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PROSTAGLANDIN E_2 INHIBITS TUMOR NECROSIS FACTOR-ALPHA RNA THROUGH PKA TYPE I

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Abstract

Tumor necrosis factor-alpha (TNF-α) is a cytokine that may contribute to the pathogenesis of septic shock, rheumatoid arthritis, cancer and diabetes. Prostaglandins endogenously produced by macrophages act in an autocrine fashion to limit TNF-α production. We investigated the timing and signaling pathway of prostaglandin-mediated inhibition of TNF-α production in RAW 264.7 and J774 macrophages. TNF-α mRNA levels were rapidly modulated by PGE_2 or carbaprostacylin. PGE_2 or carbaprostacyclin prevented and rapidly terminated ongoing TNF-α gene transcription within 15 min of prostaglandin treatment. Selective activation of PKA type I, but not PKA type II or Epac, with chemical analogs of cAMP was sufficient to inhibit LPS-induced TNF-α mRNA levels. The mechanisms by which prostaglandins limit TNF-α mRNA levels may underlie endogenous regulatory mechanisms that limit inflammation, and may have important implications for understanding chronic inflammatory disease pathogenesis.

Keywords

RAW 264.7 cells; inflammation; cAMP; Epac; PGE_2; prostacyclin; macrophage; tumor necrosis factor-alpha; lipopolysaccharide

INTRODUCTION

Induction of inflammatory genes in macrophages is necessary for effective immune responses. Equally important, inflammation must be down-regulated to prevent excess tissue damage and chronic inflammatory states. One key pro-inflammatory cytokine produced by macrophages is tumor necrosis factor alpha (TNF-α). Dysregulated production of TNF-α may lead to human diseases including septic shock, rheumatoid arthritis, inflammatory bowel disease, insulin resistance (diabetes) and cancer [1;2]. These examples of human disease may stem from a *lack* of appropriate *down-regulation* of the inflammation.

Macrophages release TNF-α, and activate cyclooxygenase-2 (COX-2) in response to stimulation with lipopolysaccharide (LPS). The subsequent release of prostaglandins (PGs) have well-known pro-inflammatory effects [3]. The limiting effects of PGs on inflammation

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are less well-appreciated. Prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) may endogenously limit stimulus-induced cytokine secretion [4;5]. Inhibition of constitutive COX-1 activity and PG synthesis in resident peritoneal macrophages (RPM) enhances TNF-α secretion from RPM responding to LPS. Adding back PGE_2 or the stable PGI_2 analog, carbaprostacyclin ($cPGI_2$) returns TNF-α secretion to levels seen with endogenously produced PGs. Thus, endogenous PG biosynthesis plays a key role in regulating TNF-α secretion from LPS-stimulated RPM [4].

In contrast to RPM, the murine macrophage cell line RAW264.7 (Raw) does not produce PGs to auto-regulate TNF-α but maintains sensitivity to TNF-α suppression by exogenous PGs [6]. Lack of auto-regulation makes the Raw cell line well-suited to study the dynamics and mechanisms by which PGs suppress TNF-α biosynthesis in macrophages, both incompletely characterized in these cells.

PGE_2 and PGI_2 increase cytosolic cAMP through G-Protein coupled receptors (EP2, EP4 and IP). PGE_2 inhibits LPS-induced TNF-α primarily at the level of gene transcription [7]. PGE_2 or dibutyryl-cAMP also inhibit TNF-α protein release and mRNA levels when added to macrophages after 90-minute LPS induction [7;8]. The major cellular effectors of increased cAMP include activation of cAMP-sensitive ion channels, exchange protein activated by cAMP (Epac) [9], and cAMP-dependent protein kinase (PKA) [10]. The two forms of PKA (types I and II) differ in regulatory subunit composition (RI or RII). In alveolar macrophages, cAMP analogs that non-specifically activate PKA, inhibit TNF-α release after LPS stimulation [11;12]. The specific roles of PKA types I and II and the dynamics of TNF-α mRNA regulation by PGs is not known. We extended our characterization of macrophage responses to inflammatory stimuli and investigated the mechanisms that limit TNF-α biosynthesis.

