Effects of autoantibodies against $\beta_1$-adrenoreceptor in hepatitis virus myocarditis on action potential and L-type Ca$^{2+}$ currents

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Abstract

AIM: To investigate the effects of autoantibodies against $\beta_1$-adrenoreceptor in hepatitis virus myocarditis on action potential and L-type Ca$^{2+}$ currents.

METHODS: Fifteen samples of autoantibodies against $\beta_1$-adrenoreceptor positive sera of patients with hepatitis virus myocarditis were obtained and IgGs were purified by octanoic acid extraction. Binding of autoantibodies against $\beta_1$-adrenoreceptor to guinea pig cardiac myocytes was examined by immunofluorescence. Using the patch clamp technique, the effects on the action potential and I$_{Ca,L}$ of guinea pig cardiac myocytes caused by autoantibodies against $\beta_1$-adrenoreceptor in the absence and presence of metoprolol were investigated. Cell toxicity was examined by observing cell morphology and permeability of cardiac myocytes to trypan blue.

RESULTS: The specific binding of autoantibodies against $\beta_1$-adrenoreceptor to guinea pig cardiomyocytes was observed. Autoantibodies against $\beta_1$-adrenoreceptor diluted at 1:80 prolonged APD$_{90}$, APD$_{95}$, and APD$_{60}$ by 39.2%, 29.1% and 15.2% respectively, and only by 7.2%, 5.3% and 4.1% correspondingly in the presence of 1 µmol/L metoprolol. Autoantibodies against $\beta_1$-adrenoreceptor diluted at 1:80, 1:100 and 1:120 significantly increased the I$_{Ca,L}$ peak current amplitude at 0 mV by 55.87±4.39%, 46.33±5.01% and 29.29±4.97% in a concentration-dependent manner. In contrast, after blocking of $\beta_1$-adrenoreceptors (1 µmol/L metoprolol), autoantibodies against $\beta_1$-adrenoreceptor diluted at 1:80 induced a slight increase of I$_{Ca,L}$ peak amplitude only by 6.81±1.61%. A large number of cardiac myocytes exposed to high concentrations of autoantibodies against $\beta_1$-adrenoreceptor (1:80 and 1:100) were turned into rounded cells highly permeable to trypan blue.

CONCLUSION: Autoantibodies against $\beta_1$-adrenoreceptor may result in arrhythmias and/or impairment of myocardiums in HVM, which would be mediated by the enhancement of I$_{Ca,L}$.

INTRODUCTION

Viral hepatitis remains a worldwide public health problem and is reported by the Chinese Ministry of Public Health as an infectious disease with the highest morbidity and mortality in China. Many complications are considered to be involved in viral hepatitis. Hepatitis virus myocarditis (HVM) as a consequence of hepatitis virus infection, secondary to Coxsackie virus myocarditis in morbidity in hepatitis virus predominated areas of China, displays severe cardiac manifestations in addition to liver impairment. It was recently found that some cases of severe fulminant hepatitis virus myocarditis were accompanied by arrhythmias and/or cardiac injury. These cases were screened with a high prevalence of circulating autoantibodies against $\beta_1$-adrenoreceptor (anti-$\beta_1$-receptor autoantibodies), which have been recognized in idiopathic dilated cardiomyopathy (DCM) and Chagas’ cardiomyopathy. Using the patch clamp technique, we examined the effects of anti-$\beta_1$-receptor autoantibodies from patients with HVM on the electrophysiological properties of cardiomyocytes so as to find the possible clinical significance of the autoantibodies.

MATERIALS AND METHODS

Samples collection

A peptide was synthesized according to the sequence of $\beta_1$-adrenoreceptor, H-W-W-R-A-E-S-D-E-A-R-R-C-Y-N-D-P-K-C-C-D-F-V-T-N-R and was used as an antigen in ELISA as described by our laboratory for screening of anti-$\beta_1$-receptor autoantibodies in serum samples of patients diagnosed as HVM according to the Chinese diagnostic criteria for adult acute myocarditis in 1999. Fifteen serum samples were selected.

