Agonistic Autoantibodies as Vasodilators in Orthostatic Hypotension
A New Mechanism

Hongliang Li, David C. Kem, Sean Reim, Muneer Khan, Megan Vanderlinde-Wood, Caitlin Zillner, Daniel Collier, Campbell Liles, Michael A. Hill, Madeleine W. Cunningham, Christopher E. Aston, Xichun Yu

Abstract—Agonistic autoantibodies to the β-adrenergic and muscarinic receptors are a novel investigative and therapeutic target for certain orthostatic disorders. We have identified the presence of autoantibodies to β2-adrenergic and/or M3 muscarinic receptors by ELISA in 75% (15 of 20) of patients with significant orthostatic hypotension. Purified serum IgG from all 20 of the patients and 10 healthy control subjects were examined in a receptor-transfected cell-based cAMP assay for β2 receptor activation and β-arrestin assay for M3 receptor activation. There was a significant increase in IgG-induced activation of β2 and M3 receptors in the patient group compared with controls. A dose response was observed for both IgG activation of β2 and M3 receptors and inhibition of their activation with the nonselective β blocker propranolol and muscarinic blocker atropine. The antibody effects on β2 and/or M3 (via production of NO) receptor-mediated vasodilation were studied in a rat cremaster resistance arteriole assay. Infusion of IgG from patients with documented β2 and/or M3 receptor agonistic activity produced a dose-dependent vasodilation. Sequential addition of the β-blocker propranolol and the NO synthase inhibitor N\textsuperscript{\textcircled{-}}-nitro-L-arginine methyl ester partially inhibited IgG-induced vasodilation (percentage of maximal dilatory response: from 57.7±10.4 to 35.3±4.6 and 24.3±5.8, respectively; P<0.01; n=3), indicating that antibody activation of vascular β2 and/or M3 receptors may contribute to systemic vasodilation. These data support the concept that circulating agonistic autoantibodies serve as vasodilators and may cause or exacerbate orthostatic hypotension. Hypertension. 2012;59:00-00."

Key Words: orthostatic hypotension ■ vasodilation ■ agonistic autoantibodies ■ β-adrenergic receptor ■ muscarinic receptor ■ nitric oxide synthase

Orthostatic hypotension (OH), generally defined as a drop in systolic/diastolic blood pressure (BP) of ≥20/10 mm Hg within 3 to 10 minutes of standing, has a diverse pathophysiological basis. It is frequently associated with autonomic dysfunction caused by a variety of primary or secondary autonomic disorders.¹ ² There is a high percentage of OH patients who have no known etiology and are assigned to the category of idiopathic OH. Several groups⁴⁻⁷ and we⁸⁻¹¹ have demonstrated previously that agonistic autoantibodies to certain autonomic G protein–coupled receptors (GPCRs) are associated with several cardiovascular diseases, including hypertension, cardiomyopathy, myocarditis, and cardiac arrhythmias, all of which have a variable degree of associated orthostasis. These autoantibodies are also found in the sera of healthy subjects but generally in a lower frequency and in lower titers.¹² They primarily target the second extracellular receptor loop and mediate physiological effects.⁴⁻¹³ We have reported recently for the first time the association of agonistic autoantibodies in 6 patients with demonstrable orthostasis.¹³ This mechanistic study demonstrated significant vasodilatory activity associated with autoantibodies directed toward the β2-adrenergic receptor (β2AR) and M3 muscarinic receptor (M3R). Although the IgG purified from these patients demonstrated marked vasodilatory activity, the combination of potentially opposing effects from other agonistic autoantibodies and their diverse presentation within the small number of patients made it difficult to develop an impression of the autoantibody frequency and physiological role in patients subject to orthostatic symptomatology.

