No Additional Effect of DPP-4 Inhibitor on Preventing Atrial Fibrosis in Streptozotocin-Induced Diabetic Rat as Compared With Sulfonylurea

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SUMMARY

Chronic inflammation is known to occur in diabetes mellitus (DM) and contributes to atrial fibrosis, possible substrates for atrial fibrillation. We tested the hypothesis that dipeptidyl peptidase (DPP)-4 inhibitors prevent the formation of atrial fibrosis through their anti-inflammatory activity, beyond the effects of controlling blood glucose.

DM models obtained by administration of streptozotocin (STZ) were divided into 3 groups: with PKF275-055, a DPP-4 inhibitor in group D, glibenclamide in group SU, and no additional drug in group P. At 8 weeks after STZ administration, the heart was subjected to Masson trichrome staining and immunohistochemistry with anti-ED2, ED3, and smooth muscle actin antibody.

The % area of fibrosis in atria of group P accounted for 14.7% ± 4.1%, showing a significant increase in fibrosis when compared with the control group. In group SU, the % area accounted for 7.9% ± 2.9%, indicating significant decreased fibrosis by sulfonylurea. Meanwhile, we could not find significant differences in group D when compared to group P or group SU. While ED3-positive cells increased in group P (1.12% ± 0.24%), they were significantly decreased in groups D and SU (0.41% ± 0.22% and 0.55% ± 0.29%, respectively). Between group D and SU, however, there were no significant differences in the amount of cells positive to ED2, ED3, and smooth muscle actin antibodies.

In STZ-induced DM rats, administration of sulfonylurea and DPP-4 inhibitors inhibited inflammation and fibrosis of the atria. However, no significant differences were observed between the 2 antidiabetic drugs. (Int Heart J 2016; 57: 336-340)

Key words: Atrial fibrillation, Diabetes mellitus, Inflammation

Atrial fibrillation (AF) is one of the most common types of arrhythmia encountered in daily clinical practice.1 Although AF infrequently impairs hemodynamics, thrombus formation in the atria is a critical problem in an aging society where stroke represents an important cause of death.2-5 Catheter ablation for this arrhythmia has been developed and recently used for the treatment of this arrhythmia. However, many aspects of the mechanisms underlying the development of AF remain unknown, and no effective and specific therapeutic strategy has been established for AF prevention.

Several established risk factors involved in the development of AF are known. Among them, diabetes mellitus (DM) is important because of the large number of patients.6-11 Chronic inflammation is known to occur in various tissues in DM,12,13 and it has also been indicated that inflammation of the atria may be one of the factors responsible for AF development.14-16 Meanwhile, dipeptidyl peptidase (DPP)-4 inhibitors, which have started to be used in recent years, are antidiabetic agents with reported anti-inflammatory activity.17,18 Thus, we formulated the following hypothesis and tested it in DM model rats: because of their anti-inflammatory activity, DPP-4 inhibitors used for the treatment of DM were more effective than sulfonylurea (SU) in reducing the substrates for AF.

METHODS

Development of DM model rats: Streptozotocin (STZ; Wako Chemicals, Osaka, Japan) dissolved in citric acid buffer was intraperitoneally administered at 60 mg/kg to 8-week-old female Wistar rats. One week after administration of STZ, rats with a fasting blood glucose level of 200 mg/dL or higher were selected and used for the experiment. The control rats were only administered citric acid buffer.

The DM model rats were divided into 3 groups. In group D, PKF275-055 (provided by Novartis Pharma K.K.), a DPP-4 inhibitor, was administered at 10 mg/kg per day for 8 weeks.
(dissolved in drinking water at a concentration of 0.025 mg/mL). In group SU, glibenclamide was administered at 8 mg/kg per day for 8 weeks (mixed with feed at a concentration of 0.005%). In group P, a standard feed was given. Each group consisted of 5 rats.

Measurement of blood glucose levels: Casual blood glucose levels were measured at 4 and 8 weeks after administration of STZ with a OneTouch UltraVue (LifeScan, Inc., Milpitas, CA, USA), a portable measuring device.

