Article

Circulating IgGs in Type 2 Diabetes with Atrial Fibrillation Induce IP$_3$-Mediated Calcium Elevation in Cardiomyocytes

Yanhong Luo, Xian Liu, Ruilian Ma, Yigang Wang, Mark Zimering, Zui Pan

mark.zimering@va.gov (M.Z.)
zui.pan@uta.edu (Z.P.)

HIGHLIGHTS

Identification of cardiomyocyte-targeting IgGs in T2DM atrial fibrillation patients

Induction of arrhythmogenic Ca^{2+} signaling by these IgGs

Independent of voltage-gated or store-operated Ca^{2+} channels

Involvement of GPCR-IP$_3$-IP$_3$R axis in IgG-evoked intracellular Ca^{2+} elevation

Article

Circulating IgGs in Type 2 Diabetes with Atrial Fibrillation Induce IP$_3$-Mediated Calcium Elevation in Cardiomyocytes

Yanhong Luo,[1,4,8] Xian Liu,[2,3,8] Ruilian Ma,[5] Yigang Wang,[5] Mark Zimering,[6,7,*] and Zui Pan[1,2,3,9,*]

SUMMARY

Higher risk of cardiac arrhythmias including atrial fibrillation (AF) associates with type 2 diabetes mellitus (T2DM) with the underlying mechanism largely unknown. The present study reported a subset of circulating immunoglobulin G autoantibodies (IgGs) from patients with T2DM with AF (T2DM/AF)-induced intracellular calcium elevation in both human induced pluripotent stem cell (iPSC)-derived and mouse atrial cardiomyocytes, whereas (identical concentrations of) IgGs from patients with T2DM without AF could not. The IgG-evoked intracellular calcium elevation was insensitive to verapamil, mibefradil, or BTP-2, indicating calcium source from neither voltage-gated calcium channels nor store-operated calcium entry. On the other hand, pharmacological antagonism or genetic knockdown of inositol triphosphate (IP$_3$) receptor significantly decreased T2DM/AF IgG-induced intracellular calcium elevation. Furthermore, pharmacological blockage of G protein-coupled receptor (GPCR), heterotrimeric G protein or phospholipase C dampened IgG-induced intracellular calcium elevation. Taken together, circulating IgGs from patients with T2DM/AF stimulated arrhythmogenic intracellular calcium elevation through IP$_3$ pathway in atrial cardiomyocytes.

INTRODUCTION

Diabetes mellitus (DM) affects 30.3 million people in the United States, i.e., 9.4% of the population, according to the 2017 National Diabetes Statistics Report (Centers for Disease Control and Prevention, 2017). Type 2 diabetes (T2DM) accounts for 90%–95% of all diabetic cases (Centers for Disease Control and Prevention, 2017). Ischemic and non-ischemic cardiovascular disease have been recognized for decades as the leading cause of morbidity and mortality in older adult T2DM (Stamler et al., 1993). Atrial fibrillation (AF) is the most common life-threatening cardiac arrhythmia in aging populations, and AF increases significantly in older persons having hypertension and T2DM (Huxley et al., 2011). Yet the underlying mechanism for the known association between AF and adult T2DM remains obscure (Sun and Hu, 2010).

Type 2 diabetes is not an autoimmune disease. Yet circulating IgG autoantibodies have been reported in patients with advanced T2DM in association with specific neurovascular complications (Zimering, 2017; Zimering and Pan, 2009, 2017). For example, in our prior report, IgG autoantibodies purified from patients with T2DM suffering with macular edema and albuminuric nephropathy potently induced stress fiber formation and Rho kinase-mediated apoptosis in endothelial cells (Zimering and Pan, 2009). Clustering of microvascular diabetic complications, i.e., painful neuropathy, maculopathy, and nephropathy triopathy, was observed in adult patients with T2DM harboring highly potent endothelial inhibitory autoantibodies (Zimering et al., 2011). Triopathy diabetic IgG autoantibodies evoked significant intracellular calcium elevation in endothelial cells as well as in differentiated, neuronal-like rat pheochromocytoma PC12 cells. Since many of the same patients manifested non-ischemic cardiomyopathy such as clinically significant AF and/or left ventricular hypertrophy, the possibility was suggested that IgG autoantibodies may alter intracellular calcium signals in cardiomyocytes as well. Disturbance in calcium homeostasis has been implicated in the pathogenesis of AF and other cardiovascular diseases (Harada et al., 2015); thus, these IgGs may be arrhythmogenic and play an important role in T2DM non-ischemic cardiomyopathy. The present study provides evidence that circulating IgG autoantibodies from patients with T2DM with AF stimulated arrhythmogenic intracellular calcium release and revealed their signaling pathway in cardiomyocytes.

Calcium signaling has been known to be involved in the development of AF. AF often progresses from an intermittent to persistent stage and finally into permanent AF, which lasts for more than a year and remains as chronic (Heijman et al., 2014). The primary arrhythmia mechanisms underlying AF can be characterized

[1]Davis Heart and Lung Research Institute, Ohio State University-Wexner Medical Center, Columbus, OH 43210, USA

[2]Department of Kinesiology, University of Texas at Arlington, Arlington, TX 76010, USA

[3]College of Nursing and Health Innovation, University of Texas at Arlington, Arlington, TX 76010, USA

[4]Department of Endocrinology, The Children's Hospital of Chongqing Medical University, Chongqing, P. R. China

[5]Division of Regenerative Medicine Research, Department of Pathology and Laboratory Medicine, University of Cincinnati, Cincinnati, OH 45219, USA

[6]Endocrinology, Veterans Affairs New Jersey Healthcare System, East Orange, NJ 07018, USA

[7]Rutgers-Robert Wood Johnson Medical School, New Brunswick, NJ 08901, USA

[8]These authors contributed equally

[9]Lead Contact

*Correspondence:
mark.zimering@va.gov (M.Z.),
zui.pan@uta.edu (Z.P.)
https://doi.org/10.1016/j.isci.2020.101036

Risk Factor	Diabetes		p Value^, *
	AF (N = 28)	No AF (N = 22)	
Age (years)	63.9 ± 8.1	65.0 ± 11.5	0.57
HbA$_1$c (%)	8.0 ± 1.4	8.2 ± 1.8	0.39
HTN (%)	82	77	0.73
Insulin use (%)	64	35	0.045
ME, AMD (%)	29	23	0.75
Nephropathy (%)	18	14	1.00

Table 1. Baseline Clinical Characteristics in Participants
HbA$_1$c, glycosylated hemoglobin; ME, macular edema.
AMD, age-related macular degeneration; HTN, hypertension.
p Value: ^T test for continuous variables (age, glycosylated hemoglobin); *Fischer's exact test for dichotomous variables (HTN, insulin use, ME, AMD, nephropathy).

into two major types, the focal ectopic firing and the re-entrant activity, for both of which calcium has been reported to be involved. First of all, both AF animal models and human patient atrial samples show abnormal atrial calcium signaling in AF pathophysiology through contributing to the ectopic activity, conduction block, and calcium-induced subcellular alternans mediated by after depolarization (Nattel and Dobrev, 2016). Second, re-entry has been identified as the other predominant mechanism that initiates and maintains arrhythmias (Nattel and Dobrev, 2016). Re-entry requires both a vulnerable substrate and a trigger to act on the substrate and initiate AF (Nattel and Dobrev, 2016). This study seeks to examine the underlying mechanism of how circulating IgG autoantibodies could trigger arrhythmogenic calcium signals in atrial myocytes.

