Potential Relevance of $\alpha_1$-Adrenergic Receptor Autoantibodies in Refractory Hypertension

Katrin Wenzel1,9, Hannelore Haase3,9, Gerd Wallukat2,9, Wolfgang Derer1, Sabine Bartel2, Volker Homuth1, Florian Herse1, Norbert Hubner2, Herbert Schulz2, Marion Janczikowski1, Carsten Lindschau3, Christoph Schroeder1, Stefan Verloehren4, Ingo Morano2, Dominik N. Muller2, Friedrich C. Luft1,2, Rainer Dietz1, Ralf Dechend1*, Peter Karczewski4*

1 Medical Faculty of the Charité, Franz-Volhard Clinic and HELIOS Klinikum-Berlin, Experimental and Clinical Research Center, Berlin, Germany, 2 Max Delbrück Center for Molecular Medicine, Berlin, Germany, 3 Department of Nephrology, Hannover Medical School, Hannover, Germany, 4 E. R. D. eV, Berlin, Germany

Abstract

Background: Agonistic autoantibodies directed at the $\alpha_1$-adrenergic receptor ($\alpha_1$-AAB) have been described in patients with hypertension. We implied earlier that $\alpha_1$-AAB might have a mechanistic role and could represent a therapeutic target.

Methodology/Principal Findings: To pursue the issue, we performed clinical and basic studies. We observed that 41 of 81 patients with refractory hypertension had $\alpha_1$-AAB; after immunoadsorption blood pressure was significantly reduced in these patients. Rabbits were immunized to generate $\alpha_1$-adrenergic receptor antibodies ($\alpha_1$-AB). Patient $\alpha_1$-AAB and rabbit $\alpha_1$-AB were purified using affinity chromatography and characterized both by epitope mapping and surface plasmon resonance measurements. Neonatal rat cardiomyocytes, rat vascular smooth muscle cells (VSMC), and Chinese hamster ovary cells transfected with the human $\alpha_1A$-adrenergic receptor were incubated with patient $\alpha_1$-AAB and rabbit $\alpha_1$-AB and the activation of signal transduction pathways was investigated by Western blot, confocal laser scanning microscopy, and gene expression. We found that phospholipase A2 group IIA (PLA2-IIA) and L-type calcium channel (Cacna1c) genes were upregulated in cardiomyocytes and VSMC after stimulation with both purified antibodies. We showed that patient $\alpha_1$-AAB and rabbit $\alpha_1$-AB result in protein kinase C alpha activation and transient extracellular-related kinase (ERK1/2) phosphorylation. Finally, we showed that the antibodies exert acute effects on intracellular Ca$^{2+}$ in cardiomyocytes and induce mesentry artery segment contraction.

Conclusions/Significance: Patient $\alpha_1$-AAB and rabbit $\alpha_1$-AB can induce signaling pathways important for hypertension and cardiac remodeling. Our data provide evidence for a potential clinical relevance for $\alpha_1$-AAB in hypertensive patients, and the notion of immunity as a possible cause of hypertension.

Introduction

Autoimmunity with agonistic autoantibodies directed at endogenous receptors can cause Graves’ disease through the thyrotropin receptor and promotes insulin release via CD38 [1,2]. Agonistic antibodies that stimulate the angiotensin (Ang) II AT1 receptor have been described in preeclampsia and humorally mediated kidney transplant rejection [3,4]. The most compelling case for agonistic autoantibody-mediated disease has been that of $\beta_1$-adrenergic receptor autoantibodies observed in patients with dilated cardiomyopathy and in a rat model fulfilling Koch’s postulates [5]. However, autoantibodies could also merely be an epiphenomenon in many instances; convincing data are still pending.

$\alpha_1$-adrenergic receptor ($\alpha_1$-AR) signaling mediates several cardiovascular actions such as vascular smooth muscle cell (VSMC) contraction, cardiac inotropy, hypertrophy, and remodeling [6]. $\alpha_1$-AR are predominantly located postsynaptically on VSMC, where they are the targets of circulating norepinephrine and regulate VSMC contraction [7]. Sympathetic over-activity in hypertension and accompanying excess stimulation of postsynaptic $\alpha_1$-AR supports the use of selective $\alpha_1$-AR inhibitors as antihypertensive drugs. However, $\alpha_2$-AR blockade was discontinued in the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) because of a putative increased risk for heart failure [8], a highly controversial decision that has been sharply criticized [9]. The Valsartan Heart Failure Trial 2 (Val-HeFT2) showed that $\alpha_1$-AR blockade was beneficial in heart failure, $\alpha_2$-AR can contribute to cardiomyocyte hypertrophy. Huang et al demonstrated a protective effect of the $\alpha_1A$-AR-subtype in cardiac myocytes and defined an extracellular-
regulated kinase (ERK) signaling pathway that is required for myocyte survival [10]. Hearts of \(\alpha_1\)-AR gene-deleted mice had increased interstitial fibrosis, increased apoptosis, and failed induction of the fetal hypertrophic gene program after pressure overload, indicating that \(\alpha_1\)-AR are required for myocardial adaptation to stress [11,12].

