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Dysfunction of mitochondria and deformed gap junctions in the heart of IL-18-deficient mice

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Submitted 3 December 2015; accepted in final form 3 June 2016

IL-18; mitochondrial autophagy; cardiac myocytes; gap junction; connexin 43

On the other hand, inflammasomes have also been shown to play pathophysiological roles in the heart. Many studies have indicated that inflammasomes are involved in exacerbating heart injury under pathological conditions (39). Pharmacological inhibition of the NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome in the early stage after ischemia-reperfusion limits the secondary inflammatory injury and reduces the infarct size (40). However, in opposition to this, hearts from NLRP3-deficient mice subjected to ischemia-reperfusion are larger than those from wild-type (WT) mice (49). Thus it is necessary to clarify the roles of inflammasomes in the heart; specifically, we need to determine whether the function of inflammasomes is restricted to inflammation through the activation of immune cells, or if they play other roles in maintaining the homeostasis of cardiac myocytes.
Notably, recent studies have revealed cross talk between inflammasomes and autophagy activation (8, 13, 19, 26, 44, 50, 56). Autophagy inhibits inflammasome activation, whereas impaired autophagy activates inflammasomes (8, 56). Conversely, inflammasome activation promotes autophagy (44, 50). Inflammasomes are activated by stimulation by metabolic products, such as reactive oxygen species (ROS), damaged DNA, and ATP, some of which are derived from dysfunctional mitochondria and are also known to promote autophagy (13, 17, 19, 24). Furthermore, signaling pathways, such as Toll-like receptor-4, unc-51-like kinase 1/beclin 1/vacuolar protein sorting 34, and mitofusin 2, that activate inflammasomes are also involved in mechanisms that regulate autophagy (8, 50). Since autophagy plays critical roles in maintaining heart function, activated inflammasomes are likely to be involved in heart function by regulating autophagy.

Furthermore, because activated inflammasomes convert IL-1β and IL-18 from inactive precursors to active forms, these molecules are likely to be involved in the cross talk between inflammasomes and autophagy. In fact, several studies suggested that IL-1β promotes autophagy (13), while another suggested that IL-1β inhibits autophagy in some diseases (19). However, to our knowledge, there are no reports about a relationship between IL-18 and autophagy.

IL-18, a member of the IL-1 family, was originally identified as a factor that induces IFN-γ and has been generally regarded as a proinflammatory cytokine (32, 35). Studies have implicated IL-18 in heart diseases (6, 37, 52); most of these studies showed that IL-18 has adverse effects that exacerbate heart injury, probably through the activation of immune/inflammatory cells. However, it was also shown that IL-18 can promote the expansion and survival of activated lymphocytes (22, 23, 51). Although the mechanism remains to be clarified, IL-18 may promote cell viability via various cellular processes, including autophagy.

In this study, we examined IL-18’s roles in cardiac myocytes using IL-18-deficient [IL-18 knockout (KO)] mice. We hypothesized that IL-18 maintains mitochondrial quality and gap-junction turnover in these cells by promoting autophagy.

MATERIALS AND METHODS

Animals

WT (BALB/c) mice were purchased from SLC, Shizuoka, Japan. BALB/c-background IL-18KO mice were generated by backcrossing the original mutant mice for the IL-18 gene (41, 48) with the BALB/c inbred background for more than eight generations, at the National Institute for Agrobiological Sciences (Ibaraki, Japan). Homozygous mutant mice were used for breeding and experiments in the animal facilities at Hyogo College of Medicine. All of the protocols for animal experiments followed the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH) and the guidelines given by the European Parliament for the protection of animals and were approved by the Animal Research Committee of the Hyogo College of Medicine. Mice were anesthetized with inhaled isoflurane (DS Pharma Animals Health, Osaka, Japan) when tissue samples were obtained.

Reagents

Recombinant murine IL-18 was kindly provided by GlaxoSmithKline (Research Triangle Park, NC). Antibodies (Ab) against phosphorylated Akt (p-Akt, Ser473, 9271), Akt (9272), phosphorylated connexin 43 (p-connexin 43, Ser368, 3511), connexin 43 (3512), autophagy protein 5 (Atg5, 12994), Atg7 (8558), lysosome-associated membrane protein 1 (Lamp1; C54H11, 3243), and 4,6-diamidino-2-phenylindole (4083) were purchased from Cell Signaling Technology (Beverly, MA). Anti-claudin 5 and anti-CD31 Ab were purchased from Abcam (Cambridge, UK), anti-LEC3 Ab (PM036) from Medical & Biological Laboratories (Nagoya, Japan), and anti-MnSOD Ab (06–984) from Upstate (Lake Placid, NY).