RESULTS

Purified IgG Autoantibodies from Patients with T2DM/AF Induced Intracellular Calcium Elevation in Human and Mouse Cardiomyocytes

To test whether IgG autoantibodies purified from patients with T2DM with AF (T2DM/AF) (Table 1) could alter intracellular calcium homeostasis in cardiomyocytes, human induced pluripotent stem cells (iPSCs) were differentiated into cardiomyocytes (Wang et al., 2014). These cells were loaded with fluorescent calcium indicator Fura-2-acetoxymethyl ester (Fura-2, AM) and intracellular calcium level (presented as F_{350nm}/F_{385nm}) was monitored using live cell imaging. As shown in Figure 1A, addition of purified IgG autoantibodies from patients with T2DM/AF could induce a rapid intracellular calcium elevation in these human iPSCs-derived cardiomyocytes. This was also seen in isolated adult mouse atrial cardiomyocytes as shown in Figure S2. Similar effects were observed in HL-1, a cardiomyocyte cell line derived from adult mouse atrium (Figure 1B). HL-1 cells contained the spontaneous calcium oscillations, and addition of purified IgGs from patients with T2DM/AF increased intracellular calcium and altered the oscillations' pattern (Figure 1B). Since HL-1 cells responded to these IgG autoantibodies similarly to human cardiomyocytes, this mouse cell line was used in the following studies.

HL-1 Cells Responded to IgG Autoantibodies from T2DM/AF but Not Control Subjects

To examine whether the intracellular calcium homeostasis disturbing-IgG autoantibodies are specific for patients with T2DM/AF, intracellular calcium levels in cultured HL-1 cells were compared using purified IgG autoantibodies from patients with T2DM with AF and patients with T2DM without non-ischemic cardiomyopathy, i.e., no AF or left ventricular hypertrophy (control subjects). Addition of 5 μg/mL of IgG autoantibodies purified from control subjects failed to stimulate significant intracellular calcium elevation in HL-1 cells, whereas IgG autoantibodies from patients with T2DM/AF at the same concentration could induce a rapid calcium elevation (Figure 2A). IgG autoantibodies purified from 24 of 28 patients with T2DM/AF had significant responses, whereas IgG autoantibodies from 21 of 22 of control subjects failed to do so. The mean level of intracellular calcium elevation (presented as $\Delta F_{350nm}/F_{385nm}$) in the autoantibodies from T2DM/AF (0.38 ± 0.01) significantly exceeded the mean level (0.08 ± 0.05) in control subjects (Figure 2B.
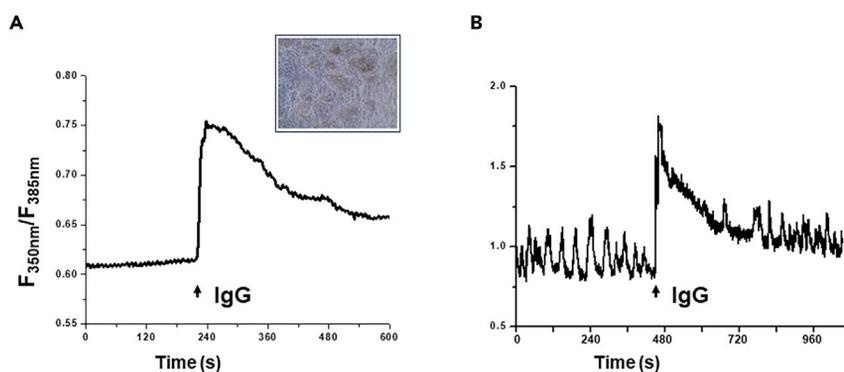
Figure 1. Purified IgG Autoantibodies from a Subset of Patients with T2DM and AF Induced Ca^{2+} Elevation in Both Human iPSC-Differentiated and Mouse Cardiomyocytes

Ratio of fluorescence of fura-2 at two excitation (F_{350nm}/F_{385nm}) was used to monitor changes in intracellular Ca^{2+} concentration.

(A) Representative trace of intracellular Ca^{2+} in iPSC-differentiated cardiomyocytes. The inserted panel is phase contract image of human iPSC-differentiated cardiomyocytes. IgGs: ~30 μg/mL, n = 8.

(B) Representative trace of intracellular Ca^{2+} in HL-1 cells. The spontaneous Ca^{2+} oscillations could be observed in most of the cardiomyocytes. IgGs: ~30 μg/mL, n = 12.

See also Figure S2.

*p < 0.01). Similar results were observed in HL-1 cells using increased concentration of IgGs (30 μg/mL) (Figures 2C and 2D).

Pharmacological Characterization of IgG Autoantibody-Induced Calcium Elevation in HL-1 Cells

To understand the mechanism underlying the IgG autoantibody-induced intracellular calcium elevation, the calcium sources were examined first using various pharmacological inhibitors. Incubation of verapamil up to 20 μM, which is known to almost completely inhibit L-type calcium channel in HL-1 cells (Xia et al., 2004), failed to block these IgG autoantibody-induced intracellular calcium elevation (Figure 3A). Mibefradil is an antagonist for both L-type and T-type calcium channels and has IC$_{50}$ at 0.4 μM for external K$^+$ induced-calcium influx in HL-1 cells. Up to 25 μM mibefradil failed to block the IgG autoantibody-induced intracellular calcium elevation (Figure 3B). BTP-2 has been shown to block store-operated calcium entry (SOCE)-mediated calcium influx in cardiomyocytes (Sabourin et al., 2018). Again, BTP-2 at concentration of 5 μM failed to block the IgG autoantibody-induced intracellular calcium elevation in HL-1 cells (Figure 3C). Interestingly, 100 μM concentration of 2-aminoethoxydiphenyl borate (2-APB) completely blocked the IgG-induced intracellular calcium elevation (Figure 3D). Addition of 20 mM caffeine at the end of experiments could induce a rapid calcium elevation (second arrow) indicating the sarcoplasmic reticulum (SR) calcium stores were intact. The quantification of the IgG autoantibody-induced calcium elevation is shown in Figure 3E. These data suggested that the IgG autoantibody-induced intracellular calcium elevation is independent of calcium influx from L-type, T-type calcium channels or SOCE.