Antibody purification

As described by Seddik et al., IgGs in anti-$\beta_1$-receptor autoantibodies-positive sera of patients with HVM were purified by octanoic acid extraction. The purity of IgGs was over 90%.

Cell preparation

Guinea pig ventricular myocytes were isolated essentially as described by Heubach et al. with a modification. An adult guinea pig weighing 200-300 g was anesthetized with sodium pentobarbital (30 mg/kg i.p.), and the trachea was cannulated for artificial respiration. The chest was opened, and the aorta was cannulated in situ. The heart was dissected and retrograde perfused on a Langendorf perfusion system at 37 °C. It was perfused first with Tyrode’s solution for about 1 min at a...
by immunofluorescence. The isolated guinea pig cardiac myocytes were fixed by acetone at 4 °C. The fixed cells were washed three times with cold PBS, incubated with anti-β1-receptors autoantibody-positive or negative sera which were absorbed by the above peptide sequence (1:10) overnight at 4 °C, and then washed 3 times in cold PBS, followed by incubation with goat anti-human IgG labeled with FITC (1:190) for 1 h at 37 °C. After rinsed with PBS, the slides were mounted with cover slips for floccosy.

Electrophysiology
Cardiac myocytes were placed in Tyrode’s solution and the Ca2+ resistant cells adhered to the coverslips of the recording chamber were selected for electrophysiological recording. Action potential and 1 Ca2+, were recorded using the whole-cell configuration of the patch-clamp technique. The cells were held in current-clamp mode or voltage-clamp mode using an Axopatch 200-A amplifier (Axon Instruments, USA). Action potential was elicited by constant current pulses of 1 nA amplitude and a 6 ms duration at a rate of 0.2 Hz.

In voltage-clamp experiments, the holding potential was set at −80 mV. Na+ and T-type Ca2+ channels were inactivated by applying a 100-ms prepulse to −40 mV immediately before each test pulse. The time course of changes in Ca2+ conductance was monitored by applying a 300-ms test pulse to 0 mV once every 5 seconds. For analysis of I-V relationship, after a 100-ms voltage step to −40 mV, 300 ms depolarizing voltage steps from −40 mV to +60 mV in 10 mV increments were used to elicit currents. Data were acquired and analyzed using the ISO2 (MFK, Germany) analysis software package.

Cell toxicity
Cell toxicity was examined by observing cell morphology and the permeability of cardiac myocytes to 20 g/L trypan blue. The live cardiac myocytes were rod-shaped and excluded trypan blue, whereas the dead cells were rounded and permeable to trypan blue.

Reagents
Tyrode’s solution contained (mmol/L) NaCl 135, KCl 5.4, MgCl2 1.0, Na2HPO4 0.33, CaCl2 1.8, HEPES 10 and glucose 10 (pH adjusted to 7.4 with NaOH). KB solution contained (mmol/L) MgCl2 5, KCl 40, KH2PO4 20, Taurine 20, Glutamicacid 50, EGTA 0.5, HEPES 10, glucose 10 (pH adjusted to 7.4 with KOH). All the chemicals for the electrophysiological experiment, enzymes and octanoic acid were purchased from Sigma Aldrich Company, USA. Metoprolol was a generous gift from AstraZeneca Company, USA. Goat anti-human IgG labeled with FITC was purchased from Wuhan Yafa Biotech Corp, China.

Statistical analysis
Data were expressed as mean±SEM and analysed using Student’s t test. P <0.05 was considered statistically significant.

RESULTS
Specific binding of anti-β1-receptors autoantibodies to cardiac myocytes
Green fluorescence in rod-shaped cardiac myocytes only in slides incubated with anti-β1-receptor autoantibodies-positive sera suggested the specific binding of anti-β1-receptor autoantibodies to cardiac myocytes.