Alexa Fluor 555 (Fab2)– (A-21430), Alexa Fluor 488 (A-11070), CellROX Green (C10444), and the ATP Determination Kit (A22066) were purchased from Molecular Probes (Life Technologies, Carlsbad, CA); the Cyto-ID Autophagy Detection Kit (ENZ-S1031) from Enzo Life Sciences; and the Envision System-horseradish peroxidase, anti-rabbit IgG (K4002), and Envision horseradish peroxidase/diaminobenzidine (K3467) from Dako (Tokyo, Japan). Minimal essential medium (M1018) was purchased from Sigma-Aldrich (Tokyo, Japan), the Cell Meter JC-10 Mitochondrial Membrane Potential Kit (22204) from AAT Bioquest, (Sunnyvale, CA), and the Mitochondrial Isolation Kit for Tissue (89801) from Thermo Scientific (Tokyo, Japan). The Complex I Enzyme Activity Microplate Assay Kit (ab109721) and Complex IV Rodent Enzyme Activity Microplate Assay Kit (ab109911) were purchased from Abcam (Tokyo, Japan), and the assay kit for GSH-to-GSSG ratio (GSH/GSSG) (GT40) from Oxford Biomedical Research, (Oxford, MI, USA). Lactate Pro 2 kit was purchased from Arkay, (Shiga, Japan), and pyruvic acid assay kit was purchased from Kyowa Medex, (Tokyo, Japan).

Exercise Loading

Male WT (n = 20) and IL-18KO (n = 27) mice aged 6–10 wk were gradually introduced to endurance training on a motor-driven treadmill (13 m/min) for 5 min/day for 3 days, followed by 10 min/day for an additional 3 days. On the next day, these mice immediately underwent endurance tests (13 m/min, 10 min/day, each day for 1 wk), in which the spontaneous running distances were measured and the average running distance was determined for each group.

Echocardiography

A Nemio 30 echocardiography system (Toshiba, Japan) was used to assess heart function of WT and IL-18KO mice, as previously described (16). Because high heart rate (HR) of mice (600–700 beats/min) made it difficult to estimate cardiac function by echocardiography, HR was reduced by anesthesia of mice (11, 15, 18, 46, 55). According to our preliminary experiments, HR was needed to be reduced to below 400 beats/min to definitely separate the peak early (E) and atrial (A) in pulse-wave Doppler. On the other hand, neither the anesthesia initiated with isoflurane (maintained at 1.5–2%) nor pentobarbital (50 mg/kg body wt by intraperitoneal injection) was successful in lowering HR below 400 beats/min (data not shown). Pentobarbital, intraperitoneally given at a dosage of 100 mg/kg body wt, provided a stable anesthetic condition for duration of 30–60 min, both in WT and IL-18KO mice. Therefore, in the present study, pentobarbital was used at the dosage of 100 mg/kg body wt to examine cardiac function by echocardiography.

Briefly, mice were given an intraperitoneal injection of pentobarbital (100 mg/kg). Under anesthesia, the heart function was monitored at the HR from 310 to 360 beats/min, in both WT and IL-18KO mice. M-mode tracings and pulse-wave Doppler spectra (E and A waves) of the mitral inflow were recorded for each group. All of the echocardiography tests were strictly performed by the same specialist and were carried out under double-blinded control. The mouse preparation, echocardiography, and data analysis were performed by different individuals.
Morphological Analysis

Paraffin sections. Heart tissue from WT and IL-18KO mice was sectioned at 5 μm and subjected to hematoxylin and eosin or Azan-Mallory staining.

Immunofluorescence staining and confocal imaging. Cardiac tissues were snap-frozen, cryosectioned at 10 μm, and placed in collagen-coated chamber slides with blocking buffer (2% skim-milk/PBS; skim milk for immunoassays, no. 31149-75; Nacalai Tesque, Kyoto, Japan). After incubation with blocking buffer for 1 h, the tissue was stained with the primary Ab, such as anti-claudin 5, anti-p-connexin 43/connexin 43, or anti-LC3, at 4°C overnight, and then with an Alexa Fluor-conjugated secondary Ab (Molecular Probes), according to the manufacturer’s protocol. For CellROX Green staining, the cryosectioned slides were incubated with 1 M CellROX Green, as described in the manufacturer’s protocol, at 4°C overnight. The slides were observed using an LSM 780 laser-scanning microscope (Carl Zeiss AG, Oberkochen, Germany). Images were analyzed using laser-scanning microscopy software (ZEN 2012, Carl Zeiss AG). To account for differences among individual mice, we evaluated data from five different visual fields for each sample, from each WT and IL-18KO group contained 4 – 6 mice, and the immunofluorescence measurements were performed under the double-blinded condition.

