Influence of the lung mechanical ventilation with injurious parameters on 7-ketocholesterol synthesis in Sus Scrofa

Oxana V. Klimenko*, Vaclav Vobruba & Pavel Martasek
Department of Pediatrics and Adolescent Medicine, 1st School of Medicine, Charles University, Prague, Czech Republic

The aim of work was to investigate changes of 7-ketocholesterol synthesis in alveolar macrophages in the dynamic of lung mechanical ventilation with injurious parameters. The goal of in vitro part of work was to observe influence of 7-ketocholesterol on iNOS and MIP1β production in bronchoalveolar lavage fluid (BALF) cells. We used 17 healthy domestic pigs randomly assigned into two treatment groups: group I with mechanical ventilation with physiological parameters; group II underwent injurious ventilation with high volume tidal (VT) and low positive end expiratory pressure (PEEP). Cells were analyzed for CYP27A1 protein and gene expression levels, 7-ketocholesterol production. In alveolar macrophages of group II, we obtained increase of production of CYP27A1 protein and 7-ketocholesterol, as well as the expression of the CYP27A1 gene at the 2nd hour of ventilation. In the in vitro experiments we show dose-dependent increase of MIP1β and decrease of CYP27A1, iNOS protein production after 7-ketocholesterol treatment. [BMB reports 2010; 43(4): 257-262]

INTRODUCTION

Many studies have demonstrated the influence of injurious methods of lung mechanical ventilation on multiple respiratory structures. Treatment with high volume tidal and low or zero PEEP leads to pathological changes, collectively termed barotrauma or ventilator-induced lung injury (VILI). Barotrauma is characterized by direct mechanical effects on lungs that lead to destructive reactions, such as primary inflammation and further immune responses in the blood alveoli barrier and in the surrounding tissues. The involvement of macrophages leads to the production of pro-inflammatory cytokines, chemokines, and the synthesis of superoxide radicals. Cholesterol and other neutral lipids are components of the hydrophobic part of surfactant and they participate in maintaining its homeostasis (1). The biochemical process of oxysterol formation is enzymatic and occurs in the mitochondria of different cells. The majority of free cholesterol utilizes with CYP27A1 enzyme which is member of the mitochondrial cytochrome P450 sterol hydroxylase C family (CYP). The goal of our work was to observe how mechanical ventilation with injurious parameters will influence on levels of expression of both CYP27A1 and product of its reaction 7-ketocholesterol in dynamics. In our in vitro experiments we investigated influence of high concentration of 7-ketocholesterol on MIP1β and iNOS production in BALF cells.

RESULTS

In vivo experiments

Influence of mechanical ventilation on CYP27A1 protein and gene expression levels in dynamics: In the western blots we detected the increase of the CYP27A1 enzyme quantity in group II at the 2nd hour of ventilation. In group I we also observed increase of CYP27A1 enzyme level. However value of this protein was significantly higher in group II than in group I. The first group had levels of 90 ± 20%, whereas group II had levels 165 ± 20% (P < 0.05). In western blots of alveolar macrophages obtained from group II we detected decrease of CYP27A1 protein at the 1st hour (53.7 ± 20% to the control 100%, P < 0.05) with minimal levels observed at the 3rd hour of ventilation (25 ± 20%, P < 0.05). In alveolar macrophages from group I we observed decrease of CYP27A1 after the 1st hour (50.5 ± 20% to the control of 85%, P < 0.05) and at the 3rd hour (4.5 ± 20%, P < 0.05) (Fig. 1).

Almost the same picture was obtained after examining CYP27A1 gene expression on gels after reverse transcriptase polymerase chain reaction. The maximal levels of cDNA template expression were observed at the 2nd hour of group II when compared to the initial level (250 ± 15% to 60 ± 20, P < 0.05) (Fig. 2). We also saw decreases of CYP27A1-gene expression at the 3rd hour in this group of pigs (20 ± 15%, P < 0.05). In the normal mechanical ventilation we did not observe any changes in cDNA template expression (data not...
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Fig. 1. Influence of mechanical ventilation with physiological and injurious parameters on production of CYP27A1 protein (western blot). In alveolar macrophages of group II underwent high volume tidal and low PEEP mechanical ventilation production of CYP27A1 increased at the 2nd hour of procedure. Data are presented as the mean ± SEM, P ≤ 0.05. *Data indicate statistically significant differences between primary level and 2nd hour ventilation level of CYP27A1 protein.

