IgG stimulated β2 adrenergic receptor activation is attenuated in patients with ME/CFS

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ARTICLE INFO

Keywords:
IgG
β2 adrenergic receptor
Autoantibodies
Myalgic encephalomyelitis
Chronic fatigue syndrome

ABSTRACT

Background: There is emerging evidence of a network of natural autoantibodies against GPCR which is dysregulated in various diseases. β2 adrenergic and M3 and M4 cholinergic receptor (β2 AdR and M3/4 mAChR) antibodies were found to be elevated in a subset of ME/CFS patients.

Methods: We comparatively analyzed the effects of polyclonal IgG on β2 AdR signaling and immune cell function in vitro. 16 IgG fractions were isolated from serum of 5 ME/CFS patients with elevated (CFS AABhigh) and 5 with normal levels (CFS AABnorm) of β2 AdR autoantibodies, and from 6 healthy controls (HC). The effect of each IgG on β-arrestin recruitment and cAMP production in β2 AdR and M3/4R reporter cell lines was studied. Further effect of each IgG on human monocyte cytokine production and on T cell proliferation in vitro was analyzed. In addition, studies on cytokine production in β2 AdR wild type and knockout mice splenocytes incubated with IgG fractions were performed.

Results: We found that IgGs from HC could stimulate β-arrestin recruitment and cAMP production in β2 AdR reporter cell lines whereas IgGs from CFS AABhigh had no effect. The IgG-mediated activation of β2 AdR was confirmed in β2 AdR wt and ko mice. In accordance with previous studies IgG fractions from HC inhibited LPS-induced TNFα and stimulated LPS-induced IL-10 production of monocytes. Further IgG fractions from HC enhanced proliferation of T-cells stimulated with anti-CD3/CD28. IgG fractions from CFS AABhigh patients had no significant effect on both cytokine production and T cell proliferation, while IgGs from CFS AABnorm had an intermediate effect. We could also observe that IgG can modulate the signaling of β2 AdR ligands isoprenaline and propranolol.

Conclusions: We provide evidence that IgG can activate β2 AdR. The β2 AdR activation by IgG is attenuated in ME/CFS patients. A dysregulation of β2 AdR function could explain many symptoms of ME/CFS.

1. Introduction

With an estimated prevalence of 0.2–0.3% Myalgic encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a frequent and severe chronic disease. Patients suffer from persistent exhaustion, cognitive dysfunctions, pain and flu-like symptoms, leading to a substantial reduction of life quality (Carruthers et al., 2011). A hallmark of ME/CFS is aggravation of symptoms by exertion. In the majority of patients ME/CFS onset is triggered by an Epstein-Barr-Virus (EBV) or another intracellular infection (Chu et al., 2019). There is ample evidence of dysregulation of the autonomic nervous and immune system (Mensah et al., 2017; Sotzny et al., 2018). Several studies focused on the role of autoimmunity in ME/CFS (Blomberg et al., 2018; Sotzny et al., 2018). Recently, a network of natural antibodies against adrenergic, cholinergic

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and other GPCR receptors has been described which is dysregulated in various autoimmune and non-autoimmune diseases such as Alzheimer Disease or ovarian cancer (Cabral-Marques et al., 2018). We found elevated antibodies against β2 adrenergic receptors (β2 AdR) and muscarinic M3 and M4 acetylcholine receptors (M3/M4 mAChR) in a subset of ME/CFS patients in accordance with a previous study (Loebel et al., 2016; Tanaka et al., 2003). In most patients both β2 AdR and M3 mAChR antibodies were elevated. In ME/CFS patients receiving rituximab we observed a sustained decline of pretreatment elevated β2 AdR antibody levels in clinical responders to rituximab treatment (Loebel et al., 2016). In a first pilot study in 10 patients with post-infectious ME/CFS we observed that immunoadsorption is effective to remove β2 AdR autoantibodies and can induce clinical improvement (Scheibenbogen et al., 2018).

