Stimulators of AMP-activated protein kinase inhibit the respiratory burst in human neutrophils

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Abstract In the present study, we have examined the potential ability of 5′-AMP-activated protein kinase (AMPK) to modulate NADPH oxidase activity in human neutrophils. AMPK activated with either 5′-aminoimidazole-4-carboxamide ribonucleoside (AICAR) or with 5′-AMP significantly attenuated both phorbol 12-myristate 13-acetate (PMA) and formyl methionyl leucyl phenylalanine-stimulated superoxide anion (O_2^-) release by human neutrophils, consistently with a reduced translocation to the cell membrane and phosphorylation of a cytosolic component of NADPH oxidase, namely $p47^{phox}$. AMPK was found to be present in human neutrophils and to become phosphorylated in response to either AICAR or other stimulators of its enzyme activity. Furthermore, AICAR also strongly reduced PMA-dependent H_2O_2 release, and induced the phosphorylation of c-jun N-terminal kinase 1 (p46), p38 mitogen-activated protein kinase and extracellular signal-regulated kinase. Present data demonstrate for the first time that the activation of AMPK, in states of low cellular energy charge (such as under high levels of 5′-AMP) or other signals, could be a factor contributing to reduce the host defense mechanisms.

Keywords: Adenine nucleotide; 5′-AMP-activated protein kinase; NADPH-oxidase; Superoxide anion

1. Introduction

Upon interaction with phagocytic stimuli, neutrophils exhibit an increase in oxygen consumption and in hexose monophosphate shunt activity. Concomitantly, neutrophils produce and secrete reactive oxygen species, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and singlet oxygen, which are important for the efficient killing of ingested microorganisms [1,2], and which may well be implicated in tissue damage in inflammation-mediated diseases. All of these changes are secondary to the activation of a membrane-bound NADPH oxidase, the enzyme responsible for O_2^- production [3]. Phagocytic NADPH oxidase is a very complex enzyme, composed of at least seven subunits: a membrane-bound component, cytochrome b558 (a heterodimer comprising the $gp91^{phox}$ and $p22^{phox}$ subunits), and the cytosolic proteins $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, rac and Rap-lA. The $p47^{phox}$ and $p67^{phox}$ polypeptides, together with the cytosolic $p40^{phox}$ component, translocate in the plasma membrane to reconstitute the active enzyme upon cell activation [4,5]. Rap-1A has been shown to specifically bind to cytochrome b558, and to become phosphorylated by the cAMP-dependent kinase protein kinase A (PKA) [6], which abrogates its interaction with cytochrome b558 [7] leading to the deactivation of NADPH oxidase [8]. This oxidase can be activated either by receptor-mediated signaling processes (e.g., those initiated by complement fragment C5a and the chemotactic tripeptide *N*-formyl-Met-Leu-Phe (fMLP)) [9], or by receptor-independent mechanisms (e.g., in response to long-chain unsaturated fatty acids or phorbol 12-myristate 13-acetate (PMA)) [10].

On the other hand, an energy-signaling protein has been described, the AMP-activated protein kinase (AMPK) [11], which is present in muscle and liver, as well as in macrophages and other tissues [12]. This enzyme appears to play a key role in regulating energy homeostasis in eukaryotes. Intracellular 5′-AMP activates AMPK via several distinct mechanisms: (i) 5′-AMP causes a direct allosteric activation of AMPK [13]; (ii) binding of 5′-AMP to AMPK renders it a better substrate for the upstream AMPK kinase (AMPKK/LKB1), or a worse substrate for protein phosphatases [13–15]; and (iii), the upstream kinase AMPKK/LKBl, which is also allosterically stimulated by 5′-AMP, in turn phosphorylates and activates AMPK [14,15]. AMPK also modulates positively other ATP-generating pathways, such as glycolysis [16] and fatty acid oxidation [17]. It has been shown that an adenosine analog, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), which enters cells and is phosphorylated by adenosine

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Abbreviations: AMPK, 5′-adenosine monophosphate-dependent protein kinase; AICAR, 5-aminoimidazole-4-carboxamide-riboside; 8-BrAMP, 8-bromoadenosine 5′-monophosphate; JNK, c-jun N-terminal kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; SOD, superoxide dismutase; BSA, bovine serum albumin; PMA, phorbol 12-myristate 13-acetate; fMLP, formyl-methionyl-leucyl-phenylalanine; DNP, 2,4-dinitrophenol; PKA, protein kinase A

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kinase to form AICAR-5′-monophosphate, is itself a potent stimulator of AMPK [16,18], and also an intermediate in the purine de novo synthesis pathway.

