Diastolic dysfunction in alveolar hypoxia: a role for interleukin-18-mediated increase in protein phosphatase 2A

Karl-Otto Larsen1,2,3*, Birgitte Lygren4, Ivar Sjaastad2,3,5, Kurt A. Krobert3,6, Kristin Arnkværn2,3, Geir Florholmen2,3, Ann-Kristin Ruud Larsen7, Finn Olav Levy3,6, Kjetil Taskén4, Ole Henning Skjønsberg1, and Geir Christensen2,3

1Department of Pulmonary Medicine, Ullevål University Hospital, University of Oslo, Oslo, Norway; 2Institute for Experimental Medical Research, Surgical Building 4th floor, Ullevål University Hospital, Kirkeveien 166, N-0407 Oslo, Norway; 3Center for Heart Failure Research, University of Oslo, Oslo, Norway; 4The Biotechnology Centre of Oslo and Centre for Molecular Medicine Norway, Nordic EMBL Partnership, University of Oslo, Oslo, Norway; 5Department of Cardiology, Ullevål University Hospital, Oslo, Norway; 6Department of Pharmacology, University of Oslo, Oslo, Norway; and 7Department of Immunology and Transfusion Medicine, Ullevål University Hospital, Oslo, Norway

Received 16 November 2007; revised 13 June 2008; accepted 24 June 2008; online publish-ahead-of-print 3 July 2008

Aims Chronic obstructive pulmonary disease with alveolar hypoxia is associated with diastolic dysfunction in the right and left ventricle (LV). LV diastolic dysfunction is not caused by increased afterload, and we recently showed that reduced phosphorylation of phospholamban at serine (Ser) 16 may explain the reduced relaxation of the myocardium. Here, we study the mechanisms leading to the hypoxia-induced reduction in phosphorylation of phospholamban at Ser16.

Methods and results In C57Bl/6j mice exposed to 10% oxygen, signalling molecules were measured in cardiac tissue, sarcoplasmic reticulum (SR)-enriched membrane preparations, and serum. Cardiomyocytes isolated from neonatal mice were exposed to interleukin (IL)-18 for 24 h. The β-adrenergic pathway in the myocardium was not altered by alveolar hypoxia, as assessed by measurements of β-adrenergic receptor levels, adenylyl cyclase activity, and subunits of cyclic AMP-dependent protein kinase. However, alveolar hypoxia led to a significantly higher amount (124%) and activity (234%) of protein phosphatase (PP) 2A in SR-enriched membrane preparations from LV compared with control. Serum levels of an array of cytokines were assayed, and a pronounced increase in IL-18 was observed. In isolated cardiomyocytes, treatment with IL-18 increased the amount and activity of PP2A, and reduced phosphorylation of phospholamban at Ser16 to 54% of control.

Conclusion Our results indicate that the diastolic dysfunction observed in alveolar hypoxia might be caused by increased circulating IL-18, thereby inducing an increase in PP2A and a reduction in phosphorylation of phospholamban at Ser16.

KEYWORDS Protein phosphatases; Phospholamban; Hypoxia; Cytokines

1. Introduction Cardiac dysfunction is frequently observed in chronic obstructive pulmonary disease (COPD), which is one of the leading causes of disability and death worldwide. Chronic hypoxia is a risk factor for COPD patients associated with an increased risk of death,1 and not only right, but also left ventricular (LV) diastolic dysfunction.2 However, little information is available regarding the molecular mechanisms for the diastolic dysfunction in chronic hypoxia. In mice subjected to chronic hypoxia, we have recently shown that LV diastolic dysfunction is associated with slower relaxation of the myocardium.3 The rate of myocardial relaxation is primarily controlled by the activity of the sarcoplasmic reticulum (SR) Ca2+ ATPase (SERCA), which pumps Ca2+ from the cytosol into the SR. Phospholamban regulates SERCA activity, and phosphorylation at the serine (Ser) 16 residue of phospholamban by cyclic AMP-dependent protein kinase (PKA) enhances relaxation rates and contractility. In our previous study, we showed that a reduction in phosphorylation of phospholamban at Ser16 in the myocardium may be an important mechanism for impaired relaxation and diastolic dysfunction in both the right ventricle (RV) and the LV in mice exposed to chronic hypoxia.3

* Corresponding author. Tel: +47 23016800; fax: +47 23016799.
E-mail address: karlottl@medisin.uio.no

Published on behalf of the European Society of Cardiology. All rights reserved. © The Author 2008.
For permissions please email: journals.permissions@oxfordjournals.org.
Reduced phosphorylation of phospholamban at Ser16 may be due either to impaired phosphorylation of the protein through the β-adrenergic pathway, involving the β-adrenoceptors, adenylyl cyclases, and PKA, or to increased dephosphorylation by protein phosphatases (PPs). It is not known which of these mechanisms reduces phosphorylation of phospholamban in alveolar hypoxia. Since reduced phosphorylation of phospholamban is present in both the RV and LV, the latter not exposed to increased afterload, an increase in circulating mediators (e.g. cytokines) could contribute to the changes found in both ventricles.

