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Akt-mediated signaling is induced by cytokines and cyclic adenosine monophosphate and suppresses hepatocyte inducible nitric oxide synthase expression independent of MAPK P44/42

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ABSTRACT

Cyclic AMP inhibits the expression of nitric oxide synthase (Harbrecht et al., 1995 [1]) in hepatocytes but the mechanism for this effect is incompletely understood. Cyclic AMP can activate several intracellular signaling pathways in hepatocytes including Protein Kinase A (PKA), cAMP regulated guanine nucleotide exchange factors (cAMP-GEFs), and calcium-mediated Protein Kinases. There is considerable overlap and cross-talk between many of these signaling pathways, however, and how these cascades regulate hepatocyte iNOS is not known. We hypothesized that Akt mediates the effect of cAMP on hepatocyte iNOS expression. Hepatocytes cultured with cytokines and dbcAMP increased Akt phosphorylation up to 2 h of culture. Akt phosphorylation was inhibited by the PI3K inhibitor LY294002 (10 μM), farnyltranferase inhibitor FTI-276, or transfection with a dominant negative Akt. The cyclic AMP-induced suppression of cytokine-stimulated iNOS was partially reversed by LY294002 and FTI-276. LY294002 also increased NFκB nucleus translocation by Western blot analysis in nuclear extracts. Cyclic AMP increased phosphorylation of Raf1 at serine 259 which was blocked by LY294002 and associated with decreased MAPK P44/42 phosphorylation. However, inhibition of MAPK P44/42 signaling with PD98059 failed to suppress cytokine-induced hepatocyte iNOS expression and did not enhance the inhibitory effect of dbcAMP on iNOS production. A constitutively active MAPK P44/42 plasmid had no effect on cytokine-stimulated NO production. These data demonstrate that dbcAMP regulates hepatocyte iNOS expression through an Akt-mediated signaling mechanism that is independent of MAPK P44/42.

1. Introduction

Hepatocyte nitric oxide synthase [1] expression is an integral part of the response to a variety of proinflammatory stimuli such as hemorrhagic shock and sepsis [1–4]. Excessive nitric oxide (NO) from induced NOS (iNOS) can produce hepatic injury and hepatic dysfunction while global elimination of all NO synthesis in vivo inhibits vascular perfusion and other important physiologic and cellular pathways [3–7]. Therefore, fully understanding the regulation and function of NO is necessary to understand hepatic physiology and function in shock and sepsis. A more thorough understanding of the role of NO synthesis in these states is essential if this pathway is to be manipulated for therapeutic purposes.

We have shown that glucagon regulates hepatic NOS both in vitro and in vivo [8–10]. Glucagon receptors lead to calcium mobilization and stimulation of adenylate cyclase to activate a number of downstream signaling cascades, protein kinases, and phosphatases that ultimately regulate intracellular gene expression [11–14]. In hepatocytes, the regulation of iNOS by glucagon appears to be due primarily to the actions of the second messenger cAMP and subsequent alterations in NF-κB and JNK [9,15]. However, other signaling pathways can be regulated by glucagon and cAMP that may also contribute to iNOS regulation. Cyclic AMP regulates mitogen activated protein (MAP) kinase activity and can either increase or decrease MAP kinase signaling depending on the different cell type [16,17]. Cyclic AMP regulates Akt phosphorylation and activation in hepatocytes [18–20]. In hepatocytes, camp decreased MAPK p44/42activation induced by epidermal growth factor [17,21,22]. cAMP also decreased MAPK p44/42activity in primary hepatocytes independent of PI3K or Akt [23] but has no effect on MAPK p44/42 in hepatoma cells [24]. Both MAP kinase and Akt exert strong regulatory effects on hepatocyte gene expression and hepatocellular function [25–30] but the role of Akt and MAP kinase, specifically MAPK p44/42, in mediating the effects of cAMP on hepatocyte iNOS expression is unknown. We therefore investigated the role of Akt in mediating the inhibition of iNOS expression produced by cAMP. Our data demonstrate that Akt regulates, in part, hepatocyte iNOS expression following exposure to cytokines and dbcAMP but does so independent of MAPK p44/42activation.

