Daily administration of interleukin-18 causes myocardial dysfunction in healthy mice

Per Reidar Woldbæk, Jørn Bodvar Sande, Tøeve Andreas Strømme, Per Kristian Lunde, Srdjan Djurovic, Torstein Lyberg, Geir Christensen, and Theis Tønnessen. Daily administration of interleukin-18 causes myocardial dysfunction in healthy mice. Am J Physiol Heart Circ Physiol 289: H708–H714, 2005. First published April 8, 2005; doi:10.1152/ajpheart.01179.2004.—Although increased levels of circulating interleukin (IL)-18 have been demonstrated in patients with cardiovascular diseases, the functional consequences of chronically increased circulating IL-18 with respect to myocardial function have not been defined. Thus we aimed to examine the effects of chronic IL-18 exposure on left ventricular (LV) function in healthy mice. Moreover, to clarify whether IL-18 has direct effects on the cardiomyocyte, we examined effects of IL-18 on cardiomyocytes in vitro. After 7 days of daily intraperitoneal injections of 0.5 μg IL-18 in healthy mice, a 40% (P < 0.05) reduction in the LV maximal positive derivative, a 25% (P < 0.05) reduction in the LV maximal rate of pressure decay, and a 2.8-fold (P < 0.001) increase in the LV end-diastolic pressure were measured, consistent with myocardial dysfunction. Furthermore, we measured a 75% (P < 0.05) reduction in β-adrenergic responsiveness to isoproterenol. IL-18 induced myocardial hypertrophy, and there was a 2.9-fold increase (P < 0.05) in atrial natriuretic peptide mRNA expression in the LV myocardium. In vitro examinations of isolated adult rat cardiomyocytes being stimulated with IL-18 (0.1 μg/ml) exhibited an increase in peak Ca2+ transients (P < 0.05) and in diastolic Ca2+ concentrations (P < 0.05). In conclusion, this study shows that daily administration of IL-18 in healthy mice causes LV myocardial dysfunction and blunted β-adrenergic responsiveness to isoproterenol. A direct effect of IL-18 on the cardiomyocyte in vitro was demonstrated, suggesting that IL-18 reduces the responsiveness of the myofilaments to Ca2+. Finally, induction of myocardial hypertrophy by IL-18 indicates a role for this cytokine in myocardial remodeling.

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IL-18 is a proinflammatory cytokine and experimentally has been shown to induce synthesis of tumor necrosis factor (TNF)-α (35), IL-1β (35), IL-6 (34), and inducible nitric oxide synthase (34), mediators that have all been associated with myocardial dysfunction (6, 13, 24). Also, activation of cytotoxic T lymphocytes (12, 33) and induction of neutrophilic cell infiltration (26), as well as stimulation of intercellular adhesion molecule (ICAM)-1 (23), have been demonstrated. Immunoinflammatory mechanisms induced by IL-18 therefore may play a role during development and progression of myocardial dysfunction.

Although IL-18 might induce myocardial dysfunction indirectly via induction of other cytokines, as is mentioned above, it might also exert direct effects on the cardiomyocyte level. Both alterations in Ca2+ handling as well as reduced myofilament responsiveness to Ca2+ have been reported as mechanisms of myocardial dysfunction (1).

Mediators of myocardial dysfunction might also induce cardiac remodeling, directly or indirectly. One hallmark of cardiac remodeling is myocardial hypertrophy. Recently, Chandrasekar et al. (10) have shown that IL-18 induces hypertrophy of cardiomyocytes in vitro following activation of phosphatidylinositol 3-kinase (PI3K), phosphoinositide-dependent kinase-1 (PDK1), Akt, and GATA-4 signaling. Also, increased atrial natriuretic peptide (ANP) expression in isolated cardiomyocytes has been demonstrated after IL-18 exposure (10, 39), suggesting that IL-18 in vivo may also activate signaling pathways leading to myocardial hypertrophy.