β2 AdR are expressed on most cell types including immune cells (Sanders, 2012). There is ample evidence for a role of β2 AdR in immune function. In monocytes, β2 AdR stimulation inhibits LPS-induced TNFx (Agac et al., 2018; Guirao et al., 1997). IL-10 release in monocytes is enhanced via β2 AdR stimulation (Agac et al., 2018). IgG from healthy persons was shown to have similar effects by reducing LPS-induced TNFx and enhancing IL-10 production (Fujii et al., 2013; MacMillan et al., 2009; Murakami et al., 2012).

There is first evidence that the β2 AdR function is impaired in ME/CFS with decreased inhibition of TNFx and reduced induction of IL-10 production by the β2 AdR agonist terbutaline (Kavelaars et al., 2000). Here we studied the effects of IgG isolated from serum of ME/CFS patients and healthy controls (HC) on β2 AdR signaling and immune cell function. Using β2 AdR transfected reporter cell lines we provide evidence that IgG from HC has an agonistic β2 AdR effect resulting in induction of β2 AdR signaling. We confirmed the β2 AdR activation by IgG using splenocytes from β2 AdR ko mice. Further in human immune cells we found that IgG from HC similar to isoprenaline reduced TNFx production, increased IL-10 production and enhanced T cell proliferation. Remarkably, IgG from ME/CFS patients with elevated β2 AdR autoantibodies (CFS AABhigh) had no significant effects neither on β2 AdR signaling nor on immune cell function.

2. Methods

2.1. Patients and controls

Patients were diagnosed at the Charité outpatient clinic for immunodeficiencies at the Institute for Medical Immunology at the Charité Universitätsmedizin Berlin. Diagnosis of ME/CFS was based on Canadian Criteria and exclusion of other medical or neurological diseases which may cause fatigue. Controls were recruited from staff. The study was approved by the Ethics Committee of Charité Universitätsmedizin Berlin in accordance with the Declaration of Helsinki. All patients and HC gave informed consent.

Symptoms of autonomic dysfunction were assessed by the Composite Autonomic Symptom Score (COMPASS-31) questionnaire examining autonomic functions in the domains of orthostatic, vasomotor, secretomotor, gastrointestinal, bladder- and pupillomotor regulation. Based on these domains a weighted total score ranging from “0” indicating no to “100” indicating severe symptoms of autonomic dysfunction was calculated (Sletten et al., 2012). Disease severity was examined by Bell score focusing on the level of restriction in daily functioning (Bell, 1995). Completely bedridden patients are classified as “0”, patients with unrestricted daily functioning as “100”.

5 ME/CFS patients with elevated level of antibodies against β2 AdR (>90% percentile of HC, CFS AABhigh) and autonomic dysfunction defined by a high COMPASS-31 score and higher heart rate, 5 patients with normal β2 AdR antibody level (<90% percentile of HC, CFS AABnorm), and 6 HC were selected. The definition of elevated antibodies (>90% percentile of HC) was based on our previous study (7). Information on the patients and HC is given in Table 1, all participants were female. All patients with elevated β2 AdR antibodies had elevated M3 and M4 mAChR antibodies as well.

2.2. Blood sampling, ELISA and isolation of IgG fractions

Venous blood samples were obtained at a morning visit to the outpatient clinic. Antibodies to β2 AdR and M3/4 mAChR were assessed using ELISA technology (CellTrend, Luckenwalde).

For preparation of IgG fractions IgG was isolated from serum by using Protein G columns (NAb Protein G Plus, Thermo Fisher) and subsequently dialyzed against PBS (Slide-A-Lyzer™ G2 Dialysis Cassette, Thermo Fisher) according to the manufacturer’s protocols. Protein concentration was determined by BCA assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher). IgG fractions were stored in Aliquots at −80 °C until used.

2.3. β2 AdR and M3/4 mAChR expressing cell lines

Analysis of β2 AdR function of IgG was performed using human β2 AdR and β-arrestin reporter Chinese hamster ovary reporter cells (CHO-K1 ADRB2, 93-0182E2, eurofins) according to the protocol (β-Arrestin eXpress GPCR Assay from DiscoverX, eurofins). Further β2 AdR activation was studied by measuring the β2 AdR dependent cAMP production using reporter CHO cells (cAMP Hunter™ eXpress ADRB2 (B2AR) CHO-K1, eurofins) according to the manufactures protocol (cAMP Hunter™ eXpress, eurofins). Analysis of M3 and M4 mAChR function was performed using human M4 mAChR β-arrestin CHO reporter cells (CHR3 CHO-K1, 93-0349E2, eurofins) or human M3 mAChR β-arrestin bone osteosarcoma epithelial reporter cells (CHR3 M3 U2OS, 93-0860E3, eurofins) respectively. Cells were cultured with 100 µg/ml IgG for indicated time points. The β-arrestin recruitment and the cAMP production are expressed in relative light units (RLU).

