Adrenergic Autoantibody-Induced Postural Tachycardia Syndrome in Rabbits

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Background—Previous studies have demonstrated that functional autoantibodies to adrenergic receptors may be involved in the pathogenesis of postural tachycardia syndrome. The objective of this study was to examine the impact of these autoantibodies on cardiovascular responses to postural changes and adrenergic orthosteric ligand infusions in immunized rabbits.

Methods and Results—Eight New Zealand white rabbits were coimmunized with peptides from the α1-adrenergic receptor and β1-adrenergic receptor (β1AR). Tilt test and separate adrenergic agonist infusion studies were performed on conscious animals before and after immunization and subsequent treatment with epitope-mimetic peptide inhibitors. At 6 weeks after immunization, there was a greater percent increase in heart rate upon tilting compared with preimmune baseline. No significant difference in blood pressure response to tilting was observed. The heart rate response to infusion of the β1-adrenoceptor agonist isoproterenol was significantly enhanced in immunized animals, suggesting a positive allosteric effect of β1AR antibodies. In contrast, the blood pressure response to infusion of the α1-adrenergic receptor agonist phenylephrine was attenuated in immunized animals, indicating a negative allosteric effect of α1-adrenergic receptor antibodies. Injections of antibody-neutralizing peptides suppressed the postural tachycardia and reversed the altered heart rate and blood pressure responses to orthosteric ligand infusions in immunized animals at 6 and 30 weeks. Antibody production and suppression were confirmed with in vitro bioassays.

Conclusions—The differential allosteric effect of α1-adrenergic receptor and β1AR autoantibodies would lead to a hyperadrenergic state and overstimulation of cardiac β1AR. These data support evidence for an autoimmune basis for postural tachycardia syndrome. (J Am Heart Assoc. 2019;8:e013006. DOI: 10.1161/JAHA.119.013006.)

Key Words: adrenergic receptors • autoimmunity • autonomic nervous system • postural orthostatic tachycardia syndrome • rabbit

Postural tachycardia syndrome (POTS) is a debilitating disorder resulting from cardiovascular autonomic dysfunction, yet its origin remains largely unknown. POTS is clinically characterized by excessive orthostatic tachycardia with symptoms of chronic intolerance to upright posture, including palpitations, lightheadedness, exercise intolerance, fatigue, headache, and cognitive impairment.1–4 POTS is likely a heterogeneous disorder with more than one underlying pathophysiology. A number of mechanisms have been proposed to explain POTS, including hypovolemia, autonomic neuropathy, hyperadrenergic state, norepinephrine transporter deficiency, mast cell activation, and autoimmunity.5–7

The potential role of autoimmunity in POTS has gained increased attention in recent years.8,9 Many patients with POTS report a viral illness or vaccination antedating the onset of their POTS symptoms,10–12 suggesting a possible autoimmune cause for POTS in some patients. Antiganglionic acetylcholine receptor antibodies were found to be variably present in POTS patients.11,13 Our group first reported the presence of activating autoantibodies (AAb) to the α1-adrenergic (α1AR) and β1/2-adrenergic receptors (β1/2AR) in a significant proportion of patients with POTS in 2014.14 Using in vitro bioassays, we demonstrated that α1AR-AAb from patients with POTS directly activated the target receptor but also partially inhibited α1AR responsiveness to its orthosteric ligand phenylephrine. In contrast, β1/2AR-AAb from patients with POTS significantly increased β1/2AR responsiveness to the β-adrenoceptor agonist isoproterenol.14,15 We subsequently reported AAb to
Clinical Perspective

What Is New?

• This study demonstrates for the first time a role of adrenergic autoimmunity in the pathophysiology of postural tachycardia syndrome in 2 related in vivo models.
• The effects of adrenergic autoantibodies are largely reversed using selective decoy peptide inhibitors for the autoantibodies.

What Are the Clinical Implications?

• These data support the concept that autoantibody-mediated changes in cardiovascular responses to catecholamines contribute to the pathogenesis of postural tachycardia syndrome.
• Pharmacologic suppression of adrenergic autoantibodies may have a therapeutic benefit in postural tachycardia syndrome.