The aim of the present study was to identify mechanisms reducing phospholamban phosphorylation in alveolar hypoxia. In addition, we wanted to examine if the observed mechanism can be regulated by a circulating mediator.

2. Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and approved by the Norwegian National Animal Research Committee. The animals were housed with a day/night cycle of 12/12 h at 21 °C, and food and water were available ad libitum.

2.1 Animal model

A total of 250 8-week-old male C57Bl/6j mice were either placed in a tightly sealed chamber under normobaric hypoxia with 10% oxygen (hypoxia group) for 1, 2, 7, or 14 days or housed under normoxic conditions (control group). The number of samples in the treatment and control groups is given as n/n. Previous studies have shown that mice breathing 10% oxygen develop pulmonary hypertension1,2 and LV diastolic dysfunction.3 Blood samples were drawn from the inferior vena cava under anaesthesia with a mixture of 2% isoflurane and 98% oxygen.3 Heart and lungs were excised, and the RV and LV (free walls) were separated from the septum and weighed.

2.2 Histology

For histological examinations, hearts from mice subjected to 14 days of hypoxia and controls were fixed in 4% paraformaldehyde and embedded in paraffin. The hearts were sectioned transversely, stained with haematoxylin and eosin, acid fuchsin orange G-stain (AFOG), and Masson trichrome, and examined in a blinded manner by two pathologists.3

2.3 Preparation of subcellular fractions

Homogenates of the RV and LV were made from hearts of mice exposed to 14 days of hypoxia and controls. SR-enriched membrane preparations were obtained from the homogenates by sucrose step gradient centrifugation,4 using an Optima Max tabletop ultracentrifuge, TLS-55 rotor, 100 000 x g for 1 h at 4 °C (Beckman Instruments, Palo Alto, CA, USA). These SR-enriched membranes form a layer at the interface between 24% and 40% sucrose. It has in previous studies by us and others5 been shown that these preparations have a high content of SR as assessed by calsequestrin content. Furthermore, the content of plasma membrane in these preparations is very low.6

2.4 Western blot and R-overlay analysis

SR-enriched membrane preparations of hearts from mice exposed to 14 days of hypoxia and controls, or homogenates of isolated neonatal cardiomyocytes, were subjected to western blotting using primary antibodies to phosphorylated phospholamban at Ser16 and threonine (Thr) 17 (Badrilla, Leeds, UK), phospholamban, SERCA2 (Affinity BioReagents, Golden, CO, USA), PP1α, PP2Ac, calsequestrin (Upstate, Lake Placid, NY, USA), PP2B, PKA regulatory (R) Is, RIIα, and catalytic (C) subunits (BD Transduction Laboratories, KY, USA), phosphodiesterase 4A (pan-PDE4 antibody (K116)),7 total Akt (or protein kinase B) (Cell Signaling Technology, Danvers, MA, USA), collagen I and III (Rockland Immunocchemicals, Gilbertsville, PA, USA), and the Na+/Ca2+ exchanger (NCX).8 Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL, USA) or Bradford (Bio-Rad Laboratories, Hercules, CA). PKA-R-overlays were conducted using [32P]labelled recombinant murine RIIα.

2.5 Protein phosphatase activity

PP activities in SR-enriched membrane preparations from the RV and LV from mice subjected to 14 days of hypoxia and from controls, and total homogenates of isolated cardiomyocytes, were measured by the Protein Serine/Threonine Phosphatase Assay System (New England BioLabs, Ipswich, MA, USA). The PP1 activity was measured using [32P]labelled glycosgen phosphorylase a as substrate in the presence of 4 nM okadaic acid (Merck, Whitehouse Station, NJ, USA), which is an inhibitor of PP2A, and 0.5 mM EDTA, which removes divalent cations that activate PP2B and PP2C.9

2.6 Radioligand binding and adenylyl cyclase activity

Membranes were prepared from RV and LV from mice exposed to 14 days of hypoxia and controls as described,5 incubated with increasing concentrations of (−)-3-(125)iodocyanopindolol in the absence (total binding) or presence (non-specific binding) of 10 μM propranolol for 1 h at 32°C. Specific β-adrenoceptor binding was determined using the same incubation buffers and filtration method as described for other receptors.10 Adenylyl cyclase activities were measured by determining conversion of [32P]ATP (Amersham Biosciences) to [32P]cAMP and after stimulation with 10 μM isoprotrenol (Sigma-Aldrich, St Louis, MO, USA) or 100 μM forskolin (Calbiochem, San Diego, CA, USA).

