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BRIEF COMMUNICATION

Enhanced Thrombotic Responses Are Associated With Striatin Deficiency and Aldosterone

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BACKGROUND: In addition to its role on blood pressure, aldosterone (ALDO) also affects the hemostatic system leading to increased experimental thrombosis. Striatin is an intermediate in the rapid, nongenomic actions of ALDO. Striatin heterozygote knockout (*Strn*$^{+/-}$) mice have salt sensitivity of blood pressure and mildly chronically increased ALDO levels. In addition, in humans, striatin polymorphic gene variants are associated with increased salt sensitivity of blood pressure. Thus, we hypothesized that striatin deficiency would be associated with an increased prothrombotic response.

METHODS AND RESULTS: *Strn*$^{+/-}$ mice and wild-type littermates were maintained on a liberal sodium diet (1.6%). We measured in vivo thrombus formation following laser-induced injury in cremaster arterioles using intravital microscopy. Mice were randomized to intravenous administration of ALDO or its vehicle. Acutely, ALDO increased thrombotic responses in wild-type mice (P<0.01) versus controls within minutes as determined by increased platelet accumulation and fibrin deposition at the site of laser injury. We then compared thrombus formation without ALDO administration in *Strn*$^{+/-}$ and wild-type mice. *Strn*$^{+/-}$ mice showed highly significant increases in laser-induced thrombosis (P<0.001), as shown by increased platelet accumulation and fibrin deposition. Interestingly, the response in the *Strn*$^{+/-}$ mice basally was far greater than the wild-type mice with ALDO administration, and ALDO administration produced no additional effect on thrombus responses in *Strn*$^{+/-}$ mice.

CONCLUSIONS: These results demonstrate a novel protective role of striatin in experimental thrombosis. Such a protective effect may be reduced in human striatin risk allele carriers, given the similar salt sensitivity of blood pressure in these individuals and *Strn*$^{+/-}$ mice.

Key Words: aldosterone ■ fibrin ■ platelet ■ striatin ■ thrombosis

Aldosterone (ALDO) and activation of its receptor, the mineralocorticoid receptor (MR), contribute to cardiovascular disease, renal disease, and stroke in humans and rodents. Traditionally it has been assumed that these cardiovascular effects are mediated via ALDO/MR. However, a growing body of data suggests that nonrenal mechanisms of ALDO/MR also may contribute to cardiovascular risk through poorly defined mechanisms. One of these is activation of thrombotic responses.[1–4] ALDO increases thrombotic responses following photochemical injury, $FeCl_3$- or laser-induced injury, and vena cava ligation in rodents.[2,4,5] Treatment with MR antagonists reduces thrombotic responses following carotid injury and platelet activation in diabetic rats.[6,7] ALDO administration increased platelet activation, degranulation, and expression of thrombogenic PAI-1 (plasminogen activator inhibitor-1), which are reversed by MR antagonists.[5]

In humans, MR antagonists have been reported to have limited effects on thrombotic risk and bleeding time in patients with hypertension and heart failure.[8] However, there are major limitations in previous clinical and preclinical reports of this association that limit our ability to interpret the underlying mechanisms. In

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human studies, ALDO's role is assumed because the conclusions are driven by correlation of ALDO and PAI-1 levels in patients with hypertension and the responses to MR antagonists where PAI-1's correlation with circulating ALDO is abolished following treatment with the MR antagonist spironolactone.[9,10] In rodents, ALDO is administered, but it is only given short term.[1,2,4,5] Such studies do not reflect what is likely occurring in humans with increased thrombotic risk, for example, those with resistant hypertension, heart failure, and renal disease, chronic, minimally elevated, but inappropriately increased, ALDO. This study was designed to address this gap in our knowledge.

We have reported that striatin (STRN), a caveolin-1-binding protein, interacts with the MR to regulate rapid and nongenomic actions of ALDO.[11] Also, using the HyperPATH (Hypertensive Pathotype) cohort, a large group of well-characterized and deeply phenotyped normotensive and hypertensive individuals, we demonstrated that individuals who carry rs2540923, a single nucleotide polymorphic gene variant of STRN, exhibit salt sensitivity of blood pressure (BP).[12] To identify the mechanisms for salt sensitivity of BP, we developed a mouse model lacking 1 functional copy of STRN (*Strn*$^{+/-}$). *Strn*$^{+/-}$ mice have ≈50% reduction in STRN levels, salt sensitivity of BP, and on a liberal salt diet, inappropriately increased ALDO levels, enhanced aortic vasoconstriction, decreased vascular relaxation, and reduced aortic eNOS (endothelial nitric oxide synthase) expression.[12,13] These results suggest that STRN-deficient mice are a model of cardiovascular disease and chronically elevated ALDO for patients with rs2540923. Whether STRN deficiency plays a role in modulating ALDO's thrombotic responses remains unexplored.