Histology: At 8 weeks after treatment, the heart was excised, perfused with physiological saline, embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek Japan, Tokyo), and then immediately frozen with liquid nitrogen. Frozen sections were prepared and stained with Masson trichrome. Tissue staining was performed with ED2 (T-3011, BMA Biomedicals, Augst, Switzerland) and ED3 antibodies (T-3013, BMA Biomedicals) for macrophage markers and with smooth muscle actin (M0851, Dako, Glostrup, Denmark) for smooth muscle and fibroblastic markers. Image-Pro Plus (Media Cybernetics, Rockville, MD, USA) application software was used to measure the proportions of areas occupied by collagen fibers stained as blue in the Masson trichrome-stainings. The proportions of areas stained as brown were measured in the immunostained samples.

Statistical analysis: Data are expressed as the mean ± SD. Differences between groups were determined by two-way repeated measure ANOVA (for blood glucose) or one-way ANOVA (for the other data) followed by the Bonferroni method. Differences were considered significant when \( P < 0.05 \).

RESULTS

Blood glucose levels: The fasting blood glucose levels in each group are shown in Figure 1. While the mean fasting blood glucose level in the control group was 119 mg/dL, that in each group of the DM model rats before administration of DPP-4 inhibitors or SU was 500 mg/dL or higher, showing marked hyperglycemia. In group D, the fasting blood glucose levels at 4 weeks after treatment were significantly decreased from that before treatment \( (P < 0.05) \). Also in group SU, a decrease in blood glucose levels was observed at 8 weeks after treatment \( (P < 0.05) \).

Atrial fibrosis: On the Masson trichrome-stained sections, the proportions of areas occupied by collagen fibers in the left atrial tissue were measured (Figures 2 and 4). There were significant differences among the 4 groups \( (P < 0.01) \). While the % area of fibrosis accounted for 6.6% ± 1.8% in the control group, the % area in group P accounted for 14.7% ± 4.1%, showing a significant increase in fibrosis in the atrium \( (P < 0.05) \). Meanwhile, in group SU, the % area accounted for 7.9% ± 2.9%, showing that fibrosis was significantly inhibited in comparison with group P \( (P < 0.05) \). In group D, the % area of fibrosis, which accounted for 10.0% ± 4.2%, was slightly decreased, but the difference did not reach statistical significance.

Immunostaining: We found significant changes in ED3 staining \( (P < 0.01) \). Although the area stained with ED3 antibody accounted for 0.34% ± 0.17% in the control group, this area in group P increased up to 0.12% ± 0.24%, showing a significant increase \( (P < 0.05) \). The areas in groups SU and D accounted for 0.55% ± 0.29% and 0.41% ± 0.22%, respectively, which were significantly lower in comparison with group P \( (P < 0.05, \text{Figure 3}) \). Meanwhile, the areas stained with ED2 antibody and smooth muscle actin were slightly increased in group P in comparison with the control group and were slightly decreased in groups D and SU in comparison with group P, but the difference did not reach statistical significance \( (\text{Figure 3}) \). Otherwise, between groups D and SU, no significant differences were observed in areas stained with ED2, ED3 and smooth muscle actin antibodies. The severity of fibrosis and macrophage infiltration is shown in Figure 4.
Atrial fibrillation (AF) is a common type of arrhythmia in the elderly, and the known risk factors include age, diabetes mellitus (DM), hypertension, heart failure, myocardial infarction, and valvular heart disease.\textsuperscript{11,19} The Framingham study showed that DM is a predictive factor of AF,\textsuperscript{6} and other observational studies also showed that DM is an independent risk factor of AF.\textsuperscript{7-9}

Fibrosis is widely known to be advanced in the atria of patients with AF,\textsuperscript{14,15} and this fibrosis is considered to be one of the important causes of AF. Regarding the mechanism for this atrial fibrosis, the involvement of chronic inflammation has recently been suggested in addition to enhancement of the local renin-aldosterone system.\textsuperscript{20-22} A well-known fact is that the AF incidence increases with exacerbation of inflammation after pericarditis, myocarditis, or cardiac operation. In addition, it has been reported that even without such inflammatory pathological conditions, biomarkers of inflammation are associated with the occurrence, recurrence, and perpetuation of AF. C-reactive protein (CRP) is produced in the liver in response to cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-\alpha. In nonsurgical patients with AF, CRP levels were 2 times higher than those in the control patients.\textsuperscript{23} Moreover, CRP levels were higher in the patients with persistent AF than in those with paroxysmal AF. In a study following a cohort of 5806 patients, high CRP levels were associated with not only the presence of AF but also future development of the disease.\textsuperscript{24} Many other reports suggest that inflammation is strongly associated with AF. For example, IL-6, an inflammation-related cytokine, is associated with AF,\textsuperscript{25} and inflammatory cells, including inflammatory cytokines, infiltrate from the

