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Chapter 7

AMP activated protein kinase activation by AICAR reduces lipoteichoic acid induced lung inflammation

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Abstract

Background:
Adenosine monophosphate-activated protein (AMP) activated kinase (AMPK) is a highly conserved kinase that plays a key role in energy homeostasis. Activation of AMPK was shown to reduce inflammation in response to lipopolysaccharide in vitro and in vivo. 5-aminoimidazole-4-carbox-amide-1-β-D-ribofuranoside (AICAR) is intracellularly converted to the AMP analog ZMP, which activates AMPK. Lipoteichoic acid (LTA) is a major component of the cell wall of gram-positive bacteria that can trigger inflammatory responses. In contrast to lipopolysaccharide, little is known on effects of AMPK activation in LTA triggered innate immune responses.

Aim:
Here, we studied the potency of AMPK activation to reduce LTA-induced inflammation in vitro and in lungs in vivo.

Results:
Activation of AMPK in vitro reduced cytokine production in the alveolar macrophage cell line MH-S. In vivo, AMPK activation reduced LTA-induced neutrophil influx, as well as protein leak and cytokine/chemokine levels in the bronchoalveolar space.

Conclusion:
AMPK activation inhibits LTA-induced lung inflammation in mice.
Introduction

Gram-positive bacteria are a frequent cause of pneumonia, among which *Staphylococcus (S.) aureus* represents a serious and emerging threat [1]. Pneumonia is a leading cause of mortality in the United States [2]. The abundant gram-positive cell wall component lipoteichoic acid (LTA) is the predominant driving force of the host inflammatory response to this type of bacteria [3,4,5].

Adenosine monophosphate-activated protein (AMP) activated kinase (AMPK) is a highly conserved kinase that classically is known for its key role in energy homeostasis. AMPK can be activated by AMP, serine/threonine kinase 11 (LKB1) and calmoduline-dependent protein kinase kinase (CaMKK) [6,7]. Activated AMPK has a strong influence on metabolic processes, by virtue of its capacity to induce glucose uptake, glycolysis, fatty acid oxidation and mitochondrial biogenesis, and to inhibit fatty acid/cholesterol synthesis, gluconeogenesis, and glycogen and protein synthesis [6]. Apart from these metabolic effector functions, AMPK signaling was recently shown to influence the inflammatory response: activation of AMPK was reported to have anti-inflammatory properties both *in vitro* and *in vivo* in response to lipopolysaccharide (LPS)-induced lung inflammation [8]. Two well-known small molecular kinase activators can modulate AMPK signaling: metformin and 5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) [9]. Metformin, a well-known drug used in patients suffering from type 2 diabetes, activates AMPK by shifting the AMP:ATP ratio. AICAR is converted intracellularly to ZMP, an AMP analogue, thus activating AMPK [9].

Here, we studied the potency of AMPK activation by AICAR to reduce inflammation *in vitro* and *in vivo* in a model of LTA-induced lung injury. Activation of AMPK *in vitro* reduced cytokine production in an alveolar macrophage cell line and gave rise to a slight enhancement of cytokine-induced neutrophil chemoattractant (KC) and interleukin (IL)-6 production in MLE-12 lung epithelial cells. *In vivo*, we validated AMPK activation through enhanced phosphorylation of Acetyl-CoA carboxylase (ACC). This increased AMPK activity was accompanied with reduced cell influx and inflammatory mediator release in the pulmonary compartment. With this study we demonstrated the potency of AMPK activation to diminish inflammatory responses in LTA-induced lung inflammation *in vivo*.