2.4. β2 AdR wt and ko mice

Splenocyte suspensions from gender and age matched β2 AdR deficient (β2 AdR ko) mice and β2 AdR wt littermates (strain name: B6.129R1-Adrb2tm1Bkk) were stimulated with 200 ng/ml LPS (Enzo) in presence or absence of human IgG fractions [100 µg/ml] for 18 h. TNFx level in supernatant was assessed by ELISA (BioLegend).

2.5. Immune cell studies

The effect of IgG fractions was assessed on TNFx and IL-10 response and on T cell proliferation of blood immune cells in vitro. For TNFx and IL-10 response whole blood immune cells were stimulated for 18 h with LPS [2 ng/ml, Enzo] in presence or absence of IgG fractions [100 µg/ml]. TNFx and IL-10 level in supernatant were assessed by ELISA (BioLegend).

For T cell proliferation PBMC were stimulated with anti-CD3/CD28 beads (Miltenyi Biotec) (1:1) IgG [100 µg/ml] was added simultaneously. Proliferation of cells was measured after 96 h by CFDA dilution assay (Thermo Fisher).

Table 1

| Characteristics of ME/CFS patients and healthy controls (HC). |
|-------------------|------------------|------------------|
|                    | CFS AABhigh | CFS AABnorm | HC |
| age (median, range) | 36 (24–61) | 43 (26–47) | 40 (27–66) |
| Bell score (median, range) | 30 (20–40) | 40 (30–40) | n.a. |
| COMPASS-31 score (median, range) | 62.1 | 29.7 | n.a. |
| heart rate (median, range) | 94 (80–103) | 69 (58–81) | n.a. |
| β2 AdR units (median, range) | 19.2 | 7.6 (4.4–8.3) | 5.2 |
| β2 AdR % (median, range) | 0 (0–33.6) | 20 (0–100) | 80 (0–100) |

| M3 mAChR units (median, range) | 13.2 | 3.6 (2.9–6.4) | 3.3 (2.2–6.1) |
| M4 mAChR units (median, range) | 13.0 | 5.7 (5.0–11.4) | 7.3 (4.3–9.0) |
Statistical data analyses were done using the software GraphPad Prism 6.0. Nonparametric statistical methods were used. Continuous variables were expressed as median and interquartile range (IQR). Univariate comparisons of two independent groups were done using the Mann-Whitney-U test, comparisons of two dependent groups were done using the Wilcoxon matched-pairs signed-rank test. A two-tailed p-value of < 0.05 was considered statistically significant.

3. Results

3.1. IgG has agonistic effects on β2 AdR signaling via β-arrestin and cAMP which is attenuated in ME/CFS

We first studied if IgG has an effect on the β2 AdR signaling using a β2 AdR-transfected β-arrestin reporter cell line. We comparatively analyzed the effects of 16 polyclonal IgG fractions isolated from serum of 5 ME/CFS patients with elevated (CFS AABhigh), from 5 ME/CFS patients with normal levels (CFS AABnorm) of β2 AdR autoantibodies and from 6 HC. We observed that all IgG fractions from HC (p < 0.01) and from CFS AABnorm (p < 0.05) induced β-arrestin recruitment to β2 AdR in the reporter cells compared to unstimulated cells (Fig. 1A). Further all IgG fractions from HC and from CFS AABnorm (both p < 0.05) induced β2 AdR dependent cAMP production (Fig. 1B). In contrast IgG fractions from CFS AABhigh had little or no effect on β-arrestin recruitment or cAMP production (Fig. 1A+B).