2.7 Quantitative real-time polymerase chain reaction

A real-time quantitative polymerase chain reaction (PCR) system (ABI 7900HT Fast Real-Time PCR System, PE Biosystems, Foster City, CA, USA) was used to measure the mRNA amounts of PP2A, collagen I and III, and the normalization gene RPL32 in RV and LV from mice exposed to 14 days of hypoxia and controls. Total mRNA was isolated by using spin or vacuum total RNA isolation system (Promega, Madison, WI, USA). All RNA samples were quality assessed by Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and RNA integrity numbers. The RNA samples were reverse transcribed by using iScript cDNA Synthesis Kit (Bio-Rad). Specific mRNA transcripts were quantified by Taqman MX assays (Applied Biosystems, Foster City, CA, USA) for PP2A (assay Mm00479816_m1, efficiency 1.88), collagen I (assay Mm00483888_m1, efficiency 1.94), collagen III (assay Mm00802331_m1, efficiency 1.87), and RPL32 (assay Mm02528467_g1, efficiency 1.94). All samples were tested in triplicate, and average values were used for quantification. Average values for PP2A and collagen I and III mRNA were normalized to RPL32 mRNA.

2.8 Luminex cytokine assays and enzyme-linked immunosorbent assay

The serum levels of 24 cytokines were assayed with Luminex cytokine assay (Bio-Rad) or ELISA (R&D Systems, MN, USA) after 1 day of hypoxia and in controls. In addition, the levels of interleukin (IL)-18 were measured with ELISA (R&D Systems) after 2, 7, and 14 days of hypoxia.
2.9 Isolation of neonatal mouse cardiomyocytes and exposure to hypoxia or cytokines

Cardiomyocytes were isolated from 1- to 3-day-old C57Bl/6j mice using a modified version of a previously described protocol. The purity of the cardiomyocyte cultures was >98%, assessed by DAPI nuclear staining and myosin light chain-2V (Synaptic Systems GmbH, Goettingen, Germany). Neonatal cardiomyocytes respond with an increase in Ser16 phosphorylation similar to adult cardiomyocytes. The neonatal cardiomyocytes were treated with recombinant IL-18 dissolved in distilled water (1000 ng/mL; R&D Systems) or vehicle (distilled water) for 24 h, or exposed to 1% oxygen for 24 h. The cells were kept in Dulbecco’s modified Eagle’s medium (Gibco-BRL) supplemented with medium 199, 1 M HEPES (Gibco-BRL), and penicillin/streptomycin/glutamine (Sigma). An IL-18 concentration of 1000 ng/mL was used based on information from previous cell studies with IL-18. Hypoxic cultures were incubated in an Invitro2 400 hypoxic workstation (Ruskinn, Cincinnati, OH, USA) in a gas mixture containing 4% CO2, 1% O2, and balanced with N2. An oxygen level of 1% has been shown to induce cellular hypoxia with an increase in the hypoxia inducible transcription factor-1α, without causing increased cardiomyocyte cell death (97% viable cells), as assessed by exclusion of trypan blue. Cardiomyocytes were treated for 24 h to allow synthesis of phosphatases as shown in previous studies on myoblasts. For measurements of phosphorylation of phospholamban at Ser16 in IL-18 treated cells, cardiomyocytes were stimulated with 1 nM isoproterenol for 5 min before harvesting. Based on our own preliminary experiments, and previous studies, a concentration of 1 nM was used as it induced a marked increase in Ser16 phosphorylation of phospholamban. In each of the above-mentioned conditions, samples from the experiments were analysed in duplicate or triplicate. Results from western blotting were normalized to calquestrin. Cardiomyocytes were obtained in separate cell isolations on several days to avoid possible errors arising from imperfect cells on 1 day. The n given in the Results section denotes the number of experiments performed. A possible limitation of the cell experiments can be that isolated neonatal cardiomyocytes respond differently than adult cardiomyocytes in a beating heart. However, both protein kinases and phosphatases are active in isolated neonatal cardiomyocytes, and phosphorylation of phospholamban occurs in response to β-adrenergic stimulation as in adult cells.

