Published in final edited form as:

Biochem Biophys Res Commun. 2012 June 22; 423(1): 85–90. doi:10.1016/j.bbrc.2012.05.087.

cAMP prevents TNF-induced apoptosis through inhibiting DISC complex formation in rat hepatocytes

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Abstract

Tumor necrosis factor α (TNF) is a pleiotropic proinflammatory cytokine that plays a role in immunity and the control of cell proliferation, cell differentiation, and apoptosis. The pleiotropic nature of TNF is due to the formation of different signaling complexes upon the binding of TNF to its receptor, TNF receptor type 1 (TNFR1). TNF induces apoptosis in various mammalian cells when the cells are co-treated with a transcription inhibitor like actinomycin D (ActD). When TNFR1 is activated, it recruits an adaptor protein, TNF receptor-associated protein with death domain (TRADD), through its cytoplasmic death effector domain (DED). TRADD, in turn, recruits other signaling proteins, including TNF receptor-associated protein 2 (TRAF2) and receptor-associated protein kinase (RIPK) 1, to form a complex. Subsequently, this complex combines with FADD and procaspase-8, converts into a death-inducing signaling complex (DISC) to induce apoptosis. Cyclic AMP (cAMP) is a second messenger that regulates various cellular processes such as cell proliferation, gene expression, and apoptosis. cAMP analogues are reported to act as anti-apoptotic agents in various cell types, including hepatocytes. We found that a cAMP analogue, dibutyryl cAMP (db-cAMP), inhibits TNF + ActD-induced apoptosis in rat hepatocytes. The protein kinase A (PKA) inhibitor KT-5720 reverses this inhibitory effect of cAMP on apoptosis. Cytoprotection by cAMP involves down-regulation of various apoptotic signal regulators like TRADD and FADD and inhibition of caspase-8 and caspase-3 cleavage. We also found that cAMP exerts its affect at the proximal level of TNF signaling by inhibiting the formation of the DISC complex upon the binding of TNF to TNFR1. In conclusion, our study shows that cAMP prevents TNF + ActD-induced apoptosis in rat hepatocytes by inhibiting DISC complex formation.

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Conflict of Interest

Authors do not have any financial relationship with the granting authority. The authors declare that they have no conflict of interest.

Keywords

Hepatocytes; TNF; db-cAMP; Apoptosis; DISC complex

1. Introduction

Tumor necrosis factor alpha (TNF) is a pleiotropic proinflammatory cytokine that mediates the inflammatory response and immune functions and triggers the apoptosis of certain tumor cells [1,2]. TNF is implicated in the excessive hepatocyte apoptosis seen in many conditions associated with damage in the liver [3,4]. Excessive production of TNF or the sustained activation of TNF signaling has been implicated in the pathogenesis of a wide spectrum of conditions in humans, including sepsis, cerebral malaria, diabetes, cancer, osteoporosis, allograft rejection, and autoimmune diseases [5-8]. The pleiotropic nature of TNF is due to the formation of different signaling complexes upon the binding of TNF to one of its receptors, TNF-receptor type 1 (TNFR1). TNFR1 is responsible for the pro-death signaling induced by TNF.

TNF signaling involves the binding of the TNF trimer to the extracellular domain of TNFR1 and the subsequent release of the inhibitory protein – silencer of death domains (SODD) – from the intracellular death domain (DD) of TNFR1. Once TNFR1 is engaged and activated, its DD rapidly binds to the adapter protein TNFR-associated death domain (TRADD), which subsequently recruits other signaling proteins such as TNFR-associated protein 2 (TRAF2) [9], receptor-associated protein kinase (RIPK)1, and cellular inhibitor of apoptosis proteins (cIAP)1 and cIAP2. The resulting complex then recruits the I κ B kinase (IKK) complex through the K63-specific polyubiquitin chain on RIPK1 and consequently triggers an NF- κ B response but does not lead to apoptosis. NF- κ B induces the activation of several anti-apoptotic proteins, including cIAP1, cIAP2 and c-FLIP that prevent apoptosis [10]. If the initially formed complex fails to initiate the expression of the anti-apoptotic factor c-FLIP through NF- κ B activation, then a second complex comprising FADD and procaspase-8 subsequently forms in the cytoplasm that leads to the formation of a death-inducing signaling complex (DISC) [11]. The activity of the second complex is inhibited by c-FLIP, a caspase-8 homologue that competes with caspase-8 for binding with FADD. The absence of c-FLIP induces FADD-mediated caspase-8 activation and apoptosis in the DISC of breast cancer cells [12]. Caspase-8-mediated activation of the mitochondrial death pathway through the cleavage of Bid plays a critical role in Aloe-emodin induced apoptosis of human nasopharyngeal carcinoma cells [13].

