Role of Leptin Signaling in the Pathogenesis of Angiotensin II - Mediated Atrial Fibrosis and Fibrillation

Running title: Fukui et al.; Leptin and Atrial Fibrillation

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Abstract:

**Background** - We examined the hypothesis that leptin signaling contributes to the atrial fibrosis and atrial fibrillation (AF) evoked by angiotensin II (AngII).

**Methods and Results** – Eight-week-old male CL57/B6 (CNT) and leptin-deficient ob/ob mice (Ob) were subcutaneously infused with AngII (2.0 mg/kg/day). Two weeks later, transesophageal burst pacing and an electrophysiological study using isolated perfused hearts were performed. Left atrial tissues were collected to determine interstitial fibrosis by Masson trichrome staining and the expressions of mRNAs related to inflammatory profibrotic signals were assessed. Left atrial fibroblasts were isolated from adult Sprague-Dawley (SD) and Zucker rats. The effects of leptin (100 ng/ml) or AngII (100 nM) treatment were evaluated. In CNT-AngII mice, leptin expression in the left atrium was upregulated (p<0.01). Transesophageal burst pacing induced AF in 88% (7/8) of CNT-AngII mice, but not in Ob-AngII mice (0/8, p<0.01). In isolated perfused hearts, AF was induced only in CNT-AngII mice (4/6, 67%). Inter-atrial conduction time was prolonged in CNT-AngII mice (p<0.01), but not in Ob-AngII mice. The upregulation of collagen 1, collagen 3, TGF-β1, α-SMA, MCP-1, F4/80 and RANTES mRNA that was seen in CNT-AngII mice, was attenuated in Ob-AngII mice. In cultured SD rat atrial fibroblasts, AngII treatment increased leptin expression (p<0.01). Addition of leptin increased TGF-β1, α-SMA, MCP-1 and RANTES expressions in SD rat atrial fibroblasts but not in Zucker rat atrial fibroblasts.

**Conclusions**- Our results demonstrate for the first time that leptin signaling is essential for the development of atrial fibrosis and AF evoked by AngII.

**Key words:** atrial fibrillation; angiotensin; collagen; electrophysiology; pathology
Introduction

Atrial fibrillation (AF) is the most common arrhythmia seen in the clinical setting, and is associated with significant morbidity and mortality.\(^1,2\) Accumulating evidence indicates that atrial structural remodeling, especially inflammatory profibrotic signals that led to the development of interstitial atrial fibrosis, have been revealed to play a crucial role in the pathogenesis of AF.\(^3-5\)

Leptin, a 16 kDa peptide hormone, that is, mainly secreted by adipocytes, regulates appetite and body fat mass, predominantly through the central nervous system.\(^6-8\) However, it has additionally been identified as a proinflammatory adipokine that can provoke inflammatory-mediated interstitial fibrosis in several peripheral organs.\(^9-12\) For instance, Honda et al.\(^11\) demonstrated that thioacetamide failed to trigger hepatic fibrosis in leptin deficient ob/ob (Ob) mice. Similarly, Tanaka et al.\(^12\) demonstrated that renal macrophage infiltration and tubulointerstitial fibrosis evoked by unilateral ureteral obstruction was reduced in Ob mice. These observations led us to hypothesize that leptin signaling plays an essential role in proinflammatory atrial fibrosis. In this regard, we have demonstrated that continuous subcutaneous infusion of angiotensin II (AngII) results in interstitial atrial fibrosis and increased AF inducibility in rats.\(^13\) We also demonstrated that AngII upregulated extracellular signal-regulated kinase 1/2 (Erk1/2) phosphorylation, \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) expression, transforming growth factor-\(\beta\)1 (TGF-\(\beta\)1) secretion, and collagen synthesis in cultured rat atrial fibroblasts.\(^13\) Thus, our continuous AngII infusion AF model can be postulated to represent AF progression associated with enhanced inflammatory profibrotic signals.

In the present study, we investigated the involvement of leptin signaling in the pathogenesis of AngII-evoked inflammatory atrial fibrosis and enhanced AF vulnerability, using
leptin-deficient Ob mice in vivo and ex vivo, and in vitro in rat atrial fibroblasts that contain a malfunctioning receptor for leptin receptor (the Zucker rat model).

Methods

All experimental procedures were conducted in accordance with the guidelines of the Physiological Society of Oita University, Japan, for the care and use of laboratory animals, following the guidelines established by the U.S. National Institutes of Health. For detailed methods, see the online-only Data Supplement.

Results

Hemodynamic characteristics

Systolic blood pressure and heart rate measurements during the experimental period are shown in Supplemental Figure 1. Prior to AngII infusion, no significant differences in these two parameters were observed between CNT and Ob mice. Continuous AngII infusion caused elevation of systolic blood pressure and heart rate in both CNT-AngII and Ob-AngII mice (both p<0.01). We observed no significant differences in these two parameters between CNT-AngII and Ob-AngII mice on days 7 and 14.

