Inducible cardiac arrhythmias caused by enhanced β₁-adrenergic autoantibody expression in the rabbit

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METHODS

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

Catheter electrophysiological study. Eight young New Zealand white rabbits (3- to 4 mo old) were anesthetized with ketamine-xylazine (35 mg/5 mg/kg) and subjected to a catheter-based electrophysiological study. Standard electrocardiograms (Leads 1-aVF) were continuously monitored. After the neck area was shaved and application of betadine antiseptic, the right jugular vein was dissected and cannulated with a 4 Fr multi-electrode catheter. Under electrographic control, the catheter was passed into the right atrium to record atrial potentials in conjunction with the standard 6-lead ECG. Atrial tachyarrhythmia susceptibility was tested by bursts of stimuli (3–5 s duration) at a high frequency (20 Hz) and voltages that were at least twice the diastolic pacing threshold before and after the infusion of ACh in three incremental concentrations (10 μM, 100 μM, and 1 mM) at a rate of 1 ml/min. These were applied to a site in the right atrium at which a discrete atrial potential with little or no far-field ventricular potentials was recorded. Nonsustained (<10 s) and sustained (≥10 s) arrhythmia occurrence was determined in response to burst pacing at 2× diastolic threshold, at baseline, and then with each of the three concentrations of ACh infusion for 2 min before initiating burst pacing. The number of burst pacing ranged from 3 to 10 (median = 6). It should be noted that the number of burst pacing in the preimmune state was most likely to be closer to 10 because it was more difficult to induce any nonsustained or sustained arrhythmia with or without ACh infusions. On the other hand, after immunization, particularly with ACh infusion two to three burst pacing events readily induced either nonsustained or sustained arrhythmias. Figure 1 shows
The flow chart depicting the protocols for these studies before and after each rabbit was immunized to produce \( \beta_1 \)AR activating autoantibodies. When this study was completed, the wound was closed and antibiotic treatment was instituted. A second electrophysiological study was performed after the 6-wk immunization interval.

**Definition of arrhythmias in the rabbit heart.** Nonsustained arrhythmia is any arrhythmia lasting <10 s. Sustained arrhythmia is any arrhythmia lasting \( \geq \)10 s. The 10-s cutoff was determined empirically since the majority of arrhythmias induced in the preimmune state did not exceed 10 s, whereas the majority of induced arrhythmias after immunization invariably exceeded 10 s, many lasting up to several minutes.

Sinus tachycardia is a regular, rapid heart rate \( \geq \)250 beats/min showing a constant A-A interval with 2:1 AV conduction with an earliest atrial electrogram occurring at least 10 ms before the onset of the P wave (Figs. 2 and 3).

Junctional tachycardia is a regular, rapid heart rate \( \geq \)200 beats/min showing 1:1 short AV interval arising from the AV junction with an altered sequence of activation and morphology of atrial electrograms compared with sinus rhythm (Fig. 4).

Atrial tachycardia is a regular, rapid heart rate \( \geq \)250 beats/min showing a constant A-A interval with 2:1 AV conduction with different P wave and electrogram morphologies compared with sinus rhythm or junctional tachycardia (Fig. 5).

Atrial fibrillation is rapid, irregular, fractionated atrial electrograms with a rapid but irregular ventricular response.

Ventricular tachycardia is three or more beats arising from the ventricles at a rate \( \geq \)250 beats/min.

**Immunization.** The eight rabbits were immunized with 1 mg of the highly conserved second extracellular loop (ECL2) peptide for \( \beta_1 \)AR (HWVRRAE5DEARRCNYPKKCDFFVTNR) in 0.5 ml of complete Freund’s adjuvant. The animals were boosted with the same peptide plus incomplete Freund’s adjuvant (1 mg/0.5 ml) at 2 and 4 wk. Pre- and postimmune sera were obtained from all animals for ELISA and activity assays of the expected antibodies generated during immunization.