Fig. 2. Changes of expression of CYP27A1 gene in alveolar macrophages under the influence of mechanical ventilation with injurious parameters in dynamics. The enhancement of CYP27A1 gene expression level was observed at the 2nd hour of ventilation in group II. We could not detect any expression of CYP27A1 in group I. Data are presented as the mean ± SEM, P ≤ 0.05. All data were normalized to gene expression levels of b-actin of cells obtained from primary BALF. This level was quantified as 100%. All samples were prepared in triplets. *Data indicate statistically significant differences between primary level and 2nd hour ventilation level of CYP27A1 gene.

Changes of 7-ketocholesterol production
We observed an increase of the 7-ketocholesterol production purified from BALF at the 2nd hour of group II and decrease at the 3rd hour of mechanical ventilation to the level, which was lower than the initial level (Fig. 3). In group I we did not observe statistically reliable changes in the dynamics of 7-ketocholesterol production during the procedure. We also used RP-HPLC to investigate the concentration of 7-ketocholesterol in BALF and in homogenates of alveolar macrophages. We did not observed statistically significant differences in intracellular production of 7-ketocholesterol.

In vitro experiments
In this part of our experiments we investigated influence of 7-ketocholesterol on some alveolar macrophage inflammatory functions. We added high dose of 7-ketocholesterol (30 μg/ml = 37.5 μM/ml) into alveolar macrophage culture medium. Changes in MIP1β, iNOS and CYP27A1 proteins in medium were detected after 24 hours of incubation.

We observed increase of MIP1β protein level (300 ± 50% to the control 100 ± 52%, P < 0.05) in alveolar macrophages treated with the highest (30 μg/ml) dose of 7-ketocholesterol (Fig. 4A). This finding indicates the pro-inflammatory activity of high concentrations of oxysterol.

We also observed a decrease of iNOS protein production in BALF cells after treatment with 7-ketocholesterol (45 ± 20% to the control 100 ± 30%, P < 0.05) (Fig. 4C). This effect may be the cause of the immunosuppressive activity of 7-ketocholesterol.

We observed statistically significant inhibition of CYP27A1 protein production after the treatment with the highest dose (30 μg/ml) of 7-ketocholesterol (50 ± 10% to the control 90 ± 10%, P < 0.05) (Fig. 4B). This finding may be connected with mechanism of the negative metabolic feedback.

DISCUSSION
Development of pathological changes in VILI in animal models may be compared with pathogenesis of acute respiratory distress syndrome (ARDS) in human (5, 6). The first investigators who used high pressure/high volume ventilation in normal lungs were Webb and Tierney (7). They ventilated rats with different volumes tidal and low or zero PEEP, and were first who observed development of severe protein-rich interstitial and airspace edema after VILI. After that time other pathological changes and mechanisms developed under the influence of high tidal volume and low PEEP were described. In spite of great number of articles devoted to VILI we could not find any publications about dynamic of 7-ketocholesterol shown).
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Fig. 4. Production of MIP1β (A), CYP27A1 (B) and iNOS (C) proteins in alveolar macrophages after treatment with 37.5 μM/ml of 7-ketocholesterol in western blots. Pre-treatment with high concentration of 7-ketocholesterol decreased iNOS and CYP27A1 protein levels, and increased MIP1β protein production in BALF cells. Data are presented as the mean ± SEM, P ≤ 0.05. *Data indicate statistically significant differences of production of MIP1β (A), CYP27A1 (B) and iNOS (C) proteins levels between cells without and after treatment of medium with 7-ketocholesterol.

Fig. 3. RP-HPLC detection of 7-ketocholesterol (UV-233 nm) (right figure). The level of 7-ketocholesterol was increased at the 2nd hour of ventilation in alveolar macrophages of group II animals. Data are presented as the mean ± SEM, P ≤ 0.05. Data were calculated to comparison with calibration curve of 7-ketocholesterol (5-cholesten-3b-ol-7-one) standard (mAU/ml units to concentration). Sample of 7-ketocholesterol and cholesterol standards chromatogram (left figure).

production, and CYP27A1 gene and protein expression in BALF.

Obtained findings we can explain by development pathological changes caused by mechanical stresses which induce changes in intra- and extracellular signaling cascades. This conversion of a physical stimulus into chemical signals inside the cell was termed mechanotransduction. Overdistention of cell
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results in activation of intracellular signaling systems. Mechanical stresses cause release calcium from the intracellular depot result in cells contraction, phosphorylation of myosin light chain kinase, tyrosine kinase, src, and others. Mechanical stresses activate expression of extracellular matrix-bound integrins. Bound integrins activate adenylate cyclase, which increases cAMP and activates protein kinase A (PKA). PKA affects gene transcription. It is may be one of the mechanisms proposed to explain CYP27A1 gene and protein enhanced expression in VILI (6).