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2. Materials and methods

2.1. Materials

Williams Medium E, penicillin, streptomycin, L-glutamine and HEPES were all from Invitrogen Corporation (Carlsbad, CA). Insulin was from Lilly (Indianapolis, IN). Polyclonal antibodies to iNOS, NFκB and IkBα were purchased from BD Bioscience (Billerica, MA). Antibodies to Raf1 (phosphorylated at serine 259 and total), Akt (total and phosphorylated at serine 473), MEK1/2 (total and phosphorylated at ser 217/ser 221), MAPK p44/42(total and phosphorylated at Thr 203/Thr 204), and actin were purchased from Cell Signaling Technology (Danvers, MA). Antibody to PCNA was purchased from Santa Cruz biotechnology (Santa Cruz, CA). LY294002 (10 mM liquid product in DMSO) and PD98059 (dissolved and re-suspended with DMSO to different concentration at 5, 10, 15 and 20 mM) were purchased from Calbiochem (San Diego, CA). Human recombinant interleukin 1β (IL-1β) was from Dupont (Boston, MA) and murine recombinant interferon γ (IFN) was from Invitrogen Life Technologies (Carlsbad, CA). The plasmid expressing dominant negative Akt was provided by Drs. Burgering and Triest from Utrecht University, Belgium. The plasmid expressing constitutively active MAPK P44/42 was provided by Dr. Wang from the University of Pittsburgh. FTI-276 (re-suspended to 10 mM with water) and all other reagents were from Sigma (St. Louis, MO).

2.2. Primary hepatocyte isolation and culture

Primary hepatocytes were isolated from male Sprague–Dawley rats (200–250 g) using the modified collagenase perfusion technique as previously described [31]. Purified hepatocytes (>98% pure with >95% viability by trypan blue exclusion) were cultured onto collagen-coated 100 mm dishes in Williams Medium E with L-arginine (0.5 mM), L-glutamine (2 mM), HEPES (15 mM) penicillin, streptomycin and 10% low endotoxin calf serum (HyClone Laboratories, Logan, UT) for 4 h, then cells were washed to remove non-attached cells and media was replaced with insulin-free media with 5% calf serum. After 16 h of further incubation, the experimental conditions were established. Conditions were performed in duplicate or triplicate and experiments were repeated three times to ensure reproducibility.

2.3. Preparation of nuclear extract

A modification of the method by Schreiber et al. [32] was used for preparation of primary hepatocyte nuclear extracts. Primary hepatocytes were washed with cold phosphate-buffered saline (PBS). After centrifugation, the cell pellets were frozen at −80 °C for at least 1 h and resuspended in 500 μl buffer A (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5% NP40). After 10 min of incubation on ice, cells were centrifuged for 10 min at 6000 rpm. Nuclei were then washed with 500 μl buffer A without NP40 and centrifuged for 10 min at 6000 rpm. The supernatant was collected as cytosolic protein and the pellet was then resuspended and salt extracted in 200 μl buffer B (20 mM HEPES [pH 7.9], 25% glycerol [v/v], 1.5 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid [EDTA], 0.5 M NaCl) on ice for 30 min. All buffers included 0.5 mM DTT (dithiothreitol), 0.2 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml chymostatin. After centrifugation at 20,000×g for 30 min, supernatants were collected, aliquoted, and stored at −80 °C. Protein concentration was measured using bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Company, Rockford, IL).

2.4. Western blot

Hepatocytes were washed with ice-cold PBS and then scraped from the plate in 500 μl of lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2-EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF). After 30 min at 4 °C, the lysates were centrifuged (15,000×g for 15 min) and stored at −80 ° C until use. Proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific binding was blocked with TBS-T (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk for 1 h. Primary antibodies were diluted and incubated with membranes for 1–2 h at room temperature or overnight at 4 °C with agitation. After washing three times with TBS-T, secondary antibodies were incubated at 1:10,000 dilution for 1 h. After 5 additional washes with TBS-T, the bands were visualized with chemiluminescence according to the manufacturer's instructions.

2.5. Plasmid transfection

Hepatocytes were plated in 6-well plates (4×10^5cells/well) and transfected with plasmids as previously described [15]. Briefly, plasmids were transfected using LipofectAMINE for 6 h, allowed to recover overnight, and the experimental conditions were established. The transfection efficiency by this method is approximately 30% for primary hepatocytes.

2.6. NO measurement

Supernatent NO_2^- was measured as an index of NO production by the Griess reaction as described [10]. Data are presented as the mean ± SD and analysis of variance (ANOVA) was used to determine statistical significance.