IgG Autoantibody-Induced Intracellular Calcium Elevation was through IP$_3$ Receptor

Since 2-APB at 100 μM could block both SOCE and inositol triphosphate (IP$_3$) receptor (IP$_3$R) (Bootman et al., 2002), an IP$_3$-mediated mechanism for IgG autoantibody-induced calcium elevation was tested using a more specific antagonist for IP$_3$R, i.e., xestsphongin C (Oka et al., 2002). Incubation of HL-1 cells with 10 μM xestospongin C nearly completely blocked the IgG autoantibody-induced intracellular calcium elevation (Figures 4B and 4C), which was unaffected in HL-1 cells treated with vehicle alone (Figures 4A and 4C). The peak of intracellular calcium elevation responding to 20 mM caffeine indicated the intact SR calcium stores in both groups. To further examine the possible involvement of IP$_3$R in the IgG autoantibody-induced intracellular calcium elevation, the genes encoding IP$_3$R type 1 or type 2 were knocked down using a short hairpin RNA (shRNA) probe targeting the common sequences on the mouse mRNAs for IP$_3$R1 and IP$_3$R2 (Tjondrokoesoemo et al., 2013). The efficacy of knocking down IP$_3$R1 and IP$_3$R2 proteins has been confirmed previously (Tjondrokoesoemo et al., 2013) and currently by qRT-PCR (Figure S1). IgG
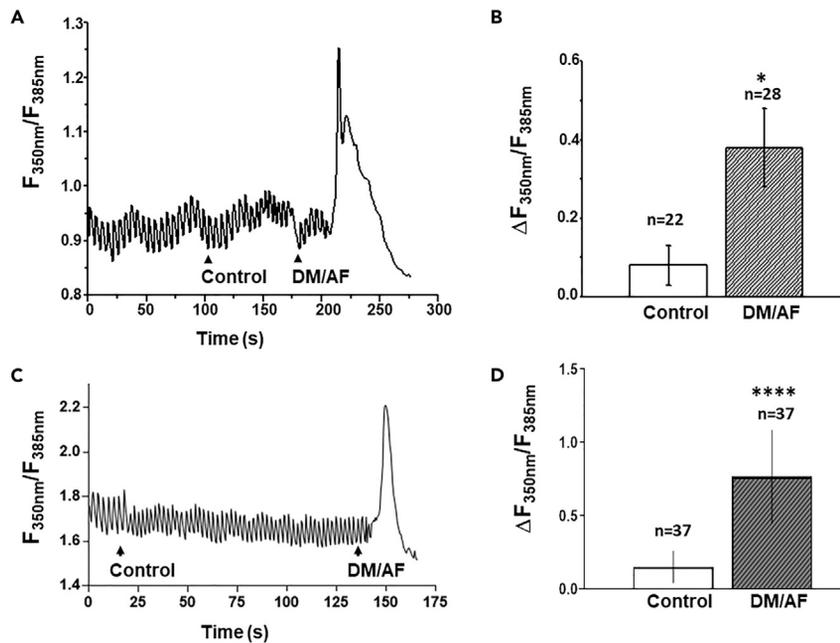
Figure 2. Intracellular Ca^{2+} Response in HL-1 Cells Treated with IgG Autoantibodies from Patients with T2DM with or without AF

(A) Representative trace of intracellular Ca^{2+}. The cardiomyocytes presenting spontaneous Ca^{2+} oscillations were selected for experiments. IgGs (2–5 μg/mL) derived from different patients were added at the indicated points (arrows). Control, T2DM without AF; DM/AF, T2DM with AF.

(B) Statistics of changes in intracellular Ca^{2+} stimulated by IgGs. *p < 0.01. Data are represented as mean ± SEM.

(C) Representative trace of intracellular Ca^{2+}. IgGs (30 μg/mL) derived from different patients were added at the indicated points (arrows).

(D) Statistics of changes in intracellular Ca^{2+} stimulated by IgGs. ****p < 0.0001. Data are represented as mean ± SEM. See also Figure S2.

autoantibodies from T2DM/AF failed to induce intracellular calcium elevation in HL-1 cells transfected with plasmids containing shRNA specifically targeting IP$_3$R1&2 (Figure 4E), but they were still able to do so in cells transfected with control plasmids (Figure 4D). Statistically, the averaged intracellular calcium changes presented as $\Delta F_{350nm}/F_{385nm}$ were 0.08 ± 0.02 in cells transfected with shRNA against IP$_3$R1&2 compared with 0.23 ± 0.02 in control cells (Figure 4F. **p < 0.001).

PLC and GPCRs Mediated IgG Autoantibody-Induced Calcium Elevation in HL-1 Cells

To reveal the mechanism underlying IgG autoantibody-induced IP$_3$R-mediated intracellular calcium elevation, the pharmacological compounds, i.e., U-73122 (Leitner et al., 2016) and SCH-202676 (Lewandowicz et al., 2006), were used to block PLC and G protein-coupled receptor (GPCR), respectively. As shown in Figures 5A and 5B, treatment with both U-73122 (10 μM) and SCH-202676 (50 μM) could significantly reduce IgG autoantibody-induced intracellular calcium elevation. Statistical data were summarized in Figure 5C.

DISCUSSION

In this study, we showed that circulating IgG autoantibodies from patients with T2DM/AF were able to induce intracellular calcium elevation in both human iPSC-differentiated cardiomyocytes and mouse adult atrial cardiomyocytes (Figure 1 and Figure S2). Such effects were not observed in the IgG autoantibodies from a control group of age-matched T2DM without atrial fibrillation, or left ventricular hypertrophy (Figure 2). The IgG autoantibody-induced intracellular calcium elevation was insensitive to L-type or T-type calcium channel blockers or SOCE blocker but was sensitive to either xestospongin c or knocking down of IP$_3$Rs, indicating that the IgG autoantibody-induced intracellular calcium elevation was not dependent on voltage-gated calcium channel or store-operated calcium channel, but rather dependent on IP$_3$Rs pathway. Both PLC and GPCR blockers were able to diminish IgG autoantibody-induced intracellular
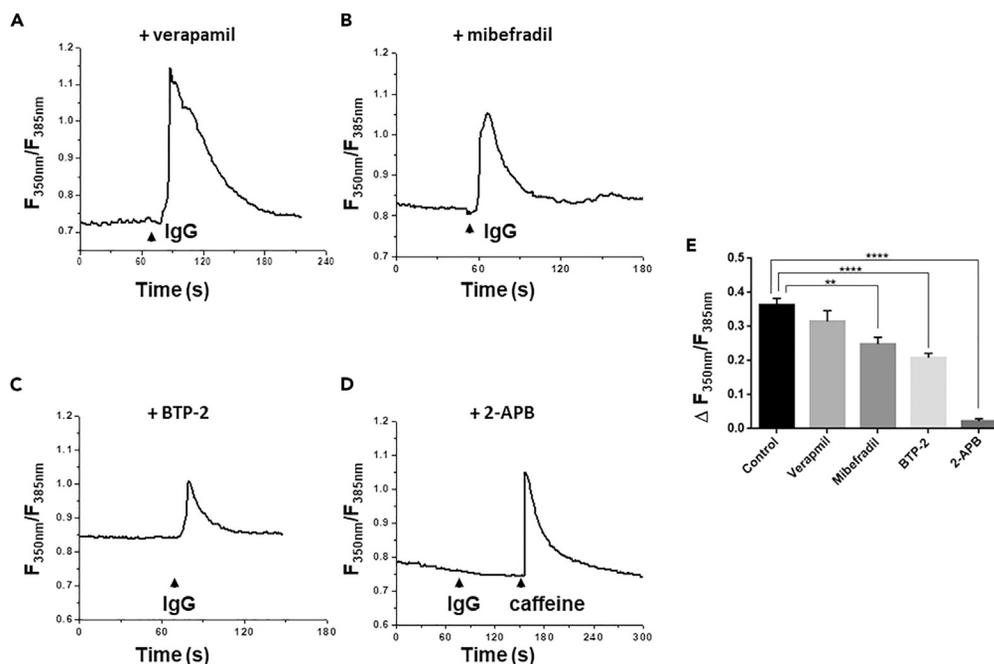
Figure 3. Pharmacological Characterization of IgG Autoantibody-Induced Ca²⁺ Elevation in HL-1 Cells

(A–D) IgGs (2 μg/mL) derived from patients with DM/AF resulted in intracellular Ca^{2+} elevation in HL-1 cells treated with mibefradil (A 25 μM), verapamil (B 20 μM), and BTP-2 (C 5 μM) but not in cells treated with 2-APB (D 100 μM). Caffeine, 20 mM, was added at the end of experiments to test whether SR Ca^{2+} stores were intact. n ≥ 3.