TNF-induced apoptosis in hepatocytes requires the addition of a sensitizing agent such as actinomycin D (Act D) or cytochrome p450. Eum et al. showed that the sensitizing agent ActD both accelerated and amplified the appearance of the DISC components in both cytosol and the mitochondria in TNF-treated hepatocytes [14]. Nevertheless, the molecular mechanisms of the shift toward apoptosis following sensitization are still not clear. It is known that cAMP prevents TNF + ActD-induced apoptosis in hepatocytes and that this occurs in association with a near complete inhibition of the upregulation of FADD via a PKA-dependent mechanism [15], but the effect of cAMP on DISC formation in hepatocytes is not known.

Cyclic adenosine monophosphate (cAMP) is an intracellular second messenger formed in response to diverse extracellular stimuli, including hormones or neurotransmitters, and its main targets of cAMP are protein kinase A (PKA) [16]. cAMP regulates numerous cellular processes, including gene expression, cell differentiation, cell cycle progression and apoptosis, both in a PKA-dependent and PKA-independent manner [17-19]. Some studies

indicate that analogues of cyclic nucleotides cause apoptosis in renal mesangial cells, multiple myeloma cells and human cancer cell-sandactivation of cAMP signaling enhances Fas-mediated apoptosis and activation-induced cell death through potentiation of caspase-8 activation [20-23]. In contrast, there have also been studies that have shown that cAMP analogues inhibit apoptosis induced by different stimuli in hepatocytes, neutrophils, smooth muscle cells, and pre-B acute lymphoblastic leukemia NALM-6 cells [15,24,25]. Previous studies have reported that cell permeable cyclic nucleotides inhibit apoptosis by modulating caspase activation, cytochrome c release, and cIAP expression, and inducing a heat shock protein. A previously study from our laboratory has also shown that cAMP prevents the elevations in FADD levels in response to TNF + ActD in hepatocytes [15]. FADD is elevated early following TNF + ActD exposure in hepatocytes suggesting that the effect of cAMP occurs at a proximal step in TNF-induced signaling. Here we assessed that the effect of cAMP on TNF + ActD-induced DISC formation in cultured hepatocytes.

2. Materials and methods

2.1. Materials

William's medium E, penicillin, streptomycin, L-glutamine and HEPES were purchased from Life Technologies Inc. Mouse recombinant TNF was obtained from R&D systems (Minneapolis, MN). Insulin was purchased from Eli Lilly (Indianapolis, IN). Benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone (Z-VAD-fmk), Sp-isomer ((Sp)-cAMPS), and KT5720 were from Alexis Corp (San Diego, CA). Actinomycin D, dibutyryl cyclic adenosine monophosphate (db-cAMP), and were obtained from Sigma (St. Louis, MO). Antibodies used in this study were purchased from BD Pharmingen for FADD, Sigma (St. Louis, MO) for β -actin, and cell signaling for caspase-8, 3, and RIPK, Biovision for caspase-8 used in immunoprecipitation (IP), Abcam for TNFR1 and caspase-8 and Santa-cruz for TRADD antibody. Anti-Rabbit Ig IP beads were bought from eBioscience. Horseradish peroxidase-linked streptavidin and Supersignal™ chemiluminescence detection reagents were from Pierce Chemical Co., (Rockford, IL). Unless indicated otherwise, all other chemicals were from Sigma.

