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Activating autoantibodies to the $\beta 1/2$ -adrenergic and M2 muscarinic receptors associate with atrial tachyarrhythmias in patients with hyperthyroidism

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Abstract

We have previously demonstrated that activating autoantibodies to β 1-adrenergic receptor (β 1AR) and M2 muscarinic receptor (M2R) facilitate atrial fibrillation (AF) in patients with Graves' disease (GD). The objectives of this expanded study were to examine the prevalence of β 1AR, β 2AR, and M2R autoantibodies in hyperthyroidism subjects. Sera from 81 patients including 31 with GD and AF, 36 with GD and sinus rhythm, 9 with toxic multinodular goiter, 5 with subacute thyroiditis, and 10 control subjects were examined for these autoantibodies by ELISA. Sera from 20 ELISA-positive GD subjects, 10 with AF and 10 with sinus rhythm, were assayed for autoantibody bioactivity using cell-based bioassays. In patients with GD and AF, 45, 65, and 77 % were ELISA positive for β 1AR, M2R, and β 2AR autoantibodies, respectively. In patients with GD and sinus rhythm, 17, 39, and 75 % were ELISA positive for β 1AR, M2R, and β 2AR autoantibodies, respectively. β 1AR and M2R autoantibodies were co-present in 39 % of patients with GD and AF compared to 14 % in GD with sinus rhythm ($p = 0.026$). Patients with toxic multinodular goiter or subacute thyroiditis had a low prevalence of autoantibodies. The mean β 1AR and M2R autoantibody activity was elevated in both GD groups but higher in those with AF than those with sinus rhythm. β 2AR autoantibody activity was also increased in both groups. In conclusion, β 1AR, β 2AR, and M2R autoantibodies were elevated in GD. β 1AR and M2R autoantibodies appear to be related to concurrent AF, while β 2AR autoantibodies were equally prevalent in those with a sinus tachycardia and those with AF.

Keywords

Activating autoantibodies; β -Adrenergic receptors; M2 muscarinic receptor; Hyperthyroidism; Atrial tachyarrhythmias

Introduction

Graves' disease (GD) is the number one cause of hyperthyroidism in the world [1]. In a recent survey of more than 586,000 individuals screened for thyroid function, approximately 3 % developed atrial fibrillation (AF) [2]. Those with evidence for subclinical hyperthyroidism had a 30 % increase in risk for developing AF during a 5.5-year follow-up period. Overt hyperthyroidism has been frequently associated with a sinus tachycardia; and other tachyarrhythmias including sustained AF have been reported in 20–30 % of patients so diagnosed [3, 4]. Some GD patients with equivalent elevated thyroid hormone levels develop

AF, while others do not, leaving an open question for the underlying pathophysiology. In the past, there was a widely accepted association of age and excess thyroid hormone with AF [5]. This relationship has been supported by evidence for excess thyroid hormone leading to shortening of the action potential duration in the atrial myocardium and thereby facilitating formation of multiple re-entry circuits [6].

GD is an autoimmune disease generally associated with the presence of thyroid-stimulating immunoglobulins to the G protein-coupled thyrotropin receptor [1]. We hypothesized and subsequently reported that activating autoantibodies to the β 1-adrenergic receptor (β 1AR) and M2 muscarinic receptor (M2R) were prevalent in GD patients with AF and at a significantly lower rate in GD without AF [7]. We presented evidence that these autoantibodies had the capacity to facilitate or induce rapid triggered firing in the atrial cuff extending into pulmonary veins and their presence served as a strong independent predictor of AF. In the present study, we have expanded our hypothesis to include activating autoantibodies to the β 2AR, since these receptors also are present in cardiac atrial tissue and have a complex relationship with atrial tachyarrhythmias as do β 1AR [8, 9].