2.10 Data analysis and statistics

Data are presented as means ± SEM. Comparisons between groups were made using an unpaired Student’s t-test or the non-parametric Mann-Whitney rank sum test in SigmaStat 3.1.1 (Systat Software, Richmond, CA, USA), or ANOVA followed by Student-Newman-Keuls’ post hoc test. Differences were considered significant for P < 0.05.

3. Results

3.1 Body, heart, and lung weights of mice exposed to alveolar hypoxia

The ratio RV weight/tibial length (TL) increased by 35% in mice exposed to 14 days of hypoxia compared with controls (P < 0.05, Table 1). There were no significant differences between the hypoxia and the control group with regard to the LV weight normalized to TL. The lung weight normalized to TL increased by 36% in the hypoxia group compared with controls (P < 0.05, Table 1).

3.2 Levels of Ca2+-handling proteins

The levels of proteins involved in the regulation of Ca2+ extrusion from the cytosol were measured in SR-enriched membrane preparations from RV and LV. Mice exposed to hypoxia had reduced phosphorylation of phospholamban at Ser16 in both the RV and LV amounting to 84 ± 6% (P < 0.05, n = 7/7) and 59 ± 9% (P < 0.05, n = 7/7) of controls, respectively (Figure 1A and B). Neither in the RV nor in the LV, significant changes in total phospholamban (RV 102 ± 5%, n = 5/5; LV 106 ± 12%, n = 5/5) or its phosphorylation at Thr17 (RV 98 ± 10%, n = 7/7; LV 121 ± 16%, n = 7/7) were observed compared with controls. The amounts of SERCA2 were not significantly changed in the RV or LV.

### Table 1 Organ weights at 14 days of hypoxia

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>86</td>
<td>85</td>
</tr>
<tr>
<td>BW (g)</td>
<td>28.1 ± 0.3</td>
<td>26.6 ± 0.2*</td>
</tr>
<tr>
<td>TL (mm)</td>
<td>16.9 ± 0.1</td>
<td>16.9 ± 0.1</td>
</tr>
<tr>
<td>RVW/TL (mg/mm)</td>
<td>0.9 ± 0.1</td>
<td>1.3 ± 0.1*</td>
</tr>
<tr>
<td>LWV/TL (mg/mm)</td>
<td>4.7 ± 0.1</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>LW/TL (mg/mm)</td>
<td>9.3 ± 0.1</td>
<td>12.6 ± 0.3*</td>
</tr>
</tbody>
</table>

Values are means ± SEM. BW, body weight; TL, tibial length; RVW, right ventricular weight; LWV, left ventricular weight; LW, lung weight. *P < 0.05 vs. control.
compared with controls (Figure 1C, n = 7/7 for RV and 5/5 for LV). The protein levels of NCX were increased to 152 ± 17% of control in the RV (P < 0.05, n = 7/7) and to 147 ± 10% in the LV (P < 0.05, n = 7/7, Figure 1D).

3.3 β-Adrenergic receptor levels and adenylyl cyclase activity

Possible mechanisms leading to reduced phosphorylation of phospholamban at Ser16 were explored by examining molecules in the β-adrenergic signalling cascade. Exposure to hypoxia did not alter the levels of β-adrenoceptors in the RV or LV (Table 2). Moreover, hypoxia did not induce any changes in the basal, isoproterenol-stimulated or forskolin-stimulated adenylyl cyclase activities in the RV or LV (Table 2).

3.4 PDE4, PKA-subunits and Akt

The β-adrenergic signalling cascade downstream of adenylyl cyclase was also examined. PDE4, as well as PDE3, represents the major cAMP hydrolytic activities in cardiomyocytes. However, in SR-enriched membrane preparations, hypoxia did not induce any significant changes in PDE4 in the RV (112 ± 5%, n = 7/7) or LV (99 ± 5%, n = 7/7) compared with controls (Table 2). In addition, alveolar hypoxia did not change the amount of PKA-C in the RV (110 ± 10%, n = 7/7) or LV (102 ± 13%, n = 7/7, Table 2). Neither were the levels of PKA-Riα and PKA-Riβ in the RV (125 ± 10%, n = 7/7 and 110 ± 9%, n = 7/7) nor LV significantly altered (109 ± 6%, n = 7/7 and 109 ± 12%, n = 7/7, Table 2). To investigate whether PKA has altered binding to A-kinase anchoring proteins in the hypoxia group, PKA-Riα-β-overlay was performed. No significant changes in A-kinase anchoring protein levels were found. Akt is an important signal transduction molecule in IL-18-mediated gene transcription, and hypoxia induced an increase in cytosolic Akt in the RV to 116 ± 5% (P < 0.05, n = 7/7) and in the LV to 110 ± 5% (P < 0.05, n = 7/7) of respective controls.