As ME/CFS patients with elevated levels of β2 AdR had elevated M3 and M4 mAChR autoantibodies as well we also studied if IgG has an effect on the M3 and M4 mAChR β-arrestin recruitment to M3 mAChR (Fig. 1C) and to M4 mAChR (Fig. 1D) was not significantly effected in reporter cells treated with IgG fractions of any cohort.

3.2. IgG modulates LPS-induced cytokine response which is attenuated in ME/CFS

It has been shown that β2 AdR stimulation inhibits LPS-induced TNFα and enhances IL-10 production in human monocytes (Agac et al., 2018; Kavelaars et al., 2000). In a similar manner polyclonal IgG was shown to inhibit TNFα production in human monocytes (Murakami et al., 2012). In line with these previous studies we observed that IgG fractions from HC and CFS AABnorm significantly reduced LPS-induced TNFα production of blood immune cells from healthy donors (both p < 0.05, Fig. 2A). In contrast the IgG fractions from CFS AABhigh had no or little inhibitory effect.

In a similar manner we analyzed the effect of IgG on IL-10 production of blood immune cells from healthy donors. The IgG fractions from HC and CFS AABnorm had a significant costimulator effect on LPS-induced IL-10, which was less and not significant with IgG from CFS AABhigh (Fig. 2B).

Taken together, these findings provide evidence that IgG can modulate human monocyte cytokine production. Thereby IgG from CFS AABhigh has less or no effect.

3.3. IgG enhances T cell proliferation which is attenuated in ME/CFS

In a next set of experiments the effect of IgG fractions was assessed on T cell proliferation in vitro. Blood immune cells from 2 healthy donors were stimulated with anti-CD3/CD28 beads and proliferating T cells were measured after 96 h by CFDA dilution assay. Incubation with IgG fractions from HC and CFS AABnorm, respectively, had a costimulatory effect on T cell proliferation while IgG from CFS AABhigh had no effect (Fig. 3A). These findings strengthens the previous results showing that IgG fractions from HC have β2 AdR activity which is low to absent in CFS AABhigh.
3.4. IgG can modulate TNFα production in wt but not in β2 AdR ko mice

To confirm that IgG regulates the cytokine production via the β2 AdR we performed experiments with β2 AdR ko mice. In murine splenocytes of wt mice human IgG could enhance the LPS-stimulated TNFα production. Here, the 5 IgGs from CFS AABhigh showed a significantly higher costimulation of TNFα production than the 6 IgGs from HC or the 5 IgGs from CFS AABnorm (Fig. 4). In ko mice all IgGs had little costimulatory effect. Taken together these findings provide evidence that human IgG stimulated TNFα production is β2 AdR dependent in mice. In contrast to experiments in human monocytes IgG stimulates TNFα production. IgG fractions from CFS AABhigh again have a differential β2 AdR activity.

3.5. Effects of IgG together with isoprenaline and propranolol on LPS-induced cytokine response

We finally studied the effect of IgG on β2 AdR ligand modulation of cytokine production. In line with a previous study (Agac et al., 2018) we observed that LPS-induced IL-10 production is enhanced by stimulation with isoprenaline (Fig. 5A) which is inhibited by addition of propranolol (Fig. 5B). The IgG fractions from patients and HC had a synergistic effect with isoprenaline on LPS-induced IL-10 production which was not different between cohorts (Fig. 5A). Interestingly, the effect of isoprenaline together with propranolol on IL-10 secretion was differentially modulated by IgG with IgGs from HC showing the strongest and IgGs from CFS AABhigh the weakest costimulatory effect (Fig. 5B). We could also observe that LPS-induced TNFα is diminished by isoprenaline but IgG had no further effect (data not shown).

4. Discussion

Various antibodies against G-protein coupled receptors (GPCR) were described to act as allosteric receptor agonists or antagonists (Dragun et al., 2009; Wallukat et al., 1999). β2 AdR agonist antibodies were described in POTS and cardiac arrhythmia (Lee et al., 2011; Li et al., 2014). We here comparatively studied the effects of IgG from ME/CFS patients and HC on β2 AdR signaling and immune cell function in various cell-based assays (Table 2). We provide first evidence for dysfunctional β2 AdR antibodies in ME/CFS.