**ELISA.** Antibodies produced in the sera were detected by ELISA. Briefly, microtiter plates were coated with \( \beta_1 \)AR ECL2 or \( \beta_2 \)AR ECL2 (for cross-reactivity testing) (16) peptide at 10 \( \mu \)g/ml in coating buffer. To determine antibody titer, sera were diluted 1:10,000 in 1% BSA in PBS and there after serially diluted twofold. Goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma, St. Louis, MO) and its substrate para-nitrophenyl-phosphate 104 were used to detect antibody binding. Titers were determined as the highest dilution with an optical density (OD) value of 0.10 at 60 min.

**Immunofluorescence microscopy.** Chinese hamster ovary (CHO) cells expressing human \( \beta_1 \)AR were cultured on glass cover slips in 6-well plates for 24 h. The cells were fixed with 4% paraformaldehyde, blocked with 5% normal goat sera, and incubated with preimmune or postimmune rabbit anti-\( \beta_1 \)AR sera (1:100) for 1 h, followed by incubation with Alexa Fluor 594-labeled goat anti-rabbit IgG (Invitrogen, Grand Island, NY). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Fluorescence images were obtained using a fluorescence microscope (Olympus).

**cAMP assay.** Rabbit sera were tested for activation of \( \beta_1 \)AR using the cAMP Hunter eXpress GPCR Assay kit (DiscoveRx, Fremont, CA). Briefly, 30,000 CHO cells expressing human \( \beta_1 \)AR were dispensed into each well of 96-well culture plate and incubated overnight. The medium was removed and assay buffer containing the cAMP antibody and rabbit sera (1:100) in the presence and absence of \( \beta_1 \)AR blocker propranolol (1 \( \mu \)M) were sequentially added and incubated for 30 min. Preincubation of sera with a 10-fold excess of \( \beta_1 \)AR ECL2 peptide was also tested for neutralization studies. cAMP standard, negative (buffer), and positive (isoproterenol 100 nM) controls were included in each assay. Samples were tested in triplicate. After sample treatment, CAMP detection reagent and solution were added, and luminescent signal was read on a TD-20/20 Luminometer (Turner BioSystems). The CAMP values are expressed as percentage of buffer baseline to normalize the individual data.

**Statistical analysis.** Data are presented as means ± SD. Differences in baseline heart rates, those induced by ACh infusions, and cAMP production were assessed by a paired Student’s t-test or repeated-measures ANOVA as appropriate. Repeated-measures logistic regression was used to assess differences in occurrence of a sustained arrhythmia for each rabbit at baseline and at all concentrations of ACh infusion before and after immunization. All of these analyses were performed using the generalized estimating equations procedure implemented in GENLIN in SPSS (IBM SPSS Statistics v. 20.0; IBM, Armonk, NY). A P value of <0.05 was considered statistically significant.

**RESULTS**

**Electrophysiological studies.** Table 1 summarizes the results of the electrophysiological studies performed before and after immunization with the \( \beta_1 \)AR ECL2 peptide to induce production of \( \beta_1 \)AR-activating antibodies. The antibody titers ranged from 1:320,000 to 1:1.28 million in the postimmune studies and were undetectable in the preimmune studies. Using each rabbit as its own control, we found that the baseline heart rate after immunization was significantly increased (preimmune: 149 ± 17 vs. postimmune: 169 ± 16 beats/min; \( P < 0.05 \)). Arrhythmias were induced by burst pacing at baseline and at each infused concentration of ACh. The rates of the induced sustained arrhythmias varied over a wide range from 250 to 385 per min. If no arrhythmia could be elicited, no response (NR) was registered. At each incremental concentration of ACh, there was a progressive increase in heart rate, each of which was significantly greater than the baseline value. The importance of these findings will be discussed below as the basis for the use of ACh as an adjunct to the provocative effect of burst pacing to induce arrhythmias in the presence of \( \beta_1 \)AR-activating antibodies.
In the preimmune studies, 15 episodes of nonsustained supraventricular tachycardia (SVT) and 1 episode of nonsustained ventricular tachycardia (VT) were induced by burst pacing during the baseline state and three incremental concentrations of infused ACh. There were five episodes of sustained SVT, including two sinus tachycardia (ST), one atrial tachycardia (AT), one junctional tachycardia (JT), and one atrial fibrillation (AF). In contrast, in the postimmune studies, there were four episodes of nonsustained tachyarrhythmias, but there were 22 burst pacing inductions of sustained tachyarrhythmias, including 15 ST, 2 JT, 1 AT, 1 AF, and 3 VT. From a pathophysiological standpoint, we compared the induction of various sustained cardiac arrhythmias between the preimmune and postimmune state for each rabbit with each rabbit serving as its own control. After the effect of dose was accounted for, the proportion of burst pacing (8 rabbits, 4 dosages) showing sustained arrhythmias before immunization was 5/32 (16%), whereas after immunization the proportion of burst pacing showing sustained arrhythmias was 22/32 (69%) ($P < 0.0001$ for the independent effect of immunization). If the results are collapsed across dose and whether a rabbit showed sustained arrhythmias at any dosage is considered then the proportion of rabbits showing sustained arrhythmias before immunization was 4/8 (50%), whereas after immunization the proportion of rabbits showing sustained arrhythmias was 8/8 (100%) ($P < 0.05$ for the effect of immunization). Similarly, we found 15 sustained ST were induced in the 32 events after immunization compared with two such sustained arrhythmias induced in the preimmune state ($P < 0.01$ for the independent effect of immunization; $P < 0.05$ after collapsing across dose: 6/8 after immunization vs. 1/8 before immunization).