In this study, we explored the hypothesis that STRN deficiency (a model of chronic, modestly increased ALDO) leads to enhanced thrombotic responses. To test our hypothesis, we assessed laser-induced thrombotic responses in the cremaster arterioles of *Strn*$^{+/-}$ and wild-type (WT) littermate control mice. Our results provide evidence for a novel role for STRN in thrombotic responses.

METHODS

The authors declare that all supporting data are available within the article.

Animals

All experimental procedures followed guidelines approved by the Institutional Animal Care and Use Committees at both Brigham and Women's Hospital and Beth Israel Deaconess Medical Center that conform with the *Guide for the Care and Use of Laboratory Animals*. Male (13 weeks old) *Strn*$^{+/-}$ mice (CSD26933) genetically engineered by the trans–National Institutes of Health Knock-Out Mouse Project, and WT littermate mice were used in the study. These mice were previously described by us.[12,13]

Aldosterone Administration

At the age of 13 weeks, WT and *Strn*$^{+/-}$ mice were randomized to acute intravenous administration of either ALDO or its vehicle, 6 mice per group. WT and *Strn*$^{+/-}$ mice had similar body mass (31.7±0.8 g and 30.2±0.9 g, respectively). Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (125 mg/kg) and xylazine (12.5 mg/kg). Mice were administered 50 µL of ALDO (100 µg/kg; Sigma-Aldrich) or 50 µL of its vehicle (0.04% ethanol) via a jugular vein cannula in a single bolus injection, 5 minutes before laser-induced thrombosis.

Laser-Injury Thrombosis

Thrombus formation in response to laser injury was measured as described previously.[14] Briefly, cremaster arterioles were injured using a MicroPoint Laser system (Photonics Instruments). Platelet and fibrin accumulation were intravitally monitored and measured by infusion of Dylight 647-labeled anti-platelet antibodies (CD42b; 0.1 mg/kg; Emfret Analytics) and Dylight 488-labeled (Thermo Fisher Scientific) anti-fibrin antibodies (59D8; 0.5 mg/kg) through a jugular vein cannula. Data acquisition was done in 2 channels (488/520 nm and 640/670 nm). Images were captured for 220 seconds at 0.5 frames per second using a charge-coupled device camera (Hamamatsu). Data were analyzed using Slidebook 6.0 (Intelligent Imaging Innovations). Image analyses were composed of data sets of 18 to 20 thrombi in 6 mice per experimental group to determine the median value of the integrated fluorescence intensity and the size of the laser-induced vessel injury to account for the variability of thrombus formation at any given set of experimental conditions. Area under the curve (AUC) was calculated for individual thrombi to evaluate statistical significance.

Measurements of Plasma Aldosterone, Plasma Renin Activity, and Salt Sensitivity of BP

Blood was collected in BD Microtainer tubes (dipotassium ethylenediaminetetraacetic acid), and plasma was separated by centrifugation. Animals were euthanized under deep anesthesia. ALDO levels were determined using a solid-phase radioimmunoassay kit (Siemens Diagnostic Products) in duplicate samples, as previously described by us.[12] Plasma renin activity was measured by radioimmunoassay, as previously described

(DiaSorin, Minneapolis, MN).[12] BP was measured in the same restrained, conscious mouse following 1 week of a 1.6% and 0.03% sodium diet using tail-cuff plethysmography following protocols previously described.[12] The difference between these BPs is the salt sensitivity of BP.