Electron microscopy. Cardiac tissues were fixed, and sections were prepared for electron microscopy. The arrangement, structure, and size of the mitochondria, Z-lines, myofilbrils, intercalated disks, and gap junctions were observed using a JEM-1400 Plus electron microscope. Five to eight different fields for each sample were examined (n = 4 – 6 mice per group), and the data were quantified and analyzed using ImageJ software (https://imagej.nih.gov/ij/; version 1.6).

Western Blotting

Cardiac tissues were homogenized in lysis buffer (M-PER Mammalian Protein Extraction Reagent, Thermo Scientific, Rockford, IL) on ice and centrifuged at 14,000 g for 10 min. The protein concentration of the extracts was determined using a Bio-Rad Protein Assay Kit. Proteins in the extracts (10 μg/10 μl) were separated by 5–20% SDS-PAGE and then transferred to a P 0.45 PVDF membrane (GE Healthcare Life Science). Proteins on the membranes were detected with Abs for p-Akt, Akt, p-connexin 43, connexin 43, MnSOD, LC3, Atg5, Atg7, and Lamp1. Target protein bands were quantified and normalized to the β-actin or GADPH level, and analyzed using ImageJ software (https://imagej.nih.gov/ij/; version 1.6).

ELISA

The ELISA kit for IL-18 (7620) was purchased from Medical & Biological Laboratories, (Nagoya, Japan), and ELISA kit for lactate dehydrogenase (LDH) was purchased from Cloud-Clone (Houston, TX).

Assay of Mitochondrial Respiratory Activity

Purified mitochondria were prepared from WT or IL-18KO heart lysate, and the enzyme activities of complex I and complex IV in the mitochondrial lysate were measured by a microplate assay kit, as described in the manufacturer’s detailed information. The GSH and GSSG in the mitochondrial lysate were also quantified.

Effect of Exogenous IL-18 on Neonatal Cardiac Ventricular Myocytes

Neonatal cardiac myocytes were prepared from WT or IL-18KO mice within 10 days of birth (29, 57). Mice were anesthetized with inhaled isoflurane, and the heart was quickly removed and placed in HBSS (−) on ice. After removal of the atrium and vessels, the ventricles were cut into sections of 2–3 mm in ice-cold HBSS (−), washed with fresh ice-cold HBSS (−), and digested by adding collagenase type IV and shaking every 3 min for 15 min at 37°C in 5% CO₂. As the tissue swelled and softened, it was gently rolled, passed through a 0.75-μm mesh, and centrifuged at 90 g for 3 min. Cell pellets were washed twice, resuspended in MEM containing 10% FCS, and cultured at 37°C in 5% CO₂. Dissociated single-cell suspensions were preplated for 1 h to remove nonmyocyte cells and transferred to COL-I coated chamber slides (IWIKA 4722-010, Asahi Glass, Japan) or plates.

The neonatal cardiac ventricular myocytes were cultured with or without IL-18. Autophagosome formation was assessed by LC3 immunostaining, and autophagy was assessed by Cyto-ID green staining.

Determination of Mitochondrial Function and Membrane Potential

Neonatal cardiac myocytes, prepared as just described, were incubated for 4 days in collagen I-coated chambers (Corning, Corning, NY) in the presence or absence of IL-18, and were assayed with a Cell Meter JC-10 Kit, according to the manufacturer’s protocol. DMSO (10%) was used as the control. Cells were observed under an LSM 780 confocal microscope.

Statistical Analysis

Statistical analyses were performed by Student’s t-test. Data are expressed as means ± SD. P < 0.05 was considered statistically significant.

RESULTS

Physiological Parameters and Cardiac Function

Because metabolic abnormalities have been demonstrated in IL-18KO mice (28), we introduced mice to running on a motor-driven treadmill (see MATERIALS AND METHODS) and then analyzed their endurance under exercise stress. The mice were tested for running endurance (13 m/min for 10 min/day), and their average running distance was calculated (Fig. 1A and Table 1). Almost all of the WT mice ran for > 10 min, while about one-half of the IL-18KO mice stopped running within 5 min (Table 1). The running distance of the IL-18KO mice was significantly less than that of WT mice, suggesting that the IL-18KO mice became exhausted more quickly than did WT mice (Fig. 1A). Meanwhile, the serum lactic acid level was significantly higher, and serum LDH level lower, in the IL-18KO mice compared with WT mice (Fig. 1B). There was no significant difference in the pyruvic acid level.