First, we could show that IgG from HC can stimulate β2 AdR signaling
via β-arrestin recruitment and cAMP production in β2 AdR-transfected reporter cell lines providing evidence for a natural functional β2 AdR antibody. This observation is in line with recent studies showing a network of natural antibodies against adrenergic, cholinergic and other GPCR receptors (Cabral-Marques et al., 2018). Using splenocytes from β2 AdR ko and littermate wt mice we could provide further evidence that IgG contains natural β2 AdR activating antibodies.

The anti-inflammatory regulation of monocyte cytokine production by the β2 AdR is well known. Various studies have demonstrated that β2 AdR agonists attenuate TNFα and increase IL-10 production (Guiro et al., 1997; Kavelaars et al., 2000). In a similar manner it was shown that IgG inhibits LPS-induced TNFα and enhances IL-10 production of monocytes (Fujii et al., 2013; Murakami et al., 2012). In accordance with these previous studies we observed that IgG from HC inhibits LPS-induced TNFα and enhances IL-10 production of monocytes. Further, we found that IgG from HC has a costimulatory effect on CD3/CD28-stimulated T cell proliferation. In a previous study an inhibitory effect of IgG on T cell proliferation was reported with an albeit 50-fold higher IgG concentration (MacMillan et al., 2009).

When we comparatively analyzed the effects of IgGs from HC and patients with ME/CFS, the IgGs from CFS AABhigh patients with elevated β2 AdR antibodies had no significant effect on β2 AdR signaling in the reporter cell lines. In line with these results we observed that CFS AABhigh IgG has little or no effect on monocyte cytokine production and T cell proliferation. Taken together these experiments provide evidence that the β2 AdR stimulating activity of IgG is attenuated in CFS AABhigh. Since the IgG in CFS AABhigh had no obvious effects neither on signaling nor on human cytokine expression and T cell proliferation, the β2 AdR antibody could be blocking or at least interplay with the physiological IgG function. We have, however, clear evidence that IgG from CFS AABhigh patients recognize the β2R and are functional from experiments in mice. In wt mice IgG from CFS AABhigh showed a higher costimulatory effect on TNFα production than IgG from HC or from CFS AABnorm. The costimulatory effect we observed in mice splenocytes was opposite to the inhibitory effect on human monocytes and may be either related to the fact that the β2R in mice is not fully homologous or that the spleen contains mostly differentiated macrophages.

Further we observed that IgG from all 3 cohorts has a synergistic effect with β2 AdR ligands isoprenaline and propranolol to enhance IL-10 production. Interestingly, this synergistic effect of isoprenaline and propranolol was least with IgG from CFS AABhigh. These findings provide first evidence that IgG can modulate the signaling of β2 AdR ligands which is altered in CFS AABhigh. Our findings are in accordance with the study by Kavelaars et al. showing a diminished stimulatory effect of the β2 AdR ligand terbutaline on monocyte IL-10 production of ME/CFS patients compared to healthy controls (Kavelaars et al., 2000).

Patients with ME/CFS frequently suffer from a severe and prolonged course of infections. Antibodies which impair adrenergic function could result in a diminished control of the proinflammatory response of monocytes. Further T cell proliferation may not adequately be controlled during infection. As β2 AdR are expressed on most cells, an impaired function of β2 AdR antibodies could explain several other findings in ME/CFS. β2 AdR play an important role in vasodilation and control blood flow to muscles during exertion. Decreased β2 AdR function in vascular endothelial cells could lead to a paradox vasoconstriction upon release of epinephrine due to the predominant activity of the α AdR mediating vasoconstriction. We and others observed capillary endothelial dysfunction in ME/CFS patients ((Newton et al., 2007) and own manuscript submitted). There is evidence from experimental studies indicating a role of β2 AdR autoantibodies in the development of endothelial dysfunction (Liu et al., 2013). We found a normalization of endothelial function in patients with elevated levels of β2 AdR antibodies who underwent immunoadsorption to remove β2 AdR antibodies (own manuscript submitted).