3.5 Protein phosphatase levels

To explore if changes in the levels of PPs could explain reduced phosphorylation of phospholamban at Ser16, PP2A and PP1 levels were measured in SR-enriched membrane preparations. Exposure to hypoxia induced an increase in the amount of PP2A in the RV to 122 ± 6% (P < 0.05, n = 7/7) and in the LV to 124 ± 12% (P < 0.05, n = 7/7) of respective controls (Figure 2A and B). Moreover, the levels of PKA1 in the hypoxia group were increased to 143 ± 16% (P < 0.05, n = 7/7) and 177 ± 27% (P < 0.05, n = 7/7) of control in the RV and LV, respectively (Figure 2C). In mice subjected to hypoxia, the amount of PP2B (calcineurin), an important mediator of cardiac hypertrophy, increased to 141 ± 19% of control in the hypertrophic RV (P < 0.05, n = 7/7). No significant alteration in the level of PP2B was found in the non-hypertrophic LV (n = 5/7, Figure 2D).

3.6 Protein phosphatase activities

PP activities were measured in SR-enriched membrane preparations. The PP2A activity was increased in the RV to 202 ± 32% (P < 0.05, n = 6/5) and in the LV to 234 ± 74% (P < 0.05, n = 6/6). In absolute values, the PP2A activity increased in both the RV (9 ± 1 pmol/min/mg in the hypoxia group vs. 4 ± 1 pmol/min/mg in the control group, P < 0.05, n = 6/5, Figure 3A) and LV (14 ± 4 pmol/min/mg in the hypoxia group vs. 6 ± 1 pmol/min/mg in the control group, P < 0.05, n = 6/6, Figure 3A). The increase in PP1 activity did not reach statistical significance (Figure 3B). The absolute PP1 activity in the RV was 32 ± 2 pmol/min/mg in the hypoxia group vs. 29 ± 6 pmol/min/mg in the control group (n = 6/5, Figure 3B) and in the LV 36 ± 6 pmol/min/mg in the hypoxia group vs. 26 ± 5 pmol/min/mg in the control group (n = 6/6, Figure 3B).

3.7 Protein phosphatase mRNA

In the LV, hypoxia induced an increase in the PP2A mRNA (144 ± 14% of control, P < 0.05, n = 6/6), whereas the PP2A mRNA was not significantly increased in the RV (115 ± 8% of control, n = 6/6).

Table 2  β-Adrenergic signaling at 14 days of hypoxia

<table>
<thead>
<tr>
<th></th>
<th>Right ventricle</th>
<th></th>
<th>Left ventricle</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hypoxia</td>
<td>Control</td>
<td>Hypoxia</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>β-Adrenergic receptor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B&lt;sub&gt;m&lt;/sub&gt; (fmol/mg protein)</td>
<td>32 ± 1</td>
<td>27 ± 2</td>
<td>24 ± 1</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>K&lt;sub&gt;a&lt;/sub&gt; (pM)</td>
<td>50 ± 10</td>
<td>50 ± 13</td>
<td>23 ± 3</td>
<td>36 ± 5</td>
</tr>
<tr>
<td>Adenylyl cyclase activity (pmol/mg protein/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>11.4 ± 1.0</td>
<td>12.0 ± 0.9</td>
<td>15.2 ± 2.0</td>
<td>12.0 ± 1.0</td>
</tr>
<tr>
<td>Isoproterenol above basal</td>
<td>19.1 ± 1.6*</td>
<td>25.0 ± 2.2*</td>
<td>29.3 ± 4.0*</td>
<td>31.3 ± 2.6*</td>
</tr>
<tr>
<td>Forskolin above basal</td>
<td>236 ± 24*</td>
<td>224 ± 17*</td>
<td>264 ± 31*</td>
<td>247 ± 27*</td>
</tr>
<tr>
<td>n</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>PDE4 (% of control)</td>
<td>100 ± 4</td>
<td>112 ± 5</td>
<td>100 ± 4</td>
<td>99 ± 5</td>
</tr>
<tr>
<td>PKA-C (% of control)</td>
<td>100 ± 12</td>
<td>110 ± 10</td>
<td>100 ± 6</td>
<td>102 ± 13</td>
</tr>
<tr>
<td>PKA-Riα (% of control)</td>
<td>100 ± 8</td>
<td>125 ± 10</td>
<td>100 ± 2</td>
<td>109 ± 6</td>
</tr>
<tr>
<td>PKA-Riβ (% of control)</td>
<td>100 ± 7</td>
<td>110 ± 9</td>
<td>100 ± 10</td>
<td>109 ± 12</td>
</tr>
</tbody>
</table>