(E) Statistical data of IgGs-induced changes in intracellular Ca^{2+} in HL-1 cells. **p = 0.0014. **p < 0.0001. Data are represented as mean ± SEM.

elevation, indicating the involvement of PLC and GPCR in the IgG autoantibody-induced arrhythmogenic effects (Figure 5). This is the first report, to our knowledge, of circulating IgG autoantibodies with activity of stimulating intracellular calcium signals in diabetic patients with AF. Taken together, these results demonstrated that the IgG autoantibodies purified from older patients with T2DM with AF could target an unknown GPCR, triggering IP_3R-dependent pathways. This discovery suggested that the circulating IgG autoantibodies might play an important role in AF (Figure 6).

This study revealed that IgG autoantibodies from patients with T2DM could trigger intracellular calcium elevation in both human iPSC-differentiated cardiomyocytes and mouse atrial cardiomyocytes. The altered intracellular calcium signals could contribute to the onset or development of AF. The significant association between intracellular calcium release in atrial cardiomyocytes evoked by IgG autoantibodies in T2DM with, but not without, co-morbid AF (Figure 2B) suggests a possible role for the IgG autoantibodies in AF causation. However, the exact contribution of these circulating IgG autoantibodies to the development of AF at different stages requires further investigation.

IP_3 receptor-dependent pathways have been reported to play an important role in hypertrophy and remodeling of cardiomyocytes. Many key factors known to induce arrhythmogenic calcium signals occur through IP_3 receptor-dependent pathways and thus regulate atrial structural and electrical remodeling associated with AF, such as angiotensin II and endothelin-1 (Berridge, 2016). For example, endothelin increases the formation of IP_3 leading to enhanced calcium signaling, thus contributing to the development of atrial arrhythmias, which can ultimately lead to sudden cardiac death (Li et al., 2005). Up-regulation of IP_3R expression has been found in atrial tissues of patients with chronic AF (Yamada et al., 2001). The G-protein signaling regulator RGS4 has been reported to be the molecular substrate for predisposition of AF (Opel et al., 2015). Mice with global RGS4 deletion show higher frequency of AF development, calcium spark under basal condition as well as upon endothelin treatment, and abnormal spontaneous calcium release events after field stimulation in the atrial cells isolated from them, compared with the control littermates. RGS4 inhibits the $G_{q/11}$, which activates the $PLC\beta$ that generates IP_3 (Tinker et al., 2016). The role of

Figure 4. Inhibition or Knockdown of IP$_3$R Diminished IgG Autoantibody-Induced Ca^{2+} Elevation in HL-1 Cells

(A and B) IgGs purified from patients with DM/AF were applied at the concentration of 2 μg/mL. The intracellular Ca^{2+} was recorded in HL-1 cells pre-treated with vehicle (A control) or 10 μM xestospongin C (B Xest C). Caffeine, 20 mM, was added at the end of experiments to test whether SR Ca^{2+} stores were intact.

(C) Statistical data of IgGs-induced changes in intracellular Ca^{2+} in HL-1 cells treated with vehicle or Xest C. *p < 0.01. Data are represented as mean ± SEM.

(D and E) Representative traces of intracellular Ca^{2+} in HL-1 cells transfected with plasmids containing shRNA-control (D control) or shRNA-α-IP$_3$R(E α-IP$_3$R).

(F) Statistical data of IgGs-induced changes in intracellular Ca^{2+} in HL-1 cells transfected with control or α-IP$_3$R plasmids. **p < 0.001. Data are represented as mean ± SEM.

See also Figure S1 and Table S1.

IP$_3$ in calcium signaling and the development of AF has also been reported in rat atrial myocytes (Mackenzie et al., 2002). The 2-APB-induced IP$_3$R inhibition at 2 μM specifically suppresses the endothelin and IP$_3$-evoked increase of calcium signal amplitude and extra calcium transients, which are absent under control conditions in rat atrial myocytes. Furthermore, in an animal study using the IP$_3$R2-deficient mice, arrhythmogenic effects are abolished in the atrial myocytes isolated from IP$_3$R2-deficient mice compared with those in the cells isolated from the wild-type littermates (Li et al., 2005). This study revealed that IP$_3$-IP$_3$R pathways mediate intracellular calcium elevation triggered by IgG autoantibodies from patients with T2DM/AF. Data suggest that pharmacologic blockers targeting IP$_3$R pathways could be new drugs to neutralize harmful effects of IgG autoantibodies in treatment or prevention of AF in patients with T2DM.

Emerging evidence has implicated the involvement of autoimmunity in the development of AF in many autoimmune diseases, together with other factors contributing to the pathogenesis of AF, ranging from electrical, structural, and neurohumoral to inflammatory processes (Kourliouros et al., 2009). Several autoantibodies that play roles in the pathogenesis of AF include autoantibodies against GPCR superfamily proteins such as myosin heavy chain (MHC), sodium-potassium pump (Na/K-ATPase), the muscarinic cholinergic type 2 (M2) receptors, and the β1-adrenergic receptor (Maixent et al., 1998). The implications of autoantibody as upstream indicators of paroxysmal AF was shown in patients with congestive heart failure (CHF) (Baba et al., 2002). In this cohort study of 95 patients with CHF and 48 age-matched control patients with hypertension, circulating autoantibodies against
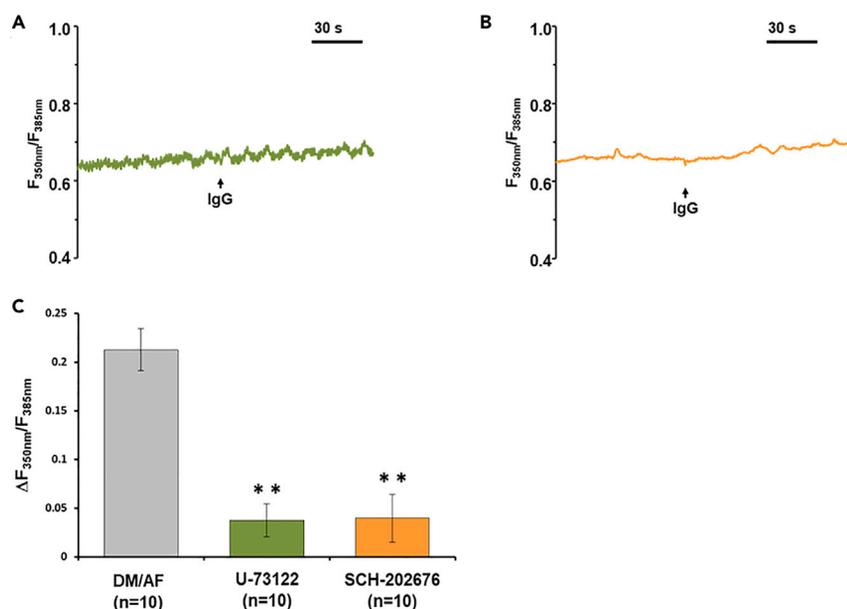
Figure 5. PLC and GPCR Are Involved in IgG Autoantibody-Induced Ca^{2+} Elevation in HL-1 Cells

IgGs from patients with DM/AF were applied at the concentration of (30 μg/mL).

