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Inhibition of Lipopolysaccharide-Induced Inducible Nitric Oxide Synthase and Cyclooxygenase-2 Gene Expression by 5-Aminoimidazole-4-Carboxamide Riboside Is Independent of AMP-Activated Protein Kinase

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Abstract Recent studies suggest AMP-activated protein kinase (AMPK), an enzyme involved in energy homeostasis, might be a novel signaling pathway in regulating inflammatory response, but the precise intracellular mechanisms are not fully understood. In this study, we have demonstrated that 5-aminoimidazole-4-carboxamide riboside (AICAR), an activator of AMPK, inhibited lipopolysaccharide (LPS)-induced protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in macrophages and microglial cells at the gene transcription level. Data obtained from electrophoretic mobility shift assay (EMSA) and promoter activity assay have further confirmed the ability of AICAR to block LPS-mediated NF-κB, AP-1, CREB, and C/EBPβ activation. However, AICAR did not affect LPS-mediated IKK, ERK, and p38 activation. Regardless of the ability of AICAR to activate AMPK, the inhibitory effects of AICAR on iNOS and COX-2 expression were not associated with AMPK. An adenosine kinase inhibitor 5′-iodotubercidin, which effectively abolished AMPK activation caused by AICAR, did not reverse the anti-inflammatory effect of AICAR. Moreover, another AMPK activator metformin was not able to mimic the effects of AICAR. Direct addition of AICAR in EMSA assay interrupted binding of NF-κB, CREB, and C/EBPβ to specific DNA elements. Taken together, this study demonstrates that the anti-inflammatory effects of AICAR against LPS-induced iNOS and COX-2 gene transcription are not associated with AMPK activation, but might be resulting from the direct interference with DNA binding to transcription factors. J. Cell. Biochem. 103: 931–940, 2008.

Key words: AICAR; AMPK; iNOS; COX-2; NF-κB; macrophages

AMP-activated protein kinase (AMPK) is an energy-sensing enzyme that is activated in response to changes in the cellular energy status, such as muscle contraction, hypoxia/ischemia, and exercise [Hardie, 2003; Carling, 2004]. Following ATP depletion or a rise in the AMP–ATP ratio within the cells, AMPK initiates multiple metabolic processes. Through increasing glucose transport, fatty acid oxidation and glycolysis, AMPK activation inhibits ATP-consuming pathways and stimulates alternative pathways for ATP regeneration [Carling, 2004]. In view of its role in improving glucose homeostasis, insulin sensitivity, and energy metabolism, AMPK has recently emerged as an attractive and novel target for the treatment of obesity, type 2 diabetes and cardiac hypertrophy [Winder, 2000; Carling, 2004].

AMPK exists as a heterotrimeric enzyme, consisting of a catalytic subunit (α) and two regulatory subunits (β and γ) [Kemp et al., 1999]. AMPK activity is absolutely dependent on the phosphorylation at a major activation site (Thr172) of the α-subunit by an upstream protein kinase, AMPK kinase (AMPKK) [Hardie and Carling, 1997]. Studies demonstrate that direct binding of AMP to the γ-subunit of AMPK, alters its susceptibility

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towards phosphorylation by AMPKK [Baron et al., 2005].

5-Aminoimidazole-4-carboxamide riboside (AICAR) is predominantly used as a pharmacological activator of AMPK and its upstream AMPKK. AICAR is cell permeable and after entering the cells, it can be converted into 5-aminoimidazole-4-carboxamide ribonucleoside (AICA ribonucleoside, ZMP) by adenosine kinase. Due to its structural similarity with AMP, ZMP can activate AMPK signaling pathway [Corton et al., 1995; Hardie et al., 1998]. However, as AICAR is likely to mimic many physiological effects of AMP, it is considered as a nonspecific activator of AMPK, and some of its biological responses are not limited to modulation of AMPK per se [Young et al., 1996; Morrow et al., 2003; Jhun et al., 2004].

Besides its crucial role in the regulation of metabolic processes, AMPK is also implicated as an anti-inflammatory target. In endothelial cells, AMPK activity is associated with phosphorylation and activation of endothelial nitric oxide synthase (eNOS), resulting in anti-inflammatory action in vascular wall [Zou et al., 2002]. Moreover, lipopolysaccharide (LPS)-induced expression of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), as well as inducible nitric oxide synthase (iNOS) in primary macrophages, microglia, and astrocytes, were also suppressed by AICAR [Giri et al., 2004; Jhun et al., 2004]. Thus, AICAR has potential to protect tissues from damage induced by sepsis [Fabian et al., 1996]. However, the molecular mechanisms underlying the anti-inflammatory effects of AICAR are still controversial. In macrophages and glial cells, inhibition of LPS-induced NF-κB and c/EBPβ activation leads to the anti-inflammatory property of AICAR [Giri et al., 2004]. In myocytes, adipocytes, and mouse bone marrow-derived macrophages, AMPK-mediated iNOS inhibition primarily results from the post-transcriptional regulation of iNOS protein [Pilon et al., 2004].