2.2. Hepatocytes isolation and culture

Primary rat hepatocytes were isolated and purified from male Sprague Dawley rats (Harlan) and cultured as described previously. Highly purified hepatocytes (>98% purity and >95% viability by trypan blue exclusion) were suspended in Williams medium E supplemented with 10% calf serum, 2 mM L-glutamine, 15 mM HEPES, pH 7.4, 100 units/ml penicillin, and 100 μ g/ml streptomycin. The cells were plated on collagen-coated tissue culture plates at a density of 2×10^5 cells/well in 12-well plates for cell viability analysis, 3×10^6 cells/60 mm dishes for Western blotting assays and 5×10^6 cells/100 mm dishes for IP. After overnight incubation, the cells were washed and further cultured in fresh medium containing 5% calf serum in the presence of various reagents as specified in the figure legends.

Apoptosis was induced by incubating hepatocytes with culture medium containing 2000 units/ml TNF and 200 ng/ml ActD for specified monolayers. TNF + ActD were added to the cells 30 min prior to db-cAMP (200 μ M) treatment. For whole cell lysates, cells were washed with cold phosphate buffered saline (PBS) and resuspended in 5-fold volume of cell lysis buffer with protease inhibitors (Sigma). After three cycles of freeze-thaw, cell debris was removed by centrifugation at 13,000g at 4 °C for 20 min. The 13,000g supernatant is termed as whole cell lysate. Protein concentration was determined using BCA assay (Pierce, Rockford, Ill.) and whole cell lysates were processed for Western blot.

2.3. Cell viability assay

Cell viability was determined by the crystal violet method. In brief, cells were stained with 0.5% crystal violet in 30% ethanol and 3% formaldehyde for 5 min at room temperature followed with six times wash with tap water. Plates were left to dry at room temperature. After drying, cells were lysed with 10% sodium dodecyl sulfate (SDS) solution, and dye uptake was measured at 550 nM using a 96-well microplate reader. Cell viability was calculated from relative dye intensity of the mean for four parallel samples and presented as percentages relative to untreated samples.

2.4. Immunoblot analysis

Protein samples were separated on 12% SDS-PAGE and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked with Tris-Buffered Saline with 0.1% Tween-20 (TBS-T, pH 7.4) containing 5% nonfat milk for 1 h of incubation with agitation at room temperature. After primary antibody incubation, the membranes were washed three times with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Following five washes with TBS-T, the membranes were incubated with Supersignal™ (Pierce, Rockford, IL) according to the manufacturer's instructions. Chemiluminescence was visualized on Kodak film (Eastman Kodak).

2.5. Cell fractionation and electrophoretic mobility shift analysis (EMSA)

To assess effects of TNF on NF- κ B activation, electrophoretic mobility shift assay (EMSA) was performed. Nuclear extracts were prepared as described previously. Briefly, for cell fractionation procedures, protease inhibitors (Sigma) were added to all the buffers in the following concentrations: pefablock 0.2 mg/ml; aprotinin 0.01 mg/ml; pepstatin 0.01 mg/ml; leupeptin 0.01 mg/ml. Cells were rinsed twice with cold PBS then harvested by scraping into 1.0 ml ice-cold PBS. Cells were spun down at 4,000g for 3 min, supernatants discarded and pellet was resuspended in Buffer A (Hepes 0.1 M; MgCl₂ 1.5 $\times$ 10⁻³ M; KCl 0.01 M; Nonidet P-40, 0.5%) and incubated on ice for 10 min. Nuclei were pelleted by centrifugation at 5,000g for 5 min at 4 °C. The supernatant fraction from Buffer A was collected and classified as cytoplasmic extract. The pellet was resuspended in Buffer B (Hepes 0.1 M; MgCl₂ 1.5 $\times$ 10⁻³ M; KCl 0.01 M) and spun down at 5,000g for 5 min at 4 °C followed by washing with buffer B for another two times. The final washed pellet was collected as nuclear extract and was lysed by C + D buffer (Hepes 0.02 M; MgCl₂ 1.5 $\times$ 10⁻³ M, KCl 1.6 M; EDTA, 2 $\times$ 10⁻⁴ M; Glycerol 10%) and incubated on ice for 1–2 h. The supernatant fraction was collected by centrifugation at 15,000g for 15 min and classified as nuclear fraction. Protein concentration was determined in all nuclear fractions using a Pierce BCA protein assay reagent as per manufacturer's instructions. Immunoblot for tubulin (Upstate Biotechnology, NY, USA) was used to confirm nuclear isolation integrity. Then nuclear extracts prepared from treated cells were incubated with ³²P-end-labeled 45-mer double-stranded NF- κ B oligonucleotide (5 μ g of nuclear extract, 10 $\times$ binding buffer, 1 μ g/ μ l poly(dI-dC), with 16 fmol DNA) for 30 min at 37 °C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.5% native polyacrylamide gels. Dried gels were incubated with X-ray films (Eastman Kodak) radioactivity was visualized.