Values are means ± SEM. B<sub>m</sub>, total number of receptors; K<sub>a</sub>, equilibrium dissociation constant ([<sup>125</sup>I]-iodocyanopindolol); PDE, phosphodiesterase; PKA, cyclic AMP-dependent protein kinase.

*Significant difference compared with basal (P < 0.05). No differences were found between control and hypoxia.
3.8 Cytokine and chemokine levels induced by alveolar hypoxia

To investigate whether alveolar hypoxia altered the levels of circulating cytokines and chemokines, of whom several are known to impair cardiac function, serum concentrations of 24 mediators were measured. One day of hypoxia increased the serum concentration of IL-18, IL-12p40, regulated on activation normally T-cell expressed and secreted, IL-5, keratinocyte chemoattractant, and granulocyte-macrophage colony-stimulating factor (*P*, 0.05 for all, *n* = 7/7, Figure 4A). The increase in IL-18 concentration was pronounced, and the IL-18 level was also increased at 2, 7, and 14 days of hypoxia (all *P*, 0.05, *n* = 6/7, 6/6, and 9/10, respectively, Figure 4B). No significant changes in the serum concentrations of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17, eotaxin, granulocyte colony-stimulating factor, interferon-γ, monocyte chemoattractant protein-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, or tumour necrosis factor-α (TNF-α) were found after 1 day of hypoxia (data not shown).

3.9 Protein phosphatases and phosphorylation of phospholamban at Ser16 in cardiomyocytes following IL-18 stimulation and hypoxia

Based on the observed increase in circulating IL-18 at 1, 2, 7, and 14 days of hypoxia together with previous observations showing that this cytokine can induce cardiac dysfunction, 19
we hypothesized that IL-18 might, at least partly, mediate the reduction in phosphorylation of phospholamban at Ser16. IL-18 has also been shown to induce signalling through Akt, which is assumed to be involved in the regulation of PP2A. Stimulation of isolated neonatal cardiomyocytes with IL-18 for 24 h increased the amount of PP2A to 133 ± 8% of control (P < 0.05, n = 6/6, Figure 5A), but the level of PP1 was not significantly changed (102 ± 7% of control, n = 6/6). IL-18 also increased PP2A activity to 114 ± 5% of control (P < 0.05, n = 6/6, Figure 5B), whereas PP1 activity was not significantly altered (102 ± 6% of control, n = 6/6). In addition, IL-18 treatment before isoproterenol stimulation reduced the amount of Ser16 phosphorylated phospholamban to 54 ± 8% of control (P < 0.05, n = 6/6, Figure 5C and D). The amounts of PP2A and PP1 in isolated cardiomyocytes exposed to 1% oxygen for 24 h were not significantly changed compared with control (100 ± 5%, n = 6/6 and 104 ± 6%, n = 6/6). Nor were changes in PP2A or PP1 activity observed in isolated cardiomyocytes exposed to 1% oxygen (97 ± 13%, n = 6/6 and 112 ± 14%, n = 6/6).

3.10 Histology and collagen content in hearts exposed to alveolar hypoxia

In the LV, hypoxia induced an increase in collagen I and III mRNA to 153 ± 16% (P < 0.05, n = 6/6) and 150 ± 13%, respectively (P < 0.05, n = 6/6). A corresponding higher amount of collagen I protein was also observed (143 ± 7%, P < 0.05, n = 7/7), whereas the amount of collagen III was not significantly changed (110 ± 8%, n = 7/7). The increase in collagen I in the LV was not associated with any visible fibrosis, as assessed by the examination of haematoxylin and eosin, AFOG, and Masson trichrome-stained sections (n = 3/3). The collagen III mRNA was significantly increased in the RV (206 ± 40%, P < 0.05, n = 6/6) in the hypoxia group, but not the collagen I mRNA (138 ± 17%, n = 6/6). Collagen I and III protein in the RV was not significantly altered (100 ± 12%, n = 7/7 and 99 ± 11%, n = 7/7, respectively), and no visible fibrosis was observed.