On the other hand, adenine nucleotides, such as ATP, have been shown to stimulate adhesive functions in neutrophils [19]. However, conflicting results have been reported regarding the activation of the respiratory burst in neutrophils after treatment with adenine nucleotides. Thus, $O_2^{\cdot-}$ release has been found to become unaffected [19], decreased [20], or enhanced [21] when compared with untreated human neutrophils. A requirement of extra energy to provide NADPH as a substrate for the oxidase reaction during phagocytosis has also been accepted [22], since a significant enhancement of glycogen degradation, glucose utilization and lactate production was observed under these conditions [23,24]. Since it is known that endogenous levels of ATP are depleted during phagocytosis [25], the purpose of the present study was, therefore, to evaluate the potential role of 5′-AMP-stimulated kinase (AMPK) on the respiratory burst in human neutrophils and to shed some light on the specific signaling pathways involved.

2. Materials and methods

2.1. Reagents

Dextran T-500 and Ficoll-Paque were obtained from Amersham-Pharmacia-Biotech (Barcelona, Spain). PMA, cytochrome *c*, horseradish peroxidase (HRP)-conjugated rabbit anti-goat, goat anti-rabbit and goat anti-mouse immunoglobulin G (IgG), 5-aminoimidazole-4 carboxamide-riboside (AICAR), scopoletin, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), 8-bromoadenosine 5′-monophosphate (8-BrAMP), rotenone, 2,4-dinitrophenol (DNP), 4-iodophenol and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (Madrid, Spain). A goat antiserum raised against $p47^{phox}$ was kindly donated by Dr. T. Leto (National Institutes of Health, Bethesda, MD) [26]. Luminol (5-amino-2,3-dihydrophthalazine-l,4-dione) was purchased from Serva Feinbiochemica GmbH (Madrid, Spain). PVDF membranes were purchased from Bio-Rad (Hercules, CA). Xanthine oxidase and xanthine were purchased from Roche Diagnostics (Barcelona, Spain). Rabbit polyclonal anti-phospho-mitogen-activated protein kinase (p38MAPK) (Thr^{180}/Tyr^{182}) and anti-phospho-extracellular signal-regulated kinase (ERK)1/2 (Thr^{202}/Tyr^{204}) and antibodies against their unphosphorylated counterparts specific antibodies were obtained from New England Biolabs (Beverly, MA). Mouse monoclonal anti-phospho-c-jun N-terminal kinase (JNK)l/2 (Thr^{183}/Tyr^{185}) (sc-6254) and anti-βactin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Preparation of human neutrophils

Human peripheral neutrophils were obtained from healthy blood donors, following their informed consent. Neutrophils were isolated from fresh heparinized blood as described [27]. Neutrophils (10^7 cells/ml) were resuspended in RPMI medium supplemented with 10% fetal bovine serum plus 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin or, where indicated, in HEPES-buffered Krebs–Ringer solution (KR–HEPES), and subjected to the drug treatments indicated in each case, washed twice with ice-cold KR–HEPES and immediately lysed.

2.3. Western blotting

Human neutrophils (10^7 cells/ml) were lysed by sonication on ice (20 W, three bursts of 5 s each separated by 30 s intervals) in 50 μl of a buffer containing Tris/HCl (50 mM), pH 7.5, NaF (50 mM), sodium pyrophosphate (5 mM), dithiothreitol (1 mM), glycerol (10%, v/v), Triton X-100 (1%, v/v), and the protease inhibitors PMSF (1 mM), leupeptin, aprotinin (10 mg/ml each) and benzamidine (156 μg/ml). The homogenate was centrifuged at $15\,000 \times g$ for 10 min at 4 °C. Proteins extracts in the cell supernatant were separated by SDS–PAGE on 7.5% (w/v) polyacrylamide gels and transferred to PVDF membranes using a semi-dry device (Bio-Rad, Richmond, USA) [27]. The membranes were probed with specific antibodies against phospho-AMPK (Cell Signaling Technology) (1:1000 dilution) in phosphate buffer saline (PBS) containing BSA (1%) and Tween 20 (0.2%), at 4 °C overnight, and after washing with PBS the membrane was incubated with HRP-conjugated goat anti-rabbit IgG, followed by enhanced chemiluminescence [27,28]. For the detection of p38MAPK, ERK1/2 and JNK1/2 phosphorylation was carried out as in [29].

2.4. $O_2^{\cdot-}$ production

Superoxide production was measured on the basis of the cytochrome *c* method [30], which is highly specific for $O_2^{\cdot-}$ released to the extracellular medium, given the inability of citochrome *c* to permeate through the plasma membrane [31]. Neutrophils (10^6 cells/ml) were preincubated for 20–30 min with or without AICAR in KR–HEPES, and cytochrome *c* (80 μM) reduction was measured spectrophotometrically following the method previously described [32]. For the measurement of ROS production, neutrophils (10^6 cells/ml) were suspended in KR–HEPES buffer and preincubated at 37 °C for 20 min with the compounds indicated in each case. Then, luminol (20 μM) and HRP (8 mU/ml) were added, and the assay was carried out as indicated previously [33]. The reaction was started by the addition of PMA to the cellular suspension, and chemiluminescence emission was recorded at different times using a Wallac 1420 Victor^2 (Perkin–Elmer) luminometer.

2.5. Extracellular levels of H_2O_2

Hydrogen peroxide production was measured by a modification of the scopoletin oxidation assay, as in [34]. Briefly, neutrophils were suspended in 300 μl of RPMI 1640 medium (1.5×10^6 cells/well) containing 10% fetal bovine serum and incubated with the indicated drugs for the indicated times. Then, HRP (2.4 U/ml), NaN_3 (1 mM) (to inhibit myeloperoxidase and catalase activities) and scopoletin (3 μM) were added. The production of H_2O_2 was triggered upon PMA addition, and the fluorescence intensity was measured for 20 min in the Wallac 1420 Victor^2 spectrofluorometer, using excitation and emission wavelengths of 355 and 460 nm, respectively.

2.6. Superoxide dismutase and glutathione peroxidase activity

Superoxide dismutase (SOD) activity was determined by measuring the inhibition of cytochrome *c* reduction by the xanthine–xanthine oxidase system [30]. This enzyme activity was measured in extracts of neutrophils previously chromatographied through a Sephadex (G25) column in order to remove small molecules. Glutathione peroxidase activity was assayed using a coupled enzyme system [35].

2.7. Mobilization of $p47^{phox}$

The neutrophil plasma membrane fraction was prepared as previously reported [27] and subjected to SDS–PAGE and electroblotted onto PVDF membranes as indicated above. Antibody probing was carried out overnight without the need for prior blocking [36] with goat anti-$p47^{phox}$ IgG (1:1000 dilution) in PBS containing BSA (1%) and Tween 20 (0.02%). Antibody binding was detected as indicated above. For immunoprecipitation experiments, incubation with anti-$p47^{phox}$ specific antibodies and subsequent procedure were carried out as indicated [28].

2.8. RT-PCR analysis of AMPK mRNA levels

Total cellular RNA was extracted using the phenolguanidinium isothiocyanate method [37]. Reverse transcription to cDNA was performed at 37 °C for 1 h in 24 μl of a reaction mixture containing 2 μg of RNA, the 4 dNTPs (1 mM each), 2.5 μM random hexamer primers, 1 mM DTT, 20 U of RNasin ribonuclease inhibitor and 100 U of M-MLV reverse transcriptase (Promega, Madison, WI). PCR amplification of cDNA was performed in 10 μl of reaction using the following AMPK-α (GenBank Accession No. 206907) specific primers at 0.5 μM each: forward, 5′-GTCGGCGTCTGTTCCAACAGA-3′; reverse, 5′-CCCTCTTCATGGGATCCACCT-3′, the 4 dNTPs (400 μM each), 5% DMSO and 1 U of *Taq* DNA polymerase (Roche Diagnostics, Barcelona, Spain). After an initial denaturation step at 95 °C for 5 min, PCR was performed for a total of 35 cycles, each of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. To ensure that equal amounts of cDNA were added to PCRs, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified as a control, using the following primers: forward, 5′-CCACCCATGGCAAATTCCATGGC-3′; reverse, 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. PCR-amplified

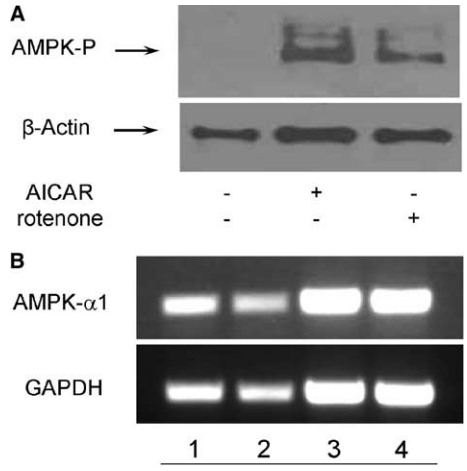

Fig. 1. AICAR and rotenone promote AMPK phosphorylation and induce AMPK-α mRNA expression. (A) Neutrophils (10^7 cells/ml) were resuspended in RPMI and then incubated in the absence or presence of AICAR (1 mM) or rotenone (3 μM) at 37 °C for 1 h. The cells were then lysed and proteins were separated electrophoretically, transferred to PVDF membranes and probed with anti-phospho-AMPK. The blot shown is representative of a set of three experiments that yielded similar results. (B) Neutrophils isolated from four healthy individuals (1–4) were lysed, and AMPK-α and GAPDH mRNA levels were analyzed by RT-PCR.

bands were visualized on 1% agarose gels stained with ethidium bromide using the BioDoe-ItTM system (UVP, Upland, CA).

3. Results

In the present work, we have studied the potential ability of AICAR to modulate $O_2^{\cdot-}$ and H_2O_2 production by phagocytic cells. As indicated previously, AICAR stimulates the phosphorylation and activation of AMPK [18]. Initial experiments were designed to analyze whether AMPK was present in human neutrophils. Fig. 1A illustrates that the enzyme became phosphorylated, and hence stimulated, when neutrophils were treated with AICAR or rotenone. Moreover, Fig. 1B shows that the mRNA of the AMPK-α subunit was expressed in human neutrophils from four healthy unrelated individuals. Subsequent experiments were addressed to study whether $O_2^{\cdot-}$ production was altered by AICAR and/or by other reagents able to activate AMPK, such as DNP and rotenone. Fig. 2 illustrates that in neutrophils treated with AICAR for 30 min, both PMA-dependent (A) and fMLP-dependent (B) release of $O_2^{\cdot-}$ became partially inhibited. This AICAR negative effect was progressive and attained its maximal value at about 10 min after PMA addition. However, AICAR inhibition was maintained after this time in fMLP-stimulated cells (B). We