Several mechanisms can impair endurance under an exercise burden, including skeletal muscle problems and neurological diseases. In this study, we focused on cardiac myopathy. The IL-18KO heart was noticeably enlarged, and the ratio of heart to body weight was greater in the IL-18KO mice than in WT mice of the same age (Fig. 1C and Table 2). Macroscopic observation under a dissecting microscope revealed thickening of the left ventricle wall and significant narrowing of ventricular spaces in the IL-18KO heart (data not shown). M-mode echocardiography defined uncoordinated and inefficient cardiomyocyte contractions (Fig. 1D). Corresponding echocardiographic evaluations, presented in Table 2, showed significant differences in ejection fraction, fractional shortening, E wave (cm/s), A wave (cm/s), and E/A, indicating abnormal contractile and diastolic functions that appeared to be related to the exercise intolerance in the IL-18KO mice. Furthermore, these abnormalities, defined by echocardiography, were age-dependently exacerbated, and arrhythmias also could be detected in IL-18KO mice at 12 mo of age (data not shown).
Because IL-18 is regarded as a proinflammatory cytokine, we also examined the effects of exercise burden on the production of inflammatory cytokines, such as IFN-γ and TNF-α. Although exercise stress caused a slight increase in the level of circulating IL-18 in WT mice (Fig. 1E), IFN-γ and TNF-α were not detected in either WT or IL-18KO heart (data not shown), suggesting that there was no inflammatory response to the exercise loading.

**Histological Analysis**

Hematoxylin and eosin staining of the IL-18KO heart tissues revealed increased interstitial spaces between cardiac myocytes compared with WT (Fig. 2A), although without any apparent accumulations of infiltratory or inflammatory cells. The IL-18KO cardiac myocytes were also significantly wider than WT cardiac myocytes (Fig. 2B). Azan-Mallory staining indicated noticeable fibrotic changes near valves and the endocardium in the IL-18KO heart; these fibrosis-like structures were not observed in WT mice (Fig. 2C). Since tight junctions between cardiac myocytes and between cardiac myocytes and vascular-endothelial cells are important to heart function, we examined the expression of CD31 and claudin 5. CD31 was located restrictively on vessels, and there was no apparent difference in CD31 expression between the WT and IL-18KO hearts (Fig. 2D). However, claudin 5 was expressed far more strongly in the IL-18KO than the WT heart and was located interstitially or between the lateral membranes of cardiac myocytes, as well as in vessels in the IL-18KO heart (Fig. 2D).

**Electron Microscopy**

Electron microscopy revealed a disordered alignment of mitochondria in the IL-18KO cardiac myocytes (data not shown). The mitochondria in the IL-18KO mice were enlarged, swollen, and elongated [Fig. 3A (dashed rectangles), B, and D]. In addition, the width of the cardiac myocytes was random, and the distance between Z-lines was shorter in the IL-18KO hearts (Fig. 3, A and E). The cristae of the swelling mitochondria in IL-18KO hearts were distributed irregularly and looked dim, fragmented, and aggregated (Fig. 3, A and B). The Z-lines of cardiac myocytes in the IL-18KO heart were loose with hala- tion (Fig. 3, A and C). The distance between Z-lines in the IL-18KO cardiac myocytes (0.9–1.38 μm), indicated by a black double arrow, was significantly shorter than that in WT mice (1.3–2.15 μm) (Fig. 3, A and E), which was consistent...
with the inefficient contraction and diastole of the IL-18KO hearts in the echocardiography data (Fig. 1D and Table 2).

In addition, morphological examination by high-magnification electron microscopy indicated that the IL-18KO hearts had rough, distorted, and disintegrated intercalated disks between sarcomeres, with an increased number of unfused, concentric pentalaminar membranes located near the intercalated disks (Fig. 3F). These observations might indicate deficient gap junction turnover in the intercalated disks of IL-18KO cardiac myocytes.