The purpose of the present study is to expand our sample size, use new cell-based bioassays with transfected target
receptors to examine the receptor-specific bioactivity of these autoantibodies, and solidify our understanding of the mechanisms by which they may alter cardiovascular homeostasis. We also have the opportunity to initiate studies to examine the potential frequency of these agonistic autoantibodies in subjects who present with OH and the possible copresence of autonomic neuropathy from a metabolic basis. Because OH is increasingly common in diabetics, we have included a group of OH patients with diabetes mellitus (predominantly type 2) in the presence or absence of apparent associated autonomic dysfunction. This study demonstrates that autoantibodies targeting the β2AR and/or M3R are present in a majority of these OH patients, and the autoantibodies possess sufficient bioactivity to alter the postural vascular response, thus contributing to the pathophysiology of OH.

Methods

Patients

Ten patients with idiopathic OH and 10 diabetic patients with OH, 5 with and 5 without concurrent gastroparesis, were selected from 50 patients referred to the Oklahoma City Veterans Affairs Medical Center, University of Oklahoma Health Sciences Center Endocrinology Section, and the Harold Hamm Diabetes Center for evaluation of OH symptoms. These 20 patients did not include the 6 idiopathic OH patients published previously and were chosen based on the criteria for OH described below. Patients with evident hypotension from administration of antihypertensive drugs or apparent primary neurological diseases were excluded. Ten voluntary healthy control subjects were examined to obtain a "low estimate" of antibody levels in a group of OH patients with diabetes mellitus (predominantly type 2) in the presence or absence of apparent associated autonomic neuropathy from a metabolic basis. Because OH is increasingly common in diabetics, we have included a group of OH patients with diabetes mellitus (predominantly type 2) in the presence or absence of apparent associated autonomic dysfunction. This study demonstrates that autoantibodies targeting the β2AR and/or M3R are present in a majority of these OH patients, and the autoantibodies possess sufficient bioactivity to alter the postural vascular response, thus contributing to the pathophysiology of OH.

cAMP Assay

IgG activation of β2AR was measured using the cAMP Hunter xXpress GPCR Assay kit (DiscoveRx, Fremont, CA), according to the manufacturer’s protocol. Briefly, 30,000 cAMP Hunter xXpress β2AR-Chinese hamster ovary cells were dispensed into each well of a 96-well culture plate and incubated overnight. The medium was then removed, and assay buffer containing the cAMP antibody and serum IgG (0.05–0.45 mg/mL) in the presence and absence of βAR blocker propranolol (1 × 10^{-8} to 1 × 10^{-6} mol/L) was sequentially added and incubated for 30 minutes. cAMP standard, negative (buffer), and positive (isoproterenol 100 nmol/L) controls were included in each assay. All of the samples were tested in triplicate. After 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 the percentage of buffer baseline to normalize the individual data.

β-Arrestin Assay

IgG activation of M3R was measured using the PathHunter xXpress β-arrestin GPCR Assay kit (DiscoveRx), according to the manufacturer’s protocol. The PathHunter β-arrestin technology monitors GPCR activity by detecting the interaction of β-arrestin with the activated GPCR using β-galactosidase enzyme fragment complementation. The β-arrestin recruitment occurs as a function of ligand activation of the target receptor. Briefly, 10,000 PathHunter xXpress β-arrestin M3R-Chinese hamster ovary cells were dispensed into each well of a 96-well culture plate and incubated for 48 hours. Assay buffer containing serum IgG (0.3–1.2 mg/mL) in the presence and absence of muscarinic blocker atropine (1 × 10^{-8} to 1 × 10^{-6} mol/L) were then added and incubated for 90 minutes. Negative (buffer) and positive (acetylcholine 100 nmol/L) controls were included in each assay. All of the samples were tested in triplicate. After sample treatment, PathHunter detection reagents were added, and chemiluminescent signal was read on the same luminometer. The β-arrestin recruitment levels are expressed as a percentage of buffer baseline to normalize the individual data.