Others and we have described agonistic autoantibodies (\(\alpha_1\)-AAB) against the \(\alpha_1\)-AR [13,14,15]. Earlier, we examined immunoglobulin fractions in 54 severely hypertensive patients and found \(\alpha_1\)-AAB in 44% [15]. However, 12% of normotensive control subjects also harbored \(\alpha_1\)-AAB. Zhou et al. immunized rats with a peptide from the \(\alpha_1\)-AR epitope [16]. The rats developed \(\alpha_1\)-AR antibodies (\(\alpha_1\)-AB) and cardiac hypertrophy. However, the link between \(\alpha_1\)-AB formation and cardiac remodeling remains unclear. We have now performed additional studies to elucidate the pathophysiological relevance of \(\alpha_1\)-AAB. We show that removal of \(\alpha_1\)-AAB with immunoabsorption is feasible and lowers blood pressure. We raised \(\alpha_1\)-AB in rabbit and purified the \(\alpha_1\)-AB from rabbits and \(\alpha_1\)-AAB from patients by affinity chromatography. After confirming binding and functional specificity of the isolated rabbit \(\alpha_1\)-AB and human \(\alpha_1\)-AAB, we performed gene expression analysis in cardiomyocytes and vascular smooth muscle cells (VSMC). We show that both rabbit \(\alpha_1\)-AB and human \(\alpha_1\)-AAB can evoke calcium signaling and contract vessel preparations via protein kinase C alpha (PKC-\(\alpha\)) activation and transient extracellular-related kinase (ERK 1/2) phosphorylation. Our data suggest that human \(\alpha_1\)-AAB can induce signaling pathways involved in hypertension and cardiac remodeling, suggesting a potential clinical relevance of \(\alpha_1\)-AAB.

**Results**

We analyzed \(\alpha_1\)-AAB in 81 patients with refractory hypertension, who required \(\geq 3\) antihypertensive medications. Severe hypertension-induced target organ damage was present in every patient, including funduscopy changes, cardiac abnormalities, and kidney damage. Forty-one patients (51%) featured \(\alpha_1\)-AAB as assessed by cardiomyocyte contraction assay. Microalbuminuria was detected in 33%, diastolic dysfunction in 87%, left ventricular (LV) hypertrophy in 85%, and reduced ejection fraction in 13% of \(\alpha_1\)-AAB positive patients. The patient characteristics are outlined in Table 1.

**Table 1. Clinical features of the patients classified as \(\alpha_1\)-AR-AA positive or negative.**

<table>
<thead>
<tr>
<th>(\alpha_1)-AAB</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>41</td>
<td>40</td>
</tr>
<tr>
<td>Age range</td>
<td>46–81 years</td>
<td>42–81 years</td>
</tr>
<tr>
<td>Male</td>
<td>59%</td>
<td>60%</td>
</tr>
<tr>
<td>Female</td>
<td>41%</td>
<td>40%</td>
</tr>
<tr>
<td>Medication classes</td>
<td>4.3</td>
<td>4.7</td>
</tr>
<tr>
<td>Goal blood pressure</td>
<td>77%</td>
<td>76%</td>
</tr>
<tr>
<td>Heart failure</td>
<td>64%</td>
<td>57%</td>
</tr>
<tr>
<td>Reduced ejection fraction</td>
<td>13%</td>
<td>24%</td>
</tr>
<tr>
<td>Diastolic dysfunction</td>
<td>87%</td>
<td>81%</td>
</tr>
<tr>
<td>Left ventricular hypertrophy</td>
<td>85%</td>
<td>76%</td>
</tr>
<tr>
<td>Microalbuminuria</td>
<td>33%</td>
<td>36%</td>
</tr>
</tbody>
</table>

Figure 1 shows data from the five \(\alpha_1\)-AAB positive patients undergoing immunoabsorption. The treatments were performed daily for 5 days. The cardiomyocyte contraction assay demonstrated gradually decreasing \(\alpha_1\)-AAB activity during the course of the five immunoabsorptions (Figure 1A, B). At the end of five days, the activity was in the normal range. The decreased \(\alpha_1\)-AAB activity persisted for the 180 day observation period (Figure 1C). Blood pressure recordings performed before and after 5 days of treatment showed a significant reduction in mean arterial pressure (MAP), compared to initial values that remained significantly reduced for the observation period of 180 days (Figure 1D).

\(\alpha_1\)-AAB were directed against the first extracellular loop (eL1) in 24% and against the second extracellular loop (eL2) in 27% of the hypertensive patients (Figure 2A). Epitope mapping for the first and second extracellular loop of the \(\alpha_1\)-AR by cardiac contraction assay is shown in Figure 2B and C. Overlapping peptide sequences were tested for competition in cardiac contraction assay. For extracellular loop 1 (eL1) the peptide sequence P1 (YWAFGR) diminished the spontaneous beating rate response completely. The peptide P2 (APEDET) competed for the antibody extracellular loop 2 (eL2) effect. Since activating autoantibodies against the second loop have already been described for the AT1-R and \(\beta_2\)-AR receptor, we analyzed \(\alpha_1\)-AAB against the second extracellular loop of \(\alpha_1\)-AR in this study.

We purified \(\alpha_1\)-AAB from patients by affinity chromatography using the peptide of the second extracellular loop of the \(\alpha_1\)-AR. We then used surface plasmon resonance (SPR) measurements to determine the binding affinity and specificity of purified autoantibody fractions. Sensorgrams, depicted in Figure 3, demonstrate that the antibodies displayed a high binding affinity (Kd~50 nM) to the biotinylated peptide corresponding to eL2 of the \(\alpha_1\)-AR immobilized to the SA-sensorchip. This affinity-purified antibody fraction also showed weak cross-reactivity with the biotinylated peptide corresponding to the eL1 of the \(\alpha_1\)-AR (Figure 3A). However, this binding was negligible compared to the strong eL2 interaction.

