To explore a novel strategy of preventing atrial fibrosis and atrial fibrillation (AF), we have established 3 appropriate experimental models of AF. Firstly, atrial fibrosis was induced by pressure overload by abdominal aortic constriction (AAC). AAC enhanced left atrial (LA) expression of monocyte chemoattractant protein-1. Scanning electron microscopy revealed that LA endothelial cells were irregularly hypertrophied, with disarrangement of lines of cells. Possible “arrested” leukocyte-derived cells were observed on the surface of LA endothelial cells. Treatment with pioglitazone, a peroxisome proliferator-activated receptor-γ agonist, resulted in attenuation of pressure overload-induced LA fibrosis. Secondly, LA fibrosis was induced by continuous infusion of angiotensin II (AII). Repeated whole-body hyperthermia led to the induction of heat shock protein (HSP) 72, which resulted in attenuation of AII-induced LA fibrosis. Thirdly, atrial fibrosis was induced by 5/6 nephrectomy as a model of AF associated with chronic kidney disease. Because the amount of nicotinamide adenine dinucleotide phosphate oxidase was increased and the potent antioxidant agent was effective, oxidative stress may be involved in the pathogenesis of LA fibrosis and enhanced AF vulnerability in this model. These observations suggest that inflammatory profibrotic processes are essential for the development of atrial fibrosis in these 3 models. Pioglitazone, induction of HSPs and antioxidant agent could be novel therapeutic approaches to preventing atrial fibrosis and AF. (Circ J 2012; 76: 2318–2326)

Key Words: Atrial fibrillation; Fibrosis; Inflammation

The “upstream” approach that targets processes involved in the development of the substrates that promote atrial fibrillation (AF) has recently attracted attention. The results obtained in animal experiments have compellingly shown the protective effect of angiotensin-converting enzyme inhibitors, angiotensin II type-1 receptor blockers (ARBs), statins, and n-3 polyunsaturated fatty acids against electrical and structural atrial remodeling in association with AF. In the clinical setting, however, the results appear discouraging. For instance, in the GISSI-AF trial, treatment with valsartan was not associated with a reduction in the incidence of recurrent AF. Nevertheless, it is possible that upstream therapies could reduce new-onset AF. Sophisticated experimental studies that have significant clinical relevance should be continued. Following the proposal of “AF begets AF”, the atrial tachypacing model has been widely used to represent clinical AF, the electrophysiologic feature of which is progressive shortening of the atrial effective refractory period (ERP). However, in a canine AF model with rapid atrial pacing for 5 weeks, it was demonstrated that rather than shortening the atrial ERP, extensive interstitial fibrosis was found in the atrial free wall in association with gradual conduction prolongation in the atria. However, the atrial tachypacing procedure in “normal” animals may be applicable only to the process of perpetuation of paroxysmal AF into permanent AF in patients without structural heart disease.

In this review, we introduce our experimental approaches to the prevention of atrial fibrosis and AF. We have induced atrial fibrosis by pressure overload, continuous infusion of angiotensin II (AII), and 5/6 nephrectomy (5/6Nx). In these 3 AF models, we have evaluated the anti-AF effects of pioglitazone, induction of heat shock protein (HSP), and an antioxidant agent, respectively.

Pressure Overload and Pioglitazone

Cardiac hypertrophy with fibrosis induced by aortic constriction has been reported to represent the outcome of the hypertrophic processes observed in patients with hypertension, which is associated with systemic inflammation. Therefore, AF induced by abdominal aortic constriction (AAC) may resemble AF in human hypertensive pathophysiologic conditions. Pioglitazone, a peroxisome proliferator-activated receptor-γ agonist, possesses antiinflammatory properties, and has been demonstrated to attenuate left ventricular hypertrophy and fibrosis in the salt-sensitive hypertensive rat. Therefore, we tested the hypothesis that pioglitazone could attenuate atrial inflammatory profibrotic signals and fibrosis, resulting in re-
Figure 1. Expression of MCP-1 and TGF-β1 in left atrial tissue. (A) Expression of MCP-1 in left atrial tissue analyzed by western blot. Top: Representative bands of MCP-1. Bottom: Quantitative expression of MCP-1 (relative density). (B) Expression of TGF-β1 in left atrial tissue analyzed by western blot. Top: Representative bands of TGF-β1. Bottom: Quantitative expression of TGF-β1 (relative density). GAPDH is an internal standard. Data are mean±SEM, and are expressed relative to an average of the Sham+VEH groups. n=5 in each group. **P<0.01. NS, not significant; AAC, abdominal aortic constriction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MCP-1, monocyte chemoattractant protein-1; PIO, pioglitazone; TGF-β1, transforming growth factor-beta1; VEH, vehicle. (Figure adapted with permission.12)