The first study included 38 subjects with GD, of which 17 had AF during the course of their hyperthyroidism [7]. Our current study was designed to expand this database with additional subjects with GD and AF. We also wished to examine the relationship of activating autoantibodies to atrial tachyarrhythmias in two separate groups of hyperthyroid patients including those with toxic multinodular goiter and whose etiology is not considered autoimmune. The second group of patients with subacute thyroiditis are generally negative of thyroid-stimulating immunoglobulins but have weak links with a previous infectious episode, and so an undefined autoimmune process has not been excluded [10].

Materials and methods

Subjects

Our study has been doubled in size to include 67 GD patients, 31 with AF and 36 in a sinus rhythm. The diagnosis of GD was based on the presence of markedly suppressed serum thyrotropin concentrations, concurrently elevated serum free thyroxine and triiodothyronine concentrations, and evidence of a diffuse goiter with increased 24-h radionuclide uptake. Measurements of thyroid-stimulating immunoglobulins were generally obtained but not required unless there was ambiguity in the diagnosis. Three of these hyperthyroid subjects were considered to have preexisting AF and had been administered amiodarone for control. These three subjects subsequently developed thyroid-stimulating immunoglobulin-positive GD. We expanded the study to include 9 patients with toxic multinodular goiter including 1 with AF. There also were 5 patients with active subacute thyroiditis, all with a sinus rhythm. Patients were diagnosed as having subacute thyroiditis based on their elevated thyroid hormone lab values, a markedly suppressed thyrotropin and suppressed 24-h radioactive iodine uptake and negative serum thyroid-stimulating immunoglobulins. A standard 12-lead electrocardiogram was performed on all patients. These were analyzed by computer using the algorithms supplied with the different instruments available in our institution. All computer-generated interpretations were reviewed by the cardiology physician on call for that week. Serum was obtained from each patient as well as from 10 healthy control

subjects. This study was approved by the University of Oklahoma Health Sciences Center Institutional Review Board, the Oklahoma City VA Medical Center Research and Development Committee, and all subjects provided written informed consent.

ELISA

Sera from 81 patients and 10 healthy control subjects were screened by ELISA for autoantibodies directed toward the β 1AR, β 2AR, and M2R as previously described [11]. Briefly, peptides corresponding to the amino acid sequence of the second extracellular loops of human β 1AR (HWWRAES DEARRCYNDPKCCDFVTNR), β 2AR (HWYRATHQEAI NCYANETCCDFFTNQ), and M2R (VRTVEDGECYIQFF SNAAVTFGTAI) were synthesized (GenScript, Piscataway, NJ) and used to coat ELISA plates at a concentration of 10 μ g/mL in coating buffer. Sera were diluted 1:100, and goat anti-human IgG conjugated with alkaline phosphatase and its substrate para-nitrophenyl-phosphate 104 were used to detect antibody binding. The optical density (OD) values were read at 405 nm at 60 min.

Antibody activity assays

Autoantibody activation of β 1AR, β 2AR, and M2R were measured using the cAMP Hunter eXpress GPCR Assay kits (DiscoverRx, Fremont, CA). Activation of the Gs-coupled β 1AR and β 2AR by autoantibodies was examined in transfected Chinese hamster ovary (CHO) cells as previously described [12]. Activation of Gi-coupled M2R was determined by autoantibody suppression of forskolin-induced cAMP production in transfected CHO cells. Briefly, 30,000 CHO cells with target receptors were dispensed into each well of a 96-well culture plate and incubated overnight. The medium was removed, and assay buffer containing the cAMP antibody and sera (1:100 dilution) in the presence and absence of the non-selective β -blocker propranolol or β 2 selective blocker ICI-118,551 (100 nM) were sequentially added for the β 1AR and β 2AR assays, respectively, and incubated for 30 min. Forskolin (20 μ M) was added to M2R-transfected cells to stimulate cAMP production. cAMP suppression by the M2R auto-antibody and its subsequent blockade by atropine (100 nM) was used to estimate M2R activity. cAMP standards, negative (buffer) and positive (isoproterenol or acetylcholine 100 nM) controls were included in each assay. All samples were tested in triplicate. Following sample treatment, cAMP detection reagent and solution were added and chemiluminescent signal was read on a TD-20/20 Luminometer (Turner BioSystems, Sunnyvale, CA). The cAMP values are expressed as percentage of buffer baseline to normalize the individual data. The intra-assay and inter-assay coefficient of variation were 5.3 % ($n = 20$) and 7.2 % ($n = 10$) for the β 1AR assay, 3.6 % ($n = 20$) and 5.4 % ($n = 10$) for the β 2AR assay, and 6.1 % ($n = 20$) and 8.1 % ($n = 10$) for the M2R assay, respectively.

