Explorations of the therapeutic potential of influencing metabolism during critical illness

Aslami, H.

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Induced hypothermia is protective in a rat model of pneumococcal pneumonia, associated with increased ATP availability and turnover

*Charlotte J.P. Beurskens¹; *Hamid Aslami¹; Maria T. Kuipers¹; Janneke Horn²; Margreeth B. Vroom²; André B.P. van Kuilenburg³; Joris J.T.H. Roelofs⁴; Marcus J. Schultz¹,²; Marcus J. Schultz¹,²; Nicole P. Juffermans¹,².

¹ Laboratory of Experimental Intensive Care and Anaesthesiology (L.E.I.C.A), Academic Medical Center, Amsterdam, the Netherlands.
² Department of Intensive Care Medicine, Academic Medical Center, Amsterdam, the Netherlands.
³ Laboratory Genetic Metabolic Diseases, Academic Medical Center, Amsterdam, the Netherlands.
⁴ Department of Pathology, Academic Medical Center, Amsterdam, the Netherlands.

*: contributed equally

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Abstract

Objective: To determine the effect of induced hypothermia on bacterial growth, lung injury and mitochondrial function in a rat model of pneumococcal pneumosepsis.

Design: Animal study

Setting: University research laboratory

Subjects: Male Sprague–Dawley rats

Interventions: Subjects were inoculated intratracheally with Streptococcus pneumoniae, controls received saline. After development of pneumonia, mechanical ventilation was started with or without induced mild hypothermia (32°C). Bacterial growth and inflammatory markers were determined in bronchoalveolar lavage fluid (BALF), blood, and organs. Oxidative phosphorylation and ATP contents were measured in mitochondria isolated from liver and soleus muscle.

Measurements and main results: Inoculation with S. pneumoniae resulted in severe pneumonia, with bacterial dissemination, distal organ injury and blunted peripheral oxygen consumption upon mechanical ventilation. Hypothermia did not affect bacterial growth in BALF and in homogenized lungs compared to normothermic controls, but was associated with reduced bacterial dissemination to the spleen, with a trend towards reduced bacterial load in blood and liver. Hypothermia reduced lung injury, exemplified by reductions in pulmonary cell influx and BALF protein levels compared to controls. Hypothermia reduced BALF levels of interleukin (IL)–1β, tended to reduce BALF CINC–3 levels, but no effect was observed on BALF TNF–α and IL–6 levels. Induced hypothermia restored the fall in oxygen consumption and ATP levels in the liver, while ATP/ADP ratios remained low. In muscle, induced hypothermia also reversed low oxygen consumption due to pneumonia, but with an increase in ATP levels, while ATP/ADP ratios were low.

Conclusion: Hypothermia did not adversely affect bacterial growth, but rather reduced bacterial dissemination in a rat model of pneumococcal pneumosepsis. Furthermore, hypothermia reduced lung injury, associated with restored ATP availability and turn–over. These findings suggest that hypothermia may reduce organ injury by preventing sepsis–related mitochondrial dysfunction.
Introduction

Sepsis triggers an uncontrolled and excessive systemic inflammatory response, which may develop into multiple organ dysfunction (1). The mechanisms by which organ failure occurs are unknown. Prolonged shock with shunting in the microcirculation may contribute to inadequate tissue perfusion, thereby reducing metabolic function (2–4). However, metabolic dysfunction has been found to persist despite adequate oxygen delivery, suggesting that the predominant defect may be a decreased use of oxygen in mitochondria.

During severe infection, the inflammatory response can result in direct damage to mitochondrial DNA, lipids and respiratory complexes, thereby inhibiting oxidative phosphorylation (5) and diminishing adenosine triphosphate (ATP) availability in organs (6). When ATP levels drop below a certain threshold, apoptotic cell death pathways are triggered (3). Energetic failure directly impacts outcome in sepsis, as skeletal muscle ATP concentrations were found to be depleted in patients with septic shock, together with structural changes in the mitochondria, associated with enhanced mortality (7). Alternatively, it was proposed that a decrease in mitochondrial activity with subsequent reduced cellular metabolism may be a functional response to excessive inflammation, protecting the cell from dying during energetic failure. Thereby, reducing metabolic demand and thus ATP requirements could be protective in case of an excessive inflammation response.