In this study, we have chosen RAW264.7, J774 macrophages, and BV-2 microglia as cell models to investigate the effects of AICAR on LPS-mediated iNOS and cyclooxygenase-2 (COX-2) gene expression. We found that AICAR indeed could reduce LPS-induced iNOS and COX-2 gene expression through a transcription mechanism. However, this action is neither related to the AMPK activation, nor to the interference with signaling pathways involving IKK, ERK, and p38. We postulate that the mechanism of anti-inflammatory action mediated by AICAR might involve a direct interruption of transcription factors on DNA binding.

MATERIALS AND METHODS

Cell Culture

Murine macrophage RAW264.7 and J774 cell lines, and BV-2 microglial cells obtained from American Type Culture Collection (Manassas, VA) were grown at 37°C in 5% CO_2 using DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Nitrite and Prostaglandin E_2 (PGE_2) Measurement

Nitrite production was measured in supernatants of RAW264.7 and BV-2 cells as we described earlier [Chen et al., 2003]. PGE_2 production was measured by commercial kits from Cayman Chemical Company according to the manufacturer's instructions.

Immunoblot Analysis

After stimulation, cells were rinsed twice with ice-cold PBS, and cell lysis buffer (20 mM Tris-HCl, pH 7.5, 125 mM NaCl, 1% Triton X-100, 1 mM $MgCl_2$, 25 mM β-glycerophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) was then added to each well. After harvesting cells, cell lysates were centrifuged, and equal protein amounts of the soluble protein, as determined by Bradford protein assay, were denatured, subjected to SDS–PAGE, and transferred to a nitrocellulose membrane. Nonspecific binding was blocked with TBST containing 5% nonfat milk for 1 h at room temperature. After immunoblotting with the first specific antibodies (1:1,000 dilution), membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000 dilution) for 1 h. After three washes with TBST, the protein bands were detected with enhanced chemiluminescence detection reagent (Beverly, MA).

Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Cells were homogenized with RNAzol B reagent; total RNA was extracted by acid

guanidinium thiocyanate–phenol–chloroform extraction; and RT was performed using a StrataScript RT-PCR kit. The oligonucleotide primers used correspond to mouse iNOS (5′-CCC TTC CGA AGT TTC TGG CAG CAG C-3′ and 5′-GGC TGT CAG AGC CTC GTG GCT TTG G-3′), and COX-2 (5′-CAG CAA ATC CTT GCT GTT CC-3′ and 5′-TGG GCA AAG AAT GCA AAC ATC-3′). PCR was performed using an initial step of denaturation (1 min at 94°C), 35 cycles of amplification (94°C for 1 min, 58°C for 30 s, and 72°C for 30 s), and an extension (72°C for 7 min). PCR products were analyzed on 1% agarose gels. The mRNA of β-actin served as the internal control for sample loading.

Transient Transfection and Luciferase Assay

We used reporter assays to assess the promoter activities of iNOS and COX-2 genes, and transcription activities of NF-κB and AP-1. The iNOS reporter plasmid, which contained binding sites for AP-1 and NF-κB, was kindly provided by Dr. C.K. Glass (University of California, San Diego, CA). COX-2 promoter plasmid was kindly provided by Dr. Byron Wingerd (Michigan State University, East Lansing, MI). Using electroporation, cells (2×10^7 cells/cuvette) were co-transfected with 0.25 μg of expression or empty vector, together with 0.25 μg of β-galactosidase expression vector. Transfected cells were cultured in 24-well plate at 2×10^6 cells/well and incubated in DMEM containing 10% FBS for 24 h. Subsequently, cells were treated with agents for 24 h and the luciferase activity was assayed using promega kit in a microplate luminometer. Luciferase activity values were normalized to transfection efficiency, monitored by β-galactosidase expression and was presented as the percentage of luciferase activity measured without stimulation.

Electrophoretic Mobility Shift Assays (EMSA)

Nuclear extraction and EMSA were performed as we previously described [Chen et al., 2003]. The supernatants containing the solubilized nuclear proteins were used for EMSA. The binding reaction mixture contained 0.25 μg of poly(dI-dC) and 20,000 dpm of [α-^{32}P]ATP-labeled DNA probe for NF-κB (5′-GATCAGTTGAGGGGACTTTCCCAGGC-3′), CRE (5′-GTCAGTCAGATGACGT CATATCGGTCAG-3′), or C/EBPβ (5′-TGCAGATTGCGCAATC TGCA-3′) in binding buffer. The binding reaction was initiated by the addition of cell extracts and continued for 1 h. Samples were analyzed on native 5% polyacrylamide gels.

Statistical Evaluation

Values were expressed as the mean ± SE of at least three experiments. Analysis of variance (ANOVA) was used to assess the statistical significance of the differences, and a *P*-value of less than 0.05 was considered statistically significant.