Figure 2 shows, as an inset, the location of the multi-electrode catheter in the right atrium and the sequence of bipolar electrograms from the superior vena cava (SVC) just above the right atrial entrance, the high right atrium, the mid-right atrium, and the area of the AV junction during sinus rhythm at a rate of 197 beats/min (bpm). ECG leads I through aVF are also shown. Figure 3 shows atrial burst pacing induced ventricular premature contractions (VPC) followed by a ST at a rate of 263 beats/min. Note the same sequence of activation as seen in the electrograms during sinus rhythm (Fig. 2). In Fig. 4, atrial burst pacing induced a JT at an initial rate of 448 beats/min. The sequence of atrial activation starts at the area of the AV junction and proceeds toward the high right atrium. Note that the atrial electrogram at the SVC is coincident with the ventricular activation until the end of the trace, when the junctional rate increased to 462 beats/min. The SVC electrogram appears before the ventricular activation. In Fig. 5, atrial burst pacing induced an AT at a rate of 509 beats/min with a constant A-A interval (118 ms) and 2:1 AV block.

**β1AR antibody binding and activity.** All eight rabbits developed high antibody titers to β1AR ranging from 1:320,000 to 1:640,000 with a geometric mean antibody titer of 1:1.28 million ($P < 0.0001$ after collapsing across dose: 6/8 after immunization vs. 1/8 before immunization).

Table 1. *Rabbit response to acetylcholine and burst pacing: preimmune and postimmune studies*

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**β1AR, β1-adrenergic receptor; AF, atrial fibrillation; AFL, atrial flutter; AT, atrial tachycardia; HR, heart rate; JT, junctional tachycardia; NR, no response; NS, nonsustained; ST, sinus tachycardia; sus, sustained; VPC, ventricular premature contraction; VT, ventricular tachycardia.**
To analyze antibody binding to β1AR, immunofluorescence was performed with CHO cells expressing human β1AR. A representative stain is shown in Fig. 6. Rabbit antisera strongly reacted with β1AR in CHO cells, whereas preimmune sera did not show any significant reactivity. Preincubation of rabbit antisera with an excess of the β1AR ECL2 peptide diminished fluorescence signal in CHO cells (data not shown), confirming the specific reactivity to β1AR.

Rabbit antisera were able to stimulate cAMP production in β1AR-transfected CHO cells in vitro (Fig. 7A). Sera-induced β1AR activation was abolished by the nonselective βAR blocker propranolol and by preincubation of the sera with the β1AR ECL2 peptide. No significant increase in cAMP production was found with the preimmune sera compared with buffer baseline.