3. Results

3.1. cAMP increases Akt phosphorylation

Cyclic AMP increased Akt phosphorylation in cultured hepatocytes [18–20] but not in hepatoma cells [24]. To investigate the role of Akt signaling in hepatocyte iNOS expression, we measured Akt phosphorylation in the presence and absence of dbcAMP in cultured hepatocytes exposed to proinflammatory cytokines (IL-1β + IFNγ). Both dbcAMP and IL-1β + IFNγ increased Akt phosphorylation but the greatest stimulation of Akt occurred in hepatocytes stimulated with IL-1β + IFNγ and dbcAMP (Fig. 1). dbcAMP induced a prolonged Akt phosphorylation lasting up to 16 h of culture, while the IL-1β + IFNγ -induced Akt phosphorylation was brief.

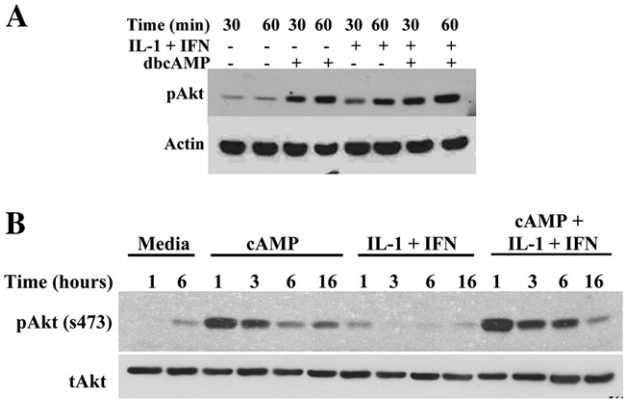

Fig. 1. IL-1β + IFNγ and dbcAMP induce Akt phosphorylation in hepatocytes. Hepatocytes were treated with IL-1β (200 U/ml) + IFNγ (100 U/ml) with or without dbcAMP (0.5 mM) and cells were harvested at the indicated time point for Western blot analysis. A: Early time point. B: Late time point. The blots shown are representative of three independent experiments.

3.2. Akt mediates the effect of cAMP on iNOS

Akt is phosphorylated by the activation of upstream kinases such as PI3K [20,25,33] To test the hypothesis that cAMP-induced activation of Akt regulates hepatocyte iNOS expression, isolated hepatocytes were stimulated to induce NOS by IL-1β + IFNγ in the presence and absence of dbcAMP (0.5 mM). LY294002 (10 μM) was used to inhibit Akt activation. IL-1β + IFN increased hepatocyte iNOS expression and this expression was inhibited by dbcAMP (Fig. 2). LY294002 partially reversed the inhibitory effect of dbcAMP on iNOS expression (Fig. 2A) and NO_2^- accumulation (Fig. 2B), while significantly inhibiting Akt phosphorylation (Fig. 2C). LY294002 was also associated with a slight increase in iNOS expression and NO_2^- accumulation in IL-1β + IFN-treated hepatocytes (Fig. 2). To confirm the results of this experiment, we transfected hepatocytes with a dominant negative Akt plasmid and then stimulated them with IL-1β + IFN in the presence and absence of dbcAMP. Overexpression of a dominant negative Akt decreased dbcAMP-induced Akt phosphorylation (Fig. 2D) and partially reversed the inhibiting effect of dbcAMP on cytokine-stimulated hepatocyte NO_2^- production (Fig. 2E). The concentration of dbcAMP (0.5 mM) used in these experiments suppressed NO production as measured by the Griess reaction (data not shown) as previously demonstrated [8,10].

3.3. Ras mediates the effect of cAMP on iNOS

Akt can also be phosphorylated by Ras [34,35]. Akt phosphorylation induced by IL-1β + IFNγ and dbcAMP was reduced by culturing hepatocytes with a farnesyltransferase inhibitor (FTI-276) 10 μM to inhibit Ras activity (Fig. 3A). Inhibiting Ras with FTI (10 μM) partially reversed the inhibitory effect of dbcAMP on IL-1β + IFNγ -induced hepatocyte iNOS expression (Fig. 3B).