To clarify the effects of prolonged IL-18 exposure on cardiac function in vivo, we assessed the effects of 7 days of daily injections of IL-18 on left ventricular (LV) contractile parameters in healthy mice. Moreover, the β-adrenergic responsiveness to isoproterenol was examined. Histological, immunohistochemical, and Western blot analyses of tissues from the LV myocardium of IL-18-treated mice were performed, evaluating the degree of lymphocyte and neutrophilic cell activation as well as the amounts of ICAM-1 and phosphorylation state of Ca2+ handling proteins. To define whether IL-18 might exert a direct effect on the cardiomyocyte, we examined the effect of IL-18 on Ca2+ homeostasis in vitro. Finally, we examined the effects of IL-18 on myocardial hypertrophy.

METHODS

Animal preparation. Thirty male BALB/c mice (Møllegaard and Bonholtgård Breeding and Research Center A/S, Ry, Denmark), 6–8 wk old, were examined in this study. A volume of 1 ml 0.9% NaCl
PCR analysis was performed to determine the relative mRNA expression levels of ANP and TNF-α. Primers and Taqman probes were designed by using the Primer Express software version 1.5 (Applied Biosystems, Foster City, CA). All probes were labeled 5’-FAM and 3’ Dark Quencher. The actual sequences of primers and probes used for RT-PCR were as follows: ANP (forward primer 5’-GTGGCTTTGGGAAAAATAGGTTG-3’; reverse primer 5’-TGTTTGTGTTGTTTGAGATCTGTTTAC-3’; probe 5’-AGATGTTCAGCATGTTCACAGTGT-3’), TNF-α (forward primer 5’-CATCTTCTAAAATCTGAGTTA-3’; reverse primer 5’-GGGGAGTAGAAGGTAACACC-3’; probe 5’-CAGCGTAGGAAACACCAGTGGAGA-3’). (Pasca et al, 2009) 

The half-life of IL-18 has been reported to be 16 h (19).

After the 7 days of injections, anesthesia was induced by injections of 0.2 ml (10 mg/ml) propofol (Diprivan; AstraZeneca, Macclesfield, Cheshire, UK) into a tail vein. A cervical midline incision was performed, and a 20-gauge intravenous cannula was inserted into the trachea. The mice were then connected to a rodent ventilator (model 874; B. Braun, Melsungen, Germany) and ventilated with a mixture of 2% isoflurane and 98% oxygen. Evaluation of the LV function was performed in a closed-chest model as previously described (41, 42). Briefly, after retrograde cannulation of the left ventricle using a 1.4-Fr Millar microtipotted transducer catheter (model SPR-671; Millar Instruments, Houston, TX), values of the LV maximal positive derivative (LV +dP/dt), LV maximal rate of pressure decay (LV -dP/dt), LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), and heart rate (HR) were obtained. To standardize hemodynamic measurements, a depth of anesthesia was chosen to maintain a HR of ~250 beats/min. All animals included in this study received approximately the same amount of anesthetic drugs; thus the influence of anesthesia on the results presented, if any, should be randomly distributed in both experimental groups examined. After the hemodynamic measurements, heart and lungs were removed, blotted dry, and immediately weighed. The remaining heart tissue was divided into right and left ventricles and rapidly snap frozen in liquid nitrogen. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996), is in accordance with the Norwegian Animal Welfare Act, and is approved by the Norwegian Animal Research Authority.

β-Adrenergic responsiveness. For assessment of the β-adrenergic responsiveness, LV pressure recordings were obtained at baseline and then 2 min after intraperitoneal injections of low (0.02 µg) and high (0.5 µg) doses of isoproterenol (Iso) (14). The LV pressure recordings were performed as described in the animal preparation section.

Histological and immunohistochemical examinations. Hearts from mice receiving IL-18 (n = 7 mice) and controls receiving NaCl only (n = 8 mice) were fixed in 4% buffered formaldehyde and embedded in paraffin. The hearts were then sectioned (5 µm) transversely from apex to the left atrium and then conventionally stained with hematoxylin and eosin. For immunohistochemical analyses of T lymphocyte phenotypes, sections from frozen hearts from mice receiving IL-18 (n = 2 mice) and NaCl (n = 2 mice) were fixed in acetone and blocked with 1% bovine serum albumin and the blocking kit (catalog no. SP-2001) from Vector Laboratories (Burlingame, CA). The sections were then immunostained using the Vectastain Elite ABC reagent (Vector) and the peroxidase substrate diaminobenzidine after incubation with rat anti-mouse CD4 or CD8 mAbs (both diluted 1:10; catalog nos. 550278 and 550281, respectively, BD Pharmingen, San Diego, CA) as primary antibodies. Slides were counterstained in hematoxylin and coverslipped.