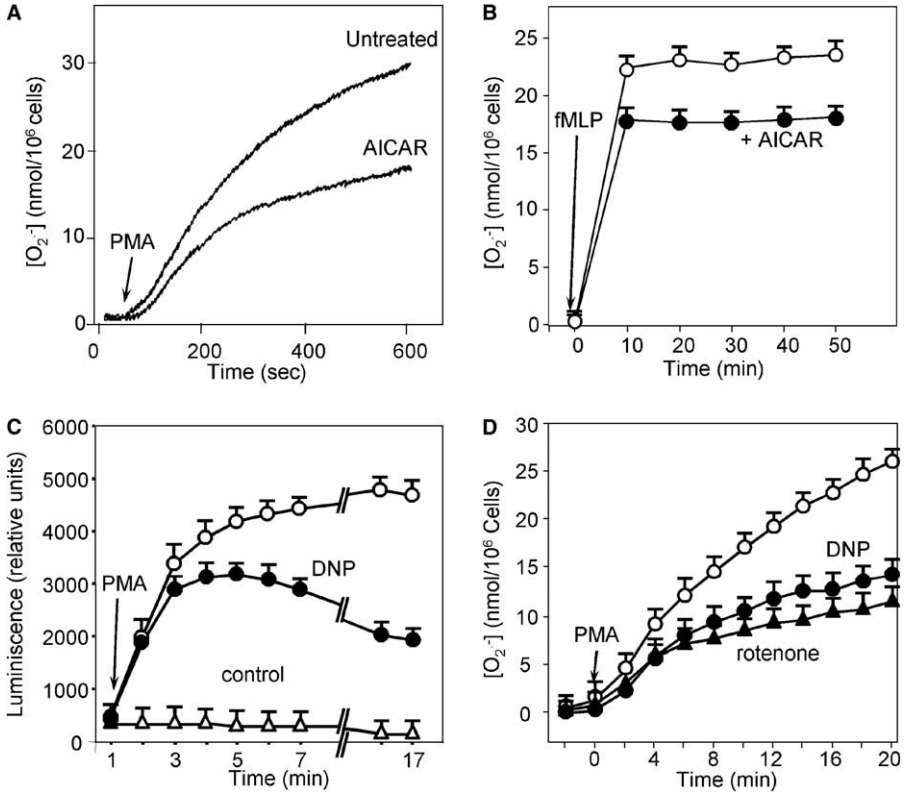

Fig. 2. AICAR, DNP and rotenone inhibit PMA-dependent ROS production in human neutrophils. (A, B) Neutrophils (10^6 cells/ml) were resuspended in KR–HEPES buffer and then preincubated with or without AICAR (1 mM) at 37 °C for 30 min. Cytochrome *c* (80 μM) was added and the reaction was started by the addition of (A) PMA (100 nM) or (B) fMLP (100 nM) as indicated (arrow). The extracellular release of $O_2^{\cdot-}$ was measured by the change in cytochrome *c* absorbance at 550 nm. (C) Neutrophils (10^6 cells/ml) were incubated in KR–HEPES buffer at 37 °C in the absence or presence of DNP (0.5 mM) for 10 min. PMA (50 nM) was then added (arrow) and ROS production was analyzed on the basis of luminol-derived chemiluminescence. (D) Neutrophils (10^6 cells/ml) were suspended in KR–HEPES buffer and then preincubated with or without DNP (0.5 mM) or rotenone (3 μM) at 37 °C for 10 min, and then $O_2^{\cdot-}$ production was analyzed as in panels A and B. Data are expressed as means ± S.E.M. of three different experiments.

have also assayed that AICAR did not scavenge $O_2^{\cdot-}$ using a $O_2^{\cdot-}$ production system (xanthine/xanthine oxidase, data not shown) Fig. 2 also shows that DNP or rotenone treatment clearly reduced both PMA-dependent ROS (C) and $O_2^{\cdot-}$ (D) production.

Furthermore, other respiratory chain uncouplers, such as oligomycin, antinomyin A and FCCP, exerted a similar inhibitory effect on ROS production by PMA-stimulated human neutrophils (data not shown).

It must be noticed that the inhibitory effect of AICAR required a previous time of cell pretreatment, since no inhibition was found when AICAR was added simultaneously to PMA (Fig. 3A). Preincubation with AICAR could be necessary to activate AMPK before PMA is able to trigger the potent and rapid phosphorylation of NADPH-oxidase components [1,38]. We show that a progressively increased preincubation time of neutrophils with AICAR resulted in a 35% inhibition of $O_2^{\cdot-}$ release after 1 h of treatment (Fig. 3A, inset).

The inhibitory effect of AICAR on $O_2^{\cdot-}$ release was dose-dependent (Fig. 3B) and reached its maximal value at 1 mM. Any concentration greater than this did not further enhance the degree of observed inhibition (data not shown). Fig. 3B illustrates that the negative effect of AICAR was specifically exerted through AMPK activation, since its inhibitory action was partially reverted by previous treatment with 8-BrAMP, an inhibitor of AMPK activity [12].

To analyze whether AICAR exerted also an effect on extracellular H_2O_2 release, neutrophils were stimulated with PMA after preincubation in the absence or presence of AICAR for 30 min. Fig. 3C illustrates that AICAR treatment inhibited by about 70% PMA-dependent H_2O_2 release, and that this effect was reversed to a 50% inhibition by 8-BrAMP. Since potential source of H_2O_2 is $O_2^{\cdot-}$ dismutation through the SOD enzyme activity, in order to explain the different rates of inhibition exerted by AICAR on $O_2^{\cdot-}$ and H_2O_2 production, we set to measure SOD activity. Experiments were performed using extracts of human neutrophils which were incubated with either AICAR or 5′-AMP at 37 °C for 30 min, and SOD activity levels measured (in U/mg protein) were 1.49 ± 0.19 in untreated extracts, 0.98 ± 0.12 with 1 mM AICAR and 1.18 ± 0.12 with 1 mM AMP. Thus, both AICAR or 5′-AMP inhibited SOD activity by 34% and 22%, respectively. Since another enzyme potentially able to remove H_2O_2 is glutathione peroxidase, we also set to analyze its activity in order to explain the AICAR effect. Glutathione peroxidase activity was found to become increased after AICAR treatment of neutrophils to 5.1 ± 0.15 U/mg protein, as compared to levels measured in untreated neutrophils (3.9 ± 0.17 U/mg protein). In summary, the observed low levels of H_2O_2 release after AICAR treatment could be a consequence of the additive effects of AICAR on three consecutive acting enzyme systems (i.e., NADPH oxidase, SOD and glutathione peroxidase).

Further experiments were addressed to analyze the effect of AICAR on the translocation to the plasma membrane of a cytosolic component of NADPH-oxidase, $p47^{phox}$, which is

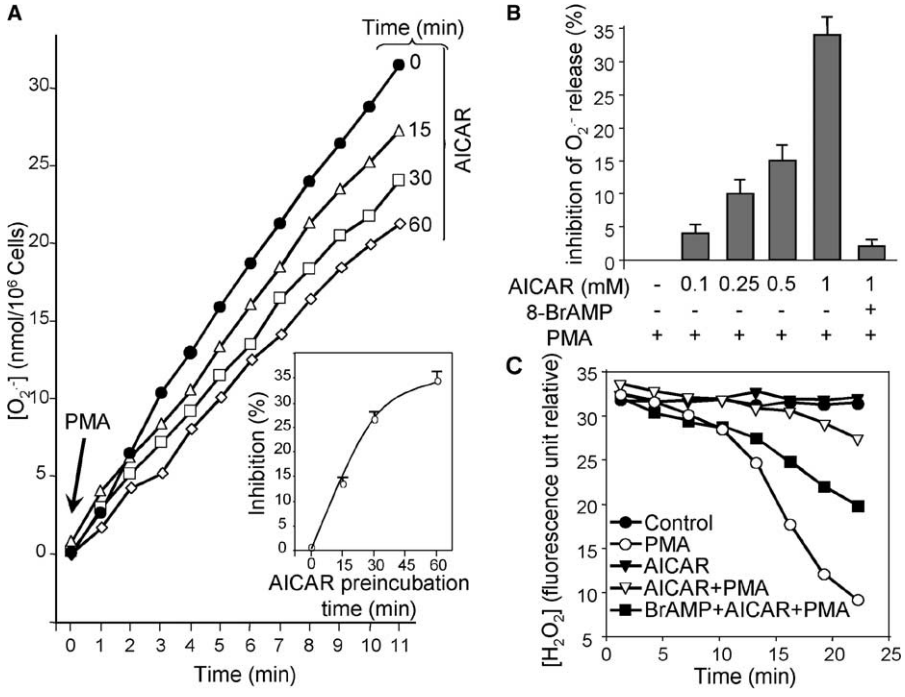

Fig. 3. AICAR inhibits $O_2^{\cdot-}$ extracellular production in a dose- and time-dependent manner and H_2O_2 release in PMA-stimulated human neutrophils. (A, B) Neutrophils (10^6 cells/ml) were resuspended in KR–HEPES buffer and then (A) preincubated in the absence or presence of AICAR (1 mM) for the indicated times, or (B) preincubated with different concentrations of AICAR for 1 h at 37 °C. Cytochrome *c* (80 μM) was added and the reaction was started by the addition of PMA (100 nM, arrow). The change of cytochrome *c* absorbance at 550 nm was then recorded. (C) Neutrophils (1.5×10^6 cells/ml) were resuspended in RPMI at 37 °C, and then preincubated with 8Br-AMP (1.5 mM) for 45 min, and further with AICAR (1 mM) for 30 min. Then, NaN_3 (1 mM), HRP (8.5 U/ml) and scopoletin (3 μM) were added and the production of H_2O_2 triggered by PMA was measured on the basis of fluorescence intensity for 20 min in a Wallac 1420 Victor^2 spectrofluorometer. Data are expressed as means ± S.E.M. of four separate experiments.

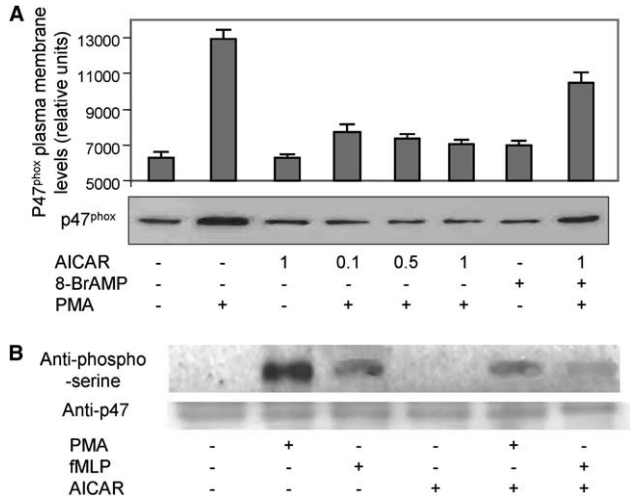

Fig. 4. Translocation of p47^{phox} to the plasma membrane (A) and its phosphorylation (B) in stimulated human neutrophils are inhibited by AICAR. Neutrophils (10^7 cells/ml) were preincubated with 8-Br-AMP (2 mM) where indicated for 45 min and then with AICAR (1 mM) for 30 min at 37 °C. Thereafter, the cells were treated with PMA (100 nM) (A) and PMA (100 nM) or fMLP (100 nM) (B). (A) Plasma-membrane fractions were separated electrophoretically and transferred to a PVDF membrane, which was then probed with anti-p47^{phox} antiserum, and the reactive bands detected by luminol-enhanced chemiluminescence. Relative p47^{phox} levels were determined by scanning densitometry analysis using the Scion Image software. (B) The cells were lysed and immunoprecipitation was carried out as indicated in Section 2. The blots are representatives of a set of three experiments yielding similar results.