The angiotensin II type 1 receptor were also present in patients with POTS. Such agonistic autoantibodies have been documented in various cardiovascular diseases, including cardiomyopathy, myocarditis, cardiac arrhythmias, and hypertension. However, the mechanisms by which they are operative are still not clear. These autoantibodies primarily target the second extracellular loop (ECL2) of their respective receptors to mediate receptor activation. For α1AR-AAb, additional epitopes on the first extracellular loop (ECL1) of α1AR have also been identified.

In the present study, we tested the hypothesis that the opposing allosteric effects of α1AR-AAb and β1AR-AAb on their specific receptors and their natural ligands may be involved in the pathophysiology of POTS in a rabbit model of induced autoimmune POTS. One of the Witebsky postulates for autoimmune diseases is reproduction of the essential features of the disease by production of autoantibodies in an animal model. This was the primary objective of this study. Additionally, we investigated the therapeutic potential of our recently designed retro-inverso (RI) peptidomimetic inhibitors that specifically target the α1AR-AAb and β1AR-AAb to block their interaction with the receptors. RI peptides, in which L-amino acids are substituted by D-amino acids in a reversed sequence, mimic the structure and antigenicity of the parent L-peptide but are resistant to protease degradation. Here, we demonstrate that the RI peptides can effectively block the effects of α1AR-AAb and β1AR-AAb both in vitro and in vivo.

Methods

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

This study protocol was approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center and conforms to international standards for animal safety and comfort.

Animal Immunization and Peptide Inhibitor Treatment

Freund’s adjuvant emulsified with antigenic peptides was used to induce α1AR-AAb and β1AR-AAb production in the rabbit. Eight male New Zealand white rabbits (2.5–3 kg; Charles River) were coimmunized with 1 mg each of α1AR ECL1 (LGYWAFGRVFCN) and ECL2 (PAPEDETICQINEE) multiple antigenic peptides and β1AR ECL2 peptide (HWWRADEARRCYNPDPCDFTVNR) in complete Freund’s adjuvant. The animals were boosted with 1 mg each of the same peptides in the less toxic incomplete Freund’s adjuvant at 2 and 4 weeks. At 6 weeks, the rabbits were treated with an intravenous bolus injection (1 mg/kg) of a combination of antibody-neutralizing, proteolytically stable RI peptides D-TEDEPA and D-DCCKPDNYCR, which mimic the functional epitopes YWAFGR from the α1AR ECL1, APEDET from the α1AR ECL2, and RCYNPDPCD from the β1AR ECL2 targeted by α1AR-AAb20 and β1AR-AAb, respectively. A monthly boost followed by RI peptide injection from weeks 6 to 30 were given to examine the long-term effect of decoy peptides on antibody inhibition. Preimmune and postimmune sera were collected from all animals for antibody detection.

Tilt Testing and Adrenergic Agonist Infusion Studies

The in vivo effects of α1AR-AAb and β1AR-AAb on cardiovascular responses to posture and adrenergic agonist infusions were examined over a 30-week period. The study protocol is shown in Figure 1. These studies were performed on conscious rabbits using a custom-made device with a rabbit restrainer secured to a tilt platform. The rabbit was placed in the restrainer and acclimatized to postural changes at least 3 times each, followed with treats during the week before the studies started. On the experimental day, the central artery of the rabbit ear was cannulated under lidocaine gel-induced topical anesthesia and the catheter connected to a pressure transducer and fixed in this position. Physiological studies included a 2-lead ECG for heart rate analysis and intraarterial blood pressure. The arterial blood pressure transducer was recalibrated before each study. The impact of tilting to 30 and 60 degrees was examined at 2-minute intervals. The heart rate and blood pressure were continuously recorded during the whole procedure using a PowerLab data acquisition system (AD Instruments, Colorado Springs, CO). The tilting test was followed by phenylephrine and isoproterenol infusion.
studies to examine the allosteric effects of α1AR and β1AR antibodies on their orthosteric ligand-induced cardiovascular responses. After 30 minutes of rest, increasing doses of phenylephrine (2.5, 5, 10, and 5 μg/kg) were injected intravenously into the rabbit placed in a ventral recumbent position at 2-minute intervals using an infusion pump. After another 30 minutes of rest, the rabbit received graded isoproterenol infusions (0.05, 0.25, 0.5, and 1 μg/kg) in a similar fashion. We recorded the blood pressure and heart rate responses at each dose of phenylephrine and isoproterenol after stabilization. The tilting and infusion studies were performed at preimmunization, 6 weeks postimmunization (before and 90 minutes after injection of the RI peptides), and 30 weeks postimmunization (Figure 1). Each rabbit served as its own control.

