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# Controls of Nuclear Factor-Kappa B Signaling Activity by 5'-AMP-Activated Protein Kinase Activation With Examples in Human Bladder Cancer Cells

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Generally, both lipopolysaccharide (LPS)- and hypoxia-induced nuclear factor kappa B (NF-κB) effects are alleviated through differential posttranslational modification of NF-κB phosphorylation after pretreatment with 5′-AMP-activated protein kinase (AMPK) activators such as 5′-aminoimidazole-4-carboxamide ribonucleotide (AICAR) or the hypoglycemic agent metformin. We found that AICAR or metformin acts as a regulator of LPS/NF-κB-or hypoxia/NF-κB-mediated cyclooxygenase induction by an AMPK-dependent mechanism with interactions between p65-NF-κB phosphorylation and acetylation, including in a human bladder cancer cell line (T24). In summary, we highlighted the regulatory interactions of AMPK activity on NF-κB induction, particularly in posttranslational phosphorylation and acetylation of NF-κB under inflammatory conditions or hypoxia environment.

**Keywords:** NF-kappa B; AMP-Activated Protein Kinases; Lipopolysaccharides; Hypoxia

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## NUCLEAR FACTOR-KAPPA B ACTIVATION: PHOSPHORYLATION AND ACETYLATION

The transcriptional factor nuclear factor kappa B (NF-κB) is a main regulator of the induction of several genes including inflammatory and immune response genes and the proliferation and death of cancer cells [1-4]. Cellular stimulation by pro-inflammatory cytokines, gram-negative bacterial lipopolysaccharide (LPS), viral infection, radiation, and hypoxia is induced by IκB kinase (IKK) complex-mediated IκB phosphorylation (predominant form, IKKβ) and ubiquitination, and degradation [5-10]. The mammalian NF-κB family consists of 5 subunits, p50-NF-κB1, p52-NF-κB2, c-Rel, p65-RelA, and RelB, which forms homo- or heterodimers of functional complexes.

NF-κB activity is regulated by its subcellular localization in the cytoplasm or nucleus. The NF-κB dimer is mainly sequestered in the cytoplasm as a complex associated with the IκB inhibitor in unstimulated resting cells, or rarely in the nucleus as a transcriptionally inactive complex shuttle [11]. In activated cells, IκB-free NF-κB translocates into the nucleus where it acti-

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vates the transcription of several genes, including newly synthesized IκB. In addition to translocation of NF-κB, the transcriptional activities of NF-κB are regulated by posttranslational modifications such as phosphorylation and acetylation of p65-NF-κB subunit for full activation [12-14]. The transcriptional activities of p65-NF-κB, which are specifically targeted by several kinases, are highly enhanced by phosphorylation of $Ser^{536}$, or $Ser^{276}$, and $Ser^{311}$ (by zetaPKC) in association with the coactivator CBP/p300 [15,16]. Additionally, Anrather et al. [17] reported 3 phosphorylation sites at $Ser^{205}$ (induced by LPS), $Ser^{276}$ (by MSK1), and $Ser^{281}$ (by LPS and unknown kinase) as essential phosphorylation sites for transcriptional activity.

Phosphorylation of p65-NF-κB at $Ser^{536}$ leads to nuclear translocation or cytoplasmic priming, and the transactivation mechanism is the target of several kinases including the important kinase IKKβ [18-20]. Phosphorylation of $Ser^{281}$ has been studied with other related phosphorylation sites, including $Ser^{205}$ and $Ser^{276}$, to evaluate transactivation [17,21-23]. Anrather et al. [17] identified $Ser^{205}$, $Ser^{276}$, and $Ser^{281}$ as potential phospho-acceptor sites within the p65 Rel homology domain, and found that both $Ser^{205}$ and $Ser^{276}$ can be mediated by Ser/Thr kinases, but $Ser^{281}$ Ser-specific kinases alter transcriptional NF-κB activities when Ser is substituted with Thr. They suggested that the phosphorylation levels of these potential sites can affect the interaction of NF-κB with coactivators, and to acetylation patterns for the full NF-κB transcriptional activities. Additionally, p65-NF-κB phosphorylation levels reflect the differential NF-κB transcriptional activity of related gene subsets; however, phosphorylation is not essential for NF-κB DNA binding [23]. It was previously shown that the transcriptional coactivators p300 and CBP mainly acetylate p65-NF-κB $Lys^{218}$, $Lys^{221}$, and $Lys^{310n}$ [24,25]. Particularly, p65-NF-κB $Lys^{310}$ acetylation stimulates the full transcriptional activity of p65-NF-κB, but is only minimally related to DNA binding or IκB assembly.

## AMPK ACTIVATION

5'-AMP-activated protein kinase (AMPK) is a highly conserved serine/threonine protein kinase that regulates energy homeostasis and metabolic stress, and exists in all eukaryotic cells as heterotrimeric complexes comprising catalytic α-subunits and regulatory β- and γ-subunits. Phosphorylation of the $Thr^{172}$ residue of the α subunit is important for maximum AMPK activity [26-28]. Three upstream kinases have been shown to activate AMPK. LKB1 stimulates AMPK in response to changes in the cellular AMP/ATP ratio, calmodulin-dependent protein kinase kinase β in response to intracellular $Ca^{2+}$ concentration, and transforming growth factor-beta-activating kinase 1 by immunological cytokines [29-32].