MATERIALS AND METHODS

Cell culture and treatments

Raw and J774 cells (ATCC) were propagated in DMEM-GlutaMax (Gibco) and 10% heat-inactivated FCS (Atlas). *E. Coli* 0111:B4 LPS (Calbiochem) and cAMP analogs (Axxora) were delivered as 100x concentrates in sterile H_2O. For cell treatments, macrophages were plated 6-well plates (60-80% confluence) and cultured overnight prior to the experiments. Cells were treated in serum free DMEM with a maximum DMSO vehicle content of 0.1%.

Real-Time RT-PCR

Total cellular RNA was primed with Oligo-dT and reverse-transcribed using Taq Man RT Reagents (Applied Biosystems). Real-time PCR reactions contained 0.1 μg cDNA, 200 nM primers, 25 μL of 2x Syber Green icycler supermix (Biorad), in a total volume of 50 μL and were run on a Biorad iCycler with melting point determination. RNA starting quantities were calculated using the C_t method (Biorad iCycler) based on known concentrations of linearized cDNA standard (PGEM-TNF or PGEM-GAPDH). Primers sequences were: TNF-α, 5′-cgtagcaaaccaccaagtgga-3′ and 5′-gctggcaccactagttggttgt-3′, GAPDH, were 5′-atggcaaagtggagattgttgg-3′ and 5′-tgccattgaatttgccgtg-3′ (Operon/Qiagen).

Plasmids

Cyclophillin cDNA was obtained from M. Magnuson, Vanderbilt University. TNF-α cDNA (GenBank accession no. **X02611**) was PCR-cloned from LPS-induced Raw cells and ligated into the PGEM easy-T vector. The amplified regions of the TNF-α mRNA and GAPDH mRNA were ligated into the PGEM easy-T vector and linearized to generate PGEM-TNF and PGEM-GAPDH. All DNA was sequenced prior to use.

Nuclear run-on transcription assay

10^8 Raw cells were washed in ice-cold PBS. Cells were lysed (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40), nuclei were pelleted, washed with lysis buffer and resuspended in 200 μL glycerol storage buffer (50 mM Tris-HCl, pH 8.3, 40% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA). 200 μL of 2x Reaction Buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 5 mM DTT, 0.3 M KCl, 1 mM ATP, 1 mM GTP, 1 mM CTP) and 10 μL of [α^{32}-P]-UTP (3000 mCi/mmol) were added to nuclei and incubated for 30 min at 30 C with shaking. Aliquots (750,000 cpm) of nuclear RNA were hybridized for 60 h at 65 C in QuickHyb to cDNA probes on Hybond N+ membranes (7 μg linearized, denatured cDNA per slot). Blots were washed (twice 2xSSC/65 C/60 min, 2xSSC/10mg/mL RNAse A/37 C/30 min, 2xSSC/37 C), dried and quantitated by phosphorimager analysis. Transcription was determined relative to murine cyclophilin A. Values were normalized to values obtained from untreated controls.

Data analysis and statistical methods

Data were analyzed in Graph Pad Prism. Error bars represent the standard error of the mean of at least three experiments with duplicate determinations. P-values were determined by paired student's T-test.

RESULTS

PGE_2 rapidly reduces TNF-α mRNA levels

To gain insight into mechanisms responsible for inhibition of TNF-α mRNA accumulation, PGE_2 or $cPGI_2$ was added to Raw cells pretreated with LPS for 1 or 6 h (Figure 1). After 1 h of LPS pretreatment, Raw cells are maximally transcribing TNF-α mRNA and after 6 h of LPS pretreatment secreted TNF-α protein levels are highest (Figure S1). Addition of PGE_2 to 1 h-pretreated cells abrogated the LPS-stimulated accumulation of TNF-α mRNA, and TNF-α mRNA levels declined by 40% within 1 h following PGE_2 addition. $cPGI_2$ also initiated a rapid decline in TNF-α mRNA levels when added to macrophages pretreated with LPS for 6 h (Figure 1B). TNF-α mRNA levels declined by 50% within 30 min of $cPGI_2$ addition, and by 80% within 1 h after $cPGI_2$ addition (Figure 1B). Thus, PGE_2 and $cPGI_2$ both rapidly lower TNF-α mRNA levels in macrophages during the early and late response to LPS (Figure 1A and 1B). These results indicate that the cellular signaling events that initiate PGE_2-mediated reductions in LPS-induced TNF-α mRNA levels must also be rapid.