**Cell-Based α1AR and β1AR Assays**

Rabbit serum-induced activation of α1AR and β1AR was assessed in α1AR-NFAT-bla CHO-K1 and β1AR-NFAT-bla CHO-K1 cells, respectively, using the GeneBLAzer FRET-based β-lactamase reporter assay (Invitrogen; Thermo Fisher Scientific, Waltham, MA) as described. Briefly, cells were plated in 384-well plates and incubated overnight. Rabbit preimmune or postimmune sera (1:50) in the presence and absence of the α1AR blocker phentolamine (10 μmol/L) or β-adrenoceptor blocker propranolol (1 μmol/L) were then added and incubated for 5 hours. Antibody neutralization was performed by incubating rabbit immune sera with an excess of the RI peptides. The β-lactamase substrate CCF4-AM (LiveBLAzer-FRET B/G Loading Kit, Invitrogen) was then added and incubated for 2 hours. The plates were read using a fluorescence microplate reader (Hidex Sense multifunctional microplate reader; LKB Instruments, Mount Waverly, Victoria, Australia). All samples were assayed in triplicate. Negative (buffer) and positive (phenylephrine or isoproterenol) controls were included in each assay. Data were calculated as the ratio of the emissions 460/530 nm (blue/green) after subtraction of the background values and expressed as fold increase over buffer baseline to normalize the individual values.

Dosage response curves for phenylephrine (10⁻⁸–10⁻⁵ mol/L) and isoproterenol (10⁻⁸–10⁻⁵ mol/L) in the absence and presence of rabbit immune sera (1:50) were constructed to examine the allosteric effects of rabbit autoantibodies on phenylephrine/isoproterenol-induced α1AR/β1AR responses in vitro.

**Statistical Analyses**

Data are expressed as mean±SEM. Comparison between 2 groups and multiple group comparisons were performed using the nonparametric Mann–Whitney test and Kruskal–Wallis test followed by Dunn’s multiple comparison test, respectively. Statistical significance was set at \( P < 0.05 \).

**Results**

**Posture and Infusion Studies**

The blood pressure and heart rate responses to tilting (Figure 2) and infusions (Figure 3) were measured before immunization, 6 weeks after immunization before and after acute RI peptide treatment, and 30 weeks after immunization with chronic RI peptide treatment. At 6 weeks after immunization, there was a greater percent increase in heart rate upon tilting to 30 degrees compared with preimmune baseline (27.8±3.3% versus 18.9±1.5%; \( P < 0.05 \)) (Figure 2A). This enhanced heart rate increase was effectively suppressed by both acute (27.8±3.3% versus 17.2±2.4%; \( P < 0.05 \)) and chronic (27.8±3.3% versus 19.0±1.8%; \( P < 0.05 \)) treatment with the antibody-neutralizing RI peptides. No significant difference in blood pressure response to 30-degree tilting was observed between preimmune and postimmune states before and after RI peptide treatment (Figure 2B). The blood pressure and heart rate responses to 60-degree tilting were similar to those to 30-degree tilting (Figure 2A and 2B).
To determine whether $\alpha$1AR-AAb and $\beta$1AR-AAb would alter the cardiovascular responses to $\alpha$1AR and $\beta$1AR orthosteric ligand infusions, we injected increasing doses of phenylephrine (2.5, 5, 10, and 15 $\mu$g/kg) and isoproterenol (0.05, 0.25, 0.5, and 1 $\mu$g/kg) separately into rabbits before and after immunization and compared their blood pressure and heart rate values. At 6 weeks after immunization, the blood pressure response to phenylephrine infusion was significantly attenuated, indicating a negative allosteric effect of $\alpha$1AR-AAb (Figure 3A). In contrast, the heart rate response to isoproterenol infusion was significantly increased after immunization, suggesting a facilitatory allosteric effect of $\beta$1-adrenergic antibodies (B). The impact of antibodies on PE and ISO responses was suppressed by acute as well as chronic treatment with the retro-inverso peptides (RIP). $^*P<0.05$ vs preimmune, $n=5$.

