O
rthostatic 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. There is a high percentage of OH patients who have no known etiology and are assigned to the category of idiopathic OH. Several groups and we 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. They primarily target the second extracellular receptor loop and mediate physiological effects. We have reported recently for the first time the association of agonistic autoantibodies to 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 presence and activity in a relatively younger population. This study was approved by the University of Oklahoma Health Sciences Center Institutional Review Board and the Veterans Affairs Medical Center Institutional Animal Care and Use Committee. All of the subjects provided written informed consent.

BP and heart rate were determined after a 5-minute period of recumbency and after 5 and 10 minutes of upright posture. For this study, OH was defined as a drop in systolic BP of >20 mm Hg or diastolic BP of >10 mm Hg and/or a lesser decrement in BP associated with an increase in heart rate of >15 bpm. All but 3 had a significant drop in systolic/diastolic BP of >20/10 mm Hg. A diagnosis of OH with partial compensation was made in those 3 whose BP dropped by 12/8 mm Hg but with an increase in pulse rate of >15 bpm demonstrating a partial cardiac compensatory response. Each recording was made in duplicate using a cuff matched for upper arm circumference. Indirect BP values were recorded by the PG3/4 fellows with experience using an automated Dinamap V100 instrument. The arm was generally supported in a slightly flexed position and the arm circumference. Indirect BP values were recorded by the PG3/4 fellows with experience using an automated Dinamap V100 instrument.

**IgG Preparation**

IgG was purified from the patient or control sera using the NAb Protein A/G Spin Kit (Pierce Biotechnology, Rockford, IL).

**cAMP Assay**

IgG activation of β2AR was measured using the cAMP Hunter eXpress GPCR Assay kit (DiscoveRx, Fremont, CA), according to the manufacturer’s protocol. Briefly, 30,000 cAMP Hunter eXpress 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 eXpress 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 eXpress 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 synthase inhibitor L-NAME-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 Ca²⁺-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.
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 stimulation, 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 2AR 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 2AR and/or M3R 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\textsuperscript{7}-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\textsuperscript{7}-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 2AR and M3R are harbored in a subgroup of idiopathic OH patients and might contribute to the pathophysiology of OH. In that size-limited study, we provided mechanistic evidence that these receptor-activating autoantibodies intrinsically possess the capability of inducing significant systemic arteriolar 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 2AR 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
different GPCRs associated with peripheral vasodilation. The present study also uses a streamlined ELISA technique with the targeted receptor peptides relevant to GPCR activation. These assays demonstrate that detection of autoantibodies against one of the studied receptors may be common; however, a small but significant number of patients have autoantibodies against both studied receptors. Although we had originally anticipated 30% to 40% positivity, we were pleasantly surprised to find the percentage as high as 70% to 75% in both the idiopathic and diabetic OH groups with more severe orthostasis. However, the ELISA technique has limitations. First, the use of linear peptides as antigens carries the risk of missing conformational epitopes. Second, it is well established that ELISA alone will not detect all of the active antibodies, and, last, it will not predict functionality of the antibodies so detected. As expected, the correlation of antibody bioactivity with the ELISA data overall was not significant. We suspect that some subjects have autoantibodies directed to the first rather than the second extracellular loop of their respective target receptor, which may explain why some functionally positive subjects were ELISA negative. The ELISA technique has limitations. First, the use of linear peptides as antigens carries the risk of missing conformational epitopes. Second, it is well established that ELISA alone will not detect all of the active antibodies, and, last, it will not predict functionality of the antibodies so detected. As expected, the correlation of antibody bioactivity with the ELISA data overall was not significant. We suspect that some subjects have autoantibodies directed to the first rather than the second extracellular loop of their respective target receptor, which may explain why some functionally positive subjects were ELISA negative.

We have examined 8 of these subjects using an isolated resistance arteriole assay and demonstrated a pattern of IgG autoantibody-induced vasodilation very similar to that observed previously with the 6 OH patients from the previous publication. None of those patients were included in this present study and, therefore, they add to the documentation of a profound vasodilation associated with these autoantibodies. As before, we have confirmed with an additional 3 OH patients for whom β-blockade without and with Nω-nitro-L-arginine methyl ester blockade of endothelial NO synthase provides substantial but not complete return toward buffer baseline conditions. These data suggest that either the combined blockade was not complete or another unidentified vasodilatory autoantibody(s) may be present. Other explana-

**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.
tions include the following: these autoantibodies may have different affinity, may interact with each other and produce conformational changes which interfere with propranolol affinity and efficacy, or vice versa.20 Because a vasoconstrictive response was not observed after combined blockade, we did not find evidence for concurrent α1AR-activating autoantibodies.7 This remains to be confirmed, because their presence might be counterbalanced by either incomplete vasodilatory blockade or the putative other vasodilatory autoantibody.

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.21 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,22 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 vascular response.

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 cokulpts in the complex cardiovascular physiopathology 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,13 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.

Sources of Funding

X.Y. has received support from National Institutes of Health grant P20RR024215 from the Centers of Biomedical Research Excellence Program of the National Center for Research Resources. This study was also supported in part by funding from National Institutes of Health grant 5R01HL056267-12 (to M.W.C. and D.C.K.), Heart Rhythm Institute, University of Oklahoma Health Sciences Center, and a Veterans Affairs Merit Review grant (to D.C.K. and X.Y.) and individual grants from Will and Helen Webster and Britani T. and Paul E. Bowman, Jr.

Disclosures

None.
References


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 and Xichun Yu

Hypertension. 2012;59:402-408; originally published online January 3, 2012;
doi: 10.1161/HYPERTENSIONAHA.111.184937
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/59/2/402

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/