Effects of anti-β1-receptor autoantibodies on action potential in cardiac myocytes in the absence and presence of metoprolol
The effects of anti-β1-receptor autoantibodies on action potential properties were tested in isolated cardiac myocytes in a current clamp mode of the patch clamp technique. After 5 min with stable action potentials, the antibodies diluted at 1:80 were superfused. The action potential duration (APD) was assessed as a duration to 20%, 50% and 90% repolarization (APD20, APD50 and APD90, respectively). Anti-β1-receptor autoantibodies diluted at 1:80 prolonged the APD in all phases of repolarization, and the increase averaged 39.2%, 29.1% and 15.2% for APD20, APD50 and APD90 vs control. Thus the plateau was markedly prolonged (Figure 2 and Table 1).

| Table 1 | Effects of anti-β1-receptor autoantibodies diluted at 1:80 on action potential phases (n=6) |
|---------|-----------------------------------|
|          | Control                     | Anti-β1-receptor autoantibodies |
| APD20(mV) | 67.3±1.8                     | 93.7±3.6                      |
| APD50(mV) | 148.8±5.4                    | 192±6.7                       |
| APD90(mV) | 195.6±8.2                    | 225±11.5                      |

*P <0.01 vs control.

Whereas exposure of cells to anti-β1-receptor autoantibodies diluted at 1:80 in the presence of 1 µmol/L metoprolol resulted in a slight prolongation of the APD. As in Table 2 shown, the increase averaged only 7.2%, 5.3% and 4.1% for APD20, APD50 and APD90, respectively.

| Table 2 | Effects of anti-β1-receptor autoantibodies diluted at 1:80 on action potential phases in the presence of 1 µmol/ L metoprolol (n=6) |
|---------|-----------------------------------|
|          | Control                             | Anti-β1-receptor autoantibodies and metoprolol |
| APD20(mV) | 65.8±1.1                           | 70.5±2.3                                   |
| APD50(mV) | 147.2±4.6                           | 155.0±6.3                                   |
| APD90(mV) | 196.2±5.1                           | 204.3±9.4                                   |
As described above, anti-β₁-receptor autoantibodies markedly prolonged the plateau, suggesting the antibodies might enhance I_{Ca-L}. We investigated the effect in the voltage clamp mode. Basal I_{Ca-L} was recorded 5-6 min after the cell membrane rupture, when I_{Ca-L} was stable. Exposure of cells to anti-β₁-receptors diluted at 1:80 led to a significant increase of the amplitude of I_{Ca-L}, as the time course of changes (Figure 3 A left) and the current traces of I_{Ca-L} (Figure 3 A right) illustrated. The enhancement of I_{Ca-L} was rapid, and generally 20-30 s were sufficient for the full effect of the antibodies to take place. The rapid run-down of I_{Ca-L} followed after around 1 min. In light microscopy, the clamped cells were observed contracting and deteriorating. Beyond this dilution, the potentiating effect of the antibodies on I_{Ca-L} was too large to make effective voltage control. In 5 cells, anti-β₁-receptor autoantibodies diluted at 1:80 caused an increase of 55.87±4.39% in the basal current (P<0.01 vs control), and the enhancement of I_{Ca-L} was in a concentration-dependent manner (Figure 3C).

I_{Ca-L} was recorded longer in cells exposed to anti-β₁-receptor autoantibodies diluted at 1:80 in the presence of 1µmol/ L metoprolol than that in the absence of metoprolol, with a slight increase of 6.81±1.61% (P<0.01 vs anti-β₁-receptor autoantibodies diluted at 1:80) (Figure 3B).

We also investigated the current-voltage (I-V) relationship by addition of anti-β₁-receptor autoantibodies in the absence and presence of metoprolol. Without shifting the I-V relationship, the antibodies caused a marked increase of current densities of I_{Ca-L}. Whereas in the presence of 1µmol/L metoprolol, a slight increase was found at positive and negative potentials (Figure 4). Overall, these results indicated that anti-β₁-receptor autoantibodies could enhance I_{Ca-L} as agonists for β₁-adrenoceptors.