2.6. Co-immunoprecipitation and Western blot

Nuclear extract was incubated with either anti-TNFR1 or anti-caspase-8 antibody overnight at 4 °C, and immune complexes were precipitated with anti-rabbit Ig IP beads (eBioscience) at 4 °C for 2 h. After the beads were washed, the IP proteins were separated by SDS-PAGE on 10% gels and then transferred to nitrocellulose membranes. Blots were probed with anti-RIPK, anti-FADD, and anti-TRADD antibody. Protein bands were identified with horse-

radish peroxidase-conjugated secondary antibodies and Supersignal™ (Pierce, Rockford, IL) chemiluminescence detection kit.

2.7. Statistics

Data are expressed as means standard error of mean. All statistical evaluations were performed using one-way ANOVA, and a probability level of $P < 0.05$ was considered to be significant.

3. Results

3.1. cAMP inhibits cell death in rat hepatocytes

We first investigated the timing and extent of TNF + ActD-induced hepatocyte death in the absence and presence of db-cAMP. Cell viability was measured by crystal violet staining at time points between 0 h (control) and 18 h following treatment. No significant cell death occurred in hepatocytes treated with 200 μ M db-cAMP alone at any time point (Fig. 1A). TNF + ActD alone caused a significant degree of cell death by 8 h (Fig. 1A). However, the co-treatment of TNF + ActD with db-cAMP inhibited cell death measured 8 h after treatment (Fig. 1A). Additionally, sp-cAMP, a specific activator of PKA, also had no effect on viability when added alone, but suppressed TNF + ActD-induced hepatocyte death (Fig. 1B).

3.2. cAMP inhibits TNF/ActD-induced expression of TNFR1 DISC components

We next determined how the levels of proteins known to be involved in DISC complex formation including TRADD, FADD and cleaved caspase-8 by immunoblot analysis changed in our experimental model. After TNF/ActD treatment, the levels of the adapter proteins TRADD and FADD increased by 3 h (Fig. 2). Cleaved caspase-8 and caspase-3 were also observed within 3–6 h (Fig. 2). Addition of db-cAMP (200 μ M) blocked the increase in TRADD and FADD levels at 6 h, and blocked the cleavage of caspase-8 and caspase-3 (Fig. 2).

3.3. cAMP blocks TNF signaling at a proximal step

In addition to caspase-8 cleavage, TNF signaling through TNFR1 also leads to NF- κ B activation. EMSA was performed on nuclear extract to determine if cAMP also blocked NF- κ B activation. As shown in Fig. 3A, TNF alone or TNF + ActD lead to a strong activation of NF- κ B compared to media alone. Of note, the addition of ActD to TNF leads to a more sustained activation of NF- κ B while ActD alone leads to only weak activation of NF- κ B. The addition of cAMP prevented NF- κ B enhanced and blocked the activation of NF- κ B by TNF + ActD (Fig. 3B). In contrast, a caspase-8 inhibitor had no effect on NF- κ B activation induced by TNF + ActD showing that NF- κ B activation is not directly downstream of caspase-8 activation.