3.4. cAMP decreases Raf1 and MAPK p44/42activation

Akt activation can alter the activity of downstream signaling pathways such as NF-κB and MAPK P44/42[19,25,36–40]. MAPK P44/42activation mediates the up regulation of iNOS expression by cytokines in macrophages [41], vascular smooth muscle cells [42], and cardiac myocytes [43]. We therefore tested the hypothesis that Akt activation regulates hepatocyte iNOS expression through regulation of the Raf/MEK/MAPK P44/42pathway. Since Akt phosphorylates Raf1 at serine 259 (pRaf1) to inhibit Raf/MEK/MAPK P44/42 signaling [44], we first measured pRaf1 phosphorylation at serine 259 in hepatocytes. Hepatocytes cultured alone or with IL-lβ + IFN had little pRaf-1 immunoreactivity (Fig. 4A). Incubation of hepatocytes with dbcAMP (0.5 mM) increased pRaf1 which was increased further in hepatocytes cultured with dbcAMP and IL-lβ + IFN (Fig. 4A). cAMP also decreased IL-1β + IFN-induced phosphorylation of MEK (Fig. 4B) and MAPK p44/42(Fig. 5A) which is consistent with the work of others [17,21–23].

3.5. cAMP-induced MAPK P44/42 inhibition does not inhibit hepatocyte iNOS

To directly test the role of MAPK P44/42 signaling on hepatocyte iNOS expression, we stimulated hepatocytes to produce iNOS with IL-1β + IFN in the presence and absence of the MEK inhibitor PD98059 to decrease MAPK P44/42activity. PD98059 decreased IL-lβ + IFN-induced MAPK P44/42 phosphorylation but did not alter IL-lβ + IFN-induced NO_2^-