RESULTS

AICAR Inhibits LPS-Induced iNOS and COX-2 Protein Expression

To investigate the effects of AICAR on LPS-induced NO and PGE_2 production, RAW264.7 macrophages were pretreated with different concentrations of AICAR (1 and 3 mM) for 30 min, followed by stimulation with 1 μg/ml LPS for 24 h. AICAR co-incubation inhibited LPS-induced NO and PGE_2 production (Fig. 1A). We also tested the effect of AICAR on BV-2 microglial cells, the resident macrophages of central nervous system. Similar to the results observed in macrophages, AICAR (1 mM) also suppressed LPS (0.1 μg/ml)-induced NO and PGE_2 production in BV-2 cells (Fig. 1B). The reason why we used 0.1 μg/ml, instead of 1 μg/ml of LPS was due to its toxicity to BV-2 cells at 1 μg/ml. Since AICAR was reported to be cytotoxic in certain cell types [Campàs et al., 2003; Lopez et al., 2003], we measured cell viability by MTT assay to determine whether the suppression of LPS-induced NO and PGE_2 production by AICAR was due to cytotoxicity. Treatment of cells with 3 mM AICAR alone or in combination with LPS (1 μg/ml for RAW264.7 cells and 0.1 μg/ml for BV-2 cells) for 24 h did not affect cell viability as assessed by MTT assay in both RAW264.7 and BV-2 cells (data not shown).

To determine whether the inhibitory effect of AICAR on LPS-induced NO and PGE_2 production resulted from the suppression of protein synthesis, we examined protein levels of iNOS and COX-2. When RAW264.7 macrophages were treated with AICAR (0.1–3 mM) for 24 h, LPS-induced expression of both iNOS and COX-2 were suppressed by AICAR in a concentration dependent manner (Fig. 1C), suggesting that the effects of AICAR on NO and PGE_2 production might be due to inhibition of iNOS

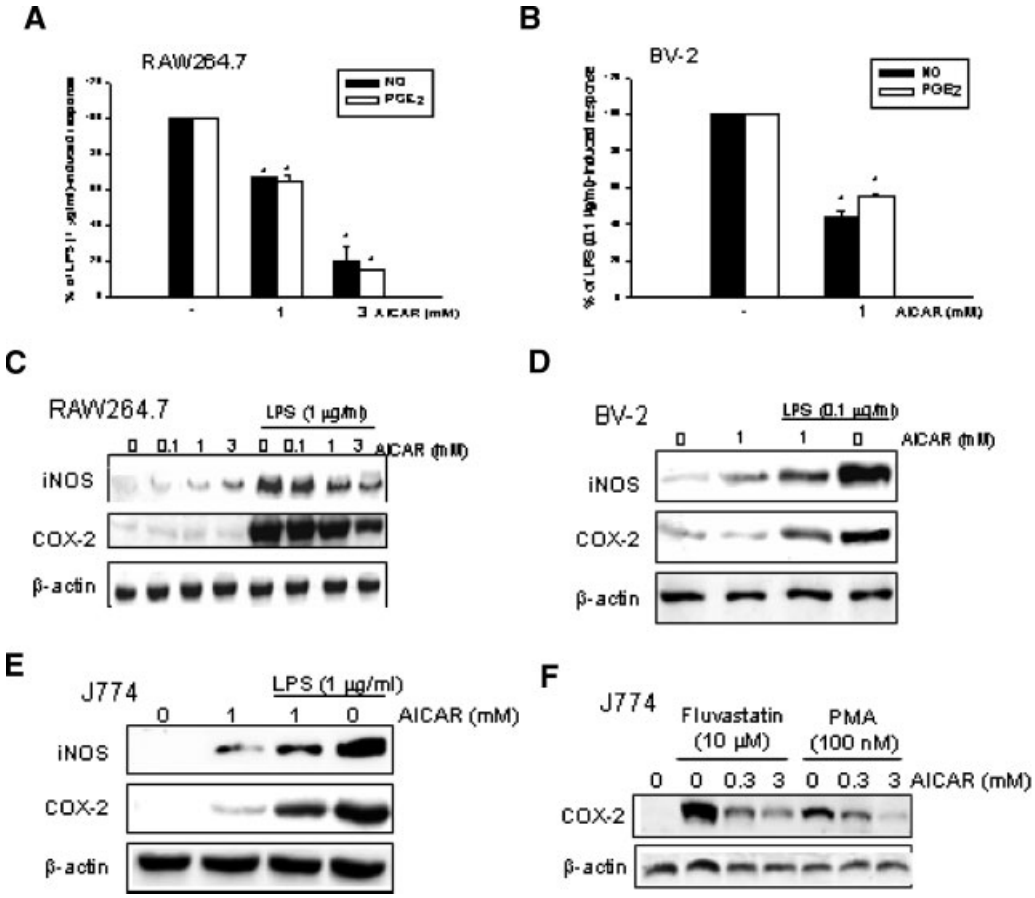

Fig. 1. AICAR inhibits LPS-mediated iNOS and COX-2 protein expression. RAW264.7 macrophages (**A**,**C**), BV-2 microglial cells (**B**,**D**), and J774 macrophages (**E**,**F**) were treated with AICAR, LPS, fluvastatin and/or PMA at the concentrations indicated for 24 h. Media were collected and assayed for NO and PGE_2 production. In 1 μg/ml LPS-treated RAW264.7 cells, nitrite was increased from 7.7 ± 0.6 to 40.5 ± 3.5 μM, and PGE_2 was increased from 0.28 ± 0.03 to 6.15 ± 0.31 ng/ml. In 0.1 μg/ml LPS-treated BV-2 cells, nitrite was increased from 9.9 ± 0.7 to 21.5 ± 0.8 μM, and PGE_2 was increased from 14.9 ± 0.8 to 27.5 ± 0.3 ng/ml. Net response of LPS was regarded as 100%, and AICAR-elicited inhibition on LPS-induced NO and PGE_2 production was shown (A,B). Data represent the mean ± SE of at least three independent experiments. $^*P < 0.05$ as compared with the LPS response without AICAR treatment. Cell lysates were also prepared for determining iNOS and COX-2 protein levels with immunoblotting (C–F). The β-actin level was measured as an internal control. Results are representative of three independent experiments.

and COX-2 protein expression. AICAR treatment also suppressed LPS-induced expression of iNOS and COX-2 protein in BV-2 and J774 macrophages (Fig. 1D,E). In order to verify whether the inhibition by AICAR was stimuli specific, we examined its effect on other COX-2 inducers in J774 macrophages. We observed that AICAR also suppressed fluvastatin (10 μM) and PMA (100 nM) induced COX-2 protein expression in a concentration dependent manner (Fig. 1F).

AICAR Suppressed LPS-Induced iNOS and COX-2 Gene Expression

To determine whether the suppressive effects of AICAR on LPS-induced iNOS and COX-2 expression resulted as a consequence of inhibition of gene expressions, we first measured iNOS and COX-2 mRNA level by RT-PCR. We found that AICAR significantly inhibited the steady state mRNA levels of iNOS and COX-2 in RAW264.7 cells treated with LPS for as short as 2 and 6 h (Fig. 2A). Similar inhibition caused by AICAR was observed in BV-2 cells treated with LPS for 2 h (Fig. 2A). To ascertain the decrease in mRNA levels resulting from gene transcription, we determined promoter activities of iNOS and COX-2 in RAW264.7 cells. Promoter activity assay using luciferase expression as an index, indicated that LPS induced iNOS and COX-2 promoter activaties were inhibited by AICAR in a concentration dependent manner

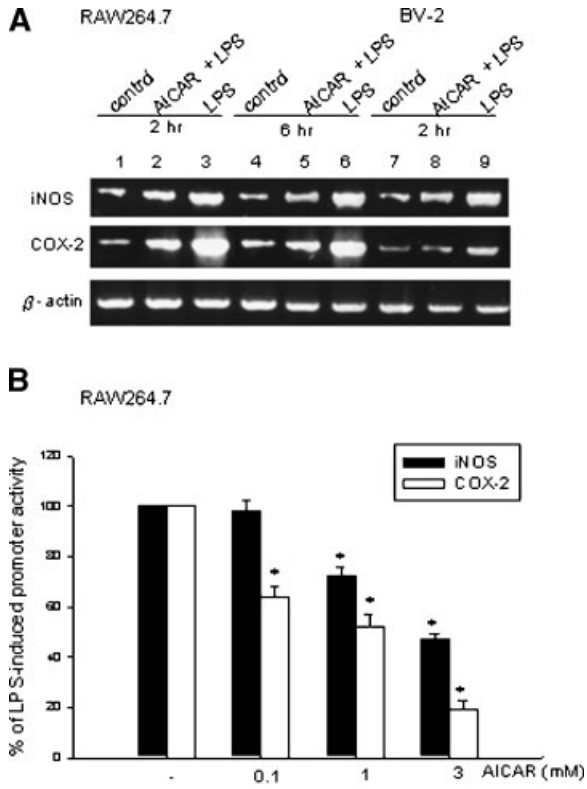

Fig. 2. AICAR inhibits steady state mRNA levels and promoter activities of iNOS and COX-2. **A**: After treatment of AICAR (1 mM) and/or LPS (0.1 μg/ml) for 2 or 6 h, iNOS and COX-2 mRNA levels in RAW264.7 macrophages (**lanes 1–6**) and BV-2 microglial cells (**lanes 7–9**) were examined by RT-PCR. The mRNA level of β-actin was determined as an internal control. Traces are representative of three independent experiments. **B**: RAW264.7 macrophages transfected with iNOS- and COX-2-luciferase promoter plasmids were treated with LPS (0.1 μg/ml) and/or AICAR at the concentrations indicated for 24 h. Net response of LPS (20-fold increase and 5.5-fold increase respectively for iNOS and COX-2 promoter activities) was regarded as 100%, and AICAR-elicited inhibition on LPS-induced responses was shown. Data represent the mean ± SE of at least three independent experiments which was performed in duplicate. $^*P < 0.05$ as compared with the LPS response without AICAR treatment.

(Fig. 2B). These results suggested that the inhibitory effects of AICAR on iNOS and COX-2 gene expressions were at the transcriptional level.

AICAR Suppressed the DNA Binding Activities of NF-κB, CREB, and C/EBPβ

NF-κB is one of the major transcription factors controlling iNOS gene expression [Lowenstein et al., 1993]. For COX-2 gene transcription, NF-κB, CREB, and C/EBPβ especially the latter two with predominant roles, have been identified in the functional regulatory regions of COX-2 gene [Inoue et al., 1995; Poli, 1998; Wadleigh et al., 2000]. Therefore, the DNA binding activities of NF-κB, CREB, and C/EBPβ were examined by EMSA in RAW264.7 cells treated with AICAR and/or LPS. Results indicated that AICAR diminished the LPS-induced DNA binding activities of NF-κB, CREB, and C/EBPβ (Fig. 3).