Figure 3. Blood pressure and heart rate (HR) responses to adrenergic agonist infusions in the rabbit. There was a significant decrease in systolic blood pressure (SBP) response to phenylephrine (PE) infusions at 6 weeks after immunization compared with preimmune baseline, indicating an inhibitory allosteric effect of $\alpha$1-adrenergic antibodies (A). In contrast, HR response to isoproterenol (ISO) infusions was significantly increased after immunization, supporting a facilitatory allosteric effect of $\beta$1-adrenergic antibodies (B). The impact of antibodies on PE and ISO responses was suppressed by acute as well as chronic treatment with the retro-inverso peptides (RIP). $^*P<0.05$ vs preimmune, $n=5$.

To confirm production of $\alpha$1AR-AAb and $\beta$1AR-AAb after immunization and examine the effect of RI peptide injections
on suppressing α1AR-AAb and β1AR-AAb, sera collected before immunization, 6 weeks after immunization before and 90 minutes after RI peptide injection, and 30 weeks after immunization after continued boost and RI peptide treatment were measured for α1AR-AAb and β1AR-AAb activity using cell-based in vitro bioassays (Figure 4). α1AR-AAb activity was evident in the serum at 6 weeks after immunization (postimmune 6 weeks: 2.8±±0.1 versus preimmune: 2.4±±0.1-fold increase over baseline; P<0.05) (Figure 4A). A single bolus injection of the RI peptides at 6 weeks suppressed the elevated α1AR-AAb activity (postimmune 6 weeks: 2.8±±0.1 versus postimmune+RI peptides 6 weeks: 2.2±±0.1-fold increase over baseline; P<0.05). α1AR-AAb suppression was also found in the serum at 30 weeks after repeated boost and RI peptide injections (postimmune 6 weeks: 2.8±±0.1 versus postimmune+RI peptides 30 weeks: 2.1±±0.1-fold increase over baseline; P<0.01). A similar production and suppression was observed for serum β1AR-AAb activity (postimmune 6 weeks: 9.4±±1.0 versus preimmune: 4.5±±1.1-fold increase over baseline; P<0.05; versus postimmune+RI peptides 6 weeks: 4.3±±1.2-fold increase over baseline; P<0.05; versus postimmune+RI peptides 30 weeks: 5.7±±0.9-fold increase over baseline; P<0.05) (Figure 4B). These results demonstrated that antibodies capable of activating α1AR and β1AR were successfully induced at 6 weeks after immunization, which were effectively blocked by acute RI peptide treatment. Chronic treatment with RI peptides following continued boost were also able to suppress antibody activity.

To further verify antibody specificity, we examined whether antibody-induced activation of α1AR and β1AR could be inhibited by receptor antagonists and by preabsorption of the antibodies with the RI peptides in the in vitro assays. Rabbit immune sera (1:50) collected at 6 weeks were tested (Figure 5). As shown in Figure 5A, sera-induced α1AR activation was effectively blocked by the α1AR blocker phentolamine (10 μmol/L) and by preincubation with the α1AR ECL1 and ECL2 RI peptides. Sera-induced β1AR activation was similarly abolished by the β-adrenergic receptor blocker propranolol (1 μmol/L) and by preincubation with the β1AR ECL2 RI peptide (Figure 5B). These results confirmed the receptor-specific activity of α1AR-AAb and β1AR-AAb produced in the rabbit and the effectiveness of the RI peptides on suppressing antibody activity in vitro.