4. Discussion

The reduction in Ser16 phosphorylation of phospholamban observed in the free wall of the LV may be a molecular mechanism contributing to the reduced relaxation rate and diastolic dysfunction observed in mice subjected to alveolar hypoxia, as well as in patients with COPD and chronic hypoxia. For the development of novel therapies of diastolic dysfunction, identification of the signalling pathways causing reduced phospholamban phosphorylation may be of importance. By examining SR-enriched membrane preparations from mice subjected to hypoxia, we found that the amounts of PP1 and PP2A and the activity of PP2A in the LV increased considerably compared with normoxic controls. Moreover, IL-18 levels were significantly increased in hypoxic mice, and IL-18 upregulated both the amount and activity of PP2A in isolated cardiomyocytes. Finally, IL-18 suppressed phospholamban phosphorylation in cardiomyocytes.

In heart failure caused by myocardial infarction or systemic hypertension, the reduced phosphorylation of phospholamban has, at least partly, been ascribed to diminished β-adrenergic signalling. In alveolar hypoxia, however, we did not observe any alterations in either the number of β-adrenergic receptors or adenylyl cyclase activity. In addition, we did not find an increase in circulating catecholamines in this hypoxia model. Thus, it would appear that signals responsible for the reduced phosphorylation of phospholamban at Ser16 in alveolar hypoxia differ from those observed in heart failure following myocardial infarction or systemic hypertension, where also disturbances in anchored pools of PKA have been found. In our study, however, the amounts of SR-associated PKA-R and -C subunits and A-kinase anchoring proteins were not altered. Additionally, the amount of PDE4, which regulates PKA activity in the heart, was not changed in alveolar hypoxia. Thus, our findings indicate that reduced β-adrenergic signalling is not an important mechanism for the reduction in phosphorylation of phospholamban at Ser16 in alveolar hypoxia.

Phosphorylation of phospholamban is a dynamic process, in which the action of protein kinases is counterbalanced by PPs. PP1 and PP2A are the main phosphatases in the heart that regulate the phosphorylation of phospholamban at Ser16. In our study, we showed that the PP activity in the SR-enriched membrane preparations from the LV free wall was substantially increased. The high PP activity was mainly caused by elevated PP2A activity, which was increased more than two-fold, accompanied by an increased amount of PP2A. This increase in PP2A activity was found in SR-enriched membrane preparations consistent with an...
increased and the time course of the decay of the Ca2+
with congestive heart failure.35,36 Moreover, daily adminis-
tration of phospholamban at Ser16 and diastolic dys-
function,26 indicating that an isolated increase in PP2A
may contribute to the reduced LV function observed in
alveolar hypoxia. In cardiac myocytes from PP2A-transgenic
mice, the diastolic Ca2+ concentration in the cytosol was
increased and the time course of the decay of the Ca2+
transients was not hastened by β-adrenergic stimulation.26
We also found increased amounts of NCX in both ventricles,
which can increase calcium extrusion and serve as a com-
pensatory mechanism for impaired SR uptake due to
reduced phospholamban phosphorylation. In addition to
being involved in the regulation of cardiac relaxation and
contractility, PP2A activity and level of expression are of
importance for growth and development of the lung.27–29
Thus, it cannot be excluded that alterations in pulmonary
PP2A during hypoxia may also play a role for hypoxia-related
changes in the lung, in addition to the alterations observed
in the heart.

Little information is available regarding extracellular
stimulators that may regulate the amount and activity of
PP2A in the heart. Since LV wall stress is not increased
during alveolar hypoxia, circulating mediators might cause
the alterations in PP2A amount and function. A link
between chronic hypoxemia and cardiovascular disease
through inflammatory pathways has been suggested.30
Moreover, accumulating evidence indicates that pro-inflamma-
tory cytokines such as TNF-α are involved in the
development of cardiac failure.31 To investigate whether
circulating mediators could cause the observed alterations
in PPs in alveolar hypoxia, we examined an array of cyto-
kines and chemokines in serum from mice exposed to alveo-
lar hypoxia. We found increased concentrations of six
cytokines, and the increase in the concentration of IL-18
was particularly pronounced. Patients suffering from
severe COPD, who are prone to having hypoxia and cardiac
dysfunction, have increased expression of IL-18 protein
in pulmonary alveolar macrophages.32 In addition to alveolar
macrophages, IL-18 is produced by airway and alveolar epili-
thelial cells.33 All these cell types can be exposed to air with
decreased partial pressure of oxygen, and thereby be stimu-
lated to generate IL-18, since hypoxia has been shown to
promote synthesis of IL-18 in cultures of other types of
cells.34