**Figure 3.** Immunostaining. The number of ED3-positive macrophages was significantly increased in the diabetic rats and significantly decreased in either group of rats treated with the antidiabetic agents. ED2-positive macrophages and smooth muscle actin-positive cells were increased in the diabetic rats. Although this increase was slightly inhibited by administration of the antidiabetic drugs, inhibition was not significant for either the macrophages or the cells.

**Figure 4.** Tissues stained with Masson trichrome and immunohistochemistry. Compared with the control group, group P showed increases in the severity of fibrosis and ED3-positive macrophage infiltration, whereas group SU showed that fibrosis and infiltration were inhibited. In group D, only infiltration of ED3-positive macrophage was significantly inhibited. No significant change was observed in ED2-positive macrophage infiltration.
endocardial side to the atrial wall in AF patients.\textsuperscript{16}

Meanwhile, DM has been revealed to exist owing to chronic inflammation of adipose tissue, and chronic inflammation is considered to occur in various tissues according to DM progression. This insight raises the hypothesis that chronic inflammation is involved in the development of AF substrates in DM patients. In fact, it has been shown that in DM model rats, advanced fibrosis in the atria causes conduction disturbance and is likely to serve as a substrate for arrhythmia.\textsuperscript{25} DPP-4 inhibitors, which are recently increasingly being used as antidiabetic agents, are reported to exert an anti-inflammatory action against arteriosclerotic diseases. In adipose tissue and the pancreas of high-fat diet obese mice, the expression levels of messenger ribonucleic acid (mRNA) for inflammatory cytokines such as IL-6 were reduced by administration of sitagliptin, and infiltration of macrophages to adipose tissue was also reduced.\textsuperscript{17} Fat-induced pancreatitis and peri-insulitis were reportedly completely prevented by long-term administration of vildagliptin in high-fat diet obese mice.\textsuperscript{18}

STZ is widely used to induce experimental diabetes mellitus in animals. STZ is taken up passively by pancreatic B cells via glucose transporter, and causes a decrease in insulin secretion.\textsuperscript{26} The plasma insulin level was significantly reduced in STZ-induced diabetic rats compared with that in control rats.\textsuperscript{27} Inflammation has been observed also in STZ-induced diabetic rats. Plasma levels of IL-6, MCP-1, and TNF-α of STZ-induced diabetic rats were significantly higher than those of control rats.\textsuperscript{28} The protein expression of IL-6, TNF-α, and IL-1β and markers of macrophages increased significantly in STZ-induced diabetic rat hearts compared with those in control rat hearts.\textsuperscript{29,30} Therefore, we chose STZ-induced diabetic rats as the chronic inflammatory diabetic model in the present study.

Based on such a scientific background, the present study was conducted to test the following hypotheses: 1) if atrial fibrosis in DM model rats is reduced as blood glucose levels are reduced; and 2) if administration of DPP-4 inhibitors, which are considered to have an anti-inflammatory activity, reduces atrial fibrosis more than administration of SU, which does not have any anti-inflammatory activity. In the atria of rats with STZ-induced DM, fibrosis was significantly increased in comparison with that in the atria of the control rats, and ED3-positive cells were increased. The ED3 antibody recognizes cluster of differentiation (CD) 169 antigen expressed on the surface of rat macrophages and is believed to detect classically activated macrophages (M1 macrophages). The ED2 antibody recognizes CD163, which is expressed on the alternatively activated macrophages (M2 macrophages). In the present study, persistent hyperglycemia due to DM increased collagen fibers and classically activated macrophages in the atria. The changes in classically activated macrophages were significantly inhibited by the hypoglycemic effects produced by administration of both SU and DPP-4 inhibitor, and the increase of collagen fibers was significantly inhibited by SU. This may support the previously reported hypothesis that persistent hyperglycemia causes chronic inflammation, which consequently leads to fibrosis. It has been observed in adipose tissue of obese women that the number of M1 macrophages was increased, whereas the number of M2 macrophages remained unchanged.\textsuperscript{31} It has also been observed that the ratio of M1 to M2 macrophages in peripheral blood monocytes is significantly increased in obese patients with DM.\textsuperscript{32} The present study shows that, similarly to adipose tissues, in the atria of DM model rats, the number of M1 macrophages is significantly increased, whereas the number of M2 macrophages remains almost unchanged.