We next raised a peptide antibody against the second extracellular loop of the \(\alpha_1\)-AR (rabbit \(\alpha_1\)-AB) and purified the antibody on the same peptide-containing affinity matrix. In SPR experiments, the rabbit \(\alpha_1\)-AB produced regular association and dissociation kinetics reaching a signal of ~2500 RU at a concentration of 80 \(\mu\)g/ml (Figure 3B). Purified \(\alpha_1\)-AAB from patients and rabbit \(\alpha_1\)-AB increased the beating rate of rat cardiomyocytes. This effect was dose-dependent (Figure 3C and D). The maximal response was obtained at an antibody dilution of 1:200 (0.5 and 0.6 \(\mu\)g/ml medium, respectively). The positive chronotropic effect was blocked by the \(\alpha_1\)-AR antagonist prazosin (1 \(\mu\)M).

To elucidate the molecular pathways induced by \(\alpha_1\)-AAB, we performed gene expression studies. We used Affymetrix microarrays for quantification of mRNA expression in cardiomyocytes and VSMC after treatment with purified \(\alpha_1\)-AAB isolated from three different patients, rabbit \(\alpha_1\)-AB, human control IgG, and the \(\alpha_1\)-AR agonist phenylephrine (PE) for 24 h. We identified two genes with increased expression in the Affymetrix array that we then verified by TaqMan RT-PCR. These genes were \(PLA2-IIA\) and \(Cacna1c\), as shown in Table 2. The upregulation of both genes was inhibited by the \(\alpha_1\)-AR antagonist prazosin while no changes in gene expression were observed after the treatment with the control IgG.

We tested whether or not \(\alpha_1\)-AAB from patients and rabbit \(\alpha_1\)-AB could elicit Ca\(^{2+}\) signals. Figure 4A shows the reaction of neonatal cardiomyocytes intracellular Ca\(^{2+}\) transients in response to the addition of the purified patient \(\alpha_1\)-AAB. There was a fast increase in the 340 nm/380 nm ratio, which peaked at less than 60 seconds and declined within 2 minutes to a plateau value.
slightly above the control level before the addition of the $\alpha_1$-AAB. Thus, $\alpha_1$-AAB potently induced a rise in intracellular Ca\(^{2+}\) in the targeted neonatal cardiomyocytes. To elucidate whether or not this Ca\(^{2+}\) response could be common to an autoantibody-$\alpha_1$-AR loop 2-type interaction, we used a rabbit $\alpha_1$-AB. As shown in Figure 4B, this rabbit $\alpha_1$-AB also generated an acute positive Ca\(^{2+}\) response in the cardiomyocytes with a similar time course as did the human antibody preparation. These responses were not elicited by a control IgG. As illustrated in Figure 4C, an IgG prepared from an $\alpha_1$-AAB negative patient was not able to raise the intracellular Ca\(^{2+}\).

These results prompted myographic experiments in mesenteric arteries displayed in Figure 5. Treatment with KCl documented brisk viability of the preparation. PE and the rabbit $\alpha_1$-AB both

Figure 1. Effects of immunoadsorption on $\alpha_1$-AAB and blood pressure. (A) Ordinate shows neonatal cardiomyocyte spontaneous beating rate; abscissa shows response to immunoadsorption performed on a representative patient. The $\alpha_1$-AAB activity decreased with every immunoadsorption with a total decrease in response over time. (B) Figure shows the initial spontaneous beating rate (closed circles). After five immunoadsorptions, this response is reduced to basal values (open circles) in all 5 patients. (C) Mean response of a representative patient in cardiomyocyte contraction assay over time to immunoadsorption is shown. (D) Mean arterial pressure (MAP) was measured 5 and 180 days after immunoadsorption. MAP was significantly reduced compared to before immunoadsorption.

doi:10.1371/journal.pone.0003742.g001

Figure 2. Epitope mapping for extracellular loop 1 and loop 2 of $\alpha_1$-AR is given. (A) $\alpha_1$-AAB were directed against the first extracellular loop (eL1) in 24% and against the second extracellular loop (eL2) in 27% of the hypertensive patients. (B) The peptide sequence P1 (YWAFGR) and P2 (GRVFCNI) (partially) for eL1 were able to diminish the spontaneous beating rate response completely. (C) Epitope mapping for loop 2 is given for the following amino acid sequences: P1: GWRQPA, P2: APEDET, P3: TICQIN, P4: INEEPG, P5: GYVLFS. The sequence P2 (APEDET) was able to diminish the spontaneous beating rate response completely.

doi:10.1371/journal.pone.0003742.g002
were able to constrict the vessel (Figure 5A). The effect of \(\alpha_1\)-AAB from two patients and from rabbit \(\alpha_1\)-AB was compared to half-maximal and maximal effective PE dosages, producing 50% and 100% of vessel constriction. Patient \(\alpha_1\)-AAB and rabbit \(\alpha_1\)-AB showed marked vasoconstriction activity. Control IgG from human and rabbits had no effect (Figure 5B).