Isolated Arteriole Assay

The vasodilatory effect of patient IgG via activation of β2AR and M3R on resistance vessels was examined using an isolated rat cremaster arteriole assay as described. Briefly, cremaster resistance arterioles (70–80 μm) were surgically removed from anesthetized Sprague-Dawley rats (180–250 g). A ∼2-mm segment of the main intramuscular arteriole was microdissected, transferred to a 5-mL temperature-regulated superfusion chamber (Living Systems, St. Albans, VT), and cannulated at each end with glass micropipettes. Vessel segments were gradually pressurized to 70 mm Hg and warmed to 34°C. The vessel preparation was positioned on the stage of an inverted microscope (Nikon TMS) equipped with a video-based imaging system (MyoCam, IonOptix, Milton, MA). Measurements of internal vessel diameter were made using a video edge detector (Model VED-205, Crescent Electronics, Sandy, UT). After equilibration and development of steady-state myogenic tone, the arterioles were perfused with serum IgG (10–300 μg/mL). The β-blocker propranolol (1 μmol/L) was then added to the perfusate containing the maximal effective dose of IgG, and the effect on vessel diameter was recorded for 5 minutes. At that point, the NO synthesis inhibitor Nω-nitro-L-arginine methyl ester (100 μmol/L) was added to the IgG and propranolol and their cumulative effects were recorded until no further change in diameter was observed. The data are reported as a percentage of maximal dilatory response to normalize the values. Maximal dilatory response was defined as the increase in diameter from basal tone to the maximal Ca^{2+}-free passive dilation at 70 mm Hg measured at the end of each preparation. This procedure was approved by the Oklahoma City Veterans Affairs Medical Center and University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee.

ELISA

The ELISA was performed as described. Briefly, a 26-mer peptide (HWYRATHQEAINCYANETCCDFFTNQ) and 25-mer peptide (KRTVPQGECFQLEPTTFTTGFA1) corresponding to the amino acid sequence of the second extracellular loop of human β2AR and M3R, respectively, were synthesized (GenScript, Piscataway, NJ) and used to coat ELISA plates at a concentration of 10 μg/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 minutes.

IgG Preparation

IgG was purified from the patient or control sera using the NAb Protein A/G Spin Kit (Pierce Biotechnology, Rockford, IL).
Among the 10 patients with idiopathic OH, 3 were positive against β2AR and M3R. These data are shown in Figure 1.

Table 1. Characteristics of Study Subjects and Their Changes in Blood Pressure and Heart Rate During the 5-min Standing Test

<table>
<thead>
<tr>
<th>Characteristics and Parameters</th>
<th>Healthy Controls (n=10)</th>
<th>Idiopathic OH (n=10)</th>
<th>Diabetic OH (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>33.1±11.7</td>
<td>52.8±24.1*</td>
<td>60.8±10.5‡</td>
</tr>
<tr>
<td>Sex, male:female</td>
<td>7:3</td>
<td>4:6</td>
<td>8.2</td>
</tr>
<tr>
<td>Delta SBP</td>
<td>0.4±7.0</td>
<td>−32.2±21.8‡</td>
<td>−29.3±19.4‡</td>
</tr>
<tr>
<td>Delta DBP</td>
<td>0.0±5.8</td>
<td>−15.3±17.6*</td>
<td>−11.1±6.5‡</td>
</tr>
<tr>
<td>Delta HR</td>
<td>5.2±4.3</td>
<td>12.2±11.6</td>
<td>7.2±7.3</td>
</tr>
</tbody>
</table>

Data are mean±SD unless otherwise specified. OH indicates orthostatic hypotension; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate.

*P<0.05 vs healthy controls by t test.
†P<0.01 vs healthy controls by t test.
‡P<0.001 vs healthy controls by t test.

Statistics

Data are expressed as mean±SD. Student t test and χ² test were used for comparison of characteristics and hemodynamic parameters between patient and healthy control groups. Autoantibody positivity by ELISA was defined as OD values above the mean +2 SD from the control group. Control mean OD values were derived from log-transformed data because the distribution of OD is skewed. Autoantibody bioactivity values in the cAMP and β-arrestin biosays were normalized to their respective baseline values. The positivity of bioactive autoantibodies was defined as bioactivity values above the mean +2 SD from the control group. Control mean bioactivity values were similarly derived from log-transformed data. Spearman rank correlation was performed to examine the relationships between ELISA and bioactivity values. Comparison between patient and healthy control groups was performed using the nonparametric Mann-Whitney test. Differences in dose effects and vasodilation were assessed by a paired or unpaired Student t test as appropriate. A Bonferroni correction was applied to adjust for multiple comparisons. A P value of ≤0.05 was considered statistically significant. Compared with the healthy control group of 10 subjects with an assumed 10% to 15% positive rate for autoantibodies, positive rates as low as 60% for patient groups of 20, 70% for patient groups of 10, and 80% for patient groups of 5 could be detected as significantly different with 80% statistical power.