Statistical Analysis

Statistical calculations were performed with GraphPad (San Diego, CA) Prism version 6.0. A Shapiro-Wilk test was used for the assessment of normality. A Mann-Whitney test was used when comparing baseline characteristics of WT and *Strn*$^{+/-}$ mice (Table). *P* values of <0.05 were considered statistically significant. Multiple comparison data from laser-induced thrombosis were analyzed by Kruskal-Wallis test followed by Dunn post hoc test. Because fluorescence intensity values derived from the laser-induced injury model are not normally distributed, we have used nonparametric analyses to evaluate fluorescence intensities, and as such, data are represented as the median AUC for individual thrombi. For multicomparison of vessel injury size, 1-way ANOVA with Bonferroni post hoc test was used.

RESULTS

General Characteristic of WT and Strn$^{+/-}$ Mice

As previously reported, *Strn*$^{+/-}$ mice when compared with WT littermate mice on a liberal salt diet had higher circulating ALDO levels and salt sensitivity of BP (Table).[12,13] However, as previously reported, there were no differences by genotype on plasma renin activity levels in these mice.

ALDO Effect on Thrombotic Responses in WT Mice

Acute ALDO administration increased thrombotic responses in WT mice. We observed an enhanced platelet and fibrin accumulation at the site of laser injury (Figure 1A). Both the fluorescence intensity of accumulated platelets and the fluorescence intensity of fibrin deposited at the site of laser injury, calculated as AUC, were significantly enhanced in WT mice injected with ALDO (5.95×10^9 for platelet AUC and 1.43×10^{10} for fibrin AUC) compared with WT mice injected with the vehicle (4.96×10^8 for platelet AUC; $P<0.01$ and 4.05×10^9 for fibrin AUC; $P<0.05$) (Figure 1B through 1E).

Thrombotic Response in WT and Strn$^{+/-}$ Mice

Strn$^{+/-}$ mice developed markedly increased thrombotic responses expressed as enhanced platelet and fibrin accumulation at the site of laser injury (Figure 1A). Both the fluorescence intensity of accumulated platelets and the fluorescence intensity of fibrin deposited at the site of laser injury, calculated as AUC, were significantly increased in *Strn*$^{+/-}$ mice (2.12×10^{10} for platelet AUC and 2.52×10^{10} for fibrin AUC) compared with WT mice (4.96×10^8 for platelet AUC and 4.05×10^9 for fibrin AUC; $P<0.001$) (Figure 1B through 1E).

Effect of ALDO on Thrombotic Response in Strn$^{+/-}$ mice

Acute ALDO administration had no effect on thrombus response in *Strn*$^{+/-}$ mice at the site of laser injury (Figure 1A). Both the fluorescence intensity of accumulated platelets and the fluorescence intensity of fibrin deposited at the site of laser injury, calculated as AUC, was not different in *Strn*$^{+/-}$ mice injected with ALDO (2.15×10^{10} for platelet AUC and 2.25×10^{10} for fibrin AUC) compared with *Strn*$^{+/-}$ mice injected with vehicle alone (2.12×10^{10} for platelet AUC and 2.52×10^{10} for fibrin AUC) (Figure 1B through 1E). However, ALDO thrombotic response was greater in *Strn*$^{+/-}$ mice (2.15×10^{10} for platelet AUC and 2.25×10^{10} for fibrin AUC) compared with WT mice (5.95×10^9 for platelet AUC and 1.43×10^{10} for fibrin AUC; $P<0.01$).

Vessel Injury Size

The size of laser-induced vessel injury was compared between experimental groups (Figure 2) and was not found to differ significantly. Thus, the hemostatic responses in WT and *Strn*$^{+/-}$ mice as well as in ALDO-infused mice did not depend on differences in vessel wall responses.

DISCUSSION

Because STRN deficient mice have chronically modestly increased ALDO levels,[12] we hypothesized that STRN deficiency would be associated with enhanced thrombotic responses. Our results support our hypothesis and confirm previous studies that acute ALDO administration increased thrombotic responses in WT mice.[2,4,5] STRN deficiency per se substantially enhanced thrombotic responses (platelet and fibrin accumulation) compared with controls or ALDO-treated WT mice. Furthermore, administering ALDO to STRN-deficient mice did not increase their thrombotic responses. Our data confirm and extend our previous findings of ALDO and thrombotic responses in rodent vessels. We recently reported that acute ALDO injection enhanced laser- and $FeCl_3$-induced thrombosis in mesenteric vessels of WT mice, as well as increased density of platelet-fibrin clots in rat plasma.[4] Therefore, in 3 rodent models, ALDO administration exerts a rapid prothrombotic effect that is associated with simultaneous activation of