Abnormal p-Connexin 43/Connexin 43 Expression and Location in the IL-18KO Heart

Many studies have shown a relationship between mitochondrial abnormalities and abnormal intercalated disks (4, 10, 14, 34, 42). Gap junction abnormalities in the intercalated disks (14, 42) are associated with impaired contractility and diastole in cardiac myocytes, which we observed in the IL-18KO heart by echocardiography (Fig. 1D and Table 2) and electron microscopy (Fig. 3, C and F). Therefore, we examined the expression of proteins related to gap junctions in the IL-18KO mice.

The mean intensity of the immunofluorescence for connexin 43 was slightly increased in the IL-18KO heart, and p-connexin 43 was apparently decreased (Fig. 4A) compared with WT. Notably, in the IL-18KO heart, connexin 43 localized eccentrically not only to intercalated disks, but also at the lateral membrane; this expression is often observed in the failing heart (Fig. 4B, top). This lateralized connexin 43 was much more frequent in IL-18KO than in WT mice, although the number of intercalated disks was similar (Fig. 4B, bottom).

Consistent with these observations, analysis by Western blotting revealed less p-connexin 43 in the IL-18KO than in the WT heart (Fig. 4, C and D). The expression of p-Akt, which mediates connexin 43 phosphorylation, was also decreased in the IL-18KO heart.

Impaired Mitochondrial Function in IL-18KO Mice

Since the IL-18KO cardiac myocytes contained morphologically abnormal mitochondria (Fig. 3, A and B), we examined the mitochondrial function in the IL-18KO heart. First, CellROX green, which specifically detects ROS, was localized primarily to the nucleus and mitochondria and was stronger in the IL-18KO than the WT heart (Fig. 5A), while the GSH/GSSG was lower in the IL-18KO mitochondrial lysate (Fig. 5B). Second, the mitochondrial MnSOD content was markedly reduced in the IL-18KO compared with the WT heart, suggesting that the scavenging of ROS was impaired (Fig. 5C). Next, we isolated mitochondria from the hearts of WT and IL-18KO mice and used Western blotting to assess the content of mitochondria-associated proteins, such as cytochrome-c oxidase, heat shock protein 60, pyruvate dehydrogenase, voltage-dependent anion channel protein, and succinate dehydrogenase. There were no apparent differences in the main protein components of WT and IL-18KO mitochondria (data not shown). However, the mitochondrial ATP content was significantly lower in the IL-18KO than in the WT heart, suggesting that ATP production was impaired in the IL-18KO mitochondria (Fig. 5D).

Finally, we analyzed the mitochondrial OXPHOS complex I (NADH dehydrogenase) and complex IV (cytochrome-c oxidase) enzyme activity. Both the complex I and complex IV enzyme activities were significantly lower in mitochondria of the IL-18KO heart than the WT heart (Fig. 5, E and F). These data suggested that the mitochondrial respiration in IL-18KO was reduced.

Reduced Production of Autophagy-Related Proteins in the IL-18KO Heart

As autophagy controls mitochondrial quality and gap-junction turnover, and, as mitochondrial functions were impaired in the IL-18KO heart, we next examined whether the synthesis of autophagy-related proteins or the autophagolysosome formation was impaired. Western blotting analysis indicated that the production of LC3-II (14 kDa) was significantly decreased in the IL-18KO heart compared with WT (Fig. 6, A and B). However, the expressions of Atg5 and Atg7 (Fig. 6A) did not differ markedly between the WT and IL-18KO hearts. Western blotting analysis of the lysosomal membrane protein Lamp1 showed a significantly greater amount of glycosylated Lamp1 (90–120 kDa) in the IL-18KO than the WT heart (Fig. 6C). However, there was little difference in nonglycosylated Lamp1 (42 kDa) between the IL-18KO and WT hearts. On the other hand, the lysosomal-membrane protein Lamp2 did not show a difference between the WT and IL-18KO heart on Western blots (data not shown).

### Table 1. Treadmill exercise test

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>IL-18KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Running Test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of mice</td>
<td>20</td>
<td>27</td>
</tr>
<tr>
<td>No. of mice</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>Total no. of</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>mice</td>
<td>27</td>
<td>20</td>
</tr>
<tr>
<td>No. of mice</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>No. of mice</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Mice were acclimated to running on a motor-driven treadmill (13 m/min) for 5 min/day for 3 days, and 10 min/day for 3 additional days. These mice were then tested for endurance by measuring how long they ran spontaneously during a period of ≤10 min (treadmill speed 13 m/min). A running duration of <5 min (one-half of the test duration) was defined as limited endurance. *P < 0.05.