Statistical analysis

Data are expressed as mean $\pm$ SEM. Autoantibody positivity by ELISA was defined as OD values above the mean + 2SD from the healthy control group [11]. χ^2 analysis was used to compare categorical variables. For continuous variables, group comparisons were performed using the nonparametric Mann–Whitney test for comparison of 2 groups or the Kruskal–Wallis test followed by Dunn’s multiple comparison test for comparison of 3 or more groups. Statistical significance was set at $p < 0.05$.

Results

Patient characteristics

Of the 67 patients with GD, 31 had AF and 36 had sinus rhythm which generally was a sinus tachycardia on presentation. One of the 9 patients with toxic multinodular goiter had AF, and all the 5 patients with subacute thyroiditis were in sinus rhythm and mild tachycardia. Most subjects were on a β -blocker by the time they were referred to our clinic. This was generally not withdrawn to avoid potentially adverse clinical sequelae. The clinical, echocardiographic, and biochemical characteristics of the hyperthyroid patients are summarized in Table 1. There was a significant difference in age among the patient groups. Patients with subacute thyroiditis were older than those in the other groups, and as expected, patients with GD and AF were older than those with GD and sinus rhythm (59.6 ± 15.3 vs. 46.8 ± 15.9 years, $p = 0.002$). Otherwise, no difference was noted for the percentage of male sex, presence of hypertension, diabetes mellitus, coronary artery disease, and congestive heart failure among the patient groups. Echo indices including left ventricular ejection fraction and left atrial diameter did not differ significantly among the patient groups. Serum thyrotropin, free thyroxine, and free triiodothyronine concentrations were similar in the patient groups. The 10 healthy control subjects (age 20–55 years) included 5 males and 5 females.

Autoantibody screening by ELISA

ELISA data are shown in Table 2 and Fig. 1. Fourteen and 20 of 31 patients with GD and AF were found to have positive autoantibodies to $\beta 1AR$ (45 %) and M2R (65 %), respectively. In patients with GD and sinus rhythm, 6/36 (17 %) and 14/36 (39 %) were observed to have positive autoantibodies to $\beta 1AR$ and M2R, respectively. Twelve of 31 (39 %) patients with GD and AF harbored both $\beta 1AR$ and M2R autoantibodies compared to only 5 of 36 (14 %) patients with GD and sinus rhythm ($p = 0.026$). We have assayed these same sera for the presence of autoantibodies to $\beta 2AR$ and found a similar prevalence between patients with GD and AF (24/31, 77 %) and patients with GD and sinus tachycardia (27/36, 75 %).

To minimize the impact of age, we examined a matched subgroup in the GD patients. Fifteen patients with AF and 15 patients with sinus rhythm (mean age = 55.3 ± 6.5 vs. 54.7 ± 7.3 years, $p = 0.81$) were compared. The co-presence of $\beta 1AR$ and M2R autoantibodies was more prevalent in patients with GD and AF than in patients with GD and sinus rhythm (47 vs. 7 %, $p = 0.035$).

Our expanded group also included subjects with non-immune-mediated forms of hyperthyroidism including 9 subjects with toxic multinodular goiter and 5 subjects with subacute thyroiditis. Two of 9 subjects with toxic multi-nodular goiter and 1 of 5 subjects with thyroiditis demonstrated low levels of autoantibodies.