Figure 2. Ultrastructure of left atrial endocardium. Vertical cross-sections of the left atrium are shown. (A) Masson-trichrome staining. (B–D) Scanning electron microscopic images. (B) Left endocardium consists of surface endothelial cells (Ec), subendothelial connective tissue (C) and atrial myocardium (M), sequentially. (C,D) Endothelial cells have peeled away from the myocytes and clearly demonstrate the presence of connective tissue (Ec).
duced vulnerability to developing AF in AAC-treated pressure overloaded rats.\textsuperscript{12} Pioglitazone (3 mg·kg\textsuperscript{-1}·day\textsuperscript{-1}) or vehicle was orally administered for 4 weeks. We observed the following. (1) AAC enhanced the protein expression of monocyte chemotactic protein (MCP)-1 (Figure 1A), transforming growth factor (TGF)-\(\beta\)1 (Figure 1B), and \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) in the left atrium (LA).\textsuperscript{12} (2) AAC also increased mRNA expression of collagen type 1 and atrial natriuretic peptide in the LA. (3) Gelatin zymography showed that the activity of promatrix metalloproteinase-9 was increased by AAC. (4) AAC induced LA interstitial fibrosis. (5) In isolated-perfused heart experiments, AAC did not alter the refractory period of either the left or the right atrium, but did prolong the interatrial conduction time. (6) Programmed extrastimuli from the right atrium induced AF in all of the AAC-treated rats. All of these changes induced by AAC were suppressed by treatment with pioglitazone. These observations suggest that inflammatory profibrotic mechanisms are involved in this model of pressure overload-induced AF. The observations also suggest that pioglitazone is effective at attenuating atrial fibrosis, possibly via suppression of MCP-1-mediated inflammatory profibrotic processes.\textsuperscript{12}

To our knowledge, there have been 2 reports demonstrating the anti-AF effects of pioglitazone. Shimano et al reported that in the rapid ventricular pacing-induced congestive heart failure (CHF) of rabbits, pioglitazone reduced the duration of induced AF, attenuated atrial fibrosis, and reduced the interatrial conduction time.\textsuperscript{21} These observations are generally in agreement with ours.\textsuperscript{12} More recently, Xu et al\textsuperscript{22} showed that pioglitazone prevents age-related arrhythmicogenic atrial remodeling and AF perpetuation by improving antioxidant capacity and inhibiting the mitochondrial apoptotic signaling pathway. Based on these observations, they suggested that PPAR-\(\gamma\) activators could become a novel upstream therapy for age-related AF.\textsuperscript{22} Thus, pioglitazone has been shown to prevent AF caused by pressure overload, CHF,\textsuperscript{13} and aging.\textsuperscript{14} It is therefore interesting to evaluate its anti-AF effects in the clinical setting. In fact, in a prospective observational cohort study of 150 consecutive patients undergoing catheter ablation for drug-refractory paroxysmal AF who had a history of type 2 diabetes mellitus, Gu et al reported that pioglitazone improved the preservation of sinus rhythm and reduced the re-ablation rate.\textsuperscript{23} Evidence has shown that inflammation is an important contributor to the progression of various cardiovascular diseases.\textsuperscript{24} It is of note that the process of atrial structural remodeling observed in AF has been revealed to mimic that of atherosclerosis.\textsuperscript{25} In this regard, by using the scanning electron microscopy, we have evaluated the morphological changes of LA endothelial cells in response to pressure overload. Figure 2 illustrates the ultrastructure of the LA surface: the vertical cross-section of the LA sequentially consists of surface endothelial cells, subendothelial connective tissue, and atrial myocardium. We investigated how atrial endothelial cells are involved in the pathogenesis of atrial fibrosis in response to pressure overload. Figure 3 shows comparative scanning electron microscopic findings at 3 days after sham (Figure 3A) or AAC pro-
Figure 4. Expression of α-smooth muscle actin (α-SMA). (A) Immunocytochemical staining of atrial fibroblasts (passage 2) using specific α-SMA primary antibodies. (B) Representative bands of α-SMA by Western blot analysis (Top) and quantitative expression of α-SMA protein (relative density) (Bottom). Data are mean±SEM. Four independent cultures were evaluated. *P<0.05, **P<0.01, All, angiotensin II; HSP, heat shock protein; HT, hyperthermia; NS, not significant. (Figure adapted with permission.)