3.4. cAMP blocks DISC formation during TNF-induced apoptosis

To examine whether cAMP directly affects the formation of the DISC complex, immunoprecipitations were performed to determine whether db-cAMP prevents the initial assembly of DISC components. After the treatment of cells with TNF + ActD, TNFR1 was immunoprecipitated using the anti-TNFR1 antibody, and different components of DISC were assessed in the immunoprecipitated complex by immunoblotting. The binding of TRADD and RIPK as initial components of complex increased 15 min after initiating the experiment. Adding db-cAMP partially blocked the recruitment of TRADD and RIPK to TNFR1 and subsequently inhibited DISC complex formation (Fig. 4A).

To determine whether cAMP also blocks the binding of DISC components to caspase-8, immunoprecipitation was performed using a caspase-8 antibody at the same time points, and the components of DISC were examined using immunoblot analysis. We found that at 30 min, FADD binds to caspase-8 upon stimulation by TNF + ActD. We also found TRADD in the caspase-8 complex, but TNFR1 was absent in the complex, thereby confirming the theory regarding formation of a second complex. db-cAMP treatment also inhibited the binding of TRADD and FADD to the caspase-8 complex (Fig. 4B).

3.5. cAMP inhibits TNF-induced apoptosis by PKA activation

We determined whether PKA-specific inhibitor, KT5720 inhibited the effects of cAMP on hepatocyte survival. We found that significant cell death occurred in hepatocytes treated with TNF + ActD, which, as before, was prevented by cAMP. KT-5720 reversed the effect of cAMP in TNF-ActD-treated cells as expected. (Data not shown).

4. Discussion

Cyclic nucleotides, including cAMP and cGMP inhibit TNF + ActD-induced apoptosis in hepatocytes. We have previously shown that cyclic nucleotides block caspase activation, cytochrome c release and signaling downstream of the initiator caspases [26], and that the inhibition of apoptosis is mediated by PKA [15]. Here we extend these previous observations to show that cAMP-mediated inhibition of TNF signaling occurs at a proximal step in DISC formation. cAMP prevented TNF-induced NF- κ B activation and suppressed the initial steps in DISC formation. This is important because it further elucidates the initiating mechanism of an important pathway involved in cell death and inflammatory signaling.

The pleiotropic nature of the TNF response has been found to be due to the sequential formation of different signaling complexes upon the binding of TNF to TNFR1 [27]. Upon TNF treatment, the death receptor TNFR1 recruits TRADD, RIPK1, FADD, and caspase-8 to form the multiprotein DISC [9]. TNFR1 stimulation leads to the rapid assembly of a complex (complex I) comprising TNFR1, TRADD, RIPK1, and TRAF2. However, complex I is devoid of FADD and caspase-8 and triggers the NF- κ B signaling pathway via recruitment of the IKK complex [28]. TRADD and RIPK associate with FADD and caspase-8, forming a cytoplasmic complex (complex II) in the second step. It is well known that the TNFR1-TRADD-RIP-TRAF2 complex initiates a pathway leading to survival through NF- κ B [29,30]. When NF- κ B is activated by complex I, complex II activity is normally inhibited by c-FLIP, a protease-dead caspase-8 homologue that competes with caspase-8 for binding with FADD. Such inhibition is relieved through the activation of a c-FLIP-degrading ubiquitin E3 ligase Itch [31], and/or termination of NF- κ B transcriptional activity by protein synthesis inhibitors that terminate c-FLIP synthesis. Both here and in our previous work [14], we have shown that the cytoplasmic DISC complex in hepatocytes involves TRADD, FADD, TRAF1 and RIPK1.

In light of the above factors, we focused on the effect of cAMP on DISC formation during the TNF + ActD-induced apoptosis. Initially, we found that cAMP blocks the activation of NF- κ B induced by both TNF and TNF + ActD treatment consistent with the previous findings of Jin et al. [32]. As the activation of NF- κ B depends on the formation of an initial complex by TNFR1, we further investigated the effect of cAMP on initial complex formation upon treatment of hepatocytes with TNF + ActD. TRADD and RIPK1-bound to TNFR1 within 15 min, and the binding of both TRADD and RIPK1 was blocked by cAMP. We also observed a decrease in FADD-caspase-8 binding triggered by cAMP within 1 h in TNF + ActD-treated rat hepatocytes. Our data indicate the disruption of initial DISC formation by cAMP. We also showed that db-cAMP stimulates PKA activity and specific

inhibitor of PKA-KT5720 partially inhibits the protective activity of cAMP against TNF + ActD-induced apoptosis.