(A) Representative trace of IgG-induced Ca^{2+} in HL-1 cells treated with 10 μM U-73122.

(B) Representative trace of IgG-induced Ca^{2+} in HL-1 cells treated with 50 μM SCH-202676.

(C) Statistical data. n ≥ 5, **p < 0.001. Data are represented as mean ± SEM.

Na/K-ATPase are suggested to be an independent risk factor for the occurrence of paroxysmal AF. Autoantibodies against M2 receptors may play roles in the development of AF in patients with dilated cardiomyopathy (DCM) (Baba et al., 2004). M2 autoantibodies are also found in patients with idiopathic AF (Baba et al., 2004). M2 autoantibodies are confirmed to be independent predictors of the presence of AF in the patients indicated by multivariable analysis (Baba et al., 2004). Autoantibodies against β1 and M2 facilitate the development of AF in patients with Graves' hyperthyroidism (Stavrakis et al., 2009). T2DM is not an autoimmune disease, yet accumulating evidence showed that circulating IgGs from patients with T2DM, especially patients at advanced diseases stage or aged, present autoimmunity (Zimering et al., 2011; Zimering and Pan, 2009). In this study, blocking GPCR could eliminate IgG autoantibody-triggered arrhythmogenic calcium signals, suggesting that these IgGs could target GPCRs. However, the exact GPCR and whether other antigens are involved in these IgG autoantibody-mediated effects in patients with T2DM/AF warrant further study.

In summary, this report highlighted that circulating IgGs from patients with T2DM/AF could induce arrhythmogenic calcium signals in cardiomyocytes. The results reported here shed light upon a possible mechanism underlying the association between increased risk of AF and T2DM. The circulating IgGs may serve as a biomarker for identifying a subset of patients with T2DM with an increased risk for the development of AF. Further identification of the receptors or antigens involved in IgG autoantibody-mediated IP$_3$R activation could result in sentinel biomarkers to assess the risk factors for AF and/or novel approaches for AF prevention in patients with T2DM.

Limitations of the Study

Some limitations to this study must be acknowledged. First, this study is limited by *in vitro* evidence. Although we have used human iPSC-differentiated, immortalized mouse atrial cardiomyocytes and isolated adult mouse atrial myocytes to reveal the arrhythmogenic calcium signaling induced by circulating IgGs purified from patients with T2DM with AF, we do not know whether the same effects can be recapitulated *in vivo*. Second, we do not know whether the IgG-evoked calcium elevations contribute to the onset or development of AF. Third, our findings have shown that the T2DM/AF IgGs could target GPCR and trigger the IP$_3$R-dependent pathway; however, the exact GPCR involved is currently unknown and remains to be further investigated.
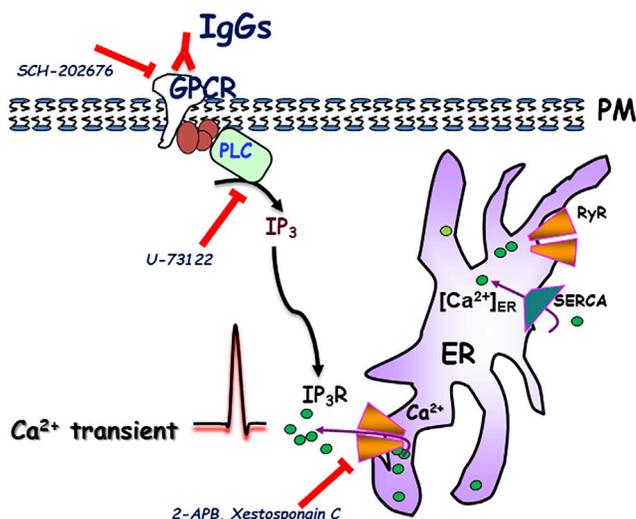
Figure 6. Working Model for Arrhythmogenic Calcium Signaling Induced by Circulating IgG Autoantibodies in Patients with T2DM/AF

Pharmacological data suggest that intracellular calcium elevation is through the GPCR-PLC-IP$_3$-IP$_3$R pathway.

AUTHOR CONTRIBUTIONS

Conceptualization, Z.P. and M.Z.; Methodology, Z.P.; Investigation, Y.L., X.L., R.M., Y.W., and Z.P.; Writing, X.L., Z.P., and M.Z.; Funding Acquisition, Z.P. and M.Z.; Resources, Z.P. and M.Z.; Supervision, Z.P.

DECLARATION OF INTERESTS

The authors declare no competing interests. The contents do not represent the views of the U.S. Department of Veteran Affairs or the United States government.

REFERENCES

Baba, A., Yoshikawa, T., Chino, M., Murayama, A., Mitani, K., Nakagawa, S., Fujii, I., Shimada, M., Koyama, T., and Akaishi, M. (2002). Autoantibodies: new upstream targets of paroxysmal atrial fibrillation in patients with congestive heart failure. J. Cardiol. 40, 217–223.

Baba, A., Yoshikawa, T., Fukuda, Y., Sugiyama, T., Shimada, M., Akaishi, M., Tsuchimoto, K., Ogawa, S., and Fu, M. (2004). Autoantibodies against M2-muscarinic acetylcholine receptors: new upstream targets in atrial fibrillation in patients

with dilated cardiomyopathy. Eur. Heart J. 25, 1108–1115.

Berridge, M.J. (2016). The inositol trisphosphate/calcium signaling pathway in health and disease. Physiol. Rev. 96, 1261–1296.

Bootman, M.D., Collins, T.J., Mackenzie, L., Roderick, H.L., Berridge, M.J., and Peppiatt, C.M. (2002). 2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca2+ entry

but an inconsistent inhibitor of InsP3-induced Ca2+ release. FASEB J. 16, 1145–1150.

Centers for Disease Control and Prevention (2017). National diabetes statistics report, 2017. https://www.cdc.gov/diabetes/pdfs/data/statistics/national-diabetes-statistics-report.pdf.

Harada, M., Tadevosyan, A., Qi, X., Xiao, J., Liu, T., Voigt, N., Karck, M., Kamler, M., Kodama, I., and Murohara, T. (2015). Atrial fibrillation activates AMP-dependent protein kinase and its

regulation of cellular calcium handling: potential role in metabolic adaptation and prevention of progression. J. Am. Coll. Cardiol. 66, 47–58.

Heijman, J., Voigt, N., Nattel, S., and Dobrev, D. (2014). Cellular and molecular electrophysiology of atrial fibrillation initiation, maintenance, and progression. Circ. Res. 114, 1483–1499.

Huxley, R.R., Filion, K.B., Konety, S., and Alonso, A. (2011). Meta-analysis of cohort and case-control studies of type 2 diabetes mellitus and risk of atrial fibrillation. Am. J. Cardiol. 108, 56–62.