To check the specificity of rabbit antisera, rabbit anti-β1AR sera were examined for cross-reactivity with β2AR by ELISA and cAMP assay. As shown in Fig. 7B and C, the anti-β1AR sera reacted specifically with the β1AR, and not the β2AR ECL2 peptide in ELISA. The anti-β1AR sera also stimulated significant cAMP production in β1AR-transfected CHO cells, whereas no significant cAMP stimulation was observed in β2AR-transfected CHO cells.

DISCUSSION

We have used ACh and burst atrial pacing for induction of arrhythmias in conjunction with β1AR-activating antibodies. The rationale for this combination is based on the observation, as shown in Table 1, that infusion of incremental concentrations of ACh consistently increases heart rate. ACh is known to have a direct action on the sinus node to slow the heart rate and also cause vasodilation which would, by a baroreflex action, increase sinus rate. In fact, within the first minute of the ACh infusion, there was a consistent increase in the heart rate compared with baseline levels, and this correlated with the concentration of ACh that was infused in the preimmune state. This pattern was qualitatively similar after immunization, which induced β1AR-activating autoantibodies. However, the baseline and ACh values for heart rate were significantly higher in response to β1AR-activating autoantibodies using each animal as its own control. We waited for 2 min when heart rate returned toward baseline values before instituting burst pacing under both circumstances. In a recent series of experiments in which we monitored both heart rate and blood pressure (unpublished data), we did indeed find the inverse baroreflex relationship so that the increase in heart rate was directly associated with a decrease in systolic and diastolic blood pressure. However, the increase in sympathetic activity based on the baroreflex effect could not account for the qualitative and quantitative differences, i.e., preponderance of induced sustained sinus tachycardia, in response to ACh and burst pacing seen in the postimmune state when each animal in the preimmune state showed no comparable arrhythmogenic effect due to the same protocol. Also, it should be noted that ACh released at presynaptic junctions can activate sympathetic neurons resulting in sinus rate acceleration. The significant increase in heart rate at all concentrations of infused ACh resulted in a positive chronotropic effect, which could also contribute to the initial sympathetic acceleration. There was a significant difference in the increased sinus rate with each concentration of infused ACh between the preimmune and postimmune state due to the direct agonistic action of the induced β1AR-activating autoantibodies. In regard to burst pacing, this standard provocative intervention was unable to
induce a substantial number of sustained arrhythmias in the preimmune state supposedly due to the tissue levels of catecholamines not being high enough even with the increase due to ACh infusions. On the other hand, with the combination of high titers of β1AR-activating antibodies and increased secondary sympathetic action of ACh infusions, burst pacing now induced significant numbers of sustained supraventricular arrhythmias particularly arising from the sinus node. It is interesting to note that some of these sustained STs terminated spontaneously (n = 3) or were terminated by burst pacing (n = 5). Others were not terminated by several attempts with burst pacing (n = 7). There were no noticeable changes in the ECG parameters such as QRS or QT interval. Some of the rabbits did show changes in T wave amplitudes associated with increased sympathetic activity after immunization due to ACh infusions or to β1AR antibody induction.

In a previous study we induced sustained atrial tachycardia in rabbits expressing high titers of β2AR-activating autoantibodies (16). In the present report, using a similar protocol but with rabbits expressing β1AR-activating autoantibodies, we were able to induce sustained supraventricular and ventricular tachyarrhythmias. Moreover, the majority of the supraventricular arrhythmias were of sinus origin (15/32 episodes) and two episodes of junctional origin. Only one episode of atrial tachycardia was observed, whereas there were three instances of sustained ventricular tachycardia noted. In the baseline state, i.e., before ACh infusion or burst pacing, there was a significant increase in the basal heart rate between the preimmune and postimmune studies. It could be argued that the increased heart rate was due to an increase in sympathetic activity associated with the initial surgery and subsequent trauma; however, in a similar protocol previously published (16) in which β2AR-activating autoantibodies were induced, there was no significant increase in the baseline sinus rate before and after immunization. Thus it indicates that β1AR-activating autoantibodies in the present study were directly associated with the significant increase in the sinus rate.