Figure 5. Expression of NADPH subunits and malondialdehyde (MDA). In each part of Figure 5, representative bands of each protein by western blot are shown at the top and its quantitative expression is shown below. (A) gp91phox expression, (B) p47phox expression, (C) Nox4 expression, (D) MDA expression. n=6 in each group. Data are mean±SEM and expressed relative to the average value in the Sham+VEH group. **P<0.01, *P<0.05. DHL, sodium zinc dihydrolipoilhistidinate; NS, not significant; 5/6Nx, 5/6 nephrectomy; VEH, vehicle. (Figure adapted with permission.)
The LA endocardial surfaces viewed from inside the LA are shown. The LA endothelial cells in the AAC-treated rats were irregularly hypertrophied with the disarrangement of lines of cells (Figure 3B). The nuclei of the LA endothelial cells were deformed in the AAC-treated rats (Figure 3B). In addition, in the AAC-treated LA endocardial surface, morphologically identified monocytes/macrophages were possibly “arrested” on the surface of LA endothelial cells (Figure 3C). These observations suggest that LA endothelial cells quickly respond to pressure overload, which may be associated with recruitment of macrophages to the endothelial surface. Our observations are of interest because the early process of atrial fibrosis in response to pressure overload is quite similar to that of atherosclerosis.

Angiotensin II and Heat Shock Protein

We initially demonstrated that oral geranylgeranylandocetone (GGA) is cardioprotective against ischemia-reperfusion injury via its induction of HSP72. Because HSPs are an important family of endogenous protective proteins that increase in response to a wide variety of stresses, including inflammation, we tested the hypothesis that atrial fibrosis and AF evoked by continuous infusion of all could be prevented by the induction of HSP72. In fact, Mandal et al previously showed that in patients undergoing elective coronary artery bypass surgery, the preoperative HSP72 concentration in right atrial tissue obtained at surgery was higher in patients who did not develop postoperative AF than in those who did develop AF. In cultured rat LA fibroblasts, pretreatment with hyperthermia (42°C for 30 min) was shown to induce HSP72 expression, peaking at 8 h following the application of hyperthermia. All-induced extracellular signal-regulated kinase (Erk1/Erk2) phosphorylation, α-SMA expression, TGF-β1 secretion, collagen synthesis, and the expressions of collagen type-1 and tissue inhibitor of metalloproteinases-1 were attenuated in fibroblasts treated with hyperthermia. A small interfering RNA (siRNA) targeting HSP72 could abolish the antifibrotic effects of hyperthermia. Figure 4 illustrates α-SMA expression in fibroblasts by immunocytochemical analysis using laser microscopy. All caused a remarkable increase in expression of α-SMA in the cytoplasm, when assessed 24 h after All application. Pretreatment with hyperthermia attenuated All-induced α-SMA expression, and the effects were abolished by pretreatment with HSP72 siRNA. Furthermore, in experiments in vivo, repeated hyperthermia (43°C for 20 min) prevented induction of LA interstitial fibrosis by continuous infusion of All. In an electrophysiological study using an isolated-perfused heart, continuous All infusion caused slowing of interatrial conduction without affecting atrial refractoriness. In the All-treated heart, right atrial extrastimuli resulted in a high incidence of AF. The high inducibility of AF was suppressed by hyperthermia treatment.
Taking all these findings together, we concluded that hyperthermia treatment is effective in suppressing AII-mediated atrial fibrosis and AF via, at least in part, induction of HSP72. Other studies have indicated that HSP27, a smaller HSP, may play an important role in AF pathogenesis. Using HL-1 myocytes derived from mouse atria, Brundel et al showed that tachypacing-induced myolysis was prevented by treatment with hyperthermia or GGA. They found that HSP27-, but not HSP72- transfection, was sufficient for protection against tachypacing-induced myolysis. The same group subsequently showed that in HL-1 cells, tachypacing-induced reduction in duration of the L-type Ca current and action potential was prevented by GGA treatment via induction of HSP27. They also showed that in dogs in vivo, atrial tachypacing shortened the atrial ERP, which was attenuated by administration of GGA. GGA also suppressed tachypacing-induced AF-promoting changes, including AF duration by burst pacing.