Autoantibody activity

Sera from 20 ELISA-positive GD subjects, 10 with AF and 10 with sinus rhythm, were tested for activation potential in transfected CHO cells. $\beta 1AR$ autoantibody-induced cAMP production in $\beta 1AR$ -transfected CHO cells, expressed as % of buffer baseline, was

significantly increased in both groups compared to healthy controls, but the mean activity was significantly higher in GD with AF than in GD with sinus rhythm (171 ± 9 vs. 129 ± 8 %, $p = 0.003$) (Fig. 2). This elevated activity was abolished in both groups by the addition of the β -blocker propranolol. M2R autoantibody activity was assayed by its ability to suppress forskolin-stimulated cAMP production in M2R-transfected CHO cells (Fig. 3). M2R autoantibody activity was observed in both groups. However, M2R autoantibodies in the sera suppressed the forskolin-induced cAMP production to 57 ± 2 % of baseline for those with AF compared to only 72 ± 2 % of baseline for those with sinus rhythm ($p = 0.0003$). This activity in both groups was reversed by the addition of the muscarinic blocker atropine.

We also examined the effect of sera from these same 20 subjects on β 2AR activation in β 2AR-transfected CHO cells. Both groups showed significantly increased β 2AR autoantibody activity compared to healthy controls (Fig. 4). However, there was no significant difference in the mean activity between GD with AF and GD with sinus rhythm (152 ± 5 vs. 143 ± 5 %, $p = 0.14$). The β 2 selective blocker ICI-118,551 decreased the activity toward baseline. All of the positive assays were related to sera taken from subjects with either AF or a significant sinus tachycardia.

Discussion

The sympathetic and parasympathetic nervous systems play a synergistic role in the initiation and maintenance of AF [13, 14]. M2 muscarinic activation decreases the action potential duration and effective refractory period, while β -adrenergic activation leads to intracellular sarcoplasmic reticulum calcium loading. These changes in combination lead to early after depolarization facilitating rapid trigger firing in cardiac atrial tissues by local autonomic nerve stimulation [15, 16]. We previously have demonstrated that autoantibodies present in sera from GD subjects activate β 1AR and M2R and facilitated ectopy in cardiac atrial tissues in vitro [7]. We recently reported studies utilizing immunized rabbits as models for the association of activating autoantibodies with atrial tachyarrhythmias [17, 18], for age [19] and for concurrent hyperthyroidism [20]. In summary, we have demonstrated that old rabbits (age 4–6 years) compared to young rabbits (age 3–4 months) are more prone to develop AF [19]. When rabbits are immunized to produce β 2AR-activating autoantibodies, they are prone to develop sustained atrial tachycardia [17]. By contrast, active immunization to produce β 1AR-activating autoantibodies in the rabbit led to sustained sinus tachycardia [18]. These data are of interest in as much as the relative densities of β 1AR and β 2AR in rabbit sinoatrial node and working atrial myocardium reflect the different origins of these tachyarrhythmias in this animal model. Although humans have the opposite expression of β 1AR and β 2AR in their atrial tissues [21], this specificity by the highly selective activating autoantibodies provides evidence that β AR targeting may affect the type of tachyarrhythmias observed in humans. Current studies in our laboratory demonstrate that these experimental autoimmunized animal models of a human disease have an even higher rate of induced receptor-specific tachyarrhythmias when thyroid hormone is added [20].

We previously demonstrated a very high co-prevalence of β 1AR and M2R autoantibodies in patients with GD [7]. These data reflected the use of a functional assay using canine

Purkinje fibers that were more sensitive than ELISA assays alone. In the present study, we have initially used ELISA data, but these assays are variably less sensitive and more importantly do not provide activity data reflecting their biological impact in vivo. For this reason, we have used a relatively expensive receptor activation assay to examine autoantibody bioactivity in a subgroup of GD patients with and without AF demonstrating positive ELISA values. The patients with AF had activity levels for β 1AR and M2R autoantibodies that were markedly higher than for those with a sinus tachycardia. These data support the concept that subjects with markedly elevated autoantibody activity are at greatest risk for developing AF.