### Table 2. Physiological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>IL-18KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>23</td>
<td>21</td>
</tr>
<tr>
<td>BW, g</td>
<td>24.29 ± 1.22</td>
<td>27.35 ± 2.15*</td>
</tr>
<tr>
<td>HW, mg</td>
<td>0.14 ± 0.02</td>
<td>0.18 ± 0.02*</td>
</tr>
<tr>
<td>HW/BW, mg·g⁻¹·%⁻¹</td>
<td>0.59 ± 0.05</td>
<td>0.67 ± 0.04*</td>
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<tr>
<td>Echocardiography, n</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>332.80 ± 18.64</td>
<td>332.80 ± 22.08 NS</td>
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<tr>
<td>IVSTd, mm</td>
<td>0.17 ± 0.06</td>
<td>0.19 ± 0.10 NS</td>
</tr>
<tr>
<td>LVIDd, mm</td>
<td>2.83 ± 0.06</td>
<td>2.83 ± 0.30 NS</td>
</tr>
<tr>
<td>LVIDs, mm</td>
<td>1.57 ± 0.25</td>
<td>1.98 ± 0.15*</td>
</tr>
<tr>
<td>LVPWd, mm</td>
<td>0.30 ± 0.01</td>
<td>0.31 ± 0.03 NS</td>
</tr>
<tr>
<td>EF</td>
<td>0.81 ± 0.05</td>
<td>0.61 ± 0.07**</td>
</tr>
<tr>
<td>FS</td>
<td>0.45 ± 0.08</td>
<td>0.28 ± 0.02ab</td>
</tr>
<tr>
<td>E/A, cm/s</td>
<td>1.93 ± 0.42</td>
<td>3.13 ± 0.54ac</td>
</tr>
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</table>

Values are means ± SD; n, no. of mice. BW, body weight; HW, heart weight; HR, heart rate under anesthesia; IVSTd, interventricular septum thickness; LVIDd, left ventricular end-diastolic dimension; LVIDs, left ventricular end-systolic dimension; LVPWd, left ventricular posterior-wall thickness; EF, ejection fraction; FS, fractional shortening. *P ≤ 0.05, NS, no significant difference.
Restoration of Cardiac Myocyte Function by IL-18

To examine whether IL-18 could restore the impaired function of IL-18KO cardiac myocytes, IL-18’s effects on mitochondrial function and autophagy were examined using neonatal cardiac myocytes. Neonatal cardiomyocytes from an IL-18KO heart were cultured with or without IL-18 for 4 days and analyzed for mitochondrial function and autophagy. The mitochondrial membrane potential measured by a JC-10 Cell Meter probe was increased by the IL-18 treatment, as indicated by stronger red fluorescence (Fig. 7A). In addition, IL-18 strongly upregulated the LC3 expression in the IL-18KO cells (Fig. 7B). Assessment by Cyto-ID Green and immunostaining for p-connexin 43 indicated that IL-18 also restored autophagy and the phosphorylation of connexin 43 in IL-18KO cells (Fig. 7C). Consistent with these findings, IL-18 promoted continuous beating, and the number of beating cells was significantly greater in the IL-18-treated than in the untreated culture (Fig. 7D). Exogenous IL-18 acted on both WT and IL-18KO cells in the same way, and there was no difference in the effect on number of beating cells (data not shown).

DISCUSSION

In the present study, we observed various abnormalities in the IL-18KO heart, but no infiltration of inflammatory cells, suggesting that IL-18 has functions distinct from its role as an inflammatory cytokine. Because IL-18 is converted to active forms by inflammasomes and because cross talk between inflammasomes and autophagy has been demonstrated, we hypothesized that IL-18 plays roles in cardiac homeostasis by regulating the interaction of inflammasomes and autophagy.
Macroscopically, the heart-to-body weight ratio was significantly greater in the IL-18KO than in the WT mice (Fig. 1C and Table 2), indicating cardiac hypertrophy. The myofibrils of the IL-18 KO heart were significantly wider than those in the WT heart (Fig. 2B). Interestingly, the continuous administration of IL-18 also induces hypertrophy in the mouse heart, indicating IL-18's activity as a pro-hypertrophic cytokine (6, 37, 52). Thus, either an excess or a deficiency of IL-18 causes hypertrophic changes in the heart: the former is accompanied by inflammatory cell infiltration and augmented inflammatory cytokines, while the latter is not (Figs. 1E and 2A). Fibrosis was observed in the cells close to valves and endocardium in the IL-18KO heart, which might be related to the hypertrophy of the IL-18KO heart (Fig. 2C). However, further analysis is required to elucidate the different mechanisms underlying the hypertrophy caused by an excess vs. a deficiency of IL-18.