**Effects of α1AR-AAb/β1AR-AAb on Phenytoin/Isoproterenol Dose Responses In Vitro**

To examine the allosteric effects of α1AR-AAb and β1AR-AAb on orthosteric ligand activation of α1AR and β1AR in cell-based bioassays, dosage response curves for phenylephrine (10⁻⁸–10⁻⁵ mol/L) and isoproterenol (10⁻⁸–10⁻⁵ mol/L) with and without immune sera (1:50) were constructed and compared (Figure 6). Rabbit immune sera collected at 6 weeks were used for these studies. In the presence of immune sera, the phenylephrine dose response was dampened (Figure 6A). When immune sera were added along with phenylephrine, there was a significant decrease in α1AR activation over phenylephrine alone at the 1 and 10 μmol/L concentrations (7.3±±0.3 versus 7.9±±0.4-fold increase over baseline, P<0.05; 7.2±±0.2 versus 8.2±±0.3-fold increase over baseline, P<0.05, respectively). Preincubation with the α1AR ECL1 and ECL2 RI peptides reversed the serum α1AR-AAb-mediated inhibitory
effect. In contrast, the immune sera significantly enhanced the isoproterenol dose response (Figure 6B). At doses of 1 and 10 μmol/L, isoproterenol alone with immune sera caused a greater increase in β1AR activation compared with isoproterenol alone (13.1±0.4 versus 11.5±0.3-fold increase over baseline, P<0.05; 13.1±0.3 versus 11.6±0.3-fold increase over baseline, P<0.05, respectively). Preincubation with the β1AR ECL2 RI peptide was also able to reverse the serum β1AR-AAb-mediated facilitatory effect.

Discussion

We have previously reported a significant number of patients with POTS harbor circulating autoantibodies that can directly stimulate α1AR and β1/2AR. These autoantibodies also exerted a positive allosteric effect on β1/2AR and a negative allosteric effect on α1AR activity in vitro.14,15 We hypothesized that these contrasting allosteric effects of α1AR-AAb and of β1/2AR-AAb might be involved in the pathophysiology of POTS. Although these autoantibodies have demonstrated in vitro activity, it is important to establish their relevance in vivo in animal models.

In the present study, we examined whether α1AR-AAb and β1AR-AAb in combination could reproduce the characteristic cardiovascular effects observed in POTS in 2 related rabbit models. The conscious rabbit model has been used commonly to study the cardiovascular responses during postural changes.27–31 Although quadruped animals do not spend most of their time in upright posture as humans do, rabbits have the ability to maintain arterial pressure against gravity-induced blood pooling, and the arterial baroreflex also plays an important role in blood pressure maintenance under orthostatic stress,32 thus providing reasonable models for studying orthostatic physiology. Moreover, we examined the pressor dose response to a relatively selective α1AR agonist phenylephrine as well as the nonselective β-adrenoceptor agonist isoproterenol in recumbent animals whereby this model of cardiovascular responsiveness is applicable to both animals and humans. We previously have used rabbit autoimmune models successfully to study the in vivo impact of sympathomimetic β1/2AR-AAb in cardiac arrhythmogene-
sis.33–35 In these prior studies, all animals developed high levels of antibodies with agonistic activity after immunization with the β1/2AR ECL2 peptides.

In the tilting studies, immunized animals demonstrated a greater postural increase in heart rate without a significant drop in blood pressure compared with their preimmune baseline values. The effect of α1AR-AAb as allosteric attenuators and β1AR-AAb as allosteric enhancers was evident by the reduced pressor response to phenylephrine and increased chronotropic response to isoproterenol in immunized animals in the infusion studies. The direct stimulatory and indirect modulatory effects of α1AR-AAb and β1AR-AAb from immunized rabbits were also documented in the in vitro assays. Rabbit immune sera were able to induce activation of both α1AR and β1AR, and were specifically blocked by their receptor antagonists. Allosterically, rabbit immune sera facilitated isoproterenol-stimulated β1AR activation and