AICAR Did Not Affect LPS-Induced IKK and MAPK Activation

In unstimulated cells, NF-κB exists as a p65/p50 heterodimer and is retained in cytoplasm by its association with IκB. After stimulation of cells with LPS, the cytosolic NF-κB/IκB complex dissociates and free NF-κB translocates to the nucleus and regulates the transcription of various genes. Phosphorylation of IκB by the upstream kinase IKK is essential for its dissociation from the NF-κB and subsequent degradation [Ghosh and Karin, 2002]. To determine whether IKK could be the target for AICAR action, RAW264.7 cells were pretreated with AICAR (3 mM) for 30 min, followed by LPS treatment. Then IκB degradation and the phosphorylation of IKK were examined using immunobloting. AICAR did not affect IκB degradation or IKK phosphorylation at 15 and 30 min of LPS stimulation (data not shown). Nuclear translocation of NF-κB subunit p65 is an important step for NF-κB binding to cognate DNA elements and thus driving gene promoter activity. Hence, we also analyzed the effects of AICAR on p65 translocation event. RAW264.7 cells were treated with AICAR and/or LPS for 30 min or 1 h. AICAR did not affect LPS-induced p65 nuclear translocation (data not shown).

Next we examined whether LPS-induced MAPKs activities were affected by AICAR. We found that LPS-induced ERK, p38 and JNK activation were not affected in the presence of 3 mM AICAR (data not shown).

Suppressive Effect of AICAR Was Independent of AMPK

To elucidate whether the anti-inflammatory effect of AICAR, in terms of downregulation of LPS-induced iNOS and COX-2 expression was associated with AMPK activation, we examined the phosphorylation level of AMPK. We found that AMPKα in RAW264.7 cells was phosphorylated by AICAR 15 min after treatment, and this event persisted for at least 3 h (Fig. 4A). Similar

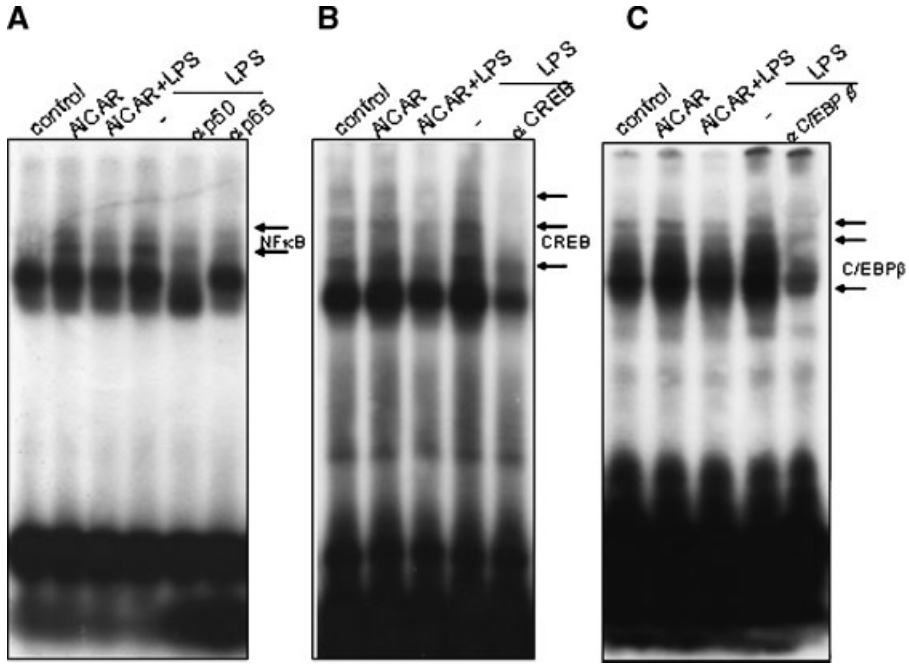

Fig. 3. AICAR inhibits LPS-induced DNA binding activity of NF-κB, CREB, and C/EBPβ. RAW264.7 macrophages were treated with AICAR (3 mM) and/or LPS (1 μg/ml) for 75 min. Nuclear fractions were extracted and assayed by EMSA for DNA binding activity of NF-κB (**A**), CREB (**B**), and C/EBPβ (**C**). To confirm the binding location and specificity of transcription factors with DNA probes, gel shift was performed in the DNA binding experiments in the presence of antibody of p50, p65, CREB, or C/EBPβ. Data are representative of three independent experiments.

results were obtained in J774 and BV-2 cells (data not shown).

Next, we used 5′-iodotubercidin, an inhibitor of adenosine kinase, whose ability to reverse AICAR's inhibitory effect could suggest involvement of AMPK. Here, RAW264.7 cells were pretreated with 5′-iodotubercidin (0.3 μM) for 30 min, and then the cells were treated with AICAR (3 mM). Results indicated that pretreatment of 5′-iodotubercidin indeed prevented AICAR-mediated AMPK phosphorylation (Fig. 4A), but did not reverse the suppressive effects of AICAR on LPS-induced iNOS or COX-2 protein expression (Fig. 4B). To further elucidate the role of AMPK in regulating iNOS and COX-2 gene expressions, we tested the effect of 5′-iodotubercidin on NF-κB and AP-1 promoter activity. AP-1 binding on CRE site in COX-2 promoter was demonstrated as a crucial element for gene induction [Degner et al., 2006]. Moreover, a recent study demonstrated an additional functional AP-1 site for COX-2 induction [Kang et al., 2006]. As shown in Figure 4C, 5′-iodotubercidin (0.3 μM) did not reverse the suppressive effect of AICAR on LPS-induced NF-κB and AP-1 activity. All these evidences suggest that the suppressive effect of AICAR on LPS-induced event was not mediated through the activation of AMPK.

To further support this notion, we tested metformin, a known AMPK activator. Although metformin (1 mM) could induce AMPK phosphorylation in RAW264.7 macrophages (Fig. 5A), it did not inhibit LPS-induced iNOS and COX-2 protein expression (Fig. 5B) as well as NO and PGE_2 production (data not shown). Similar results were observed in BV-2 cells, where LPS-induced iNOS and COX-2 expression was unaffected by the presence of metformin (Fig. 5B).

AICAR Interfered With DNA Binding of NF-κB, CREB and C/EBPβ

Since AICAR did not affect LPS-induced IκB degradation, IKK phosphorylation, and p65 nuclear translocation, but inhibited DNA binding activity of NF-κB, CREB, and C/EBPβ, we wondered whether the latter effect was as a result of direct interruption of DNA binding activities. To examine this possibility, RAW264.7 cells were stimulated with LPS (1 μg/ml) for 75 min, and then nuclear fractions

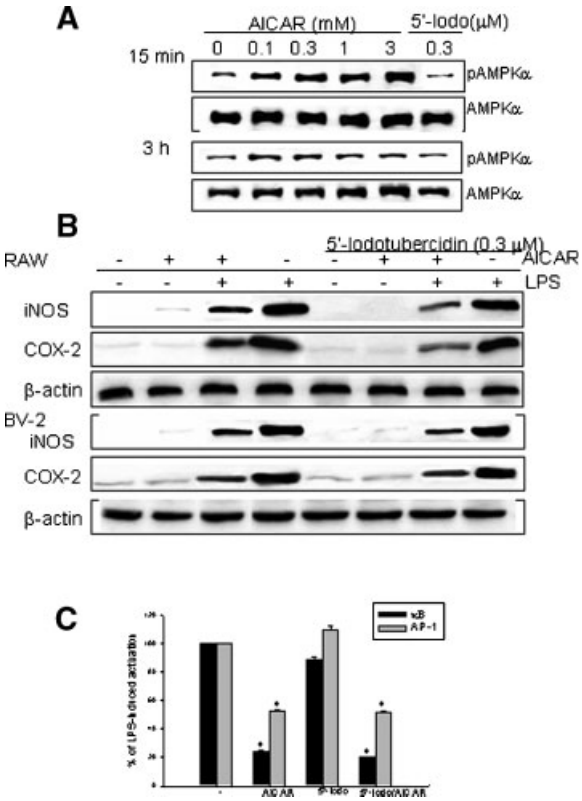

Fig. 4. Effects of AICAR in downregulating iNOS and COX-2 protein expression, NF-κB and AP-1 activation are AMPK-independent. **A**: After RAW264.7 macrophages were treated with AICAR at the indicated concentrations and time intervals, AMPK phosphorylation was examined by immunoblot. In some experiments, cells were pretreated with 5′-iodotubercidin (0.3 μM) for 30 min, and then the cells were treated with AICAR (3 mM). **B**: RAW264.7 macrophages and BV-2 cells were treated with 5′-iodotubercidin (0.3 μM), AICAR (3 mM in RAW264.7 and 1 mM in BV-2), and/or LPS (1 μg/ml in RAW264.7 and 0.1 μg/ml in BV-2) for 24 h. Immunobloting analysis was performed to examine iNOS and COX-2 protein expression. **C**: Promoter activities of NF-κB and AP-1 in RAW264.7 cells treated with 5′-iodotubercidin (0.3 μM), AICAR (3 mM), and/or LPS (1 μg/ml) were assayed. Data represent the mean ± SE of at least three independent experiments which were performed in duplicate. $^*P < 0.05$ as compared with the control LPS response without AICAR or 5′-iodotubercidin treatment.