Material and methods

Cell line experiments

The effect of AICAR on cytokine responses of resident macrophages and lung epithelium was tested as follows: 1x10^5 MH-S (alveolar macrophage cell line; American Type Culture Collection, Rockville, MD) and MLE-12 (mouse lung epithelial cells; kindly provided by Jeffrey Whitsett, Division of Pulmonary Biology, Department of Pediatrics, Cincinnati Children’s Hospital Medical Center and the University of Cincinnati College of Medicine, Cincinnati, Ohio) cells were seeded in a 24-well plate (10^5 cells per well) (Millipore, Amsterdam, the Netherlands). After 24 hours of culture, cells were stimulated with 10 μg/ml LTA (purified...
AICAR reduces lipoteichoic acid induced lung inflammation from *S. aureus*; endotoxin level: <1.25 EU/mg; Invivogen, San Diego, CA). Simultaneously, cells were treated with 1 mM AICAR or vehicle (PBS). At 4 and 8 hours, supernatant was harvested for ELISA. Cell viability was assessed by adding MTT ([3-(4,5-methylthiazol-2-yl)-2,5-dipheyl-tetrazolium bromide]; Sigma-Aldrich) reagent to all wells for 60 minutes. Supernatant was discarded and the cells were lysed in acidic isopropanol (Merck, Darmstadt, Germany). Absorbance was measured at 570 nm.

**Mice**

For all experiments female C57BL/6 mice (aged 10-11 weeks) were purchased from Charles River (Maastricht, The Netherlands). The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

**Induction of lung inflammation and AICAR administration**

Acute lung inflammation was induced as described previously [4,10]. Briefly, mice were anesthetized with isoflurane (Upjohn) and 100 μg LTA (Invivogen) diluted in 50 μl of sterile saline was instilled intranasally. 500 mg/kg of AICAR in 200 μl saline or 200 μl saline (vehicle) was administered intraperitoneally at the start of the experiment. After 6 and 24 hours mice were anesthetized with Domitor (Pfizer Animal Health Care, Capelle aan der IJssel, The Netherlands: active ingredient medetomidine) and Nimatek (Eurovet Animal Health, Bladel, The Netherlands, active ingredient ketamine) and sacrificed by cardiac puncture followed by cervical dislocation.

**Bronchoalveolar lavage**

Through a midline incision the trachea and lungs were exposed; the right lung was isolated from the airways via a suture. The trachea was cannulated with a 22G Abbocath-T catheter (Abbott, Sligo, Ireland) and the left lung was instilled with two times 0.4 ml sterile PBS. The fluid was retrieved, weighed and total cell counts were determined with a Coulter cell counter (Beckman Coulter, Fullerton, CA). Differential cell counts were determined by counting 100 cells on Giemsa stained cytospin preparations. BAL was centrifuged at 1500 rpm for 10 minutes at 4 °C, supernatant was stored at -20 °C until assays were performed, the remaining cell pellet was used for western blot analysis.

**Assays**

IL-1β, IL-6, tumor necrosis factor α (TNF-α), KC, macrophage inflammatory protein (MIP)-2 and soluble receptor for advanced glycation (RAGE) were measured using ELISAs (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Detection limits were: TNF-α: 62.5 pg/ml, 31.3 pg/ml for IL-6, 32.9 pg/ml for MIP-2, 11 pg/ml for KC and 250 pg/ml for soluble RAGE. Total protein concentrations were measured using a DC protein assay (Bio-Rad Laboratories, Veenendaal, The Netherlands).

**Western blotting**

Samples for western blotting were boiled at 95 °C for 5 minutes in Laemmli buffer and loaded onto SDS-PAGE gels. After electrophoresis the content of the gels was transferred onto Immobilon-PVDF membranes (Millipore, Billerica, MA). The membranes were
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blocked in 5% BSA (Roche, Basel, Switzerland) in TBS-T at room temperature for 60 minutes. Phosphorylated ACC (Cell signalling Technology, Boston, MA) antibodies were diluted 1:500; β-actin (Santacruz Biotechnology, Santa cruz, CA) was diluted 1:4000. The membranes were incubated overnight at 4 °C. Next, the membranes were incubated for 60 minutes with anti-rabbit-HRP conjugated secondary antibody (Cell signalling Technology) and blots were imaged using LumiLight Plus ECL (Roche, Basel, Switzerland) on a LAS 4000 chemiluminescence imager (GE Healthcare Biosciences, Pittsburgh, PA, USA). Quantification was performed using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, http://rsb.info.nih.gov/ij/, 1997-2009).