Kourliouros, A., Savelieva, I., Kiotsekoglou, A., Jahangiri, M., and Camm, J. (2009). Current concepts in the pathogenesis of atrial fibrillation. Am. Heart J. 157, 243–252.

Leitner, M.G., Michel, N., Behrendt, M., Dierich, M., Dembla, S., Wilke, B.U., Konrad, M., Lindner, M., Oberwinkler, J., and Oliver, D. (2016). Direct modulation of TRPM4 and TRPM3 channels by the phospholipase C inhibitor U73122. Br. J. Pharmacol. 173, 2555–2569.

Lewandowicz, A.M., Vepsalainen, J., and Laitinen, J.T. (2006). The 'allosteric modulator' SCH-202676 disrupts G protein-coupled receptor function via sulphydryl-sensitive mechanisms. Br. J. Pharmacol. 147, 422–429.

Li, X., Zima, A.V., Sheikh, F., Blatter, L.A., and Chen, J. (2005). Endothelin-1–induced arrhythmogenic Ca2+ signaling is abolished in atrial myocytes of inositol-1, 4, 5-trisphosphate (IP3)–receptor type 2–deficient mice. Circ. Res. 96, 1274–1281.

Mackenzie, L., Bootman, M.D., Laine, M., Berridge, M.J., Holmes, A., Li, W.H., and Lipp, P. (2002). The role of inositol 1, 4, 5-trisphosphate receptors in Ca2+ signalling and the generation of arrhythmias in rat atrial myocytes. J. Physiol. 541 (Pt 2), 395–409.

Maixent, J.M., Paganelli, F., Scaglione, J., and Levy, S. (1998). Antibodies against myosin in sera of patients with idiopathic paroxysmal atrial fibrillation. J. Cardiovasc. Electrophysiol. 9, 612–617.

Nattel, S., and Dobrev, D. (2016). Electrophysiological and molecular mechanisms of paroxysmal atrial fibrillation. Nat. Rev. Cardiol. 13, 575–590.

Oka, T., Sato, K., Hori, M., Ozaki, H., and Karaki, H. (2002). Xestospongin C, a novel blocker of IP3 receptor, attenuates the increase in cytosolic calcium level and degranulation that is induced by antigen in RBL-2H3 mast cells. Br. J. Pharmacol. 135, 1959–1966.

Opel, A., Nobles, M., Montaigne, D., Finlay, M., Anderson, N., Breckenridge, R., and Tinker, A. (2015). Absence of the regulator of G-protein signalling, RGS4, predisposes to atrial fibrillation and is associated with abnormal calcium handling. J. Biol. Chem. 290, 19233–19244.

Sabourin, J., Boet, A., Rucker-Martin, C., Lambert, M., Gomez, A.M., Benitah, J.P., Perros, F., Humbert, M., and Antigny, F. (2018). Ca(2+) handling remodeling and STIM1L/Orai1/TRPC1/TRPC4 upregulation in monocrotaline-induced right ventricular hypertrophy. J. Mol. Cell. Cardiol. 118, 208–224.

Stamler, J., Vaccaro, O., Neaton, J.D., and Wentworth, D. (1993). Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. Diabetes Care 16, 434–444.

Stavrakis, S., Yu, X., Patterson, E., Huang, S., Hamlett, S.R., Chalmers, L., Pappy, R., Cunningham, M.W., Morshed, S.A., and Davies, T.F. (2009). Activating autoantibodies to the beta-1 adrenergic and m2 muscarinic receptors facilitate atrial fibrillation in patients with Graves' hyperthyroidism. J. Am. Coll. Cardiol. 54, 1309–1316.

Sun, Y., and Hu, D. (2010). The link between diabetes and atrial fibrillation: cause or correlation? J. Cardiovasc. Dis. Res. 1, 10–11.

Tinker, A., Finlay, M., Nobles, M., and Opel, A. (2016). The contribution of pathways initiated via the Gq\11 G-protein family to atrial fibrillation. Pharmacol. Res. 105, 54–61.

Tjondrokoesoemo, A., Li, N., Lin, P.-H., Pan, Z., Ferrante, C.J., Shirokova, N., Brotto, M.,

Weisleder, N., and Ma, J. (2013). Type 1 inositol (1, 4, 5)-trisphosphate receptor activates ryanodine receptor 1 to mediate calcium spark signaling in adult mammalian skeletal muscle. J. Biol. Chem. 288, 2103–2109.

Wang, Y., Huang, W., Liang, J., Wen, Z., Chang, D., Kang, K., Wang, J., Xu, M., Millard, R.W., and Wang, Y. (2014). Suicide gene-mediated sequencing ablation revealed the potential therapeutic mechanism of induced pluripotent stem cell-derived cardiovascular cell patch post-myocardial infarction. Antioxid. Redox Signal. 21, 2177–2191.

Xia, M., Salata, J.J., Figueroa, D.J., Lawlor, A.M., Liang, H.A., Liu, Y., and Connolly, T.M. (2004). Functional expression of L- and T-type Ca2+ channels in murine HL-1 cells. J. Mol. Cell. Cardiol. 36, 111–119.

Yamada, J., Ohkusa, T., Nao, T., Ueyama, T., Yano, M., Kobayashi, S., Hamano, K., Esato, K., and Matsuzaki, M. (2001). Up-regulation of inositol 1, 4, 5 trisphosphate receptor expression in atrial tissue in patients with chronic atrial fibrillation. J. Am. Coll. Cardiol. 37, 1111–1119.

Zimering, M.B. (2017). Diabetes autoantibodies mediate neural-and endothelial cell-inhibitory effects via 5-hydroxytryptamine-2 receptor coupled to phospholipase C/inositol triphosphate/Ca2+ pathway. J. Endocrinol. Diabetes 4, 1–10.

Zimering, M.B., Alder, J., Pan, Z., and Donnelly, R.J. (2011). Anti-endothelial and anti-neuronal effects from auto-antibodies in subsets of adult diabetes having a cluster of microvascular complications. Diabetes Res. Clin. Pract. 93, 95–105.

Zimering, M.B., and Pan, Z. (2009). Autoantibodies in type 2 diabetes induce stress fiber formation and apoptosis in endothelial cells. J. Clin. Endocrinol. Metab. 94, 2171–2177.

Zimering, M.B., and Pan, Z. (2017). Increased neuronal depolarization evoked by autoantibodies in diabetic obstructive sleep apnea: role for inflammatory protease (S) in generation of neurotoxic immunoglobulin fragment. J. Endocrinol. Diabetes 4, 1–10.