Physiological and echocardiographic findings

The physiological and echocardiographic findings on day 14 are presented in Table 1. Compared to the corresponding VEH-treated mice, body weights were significantly lower in the CNT-AngII and Ob-AngII mice (both p<0.01). However, AngII infusion led to increased heart weight in CNT-AngII and Ob-AngII mice compared to the corresponding VEH-treated mice (both p<0.01). The heart-to-body weight ratio was significantly greater in the CNT-AngII and Ob-AngII groups relative to the corresponding VEH-treated mice (both p<0.01). Echocardiographic findings
revealed that AngII caused concentric hypertrophy in both CNT and Ob mice, characterized by a decrease in LVDd (both p<0.05) and an increase in LVPWth (both p<0.01). LVEF was not significantly influenced by AngII in either the CNT-AngII or Ob-AngII groups.

**Serum parameters**

Serum blood chemistry data are presented in Table 1. In CNT mice, AngII did not cause significant changes in the plasma levels of glucose and insulin. In contrast, AngII infusion in Ob mice resulted in a significant decrease in plasma glucose (p<0.05) and insulin (p<0.01). As expected, plasma leptin was not detected in Ob mice. In CNT mice, AngII infusion resulted in a decreased level of plasma leptin (p<0.05). Plasma adiponectin level was not significantly different between the four experimental groups.

**Left atrial tissue expression of leptin**

Representative immunohistochemical staining using leptin antibody is depicted in Figure 1A. Brown staining indicates leptin expression. Leptin was expressed at low levels in the CNT-VEH mouse left atrium but was abundant in the AngII-CNT mouse left atrium. On the other hand, leptin was not observed in the Ob mouse left atrium, irrespective of AngII infusion. Figure 1B displays quantitative analysis of leptin mRNA in left atrial tissue. Compared with CNT-VEH mice, CNT-AngII mice showed a 4.79-fold increase in leptin mRNA (p<0.01). The protein levels of leptin in the whole heart were greater in CNT-AngII mice than in CNT-VEH mice (1.7-fold, p<0.01; Supplemental Figure 2).

**Messenger RNAs related to profibrotic signals**

Quantitative analysis of the mRNA levels of molecules related to profibrotic and proinflammatory signals is presented in Figure 2. Messenger RNAs of collagen 1 and collagen 3 (Figures 2A, B) were increased by AngII infusion, both in CNT-AngII (3.01- and 3.50-fold,
respectively; both p<0.01) and Ob-AngII mice (1.78- and 1.70-fold, respectively; p<0.01 and p<0.05, respectively) compared with the CNT-VEH group. Notably, mRNA expression levels were significantly lower in Ob-AngII than CNT-AngII mice (p<0.05 and p<0.01, respectively). Similarly, in CNT mice, AngII induced a significant increase in the expression of TGF-β1 (1.87-fold, p<0.01), α-SMA (2.50-fold, p<0.01), F4/80 (2.23-fold, p<0.01), MCP-1 (3.34-fold, p<0.01), and RANTES (3.76-fold, p<0.01), as shown in Figures 2C-G. However, the increase did not reach statistical significance in Ob mice. Therefore, the mRNA expression of TGF-β1, α-SMA, F4/80, MCP-1, and RANTES were significantly lower in the Ob-AngII mice than in CNT-AngII mice (p<0.05, p<0.01, p<0.01, p<0.01, p<0.01, respectively). The corresponding protein levels of these mRNAs isolated from whole heart in CNT mice were significantly increased in response to continuous AngII infusion while such a response was significantly attenuated in Ob mice (Supplemental Figure 3).

Left atrial interstitial fibrosis

Figure 3A shows the representative results of Masson trichrome staining of the left atrium. The blue staining indicates interstitial fibrosis. The quantitative ratio of the area of fibrosis to the area of the reference tissue is summarized in Figure 3B. Masson trichrome staining revealed inhomogeneous interstitial fibrosis in CNT-AngII mice left atrium (p<0.01). However, such fibrosis was not observed in Ob-AngII mice left atrium. By quantification, interstitial fibrosis was attenuated in Ob-AngII mice compared with CNT-AngII mice (p<0.05).

Hydroxyproline assay

Supplemental Figure 4 shows the collagen content in the left atrium as evaluated by quantification of hydroxyproline. Continuous AngII infusion significantly increased left atrial collagen content in CNT-AngII mice (p<0.01). This increase was attenuated in Ob-AngII mice.
Incidence and duration of AF by transesophageal burst pacing

Representative ECG recordings before and after the transesophageal burst pacing are presented in Figure 4A. In Figure 4A, burst pacing was followed by induction of AF. AF was equally induced in 1/8 (13%) of both the CNT-VEH and Ob-VEH groups, as shown in Figure 4B. Burst pacing induced AF in 7/8 (87%) of CNT-AngII mice (p<0.01 vs. CNT-VEH), but not in Ob-AngII mice (0/8; p<0.01 vs. CNT-AngII). During the AF induced in CNT-AngII mice, the mean ventricular cycle length (CL) was 172±8 ms. The analysis of AF duration is depicted in Figure 4C. The mean duration of AF was significantly longer in CNT-AngII mice compared with the CNT-VEH mice (p<0.01).