It has been documented that increased age as well as increased thyroid hormone concentrations is associated with increased AF. We have shown that activating autoantibodies to β 1AR and M2R were also determinants of AF in GD [7]. This concurrence has again been observed in the present study in an age-matched subgroup of our GD patient population. The association between the co-presence of β 1AR and M2R autoantibodies and AF remains statistically significant.

We examined separate groups of subjects with hyperthyroidism not associated with autoimmune issues. Only one in the 9 subjects with toxic multinodular goiter had AF, and this subject as well as one other in this group and one in the 5 subjects with subacute thyroiditis had relatively low levels of autoantibodies as measured by ELISA. Larger numbers of these subjects will be needed to provide a clear impression as to whether activating autoantibodies are less prevalent in these circumstances.

We previously demonstrated a significant prevalence of β 1AR-activating autoantibodies in patients with GD [7], and in the present study this relationship appears to also include β 2AR-activating autoantibodies. We have demonstrated that there is no cross reactivity between these two antibodies [18]. The lack of a significant relationship of β 2AR autoantibodies to AF in this study may reflect previous observations that this receptor may play a more significant role in the sinus tachycardia frequently observed in GD. We have reported that both β 1AR and β 2AR autoantibodies not only have an intrinsic ability to activate their respective receptors but also facilitate receptor activation by their normal orthosteric ligand [12]. Thus, it is possible that the co-presence of high thyroid hormone and β 1/2AR autoantibody-facilitated activity of endogenous norepinephrine may play a synergistic role in induction of sinus tachycardia as well as induction of AF. Our data in a rabbit model favor this interpretation [20].

In conclusion, these data expand and confirm our previous study demonstrating a concordance of autoantibodies to β 1AR and M2R in GD patients with AF. It seems likely that the co-presence of old age, thyroid hormone excess, and activating autoantibodies to β 1AR and M2R place a patient in the highest probability for developing AF. Patients with toxic multinodular goiter and subacute thyroiditis appear to have a relatively low prevalence of autoantibodies compatible with their non-autoimmune pathogenesis. We have shown in an animal model that β 2AR-activating autoantibodies cause an atrial tachycardia syndrome. β 2AR autoantibodies in the co-presence of excess thyroid hormone create the environment for induction of supraventricular tachycardias. Studies using auto-immune animal models

confirm the additive effect of activating autoantibodies, excess thyroid hormone, and old age in initiating and sustaining AF. It is likely that future therapies that can block and/or remove these pathological autoantibodies may improve or prevent the persistence of AF in subjects with GD.

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Fig. 1.

ELISA detection of autoantibodies to $\beta 1$ -adrenergic receptor ($\beta 1$ AR), $\beta 2$ -adrenergic receptor ($\beta 2$ AR), and M2 muscarinic receptor (M2R) in patients with Graves' disease (GD) and atrial fibrillation (AF, $n = 31$), patients with GD and sinus rhythm ($n = 36$), patients with toxic multinodular goiter (TMNG, $n = 9$), patients with subacute thyroiditis ($n = 5$), and healthy control subjects ($n = 10$). Median and interquartile range are shown for each group. The *dashed line* is the threshold derived from the mean optical density (OD) values + 2SD of the healthy controls

Fig. 2.

Effects of sera (1:100) from 20 ELISA-positive patients with GD (10 with AF and 10 with sinus rhythm) and 10 healthy control subjects on activation of β1AR in transfected Chinese hamster ovary (CHO) cells. The cAMP values are expressed as % of buffer baseline. There was a significant increase in β1AR activity in both GD groups compared to control values (* $p < 0.01$, ** $p < 0.001$). However, the β1AR activity was significantly higher in GD and AF than in GD and sinus rhythm ($p = 0.003$). The addition of β-blocker propranolol suppressed the elevated values to control levels (# $p < 0.01$)

Fig. 3.