Supplemental Information

Circulating IgGs in Type 2 Diabetes

with Atrial Fibrillation Induce IP$_3$-Mediated

Calcium Elevation in Cardiomyocytes

Yanhong Luo, Xian Liu, Ruilian Ma, Yigang Wang, Mark Zimering, and Zui Pan

TRANSPARENT METHODS

Subjects

All participants signed a local Veterans Affairs New Jersey Healthcare System (VANJHCS) Investigational Review Board (IRB)-approved informed consent prior to blood drawing for the study. Participants included twenty-one adult T2DM patients from the Veterans Affairs Diabetes Trial (Zimering et al., 2008, Zimering and Pan, 2009); and twenty-nine adult T2DM participants enrolled in a VANJHCS study of diabetic complications. Patients having diabetes with AF were excluded if they had a co-morbid condition associated with an independent increased risk of AF (severe pulmonary disease, heavy alcohol use, valvular heart disease or AF-onset following episode of ischemic heart disease). Diabetes without AF was excluded for these same co-morbidities or for having left ventricular hypertrophy, or sinus arrythmia on a recent electrocardiogram. Diabetes (with or without atrial fibrillation) did not differ significantly in their baseline age, glycosylated hemoglobin or presence of nephropathy or retinopathy (Table 1). Diabetes with atrial fibrillation had significantly higher baseline prevalence of insulin use (64% vs 35%, p=0.45) compared to diabetes without atrial fibrillation (**Table 1**). The circulating IgGs were purified from each patient's stored serum samples using protein-A columns as previously described (Zimering and Pan, 2017). The purified IgG samples were coded and shipped on dry ice to Dr. Pan's laboratory. Dr. Pan was blinded to the study participants' diagnosis until the conclusion of the calcium elevation experiments.

Cell lines and cell culture

HL-1 cells were maintained in Claycomb medium supplemented with 10% FBS, 100U/ml penicillin, 100μg/ml streptomycin, 0.1mM norepinephrine, 2mM L-glutamine (Claycomb et al., 1998). The cell line was cultured at 37°C in a humidified 5% CO_2 incubator. Human induced pluripotent stem cells (iPSCs) were cultured and differentiated into cardiomyocytes following the published procedure (Wang et al., 2014).

Measurement of intracellular calcium concentration

Intracellular calcium concentration in HL-1 cell line, human iPSCs and isolated adult mouse atrial myocytes was monitored by fluorescence microscopy with a 40x objective (Nikon TE200 Super Fluo, N.A. 1.3) in a dual-wavelength spectrofluorometer (Photon Technology International, Monmouth Junction, NJ), with excitation wavelengths at 350 and 385nm and emission at 510nm (Zimering and Pan, 2017). Purified IgGs from T2DM AF patients were added into bath saline solution (140mM NaCl, 2.8mM KCl, 2mM $MgCl_2$, 2mM $CaCl_2$, 10mM HEPES, pH 7.2) at indicated time. The intracellular calcium elevation was presented as $\Delta F_{350nm}/F_{385nm}$.

Plasmids and shRNAs

The plasmid containing RFP reporter gene and a short hairpin RNA (shRNA) probes targeting common sequences of mouse mRNAs for both IP_3R1 and IP_3R2 were transfected into HL-1 cells. Same plasmids containing scramble shRNA were used as control. The efficacy in knocking down IP_3R1 and IP_3R2 protein has been confirmed previously verified by Western blotting analysis (Tjondrokoesoemo et al., 2013) and by current experiment examining the mRNA levels using quantitative reverse transcription polymerase chain reaction (qRT-PCR) two days after plasmid DNA transfection.

Statistical analysis

Data were analyzed using Origin Pro7 and Graphpad5 software. Values are Mean ± SEM or otherwise indicated. Significance was determined by one-way analysis of variance (ANOVA). A value of $p<0.01$ was used as criterion for statistical significance.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNAs were extracted from HL-1 cells using Illustra RNAspin MiniRNA Isolation Kit and measured photometrically at 260/280nm. Primers were obtained from Sigma Aldrich. 200ng of total RNA was applied for reverse transcription using qScript microRNA Synthesis Kit (QuantaBio) according to the manufacturer's protocol. cDNA was diluted 1:5 in DNase-, RNase- and protease-free water and 2μl

template was used. Primer pairs of IP_3R1, IP_3R2 and GAPDH were used. The sequences are indicated in **Table S1**. For qRT-PCR QuantaBio PerfecTa SYBR Green FastMix ROX was used based on the manufacturer's procedure. Signals generated by integration of SYBR Green into the amplified DNA were detected in a real-time PCR system (StepOnePlus) and normalized to GAPDH gene expression. Data were expressed as $2^{-\Delta\Delta CT}$.

Adult mouse atrial myocyte isolation

All animal protocols were approved by the University of Texas at Arlington Institutional Animal Care and Use Committee (IACUC) in accordance with the Animal Welfare Act (AWA) and PHS Policy on Humane Care and Use of Laboratory Animals of United States. C57BL/6J mice with both genders, at age of 5-16 months old were used. Primary atrial myocytes were isolated according to the published protocol (Jansen and Rose, 2019) using a combination of enzymatic digestion and mechanical dissociation of these tissues. Briefly, the mouse atrial appendage was excised and put into modified Tyrode's pH 7.4 solution and cut into 8-10 strips and then transferred into 2.5ml modified Tyrode's pH 6.9 solution and incubated for 5min. The atrial tissues were washed three times in 2.5ml modified Tyrode's pH 6.9 solution, followed by enzymatic digestion for 30min in 5ml Enzymatic solution. Three washes were then performed in 2.5ml Kraft-Brühe (KB) solution (HEPES balanced, pH 7.2) followed by 5min incubation in 2.5ml KB solution. The atrial cardiomyocytes were then triturated for 7.5min in 2.5ml KB solution and incubated for 1hour at room temperature in 29mm glass-bottom dishes pre-coated with laminin overnight. Right before calcium measurement, the external solution was changed to $BSS-Ca^{2+}$ solution.

SUPPLEMENTAL REFERENCES

CLAYCOMB, W. C., LANSON, N. A., STALLWORTH, B. S., EGELAND, D. B., DELCARPIO, J. B., BAHINSKI, A. & IZZO, N. J. 1998. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proceedings of the National Academy of Sciences, 95, 2979-2984.

JANSEN, H. J. & ROSE, R. A. 2019. Isolation of Atrial Myocytes from Adult Mice. J Vis Exp.

TJONDROKOESOEMO, A., LI, N., LIN, P.-H., PAN, Z., FERRANTE, C. J., SHIROKOVA, N., BROTTO, M., WEISLEDER, N. & MA, J. 2013. Type 1 inositol (1, 4, 5)-trisphosphate receptor activates ryanodine receptor 1 to mediate calcium spark signaling in adult mammalian skeletal muscle. Journal of Biological Chemistry, 288, 2103-2109.

WANG, Y., HUANG, W., LIANG, J., WEN, Z., CHANG, D., KANG, K., WANG, J., XU, M., MILLARD, R. W. & WANG, Y. 2014. Suicide gene-mediated sequencing ablation revealed the potential therapeutic mechanism of induced pluripotent stem cell-derived cardiovascular cell patch post-myocardial infarction. Antioxidants & redox signaling, 21, 2177-2191.

ZIMERING, M. B., ANDERSON, R. J., LUO, P., MORITZ, T. E. & TRIAL, I. F. T. V. A. D. 2008. Plasma basic fibroblast growth factor is correlated with plasminogen activator inhibitor–1 concentration in adults from the Veterans Affairs Diabetes Trial. Metabolism, 57, 1563-1569.

ZIMERING, M. B. & PAN, Z. 2009. Autoantibodies in type 2 diabetes induce stress fiber formation and apoptosis in endothelial cells. The Journal of Clinical Endocrinology & Metabolism, 94, 2171-2177.