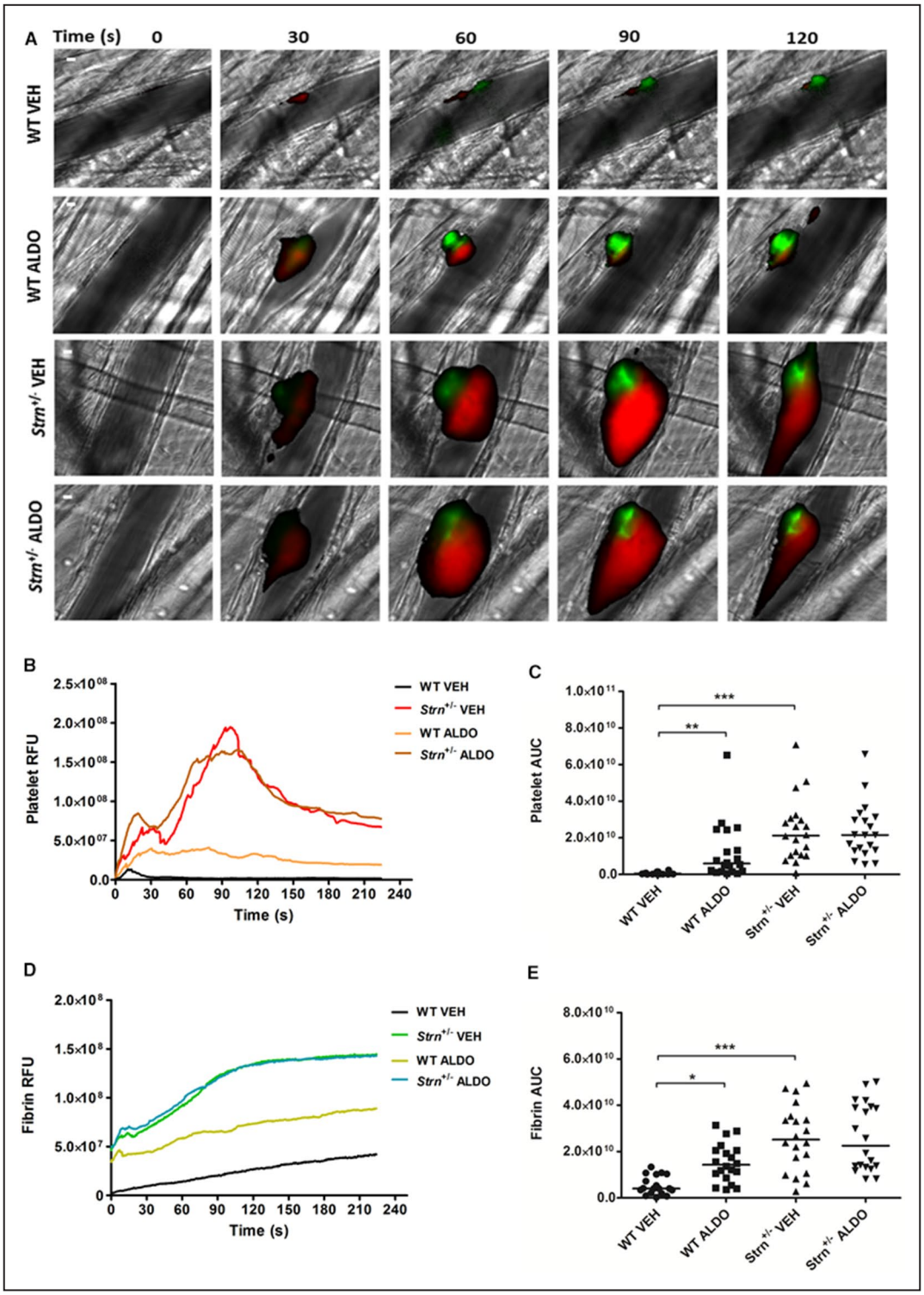

Figure 1. Acute aldosterone (ALDO) administration and striatin deficiency increases thrombotic response. Platelet and fibrin accumulation at the site of laser injury was monitored in wild-type (WT) mice and striatin heterozygous (*Strn*$^{+/-}$) mice administered with ALDO or its vehicle (VEH), using anti-platelet Dylight 649-labeled and anti-fibrin Dylight 488-labeled antibodies. **A**, Representative images of a single thrombus (platelet=red; fibrin=green) at the time of laser injury (0 seconds) and 30 to 120 seconds following laser injury. Median integrated fluorescence intensities (RFU) and area under the curve (AUC) were calculated for platelets (**B** and **C**) and fibrin (**D** and **E**). **C** and **E**, Data are represented as medians and individual values (WT VEH, n=18 thrombi/6 mice per group; WT ALDO, n=20 thrombi/6 mice per group; *Strn*$^{+/-}$ VEH, n=20 thrombi/6 mice per group; *Strn*$^{+/-}$ ALDO, n=20 thrombi/6 mice per group). $^{**}P<0.01$; Kruskal-Wallis test followed by Dunn post hoc test for multiple comparison. Scale bars: 10 μm.

platelets, fibrin generation, and secretion of procoagulant and antifibrinolytic factors. In contrast, STRN deficiency alone has a profound effect on thrombus formation. These results provide evidence for either a novel ALDO-independent role of STRN in regulating the hemostatic system, or the chronic modestly increased ALDO, associated with STRN deficiency, sensitizes the mouse to thrombotic responses.

Our studies suggest that STRN likely plays a protective role in the hemostatic system, because STRN deficiency significantly enhanced thrombosis in our model. STRN deficiency alone produces a 3.5-fold higher platelet accumulation than that seen with acute ALDO administration in WT mice. Furthermore, ALDO had no additional effects on