We then examined signal transduction. We concentrated on PKC-\(\alpha\) and ERK 1/2 as both have been shown to be important in \(\alpha_1\)-AR-stimulated hypertension-induced target organ damage [6]. As shown in Figure 6, we found that \(\alpha_1\)-AAB and rabbit \(\alpha_1\)-AB exposure to cardiomyocytes or VSMC resulted in PKC-\(\alpha\) activation, as did the positive PE control. These effects were blocked completely by the specific PKC-\(\alpha\) blocker, Go 6976. Additionally, the incubation of Chinese hamster ovary (CHO) cells stably transfected with human \(\alpha_1\)A-AR (CHO/\(\alpha_1\)A-AR) with \(\alpha_1\)-AAB resulted in PKC-\(\alpha\) activation, which was blocked by the peptide P2, corresponding to the binding site of the \(\alpha_1\)-AAB (Figure 7A).

We then studied ERK 1/2 phosphorylation and the upstream phosphatidylinositol 3 (PI3)-kinase activity in CHO/\(\alpha_1\)A-AR cells. Incubation with human \(\alpha_1\)-AAB resulted in ERK 1/2 phosphorylation. The PI3-kinase inhibitor, LY294002, inhibited the ERK 1/2 phosphorylation (Figure 7B). Additional hypertensive patients, who were either \(\alpha_1\)-AAB positive or negative in cardiomyocyte contraction assay, were characterized by ERK 1/2 activation with immunoblotting (Figure 8). \(\alpha_1\)-AAB from three patients induce a transient ERK 1/2 phosphorylation similar to that evoked by PE and rabbit \(\alpha_1\)-AB in neonatal cardiomyocytes. Phosphorylation was blocked by prazosin (Figure 8A). A similar activation was obtained in CHO/\(\alpha_1\)A-AR cells. The activation was specifically blocked by peptide P2 (Figure 8B). Fractions eluted from patients, who were negative in the cardiomyocyte contraction assay, failed to activate ERK 1/2.

doi:10.1371/journal.pone.0003742.g003

doi:10.1371/journal.pone.0003742.t002

**Table 2.** Differential expression of PLA2-IIA and Cacna1c in cardiomyocytes and VSMC after treatment with patient \(\alpha_1\)-AAB, rabbit \(\alpha_1\)-AB or PE (Fold changes in TaqMan analysis).
Discussion

The important findings in this study are that isolated α1-AAB and generated α1-AB show similar binding characteristics by surface plasmon resonance sensorgram and functional testing. The antibodies induce a Ca²⁺ dependent signal transduction cascade in VSMC and cardiomyocytes that are both important to the pathophysiology of hypertension and cardiac remodeling. By gene array studies, we showed that the isolated patient α1-AAB and rabbit α1-AB both upregulate two gene types, namely PLA2-IIA and Cacna1c. In a proof-of-concept, non-controlled trial, we show that removal of α1-AAB by immunoadsorption lowers blood pressure in α1-AAB positive patients with intractable primary hypertension.

The entire idea of “agonistic” autoantibodies contributing to cardiovascular disease is controversial [17]. Antibodies directed at receptors can block or stimulate their targets. Hallmark example of the latter action is Graves’ disease where antibodies directed at the thyroid stimulating hormone receptor exert an agonistic action. Recently, compelling evidence has been presented regarding agonistic antibodies directed against the α1-AR, the β-ARs, the Ang II AT1-R, and the platelet-derived growth factor-alpha receptor, as reviewed elsewhere [17]. The antibodies could play a pathogenic role in various cardiovascular diseases, including hypertension, cardiomyopathy, preeclampsia, acute humoral rejection, and connective tissue disease.

Earlier, we speculated that α1-AAB could contribute to hypertension [15]. Our goal here was to give the notion of α1-AAB a more robust scientific basis. Thus, we verified our candidate by generating α1-AB by immunization. Concomitantly, we subjected patients from our collective who harbored α1-AAB to immunoadsorption. We found in our uncontrolled trial that lowering α1-AAB titers was associated with a lower blood pressure. We did not perform a randomized double blind, crossover trial. Nonetheless, our study meets class I (safety) criteria and suggests that a future class II (efficacy) trial might be warranted. We cannot exclude the possibility that short-term effects of the immunoadsorption, such as circulating blood volume changes during apheresis or other confounders contributed to the reduction in blood pressure. Since the reduction in blood pressure persisted for the observation period of 180 days, we believe that removal of α1-AAB by apheresis was the main cause for the blood pressure reduction. Within the five days immunoadsorption period, the level of α1-AAB decreased gradually. Thereafter, α1-AAB could not be detected after successful immunoadsorption in these patients for the entire observation period.

Figure 4. Representative traces of responses in intracellular Ca²⁺ of cultivated neonatal rat cardiomyocytes exposed to isolated patient α1-AAB and rabbit α1-AB are shown. (A) α1-AAB isolated from the serum of a patient with refractory hypertension elicited a Ca²⁺ signal. (B) The rabbit α1-AB gave a similar Ca²⁺ signal. (C) A human control IgG preparation was unable to affect intracellular Ca²⁺. Cardiomyocytes were electrically stimulated at 1 Hz, and the peak Ca²⁺ was monitored.

