other Lipid Mediat*. 2012;98:39–47. doi: 10.1016/j.prostaglandins.2011.12.002
32. Hashmi AZ, Hakim W, Kruglov EA, Watanabe A, Watkins W, Dranoff JA, Mehal WZ. Adenosine inhibits cytosolic calcium signals and chemotaxis in hepatic stellate cells. *Am J Physiol Gastrointest Liver Physiol*. 2007;292:G395–G401. doi: 10.1152/ajpgi.00208.2006
33. Wen J, Jiang X, Dai Y, Zhang Y, Tang Y, Sun H, Mi T, Phatarpekar PV, Kellems RE, Blackburn MR, et al. Increased adenosine contributes to penile fibrosis, a dangerous feature of priapism, via A2B adenosine receptor signaling. *FASEB J*. 2010;24:740–749. doi: 10.1096/fj.09-144147
34. O'Rourke MF, Nichols WW. Aortic diameter, aortic stiffness, and wave reflection increase with age and isolated systolic hypertension. *Hypertension*. 2005;45:652–658. doi: 10.1161/01.HYP.0000153793.84859.b8
35. Mitchell GF, Guo CY, Benjamin EJ, Larson MG, Keyes MJ, Vita JA, Vasan RS, Levy D. Cross-sectional correlates of increased aortic stiffness in the community: the Framingham Heart Study. *Circulation*. 2007;115:2628–2636. doi: 10.1161/CIRCULATIONAHA.106.667733
36. Coutinho T. Arterial stiffness and its clinical implications in women. *Can J Cardiol*. 2014;30:756–764. doi: 10.1016/j.cjca.2014.03.020
37. DuPont JJ, Kenney RM, Patel AR, Jaffe IZ. Sex differences in mechanisms of arterial stiffness. *Br J Pharmacol*. 2019;176:4208–4225. doi: 10.1111/bph.14624
38. DuPont JJ, Kim SK, Kenney RM, Jaffe IZ. Sex differences in the time course and mechanisms of vascular and cardiac aging in mice: role of the smooth muscle cell mineralocorticoid receptor. *Am J Physiol Heart Circ Physiol*. 2021;320:H169–H180. doi: 10.1152/ajpheart.00262.2020
39. Machin DR, Auduong Y, Gogulamudi VR, Liu Y, Islam MT, Lesniewski LA, Donato AJ. Lifelong SIRT-1 overexpression attenuates large artery stiffening with advancing age. *Aging (Albany NY)*. 2020;12:11314–11324. doi: 10.18632/aging.103322

SUPPLEMENTAL MATERIAL

Figure S1. miRNAScope for RNU6.



To determine the RNA quality in the human aorta section miRNAScope was performed for RNU6 mRNA. Representative staining of RNU6 in the entire vascular layers including intima and outer layer (top panel) and zoomed in the media (lower panel) from 34 y age and 68 y old male aorta. The original pictures were taken in 4X (top panel) and 40X magnification (lower panel). n=3-4/group was used to stain for RNU6.