thrombosis in *Strn*$^{+/-}$ mice, suggesting that platelet activation may have been maximal basally. However, as noted above, STRN deficiency is associated with a modest increase in circulating ALDO levels on a liberal salt diet. Potentially, the apparent maximal thrombotic response induced in *Strn*$^{+/-}$ mice was secondary to the chronic modestly increased ALDO levels associated with STRN deficiency. Thus, acutely adding more ALDO did not modify an already sensitized prothrombotic state.

We posit that the mechanism of strong platelet and fibrin responses to laser injury in STRN deficient mice is endothelial dependent. We have reported that *Strn*$^{+/-}$ mice have enhanced vasoconstriction and decreased vascular relaxation as compared with WT littermate mice. The reduced aortic STRN expression was paralleled by reduced aortic expression of eNOS, suggesting a critical role for STRN in regulating vascular function by modulation of the endothelial NO-cGMP pathway.[13] Reduced NO bioavailability may in turn affect platelet responses, because NO is a potent inhibitor of platelet adhesion and aggregation. Thus, reduced NO-cGMP signaling, as observed in *Strn*$^{+/-}$, mice may contribute to increased thrombotic responses in this mouse model. Whether STRN risk allele carriers in humans have NO-cGMP alterations is unknown.

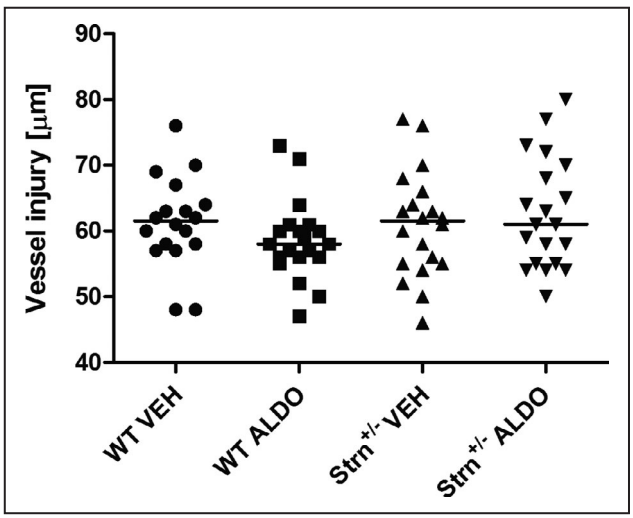

Figure 2. Injury size.
The size of laser-induced vessel damage was measured at the time of laser-induced injury for individual thrombi in wild-type (WT) littermate controls and striatin heterozygous (*Strn*$^{+/-}$) mice administered with aldosterone (ALDO) or its vehicle (VEH). Injury sizes were measured using image analyses with Slidebook 6.0. Data represent medians and individual values (n=18–20 injuries/6 mice per group), 1-way ANOVA with Bonferroni post hoc test.