Statistical analysis
Data are expressed as mean ± SEM. In vitro analysis was performed by ANOVA. For in vivo data, two sample comparisons were performed by Mann Whitney U tests using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). Comparisons between multiple groups were done using Kruskall-Wallis test; if overall significant individual groups where assessed by Mann Whitney-U tests. P < 0.05 was considered to be statistically significant.

Results

AICAR has dual effects on cytokine and chemokine production in vitro
AMPK activation through AICAR pretreatment was previously shown to reduce TNF-α production in LPS stimulated RAW264.7 murine macrophages [11]. We tested the anti-inflammatory property of AICAR in response to LTA stimulation in two typical pulmonary cell lines: MH-S (alveolar macrophages) and MLE-12 (respiratory epithelial cells). Cells were stimulated with 10 μg/ml LTA and cytokine production was measured after 0, 4, 8 and 24 hours.

In MH-S cells treated with AICAR, TNF-α and IL-6 production was strongly reduced throughout the 24 hours of stimulation (P<0.001, figure 1A and B). In MLE-12 cells activation of AMPK resulted in a statically significant enhancement of KC (P<0.05, figure 1C) and IL-6 levels (P<0.001, figure 1D) production. Cell viability was not altered by AICAR treatments (data not shown).

AICAR enhances ACC phosphorylation in vivo
Next we set out to assess the inflammatory effects of treatment with AICAR in LTA-induced pulmonary inflammation. Inflammation was induced by intranasal instillation of 100 µg LTA [4,10]. Simultaneously, 500 mg/kg of AICAR in 200 µl saline or 200 µl saline (vehicle) was administered intraperitoneally. Cells present in the airways were obtained through bronchoalveolar lavage (BAL). In the cell fraction of BAL fluid, AMPK activation was determined through measuring the phosphorylation level of ACC, a downstream substrate of AMPK [8]. ACIAR treatment enhanced the levels of phosphorylated ACC statistically significantly at 6 hours (P<0.05) compared to vehicle. After 24 hours this effect of AICAR treatment was no longer present and pACC levels were back to baseline (figure 2A and B).
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Treatment with AICAR resulted in reduced cellular counts in BAL fluid 6 hours after LTA administration (P<0.05, figure 3A). Cellular differentiation showed that this reduction was based on a reduction of polymorphonuclear cell (PMNs) (P<0.01, figure 3C) and lymphocyte (P<0.01, figure 3C) numbers, while macrophage counts were not influenced by AICAR. At 24 hours no differences in BAL fluid cellular composition were present between treatment and vehicle groups.

Figure 1: AICAR reduces macrophage cytokine but increases epithelial cell KC and IL-6 production in vitro
The effect of AICAR on cytokine responses of MH-S (alveolar macrophage cell line) (A:TNF-α, B: IL-6) and KC production in MLE-12 (C:KC, D: IL-6) (mouse lung epithelial cell line) was assessed. Cells were stimulated with 10 μg/ml S. aureus LTA and simultaneously treated with 1 mM AICAR (closed symbols) or vehicle (open symbols) for 0, 4, 8 and 24 hours. Data are representative for 2 independent experiments and expressed as mean ± SEM, N=4 per treatment. * P<0.05, *** P<0.001.
As a measure of vascular leak, total protein levels were determined in BAL fluid. In the ACIAR treated group protein levels were lowered by 45% relative to vehicle controls after 6 hours (table 1, P<0.01). At 24 hours, protein levels in BAL fluid were similar in both treatment groups. To further assess lung damage we measured soluble RAGE in BAL fluid. Soluble RAGE was shown to be a marker of lung epithelial injury based on animal studies in rats and mice of lung injury and clinical measurements of acute lung injury patients [12,13,14]. Soluble RAGE levels were high at 6 hours, decreasing thereafter; no differences were detected between AICAR and vehicle treatment.