Effects of sera (1:100) from 20 ELISA-positive patients with GD (10 with AF and 10 with sinus rhythm) and 10 healthy control subjects on activation of M2R in transfected CHO cells. The cAMP values are expressed as % of buffer baseline. Sera from both GD groups significantly suppressed forskolin-induced cAMP production compared to controls (** $p < 0.001$). However, the M2R activity was significantly greater in GD and AF than in GD and sinus rhythm ($p = 0.0003$). This activity in both groups was reversed by the addition of muscarinic blocker atropine (# $p < 0.01$)

Fig. 4.

Effects of sera (1:100) from 20 ELISA-positive patients with GD (10 with AF and 10 with sinus rhythm) and 10 healthy control subjects on activation of β 2AR in transfected CHO cells. The cAMP values are expressed as % of buffer baseline. Both GD groups showed significantly increased β 2AR activity compared to controls (** $p < 0.001$). No significant difference in β 2AR activity was observed between the two GD groups ($p = 0.14$). The β 2 selective blocker ICI-118, 551 blocked the activity back to control levels ($^{\#}p < 0.01$)

Table 1

Clinical, echocardiographic, and biochemical characteristics of hyperthyroid patients

	GD AF (<i>n</i> = 31)	GD sinus rhythm (<i>n</i> = 36)	TMNG (<i>n</i> = 9)	Thyroiditis (<i>n</i> = 5)	<i>p</i> value
Age (years)	59.6 ± 15.3	46.8 ± 15.9	60.2 ± 13.8	71.0 ± 9.9	<0.001
Male (%)	61.3	38.9	55.6	80.0	0.16
Hypertension (%)	48.4	38.9	33.3	40.0	0.81
Diabetes mellitus (%)	16.1	11.1	11.1	20.0	0.90
Coronary artery disease (%)	12.9	5.6	11.1	20.0	0.64
Heart failure (%)	35.5	16.7	11.1	20.0	0.23
Ejection fraction (%)	52.1 ± 17.8	53.0 ± 15.1	56.2 ± 5.5	58.4 ± 4.2	0.83
Left atrium (mm)	43.3 ± 8.8	39.2 ± 6.3	35.8 ± 7.8	40.2 ± 4.3	0.22
Serum thyrotropin (mU/L)	0.055 ± 0.12	0.029 ± 0.03	0.073 ± 0.08	0.116 ± 0.07	0.19
Serum-free T4 (ng/dL)	3.30 ± 2.6	3.53 ± 2.5	1.97 ± 2.1	2.36 ± 1.4	0.35
Serum-free T3 (ng/dL)	4.35 ± 2.0	6.34 ± 4.5	4.46 ± 1.8	3.60 ± 1.4	0.16

Data are expressed as mean ± SD. Statistical comparisons among groups were performed by χ^2 test or Kruskal–Wallis test as appropriate

GD Graves' disease, TMNG toxic multinodular goiter, AF atrial fibrillation, T4 thyroxine, T3 triiodothyronine

Table 2

ELISA autoantibody screening

	GD AF	GD sinus rhythm	TMNG	Thyroiditis	Control	p value
Anti-β1AR positivity	14/31 (45 %)	6/36 (17 %)	2/9 (22 %)	1/5 (20 %)	1/10 (10 %)	0.06
Anti-M2R positivity	20/31 (65 %)	14/36 (39 %)	2/9 (22 %)	1/5 (20 %)	1/10 (10 %)	0.01
Anti-β2AR positivity	24/31 (77 %)	27/36 (75 %)	2/9 (22 %)	1/5 (20 %)	0/10 (0 %)	<0.001

Statistical comparisons among groups were performed by χ^2 test

β1AR β1-adrenergic receptor, β2AR β2-adrenergic receptor, M2R M2 muscarinic receptor