Results

Patient Characteristics

The OH patients were older than the healthy controls. These patients had a history of orthostatic symptoms and demonstrated a significant decrease in BP and/or a significant increase in pulse rate with only partial compensation of BP. Certain patients had a significant drop in BP and an inappropriate bradycardia demonstrating an inability to compensate for the drop in BP by an increase in cardiac output. The posture test results of all of the subjects are summarized in Table 1.

Autoantibody Screening by ELISA

Sera were examined by ELISA for autoantibodies directed against β2AR and M3R. These data are shown in Figure 1. Among the 10 patients with idiopathic OH, 3 were positive for β2AR antibodies, 7 were positive for M3R antibodies, and 3 were positive for both antibodies. A similar percentage of antibody positivity was observed in the 10 diabetic OH patients (β2AR antibodies: 4; M3R antibodies: 6; β2AR and M3R antibodies: 2). In this small sample of diabetic patients with OH, the presence of gastroparesis, a marker of autonomic neuropathy, did not seem to influence results. Because the statistical power for this comparison was low, this observation requires replication in a larger study. Overall 75% (15 of 20) of the OH patients showed positivity for β2AR and/or M3R antibodies, and 25% (5 of 20) had coexisting antibodies.

IgG Activation of β2AR and M3R in Cell-Based Assays

To examine the dose-responsive biological activity of the autoantibodies, IgG from 3 OH patients (2 idiopathic OH and 1 diabetic OH), who were strongly ELISA positive for autoantibodies to β2AR and M3R, were tested for their ability to activate β2AR and M3R in cultured cells using the cAMP Hunter and PathHunter β-arrestin technology. These assays provide an important parameter of cellular function relevant to the intrinsic activity of these autoantibodies. There was a significant dose effect on activation of both β2AR and M3R for these IgG samples (Figure 2). In addition, the IgG-induced activation of β2AR and M3R was effectively blocked in a dose-dependent fashion by the nonselective β-blocker propranolol and muscarinic blocker atropine, respectively (Figure 3).

IgGs from all 20 of the OH patients were then tested and demonstrated a variable but significant capacity to activate β2AR and M3R (Figure 4 and Table 2). The idiopathic OH and diabetic OH groups both showed significantly increased β2AR activation compared with healthy controls (P=0.007...
and $P=0.014$, respectively). The increases in M3R activation in the idiopathic OH and diabetic OH groups were even more significant ($P=0.002$ and $P=0.003$, respectively) compared with healthy controls. There were no significant differences in autoantibody activity or frequency between the idiopathic OH group and diabetic OH group with or without concurrent gastroparesis, so the 2 subgroups with diabetic OH were combined for analysis with the caveat that the statistical power to detect all but extreme differences between these subgroups was low. An isoproterenol or acetylcholine stimulus, not shown, was performed with each assay as a positive control. Pooled normal human IgG (Sigma) also was tested and did not show any significant activation of $\beta2$AR and M3R (Figure 4).

We have plotted the correlation of the individual OH subjects with their bioactivity measured by the receptor activation assays compared with their ELISA OD values in Figure 5. Although individuals with elevated ELISA values more frequently had significant bioactivity, others did not, and the Spearman correlation coefficients failed to reach significance.