Western blot analysis. Homogenates of the LV were prepared as previously described by Semb et al. (38), with minor modifications. The following antibodies were used: ICAM-1 (7% SDS gel, catalog no. SC-1511; Santa Cruz Biotechnology, Santa Cruz, CA), sarcoplasmic reticulum Ca2+-ATPase (SERCA2) (7% SDS gel, catalog no. MA-919; Affinity Bioreagents), serine-16-phosphorylated phospholamban (15% SDS gel, catalog no. A010-12; Baddrila, Leeds, UK), threonine-17-phosphorylated phospholamban (15% SDS gel, catalog no. A010-13; Baddrila), and phospholamban (15% SDS gel, catalog no. A010-14, Baddrila).

Real-time quantitative PCR. Real-time (RT) quantitative Taqman PCR analysis was performed to determine the relative mRNA expression of the animals. Mean body weights, heart weights, lung weights, and heart and lung-to-body weight ratios are shown in Table 1. After 7 days of IL-18 administration, body weights remained unchanged compared with controls. However, a significant increase in lung weights and in the heart and lung-to-body weight ratios was found in mice receiving IL-18, consistent with pulmonary edema and cardiac hypertrophy.
Effects of IL-18 on LV function. Figure 1, A–E, shows the in vivo effects of chronic IL-18 administration on LV function (LVSP, LVEDP, LV +dP/dt, LV −dP/dt, and HR). Mice receiving IL-18 demonstrated significantly reduced values of both LV contractility (LV +dP/dt; 2,590.3 ± 202.1 vs. 4,326.2 ± 297.9, *P < 0.001) and LV relaxation (LV −dP/dt; 2,101.0 ± 237.7 vs. 2,794.8 ± 189.1, **P < 0.001) compared with controls. In addition, mice receiving IL-18 demonstrated a 2.8-fold (*P < 0.05) increase in LVEDP, which is consistent with LV dysfunction. We found, however, no significant changes in LVSPs or HRs when comparing the two experimental groups.

β-Adrenergic responsiveness. Figure 2 shows the effects of Iso on LV +dP/dt in controls and in IL-18-treated mice. When compared with baseline conditions, control mice demonstrated a 3.6-fold (*P < 0.05) and a 4.1-fold (**P < 0.001) higher increase in LV +dP/dt following low and high doses of Iso compared with the response in IL-18-treated mice, indicating a substan-

Table 1. Body, heart, and lung weights

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<tr>
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<th>NaCl</th>
<th>IL-18</th>
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<tr>
<td>BW, g</td>
<td>21.270 ± 0.894</td>
<td>20.671 ± 0.790</td>
</tr>
<tr>
<td>HW, g</td>
<td>0.088 ± 0.002</td>
<td>0.093 ± 0.003</td>
</tr>
<tr>
<td>LW, g</td>
<td>0.141 ± 0.006</td>
<td>0.166 ± 0.004*</td>
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<tr>
<td>HW/BW ratio (10⁻³)</td>
<td>4.169 ± 0.106</td>
<td>4.648 ± 0.107*</td>
</tr>
<tr>
<td>LW/BW ratio (10⁻³)</td>
<td>6.639 ± 0.168</td>
<td>8.326 ± 0.240†</td>
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Values are means ± SE; n = 10 mice in each group. IL-18, interleukin-18; BW, body weight when killed; HW, heart weight; LW, lung weight. *P < 0.005; †P < 0.001.
tial blunted LV β-adrenergic response to Iso in mice receiving IL-18.

**Histological examination.** Histological examination of the excised hearts from mice receiving IL-18 showed no increase in the number of infiltrating lymphocytes, granulocytes, or macrophages in the LV myocardium compared with controls. Neither were there any signs of increased adhesion of leukocytes to the endothelial surface of the myocardial microvessels. The few lymphocytes demonstrated in each histological section were found almost exclusively intravascularly, and the CD4⁺/CD8⁺ ratio was not different in IL-18-treated mice and controls and was well within the normal range.