![Figure 5. Specific activity of antibodies produced in the rabbit. The immune serum collected at 6 weeks was tested. In cell-based bioassays, rabbit immune serum-induced α1-adrenergic receptor (α1AR) activation was completely blocked by the α1AR blocker phentolamine and by preincubation with the α1AR retro-inverso peptides (RIP) (A). Serum-induced β1-adrenergic receptor (β1AR) activation was similarly inhibited by the β-adrenoceptor blocker propranolol and by preincubation with the β1AR RIP (B). *P<0.05, α1AR activity: n=8; β1AR activity: n=5. Data are expressed as fold increase over buffer baseline.](https://doi.org/10.1161/JAHA.119.013006)
attenuated phenylephrine-induced α1AR activation, which was consistent with the in vivo findings. From a pathological viewpoint, an α1AR-AAb-mediated impaired pressor response to α1AR endogenous ligand norepinephrine would be expected to compensatorily increase the sympathetic output to normalize vasoconstriction and blood pressure. The relatively unprotected cardiac chronotropic β1AR would respond to this increased adrenergic activity with an enhanced tachycardia, which would be exaggerated by the β1AR-AAb, and might explain the clinical characteristics of POTS. There are few comparable studies in humans with POTS. Jacob et al. reported administration of the α1AR agonist midodrine to a group of POTS subjects characterized as “hyperadrenergic” with elevated plasma catecholamines. The acute administration produced some improvement in the heart rate, but there appeared to be a blunted blood pressure response to the agonist. They did not have control subject responsivity for comparison. Miller and Streeten reported a variable but mostly “normal” venous contractility response to infusion of norepinephrine in the hand vessels of a group characterized as having sympathetic POTS. The difficulty with this preparation is the absence of arteriolar measurements and the mixed impact of norepinephrine on both β1/2AR and α1-3AR activation in the veins and systemic vasculature. The heart rate response to infusion of the β-adrenoceptor agonist isoproterenol has been shown to be significantly greater in patients with POTS than in age-matched control subjects.38

The importance of the autoantibodies to the pathophysiology of POTS will ultimately depend on our ability to remove or inactivate the specific antibodies from patients with POTS, and to determine if it will reverse or improve their clinical conditions. We have developed and validated the use of proteolytically resistant, epitope-mimicking RI peptides that specifically target the β1AR-AAb and angiotensin receptor-activating autoantibodies. These peptidomimetic inhibitors effectively suppressed autoantibody-mediated cardiac tachyarrhythmias and hypertension in immunized rabbits.25,39 Small peptide inhibitors as therapeutic agents have several advantages over other small molecules, including high specificity and low levels of toxicity and immunogenicity.23 They also have the potential to induce antigen-specific immune tolerance leading to suppression of autoantibody production.40 Orthosteric receptor antagonists such as β-blockers produce variable outcomes, as they also block the normal ligand and thereby impair the normal physiological responses. In this study, we tested similarly designed RI peptides that specifically target α1AR-AAb and β1AR-AAb. These peptides demonstrated effective antibody-blocking effect both in vivo and in vitro. They suppressed the postural tachycardia, and reversed the impaired blood pressure response and heightened chronotropic response to orthosteric ligand infusions in immunized animals. Immune sera-stimulated activation of α1AR and β1AR as well as their differing allosteric effects were similarly blocked in cultured cells. Antibody suppression and prevention of POTS-like phenotype were also observed at the end of the 30-week study with chronic RI peptide treatment.

**Figure 6.** Effects of rabbit immune sera on orthosteric ligand dose responses in cell-based bioassays. The immune serum collected at 6 weeks was tested. In the presence of immune sera, phenylephrine (PE)-induced α1-adrenergic receptor (α1AR) activation was significantly reduced at the 1 and 10 μmol/L concentrations, which was completely reversed by preincubation with the α1AR retro-inverso peptides (RIP) (A). In contrast, the immune sera significantly increased isoproterenol (ISO)-induced β1-adrenergic receptor (β1AR) activation at the 1 and 10 μmol/L concentrations, which was also blocked by preincubation with the β1AR RIP (B). *P<0.05 vs PE alone, n=8; *P<0.05 vs ISO alone, n=5. Data are expressed as fold increase over buffer baseline.
There are currently no animal models that exhibit the characteristic postural and cardiovascular manifestations of POTS seen in humans. Our model, as the first animal model linking adrenergic autoantibodies to development of POTS, provides a valuable tool for in vivo studies of autoimmune pathophysiology of POTS as well as therapeutic interventions. Antibody-neutralizing, proteolytically stable RI peptides serve as a decoy and prevent the autoantibodies from binding to and activating the membrane receptors. This would potentially lead to the body developing tolerance to the epitopes and suppression of autoantibody production. These peptides, therefore, have the potential for a personalized medicine approach to the treatment of POTS based on the patient's autoantibody profile.

This study supports the concept that cardiovascular autoantibodies play an important role in POTS pathophysiology. Pharmacologic blockade of pathological autoantibodies with peptide inhibitors without altering the underlying autonomic control system may open a new avenue for the prophylaxis and/or treatment for patients who harbor these autoantibodies.

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Disclosures
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