Intra- and extracellular processes in VILI also promote development of oxidative stress (8). Oxidative stress accompanying with expression of mitochondrial enzymes, one of them is CYP27A1 (9, 10). Accumulation of superoxide radicals changes intracellular and intra-mitochondrial metabolic and cell respiratory reactions. As the result expression of many genes with different metabolic and respiratory functions changes. We detected increase of protein and gene expression of CYP27A1 in the alveolar macrophages in the group II of animals at the 2nd hour of ventilation. As it was written above big amount of cholesterol which is substrate for CYP27A1 and high expression of CYP27A1 result in production of big amount of 27-α hydroxycholesterol and 7-ketocholesterol, which may penetrate the cell (11). We observed increase of oxysterol levels in the group II of animals at the 2nd hour of ventilation to compare with control level. The high level of cholesterol oxidation in condition of oxidative stress was also observed by Pappolla and co-workers (12). Finally, oxysterols were identified as markers of oxidative stress (13).

Accumulation of big amount of oxysterols starts up the cascade of wide spectrum of metabolic reactions. In the cells 7-ketocholesterol activates liver X receptors (LXR), PPARα (14-16), IκKα, IκKβ receptors (17, 18). All these nuclear receptors influence on gene expression of NF-κB in nucleus. The main function of NF-κB is regulation of immune reactions (19). NF-κB changes expression of genes, which control production of cytokines and chemokines (MIP1β), iNOS, etc. In our in vitro experiments we observed increase the expression of MIP1β protein in alveolar macrophage cells treated with high concentrations of 7-ketocholesterol. This finding indicates the influence of 7-ketocholesterol on stimulating of CXC chemokine protein expression in macrophage cells. We couldn’t find some publications about influence of oxysterols on expression of MIP1β protein in alveolar macrophages. We observed inhibition of iNOS production 24 hours after the addition of 7-ketocholesterol to alveolar macrophage cell cultures. Deckert and co-workers also obtained data demonstrating the inhibition of nitric oxide release by 7-ketocholesterol in human vascular endothelial cells (20). The same observation was made by Kim and co-workers. (21). Diestel and co-workers also observed a decrease of iNOS expression after treating microglia cells with high amount of 7-ketocholesterol (22). The same effect on the expression of iNOS was detected by Lee et al. They found down-expression of iNOS by oxysterol in astrocytes pre-stimulated with LPS (23).

All these effects of oxysterols are important for further investigations. Dynamic of oxysterol synthesis as a marker of pathologic disturbances degree would help in planning of therapeutic manipulations in patients with ARDS.

In the dynamic of lung mechanical ventilation increases production of 7-ketocholesterol and CYP27A1 at the 2nd hour of ventilation. This fact indicates development of changes in intracellular metabolic processes at the 2nd hour of mechanical ventilation with injurious parameters. Oxysterol (7-ketocholesterol) has influence on some inflammatory functions of alveolar macrophages, and changes production of iNOS and MIP1β.

MATERIALS AND METHODS

Experimental procedure
For the in vivo study, 17 healthy pigs (large white, 6-8 weeks old, weight 22-30 kg) were randomly assigned into two treatment groups. Animals from group I [8] were intubated and ventilated with the tidal volume (TV) of 7 ml/kg and PEEP of 5 cmH2O. Pigs of the group II [9] were treated with mechanical ventilation with TV of 15 ml/kg and PEEP of 0 cmH2O. Other parameters of mechanical ventilation in groups I and II were: respiratory rate of 20/min and FiO2 of 0.21. Siemens SV 900 C ventilator was used in our experiments. In the end of experiments all animals were euthanized with a bolus of 10% Thomas solution in the right atrium using an average dose of 2 ml/kg. Deceased animals were removed by professional employees according to a standard procedure and brought to the incinerator. Azaperon, thiopental and ketamine were used as anesthetic drugs. All experiments were performed with the approval of the ethical committee, in accordance with Section 12 of Act No. 311/97 Coll. "On breeding and using experimental animals" at the accredited Experimental Centre of the Charles University in Prague, Faculty of Medicine in Pilsen, the principles of the Guide for care and use of laboratory animals (24).