**ICAM-1 protein.** Western blot examinations revealed no differences in the amounts of ICAM-1 protein between tissues from LV myocardium in mice treated with IL-18 (112 ± 5.5 arbitrary units; n = 6 mice) and controls treated with NaCl only (100 ± 3.3 arbitrary units; n = 6 mice).

**Phospholamban and SERCA2 protein.** Figure 3, A–E, shows the relative levels of the monomeric and pentameric forms of serine-16-phosphorylated phospholamban (PLB), threonine-17-phosphorylated PLB, total PLB, and SERCA2 proteins from the LV myocardium in controls and IL-18-treated mice. Mice receiving IL-18 showed a significant increase in both the monomeric (1.8-fold; P = 0.001) and pentameric (1.9-fold; P < 0.05) forms of the serine-16-phosphorylated PLB when compared with controls. In contrast, there were no significant differences between the two experimental groups regarding the amount of threonine-17-phosphorylated PLB, total PLB, or SERCA2 protein.

**ANP and TNF-α gene expression.** RT-PCR analysis showed a significant increase in the ANP mRNA expression in the LV myocardium in mice receiving IL-18 compared with controls (0.56 ± 0.16 vs. 0.19 ± 0.06 arbitrary units; P < 0.05). In contrast, the analysis revealed no significant differences in the levels of TNF-α mRNA when comparing IL-18-treated mice (0.023 ± 0.012 arbitrary units) and controls (0.017 ± 0.008 arbitrary units).

**Effects of IL-18 on Ca²⁺ transients in rat cardiomyocytes.** Figure 4, A–C, shows the effects of IL-18 on Ca²⁺ transients (peak and diastolic) and on the rate of decline in Ca²⁺ transients.
IL-18 CAUSES MYOCARDIAL DYSFUNCTION

Fig. 4. In vitro effects of IL-18 on peak Ca$^{2+}$ transients (A), rate of decline in Ca$^{2+}$ transients (B) and diastolic Ca$^{2+}$ (C) in isolated cardiomyocytes (n = 8) after a stabilization period of 4 min (Ctr), then 4 min after stimulation with IL-18 (0.1 μg/100 ml), and finally 4 min after ending the IL-18 stimulation (recovery) are shown. Data represent means ± SE. *P < 0.05 vs. Ctr; †P < 0.05 vs. IL-18.

DISCUSSION

Heart failure of different etiologies is commonly characterized by depressed myocardial contractility and relaxation (7). In our study we are demonstrating that daily administration of IL-18 in healthy mice diminishes LV function. Moreover, IL-18 increases lung weight and induces myocardial hypertrophy. These findings are consistent with the animals being in heart failure. Our observations may explain why Mallat et al. (27) reported in patients with acute coronary artery disease a correlation between increased circulating IL-18 and reduced LV ejection fraction, although in that study a causal relationship was not demonstrated. Furthermore, Raeburn et al. (36) showed in an experimental model of sepsis in mice that neutralization of IL-18 protects against LPS-induced myocardial dysfunction. These findings indicate that IL-18 activates cardiodepressive pathways within the LV myocardium, thus contributing to development of cardiac contractile dysfunction.

IL-18 may directly activate cytotoxic T cells (12, 33) and inflammatory cells (26), as well as ICAM-1 (23), within the LV myocardium. Activation of cytotoxic T cells and ICAM-1 has been shown to be associated with cardiomyocyte injury (40, 43). However, in our study, neither increased accumulation of neutrophils or cytotoxic T cells within the myocardial tissue nor evidence for increased leukocyte-endothelium adhesion within myocardial vessels or increased amounts of ICAM-1 were demonstrated in IL-18-treated mice. Similarly, Okamota et al. (32) demonstrated no changes in inflammatory cells in lung tissue following chronic exposure to a lower dose of IL-18 (0.1 μg). However, that study showed that IL-18 in synergy with IL-2 induced lethal lung injury because of activation of mononuclear lymphocytes such as cytotoxic T cells. Consequently, the lack of cardiac neutrophils or cytotoxic T cells observed in our experimental model does not exclude that IL-18 may act in synergy with other cytokines such as IL-2 in promoting cardiomyocyte injury.