**Table 1: AICAR reduces protein content in bronchoalveolar lavage fluid**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein BAL Fluid</th>
<th>Soluble RAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham³</td>
<td>0.35 ± 0.03</td>
<td>0.49 ± 0.10</td>
</tr>
<tr>
<td>6 hours</td>
<td>1.44 ± 0.18</td>
<td>5.69 ± 0.78</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.64 ± 0.08 **</td>
<td>4.05 ± 0.90</td>
</tr>
<tr>
<td>ACIAR</td>
<td>0.91 ± 0.09</td>
<td>2.67 ± 0.67</td>
</tr>
<tr>
<td>24 hours</td>
<td>1.01 ± 0.13</td>
<td>2.52 ± 0.60</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.91 ± 0.09</td>
<td>2.67 ± 0.67</td>
</tr>
<tr>
<td>ACIAR</td>
<td>1.01 ± 0.13</td>
<td>2.52 ± 0.60</td>
</tr>
</tbody>
</table>

¹Lung inflammation was induced by intranasal instillation of 100 μg LTA. Simultaneously 500 mg/kg AICAR or vehicle (saline) was administered intraperitoneally. After 6 and 24 hours samples were harvested. ²Total protein (mg/ml) and ³Soluble RAGE (ng/ml) levels were measured in BAL fluid. ⁴Sham mice reference values. Data are expressed as mean ± SEM. N=8. ** P<0.01 vs. vehicle.

**Figure 2: AICAR enhances ACC phosphorylation in BAL cells**

Acute lung inflammation was induced by instillation 100 μg LTA intranasally. 500 mg/kg AICAR or vehicle (saline) was administered simultaneously intraperitoneally. After 6 and 24 hours mice were sacrificed. The cell fraction obtained from pelleted BAL fluid was lysed and pACC levels were determined by western blot. For sham reference naïve mouse samples used. Representative western blot is shown (A). The bar graphs (B) represent densitometric quantification of the relative amounts of pACC of 4-8 mice per group normalized for β-actin. Graph data are expressed as mean ± SEM. * P<0.05 vs. vehicle.
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Relative to vehicle controls, AICAR treated mice displayed strongly reduced lung TNF-α levels at 6 hours after LTA instillation (P<0.01, figure 4A). At 24 hours pulmonary TNF-α levels had decreased substantially in both groups but were still significantly lower in AICAR administered animals (P<0.05). Similar differences between groups were found for MIP-2 (Figure 4D). AICAR also diminished pulmonary KC (P<0.05, figure 4C) and reduced IL-6 (non-significant, figure 4B) levels at 6 hours after LTA instillation.

**Figure 3:** AICAR treatment reduces cell influx into the bronchoalveolar space

Lung inflammation was induced by intranasal instillation of 100 μg LTA. Simultaneously 500 mg/kg AICAR or vehicle (saline) was administered intraperitoneally. After 6 and 24 hours samples were harvested. For sham reference naïve mouse samples used. Total cell count of BAL fluid (A). Macrophage (B), PMN (C) and lymphocyte (D) counts were determined by counting Giemsa stained cytospin preparations from BAL fluid. Data are expressed as mean ± SEM, N=8 per group, ** P<0.01
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**Discussion**

A potent pulmonary inflammatory response is crucial to host defense against invading pathogens. However, un-adapted inflammation can be detrimental to the outcome of infection [15]. The gram-positive cell wall component LTA is a major contributor to the inflammation triggered by gram-positive bacteria, such as *S. aureus*, an emerging pathogen in pulmonary infections [1,3].

Here, we studied the effects of AMPK activation by AICAR on pulmonary inflammation
induced by administration of LTA via the airways. AICAR was previously shown to reduce LPS-induced lung inflammation [8]. We show that AMPK activation by AICAR diminished cellular influx into the bronchoalveolar space as well as protein leak and inflammatory mediator production at the early phase of LTA elicited lung inflammation.