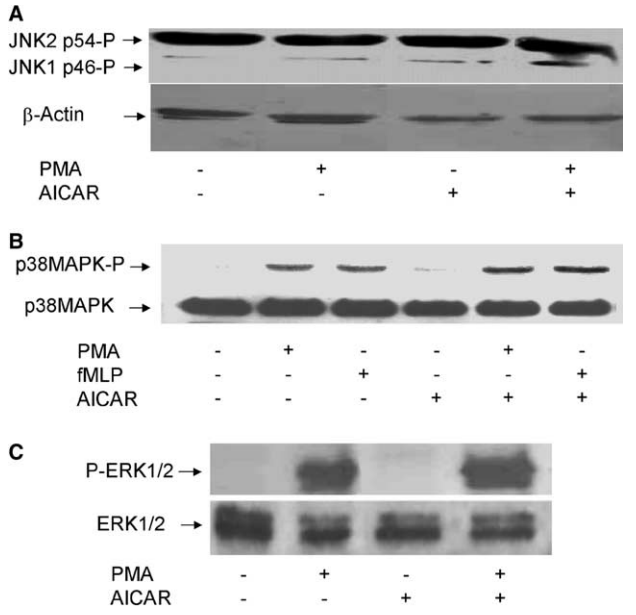

Fig. 5. AICAR induces JNK phosphorylation and potentiates PMA- and fMLP-induced p38 and ERK phosphorylation. Neutrophils (10^7 cells/ml) were resuspended in RPMI and preincubated with AICAR (1 mM) for 1 h, and then treated with PMA (100 nM) or fMLP (100 nM) for 10 min, as indicated. Cells were lysed, proteins were separated electrophoretically, transferred to a PVDF membrane and probed with anti-phospho-JNK1/2 (A), p38MAPK (B), and ERK1/2 (C). To verify even protein loading, the blots were subsequently stripped and reprobed with anti-β-actin, anti-p38MAPK and anti-ERKl/2 as shown. Each blot is representative of a set of 3 experiments yielding similar results.

essential for the activation of this enzyme. AICAR was able to reduce the PMA-stimulated p47^{phox} translocation in a dose-dependent manner (Fig. 4A). This AICAR effect was partially prevented by previous treatment with 8-BrAMP. However, neither AICAR nor 8-BrAMP exerted by itself any effect on p47^{phox} mobilization. The phosphorylation of p47^{phox} has been implicated in the process of activation of NADPH oxidase, during which the p47^{phox} subunit is extensively phosphorylated at 8–9 C-terminal serine residues [39]. In particular, the phosphorylation of S379 is necessary for both translocation of p47^{phox} and activation of the oxidase [40]. Therefore, we set to analyse the levels of p47^{phox} phosphorylation in serine residues. By means of immunoprecipitation experiment, we found that AICAR treatment significantly decreased p47^{phox} serine phosphorylation levels in both PMA- and fMLP-stimulated neutrophils (Fig. 4B).

With regard to the signal-transducing molecules involved in the AICAR inhibitory effect on AMPK-dependent ROS production, the relationship between NADPH oxidase and MAPK activation is well known [39]. It has been described that ERK and p38 MAPK phosphorylate p47^{phox} in human neutrophils [41] and that inhibitors of these kinases attenuate the neutrophil respiratory burst stimulated by a wide array of agonists [42,43]. Moreover, our group has shown that angiotensin II-dependent $O_2^{\cdot-}$ production becomes suppressed by specific inhibitors of the p38MAPK and ERK1/2 pathways [29]. In the present work, we found that JNK1 becomes phosphorylated upon AICAR treatment in human neutrophils (Fig. 5A). Moreover, this compound was able to enhance PMA-dependent phosphorylation of JNK1, although with no observable change in JNK2 phosphorylation. We also found that AICAR increased both PMA- and fMLP-dependent effects on p38MAPK phosphorylation, although AICAR exerted no effect by itself (Fig. 5B). A similar enhancing effect of AICAR on PMA-dependent ERK1/2 phosphorylation was also found (Fig. 5C).