Plasma ALDO levels correlate with thrombotic markers such as plasma fibrinogen levels in hypertensive subjects.[10] We observed a strong baseline fibrin signal at the beginning of the experiments in *Strn*$^{+/-}$ mice and WT mice infused with ALDO. These results underscore the hypothesis that ALDO stimulates a procoagulant phenotype, as baseline fibrin levels were similar in (1) mice with reduced STRN levels (and increased circulating plasma ALDO levels), (2) in WT mice in response to acutely increased plasma ALDO levels, and (3) in *Strn*$^{+/-}$ mice in response to acutely increased plasma ALDO levels.

We used a laser-induced thrombosis model with intravital imaging capture, which allowed for observation of the first stage of thrombus formation and rapid effects on hemostasis. The laser-induced thrombosis was performed in cremaster arterioles with real time visualization of thrombus formation according to the original method of Falati et al.[14] Thus, only male mice were used. It was shown that a single intensity arteriolar injury spares the endothelium and generates a non-occlusive thrombus.[14] Although, we did not evaluate the precise mechanism of platelet activation, it has been reported that in this model, platelet activation leading to platelet thrombus formation and fibrin generation is endothelium dependent.[15]

We did not evaluate sex-dependent differences in thrombus formation in this study because accumulated peritoneal fat precluded using mesenteric vessels. Thus, based on our novel findings and the results and clinical evidence of substantial sex differences in the rate of thrombotic events (eg, myocardial infarction, stroke, and venous thromboembolism), studies in *Strn*$^{+/-}$ female mice and humans and sex-based comparisons are warranted. Our results provide a rational for such studies.

We showed that STRN deficiency in mice was associated with salt sensitivity of BP with defective aortic vasorelaxation and contraction, decreased eNOS levels, and inadequate ALDO suppression.[12,13] Of clinical relevance, we have documented that polymorphic STRN gene variants in humans are associated with salt sensitivity of BP.[12] Here, we show that in mice, STRN is involved in regulation of hemostasis via 2 pathways,

Table 1. Comparison of Baseline Characteristics of WT and *Strn*$^{+/-}$ Untreated Mice

Characteristic	WT	*Strn*$^{+/-}$
Plasma aldosterone level, ng/dL	74.84 (64.02–98.72), n=9	98.94 (82.02–158.80), n=11*
Plasma renin activity, ng/mL per h	29.31 (23.97–37.30), n=12	30.01 (25.24–41.02), n=13
Systolic salt sensitivity of blood pressure, mm Hg	6.0 (–2.0 to 7.0), n=7	13.0 (7.0–14.0), n=7†

Data represent medians (25th percentile–75th percentile). The Mann-Whitney test was used. n indicates number of mice in each experimental group; *Strn*$^{+/-}$, striatin heterozygous mice; and WT, wild-type mice.
*P=0.028.
†P=0.008.

ALDO increased level and/or STRN deficiency. Also, we have chosen to study *Strn*$^{+/-}$-deficient mice rather than full STRN knockout mice because humans who lack STRN have not been reported. Thus, we posit that STRN plays a protective role in the cardiovascular system for thrombotic responses and that polymorphic STRN gene variants in humans may represent a novel marker for a procoagulant phenotype and increased thrombotic risk.

However, there are limitations to our study. First, we did not perform an ALDO dose-response curve in WT mice, and the dose used was high. However, unlike other steroids, ALDO has little binding to circulating proteins. Thus, much of the acutely administered dose is excreted in the urine with first renal passage. Second, we did not chronically block the MR in the STRN-deficient mice. Finally, these studies were performed in a genetically engineered male mouse model whose phenotype is like human STRN risk allele carriers. However, it is unknown if a similar prothrombotic phenotype exists in these individuals.

In conclusion, ALDO and STRN deficiency enhance thrombotic responses in mice potentially by different mechanisms. However, increased ALDO levels are among the vascular and endocrine mechanisms associated with STRN deficiency. Finally, our results suggest that STRN likely serves a protective role in the hemostatic system. Such a protective effect also may be reduced in humans with STRN risk allele carriers, given the similar SSBP in these individuals and *Strn*$^{+/-}$ mice.

ARTICLE INFORMATION

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Disclosures

None.

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