Previously, ACIAR pretreatment (4 hours) was shown to inhibit TNF-α production in RAW264.7 murine macrophages in response to LPS [11]. Moreover, silencing of AMPK in murine bone marrow derived macrophages (BMDMs) resulted in enhanced TNF-α, IL-6 and cyclooxygenase-2 mRNA levels in response to LPS stimulation [16]. In accordance, transfection of a constitutively active form of AMPK decreased TNF-α and IL-6 production in LPS stimulated BMDMs [16]. We assessed the impact of direct (no pretreatment) AICAR treatment on cell lines with relevance for the cell types that initiate lung inflammation upon LTA instillation via the airways. We observed a strong reduction in TNF-α and IL-6 levels in alveolar macrophages (MH-S) due to AICAR treatment. This is in accordance with the previously described results on RAW264.7 cells and bone marrow derived macrophages [11]. However, in the epithelial MLE-12 cell line AICAR increased KC and IL-6 production; these cells do not produce TNF-α. Recently, in human primary bronchial epithelial cells, transfection of constitutively active AMPK resulted in reduced mRNA levels of monocyte chemotactic protein-1 in response to combined TNF-α and interferon-γ stimulation [17]. The discrepancy with our data might be due to differences between the bronchial epithelium and distal lung epithelium (from which MLE-12 cells were derived [18]).

Our in vivo results are based on applying AICAR treatment concurrently with LTA administration. Other studies applied AICAR in several different models of inflammation. In murine lung inflammation, 500 mg/kg AICAR, given 4 hours prior to intratracheal administration of 1 mg/kg LPS, reduced neutrophil accumulation and TNF-α and IL-6 protein levels [8]. In an OVA and poly I:C based asthmatic exacerbation model, repeated treatment with AICAR (3x100 mg/kg) lowered macrophage and eosinophil influx as well as BAL fluid protein levels of IL-5, IL-13 and TNF-α [19]. Moreover, intravenous infusion of AICAR (0.2 mg/kg/minute) reduced BAL fluid protein levels, edema scores and improved survival in porcine mechanical ventilation chest trauma [20]. Apart from usage in lung inflammation models, AICAR was also applied in murine trinitrobenzene sulphonic acid induced colitis. In this model, daily administration of 500 mg/kg of AICAR reduced bodyweight loss and TNF-α, IFN-γ, IL-17 levels, and partially prevented colon length shortening [21]. Although diverse in experimental settings, inflammation types and dosing strategies, all these studies described anti-inflammatory effects of AICAR treatment and AMPK activation. The anti-inflammatory effects of AICAR reported here in LTA-induced lung inflammation are in accordance with these earlier studies. In addition, we show that pre-treatment is not necessary for AICAR to exert its effects.

Different pro-inflammatory (i.e. LPS, LTA, poly I:C) stimuli have been used to assess the effects of AMPK activation in inflammation, each acting on different Toll-like receptors (TLR). LTA signals via TLR2, with Myeloid differentiation primary response gene 88 (MyD88) mediating downstream signaling [22]. TIR-domain-containing adapter-inducing interferon-β (TRIF) is the adaptor protein for TLR3 (triggered by poly I:C used in [19]). TLR4 stimulation
via LPS (used in [8,11]) acts upon both MyD88 and TRIF [22]. The exact mechanism of AMPK modulation on inflammation is unclear. Given the differences in upstream signaling cascades and similar AICAR induced anti-inflammatory outcomes, it seems that the effect of AICAR and AMPK activation is based on a downstream target of inflammatory signaling cascades. It has been described that AICAR inhibits IκBα degradation and thereby reduces NFκB translocation in LPS stimulated neutrophils [8]. Moreover, AMPK activation may also reduce translocation to the cytosol of (embryonic lethal, abnormal vision, Drosophila)-like 1 (ELAVL1), which regulates translation of proinflammatory gene mRNA’s (e.g. TNF-α, IL-6) by binding the 3’UTR [17]. Another mechanism by which AMPK activation may contribute to inhibition of pulmonary inflammation is by reducing eNOS dependent leukocyte rolling and adhesion to endothelium [23].

In the current study we show that AMPK activation by AICAR treatment in vivo reduced LTA induced murine lung inflammation. We observed reduced pulmonary cell influx and diminished inflammatory mediator production at the early phase of TLR2 dependent lung inflammation (6 hours). Based on the current and previous data, activated AMPK immune inhibition is present in a broad range of inflammatory settings and thus may represent an effective strategy in reducing pulmonary inflammation.

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