IgG-Induced Vasodilation

The vasodilator response to a 3-point dosage of IgG from 8 OH patients with documented $\beta2$ and/or M3 receptor agonistic activity from the cell bioassays was examined using a rat cremaster arteriolar assay (Figure 6). A significant dose effect on vasodilation was observed for all of the tested IgG. Pooled, dialyzed normal human IgG (Sigma) and IgG from 3 healthy control subjects failed to produce any significant vasodilation. The effects of sequential addition of propranolol and $N^\text{G}$-nitro-L-arginine methyl ester on vasodilation induced by IgG from 3 OH patients are shown in Figure 7. There was a significant decrease in IgG-induced vasodilation with non-selective $\beta$-blockade and a further decrease with blockade of NO synthase by $N^\text{G}$-nitro-L-arginine methyl ester (from $57.7\pm10.4$% to $35.3\pm4.6$% and $24.3\pm5.8$%, respectively; $P<0.01$; $n=3$).

Discussion

OH is associated with increased mortality, causing falls and injury, impairing quality of life, and complicating concurrent medication usage. Any pharmacological or endogenously produced autonomic vasodilation involving the systemic peripheral resistance will cause or accentuate orthostasis in susceptible subjects. Although patients with obvious central or peripheral neuropathies have reason to demonstrate significant orthostasis, many other subjects have either minimal or no evidence for such a severe autonomic deficiency yet present with clinically relevant symptoms and signs of OH.

We previously hypothesized and subsequently demonstrated that autoantibodies to $\beta2$AR and M3R are harbored in a subgroup of idiopathic OH patients and might contribute to the pathophysiology of OH.\(^{14}\) In that size-limited study, we provided mechanistic evidence that these receptor-activating autoantibodies intrinsically possess the capability of inducing significant systemic arterial vasodilation and could be selectively antagonized by $\beta$-blockade, inhibition of endothelial NO synthase, and clinically by generalized muscarinic blockade.

We have used 2 commercially available specific receptor-transfected cell-based microassay kits to demonstrate that small amounts of IgG from such subjects are capable of activating $\beta2$AR and M3R. These kits provide the means for the assay of larger numbers of subjects and will be useful for studies of intrinsic bioactivity because they respond appropriately to orthosteric agonists and antagonists. Using these assays, we have demonstrated that some patients with idiopathic and diabetic OH have significant activation of 2
Table 2. Comparison of IgG-Induced Activation of β2AR and M3R in Cell-Based Bioassays Between OH Patients and Healthy Control Subjects

<table>
<thead>
<tr>
<th>IgG Source</th>
<th>β2AR Activation, Median (Interquartile Range)</th>
<th>M3R Activation, Median (Interquartile Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls (n=10)</td>
<td>104 (98–115)</td>
<td>98 (89–108)</td>
</tr>
<tr>
<td>Idiopathic OH (n=10)</td>
<td>122 (108–132)†</td>
<td>144 (110–180)†</td>
</tr>
<tr>
<td>Diabetic OH (n=10)</td>
<td>117 (113–123)†</td>
<td>122 (108–142)†</td>
</tr>
<tr>
<td>All OH (n=20)</td>
<td>120 (111–125)†</td>
<td>131 (111–161)‡</td>
</tr>
</tbody>
</table>

Data are expressed as percentage of baseline. β2AR indicates β2-adrenergic receptor; M3R, M3 muscarinic receptor; OH, orthostatic hypotension.

*p<0.05 vs healthy controls by Mann-Whitney test.
†*p<0.01 vs healthy controls by Mann-Whitney test.
‡*p<0.001 vs healthy controls by Mann-Whitney test.

Figure 4. Effects of IgG from orthostatic hypotension (OH) patients and healthy control subjects on activation of β2-adrenergic receptor (β2AR) and M3 muscarinic receptor (M3R) in specific receptor-transfected cultured cells. Idiopathic OH, ▲; diabetic OH, ▼; healthy ctrl, ■. n=10 in each group. Pooled normal human IgG (●) also was included as a negative control. IgG was tested at a concentration of 0.15 mg/mL and 0.6 mg/mL for activation of β2AR and M3R, respectively. Values are expressed as percentage of baseline. Median values and interquartile ranges are shown. Group comparisons were performed by the Mann-Whitney test.