Meanwhile, an issue that could not be solved by the above-mentioned explanation was also revealed. The number of cells expressing smooth muscle actin, a fibroblastic marker, was increased by DM. However, the decrease in the number of the cells by the hypoglycemic action of the antidiabetic agents was mild, and the decrease was not significant and diverged from the decrease in ED3 antibody-positive cells. Atrial fibrosis is mainly caused by myofibroblasts containing abundant c-smooth muscle actin.\textsuperscript{33} However, cytokines including transforming growth factor (TGF) β, endothelin-1, angiotensin II, connective tissue growth factor, and platelet-derived growth factor (PDGF) are intricately involved in actual fibrogenesis, and many details remain unknown. This indicates that the mechanism for the development of atrial fibrosis in DM is not simple as hypothesized in the present study.

In the present study both SU and DPP-4 inhibitor decreased the number of M1 macrophages, but only SU reduced fibrosis, which seems to indicate the superiority of SU. However, there were no significant differences between SU and DPP-4 inhibitor in blood glucose level, collagen fiber, or M1 macrophages. It is inappropriate to conclude that SU is superior to DPP-4 inhibitor based on these results. Further studies will be needed to discuss the relative merits of both drugs.

In addition, this study did not confirm that DPP-4 inhibitors, which have an anti-inflammatory activity, exerted additional effects on fibrosis and macrophage infiltration, compared with SU. Although this finding does not completely deny the anti-inflammatory activity of DPP-4 inhibitors in atria, it seems to suggest that control of blood glucose levels is more important than the pleiotropic effects of the inhibitors to inhibit substrate formation for AF.

The present study has the following limitations: 1) although we determined the drug concentrations in feed and drinking water after investigating beforehand according to the consumed feed and drinking water of the rats, precise ingested doses might have varied. 2) The blood glucose levels at 4 weeks after treatment differed between groups SU and D. This study aimed to evaluate the superiority of DPP-4 inhibitors. Because the blood glucose levels were somewhat lower in group D, we do not believe that the differences in the levels affect the conclusion about the failure to detect any additional effect of DPP-4 inhibitors. 3) The experiment was discontinued after 8 weeks of treatment because of increased mortality. However, this short duration of treatment might have contributed to the failure to detect differences between DPP-4 inhibitors and SU. 4) Although STZ was used to develop DM models, the models developed with STZ exhibited the pathological conditions of type 1 DM instead of DM caused by obesity. However, these models were also reported to exhibit chronic organ inflammation.\textsuperscript{28,30,31}

Conclusion: In STZ-induced DM model rats, administration of SU and DPP-4 inhibitors prevented inflammation and fibrosis of the atria. However, no additional effect due to the anti-inflammatory activity of DPP-4 inhibitors was confirmed. For the inhibition of atrial fibrosis, the control of blood glucose levels seemed to be more important.
DISCLOSURES

Dr. Hayami received remuneration from Boehringer Ingelheim, Bristol-Myers Squibb, Sanofi, Daiichi-Sankyo, and Astellas Pharma. Dr. Sekiguchi has no conflict of interest. Dr. Iwasaki received remuneration from Boehringer Ingelheim, Daiichi-Sankyo, Pfizer, and Ono Pharmaceutical. Dr. Murakawa received remuneration from Boehringer Ingelheim, Daiichi-Sankyo, and Bayer Healthcare. Dr. Yamashita received research funding from Boehringer Ingelheim and Daiichi-Sankyo, and remuneration from Boehringer Ingelheim, Daiichi-Sankyo, Bayer Healthcare, Pfizer, Bristol-Myers Squibb, Eisai, Toa-Eiyo, and Ono Pharmaceutical.

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