Heart failure of different etiologies has been shown to be associated with reduced inotropic responsiveness to β-adrenergic stimulation (7). Mice receiving IL-18 chronically show a substantially blunted LV β-adrenergic responsiveness to Iso. It is well recognized that other inflammatory mediators such as TNF-α, IL-1β, and nitric oxide (NO) may contribute to β-adrenergic hyporesponsiveness because of impaired coupling of the β-adrenergic receptor to adenylyl cyclase and/or alterations in G proteins (2, 4, 16). Previously, IL-18 has been shown to facilitate the synthesis of TNF-α mRNA in the LV myocardium after prolonged IL-18 exposure. Moreover, Ogura et al. (31) have shown that daily administration of IL-18 failed to induce IL-1β in sera of mice. On the other hand, that study demonstrated increased circulating levels of IL-6, a cytokine, which previously has been shown to depress myocardial contractility via NO production (13). It is therefore possible that the observed cardiodepressive effect from IL-18 may result from increased production of the myocardial depressant NO.

In the present study we have shown a direct effect of IL-18 on the cardiomyocyte. After short-time IL-18 exposure in vitro, we demonstrated increased Ca$^{2+}$ transients (diastolic and peak) and reduced contractility of cardiomyocytes (42). The precise mechanisms underlying the IL-18-induced increase in cytosolic Ca$^{2+}$ are not presently defined. However, IL-1β has been shown to increase the influx of Ca$^{2+}$ into the cardiomyocytes because of an interaction with L-type Ca$^{2+}$ channel proteins (4). Because IL-18 structurally belongs to the IL-1 receptor...
family sharing common intracellular signal pathways with IL-1β (3), we find it conceivable that IL-18 also may increase Ca\(^{2+}\) through the same mechanism. In support of our results it should be noted that Wyman et al. (44) reported a rapid increase in intracellular Ca\(^{2+}\) when stimulating neutrophils with IL-18. The immediate reduction of cardiomyocyte contractility in the presence of increased intracellular Ca\(^{2+}\) is intriguing, and it might be speculated that increased cytosolic Ca\(^{2+}\) initiates reduced responsiveness of the myofilaments to Ca\(^{2+}\) and promotes myofilament proteolysis (15, 22).

Mice treated with IL-18 chronically demonstrated a significant increase in the phosphorylation state of PLB at serine-16 in the LV myocardium compared with mice receiving NaCl only. Regarding the physiological significance of PLB serine-16 phosphorylation, previous studies (17) have clearly demonstrated that serine-16 is phosphorylated by cAMP-dependent protein kinase and that this phosphorylation process reflects a β-adrenoceptor-mediated response. As IL-18-treated mice are in heart failure and a common physiological response to cardiac dysfunction is an increased activation of the sympathetic nervous system (11), we are suggesting that increased phosphorylation of PLB at serine-16, as observed in this study, most likely has to be interpreted as a compensatory regulation of the β-adrenergic signaling pathway in restoring cardiac function.

Cardiac hypertrophy is an important adaptive response to cardiac dysfunction (7). Recent studies have shown that IL-18 activates intracellular signaling proteins such as p38 mitogen-activated protein kinase (44), extracellular signal-regulated kinase (29), and signal transducer and activator of transcription STAT3 (20), all of which have been shown to be associated with myocardial hypertrophy (8, 25, 45). More recently, one in vitro study (10) has convincingly demonstrated that IL-18 induces cardiomyocyte hypertrophy through activation of the PI3K-PDK1-Akt-GATA4 signaling pathway. Interestingly, mice receiving IL-18 chronically showed both increased heart-to-body weight ratios and increased expression of the myocardial hypertrophy marker ANP. In accordance with this finding, increased expression of ANP mRNA has previously been reported after IL-18 exposure to isolated cardiomyocytes in vitro (10, 39). Consequently, our in vivo data from IL-18-treated mice confirm that IL-18 is a prohypertrophic cytokine.

In conclusion, our study shows that daily administration of IL-18 in healthy mice induces LV contractile dysfunction and blunted β-adrenergic responsiveness to isoproterenol. Moreover, induction of myocardial hypertrophy by IL-18 indicates a role for this cytokine in myocardial remodeling. We have also demonstrated a direct effect of IL-18 on the cardiomyocyte in vitro, suggesting that IL-18 reduces the responsiveness of the myofilaments to Ca\(^{2+}\). However, further in vivo studies are required to elucidate the precise role of IL-18 in the pathogenesis of myocardial dysfunction.

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