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

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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.

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 cytosolic 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 directly 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 also may 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 Bomholtgård 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 was injected daily for 7 days into the peritoneal cavity. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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’-6-FAM and 3’ Dark Quencher. The actual sequences of primers and probes used for RT-PCR were as follows: ANP (forward primer 5’-TGGGCTTGGGGAAATATGTGTA-3’; reverse primer 5’-CCGGTCTGATGGAAACGTTATATCC-3’), TNF-α (forward primer 5’-CATCTTTCTAAAAATTCGAGTCGAA-3’; reverse primer 5’-TGGGGTTGAGCAAGGTAACACCAC-3’; probe 5’-CAAGCTGTAAGGCAACACCAACTGGG-3’) (forward primer 5’-CATTGGAATCTCTGAGTGTG-3’; reverse primer 5’-AGATGGTTCAAGCAGTTCCAGTGTG-3’; probe 5’-CTG-GCTCCACACTTTGCTCCAGTCTTATC-3’). RT-PCR (Ambion, Austin, TX) and Taqman PCR (Applied) were performed according to the manufacturers’ instructions, using 2× Taqman Universal Master Mix (Applied) and 20 μM sense and antisense primers. Quantification of mRNA was done using the Sequence Detector version 1.6.3 program (Applied). Gene expression of the 60S acidic ribosomal protein P0 (Applied) was used for normalization.

**Myocyte isolation and intracellular Ca<sup>2+</sup> measurements.** Both isolation of adult rat myocytes and Ca<sup>2+</sup> measurements were performed as described in previous articles from our laboratory (18, 37). Briefly, myocytes were plated on coverslips and loaded with fluo-3 acetoxyethyl ester (10 μmol/l; Molecular Probes, Eugene, OR) at room temperature for 40 min. After being washed for 15 min in standard Tyrode solution (in mmol/l: 5 HEPES, 140 NaCl, 1.8 CaCl<sub>2</sub>, 5.4 KCl, 0.5 MgCl<sub>2</sub>, 5.5 glucose, 0.4 NaH<sub>2</sub>PO<sub>4</sub>; pH was adjusted to 7.4 with NaOH), the coverslips were placed in an open perfusion chamber located on an inverted microscope (Diaphot-TMD; Nikon, Tokyo, Japan), which was attached to a PTI Deltaram fluorescence system (Photon Technology International, Monmouth Junction, NJ). The myocytes were stimulated at 0.5 Hz, and the temperature was 32 ± 0.5°C. The protocol used for measuring Ca<sup>2+</sup> transients (peak and diastolic) and the rate of decline in Ca<sup>2+</sup> transients was performed as follows: Myocytes were superfused with HEPES-Tyrode solution (recovery phase). The dose of IL-18 used in this experiment was based on previous studies in which IL-18 was shown to exert effects in vitro (26, 42, 44). The Ca<sup>2+</sup> transients were calculated as a mean of four consecutive transients, and the intracellular calcium concentration values measured in the IL-18 and recovery phases were all normalized to the control phase. The mean intracellular calcium concentration values of the control phase were set to 100%. Eight myocytes from four separate isolations were included in the study.

**Statistical analysis.** The data are expressed as means ± SE. Statistical analysis was performed using Student’s t-test and one-way analysis of variance when appropriate (SigmaStat version 2.0; Jandel Scientific, Erkrath, Germany). Multiple comparisons were corrected for by using the Student-Newman-Keuls test. A P < 0.05 was considered statistically significant.

**RESULTS**

**Characterization 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 < 0.05) 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.

\[\text{IL-18 CAUSES MYOCARDIAL DYSFUNCTION}\]

\[\begin{array}{|c|c|c|}
\hline
\text{Parameter} & \text{NaCl} & \text{IL-18} \\
\hline
\text{BW, g} & 21.270 ± 0.894 & 20.671 ± 0.790 \\
\text{HW, g} & 0.088 ± 0.002 & 0.093 ± 0.003 \\
\text{LW, g} & 0.141 ± 0.006 & 0.166 ± 0.004* \\
\text{HW/BW ratio (10^{-3})} & 4.169 ± 0.106 & 4.648 ± 0.107* \\
\text{LW/BW ratio (10^{-3})} & 6.639 ± 0.168 & 8.326 ± 0.240† \\
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\end{array}\]

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.

\[\text{β-Adrenergic responsiveness.}\]

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 < 0.05) 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-
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+ to 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.
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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 cardiomycocyte 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 adenylyt 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 cardiomycocyte. After short-time IL-18 exposure in vitro, we demonstrated increased Ca2+ transients (diastolic and peak) and reduced contractility of cardiomycocytes (42). The precise mechanisms underlying the IL-18-induced increase in cytosolic Ca2+ are not presently defined. However, IL-1β has been shown to increase the influx of Ca2+ into the cardiomycocytes because of an interaction with L-type Ca2+ channel proteins (4). Because IL-1β 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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