Electrophysiological studies using isolated perfused hearts

No significant differences were observed in left atrial ERP between the four experimental groups (Figure 5A). When compared with the CNT-VEH and Ob-VEH groups, the IACT in the CNT-AngII mice was prolonged at all the basic CLs tested (p<0.01; Figure 5B). This prolongation was suppressed in Ob-AngII mice (p<0.01 for each CL). Figure 5C shows a representative AF induced by the burst pacing, which was observed in a CNT-AngII mouse. Figure 5D shows the inducibility of AF. In the CNT-AngII group, S3 extrastimuli and/or burst pacing induced AF in 4/6 rats (67%). In contrast, AF was not induced at all in hearts from CNT-VEH, Ob-VEH or Ob-AngII mice (0/6 in each group). Figure 5E illustrates the AF duration while Figure 5F plots the atrial and ventricular CLs during AF. During the AF induced in CNT-AngII mice, the atrial and ventricular CLs were 58.3±15.3 and 244.0±39.0 ms, respectively.

Leptin expression in rat atrial fibroblasts

In CNT rat atrial fibroblasts, the addition of AngII (100 nM) into the culture medium increased
leptin expression at both the mRNA (Figure 6A) and protein levels (Figure 6B) (both p<0.01).

**Experiments using Zucker rat atrial fibroblasts**

The distinct responses between SD and Zucker rat atrial fibroblasts to leptin (100 ng/ml) in terms of profibrotic signals are shown in Figure 7. TGF-β1 secretion was increased by leptin in SD rat atrial fibroblasts (p<0.05; Figure 7A). Expression of α-SMA resulted in a 1.56-fold increase in SD rat atrial fibroblasts (p<0.01; Figure 7B). The expressions of MCP-1 by leptin also increased 2.02-fold in SD rat atrial fibroblasts (p<0.01; Figure 7C). The expression of RANTES in response to leptin showed a 1.63-fold increase in SD rat atrial fibroblasts (p<0.01, Figure 7D). In sharp contrast, these positive responses to leptin were lacking in atrial fibroblasts isolated from Zucker rats (Figures 7A-D).

**Discussion**

The core findings of the present study are as follows: (1) in the mouse in vivo experiments, naturally occurring leptin-deficient Ob mice did not develop left atrial interstitial fibrosis and AF inducibility in response to continuous infusion of AngII; (2) in an electrophysiological study using isolated perfused hearts, neither the high inducibility of AF nor the atrial conduction delay observed in CNT-AngII mice was observed in Ob-AngII mice; (3) left atrial tissue isolated from CNT mice showed abundant leptin expression in response to continuous AngII infusion, which led to upregulated leptin expression in cultured SD rat atrial fibroblasts; and (4) left atrial tissue isolated from Ob mice showed a reduced effect of AngII inflammatory profibrotic signal-related mRNA. Consistent with these findings, cultured atrial fibroblasts isolated from Zucker (leptin receptor malfunctioning) rats did not express molecules associated with inflammatory profibrotic signals in response to leptin treatment. The lack of these responses to AngII was also confirmed in whole heart isolated from Ob mice. To our knowledge, this is the first report to demonstrate
the essential role of leptin and its receptor-mediated signaling in the pathogenesis of atrial fibrosis and AF. Enhanced local atrial expression of leptin is also suggested to play an autocrine role in AngII-induced atrial fibrosis, which is absent in Ob mice and Zucker rats. Although there is no direct evidence, the mechanisms of AF observed in CNT-AngII mice can be postulated to be reentrant due to atrial conduction disturbance, which is associated with AngII-induced atrial interstitial fibrosis.

The relationship between leptin signaling and fibrogenesis has been extensively investigated in the liver.9-11 Ikejima et al.9 first demonstrated that intraperitoneal administration of leptin, administered with hepatotoxic chemicals including carbon tetrachloride and thioacetamide, enhanced hepatic fibrosis in association with enhanced expression of α-SMA and TGF-β1. The authors concluded that upregulation of TGF-β1, leading to activation of hepatic stellate cells (HSCs), is an essential leptin-mediated fibrogenic response in the liver.9 Subsequently, the same group demonstrated near-complete prevention of thioacetamide-induced hepatic fibrosis in Zucker rats, where induction of TGF-β1 and activation of HSCs were abolished as well.14 These observations are consistent with those of our present study. In our AngII infusion mouse model, atrial fibroblasts appear to play a key role in the promotion of profibrotic signals, similar to HSCs in the liver. Interestingly, the above authors demonstrated that although the normal liver does not produce leptin, activated HSCs produce leptin in vivo during fibrogenesis caused by thioacetamide.14 This finding is also in agreement with our present observations that left atrial tissue (Figure 1A) and atrial fibroblasts stimulated by AngII (Figure 6) express abundant leptin. The same group recently demonstrated that hepatic fibrosis caused by thioacetamide does not develop in Ob mice.11