ZIMERING, M. B. & PAN, Z. 2017. Increased Neuronal Depolarization Evoked by Autoantibodies in Diabetic Obstructive Sleep Apnea: Role for Inflammatory Protease (S) in Generation of Neurotoxic Immunoglobulin Fragment. Journal of endocrinology and diabetes, 4.
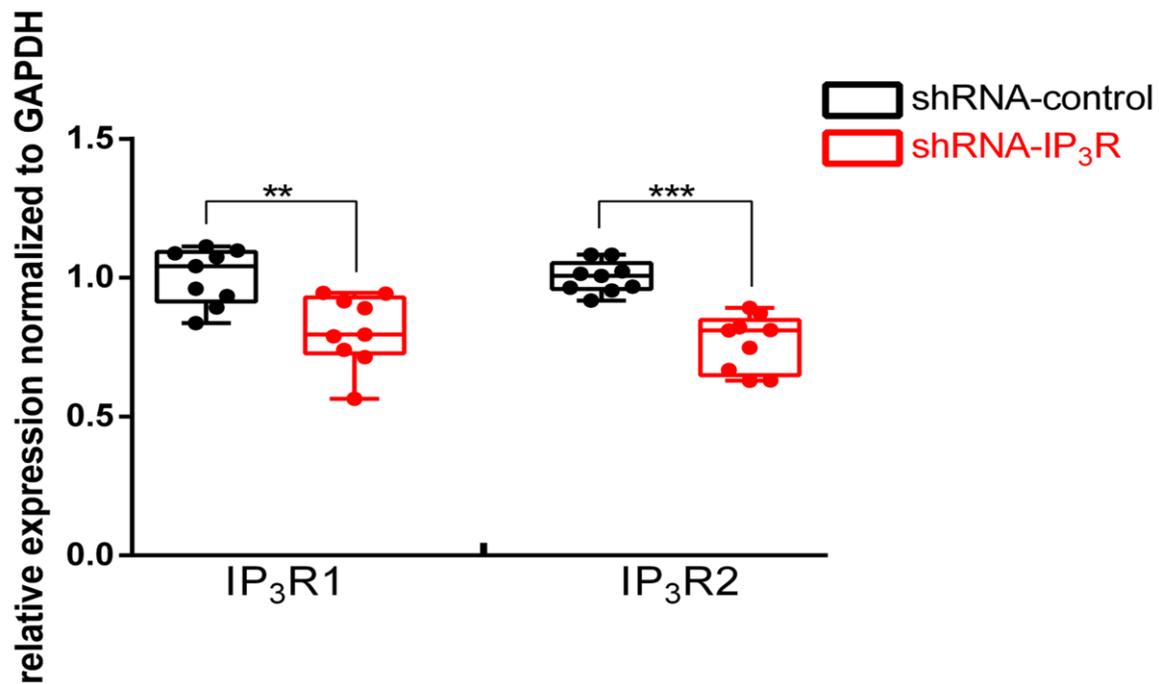
Figure S1. shRNA-mediated knockdown of IP$_3$R1 and IP$_3$R2 in HL-1 cells, Related to Figure 4. shRNA-IP$_3$R was designed to target a common sequence on IP$_3$R1 and IP$_3$R2. Quantitative RT-PCR was performed using total RNAs from HL-1 cells two days after plasmid DNA transfection and showed that shRNA-IP$_3$R resulted in significant knockdown of IP$_3$R1 and IP$_3$R2 in HL-1 cells. n=3, **p=0.0013. *** p=0.0001.
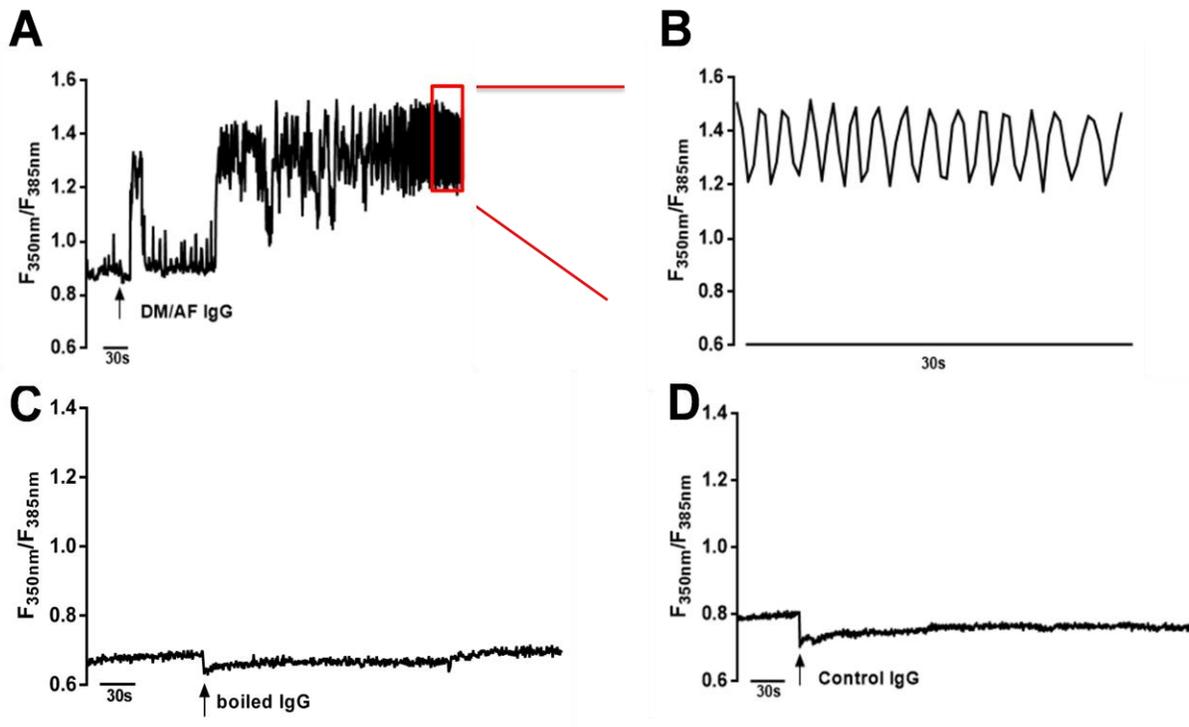
Figure S2. Intracellular calcium response in isolated adult mouse atrial myocytes treated with IgGs from T2DM patients with and without AF, **Related to Figure 1 and Figure 2.**
A. Representative trace of intracellular calcium signal. IgGs (5μg/ml) derived from T2DM/AF patients were added at the indicated point (arrow).
B. Enlarged calcium oscillation signals in the red rectangle of **A**.
C. Representative trace of intracellular calcium signal treated with boiled IgGs (same as used in **A**) as negative control.
D. Representative trace of intracellular calcium signal treated with 5μg/ml IgG from diabetic patients without AF. The same cells were later treated with 5μg/ml T2DM/AF IgGs and demonstrated similar trace as in **A**.

Table S1. Primer sequences for qRT-PCR, Related to Figure 4.

Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')
IP$_3$R1	CGTTTTGAGTTTGAAGGCGTTT	CATCTTGCGCCAATTCCCG
IP$_3$R2	CCTCGCCTACCACATCACC	TCACCACTCTCACTATGTCGT
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA