AMP-activated Protein Kinase Activation by 5-Aminoimidazole-4-carbox-amide-1-β-D-ribofuranoside (AICAR) Reduces Lipoteichoic Acid-induced Lung Inflammation

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 the effects of AMPK activation in response to LTA.

**Background:** AMPK is a highly conserved energy homeostasis-regulating kinase.

**Results:** Activation of AMPK by AICAR in vitro reduced cytokine production in alveolar macrophage cell line and in vivo reduced LTA-induced neutrophil influx, protein leak and cytokine/chemokine levels.

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

**Significance:** AICAR reduces LTA inflammation.

Gram-positive bacteria are a frequent cause of pneumonia, among which *Staphylococcus aureus* represents a serious and emerging threat (1). Pneumonia is a leading cause of mortality worldwide (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 (3–5).

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2 The abbreviations used are: LTA, lipoteichoic acid; ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carbox-amide-1-β-D-ribofuranoside; AMPK, AMP-activated kinase; BAL, bronchoalveolar lavage; MIP, macrophage inflammatory protein; mTOR, mammalian target of rapamycin; RAGE, receptor for advanced glycation; TLR, Toll-like receptor; KC, keratinocyte chemotactant; ZMP, AICAR monophosphate.

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 calmodulin-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-aminoimidazole-4-carbox-amide-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 analog, 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 independent of mTOR signaling. 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.

**EXPERIMENTAL PROCEDURES**

**Cell Line Experiments**—The effect of AICAR on cytokine responses of resident macrophages and lung epithelium was tested as follows: 1 × 10⁵ MH-S (alveolar macrophage cell line;
American Type Culture Collection) cells were seeded in a 48-well plate (10^5 cells/well) (Millipore). After 24 h of culture, cells were stimulated with 10 μg/ml LTA (purified from S. aureus; endotoxin level: <1.25 enzyme units/mg; Invivogen). Simultaneously, cells were treated with 1 mM AICAR, 100 nM rapamycin, 1 mM AICAR + 100 nM rapamycin or vehicle (0.3% dimethyl sulfoxide/PBS). At 6 and 24 h, supernatant was harvested for ELISA.

Cell lysates were prepared by treating the cells in cell lysis buffer (Cell Signaling Technology). Cell viability was assessed by adding (3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT; Sigma-Aldrich) reagent to all wells for 60 min. Supernatant was discarded, and the cells were lysed in acidic isopropyl alcohol (Merck). Absorbance was measured at 570 nm.

**Results**

Acute lung inflammation was induced as described previously (4, 10). Briefly, mice were anesthetized with isoflurane (Upjohn), and 100 μl of saline (Invivogen) diluted in 50 μl of sterile saline was instilled intranasally. 500 mg/kg AICAR in 200 μl of saline or 200 μl of saline (vehicle) was administered intraperitoneally at the start of the experiment. After 6 and 24 h mice were anesthetized with Domitor (Pfizer Animal Health Care; 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 (BAL)**—Through a midline incision the trachea and lungs were exposed; the right lung was isolated by adding ((3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT; Sigma-Aldrich) reagent to all wells for 60 min. Supernatant was discarded, and the cells were lysed in acidic isopropyl alcohol (Merck). Absorbance was measured at 570 nm.

**RESULTS**

**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 of LTA (4, 10). Simultaneously, 500 mg/kg AICAR in 200 μl of saline or 200 μl of saline (vehicle) was administered intraperitoneally. Cells present in the airways were obtained through BAL. In the cell fraction of BAL fluid, AMPK activation was determined by measuring the phosphorylation level of ACC, a downstream substrate of AMPK (8). AICAR treatment enhanced the levels of phosphorylated ACC (Cell Signaling Technology) antibodies were diluted 1:500; phosphorylated and total p70/S6K, phosphorylated AMPK, total AMPK, and phosphorylated mTOR (all Cell Signaling Technology) were diluted 1:100. β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:4000. The membranes were incubated overnight at 4 °C. Next, the membranes were incubated for 60 min with anti-rabbit-HRP-conjugated secondary antibody (Cell Signaling Technology), and blots were imaged using LumiLight Plus ECL (Roche Applied Science) on a LAS 4000 chemiluminescence imager (GE Healthcare). Quantification was performed using Image (software (National Institutes of Health).

**Statistical Analysis**—Data are expressed as mean ± S.E. In vitro analysis was performed by ANOVA and Bonferroni’s multiple comparison test post tests. For in vivo data, two sample comparisons were performed by Mann-Whitney U tests using Prism version 5.01 (GraphPad Software). Comparisons between multiple groups were done using the Kruskall-Wallis test. Overall significant individual groups were assessed by Mann-Whitney U tests. p < 0.05 was considered to be statistically significant.

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of phosphorylated ACC statistically significantly at 6 h ($p < 0.05$) compared with vehicle. After 24 h this effect of AICAR treatment was no longer present, and $p$ACC levels were back to baseline (Fig. 2, A and B).

**AICAR Reduces LTA-induced Lung Inflammation**—Treatment with AICAR resulted in reduced cellular counts in BAL fluid 6 h after LTA administration ($p < 0.05$, Fig. 3A). Cellular differentiation showed that this reduction was based on a reduction of polymorphonuclear cell ($p < 0.01$, Fig. 3C) and lymphocyte ($p < 0.01$, Fig. 3C) numbers, whereas macrophage counts were not influenced by AICAR. At 24 h no differences in BAL fluid cellular composition were present between treatment and vehicle groups.

As a measure of vascular leak, total protein levels were determined in BAL fluid. In the AICAR-treated group protein levels were lowered by 45% relative to vehicle controls after 6 h (Table 1, $p < 0.01$). At 24 h, 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 (12–14). Soluble RAGE levels were high at 6 h and decreasing thereafter; no differences were detected between AICAR and vehicle treatment.

Relative to vehicle controls, AICAR-treated mice displayed strongly reduced lung TNF-α levels at 6 h after LTA instillation.
AICAR reduces protein content in bronchoalveolar lavage fluid

Lung inflammation was induced by intranasal instillation of 100 μg of LTA. Simultaneously, 500 mg/kg AICAR or vehicle (saline) was administered intraperitoneally. After 6 and 24 h samples were harvested. Total protein and soluble RAGE levels were measured in BAL fluid. Sham mice were used for reference values. Data are expressed as mean ± S.E. (*, p < 0.05; **, p < 0.01).

| Harvest time | Treatment | Protein (mg/ml) | Soluble RAGE (ng/ml) |
|--------------|-----------|----------------|---------------------|
| 6 h          | Vehicle   | 0.35 ± 0.03    | 0.49 ± 0.10         |
|              | AICAR     | 1.44 ± 0.18    | 5.69 ± 0.78         |
| 24 h         | Vehicle   | 0.64 ± 0.08**  | 4.05 ± 0.90         |
|              | AICAR     | 0.91 ± 0.09    | 2.67 ± 0.67         |
|              | Vehicle   | 1.01 ± 0.13    | 2.52 ± 0.60         |
(16). We assessed the impact of direct (no pretreatment) AICAR treatment on an alveolar macrophage cell line. 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). To assess whether the effects of AICAR were dependent on mTOR signaling, we combined AICAR treatment with rapamycin. As AICAR (unlike rapamycin) did not reduce mTOR or p70/S6K phosphorylation and rapamycin did not affect cytokine production, the effects of AICAR seem to be mediated independent of mTOR.

Our observation on the lack of AICAR effects on p70/S6K phosphorylation are in line with previous findings (17). 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 h 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 (3 × 100 mg/kg) lowered macrophage and eosinophil influx as well as BAL fluid protein levels of IL-5, IL-13 and TNF-α (18). Moreover, intravenous infusion of AICAR (0.2 mg/kg/min) reduced BAL fluid protein levels and edema scores and improved survival in porcine mechanical ventilation chest trauma (19). Apart from usage in lung inflammation models, AICAR was also applied in murine trinitrobenzene sulfonic acid-induced colitis. In this model, daily administration of 500 mg/kg AICAR reduced body weight loss and TNF-α, IFN-γ, IL-17 levels, and partially prevented colon length shortening (20). Although diverse in experimental settings, inflammation types and dosing strategies, all of 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 pretreatment 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 (TLRs). LTA signals via TLR2, with myeloid differentiation primary response gene 88 (MyD88) mediating downstream signaling (21). TIR domain-containing adapter-inducing interferon-β (TRIF) is the adaptor protein for TLR3 (triggered by poly(I:C) used in Ref. 18). TLR4 stimulation via LPS (used in Refs. 8, 11) acts upon both MyD88 and TRIF (21). The exact mechanism of AMPK modulation on inflammation is unclear. However, given our in vitro results, the effect of AICAR seems to be driven by AMPK in an mTOR-independent manner.

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 IkBα degradation and thereby reduces NFκB translocation in LPS-stimulated neutrophils (8). Moreover, AMPK activation may also reduce translocation to the cytosol of ELAVL1 (embryonic lethal, abnormal vision, Drosophila)-like 1, which regulates translation of pro-inflammatory gene mRNAs (e.g. TNF-α, IL-6) by binding the 3′-UTR (22). 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 showed 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 h). 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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