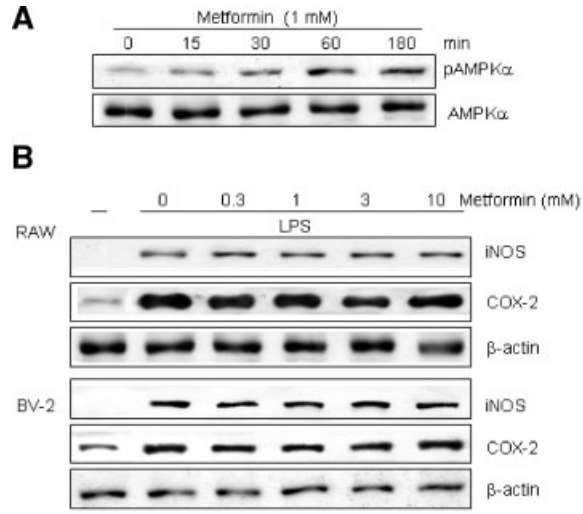

Fig. 5. Metformin activates AMPK, but doesn't affect LPS-induced iNOS and COX-2 protein expression. **A**: After RAW264.7 macrophages were treated with metformin (1 mM) at indicated time intervals, AMPK phosphorylation was examined by immunoblot. **B**: RAW264.7 and BV-2 cells were treated with metformin and/or LPS at the concentrations indicated for 24 h, and then iNOS and COX-2 protein induction were determined. Results are representative of three independent experiments.

were collected for EMSA assay in the presence of exogenous AICAR at different concentrations. Results indicated that AICAR was able to abolish in vitro NF-κB DNA binding at concentrations higher than 30 μM (Fig. 6A). The inhibition was not observed when the concentration of AICAR was 6 μM or lower. The same effect of AICAR was also seen in CREB and C/EBPβ. AICAR attenuated CREB-DNA binding at concentrations between 300 and 1,500 μM, and this effect was not apparent at 120 μM and lower concentration (Fig. 6B). Meanwhile, results showed that AICAR sufficiently attenuated C/EBPβ-DNA binding at concentration as low as 1.5 μM (Fig. 6C). Although different in vitro susceptibility for inhibition of DNA binding was observed, these results imply the direct interference of AICAR on DNA binding capability of target genes.

DISCUSSION

AMPK, an energy-sensing enzyme activated in response to cellular stress, is a critical signaling molecule for the regulation of multiple metabolic processes. Besides its role in metabolic process, AICAR is also reported to regulate inflammatory gene transcription, while the action mechanism remains paradoxical and unclear. In macrophages and glial cells, AICAR inhibits LPS-induced NF-κB and c/EBPβ activation, accounting for its anti-inflammatory action [Giri et al., 2004]. In myocytes, adipocytes, and mouse bone marrow-derived macrophages, AMPK-mediated iNOS inhibition results from post-transcriptional regulation of iNOS protein [Pilon et al., 2004]. In this study, using macrophages and microglial cells, we observed that AICAR can transcriptionally

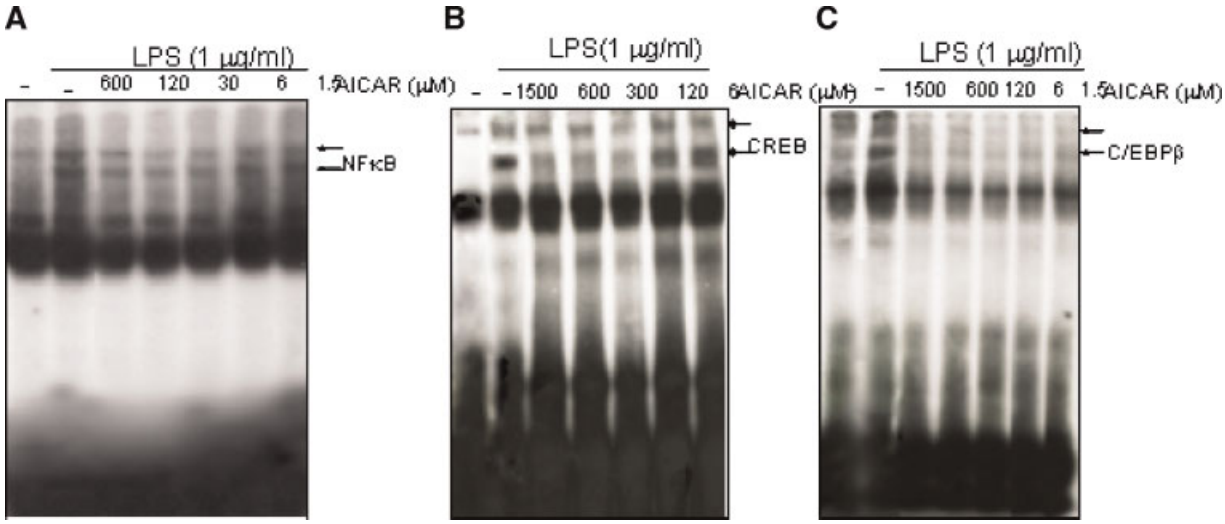

Fig. 6. AICAR directly inhibits DNA binding abilities of NF-κB, CREB, and C/EBPβ. RAW264.7 cells were stimulated with 1 μg/ml LPS for 75 min, and nuclear fractions were then prepared to determine the in vitro DNA binding abilities of NF-κB (**A**), CREB (**B**), and C/EBPβ (**C**) in the presence of various concentrations of AICAR ranging from 1.5 to 1,500 μM. In the LPS control group (**lane 2** of each panel), vehicle was examined. Running on polyacrylamide gel was used for sample analysis. Data are representative of three independent experiments with similar results. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

inhibit LPS-induced iNOS and COX-2 gene regulation. This action was independent of AMPK activation, suggestive of a direct interruption of DNA binding with various transcription factors, which are essential for iNOS and COX-2 gene expression.