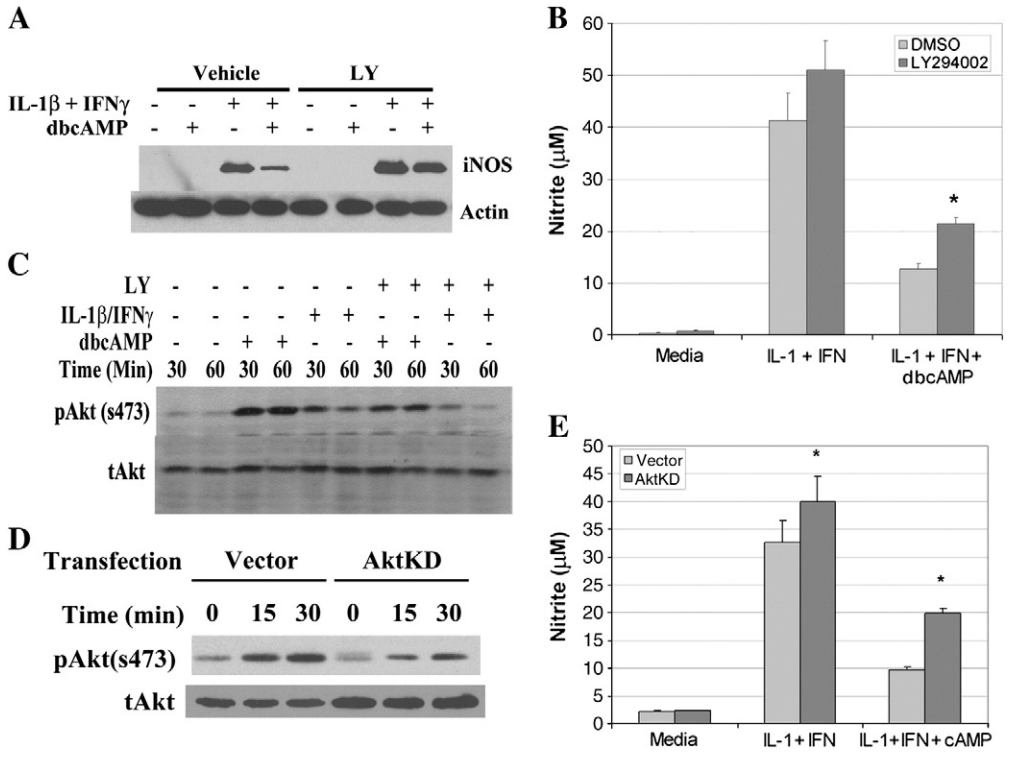

Fig. 2. PI3K mediates the effects of cAMP on Akt phosphorylation and iNOS production. A: Hepatocytes were pre-incubated with 10 μM of the PI3K inhibitor, LY 294002, and then stimulated with IL-1β + IFNγ with or without 0.5 mM dbcAMP. After 24 h, cell lysates were subjected to Western blot analysis using a specific iNOS antibody. The blot shown is representative of three independent experiments. B: Hepatocytes were treated as in A and superantants analyzed for nitrite using the Griess assay. C. Hepatocytes were pre-incubated with 10 μM P13K inhibitor (LY294002) for 30 min, and then stimulated with IL-1β + IFNγ with or without 0.5 mM dbcAMP for 30 and 60 min. Cells were lysed for Western blot analysis and probed with antibodies against phosphorylated Akt (s473) and total Akt. The blot shown is representative of three independent experiments. D. Hepatocytes were transfected with a dominant negative Akt expression plasmid (AktKD) for 24 h and stimulated with 0.5 mM dbcAMP for 0, 15 or 30 min. The cell lysates were subjected to Western blot with Akt antibody. The blot shown is representative of three independent experiments. E: Hepatocytes were transfected with a plasmid that expresses a dominant negative Akt (Akt KD). The cells were stimulated with IL-1β + IFN with or without 0.5 mM dbcAMP. After 24 h, the supernatant was analyzed for nitrite using the Griess assay. In B and E, the data represent the mean ± S.D. from three independent experiments. $^{**}p < 0.05$ versus vector transfection group, n = 6.

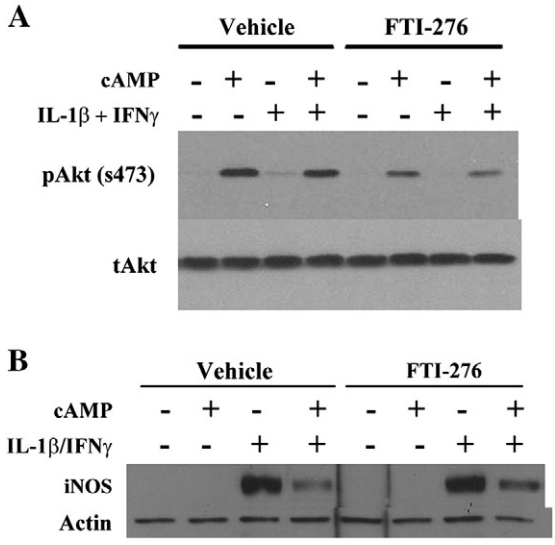

Fig. 3. Ras is involved in cAMP-induced Akt phosphorylation and its inhibitory effect on iNOS expression. A: Hepatocytes were pre-incubated with 10 μM farnesyltransferase inhibitor FTI-276 (dissolved in water accounting to manufacturers' instruction) for 30 min, and then stimulated with IL-1β + IFNγ with or without 0.5 mM dbcAMP for 60 min. Cells were lysed for Western blot analysis and probed with antibodies against phosphorylated Akt (s473) and total Akt. B: Hepatocytes were pre-incubated with 10 μM of the Ras inhibitor, FTI-276, and then stimulated with IL-1β + IFNγ with or without 0.5 mM dbcAMP. After 24 h, cell lysates were subjected to Western blot analysis using a specific iNOS antibody. The blots shown are representative of three independent experiments.

production and iNOS expression (Fig. 5B). PD98059 did not enhance the effect of dbcAMP on iNOS expression and production. We confirmed the effect of PD98059 on iNOS regulation by transfecting hepatocytes with a plasmid that expresses constitutively active MAPK P44/42 (Fig. 5C). Overexpressing MAPK P44/42 had no effect on cytokine-stimulated iNOS activation or on the inhibition of iNOS produced by dbcAMP (Fig. 5C). These data demonstrate that the cytokine- and dbcAMP-induced activation of Akt decreases MAPK P44/42in cultured hepatocytes but that MAPK P44/42does not mediate the inhibition of iNOS expression produced by cAMP.

3.6. PI3K inhibitor affects NFκB nucleus translocation

We previously showed that cAMP decreased IL-lβ + IFNγ-induced NFκB DNA binding and p65 translocation [9,45]. To test if cAMP/Akt signaling mediates the effect of cAMP on p65 translocation, hepatocytes were pre-incubated with the PI3K inhibitor to attenuate Akt activation and then treated with IL-lβ + IFNγ with or without dbcAMP. The nuclear extracts and cytosolic proteins were prepared after 60 min and subjected to Western blot analysis (Fig. 6A). Similar to our previous work [9,45], IL-lβ + IFNγ increased p65 in nuclear extracts and this was reduced by dbcAMP (Fig. 6A and B). Inhibition of PI3K and Akt signaling with LY294002 increased p65 in the nucleus in IL-lβ + IFNγ-stimulated hepatocytes and reversed the dbcAMP-induced suppression of nuclear p65 (Fig. 6A and B).