Induced mild hypothermia is applied in patients with cardiac arrest to prevent ischemia–reperfusion injury, resulting in a better neurologic outcome and reduced mortality (8). The underlying mechanisms of the protective effect of hypothermia include a slower metabolism, as well as prevention of mitochondrial dysfunction (9). Induced mild hypothermia may also exert its protective effect by inhibition of leukocyte migration, phagocytosis and pro–inflammatory cytokine production (10;11). A protective effect of mild hypothermia was found in murine models of sterile acute lung injury (12–15). In patients with severe acute lung injury, hypothermia applied as a last resort was found to reduce mortality (16). The downside of hypothermia however, may be an increased risk of infections (17;18), due to an impaired host response (19;20).

In this study, we investigated the effect of induced mild hypothermia in a model of pneumococcal pneumosepsis. Pneumococcal pneumonia is the most common pathogen causing pneumonia with organ failure, requiring ICU admission (21;22). We hypothesized that hypothermia would reduce ATP requirements during pneumosepsis by reducing inflammation, thereby reducing organ injury. Also, we investigated the effect of hypothermia on bacterial dissemination to distant organs.
Materials & Methods
This study was approved by the animal care and use committee of the Academical Medical Center, Amsterdam, The Netherlands. Animal procedures were carried out in compliance with Institutional Standards for Human Care and Use of Animal Laboratory Animals.

Induction of pneumococcal pneumonia
Male Sprague–Dawley rats (Harlan, The Hague, The Netherlands) weighing 350–400 grams were inoculated intratracheally with ~8 x 10^6 colony forming units (cfu’s) of aerosolized Streptococcus pneumoniae (ATCC 6303; Rockville, MD, USA) using a trans–oral miniature nebulizer under light anesthesia with isoflurane 3%. Controls received saline. Supplemental fluid (10 ml/kg Ringers Lactate) was given intraperitoneally 16 hours after inoculation.

Anesthesia, instrumentation and induction of hypothermia
After 40 hours, anesthesia was induced by an intraperitoneal injection of an anesthesia mix (200μL/100g body weight) containing 90 mg/kg ketamine (Eurovet Animal Health B.V., Bladel, the Netherlands), 0.5 mg/kg medetomidine (Pfizer Animal Health B.V., Capelle a/d IJssel, the Netherlands) and 0.05 mg/kg atropine (Pharmachemie, Haarlem, the Netherlands). Anesthesia was maintained by infusion of 90 mg/kg ketamine at 0.5 ml/100g/hr through a venflon cannula, which was inserted in the tail vein. Tracheotomy was performed, a metal cannula was inserted in the trachea, tied down in situ and connected to a ventilator (Servo 900C, Siemens, Sweden). Rats were pressure controlled ventilated with 13 cmH₂O over 5 cmH₂O positive end–expiratory pressure, an fraction of inspired oxygen (FiO₂) of 50%, an I:E ratio of 1:2 and a respiratory rate of 35 to 50 breaths per minute in respectively healthy or infected rats. Hemodynamic monitoring was done by inserting a polyethylene catheter into the right carotid artery (Braun, Melsungen, Germany) which was connected to a monitor (Siemens SC900, Danvers, USA). Arterial blood gas analysis was performed hourly (Rapidlab 865 blood gas analyzer, Bayern, Mijdrecht, the Netherlands, alphastat).

After baseline measurements, hypothermia (32°C) was induced in infected and non–infected animals using icepacks on the abdomen (n=8 per group). In infected and non–infected controls, normothermia (37°C) was maintained by a thermo mattress. Temperature was monitored rectally (ama–digit ad 15th, Amarell, Kreuzwertheim, Germany).

Bronchoalveolar lavage and measurements
After 4 hours of mechanical ventilation, rats were bled. The lungs were removed en block; the right lung was ligated, followed by bronchoalveolar lavage (BAL) of the left lung (3 x 2.5 ml NaCl). The right lung was used to determine wet weight, as was the right kidney. Cell counts were determined using a hematocytometer (Z2 Coulter Particle Counter, Beckman Coulter Corporation; Hialeah, Florida, USA) in BAL–fluid (BALF). Differential counts were done on
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cytospin preparations stained with Giemsa stain (Dade Behring AG, Dudingen, Switzerland). CINC–3, IL1β, IL–6, TNF–α and IL10 levels were measured by ELISA according to instructions from the manufacturer (R&D Systems; Abingdon, United Kingdom), as were protein levels in BALF and urine (Oz Biosciences, Marseille, France). Creatinine was measured in blood samples using standard techniques. Glomerular filtration rate was calculated by dividing the product of urine creatinine level and urine volume by the plasma creatinine level. The left lung was fixed in 4% buffered formaldehyde and subsequently embedded in paraffin. Hematoxylin and eosin–stained lung sections were analyzed by a pathologist who was blinded to group identity. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following variables: interstitial inflammation, endothelialitis, bronchitis, edema, pleuritis, and thrombus formation, as described previously (23). Each variable was graded on a scale of 0 to 4 (0, absent; 1, mild; 2, moderate; 3, severe; 4, very severe). The total histopathology score was expressed as the sum of the scores for all variables.