Recently, we have reported that PD98059 and SB203580 inhibit angiotensin II-dependent ROS production [29] by human neutrophils. However, in the present work we observed that these MAPK inhibitors neither had any effect on the PMA-dependent ROS or H_2O_2 production (data not shown), nor did they affect the inhibitory effect of AICAR on these processes. These data suggested that AICAR inhibition of ROS and H_2O_2 production is exerted by mechanisms independent of MAPK activation.

4. Discussion

AMP-activated protein kinase is a downstream component of a protein kinase cascade acting as an intracellular energy sensor and responsible for maintaining the energy balance within the cell. Once activated, AMPK phosphorylates several downstream substrates, the overall effect of which is that ATP-consuming pathways are switched off and ATP-generating pathways are switched on [15]. Although the participation of activated-AMPK in energy processes has been described, its potential interaction with an energy-consuming system, such

as the neutrophil NADPH oxidase, had not been previously investigated. Present data illustrate that AMPK is located in the cytosol of human neutrophils and that becomes phosphorylated, and hence activated, upon AICAR or rotenone treatment. Moreover, the catalytic subunit of AMPK present in neutrophils is the AMPK-α. In this paper, we describe that the activation of AMPK promoted a decrease in the levels of total ROS, and specifically of $O_2^{\cdot-}$ and H_2O_2 release. These observations were in agreement with the fact that other compounds known to set as respiratory-chain inhibitors, such as oligomycin, antinomycin A and FCCP, also inhibited ROS production in neutrophils. We also found that AICAR inhibited both PMA- and fMLP-elicited $O_2^{\cdot-}$ production, this suggesting that AICAR could act at some common step of the signaling pathways triggered by PMA and fMLP. The inhibition of both $O_2^{\cdot-}$ and ROS production by AICAR ran parallel to the translocation to the plasma membrane and phosphorylation in serine residues of the $p47^{phox}$ subunit NADPH oxidase, which represent pivotal steps in the activation of this enzyme [4,5]. The specific participation of AMPK on NADPH oxidase inhibition was demonstrated by the fact that AICAR-dependent inhibition of $O_2^{\cdot-}$ production was canceled by 8-BrAMP, which acts as an inhibitor of AMPK. There is the possibility that an increase of 5′-AMP could enhance intracellular adenosine levels, a nucleotide previously shown to strongly inhibit ROS production by neutrophils when these are stimulated by fMLP, but not by PMA [44]. However, we here show that AMPK activation by 5′-AMP/AICAR inhibited ROS production in cells stimulated by either fMLP or PMA. Therefore, the observed inhibition by AICAR of NADPH oxidase activity is unlikely to be exerted by adenosine itself.

The functional significance of present findings could be that the inhibition of NADPH oxidase, in a status of low energy charge (i.e. high 5′-AMP levels), could contribute to save ATP. Early reports on phagocytic cells have reported an enhanced requirement of energy when these become activated during phagocytosis, or by treatment with PMA and lipopolysaccharides [23]. It is known that endogenous levels of ATP are depleted during phagocytosis [23,25], and hence the requirement of extra energy to provide NADPH as a substrate for the oxidase reaction during phagocytosis could be provided by an enhancement of glycogen degradation and glucose oxidation through glycolysis [23]. We have reported that activated peritoneal macrophages from *Escherichia coli*-treated rats showed an enhanced glycolytic rate, as measured on the basis of fructose 2,6-bisphosphate levels, a stimulator of this metabolic pathway, as well as by lactate production [45]. Moreover, the response to PMA of key glycolytic enzymes, such as 6-phosphofructo-1-kinase and 6-phosphofructo-2-kinase, was greater in activated macrophages as compared to resting macrophages from untreated animals [45]. It has been previously shown that AMPK is able to detect an increase of 5′-AMP intracellular levels and then enhances the cells capacity to produce ATP [15]. In this regard, it has been recently demonstrated that monocytes treated with lipopolysaccharides show increased fructose 2,6-bisphosphate levels, and that the activation of these cells by different stimuli required the activation of 6-phosphofructo-2-kinase by AMPK [16].

In summary, AMPK likely acts as an intracellular energy sensor responsible for maintaining the intracellular energy balance by activating those processes leading to ATP synthesis (e.g., glycolysis) and by reducing ATP-consuming reactions (e.g., $O_2^{\cdot-}$ production). Additionally, present data suggest that the activation of AMPK in states of low cellular energy charge is an additional factor in the modulation of the host defense mechanisms that become triggered upon $O_2^{\cdot-}$ release by phagocytic cells.

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