4. Discussion and conclusion

Hepatocyte iNOS expression is regulated primarily by control of gene transcription [46]. While several transcription factors that regulate iNOS expression have been identified [46–48], the intracellular signaling pathways leading to iNOS expression and their specific mechanisms are incompletely defined. We have shown that increasing cAMP suppresses cytokine-induced iNOS expression and NO synthesis in hepatocytes both in vitro and in vivo [8–10].

In hepatocytes, cAMP regulates gene expression and cell function in via several downstream signaling mechanisms. cAMP induced hepatic HO-1 gene transcription in a PKA-dependent manner [49,50] and induced glucose output through Ca^{2+} [51]. PKA is the most thoroughly studied pathway in hepatocytes mediating cAMP-induced changes in gene expression [11–14]. The effect of cAMP on iNOS in hepatocytes is primarily PKA-independent and involves inhibition of NF-κB DNA binding and an increase in JNK activation [9,15], but cAMP also regulates Akt and MAP kinase signaling [16,17,20]. We therefore investigated the role of Akt and MAPK P44/42 in regulating the inhibitory effects of cAMP on hepatocyte iNOS expression. Our data demonstrate that dbcAMP and IL-1β + IFN induce a sustained increase in Akt phosphorylation and activation. Both cytokine- and cAMP-induced Akt phosphorylation appear to involve PI3K and Ras since inhibiting both proximal kinases decreases Akt phosphorylation. Inhibition of PI3K and Ras also reversed the camp-induced suppression of iNOS expression. MAP kinases regulate iNOS expression in other cell types [41–43] and are themselves regulated by Akt [37–39]. Despite demonstrating that cAMP-induced Akt activation regulates Raf/MEK/MAPK P44/42 signaling in hepatocytes, however, we could find no role for MAPK P44/42 activation in the regulation of iNOS expression by cytokines or dbcAMP. MAPK P44/42 can regulate the expression of a number of genes [27–29]. It is possible that other hepatocyte genes are regulated by Raf/MEK/MAPK P44/42 signaling and are altered by cAMP through this pathway but identifying those genes will require further investigation.

The specific mechanism for the regulation of iNOS expression by Akt has not yet been defined. Akt can regulate the activation of other signaling pathways besides Raf/MEK/MAPK P44/42including NF-κB and JNK [25,29,52,53]. Akt phosphorylation increases NF-κB

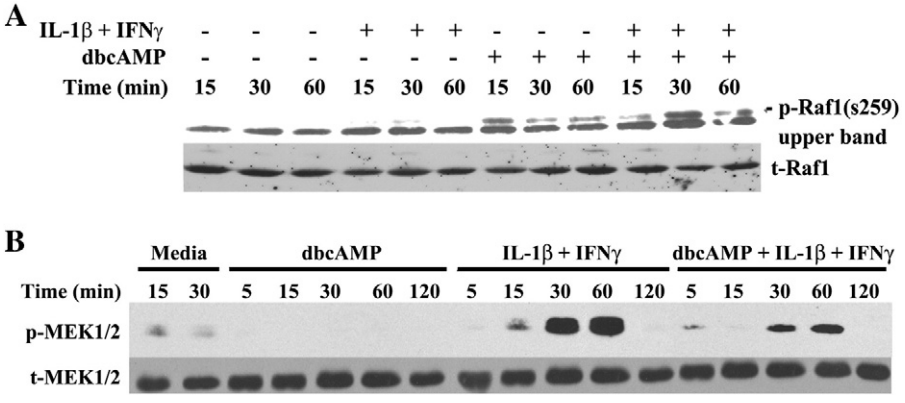

Fig. 4. dbcAMP increases hepatocyte Raf1 (s259) phosphorylation and decreases MEK1/2 phosphorylation. Hepatocytes were treated with IL-1β (200 U/ml) + IFN (100 U/ml) with or without dbcAMP (0.5 mM) for the indicated time and cell lysates were harvested for Western blot analysis. The membranes were probed with antibodies specific for p-Raf1 (ser259) (A), p-MEK1/2 and total MEK1/2 (B). The blots shown are representative of three independent experiments.