**Bacterial growth**

Undiluted blood (100 μL) and serial 10–fold dilution of BALF, homogenized lung, liver and spleen were plated on blood agar plates and incubated overnight at 37°C with 5% CO₂. The number of cfu’s was counted the next day.

**Mitochondrial isolation**

Mitochondria were isolated from the liver and right calf muscle (gastrocnemius and soleus muscle) by a differential centrifugation technique. Approximately ¼ of the liver or gastrocnemius muscle was excised and submerged in isolation buffer (200 mmol L–1 mannitol, 50 mmol L–1 sucrose, 5 mmol L–1 KH2PO4, 5 mmol L–1 3–(n–morpholino) propanesulfonic acid (MOPS), 1 mmol L–1 Ethylene glycol–bis (2–aminoethylether)–N,N,N′,N′– tetraacetic acid (EGTA), 0.1% bovine serum albumin (BSA), pH 7.15 adjusted with KOH) and minced into small pieces. In muscle samples, protease (Sigma–Aldrich, Steinheim, Germany) was added. The suspension was washed twice, homogenized in isolation buffer and centrifuged at 3220 g for 10 min. The pellet was resuspended in isolation buffer and centrifuged at 800 g for 10 minutes. The supernatant was centrifuged at 3220 g for 10 min. The final pellet was suspended in isolation buffer and kept on ice. All isolation steps were conducted at 4°C. Protein content was determined by the Bradford method (Bio–Rad, München, Germany).

**Mitochondrial Oxygen consumption**

Mitochondria (2 mg protein/ml) were suspended in respiration buffer (130 mmol/L KCl, 5 mmol/L K₂HPO₄, 20 mmol/L MOPS, 2.5 mmol/L EGTA, 1 μmol/ L Na₄P₂O₇, 0.1% BSA, pH 7.15 adjusted with KOH). Mitochondrial respiration was initiated by administration of complex 1 enhancers glutamate and malate (both 20mM, Aldrich, Steinheim, Germany) in a respiration
chamber (System S 200A, Strathkelvin Instruments, Glasgow, Scotland). Exactly 60 seconds later, state 3 respiration was initiated by 200 μmol/L adenosine–diphosphate (ADP) injected into the respiration chamber. Respiration rates were recorded polarographically at 37°C under state 3 conditions and after complete phosphorylation of ADP to adenosine–triphosphate (ATP) (state 4 respiration). The respiratory control ratio (RCR, state 3 / state 4) was calculated as a parameter of mitochondrial coupling between respiration and oxidative phosphorylation.

ATP measurements
Samples of liver and the left calf muscle were taken using a freeze–clamp, snap–frozen and stored at −80°C. For nucleotide extraction, samples were grinded in liquid nitrogen using a ceramic mortar. Before the nitrogen liquid had evaporated, the semi–viscous tissue powder was added to 200 μl 0.4 M perchloric acid (HClO₄), stirred, placed on ice for 10 minutes and centrifuged at 10000 g for 10 minutes, at 4°C. The supernatant was saved and used for HPLC analysis. The pellet containing total protein was dissolved in 1000 µl 0.2 M NaOH and the protein content was measured with a copper–reduction method using bicinchoninic acid assay (Pierce, Rockford, USA).

Nucleotide profiles were determined by ion–exchange, using Whatman Partisphere SAX 4.6 x 125 mm column (5 μm particles) in combination with a Whatman 10 x 2.5 mm AX guard column (Clifton, NJ, USA). The buffers used were 9 mM NH₄H₂PO₄, pH 3.5 (buffer A) and 325 mM NH₄H₂PO₄, 500 mM KCL, pH 4.4 (buffer B). Nucleotides were eluted with a gradient from 100% buffer A to 90% buffer B in a total run time of 60 min, at a flow–rate of 1 ml/min. The concentration of ATP and ADP were corrected for protein concentrations.