## INTERACTIONS BETWEEN NF-κB INDUCTION AND AMPK ACTIVATION

In recent years, numerous studies have reported the effects of AMPK activities on inflammatory NF-κB activity [33-48]. The major function of AMPK in inhibiting inflammation has been demonstrated using a known AMPK activator and the pharmacological mimetic 5′-aminoimidazole-4-carboxamide ribonucleotide (AICAR). Other studies showed that AICAR inhibits tumor necrosis factor (TNF)-α and interleukin-β-induced NF-κB activities in immune cells [49-52] and inducible nitric oxide synthase and cyclooxygenase (COX-2) expression levels in LPS- or cytokine-stimulated myocytes, adipocytes, or macrophages grown in culture [53,54]. However, the anti-inflammatory effects of AICAR were also found to be AMPK-independent or nonspecific activators of AMPK in several studies [52,55].

In addition to AICAR, there are several AMPK activators; metformin, used to treat type 2 diabetes; berberine, a natural product used in traditional Chinese medicine; and A-769662, derived from a high-throughput screen for AMPK activators [56,57]. Aspirin and salicylate also inhibit the inflammatory NF-κB pathway, and it has been proposed that this results from inhibition of the upstream kinase IKK-β [58]. However, they suggested that inhibition of the NF-κB pathway is mediated by AMPK activation, rather than by direct inhibition of IKK-β. Overall, all AMPK activators described above have been reported to inhibit inflammatory responses in various model systems.

### Example 1 (Unpublished): Effects of AMPK Activator on LPS- or Hypoxia-Induced NF-κB Phosphorylation and Acetylation Activities in the Human Bladder Cancer Cell Line T24

We recently investigated the effects of LPS and the AMPK activator AICAR on COX-2 induction, two specific p65-NF-κB phospho-activities ($Ser^{536}$ and $Ser^{281}$), and the acetylation activity of p65-NF-κB $Lys^{310}$. Particularly, we proposed that the expression levels of p65-NF-κB $Ser^{281}$ phosphorylation and p65-NF-κB $Lys^{310}$ acetylation were inversed, suggesting potential inhibitory activity of p65-NF-κB $Ser^{281}$ phosphorylation. Both LPS- and hypoxia-induced NF-κB activities in a human bladder can-

cer line T24 were alleviated by pretreatment with AICAR. Additionally, AMPK siRNA-mediated suppression enhanced NF-κB-mediated COX-2 induction by LPS or hypoxia. Particularly, we suggested that direct interactions and colocalization occur between p-AMPK (at phospho-activation site $Thr^{172}$) (p-AMPK) and IκBα-free NF-κB, especially in nucleus. LPS-induced full transcriptional activity of NF-κB, as indicated by a critical acetylation level (Ac-K310 p65-NF-κB), was decreased by AICAR pretreatment, whereas the phosphorylation level at p65-NF-κB $Ser^{281}$ was increased [35].

### Example 2 (Unpublished): Transient Inactivation of AMPK and ROS Participation After LPS Treatment in the Human Bladder Cancer Line T24

We also found that AMPK phosphorylation as well as the ability of AICAR to enhance phosphorylation of AMPK was decreased only at the early time (~1 hour) after LPS stimulation. This effect of LPS stimulation on p-AMPK levels was abolished or showed a greater increase at the later time (after 16 hours). Recently, Sag et al. [59] and Tadie et al. [60] demonstrated that the transient suppression of AMPK phosphorylation diminished the ability of AICAR to increase AMPK phosphorylation in LPS-stimulated cells. In particular, Tadie et al. [60] suggested that HMGB1 released form injured or necrotic cells was involved in decreasing LPS-treated AMPK phospho-activity based on their results showing an inverse relationship between accumulated HMGB1 in cytoplasm and AMPK phosphorylation levels. Furthermore, we examined *N*-acetyl cystein (NAC) pretreatment under the above experimental conditions to immediately inhibit reactive oxygen species (ROS) release at the early time, which resulted in increased p-AMPK levels and decreased COX-2 induction. Thus, our finding may also be explained by the influence of early released ROS on AMPK phospho-activity in LPS-stimulated bladder cancer cells.

Based on our findings, AICAR pretreatment partially decreased both LPS- and hypoxia-treated COX-2 induction. Additionally, LPS-induced NF-κB p65 $Ser^{536}$ phospho-activity was decreased by AICAR pretreatment, but highly increased by AMPK-siRNA, suggesting an AMPK-dependent mechanism. Consistent with this result, recent studies reported the ability of AICAR to suppress NF-κB activation in response to LPS or pro-inflammatory cytokines through an AMPK-dependent or -independent mechanism [52,59-61].

### Example 3 (Unpublished): Differential Activities of AMPK and Activation of NF-κB Signaling Pathways Under Inflammatory or Hypoxia Conditions

It is generally known that the TLR4/NF-κB signaling pathway is activated under hypoxic conditions, increasing the gene expression of downstream inflammatory mediators [7,8,57,62-67]. Additionally, hypoxia induces AMPK activation in cancer cells as a survival mechanism by ATP-depletion (Laderoute et al. [66], 2006; Miller et al. [67], 2008; Kim et al. [57], 2012). In contrast, the phospho-activities of the serial enzymes p-LKB1, p-AMPK, and p-ACC in our study were time-dependently diminished under hypoxia conditions and HIF1α expression was increased (unpublished). In addition, p-AMPK levels after LPS, AICAR, or NAC treatment and even in untreated cells were decreased under hypoxia conditions, and hypoxia-induced COX-2 expression was synergistically enhanced by additional treatment with LPS and blocked or decreased by AICAR pretreatment; these results are similar to those of other recent reports [9,10].

## CONCLUSIONS

In this short review, we highlighted the regulatory interactions of AMPK activity on NF-κB induction, particularly in post-translational phosphorylation and acetylation of NF-κB under inflammatory conditions or in hypoxia environments, providing examples in the human bladder cancer cell line T24.

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