Norepinephrine and epinephrine levels were higher in ME/CFS than controls in two studies (Kavelaars et al., 2000; Wyller et al., 2016). Thus, one may speculate that patients with ME/CFS suffering from an impaired peripheral β2 AdR function have a compensatory upregulation of (nor) epinephrine levels. Oppositely it may be, that chronically elevated epinephrine levels result in β2 AdR antibody dysfunction. The main effect of β2R autoantibodies may not be the direct activation of the receptor but rather modulation of the action of the ligands. This would fit to the

Fig. 5. Effect of IgG on LPS-induced IL-10 production in blood immune cells from healthy donors in the presence of (A) isoprenaline [0.001 μM] alone or with (B) propranolol [10 μM]. 16 different IgG fractions [100 μg/ml] from ME/CFS patients (CFS AABhigh, n = 5; CFS AABnorm, n = 5) and healthy controls (HC, n = 6) were analyzed. One representative experiment of two is shown. For statistical analysis Mann-Whitney test was performed and median with interquartile range is shown. *p ≤ 0.05, **p ≤ 0.01.

Table 2

| Assay target          | IgG HC | IgG CFS AABhigh |
|-----------------------|--------|-----------------|
| β2 AdR reporter CHO-K1 cells | β-arrestin | ++ | n.s. |
| β2 AdR reporter CHO-K1 cells | cAMP | + | n.s. |
| human monocytes TNFα | - | * | n.s. |
| human monocytes IL-10 | + | n.s. |
| human T cells proliferation | + | n.s. |

Stimulation ++ ≤ 0.01, + ≤ 0.05, inhibition - ≤ 0.05, n.s. non-significant effect compared to without IgG.
observation that symptoms of ME/CFS aggravate upon exertion. Adreno-
ergic dysregulation could cause or contribute to the many enigmatic
findings and symptoms of ME/CFS. It needs to be further investigated
how β2 AdR antibodies modulate the ligand activity.
We and others observed elevated levels of antibodies against mAChR
as well (Loebel et al., 2016; Tanaka et al., 2003). In our study we found
no effect of IgG on the M3 and M4 mAChR in reporter cell lines although
CFS AAbs had elevated M3 and M4 AChR antibodies as well. Further
experiments with higher IgG concentrations and combinations with li-
gands are, however, needed to provide clear evidence that IgG has no
effect on M3 or M4 mAChR. As M3 mAChR have vasodilatory effects,
dysfunctional M3 mAChR could aggravate the β2 AdR
antibody-mediated vascular dysfunction.
In our study we observed a diminished immunomodulatory effect of
IgG in patients with normal levels of β2 AdR antibodies as well, which
suggests that dysfunctional β2 AdR antibodies may be present in ME/CFS
patients despite normal β2 AdR antibody levels. We do not know the
epitope of the β2 AdR antibodies yet. In this study we have measured the
antibodies against the whole receptor by ELISA. β1 AdR antibodies in
cardiomyopathy are described to specifically bind to the 1st and 2nd
extracellular loop of the β1 AdR (Wallukat and Schinke, 2014).

5. Conclusion

Our data provides evidence that IgG physiologically stimulates the β2
AdR and that this function is attenuated in ME/CFS patients. Further
there is first evidence that IgG from ME/CFS patients with elevated β2
AdR antibodies differentially modulate β2 AdR ligand signaling. Thus, it
is conceivable that various symptoms of ME/CFS including immune
activation and autonomic dysregulation could be mediated or aggravated
by dysfunctional autoantibodies against β2 AdR. First clinical studies
targeting autoantibodies were shown to be effective in ME/CFS
(reviewed in (Sotzny et al., 2018). Further studies are required to study
the function and targets of the dysfunctional β2 AdR antibodies and how
this can be overcome. This may open a perspective for specific targeting
of adrenergic dysfunction as treatment of ME/CFS.

Declaration of competing interest

CellTrend GmbH holds a patent on the use of β adrenergic receptor
antibodies in diagnosis of ME/CFS.

List of abbreviations

| ME/CFS | Chronic Fatigue Syndrome |
| β2 AdR | β2 adrenergic receptors |
| M3/M4 mAChR | M3 and M4 muscarinic acetylcholine receptors |
| AAB | autoantibody |

Funding

This work was supported by grants from ME Research UK, from
German Ministry of Economy (grant no. 16K041848 to CS and HH) and the
Weidenhammer-Zöfel Foundation.

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