A role for leptin signaling in macrophage infiltration has been reported in the kidney.12
Tanaka et al.\textsuperscript{12} showed reduced renal macrophage infiltration after unilateral ureteral obstruction in Ob mice, a situation that was reversed by leptin administration. In the present study, we evaluated the transcript levels of F4/80, a marker of mature macrophages, as well as MCP-1 and RANTES, which are chemokines required for monocyte accumulation. We have recently demonstrated that expression of MCP-1 in the left atrium was enhanced by pressure overload via abdominal aortic constriction (AAC) in rats, which was suppressed by pioglitazone.\textsuperscript{15} Based on these findings, we concluded that MCP-1 is a key molecule in AAC-induced inflammatory fibrotic processes in the left atrium and that pioglitazone attenuates atrial fibrosis, possibly via suppression of MCP-1 expression.\textsuperscript{15} Our current findings that the upregulation of F4/80, MCP-1 and RANTES witnessed in CNT mice and SD rat atrial fibroblasts is blocked by AngII in Ob mice and leptin in Zucker rat atrial fibroblasts also suggest that monocyte/macrophage infiltration into left atrial tissues is promoted by leptin signaling.

Although limited studies have investigated the role of leptin in cardiac fibrosis, its hypertrophic effects have been demonstrated in experiments using cardiomyocytes.\textsuperscript{16,17} Rajapurohitam et al.\textsuperscript{16} initially reported that exposure of cultured neonatal rat ventricular myocytes to leptin for 24 h produced cell hypertrophy, in association with enhanced expression of \(\alpha\)-SMA and myosin light chain. Subsequently, the same group demonstrated that the hypertrophic effects of AngII on rat ventricular myocytes can be prevented by leptin antagonists. Because AngII caused a five-fold increase in leptin in the culture medium, the authors concluded that leptin plays an autocrine role in mediating the hypertrophic effects of AngII.\textsuperscript{17} These findings support our observation that AngII promotes leptin expression in CNT mice left atria and SD rat atrial fibroblasts, although it is unclear why AngII infusion results in decreased serum levels of leptin in CNT mice \textit{in vivo} (Table 1).
Ob mice develop morbid obesity and the characteristic hyperinsulinemia and hyperglycemia of type 2 diabetes by 8 weeks of age. In agreement, the serum levels of glucose and insulin were remarkably increased in the Ob mice at the start of the continuous AngII infusion (8 weeks old), as shown in Table 1. Although we did not perform the analysis, the serum levels of triglycerides and free fatty acids are reportedly elevated in Ob mice. In addition, Ob mouse heart rapidly modifies its energy metabolism, resulting in augmented fatty acid and decreased glucose consumption. Thus, it remains to be elucidated whether our finding that Ob mice are resistant to atrial fibrosis by continuous AngII infusion is due to the direct effects of leptin or due to indirect metabolic changes characteristic to the mice.

On the other hand, leptin has been shown to regulate systemic immune responses, which may explain the lack of fibrotic responses to AngII in Ob mice and Zucker rat atrial fibroblasts. For instance, Gainsford et al. suggested that leptin regulates aspects of hemopoiesis and macrophage function. Santos-Alvarez et al. demonstrated that human leptin stimulates proliferation in a dose-dependent manner and functionally activates circulating monocytes in vitro by inducing the production of cytokines, such as TNF-α and interleukin-6. In fact, leptin-deficient mice are less prone than non-leptin-deficient mice to inflammatory diseases, including experimentally induced colitis, experimental autoimmune encephalomyelitis, type I diabetes and experimentally induced hepatitis. Attenuation of systemic immune responses in Ob mice may, in turn, reduce the development of atrial fibrosis evoked by AngII, as observed in the present study.

From the clinical viewpoint, the serum levels of leptin and AngII are elevated in patients with metabolic syndrome. In the Niigata preventive medicine study, metabolic syndrome was shown to increase the risk of AF development. Our results may thus provide an explanation,
at least partly, for why AF is developed more frequently in patients with metabolic syndrome and additionally support the use of leptin as a novel therapeutic target to prevent atrial fibrosis and AF in this population.

Conclusions

Our results demonstrate for the first time that leptin and its receptor-mediated signaling are fundamental to the pathogenesis of atrial fibrosis and AF evoked by AngII. Further studies are required to confirm the efficacy of leptin as a novel therapeutic target to prevent atrial fibrosis and AF in some populations, including those with metabolic syndrome.

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Conflict of Interest Disclosures: None.

References:


Table 1. Physiological, echocardiographic and serum parameters.