Statistical analysis
Data are presented as mean ± SD or median [IQR] according to distribution. To test groups Student’s t–test or analysis of variance (ANOVA) and Bonferroni’s post–hoc test will be used. If continuous data is not normally distributed a Kruskal–Wallis test will be used, followed by a Mann–Whitney U test. Categorical variables will be compared with the Chi–square test. A p value of < 0.05 was considered significant. Statistical analyses were carried out using GraphPad Prism version 5 (GraphPad Sofware inc.).
Results

*Inoculation with S. pneumoniae resulted in severe pneumonia and inflammation*

Pneumonia was demonstrated by an increased respiration rate, macroscopic lung infiltrates and bacterial growth in lungs and BALF (figure 1). Of the infected animals, 25% had positive blood cultures at the start of mechanical ventilation. All animals survived mechanical ventilation of 4 hours. Induced mild hypothermia resulted in a drop in heart rate in infected and healthy animals. Mean arterial pressure remained between 100 and 130 mmHg throughout the experiment and did not differ between groups. Acid–base balance remained within normal limits in all groups (table). Pneumonia resulted in a decrease in arterial oxygen tension.

![Figure 1: Bacterial growth in rats infected with S. pneumoniae, with induced hypothermia (P32°C) or normothermia (P). Dots represent the number of colony forming units (cfu's) in bronchoalveolar lavage fluid (BALF) (A), lung (B), spleen (C) and liver tissue (D). Horizontal bars are means; (A) and (B) are log scales. *: P<0.05).](image-url)
Pneumonia induced pulmonary vascular leakage, indicated by an increase in BALF protein levels and lung wet weight compared to non–infected controls (figure 2), together with an increase in pulmonary cell influx and a trend for increased neutrophil influx. Pneumonia was associated with increased levels of IL–1β, IL–6 and TNFα in BALF; levels of CINC–3 were also higher with pneumonia as compared to controls, although differences did not reach statistical significance (figure 3). Pneumonia resulted also in significantly increased lung injury scores (figure 4). In plasma, levels of IL–6 were increased compared to controls (figure 5).

Pneumonia increased protein levels in urine and kidney wet weight. Plasma creatinine was not significantly affected (figure 6).

**Table:** Arterial blood analysis at baseline and after 4 hours of mechanical ventilation in both hypothermia (32°C) and in normothermia (37°C), in animals infected with *S. pneumoniae* and healthy controls.

<table>
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<tr>
<th></th>
<th>Healthy 37°C</th>
<th>Healthy 32°C</th>
<th>Pneumonia 37°C</th>
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<td>7.45±0.06</td>
<td>7.35±0.06†</td>
<td>7.44±0.08†</td>
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</table>

Data are mean ± SD. *: Healthy (37°C) vs. Healthy (32°C); †: Pneumonia (37°C) vs. Pneumonia (32°C) and ‡: Healthy (37°C) vs. Pneumonia (37°C).
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Effects of induced mild hypothermia on bacterial growth

Induced mild hypothermia was not associated with a change in bacterial growth from BALF or lung tissue when compared to normothermic controls (figure 1). Mild hypothermia was associated with fewer rats with positive blood cultures compared to normothermic rats (50 vs. 38%), albeit not reaching statistical significance. Mild hypothermia reduced bacterial dissemination to the spleen and tended to reduce dissemination to the liver compared to normothermic controls (figure 1).

Effects of induced mild hypothermia on organ injury

Hypothermia reduced BALF protein levels and cell count (figure 2) and tended to reduce the amount of neutrophils during pneumonia (figure 2). No differences were seen in lung wet weights between groups. Hypothermia reduced BALF IL–1β levels, but not levels of IL–6, CINC–3 and TNF–α. Induced hypothermia showed a trend to lower urine protein levels, but this was not statistically significant. Kidney wet weight was not affected by hypothermia.

Figure 2: Lung wet weight (A), cell count in bronchoalveolar lavage fluid (BALF), total protein in BALF (C) and BALF neutrophil influx (D) in rats infected with S. pneumoniae (P) and healthy controls (H), with induced hypothermia (32°C) or normothermia (37°C). Data are MEAN ± SD. *: P<0.05; **: P<0.01; ***: P<0.001.
Plasma creatinine level was reduced by hypothermia (figure 6), with unchanged glomerular filtration rates compared to normothermic controls.