Figure 5. Correlation analysis between ELISA and cell-based bioassays for autoantibodies to β2-adrenergic receptor (β2AR) and M3 muscarinic receptor (M3R) in the 20 orthostatic hypotension (OH) patients. The vertical and horizontal dashed lines represent the cutoff values (mean ±2 SD of healthy controls) for ELISA and bioassay positivity, respectively. No significant correlation was detected between ELISA optical density (OD) values and bioactivity values.
antibodies. This remains to be confirmed, because their allosteric stimulatory autoantibodies in in vitro studies, and, for the agonist’s intended use, such as in asthma, as well as desensitize their target receptors resulting in limited function. There are several reasons to believe that this is not a parallel response was not observed after combined blockade, we did not find evidence for concurrent 

Figure 6. Dose effects of IgG from 8 orthostatic hypotension (OH) patients on basal spontaneous myogenic tone of rat cremaster arterioles. Values are expressed as percentage of maximal dilatory response, which is the increase in diameter from basal tone to the maximal Ca²⁺-free passive dilation at 70 mm Hg measured at the end of each preparation. *P<0.01 vs buffer control. Pooled normal human IgG and IgG from 3 healthy control subjects also were tested and did not show any significant vasodilatory effect.

Orthostatic changes are not generally reported with use of orthosteric β2AR agonists, such as in treatment of asthma. There are several reasons to believe that this is not a parallel situation. Orthosteric ligands for virtually all GPCRs quickly desensitize their target receptors resulting in limited function for the agonist’s intended use, such as in asthma, as well as for adverse effects. This desensitization is not observed for allosteric stimulatory autoantibodies in in vitro studies, and, therefore, these autoantibodies have the potential for sustained activation. Secondly, vasodilation by β2AR autoantibodies will elicit a baroreceptor-mediated sympathetic response to compensate for the systemic effects. These changes at first may not be sufficient to exhaust the “compensatory homeostatic reserve,” but in conjunction with other autoantibodies causing vasodilation (eg, M3R activation) or limiting the cardiac rate (eg, M2R activation), associated autonomic neuropathy (eg, diabetes mellitus), or antibodies directed toward neural elements, patients may become symptomatic because of the presence of bioactive autoantibodies. We have observed some patients have coexisting autoantibodies, and, therefore, complex interactions might be expected and vary from subject to subject. Our finding that vasodilatory autoantibodies to β2AR and M3R are present in a high proportion of OH patients with and without apparent autonomic dysfunction suggests that these autoantibodies may cause or exacerbate orthostasis by altering the compensatory postural vascula

Figure 7. Effects of β-adrenergic blockade and NO synthase inhibition on IgG-induced arteriolar dilation. Values are expressed as percentage of maximal dilatory response. Sequential addition of the β blocker propranolol (Prop, 10⁻⁶ mol/L) and NO synthase inhibitor N³-nitro-L-arginine methyl ester (L-NAME, 10⁻⁴ mol/L) after IgG infusion led to a progressive decrease in vasodilation (from 57.7±10.4% to 35.3±4.6% and 24.3±5.8%, respectively; *P<0.01; n=3).

Perspectives

We have confirmed and extended our observation that a subgroup of patients with OH demonstrates vasodilatory autoantibodies to β2AR and M3R, which dilate resistance arterioles. Although isolated vasodilation is unlikely the sole cause of OH, these autoantibodies, when present, may be important culprits in the complex cardiovascular pathophysiology of OH. Our data reinforce the need for an expanded patient study with careful identification of subgroups, their pathophysiology, and association with specific autoantibody activity. The inconsistent correlation between the peptide-based ELISA and functional assays indicates that both assays must be performed in parallel at the same time. Because orthosteric antagonists may not provide total protection against allosteric effects of these autoantibodies, specific removal of pathological autoantibodies or use of selective autoantibody antagonists will be a desirable goal and permit more definitive assessment of the importance of these autoantibodies.

These data must be interpreted with caution until confirmation by larger studies and by use of specific antagonists to counteract their activity in vivo. However, we believe that this study should raise the hopes of patients and their physicians that newer approaches may improve the identification of previously unrecognized causes for OH and lead to new and improved pharmacological management of OH.

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Disclosures

None.
References
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