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<tr>
<td></td>
<td>CNT</td>
<td>Ob</td>
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<tr>
<td>BW (g)</td>
<td>24.8 ± 0.5</td>
<td>47.7 ± 1.6</td>
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<tr>
<td>HW (mg)</td>
<td>109.3 ± 0.6</td>
<td>109.5 ± 3.2</td>
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<td>HW/BW ratio (mg/g)</td>
<td>4.41 ± 0.08</td>
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<td>LVDd (mm)</td>
<td>3.10 ± 0.07</td>
<td>3.16 ± 0.11</td>
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<td>LVDs (mm)</td>
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<td>1.89 ± 0.05</td>
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<td>LVEF (%)</td>
<td>76.1 ± 0.06</td>
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<td>LVPWth (mm)</td>
<td>0.65 ± 0.03</td>
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<td>Glucose (mg/dl)</td>
<td>196.3 ± 9.1</td>
<td>309.3 ± 21.9††</td>
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<td>Insulin (ng/ml)</td>
<td>1.60 ± 0.06</td>
<td>71.82 ± 9.32††</td>
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<td>Leptin (ng/ml)</td>
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<td>N.D.</td>
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<tr>
<td>Adiponectin (µg/ml)</td>
<td>25.8 ± 5.6</td>
<td>28.0 ± 4.7</td>
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n=8 for each group. Data are presented as mean±SEM. BW, body weight; HW, heart weight; HW/BW, heart-to-body weight ratio; ND, not detected. *p<0.05 vs. corresponding VEH, **p<0.01 vs. corresponding VEH, †p<0.05 vs. corresponding CNT, ††p<0.01 vs. corresponding CNT.

Figure Legends:

Figure 1: Leptin expression in the left atrium. Immunohistochemical (A) and RT-PCR (B) analyses revealed that leptin expression was upregulated by continuous AngII infusion in CNT mice. No leptin expression was observed in Ob mice irrespective of AngII infusion. In A, brown staining indicates expression of leptin. In A and B, n=5 for each group. Data are presented as mean±SEM. Scale bar=20 µm. **p < 0.01 vs. CNT+VEH.
**Figure 2:** Messenger RNA levels in the left atrium. Messenger RNA levels of collagen 1 (A), collagen 3 (B), TGF-β1 (C), α-SMA (D), F4/80 (E), MCP-1 (F), and RANTES (G) were significantly upregulated by continuous AngII infusion in CNT mice. In Ob-AngII mice, the increase was significantly attenuated. n=5 or 6 for each group. Data are presented as mean±SEM. *p<0.05 vs. CNT+VEH, **p<0.01 vs. CNT+VEH, †p<0.05 vs. CNT+ATII, ††p<0.01 vs. CNT+ATII.

**Figure 3:** Left atrial interstitial fibrosis. Representative results of Masson trichrome staining of the left atrium (A) and quantitative analysis of interstitial fibrosis (B). Blue staining indicates interstitial fibrosis. Masson trichrome staining revealed inhomogeneous interstitial fibrosis in the CNT-AngII left atrium. In Ob-AngII mice, the increase was significantly attenuated. n=6 for each group. Data are presented as mean±SEM. Scale bar=100 μm. **p<0.01 vs. CNT+VEH, †p<0.05 vs. CNT+ATII.

**Figure 4:** Transesophageal burst pacing. Representative AF induction (A). Incidence of AF (B). AF duration (C). AF was frequently induced in CNT-AngII mice while it was never induced in Ob-AngII mice. n=8 for each group. Data are presented as mean±SEM. Arrows, P waves; SR, sinus rhythm. *p<0.05 vs. CNT+VEH, **p<0.01 vs. CNT+VEH, ††p<0.01 vs. CNT+ATII.

**Figure 5:** Electrophysiological study using isolated perfused hearts. Left atrial ERP (A). IACT (B). Representative AF induction (C). Incidence of AF (D). AF duration (E). Atrial and ventricular CL during induced AF (F). IACT was prolonged in CNT-AngII mice, which was not
observed in Ob-Ang II mice. AF was induced only in the CNT-AngII group (4/6, 67%). n=6 for each group. Data are presented as mean±SEM. BCL, basic cycle length; SR, sinus rhythm.

**p<0.01 vs. CNT+VEH, ††p<0.01 vs. CNT+ATII.

**Figure 6:** Leptin expression in SD rat atrial fibroblasts. Addition of AngII (100 nM) to culture medium increased leptin at both mRNA (A) and protein (B) levels. n=5 or 6 for each group. Data are mean±SEM. **p<0.01 vs. control.