Figure 5. Contractile effect of patient α1-AAB and rabbit α1-AB in mesenteric arteries is given. A brisk KCl response is documented. (A) Representative experiment showing the contractile response to phenylephrine (PE, 10 nM–10 μM) and rabbit α1-AB (50 μg in 5 ml PBS buffer). The PE and antibody responses were similar in kind. (B) Contractile response of human α1-AAB isolated from two patients (5 μg in 5 ml PBS buffer) and rabbit α1-AB (50 μg in 5 ml PBS buffer) are compared to the PE response (300 nM and 10 μM, respectively) is shown. Contractions are expressed as % KCl response from patient 1 and patient 2. The human antibody responses were less than the low and high-dose PE responses. The rabbit α1-AB responses approached the PE responses. Two columns on the right are control human and control rabbit IgG showing no response.
of 180 days. A similar state-of-affairs has been described for β1-AAB in dilated cardiomyopathy after immunoadsorption. These data suggest that immunomodulation by removal or neutralization of the AAB might represent a potential therapeutic strategy [18].

We isolated the detected α1-AAB and the α1-AB that we generated against the same epitope in rabbits, using the same affinity purification protocol. Since we used the same peptide sequence for epitope mapping in the cardiomyocyte contraction assay, immunization in the rabbit, and isolation in the affinity column, we are confident that we isolated solely α1-AB. Before using this material to learn more about α1-AR signaling in VSMC and cardiomyocytes, we confirmed binding specificity by functional (cardiac contraction assay and ERK 1/2 phosphorylation) and biochemical (surface plasmon resonance measurement) verification methods. We then performed an Affymetrix gene expression study. We followed up on the results to pursue two candidate genes, PLA2-IIA and Cacna1c that were upregulated in the display, as verified by RT-PCR. Both genes contribute to signaling pathways in hypertension and atherosclerosis. Phospholipases A2 are acute-phase reactants and play an important role in digestion and metabolism of phospholipids, as well as in production of precursors for inflammatory reactions. Plasma PLA2-IIA levels are increased in systemic inflammation, including, rheumatoid arthritis and cardiovascular diseases [19,20,21]. In infarcted hearts, expression of PLA2-IIA was markedly increased in damaged cardiomyocytes [22]. Inhibition of PLA2-IIA also prevented cardiac fibrosis in spontaneously hypertensive rats [23].

Another important finding accrued is the fact that both the α1-AAB from patients and the rabbit α1-AB affected intracellular Ca2+ at two different levels, namely the acute, short-term elevation of intracellular Ca2+, and the increased transcript expression of the voltage-gated L-type Ca2+ channel pore subunit. Acute administration of the purified antibodies to neonatal cardiomyocytes produced a typically shaped Ca2+ transient. The onset of the cytosolic Ca2+ response occurred within few seconds reaching its maximum at less than one minute. α1-AR stimulation potentiates L-type Ca2+ current through CaMK II activation in rat ventricular myocytes [24].

Figure 6. Protein kinase C alpha (PKC-α) activation in cardiomyocytes and vascular smooth muscle cells (VSMC) after 2 min incubation with PE, α1-AAB from patients, and rabbit α1-AB is demonstrated. Horizontal rows show regimens, namely, untreated control, PE, patient α1-AAB, and rabbit α1-AB. Vertical columns show cardiomyocyte and VSMC responses. Yellow shows PKC-α activation; red is more intense activation. G6 6976 is a PKC-α inhibitor, which blocked all responses. doi:10.1371/journal.pone.0003742.g006

Figure 7. PKC-α and ERK 1/2 activation in CHO cells stably transfected with human α1A-AR (CHO/α1A-AR) by α1-AAB is demonstrated. (A) Incubation of CHO/α1A-AR cells with α1-AAB for 2 min resulted in a PKC-α activation, which was blocked by the peptide P2, but not by the peptide P5. (B) ERK 1/2 phosphorylation after incubation with α1-AAB for 5 min is shown. Inhibition of the upstream phosphatidylinositol 3 (PI3)-kinase activity by the inhibitor LY294002 strongly reduced ERK 1/2 phosphorylation. Yellow shows PKC-α and ERK 1/2 activation, respectively; red is more intense activation. doi:10.1371/journal.pone.0003742.g007
Figure 8. Activation of ERK 1/2 in response to treatment with PE, α1-AAB from patients or rabbit α1-AB is shown. (A) Cardiomyocytes were treated for 5 and 15 min with 10 μM PE, 2.5 μg/ml α1-AAB or rabbit α1-AB, respectively. Equivalent amounts of protein were analyzed by Western blotting with anti-pERK 1/2 antibody (44/42 kD) and the amount of ERK was analyzed with anti-ERK 1/2 antibody. PE, patient α1-AAB, and rabbit α1-AB caused ERK 1/2 phosphorylation. (B) CHO/α1-AR cells were incubated with patient α1-AAB for 5 min. Lane 1 and 2 represent untreated cells. Patients 1, 4–8 were α1-AAB-positive in the cardiomyocyte contraction assay. "Patient" 9 represents a pool of five α1-AAB positive patients. Patients 10–12 were α1-AAB negative in the cardiomyocyte contraction assay. Their serum samples were processed identically to those of α1-AAB-positive patients. ERK 1/2 phosphorylation was inhibited by peptide P2, but not by peptide P5. Eukaryotic initiation factor 4E (eIF4E, 25 kD) antibody was used as translational control, the amount of ERK was analyzed with anti-ERK 1/2 antibody.

doi:10.1371/journal.pone.0003742.g008

Furthermore, rabbit antibody to the α1AR and autoantibodies against the AT1-receptor could activate the Ca\(^{2+}\) current [25,26]. The peak response of cytosolic Ca\(^{2+}\) to the antibodies apparently comprises a temporary imbalance of Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels and the sarcoplasmic reticulum Ca\(^{2+}\) release on one hand and Ca\(^{2+}\) sequestration into the sarcoplasmic reticulum and Ca\(^{2+}\) extrusion via the Na\(^{+}\)/Ca\(^{2+}\) exchanger on the other hand.