In summary, our study shows that cAMP blocks TNF + ActD-induced apoptosis in rat hepatocytes. cAMP down-regulates the increased levels of apoptotic proteins such as TRADD, FADD, and caspase-8 and also NF- κ B activation in hepatocytes. cAMP exerts this effect by blocking the initial events in DISC complex formation. Because PKA inhibitors reverse the anti-apoptotic effects of cAMP, it is likely that the effects of cAMP of DISC formation are mediated through PKA.

Acknowledgments

This work has been supported by the National Institutes of Health Research Grant R37 GM44100.

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Abbreviations

TNF	tumor necrosis factor α
TNFR1	TNF-receptor
SODD	silencer of death domains
DD	death domain
TRADD	TNFR-associated death domain
TRAF2	TNFR-associated protein 2
RIPK1	receptor-associated protein kinase 1
cIAP1/2	cellular inhibitor of apoptosis proteins 1/2
cFLIP	cellular FLICE inhibitory protein
DISC	death-inducing signaling complex
ActD	actinomycin D
cAMP	cyclic adenosine monophosphate
PKA	protein kinase A
IP	immunoprecipitation
FADD	Fas-associated protein with death domain

Fig. 1.

Effect of TNF/ActD, db-cAMP and sp-cAMPS on hepatocyte cell death. (A) Primary rat hepatocytes were exposed to TNF (2000 U/mL) + ActD (200 ng/mL), TNF + ActD + db-cAMP (200 M), or db-cAMP alone, and cell viability was assessed at time points from 0 to 18 h by crystal violet staining. (B) Primary rat hepatocytes were treated with sp-cAMPS (200 M) with and without TNF + ActD for 18 h and cell viability was measured by crystal violet staining. * $P < 0.01$, group treated with TNF + ActD and TNF + ActD + Sp-cAMPS compared with unstimulated cells. Experiments performed in triplicate and representative of at least three separate repeats.

Fig. 2. cAMP alters expression of TNFR1 DISC components. Rat primary hepatocytes were treated with TNF (2000 units/ml) + ActD (200 ng/mL) with or without db-cAMP (200 μ M). Western blotting determined levels of apoptotic proteins FADD, caspase-8 and caspase-3 in whole cell lysates at 6 h after treatment.

Fig. 3. cAMP blocks TNF signaling at a proximal step. (A) Primary rat hepatocytes were treated with TNF (2000 units/ml), ActD (200 ng/mL), or TNF + ActD and NF- κ B activation was examined by EMSA at 1, 3 and 6 h treatment with TNF + ActD. (B) Primary rat hepatocytes were treated with TNF + ActD (TA) alone or in combination with cAMP (200 μ M), caspase-8 inhibitor (20 μ M) of both cAMP and caspase-8 inhibitor. NF- κ B activation was measured by EMSA at 1, 3 and 6 h after treatment. Images are representative of at least two repeated experiments.

A IP:TNFR1 **B** IP:caspase-8 **Fig. 4.**

cAMP blocks DISC formation during TNF-induced apoptosis. (A) Primary rat hepatocytes were treated with TNF (2000 units/mL) + ActD (200 ng/mL) (TA), cAMP (200 μ M) or TA + cAMP for time points up to 60 min. Whole cell lysates were immunoprecipitated using anti-TNFR1 antibody and samples were then immunoblotted with anti-TRADD, anti-RIPK or anti-FADD antibodies. (B) Whole cell lysates from primary rat hepatocytes treated as above were immunoprecipitated with anti-caspase-8 antibody and samples were then immunoblotted with anti-TNFR1, anti-TRADD or anti-FADD antibodies. Images are representative of at least three repeated experiments.