**Figure 7:** Effects of leptin on TGF-β1 secretion and on proteins relating to profibrotic and proinflammatory signals in SD and Zucker rat atrial fibroblasts. Addition of leptin (100 ng/ml) increased TGF-β1 secretion (A), and the expression levels of α-SMA (B), MCP-1 (C) and RANTES (D) in SD rat atrial fibroblasts (white bars). In contrast, such increases in response to leptin were not observed in Zucker rat atrial fibroblasts (black bars). n=6 for each group. Data are presented as mean±SEM. *p<0.05 vs. control, **p<0.01 vs. control.
Figure 1

A

VEH

AngII

CNT

Ob

B

leptin mRNA

leptin/Actin mRNA level (fold over control)

CNT

VEH

AngII
Figure 2

A) Collagen 1 mRNA level (fold over control)

B) Collagen 3 mRNA level (fold over control)

C) TGF-β1 mRNA level (fold over control)

D) α-SMA mRNA level (fold over control)
Figure 2

E F4/80 mRNA level (fold over control)

F MCP-1 mRNA level (fold over control)

G RANTES mRNA level (fold over control)
Figure 3

A

VEH AngII

CNT Ob CNT Ob

B

Interstitial fibrosis (%)

CNT Ob CNT Ob

VEH AngII

** †
**Figure. 4**

(A) Diagram showing the sequence of events: SR (sustained rhythm), Burst pacing, Induction of AF (Atrial Fibrillation), and AF (Atrial Fibrillation). The timeline is marked with 100 ms intervals.

(B) Bar chart showing the incidence of AF (%): CNT (Control), Ob (Obese) for VEH (Vehicle) and AngII (Angiotensin II). The incidence is marked with asterisks (*).

(C) Bar chart showing the AF duration (s): CNT (Control), Ob (Obese) for VEH (Vehicle) and AngII (Angiotensin II). The duration is marked with p-p values (††) for none and without any significant difference.**
Figure 5

A. ERP (ms) vs. BCL (ms)

B. IACT (ms) vs. BCL (ms)

C. LA-EGM and LV-EGM recordings during SR, Burst pacing, AF, and SR

Graphs and figures showing the effects of different conditions on ERP, IACT, LA-EGM, and LV-EGM.
Figure. 5

(A-B) Incidence of AF (%)

(C-D) AF duration (s)

(E-F) Cycle length during AF (ms)

** p < 0.01
†† p < 0.001

Table:

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<th>Treatment</th>
<th>CNT</th>
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<td>VEH</td>
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<tr>
<td>AngII</td>
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<td>none</td>
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Comparison:

- ** A-A
- V-V
Figure 6

**A**

leptin mRNA

\[ \text{leptin/GAPDH mRNA level (fold change over control)} \]

- SD rat fibroblasts

AngII (nM) 0 100

**B**

leptin → [Image of leptin band]

GAPDH → [Image of GAPDH band]

SD rat fibroblasts

AngII (nM) 0 100

**"**
Figure 7

A

Active TGF-β1 (pg/ml)

SD rat fibroblasts
Zucker rat fibroblasts

leptin (ng/ml) 0 100

B

α-SMA/GAPDH (fold change over control)

SD rat fibroblasts
Zucker rat fibroblasts

leptin (ng/ml) 0 100

C

MCP-1/GAPDH (fold change over control)

SD rat fibroblasts
Zucker rat fibroblasts

leptin (ng/ml) 0 100

D

RANTES/GAPDH (fold change over control)

SD rat fibroblasts
Zucker rat fibroblasts

leptin (ng/ml) 0 100

* p < 0.05
** p < 0.01
Role of Leptin Signaling in the Pathogenesis of Angiotensin II - Mediated Atrial Fibrosis and Fibrillation

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Methods

Mice and surgical procedures

Eight week old male control CL57/B6 (CNT) and age-matched leptin-deficient ob/ob (Ob) mice were purchased from SLC Japan (Hamamatsu, Japan). Each mouse was randomly assigned to either the AngII infusion or vehicle (VEH) infusion group. An osmotic mini-pump (Alzet 1002, Alzet, Cupertino, CA, USA) was implanted subcutaneously for constant infusion of AngII (2.0 mg/kg/day) or VEH for 2 weeks, as described previously.¹

Hemodynamic parameters

Systolic blood pressure and heart rate were measured before AngII infusion and on days 7 and 14 using the tail cuff method.

Plasma glucose, insulin, leptin, and adiponectin measurement

On day 14, blood samples were collected from the inferior vena cava. Glucose plasma levels were measured with the Medisafe Mini system (Teromo, Tokyo, Japan) while the plasma levels of insulin (Shibatagi, Gunma, Japan), leptin (Bio Vender, Brno, Czech Republic), and adiponectin (Otsuka, Tokyo, Japan) were determined using enzyme-linked immunosorbent assay (ELISA) kits.

Echocardiography

On day 14, transthoracic echocardiography (Aloka, Tokyo, Japan) was performed. The left ventricular end-diastolic diameter (LVDd), left ventricular end-systolic diameter (LVDs), left ventricular ejection fraction (LVEF), and left ventricular end-diastolic posterior wall thickness (LVPWth) measurements were determined.
**Immunohistochemistry**

Isolated left atrium were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 µm sections. Paraffin-embedded sections were subjected to immunohistochemistry using an antibody specific for leptin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a commercially available detection system (Dako, Glostrup, Denmark). *In situ* bromodeoxyuridine (BrdU) labeling was performed to detect the proliferating cells.