In addition to acute Ca\(^{2+}\) current stimulation, we found that long-term activation of the α1-AR pathway by patient α1-AAB and rabbit α1-AR increases transcript levels of the voltage-dependent L-type Ca\(^{2+}\) channel and the sarcoplasmic reticulum Ca\(^{2+}\) release on one hand and Ca\(^{2+}\) sequestration into the sarcoplasmic reticulum and Ca\(^{2+}\) extrusion via the Na\(^{+}\)/Ca\(^{2+}\) exchanger on the other hand.

We isolated immunoglobulin fractions from serum samples as described earlier [4,15]. For detection of autoantibodies, the immunoglobulin fractions were added to neonatal rat cardiomyocytes at a dilution of 1:20. For the neutralization experiments, synthetic peptides corresponding to the sequence of the first extracellular loop (YWAFGRVFCNIWA), and the second extracellular loop (PAPDECTICQINEE) of the human α1C-AR were each added in excess (0.05 to 0.1 μg) to the immunoglobulin fraction. The mixtures were shaken and placed in a refrigerator for 1 h. The 100 μl samples were then added to neonatal rat heart muscle cells cultured in 2 ml of medium to a final dilution of 1:40. The beating rate was counted for 15 sec, 5 and 60 min after the addition of the peptide/immunoglobulin mixture. We tested α1-AAB activity in a neonatal cardiomyocyte assay without and with

α1-AR Autoantibodies

Materials and Methods

Patients

Patients requiring three or more medication classes to achieve goal control blood pressure values were recruited from the Franz-Vollhard Clinic outpatient department. All had been studied intensively and secondary causes of hypertension had been ruled out. Home and clinic blood pressures in these patients were determined with an automated oscillometric device under standardized conditions. The internal review board approved the study and written informed consent was obtained from all participants. Five patients harboring α1-AAB were asked to undergo five immunoabsorption treatments with an especially prepared column according to procedures described elsewhere [34].

α1-AAB detection

We isolated immunoglobulin fractions from serum samples as described earlier [4,15]. For detection of autoantibodies, the immunoglobulin fractions were added to neonatal rat cardiomyocytes at a dilution of 1:20. For the neutralization experiments, synthetic peptides corresponding to the sequence of the first extracellular loop (YWAFGRVFCNIWA), and the second extracellular loop (PAPDECTICQINEE) of the human α1C-AR were each added in excess (0.05 to 0.1 μg) to the immunoglobulin fraction. The mixtures were shaken and placed in a refrigerator for 1 h. The 100 μl samples were then added to neonatal rat heart muscle cells cultured in 2 ml of medium to a final dilution of 1:40. The beating rate was counted for 15 sec, 5 and 60 min after the addition of the peptide/immunoglobulin mixture. We tested α1-AAB activity in a neonatal cardiomyocyte assay without and with
peptide inhibition. Pulsation rate was exhibited 1 h or 24 h, respectively after incubation with α1-AR AAB and compared with the spontaneous basal pulsation rate. Eight synchronously contracting cell clusters per flask were counted for 15 sec on a heat table stage.

**α1-AB generation in rabbit and antibody purification**

We immunized rabbits against a peptide corresponding to the amino acid sequence of the second extracellular loop of the isoform A of human α1-AR (PAPEDETICQINEE) by BioGenes GmbH (Berlin, Germany). The corresponding peptide of the second extracellular loop of the α1-AR was covalently bound to ε-aminoacryl agarose (Sigma-Aldrich, Munich, Germany) to yield epitope-specific affinity beads. For coupling the agarose gel matrix (~1 ml of packed gel) was activated by glutaraldehyde (1% solution, freshly prepared) for 20 min at room temperature on a rotating wheel. The activated gel was extensively washed with PBS and reacted with α1-AAR for 3 h at room temperature or overnight at 4°C on a rotating wheel. The coupling reaction was terminated by incubation with 200 mM Tris/Glycine buffer pH 7.2. The affinity beads were poured into a column (Bio-Rad, Munich, Germany) and stored with 0.02% sodium acid in buffer A consisting of 50 mM Tris/HCl, 0.5 M NaCl, pH 7.4, at 4°C.

The affinity beads were washed with buffer A to remove the sodium acid. Serum samples (5–15 ml) from patients with refractory hypertension or from immunized rabbits were incubated with the affinity beads overnight at 4°C on a rotating wheel. The beads were allowed to settle and the sera were removed and stored for a second round of affinity purification at a given matrix. The beads were washed with buffer A until baseline levels of protein are detected at 280 nm. The antibodies were eluted at room temperature in 1 ml fractions with 50 mM Tris/Glycine, 0.5 M NaCl, pH 2.5. Antibody fractions were immediately neutralized with 0.5 ml of 0.5 M Tris/HCl, 0.5 M NaCl, pH 7.4. The antibody concentration was calculated by measurement the absorption at 280 nm.