Consistent with the hypertrophy, echocardiographs indicated uncoordinated, deficient systolic and diastolic function in the
Fig. 4. Abnormal gap junctions in IL-18KO mice. A: impaired phosphorylation of connexin 43 in IL-18KO mice. Cryosections (10 μm) were prepared from the snap-frozen cardiac tissue of IL-18KO and WT mice and placed in collagen-coated chambers. After incubation in a blocking solution, the sections were stained for p-connexin 43 (1/1,000) and connexin 43 (1/1,000) overnight at 4°C. Alexa Fluor 555F (ab=) and 4,6-diamidino-2-phenylindole (DAPI) were used according to the manufacturers’ protocols. The slides were observed using an LSM 780 laser-scanning microscope. The region in the white rectangle was further magnified threefold. Fluorescence intensity of the stained parts was measured using soft histograms, with software in the laser-scanning microscope, 3–4 visual fields per mouse. B: accumulation of connexin 43 in the lateral membranes between myocardial cells. Slides were observed using an AX80/DP72 scanning microscope. Scale bar, 20 μm. Red stars, lateralized connexin 43; pink arrows, intercalated disks. The amounts of lateralized connexin 43 and intercalated disks were quantified from 10 visual fields/mouse, using ImageJ software. The images in A and B are representative of 3–5 mice from each group. C and D: Western blotting and quantification of p-connexin 43/connexin 43 and p-Akt/Akt. Images of 3 selected lanes from 5 independent experiments at same gel are shown. Values are means ± SD. *P < 0.05. NS, not significant.
heart of IL-18KO mice (Fig. 1, C and D, and Table 2), which may contribute to the intolerance of these mice for exercise (Fig. 1A and Table 1). Echocardiography demonstrated marked differences in ejection fraction, fractional shortening, and E/A between the WT and IL-18KO mice, as implied by their impaired contractile and diastolic ability. However, echocardiograms of the IL-18KO heart did not show arrhythmias at the age of 8 wk, although they exhibited arrhythmias at ages older than 1 yr (data not shown).

Thus cardiac myocyte dysfunction in the IL-18KO heart was suggested by abnormalities observed by echocardiography and was further supported by various abnormalities in the structures and functions of these cells, especially in the mitochondria. A marked accumulation of damaged mitochondria with swelling, elongated, and concentrated cristae was observed by electron microscopy (Fig. 3). Furthermore, decreased respiratory activity (Fig. 5, E and F), reduced ATP production (Fig. 5D), impaired membrane potential (Fig. 7A), excess ROS and GSSG productions (Fig. 5, A and B), and reduced MnSOD (Fig. 5C) were also observed in the IL-18KO heart compared with WT.

In addition to the abnormalities of the mitochondria, the IL-18KO hearts showed disintegrated intercalated disks between sarcomeres and multilamellar membranous material around the intercalated disks (Fig. 3F). Such unfused, concentric multilamellar membrane structures are often observed in the intercalated disks of the failing heart (4, 14, 42). These structures may indicate the impaired degradation of gap junctions in the IL-18KO heart. We also observed poorly situated gap junctions in the IL-18KO heart. Immunostaining showed excessive connexin 43 at the lateral membrane in IL-18KO cardiomyocytes, while the connexin 43 phosphorylation was...
reduced (Fig. 4, A and B). p-Connexin 43 is normally localized to intercalated disks, where it is rapidly recycled, and lateraledized connexin 43 has been regarded as nonrecycling connexin 43 caused by deficient lipid metabolism, which is often observed in heart failure (14).

It is well known that autophagy controls the quality of mitochondria and mediates gap-junction turnover, and that defective autophagy is associated with heart dysfunction (4, 10, 14, 20, 25, 33, 36, 42). Therefore, the impaired mitochondria and gap junctions might be due to dysregulated autophagy in the IL-18KO heart. In the present study, Western blotting analysis and immunofluorescence staining indicated decreased productions of autophagy-related proteins, such as LC3 II and p-Akt, in addition to p-connexin 43, in the IL-18KO heart (Figs. 6 and 4C), while the expressions of Atg5 and Atg7 were comparable between the WT and IL-18 KO heart (Fig. 6A). In addition, Lamp1 was expressed far more strongly in the IL-18KO than WT heart, which may suggest that the autophagosome-lysosome fusion was impaired in IL-18KO cardiac myocytes (Fig. 6C). Furthermore, the activation of phosphatidylinositol 3-kinase/Akt has been shown to promote autophagy (38) and the phosphorylation of connexin 43 (9, 45), and IL-18 has been shown to activate phosphatidylinositol 3-kinase/Akt (16, 22, 23). These findings may indicate that autophagy was decreased in the IL-18KO heart and support the likelihood that IL-18 promotes autophagy flux. Since pathways for degradation of connexins and gap junctions are also mediated by protein kinase C(PKC)-ε and -δ (43, 53), and, in addition, IL-18 upregulates activity of PKC-ε and -δ in activated natural killer and T cells (2, 54), we examined whether there was a difference in the expression of PKC, but failed to detect the difference (data not shown). This must be further examined in the future studies.