We have demonstrated that AICAR inhibits LPS-induced NO and PGE_2 production in macrophages and microglia. LPS-induced iNOS and COX-2 protein expression levels were accordingly abolished by AICAR in these cells. Besides inhibiting LPS-induced inflammatory responses, AICAR also inhibited the increased COX-2 protein expression by other inducers, such as fluvastatin [Chen et al., 2004] and PMA, in a concentration dependent manner. Our previous study has demonstrated that statins can upregulate COX-2 gene expression through activation of CRE and C/EBPβ, but not NF-κB, promoter sites [Chen et al., 2004]. PMA is a potent PKC activator, which activates AP-1 leading to a dramatic increase in COX-2 induction [Matthews et al., 2007]. Using RT-PCR and promoter assays, we confirmed that the suppressive action of AICAR on LPS-induced expression of both iNOS and COX-2 proteins was through the inhibition of gene transcription. The inhibition, irrespective of the source of stimuli, suggested that AICAR might influence downstream target of gene transcription.

Since NF-κB, CREB, and C/EBPβ play important roles in the functional regulatory regions of iNOS and/or COX-2 genes [Inoue et al., 1995; Wadleigh et al., 2000], we examined their DNA binding activities in AICAR- and LPS-treated cells. Results obtained from EMSA indicated that AICAR diminished the LPS-induced DNA binding activities of these transcription factors in macrophages. Moreover, reporter driven transactivation assay also confirmed that inhibition of NF-κB and AP-1 activities were due to AICAR-mediated suppressive effects.

Consistent with our results, recent studies have demonstrated the ability of AICAR to inhibit NF-κB activation in response to LPS and TNF-α [Giri et al., 2004; Prasad et al., 2006]. Although IκB degradation and p65 nuclear translocation are considered important events for NF-κB activation and thus driving its promoter activity, AICAR did not affect LPS-induced IKK activation, IκB degradation, and p65 nuclear translocation. Therefore, we postulate that the inhibition of NF-κB activity by AICAR, as described above, might have occurred at the level of nuclear NF-κB binding to κB DNA element. Notably, we found weak responses of IKK activation, IκB degradation, and p65 nuclear translocation, after AICAR treatment. This suggested the ability of AICAR to increase IKK-dependent NF-κB activity and

similar effects of AICAR (Jung et al., 2004) were also observed in neuroblastoma cells. The molecular mechanism and functional intervention of such weak NF-κB activation elicited by AICAR need to be clarified further. However, we suggest the opposite effects of AICAR dependent on inflammatory status, might result from its multiple action mechanisms.

For LPS-mediated COX-2 gene induction, both ERK and p38 signaling pathways have been shown to contribute to AP-1, C/EBPβ, and CREB activation [Mestre et al., 2001; Chen et al., 2004; Kundu et al., 2006; Ouyang et al., 2007]. In this study, we found AICAR did not affect both signaling cascades. The direct inhibition of DNA binding activities of CREB and C/EBPβ by AICAR indicated that AICAR could exert its anti-COX-2 effects through targeting on DNA binding of these transcription factors, while the precise mechanism of action, warrants further study. AMPK-independent inhibition of PI3K/Akt signaling pathway was suggested for AICAR-mediated inhibition of TNF-α production in LPS-stimulated RAW264.7 macrophages [Jhun et al., 2004]. However, experiments conducted in RAW264.7 macrophages using wortmannin, a potent PI3K inhibitor, did not reverse the suppressive effects of AICAR on LPS-induced iNOS and COX2 expression, further ruling out the possible involvement of Akt pathway (data not shown).

While AICAR treatment significantly increased AMPK phosphorylation in macrophages and microglial cells, we suggest that the suppressive action of AICAR on LPS-induced iNOS and COX-2 protein expression as well as NF-κB and AP-1 activation were not mediated by AMPK. This conclusion was drawn based on the inability of 5′-iodotubercidin to revert the above inhibitory effects of AICAR. As reported, 5′-iodotubercidin was able to inhibit adenosine kinase to process intracellular AICAR to a metabolite essential for AMPK activation, while the suppression of LPS induced iNOS and COX-2 protein expression was not reverted. Confirming this notion, we detected the ability of 5′-iodotubercidin to diminish AICAR-induced AMPK phosphorylation. Yet another data to support this notion was based on the nonmimetic effect of metformin. AMPK activation was already reported to contribute to the insulin sensitization and anti-diabetic action of metformin [Hawley et al., 2002].

In conclusion, we demonstrated the actions of AICAR in downregulating iNOS and COX-2 gene expression. This anti-inflammatory action of AICAR neither involved AMPK activation nor interfered with IKK, ERK, p38, or AKT pathways, but primarily resulted through a blockade in DNA binding activities of NF-κB, CREB, and C/EBPβ. These results suggest that in addition to its application for treating metabolic diseases, AICAR may also have therapeutic potential for treating inflammatory diseases.

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