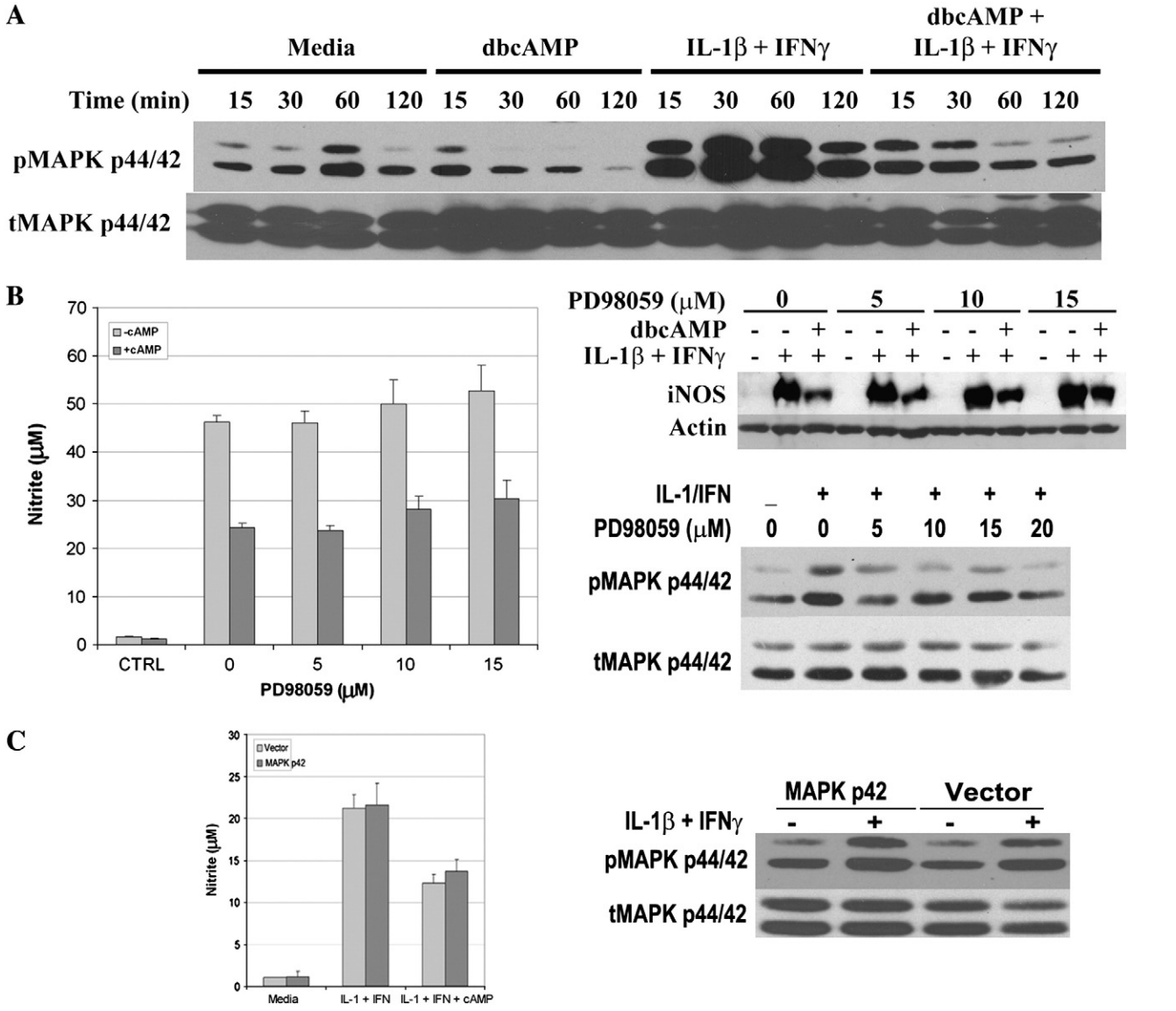

Fig. 5. Decreased hepatocyte MAPK P44/42phosphorylation by cAMP does not regulate hepatocyte iNOS expression. A: Hepatocytes were treated with IL-1β (200 U/ml) + IFN (100 U/ml) with or without dbcAMP (0.5 mM) for the indicated time and cell lysates were harvested for Western blot analysis. The membranes were probed with antibodies specific for and pMAPK P44/42 and tMAPK P44/42. B. Hepatocytes were incubated with PD98059 at indicated concentration for 30 min and then stimulated with IL-1β (200 U/ml) + IFN (100 U/ml) for 24 h for the Griess assay (left panel) and Western blot analysis to iNOS (right panel, upper) or 60 min for Western blot analysis with antibodies against phosphorylated MAPK P44/42 or total MAPK P44/42. C: Hepatocytes were transfected with a control plasmid (light gray) or plasmid that expresses a constitutively active MAPK P44/42 (dark gray) and then stimulated to produce NO in the presence or absence of dbcAMP (0.5 mM). Supernatants were collected after 24 h and analyzed for nitrite (left panel) or harvest cells after 60 min for Western blot analysis (right panel). Data indicate mean ± S.D. from three independent experiments, $n = 6$. The blots shown are representative of three independent experiments.

activation [25,40] and NF-κB binding is an important inducer of iNOS expression [46]. In our experiments, LY294002 increased NF-kB nucleus translocation and may partially mediate cAMP's effects on iNOS. This is consistent with our previous finding that dbcAMP produced a decrease in NF-κB DNA binding in cytokine-stimulated hepatocytes [9,45].