Myocardial ischemia is associated with an increased risk of AF. Sinno et al reported that in dogs, experimental atrial ischemia resulted in slow conduction, which stabilized the re-entrant AF circuit. Subsequently, by using the same atrial ischemia model, it was reported that HSP72 induction by orally administered GGA suppressed conduction heterogeneity and burst pacing-induced AF duration. In the ischemic and non-ischemic regions, HSP72 was intensively induced by GGA, whereas HSP72 induction was not significant. Our research group showed that HSP72 induction attenuated ventricular ischemia-reperfusion injury. Thus, HSP72 rather than HSP27 appears to have a more important role in the prevention of ischemia-induced production of AF substrate.

**Chronic Kidney Disease (CKD) and Novel Antioxidant Agent**

Recent studies have revealed a close relationship between AF and CKD. An analysis based on the Chronic Renal Insufficiency Cohort (CRIC) study reported that the prevalence of AF was 18% in participants with mild-to-moderate CKD, suggesting that the prevalence of AF was 2- to 3-fold higher than that of the general population. Thus, it is necessary to elucidate the mechanisms responsible for development of AF among people with CKD and to explore preventive strategies. However, there have been few reports exploring the pathogenesis of CKD-related AF, because no appropriate animal model of AF related to CKD has been established. In this regard, we have recently established a model of AF associated with CKD in rats. The 5/6 nephrectomy (5/6Nx) rat model has been used widely to represent the pathogenesis of clinical CKD. In 5/6Nx rats, a major contributor to the elevation in circulating levels of inflammatory biomarkers may be enhanced oxidative stress, the mechanisms of which involve activation of nicotinamide adenine dinucleotide phosphate oxidase (NADPH) oxidase.

We therefore tested the validity of 5/6Nx as an appropriate model of AF associated with CKD and investigated the role of oxidative stress. Male Sprague-Dawley rats underwent 5/6Nx. To evaluate the involvement of oxidative stress, a novel de-
Derivative of lipoic acid, sodium zinc dihydrolipoylhistidinate (DHLHZn), was subcutaneously infused and 4 weeks later, the hearts were isolated and evaluated: 5/6Nx induced renal dysfunction corresponding to stage 4 CKD. In the LA, expressions of α-SMA and collagen type 1, the compositional proteins of NADPH oxidase, and malondialdehyde (MDA) were increased by 5/6Nx and was reversed by DHLHZn treatment. Figures 5A–C demonstrate the protein expression of the NADPH oxidase subunit; 5/6Nx resulted in the expression of gp91\textsuperscript{phox}, Nox4 and p47\textsuperscript{phox} and treatment of 5/6Nx-treated rats with DHLHZn reduced these expressions. In addition, 5/6Nx increased the expression of MDA (Figure 5D), which treatment with DHLHZn reduced. In the LA, the tissue content of AII was elevated by 5/6Nx and reversed by DHLHZn. Masson-trichrome staining demonstrated that heterogeneous LA interstitial fibrosis was induced by 5/6Nx and attenuated by DHLHZn (Figure 6). In isolated-perfused heart experiments, 5/6Nx caused the slowing of interatrial conduction. In the hearts of the rats from the 5/6Nx group, right atrial extrastimuli invariably induced AF, which was suppressed by DHLHZn (Figure 7). In experiments in vivo, the P-wave duration was prolonged in 5/6Nx-treated rats and this was suppressed by DHLHZn (Figure 8).