Figure 3: CINC–3 (A), IL1β (B), IL–6 (C) and TNFα (D) levels in bronchoalveolar lavage fluid (BALF) of rats infected with *S. pneumoniae* (P) and healthy controls (H), with induced hypothermia (32°C) or normothermia (37°C). Data are MEAN ± SD. *: P<0.05; **: P<0.01; ***: P<0.0001.

**Effects of induced mild hypothermia on mitochondrial respiration and ATP availability during pneumonia**

During pneumonia, liver mitochondrial oxygen consumption, as measured by state 3 respiration, was markedly decreased compared to healthy controls, whereas state 4 respiration and respiration control index (RCI) were unaltered (figure 7). The decrease in oxygen consumption was accompanied by a decrease in the amount of ATP in the liver, while ATP/ADP ratio remained low (figure 8). Induced mild hypothermia reversed the fall in oxygen consumption of liver mitochondria observed during pneumosepsis, without an effect on state 4 or RCI (figure 7), with a concomitant reversal of the fall in ATP content in liver, while ATP/ADP ratio remained low during induced hypothermia (figure 8).

In muscle, pneumosepsis decreased mitochondrial state 3 respiration, comparable to the effect on liver mitochondria, but this was accompanied by an increase in ATP levels.
and ATP/ADP ratio compared to healthy controls, suggesting increased ATP availability, but no conversion to ADP. This may not have been due to uncoupling of mitochondria, as pneumonia did not affect RCI. Induced mild hypothermia further increased muscle ATP levels in all groups. In pneumosepsis, high ATP levels were accompanied by a decrease in ATP/ADP ratio, suggesting increased ATP availability and ATP conversion to ADP. This was not observed in muscle from healthy controls.

Figure 4: Lung Injury Score in the lung and histopathology pictures of rats infected with *S. pneumoniae* (P) and healthy controls (H), with induced hypothermia (32°C) or normothermia (37°C). Horizontal bars are means. **: P<0.01.

Figure 5: IL1β (A) and IL–6 (B) levels in plasma of rats infected with *S. pneumoniae* (P) and healthy controls (H), with induced hypothermia (32°C) or normothermia (37°C). Data are MEAN ± SD. *: P<0.05.
Figure 6: Levels of protein in urine (A), kidney wet weight (B), plasma creatinine (C) and glomerular filtration rate (D) of rats infected with *S. pneumoniae* (P) and healthy controls (H), with induced hypothermia (32°C) or normothermia (37°C). Data are MEAN ± SD. *: P<0.05.
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Figure 7: Mitochondrial state 3, state 4 and respiratory control index (RCI, calculated as the ratio between state 3 and state 4) during oxidative phosphorylation of mitochondrial complex I substrate glutamate/malate in rats infected with *S. pneumoniae* (P) and healthy controls (H), with induced hypothermia (32°C) or normothermia (37°C). Upper graphs A, B and C: liver mitochondria. Lower graphs D, E and F: muscle mitochondria. Horizontal bars are means. *: P<0.05; **: P<0.01; ***: P<0.0001.
Discussion

In this model of pneumococcal pneumonia in rats, induced mild hypothermia reduced bacterial dissemination and parameters of lung injury, possibly by preserving mitochondrial oxygen consumption, thereby improving ATP availability and maintaining ATP conversion to ADP. Hypothermia had a modest effect on kidney function.

We used an infectious model to study effects of hypothermia, which is relevant for the ICU, as hypothermia is induced for prolonged time periods in mechanically ventilated survivors of a cardiac arrest. These critically ill patients frequently have infectious complications, e.g. pneumonia due to aspiration or due to exogenous infections (24).

The effect of hypothermia on host response to infections is not known. It is generally feared that hypothermia may increase the risk of acquiring or aggravating infections, due to an impairment of the immune system (17). Although most studies do not show an increase in infections (8;11;25), prolonged hypothermia (>24h) was found to be associated with an increase in inflammatory parameters(17;26) as well as with an increase in infections (11;17;27). We found however that hypothermia does not increase local bacterial growth. Induced mild hypothermia even reduced bacterial dissemination to the spleen. Although statistical significance was not noted for dissemination to other organs, the consistency of findings may suggest a trend for local bacterial containment.

The mechanism behind these results could be prevention of translocation via the pulmonary capillaries due to a decrease in endothelial cell damage or pulmonary vascular permeability. Alternatively, bacterial replication may be reduced during hypothermia. Our results suggest that short term hypothermia does not aggravate severe infection. It can not be excluded however, that bacterial growth may show a rebound after discontinuation of hypothermia as only a short period of hypothermia was studied. Thereby, results should be interpreted within the limits of our short–term model.