**Quantitative RT-PCR analysis of left atrial tissues**

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed as described previously. Total RNA was extracted using the RNeasy Fibrous Tissue Mini Kit (QIAGEN, Hilden, Germany) for mouse left atrial tissue and RNeasy Mini Kit (QIAGEN) for cultured atrial fibroblast according to the manufacturer’s protocols. Single-stranded cDNA was transcribed using the QuantiTect Reverse Transcription Kit (Roche Diagnostics, Germany) according to the manufacturer’s protocol. Quantitative RT–PCR was performed with the Universal Probe Library (Roche Diagnostics) and LightCycler 480 probe master (Roche Diagnostics) using the TaqMan method. Messenger RNA expression levels relative to actin for left atrial tissue and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for cultured atrial fibroblasts were obtained from a standard curve. Primer sequences are provided in the online-only data supplement.

**Western blot analysis using whole mouse heart**

Whole mouse hearts was removed on day 14, Western immunoblotting was performed as previously described. The protein expression levels of leptin, collagen 1, collagen 3, TGF-β1, α-SMA, F4/80, monocyte chemoattractant protein-1 (MCP-1) and regulated upon activation normal T-cell expressed and secreted (RANTES) were evaluated. Equal amounts of total protein were subjected to sodium
dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred electrophoretically onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). After blocking, membranes were incubated with anti-Leptin (Ob) (Santa Cruz), anti-α-SMA (Sigma-Aldrich, St. Louis, MO, USA), anti-MCP-1 (Abcam, Cambridge, United Kingdom), anti-collagen 1 (Santa Cruz), anti-collagen 3 (Santa Cruz), anti-TGF-β1 (Santa Cruz), anti-F4/80 (Santa Cruz), anti-RANTES (Abcam), or anti-GAPDH antibody (Sigma-Aldrich). After washing, membranes were incubated with horseradish peroxidase–tagged secondary antibodies (GE Healthcare, Amersham, United Kingdom). And the proteins were visualized using ECL (GE Healthcare).

**Histological studies**

Isolated left atria were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 µm sections. Masson trichrome staining was used to evaluate interstitial fibrosis. Micrographs were digitized using Photoshop 7.0 (Adobe, San Jose, CA, USA), and areas of fibrosis were analyzed using Scion image software (Scion Corp., Frederick, MD, USA). In each atrium, five images with a magnification of ×400 were analyzed and averaged (the number of mice in each group was six).

**Hydroxyproline assay**

Collagen content in the left atrium was evaluated by quantification of hydroxyproline using a hydroxyproline assay kit (BioVision, Mountain View, CA, USA) according to the manufacturer’s protocol.

**Transesophageal burst pacing**

On day 14, mice were anesthetized and the body temperature was kept at 37°C. A 4-French catheter electrode (Japan Lifeline, Tokyo, Japan) was placed at the esophageal position dorsal to the left atrium. Surface electrocardiogram (ECG) was simultaneously recorded using electrodes in a lead-II
configuration. Inducibility of AF was tested by applying 3 trains of a 2-s burst pacing using the
automated stimulator. Namely, the first 2-s burst had a cycle length (CL) of 40 ms (pulse duration=5 ms). Following 3 min of stabilization, second 2-s burst was applied with a CL of 20 ms (pulse duration=5 ms). After 3 min of stabilization, the last 2-s burst with a CL of 20 ms was applied with a 10 ms pulse duration. AF was defined as a rapid irregular atrial rhythm with irregular R-R intervals lasting at least 1 s. The duration of AF was measured from the end of burst pacing to the first P wave detected after the rapid irregular atrial rhythm.

**Electrophysiological studies using isolated perfused hearts**

On day 14, electrophysiological studies were carried out using isolated perfused hearts using a Langendorff apparatus with Krebs-Henseleit buffer equilibrated with a 95% O₂/5% CO₂ gas mixture at 37 °C and at a constant pressure of 60 mmHg as described previously. All isolated hearts were stabilized for 10 min by perfusion at a constant flow before programmed electrical stimulation. Teflon-coated (except their tips) silver bipolar electrodes were placed on the appendages of the right atrium, left atrium and left ventricle. The inter-electrode distance between the right atrium and left atrium was set at 10 mm to measure the interatrial conduction time (IACT). The effective refractory period (ERP) of the left atrium was measured by the S2 extrastimulus method using 8 regularly paced beats with cycle lengths (CLs) of 150, 120, and 90 ms. IACT was measured during right atrial pacing. Atrial fibrillation (AF) inducibility was tested by both the S3 extrastimulus pacing and burst pacing methods. In S3 extrastimulus pacing, the intervals of S1 to S2 and S2 to S3 were the same and decremented from 100 ms to the ERP of the right atrium. In burst pacing, we used the same protocol as transesophageal burst pacing. AF was defined as rapid, irregular atrial excitations lasting at least 1 s. During AF, we measured atrial and ventricular CLs.