As discussed above, Akt also regulates JNK in some cells and can both downregulate [52–54] and upregulate [55] JNK activation. How Akt and JNK interact in hepatocytes and mediate inflammation-induced gene expression is unknown. Cytokines increase JNK phosphorylation and subsequent c-Jun-mediated gene regulation [15] in cultured hepatocytes. Cyclic AMP also increases JNK signaling and this pathway mediates, in part, the inhibition of iNOS expression by dbcAMP [15]. Whether cAMP regulates JNK activation through Akt is not yet known. There is evidence that cAMP activate PI3K/Akt signaling through cAMP-GEF pathway [18]. Akt can also regulate the activity, either directly or indirectly, of several transcription factors such as CREB, C/EBP, and Foxo1 that could potentially contribute to the regulation of iNOS expression [26,56,57]. Determining whether Akt regulates hepatocyte gene expression in general, and hepatocyte iNOS expression more specifically, through these transcription factors is currently being investigated.

Disclosures

The authors declare that there are no financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

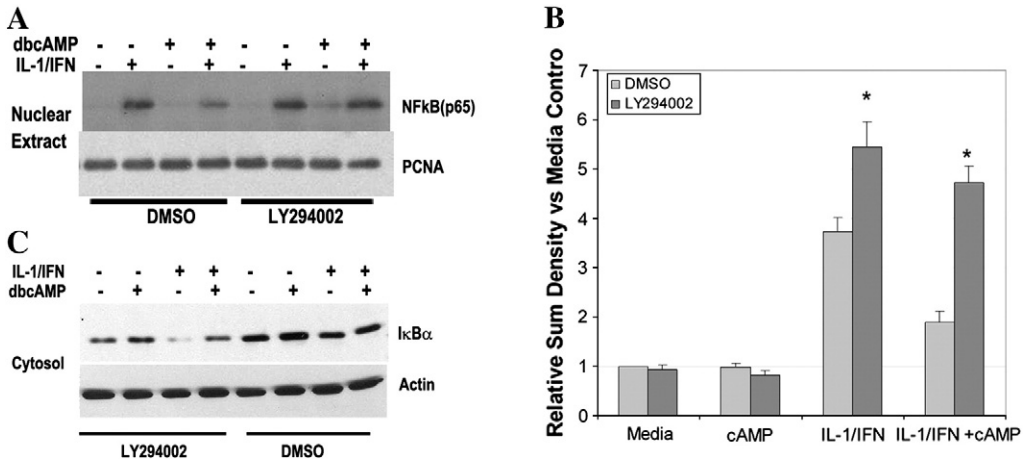

Fig. 6. PI3K inhibitor regulates NFκB nuclear translocation. Hepatocytes were pre-incubated with 10 μM LY294002 for 30 min and then stimulated with IL-1β (200 U/ml) + IFNγ (100 U/ml) with or without 0.5 mM dbcAMP. The cells were collected after 60 min for nuclear extracts and cytosolic proteins. Proteins were subject to Western blot analysis by polyclonal antibodies against NFκB (p65) (A) and IκBα (B) antibodies. B shows digitalized results from 3 blots of A. The ratios of sum density of p65 bands to PCNA bands were calculated and then normalized by Media with DMSO group. The data represent the mean ± S.D. from three independent scans, $^{*}p<0.05$ vs. DMSO. The blots shown are representative of three independent experiments.

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