**Effects of anti-β₁-receptor autoantibodies on I_{Ca-L} channels in the absence and presence of metoprolol**

A large number of cardiac myocytes were exposed to high...
concentrations (1:80 and 1:100) of anti-β₁-receptor autoantibodies. Approximately over 90% rod-shaped cells were turned into rounded cells highly permeable to the dye within 2 min (Figure 5).

Figure 5 Cell toxicity effect of cardiac myocytes exposed to high concentrations of anti-β₁-receptors autoantibodies. A: rod-shaped cells excluding trypan blue. B: rounded cells permeable to trypan blue.

DISCUSSION

It was documented that there was a high homology between mouse hepatitis virus and β₁-adrenoceptors. Therefore, autoimmune response directed against β₁-adrenoceptors can be initiated by cross reaction between these two molecules. In our clinical investigation, we found that the positive rate of hepatic virus antibodies and anti-β₁-receptor autoantibodies was highly consistent in patients with HVM. Photomicrographs of guinea pig cardiac myocytes treated with anti-β₁-receptor autoantibodies before and after absorbed by the peptide sequence of β₁-adrenoceptor epitope and subsequently a fluorescently-tagged second antibody revealed the specific binding of autoantibodies in HVM to cardiac myocytes.

In heart diseases, interaction between catecholamine and β₁-receptors could result in various arrhythmias especially tachycardia and was the main cause of sudden death. Based on our study, anti-β₁-receptor autoantibodies in HVM, as isoprenaline-like agonists for β₁-adrenoceptors were able to prolong the plateau of APD and enhance the Ca²⁺ permeability of cardiac myocytes via L-type Ca²⁺ channel, which triggers early after depolarization (EAD).

Ca²⁺ disorder was considered to be associated with myocarditis and cardiomyopathy. It was reported anti-β₁-receptor autoantibodies in DCV could display a positive chronotropic effect on cultured neonatal rat ventricular cells without desensitization, thus increasing the metabolic pressure of myocardial cells, which was further demonstrated to be mediated by I_Ca,1. The time-course changes of I_Ca,1 in the presence of anti-β₁-receptor autoantibodies in HVM might reflect the process of the impairment of cardiac myocytes. About one minute after reaching the maximal peak value within 20-30s, I_Ca,1 was observed running down and accompanied by the clamped cells contracting and deteriorating in the presence of anti-β₁-receptor autoantibodies diluted at 1:80. Whereas I_Ca,1 was recorded longer in cells exposed to anti-β₁-receptor autoantibodies diluted at 1:80 in the presence of 1 μmol/L metoprolol. Furthermore, cell toxicity was observed by cell morphology and the permeability of cells to trypan blue. Rod-shaped cardiac myocytes exposed to high concentrations (1:80 and 1:100) of the antibodies were turned into rounded cells highly permeable to trypan blue. The overall influx of I_Ca,1 caused by anti-β₁-receptor autoantibodies in triggered electrophysiological and biochemical changes of cardiac myocytes and β₁-adrenoceptor blocker (metoprolol) could inhibit these effects. These results may in fact contribute to the proper explanation of the findings in our clinical investigation. The clinical investigation indicated that myocardial injury of patients with positive anti-β₁-receptor autoantibodies was more severe than that of patients with negative anti-β₁-receptor autoantibodies. Some arrhythmias such as sinus tachycardia or ventricular arrhythmias were associated with anti-β₁-receptor autoantibodies. These patients benefited from the use of β₁-adrenoceptor blocker.

In conclusion, autoantibodies against β₁-adrenoceptors can result in arrhythmias and/or the impairment of myocardiums in HVM, which would be mediated by the enhancement of I_Ca,1. As a basic research, our investigation would help to give early diagnosis by examining the presence of autoantibodies against β₁-adrenoceptor and beneficial treatment by properly utilizing selective β₁-adrenoceptor blocker for hepatitis virus myocarditis.

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