These findings correspond to previous studies of the βAR densities. In the rabbit, as opposed to human (18), the highest density of β1AR was found in the specialized tissues, i.e., sinus node and other sites of pacemaker activity (20). Although β1AR and β2AR coexist in the rabbit atria, β1AR is predominant in the rabbit ventricles (2, 4). It is interesting to note that there were three episodes of sustained VT after expression of β1AR autoantibodies, whereas no sustained VT was induced in the preimmune state. These differences should be taken into consideration when applying these observations to the human. An important issue is the marked specificity of the autoantibodies, and the dependence of the site of the arrhythmia appears to be at least in part associated with the preponderance of the β1/2AR subtype for that region. These observations have been more difficult to demonstrate in previous studies that use only partially specific orthosteric agonists and antagonists.

Lee et al. (15) addressed the evidence that autoantibodies targeting the cardiac autonomic receptors are involved in the development of various cardiovascular disorders and specifi-
preincubation with the second extracellular loop (ECL2) peptide for arhythmogenic effects of the activating autoantibodies.

Furthermore, these models to provide a model with direct evidence linking specific autoantibodies on human disease (3, 9, 11, 12, 17). These autoantibodies have been reported variously associated cardiac arrhythmias. These autoantibodies display agonist-like properties in vitro and primarily target the second extracellular loops of the respective receptors. The sympathomimetic anti-βAR autoantibodies have been reported to be associated with primary ventricular arrhythmias (7), inappropriate sinus tachycardia (6), and a high incidence of VT and sudden death in dilated cardiomyopathy (10), whereas the parasympathomimetic autoantibodies to M2 muscarinic receptor are associated with both bradyarrhythmias and tachyarrhythmias, such as idiopathic sinus node dysfunction (5) and AF (1).

The importance of the previous report and the present study is to provide a model with direct evidence linking specific forms of supraventricular arrhythmias to receptor-specific activating autoantibodies as opposed to the indirect or observed associations described clinically. Furthermore, these models may provide specific targets for drugs that will neutralize the arrhythmogenic effects of the activating autoantibodies.

Limitations of the study. Animal models have played an important role in our understanding of the impact of these autoantibodies on human disease (3, 9, 11, 12, 17). These studies have primarily focused on their cardiomyopathic effects, whereas study of their impact on cardiac arrhythmias has been more complicated owing to the small heart size of smaller rodents and their difficulty in sustaining complex tachyarrhythmias compared with that observed in larger hearts in animals such as the dog or pig. However, these latter animals do not lend themselves to immunological study for a variety of reasons, and this has impaired research in this venue. We have found the immunized rabbit presents a fertile avenue for examination of a variety of tachyarrhythmias (16). However, it is important to recognize there may be species-related differences in their manifestations of the tachyarrhythmias to make appropriate conclusions from the available data in regard to the human.

The previous and present studies were intended to provide prototypical models to examine the specific role of β-adrenergic activation on triggering and perpetuation of the arrhythmias. They also provide evidence for alteration of the substrate through remodeling associated with the persistent β1AR antibody-induced tachycardia. Future studies will focus on using combinations of β1AR- and β2AR-activating autoantibodies with other receptor activators to simulate more complex arrhythmias present in chronic human autoimmune diseases, application of more selective testing of alterations in substrate susceptibility, and use of specific peptide antagonists for the activating autoantibodies to block their activity without altering their target receptor function.

We recognize that anesthesia can have an effect on cardiac electrophysiology and arrhythmogenesis. It should be noted that the induction and maintenance of anesthesia was the same in the pre- and postimmune states, thus, it seems unlikely that the differences in baseline heart rates and induction of sustained arrhythmias using each animal as its own control was based on the anesthetic employed. Moreover, in a previous report in which a similar protocol was instituted and β2AR-activating autoantibodies were induced, burst pacing significantly increased the incidence of sustained atrial tachycardia rather than sinus tachycardia.

Conclusions

In summary, we have demonstrated the presence of β1AR-activating autoantibodies predisposes the heart to tachyarrhythmias arising mainly at the sinus node. These data are consistent with the previous demonstration of a relative predominance of β1AR in this region of the rabbit heart. The high specificity of these receptor-activating autoantibodies makes them useful for examination of the regional pathophysiology and mechanistic basis for cardiac arrhythmias.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