Collection of BALF and macrophages
Physiological solution (50 ml) warmed to 37°C was used for the bronchoalveolar lavage procedure. BALF was collected from pigs immediately after intubation and at the end of the 1st, 2nd and 3rd hours of ventilation. Obtained fluid was filtered through sterile filters (100 μm) to remove mucus, and gently mixed. Alveolar macrophages were collected by spinning the lavage fluid at 1,000 rpm for 10 min. Supernatants from BALF were immediately frozen until they were used for assays. The cell pellet was resuspended in Dulbecco’s modified Eagle’s medium (DMEM), washed, and cultured in medium (DMEM - 4 ml, mixed with 1% solution of antibiotic mix (Sigma), and 10% fetal bovine serum). BAL cells were incubated for 2-3 hours in a 5% CO2-humidified atmosphere at 37°C. After that nonadherent cells were gently removed. The adherent cells were cultured in 25 cm² flasks over a period of 18-24 hours.
HPLC detection of oxysterols in total cholesterol

Total cholesterol was obtained from homogenized macrophages using the method described by Folch, 1957 (25). For the HPLC procedure we used tert-butyl methyl ether and acetoneitrile from Aldrich Chromasolv (Sigma-Aldrich), with 5-cholenest-3β-ol-7-one (7-ketocholesterol) from Sigma-Aldrich as a control. The bronchoalveolar fluid lipids were isolated using the method of Ferraz T.P.L., et al., 2004 (26). HPLC detection was performed with 13% of MTBE in acetonitrile to 25% water (87 : 13 : 25 (acetoneitrile : MTBE : H2O ratio). For the separation and quantification of 7-ketocholesterol we used reverse-phase HPLC column C18 (Microsorb-MV 100 C18, Varian, USA) with the following technical parameters: 250 × 4.6 mm with 5 μm particle size and guard column (MetaGuard 1.0 mm Microsorb 100A 5u C18, Varian, CA, USA) in accordance with Hui Shan, JiHai Pang, et al., 2002; Schroepfer J. G., 2000; Rodrigues I., 2004 (27-29). For the detection of oxysterols, we used UV detector set for 233, 210 and 214 nm wavelengths. Maximal peaks detected at 233 nm. The chromatographic procedure was done using the ÄKTA purifier HPLC system (Amersham Biosciences).

Semi-quantitative reverse transcriptase polymerase chain reaction

Total RNA was extracted from alveolar macrophages using the "Cells-to-cDNA II Kit" (Ambion, Austin, USA) according to the manufacturer's protocol. In our experiments we used a two-step RT-PCR procedure with some modifications of the PCR amplification step. Amplification of CYP27A1 or β-actin cDNA (as an internal control) was performed with an automatic thermocycler (Bio-Rad, CA) in a reaction mixture with 12.5 μl PPP Master Mix (Top-Bio s.r.o., Czech Republic), 6.25 μM primer (sense and antisense), template, and H2O. The CYP27A1 primer sets were 5'-TGGAGCTATGGAAGGACAC-3' (sense) and 5'-AGCTGGTCCAGTCGAGTCAT-3' (antisense). The β-actin primer sets were 5'-TCCCTGGAGAAGAGCTACGA-3' (sense) and 5'-AGCAGCTGTGTTGGCGTACAG-3' (antisense). Amplification proceeded according to the following protocol: 95°C for 30 s, 58°C for 40 s, and 72°C for 30 s followed by a 30 sec final extension at 72°C. The CYP27A1 product (187 bp) and β-actin (194 bp) were amplified for 35 cycles. PCR products were loaded on 1.5% agarose gel and electrophoresed then colored with EtBr, exposed to a VersaDoc (Bio-Rad) and quantified with Quantity One Software (Bio-Rad). For comparison we used: internal control - expression of β-actin gene, and external control for which we used cells after the first harvesting of BALF.

Cell treatment

We treated the 24-hour alveolar macrophages cell culture from the controls with maximal permissible for cells survival concentration of 7-ketocholesterol (5-cholenest-3β-ol-7-one) (Sigma-Aldrich) (30). To prepare the initial solution, 800 μg of 7-ketocholesterol was dissolved in 50 μl of absolute ethanol and 950 μl of culture medium was added. To obtain 30 μg/ml final concentration, 37.5 μl of the initial solution was added per milliliter of culture medium at the beginning of incubation (31).

Western blot analysis of CYP27A1, MIP-1β, and iNOS proteins

For the detection of CYP27A1 protein levels we used primary polyclonal human anti-donkey antibodies for CYP27A1 and anti-donkey IgG-HRP secondary antibodies (Santa Cruz biotechnologies Co., CA, USA). For detection of iNOS protein value we used primary rabbit anti-mouse iNOS polyclonal antibodies (Alexis), diluted 1 : 500 in blocking solution and secondary anti-rabbit IgG-HRP antibodies (Sigma). For the detection of MIP-1β protein we used primary rabbit anti-human MIP-1β polyclonal antibodies (Alexis), diluted 1 : 500 in blocking solution and horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (Sigma).

Statistics

Data are presented as the mean ± SEM. Two-tailed Student's t-test was used for analyses comparing the groups. The observed differences between study groups were considered statistically significant if p-values were ≤ 0.05. Levels measured parameters at the first hour of ventilation were used as control. All gene expression data were normalized to gene expression levels of β-actin. All samples were prepared in triplets.

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