To our knowledge, the relationship between circulating
IL-18 and alveolar hypoxia or pulmonary hypertension has
not yet been studied. We found increased serum concen-
trations of IL-18 throughout the investigation period of 2
weeks of hypoxia, showing that the heart in alveolar hypoxia
is chronically exposed to increased concentrations of
IL-18. Increased circulating IL-18 has also been found in
a post-infarction model of heart failure and in patients
with congestive heart failure.35,36 Moreover, daily adminis-
tration of IL-18 to healthy mice caused LV myocardial dys-
function,19 but little is known about the mechanism
caus ing this dysfunction. In the present study, IL-18
increased both the amount and activity of PP2A in isolated
cardiomyocytes. Importantly, we also showed that in cardi-
omyocytes treated with IL-18 for 24 h, there was an ~50%
decrease in phosphorylation of phospholamban at Ser16 in
response to submaximal β-adrenergic stimulation. The
intracellular pathway by which IL-18 exerts its effect on
cardiac hypertrophy has been shown to be via Akt.18 We
found elevated amounts of cytosolic Akt, which may be
induced by IL-18 and possibly lead to increased synthesis
of PP2A. However, although our findings indicate a role for
IL-18, we cannot exclude that also other circulating
mediators may influence the amount and activity of PP2A
acting on phospholamban. Another possible regulator of
PP2A or PP1 could be hypoxia per se. However, in isolated
cardiomyocytes exposed to 1% oxygen, neither the amount
nor the activity of PP1 and PP2A was altered.

Another possible mechanism for the LV diastolic dysfunc-
tion in alveolar hypoxia is myocardial fibrosis. We did
not observe myocardial fibrosis by histological examination,
although increased content of collagen I mRNA and protein
was observed in the LV. The increase in collagen con-
tent might contribute to increased passive chamber stiff-
ness and altered late-diastolic filling. We have previously
measured slower relaxation in the early phase of disas-
tole in alveolar hypoxia,3 and the relaxation in this phase
of the diastole is predominantly regulated by SERCA
activity.37

PP2B (calcineurin) has been shown to mediate cardiac
hypertrophy.38 In our study, calcineurin was significantly
increased in the RV, consistent with a role as a mediator
of hypertrophy in the RV, which is exposed to increased
afterload. No significant increase in calcineurin was found
in the LV, which has a normal afterload and no hypertrophy.
In contrast to the PP2A activity that was increased in both
ventricles, possibly due to circulating mediators, the
expression of PP2B seems to be stronger related to mechan-
ical stress, such as increased afterload. In a rat model with
chronic hypoxia, increased mRNA of the modulatory
calcineurin-interacting protein-1, an indicator of calci-
neurin activity, was found in the RV.39 However, to our
knowledge, the amount of PP2B has not previously been
measured in alveolar hypoxia or in any types of pulmonary
hypertension.

In summary, alveolar hypoxia leads to decreased phos-
phorylation of phospholamban at Ser16. Changes in the
β-adrenergic signalling cascade could not account for this
hypophosphorylation. However, alveolar hypoxia induced
an increase in the amount and activity of PP2A, which may
lead to reduced phospholamban phosphorylation in the myo-
cardium. Serum concentrations of IL-18 were substantially
increased in alveolar hypoxia, and IL-18 stimulation of iso-
lated cardiomyocytes reduced phosphorylation of phospho-
lamban at Ser16 and increased the amount and activity of
PP2A. Thus, the chronically elevated serum levels of IL-18
in alveolar hypoxia may contribute to diastolic dysfunction
via increased PP2A activity causing reduced phosphorylation
of phospholamban.

Acknowledgements

We are grateful to Bjørg Austbe, Hilde Dishington, Liv Marit Skaug,
Dina Behmen, and Almira Karahasan for skilful laboratory work, to
Else Marit Leberg for heading the histological examinations, to Siv
Leng Tran, Siv Rong Tran, and Carsten Lund for animal care, and
to Roy Trondsen for expert technical help.

Conflict of interest: none declared.
**Funding**

This study was supported by Anders Jahre’s Fund for Promotion of Science, The Research Council of Norway, The Norwegian Council for Cardiovascular Diseases and The Norwegian Cancer Society.

**References**