**Surface plasmon resonance measurements**

Binding experiments were performed in a BIAcore 2000 Instrument (Uppsala Sweden) at 25°C. N-terminally biotinylated peptides corresponding to the first and second extracellular loop of the α1A-AR were immobilized at binding levels of 100 relative units (RU) each on parallel lanes of a SA-biosensor chip. Affinity-purified antibodies from patient blood samples were injected in the flow cells at a rate of 20 µl/min in HBSE running buffer consisting of 10 mM HEPES, pH 7.4, 150 mM NaCl, and 3 mM EDTA.

The regeneration was performed after binding measurements using 5 mM Tris/Glycine, 50 mM NaCl, pH 2.5, with no decrease in extent measurements over the duration of an experiment. Data were analyzed using the software corrects for baseline drift during measurements. The curves were fitted to a single-site interaction model. Kd values were calculated using the formula Kd = k_off / k_on, which k_off and k_on is the rate constant of dissociation and association kinetics, respectively.

**Cell culture and autoantibody incubation**

Rat neonatal cardiomyocytes were prepared from ventricles of 1–2 day-old Wistar rats using a modified method [35]. The cells were cultured as monolayers for 4 days at 37°C in SM 20-1 medium supplemented with 10% heat-inactivated calf serum, 2 mM fluorodeoxyuridine and penicillin/streptomycin. Aortic VSMC were isolated from Sprague Dawley rats as described previously [36]. CHO cells were stably transfected with human α1A-AR (CHO/α1A-AR) using a pSW104 vector and were cultured in F12 HAM medium supplemented with glutamine, 10% FCS and 1% penicillin/streptomycin as described earlier [37]. For gene expression analysis cardiomycocytes and VSMC, respectively were incubated with human control IgG endogin (5 µg/ml medium, Baxter, Wien, Austria), α1-AAB from different patients (2.5 µg/ml medium), rabbit α1-AB (2.5 µg/ml medium), and with the α1-AR agonist PE (10 µM, Sigma-Aldrich) for 24 h in DMEM medium containing 1% serum. Experiments were repeated with three different cardiomycocytes and VSMC preparations. For investigation of protein phosphorylation, cardiomycocytes and CHO/α1A-AR cells were maintained in serum-free media for 24 h or 4 h, respectively and treated with PE, human α1-AAB or rabbit α1-AB for 5 and 15 min, respectively. For inhibition experiments, prazosin (1 µM, Sigma-Aldrich) was added. Five µg of the peptides P2 (APEDET) or P5 (GYLVLF) were given to 2.5 µg of α1-AAB 1 h before cell treatment. For the inhibition of ERK 1/2 activation, CHO/α1A-AR cells were pre-incubated with PI3-kinase inhibitor LY294002 for 10 min.

**Gene expression analysis**

We extracted total RNA from cardiomycocytes treated with human control IgG, α1-AAB, rabbit α1-AB or PE using the RNeasy Purification Kit (Qiagen GmbH, Hilden, Germany). RNA was treated by deoxyribonuclease I (Qiagen). Two µg RNA of cells were transcribed in cRNA with One-Cycle Target labeling and Control Reagents (Affymetrix, Santa Clara, CA, USA). Non-pooled microarray experiments were performed with cRNA prepared from independent cardiomycocyte cell preparations using Rat Genome 230 2.0 Arrays (31,099 probe sets, Affymetrix). After passing the quality control for each experiment a set of RNA normalized expression values have been produced. The log scale robust multi-array analysis (RMA) estimates are based upon a robust average of log2 (B (PM)), where B (PM) are background corrected perfect match intensities [38]. For statistical comparison of expression data student’s t-test was used.

**Quantitative Real-Time Reverse Transcriptase PCR (TaQMan)**

cDNA was synthesized from 2 µg of total RNA isolated from cardiomycocytes and VSMC, respectively using PowerScript Reverse Transcripase (BD Bioscience Clontech, Palo Alto, USA) and an Oligo (dT)18 primer. Real-time PCR experiments were done using the Mx3000P® real-time PCR system (Stratagene Europe, Amsterdam, NL) and the Brilliant QPCR master mix (Stratagene Europe). Real-time PCR was performed with non-pooled samples from cardiomycocytes and VSMC respectively. Beta-2-microglobulin (B2M) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were used as endogenous references to normalize expression of a target gene. For every sample three independent runs in triplicates and the relative changes in gene expression were quantified by comparative Ct method [39]. Primer sequences are listed in Table 3.

**Immunocytochemistry**

We described the techniques for confocal microscopy and immunocytochemistry as referenced previously [4,10]. The cells were fixed with 4% paraformaldehyde and permeabilized with 80% methanol at ~20°C. After incubation with 2% BSA in PBS for 60 minutes, the preparation was incubated for 1 hour at room temperature with the monoclonal anti-PKC-α antibody from UBI (clone M4) diluted in PBS with 1% BSA (1:80) or pERK 1/2 antibody (1:200, Cell Signalling Technology, Boston, MA, USA), respectively, washed twice with PBS, and then exposed to the secondary antibody (Alexa-480-conjugated anti-mouse IgG at 1:200,
Table 3. Primer and probe sequences used for TaqMan RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences (5′−3′)</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2M</td>
<td>for GCT CCG TGA CCG TGA TCT TT</td>
<td>NM_012512</td>
</tr>
<tr>
<td></td>
<td>rev GAG TTT TCT GAA TGG CAA GCA</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>for CAA CCG CAC AGT CAA G</td>
<td>NM_017008</td>
</tr>
<tr>
<td></td>
<td>rev TGG TCT CTT GAA CAT G</td>
<td></td>
</tr>
<tr>
<td>PLA2-IA</td>
<td>for GGA CTC CTT CCG GAA ACA G</td>
<td>NM_031598</td>
</tr>
<tr>
<td></td>
<td>rev TTC CCG GCA AAA CAT TCA G</td>
<td></td>
</tr>
<tr>
<td>Cacna1C</td>
<td>for TCA CTG CTG TGG GAA TAA GC</td>
<td>NM_02517</td>
</tr>
<tr>
<td></td>
<td>rev GCC CTT CTC CCC GAA AAA G</td>
<td></td>
</tr>
</tbody>
</table>