The cardiovascular pathologic features of our CKD model, including cardiac hypertrophy with left ventricular hyperkinetic motion, were consistent with those of previous studies.\textsuperscript{45,46} The LA of 5/6Nx-treated rats showed heterogeneous interstitial fibrosis. Interatrial conduction was delayed and AF inducibility was increased. However, the ERP of the LA was not affected. These observations were similar to those in 2 AF models that we reported recently, namely, continuous infusion of angiotensin II\textsuperscript{13} and pressure overload by AAC.\textsuperscript{12} Taken together, the results suggest that the 5/6Nx procedure provokes structural remodeling of the atrium characterized by interstitial fibrosis, leading to an appropriate animal model of CKD-related AF. Numerous factors underlying this model, including hypertension, inflammation, oxidative stress, and activation of the sympathetic nervous system and renin-angiotensin system, could participate in the development and progression of atrial fibrosis. Of these, we focused on oxidative stress, because in kidney tissue, 5/6Nx is reportedly associated with increased activity of oxygen free radicals, depressed expression of superoxide dismutase and elevated expression of NADPH oxidase.\textsuperscript{43,44} Clinically, in samples of right atrial appendages obtained from 170 patients undergoing coronary bypass surgery, the activity of NADPH oxidase was increased in patients who developed postoperative AF.\textsuperscript{47,48} In gp91\textsuperscript{phox} knockout mice, neither cardiac hypertrophy nor interstitial ventricular hypertrophy was induced by continuous infusion of AII.\textsuperscript{49} Nox4 is expressed primarily in mitochondria in cardiomyocytes.\textsuperscript{50} Nox4 in cardiac myocytes was recently demonstrated to be a major source of mitochondrial oxidative stress, and thereby mediates mitochondrial and cardiac dysfunction, as well as fibrosis during pressure overload.\textsuperscript{51} In experimental liver fibrosis induced by bile duct ligation, p47\textsuperscript{phox} knockout mice showed attenuated liver fibrosis and expression of α-SMA.\textsuperscript{52} Using hepatic cellular...
stead cells, it was also demonstrated that phosphorylation of p47phox by AII induced the formation of reactive oxygen species via the enhanced activity of NADPH oxidase. Thus, profibrotic signals of gp91phox, Nox4 and p47phox in response to excessive tissue levels of AII might underpin the progression of LA interstitial fibrosis in 5/6Nx-treated rats. MDA is the main product of peroxidation of polyunsaturated fatty acids. MDA is highly toxic and is considered to be a marker of lipid peroxidation. Cardiac ventricular MDA levels are reportedly increased in 5/6Nx rats. Circulating levels of MDA have been reported to be elevated in patients with AF, and higher baseline values of MDA can be used to predict AF recurrence after cardioversion. DHLH/Zn, used in our study, has been shown to attenuate hepatic ischemia-reperfusion injury in rats in association with a reduction in MDA levels in the liver. It also prevented cardiac and cellular mitochondrial dysfunction in an isolated-perfused rat heart model. DHLH/Zn successfully attenuated increases in the levels of gp91phox, Nox4, p47phox and MDA in the LA in our rat CKD-related AF model. Antioxidant interventions can be expected to be a preventive strategy for AF associated with CKD.

Conclusions

Our observations demonstrated that inflammatory profibrotic signals are essentially involved in the pathogenesis of atrial fibrosis induced by pressure overload, excessive AII and CKD. Pioglitazone, induction of HSPs and antioxidant agents could be potential therapeutic approaches for these fibrotic conditions and AF vulnerability, respectively. We would like to emphasize that the early process of atrial interstitial fibrosis induced by pressure overload is quite similar to that of atherosclerosis. Innovative therapeutic approaches for atherosclerosis may lead to discovery of novel strategies to prevent atrial fibrosis and AF.

Disclosures

Conflict of Interest for All Authors: None.

References


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