Fig. 6. IL-18 deficiency causes abnormal levels of autophagy-related proteins. A: Western blots for LC3-1/2, Atg5, and Atg7 in the lysates from WT and IL-18KO hearts. B: reduction of LC3 in the IL-18KO heart. LC3 expression was detected by confocal microscopy. Images were representative of 3 independent experiments. C: Western blots for Lamp1 expression. Images in A and C are representative of 5 independent experiments. Values are means ± SD. *P < 0.05. NS, not significant.
In addition to the abnormalities of mitochondria and gap junctions, there were several other abnormalities in the IL-18KO heart. Claudin 5 was unusually localized along the lateral membranes in the IL-18KO but not WT cardiac myocytes (Fig. 2D). Cellular-membrane or cellular-adhesion proteins, such as the ephrin and claudin families, are considered to be involved in the maintenance of cardiomyocyte homeostasis and function (7, 12). Claudin 5, which regulates transendothelial electrical resistance and is critical for the barrier function of neutral vasculature (47), is reduced in human end-stage cardiomyopathy (21, 27). Abnormal accumulations of claudin 5 are usually observed in hypoxic conditions (7, 21). However, the relationship between IL-18 deficiency and the unusual localization of claudin 5 was not explored in this study. The blurred, thickened Z-lines and reduced distances between Z-lines (Fig. 3, A and E), and the reduced LDH and increased lactic acid in the serum of IL-18KO mice (Fig. 1B) may all be the result of mitochondrial dysfunction.

Neonatal cardiac myocytes may not be ideal for studying mitochondrial function, because the way of energy production in neonatal cardiac myocytes depends on glycolysis, while that in adult hearts depends on fatty acid. In the present study, we used neonatal cardiac myocytes to examine whether IL-18 can directly influence autophagy. IL-18 elevated the mitochondrial membrane potential and restored the LC3 expression in cardiac myocytes from neonatal IL-18KO mice (Fig. 7, A and B). IL-18 also upregulated the autophagic activity as assessed by Cyto-ID green and increased the connexin 43 phosphorylation in these cells (Fig. 7C). However, these cultured cells were a mixture of various cells, and further experiments are required to obtain direct evidence demonstrating that IL-18 promotes autophagy.

In terminally differentiated cells, such as cardiac myocytes and neurons, autophagy contributes to the housekeeping and to metabolic homeostasis (1, 30, 31, 33, 34, 36). Dysfunctional mitochondria, impaired gap junction turnover, and excess lactic acid production are all related to impaired autophagy (4, 5, 10, 14, 33, 42). Autophagy is regulated in a variety of ways. Recent findings about the relationship between inflammasomes and autophagy prompted us to examine the involvement of IL-18 in autophagy. In the present study, we documented various abnormalities in the IL-18KO heart and showed that these abnormalities could be due to impaired autophagy in IL-18KO mice. Although IL-18 is primarily recognized as a proinflammatory factor, this cytokine may also have a protective role in the heart under physiological conditions.

ACKNOWLEDGMENTS

We thank H. Seki, F. Yoshida, A. Tamamoto, E. Yamakuchi, N. Gamachi, D. Nagata, and H. Fujimoto for valuable technical support.

GRANTS

This work was supported by JSPS KAKENHI Grant 24390306.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

W.L. and H.O. conception and design of research; W.L., D.J., and M.H. performed experiments; W.L., D.J., and M.H. analyzed data; W.L., K.Y., W.S., and H.O. interpreted results of experiments; W.L., K.Y., and W.S. prepared figures; W.L., K.Y., and W.S. drafted manuscript; W.L., D.J., M.H., S.T.,
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K.Y., W.S., Y.E.-D., H.Y., and H.O. edited and revised manuscript; W.L. and H.O. approved final version of manuscript.

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