Used annealing temperature was 58°C.

doi:10.1371/journal.pone.0003742.t003

1% BSA/PBS; Invitrogen, Life Technologies, Carlsbad, CA, USA) for 60 minutes. The preparation was mounted with AquaPolymount (Polyscience, Niles, IL, USA) under a glass coverslip on a Nikon-Diaphot microscope. An MRC 1024 confocal imaging system (Bio-Rad) with an argon/krypton laser was used. At least 25 to 40 cells from each of at least three experiments were examined under each experimental condition.

Western blotting

Cardiomyocytes and CHO/γ1-AR cells were scraped in lysis buffer [20 mM HEPES (pH7.9), 350 mM NaCl, 20% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP40, complete protease inhibitor cocktail (Roche Diagnostic GmbH, Mannheim, Germany) and phosphatase inhibitor cocktail (Sigma-Aldrich)] and centrifuged at 10,000 rpm for 10 min. Protein concentrations were measured with Bradford method and equal amounts of proteins were analyzed by Western blotting. ERK phosphorylation was detected by pERK 1/2 antibody (1:1000, Cell Signaling Technology). As translational control an ERK antibody (1:1000, Cell Signaling Technology) and eukaryotic initiation factor 4E (eIF4E) antibody (1:7000, Cell Signaling Technology) were used. HRP-conjugated goat anti-rabbit secondary antibody (1:5000, Jackson ImmunoResearch Europe, Suffolk, UK) was used. Detection was performed with the ECL™-substrate (Lumigen, Southfield, MI, USA) according to the manufacturers instructions.

Measurement of cytosolic Ca2+ transients

Neonatal cardiomyocytes were plated onto Labtek four chamber slides (Nunc GmbH & Co, Wiesbaden, Germany) suited for fluorescence measurements at a density of 0.2×10⁶ cells per chamber. After cultivation for four days the medium was removed and cells were washed twice with 10 mM Hepes-buffered Hank’s salt solution, pH 7.4 (HBSS). Cardiomyocytes were incubated on HBSS for 60 minutes at 37°C. Then the solution was replaced by 0.5 ml HBSS containing 2.5 μM final concentration of Fura 2-AM (Calbiochem AG, Luzern, Switzerland) and left for loading in the dark at room temperature. After 30 min the loading solution was aspirated, the cells washed and kept on 0.5 ml HBSS in the dark and at room for another 30 minutes before use. Measurements of cytosolic Ca2+ transients were performed on an IonOptix Fluorescence and Contractility System (Milton, MA, USA) equipped with a Leica microscope with a heatable stage. All measurements were carried out at 37°C. Cardiomyocytes were electrically stimulated at 1 Hz and the ratio of 340 nm/380 nm was recorded. After stabilization of the Fura signal the control trace was taken. Antibody preparations were applied in a constant volume of 100 μl of prewarmed HBSS to give a final concentration of 2 μg/ml. For evaluation of Ca2+ changes peak values were derived from the trace recordings representing the maximum cytosolic Ca2+ achieved in the contracted cell.

Contraction of mesenteric arteries

Vessel rings from superior mesenteric arteries of male Sprague-Dawley rats (200 to 300 g, 6 to 8 weeks) were prepared and intracellular membrane potential was measured as earlier described [41]. In the first series of experiments, the rings were exposed to increasing doses of PE (10 nM–10 μM) and with acetylcholine (10 nM–10 μM) for relaxation. Following, vessel rings were incubated with γ1-AAB (5 μg in 5 ml PBS buffer), rabbit γ1-AR (50 μg in 5 ml PBS buffer), rabbit IgG (50 μg in 5 ml PBS buffer, Dumn Labortechnik GmbH, Asbach, Germany) and human control IgG endobulin (50 μg in 5 ml PBS buffer).

Statistics

We relied on student’s t-tests (adjusted as necessary) for normally distributed data and performed analysis of variance (repeated measures were indicated). P<0.05 was accepted as significant. Data are expressed as mean±SD.

Acknowledgments

We thank Karin Karczewski and Petra Hempel for the cultivation of cardiomyocytes and Steffen Luther for the surface plasmon resonance measurements. We are indebted to Prof. Martin Michel, Dept. Pharmacology & Pharmacotherapy the University of Amsterdam, Netherlands for providing CHO cells stably transfected with human γ1-AR.

Author Contributions

Conceived and designed the experiments: KW HH GW DNM FCL RD PK. Wrote the paper: KW HH WD SB IM PK. Contributed reagents/materials/analysis tools: KW HH GW DNM FCL RD RD PK. Acknowledgments

References