**Isolation and culture of adult rat atrial fibroblasts**
For further confirmation of the role of the leptin receptor, we used leptin receptor-deficient Zucker rats. Adult male Sprague–Dawley (SD) and Zucker rats weighing 250 to 300 g were purchased from SCL Japan. Rats were anesthetized and the left atria were removed and minced in phosphate-buffered saline (PBS). After digestion with trypsin (2 mg/ml) at 37°C, fibroblasts were pelleted at 1000 rpm for 10 min and resuspended in DMEM supplemented with 5% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml), vitamin B12 (2 g/ml), and bromodeoxyuridine (0.1 mmol/l). After 2 to 3 days, confluent cultures were passaged by trypsinization and re-plated and atrial fibroblasts at their second passage were used in the experiments. After 24 h of incubation in serum-free DMEM, 100 ng/ml leptin or 100 nM Ang II was added. Distilled H$_2$O was used as control. Incubated cells were collected after 24 h for evaluating the expression of α-SMA, MCP-1, RANTES and leptin. The supernatant was also collected for assaying TGF-β1 using ELISA. Western blots were performed as described above. Whole cell lysates were prepared in cell lysis buffer and homogenized. Membranes were assayed using anti-leptin (Santa Cruz), anti-α-SMA (Sigma-Aldrich, St. Louis, MO, USA), anti-MCP-1 (Abcam, Cambridge, United Kingdom), anti-RANTES (Abcam), or anti-GAPDH antibody (Sigma-Aldrich).

**TGF-β1 secretion in the supernatant of atrial fibroblasts**

The secretion of TGF-β1 from atrial fibroblasts to the supernatant was measured using a TGF-β1 quantitative ELISA kit (R&D Systems, Minnesota, USA).

**Statistical analysis**

Continuous data were assessed for normality using the Shapiro-Wilk test. Normally-distributed data are expressed as mean±SEM. Non-normally distributed variables are expressed as medians and interquartile ranges. Four-group comparisons were obtained with one-way ANOVA followed by Bonferroni-Dunn test (for normally-distributed data) or Kruskal-Wallis test with Dunn's Multiple
Comparison test (for non-normally distributed data) for non-repeated measures. Repeated-measures analyses were performed for normally-distributed data with two-way ANOVA and Bonferroni-Dunn test. Two-group comparison was performed by one-way ANOVA, followed by Mann-Whitney’s U test. The incidence of AF among four groups was compared using Fisher’s exact test. Two-tailed p<0.05 indicated statistical significance.
### Supplemental Table

#### Mouse primers for RT-PCR

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<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>Leptin</td>
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<td>Collagen 3</td>
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<td>5’-TGAGTCAATTGAGGAGAAT-3’</td>
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<td>TGF-β1</td>
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<td>5’-CAGCAGCAGTTACCAAG-3’</td>
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<td>5’-ACCAGAGGCATAACAGGGACA-3’</td>
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α-SMA, α-smooth muscle actin; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated upon activation normal T-cell expressed and secreted; TGF-β1, transforming growth factor-β1.
Supplemental Figures

Supplemental Figure 1

Hemodynamic parameters during the experimental period. Continuous infusion of AngII increased systolic blood pressure (A) and heart rate (B) in both CNT and Ob mice. n = 8 for each group. Data are presented as mean ± SEM. **p<0.01 vs. corresponding VEH. ††p<0.01 vs. day 0.
Supplemental Figure 2

Leptin expression in response to continuous angiotensin II (AngII) infusion in mouse whole heart. Western blot analysis revealed that leptin expressions were upregulated by continuous AngII infusion in CNT mice. No leptin expression was observed in Ob mice irrespective of AngII infusion (data not shown). n=6 for each group. Data are reported as mean±SEM. CNT, control; VEH, vehicle.

**p<0.01 vs. CNT+VEH.
Supplemental Figure 3

Western blot analyses using mouse whole heart. The protein levels of collagen 1 (A), collagen 3 (B), TGF-β1 (C), α-SMA (D), F4/80 (E), MCP-1 (F), and RANTES (G) were significantly upregulated by continuous AngII infusion in CNT mice. However, in Ob-AngII mice, the increase was significantly attenuated. n=6 for each group. Data are reported as mean±SEM. α-SMA, α-smooth muscle actin; AngII, angiotensin II; CNT, control; MCP-1, monocyte chemoattractant protein-1; Ob, ob/ob; RANTES, regulated upon activation normal T-cell expressed and secreted; TGF-β1, transforming growth factor-β1; VEH, vehicle. *p<0.05 vs. CNT+VEH, **p<0.01 vs. CNT+VEH, †p<0.05 vs. CNT+ATII, ††p<0.01 vs. CNT+ATII.
Supplemental Figure 4

Collagen content in the left atrium as evaluated by quantification of hydroxyproline. Continuous angiotensin II (AngII) infusion increased left atrial collagen content in CNT-AngII mice, an increase that was attenuated in Ob-AngII mice. n=6 for each group. Data are reported as mean±SEM. AngII, angiotensin II; CNT, control; Ob, ob/ob; VEH, vehicle. **p<0.01 vs. CNT+VEH, †p<0.05 vs. CNT+ATII, ††p<0.01 vs. CNT+ATII.
References