Hypothermia reduced pulmonary inflammation during pneumonia, which was associated with less cell influx and reduced protein leakage into the bronchoalveolar compartment. These findings are in line with studies of the effect of induced hypothermia in sterile models of acute lung injury due to smoke inhalation or endotoxemia, in which it was found that hypothermia reduced neutrophil–influx, delayed the pro–inflammatory response and may reduce neutrophil–mediated endothelial damage and prevent leakage (12–14). Of note, hypothermia also reduced cytokine levels in healthy animals, suggesting that mechanical ventilation may have contributed to an inflammatory reaction. In our study, induced hypothermia may reduce interstitial kidney tissue damage, as our results show a trend towards less protein leakage. Thereby, glomerular filtration rate may have improved (28), consistent with a reduction in plasma creatinine levels. Of note, not all parameters of organ injury were affected. Thereby, results suggest, but do not prove, that hypothermia protects
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against sepsis–induced organ failure. Unfortunately, survival experiments which may have underlined a protective effect of hypothermia were not feasible in this lethal model. Whether prolonged courses of hypothermia would confer protection more clearly remains to be established, as is the optimal cooling temperature by which protection is established with minimal adverse effects.

The mechanism of the observed effects of hypothermia may be a change in mitochondrial function. Most studies report a drop in mitochondrial oxygen consumption during sepsis (3;6;29), consistent with our findings (3). There are two contrasting propositions about decreased mitochondrial function. The first hypothesis is that mitochondrial ‘shutdown’ may be an adaptive response. In this view, hypothermia may result in decreased ATP demands, thereby preventing the levels of ATP from falling below a crucial threshold. In favour of this view, hypothermia reduced pCO₂ levels in our study, with lower ATP/ADP ratios, which may imply a lower rate of ATP turnover. The second hypothesis suggests that decreased respiration and low energetic status is due to inflammatory damage to the mitochondria. In our study, hypothermia increased oxygen consumption, with concomitant increased ATP levels, while ATP/ADP ratio was maintained or even decreased, indicative of a higher conversion of ATP into ADP, whereas uncoupling of respiration to ATP production may be less likely (30).

We suggest that hypothermia is not associated with reduced ATP demand in this study, but may have limited energetic failure by increasing ATP availability, which does not support the theory of an adaptive response but rather may be the result of less mitochondrial damage. Of note, the centrifugation technique to isolate mitochondria may have favoured isolation of intact mitochondria while excluding damaged mitochondria, which may have contributed to lower ATP ratios in pneumonia. However, slow centrifugation speed can not account for the differences between hypo– and normothermia in the diseased animals.

Mitochondria from a central (liver) and distant (muscle) organ showed differential results. In muscle, we found an increase in ATP levels during pneumonia. This is in contrast with a previous study in sepsis patients in which a decrease in ATP in skeletal muscle was observed (7). A conceivable explanation might be a generally very high concentration of ATP in rat muscle cells during inflammation (31). Hypothermia resulted in high ATP levels in the muscle, in contrast to the effect on liver mitochondria. This might suggest that mitochondrial function is differentially regulated in various organs and even in different muscles. Of note, ATP measurements in this study do not distinguish between extracellular ATP from damaged mitochondria and cytosolic ATP. However, during infection, the effect of hypothermia was the same in liver as in muscle mitochondria, resulting in an increase in ATP and low ATP/ADP ratio.
Figure 8: ATP levels (A) and ATP/ADP ratio (B) in the liver of rats infected with *S. pneumoniae* (P) and healthy controls (H), with induced hypothermia (32°C) or normothermia (37°C). ATP levels (C) and ATP/ADP ratio (D) in the muscle of rats infected with *S. pneumoniae* (P) and healthy controls (H), with induced hypothermia (32°C) or normothermia (37°C). Horizontal bars are means. *: P<0.05; **: P<0.01; ***: P<0.0001.

**Conclusion**

Induced mild hypothermia reduced bacterial dissemination in a rat model of pneumococcal pneumosepsis and reduced lung injury, with a concomitant reversal of a fall in ATP levels and preserved mitochondrial oxidative phosphorylation. Results are encouraging in further exploring the possibilities of induced hypothermia as a strategy to control sepsis–related organ injury.
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References


Chapter VI