PGE_2 and $cPGI_2$ rapidly halt TNF-α gene transcription

In response to inducing stimuli, TNF-α mRNA levels are increased by alterations in gene transcription and mRNA stability [1;13]. Nuclear run-on transcription assays were conducted to directly measure TNF-α gene transcription (Figure 2). After 6 h of exposure to LPS, TNF-α gene transcription was 3.5 times higher in LPS-stimulated cells than untreated macrophages (Figure 2 bars A, B). Both PGE_2 and $cPGI_2$ completely prevented transcription when co-administered with LPS (bars C, F). To determine the effect of PGE_2 and $cPGI_2$ on ongoing transcription, macrophages were pretreated with LPS for 6 h, and PGE_2 or $cPGI_2$ was added to the cell culture medium for 15 or 30 min after the pretreatment and nuclear run-on analysis was performed (Figure 2 Bars D, E, G). Within 15 min of adding either prostanoid, the level of TNF-α transcription returned to unstimulated levels; PGE_2 and $cPGI_2$ completely halted ongoing TNF-α transcription.

The effects of PGE_2 to inhibit TNF-α gene transcription are rapid and occur immediately following the addition of PG to LPS-pretreated macrophages. These findings indicate that the cellular mechanism leading to inhibition of TNF-α gene transcription likely involves rapid intracellular signaling and post-translational modification of cellular protein function rather than new protein synthesis. Therefore, we decided to investigate proximal PGE_2 signaling to determine the cellular effectors of PGE_2-mediated inhibition of TNF-α mRNA levels in Raw cells.

Activation of PKA type I inhibits LPS-induced TNF-α mRNA levels

PKA is activated by cAMP binding to regulatory subunits and subsequent dissociation of the regulatory and catalytic subunits. There are two types of PKA (I, II) classified based on regulatory subunit isoform. Each regulatory subunit contains two cAMP binding sites (A, B). Analogs of cAMP have been successfully employed to determine PKA type specificity for biologic actions [14]. Cyclic-AMP also binds to the exchange protein activated by cAMP (Epac) and activates Rap1. $cPGI_2$ stimulation of Raw cells rapidly increases intracellular cAMP corresponding temporally to decreases in TNF-α mRNA levels following PGs (Figure 1, S2) It is not known whether PGs inhibit TNF-α through PKA type I or II. Synergistic pairs of cAMP analogs were employed to determine the involvement of PKA type I, PKA type II or Epac to mediate the inhibitory action on TNF-α mRNA levels of agents that increase cellular cAMP. The binding specificities and combinations of cAMP analogs used in these studies are listed in Table 1. J774 and Raw cells were stimulated with LPS and 250 μM of the respective cAMP analogs for 1 h. In both Raw cells and J774 cells, individual cAMP analogs did not significantly inhibit LPS-induced TNF-α mRNA levels (Figure 3A, 3C). Selective activation of PKA type I with 6-Bnz and 8-HA inhibited LPS-induced TNF-α mRNA levels by 50% in Raw and J774 macrophages (Figure 3A, 3C). Activation of PKA type II with 6-Bnz and 8-PIP did not significantly inhibit TNF-α mRNA levels induced by LPS stimulus in Raw or J774 macrophages (Figure 3A, 3C). Likewise, activation of Epac with 8-CPT did not inhibit TNF-α mRNA levels in either Raw or J774 macrophages (Figure 3B, 3D). In both cell lines, the inhibitory effect of PKA type I activation was constant in the presence of the Epac activator (Figure 3B, 3D).

Dibutyryl-cAMP inhibits TNF-α secretion from LPS-stimulated murine peritoneal macrophages and Raw 264.7 cells [8]. To test whether increases in cAMP and subsequent dissociation of the PKA holoenzyme are sufficient to decrease TNF-α mRNA levels, J774 macrophages were stimulated with LPS for 1 h in the presence of a cell permeable cAMP analog and activator of PKA, Sp-8-Bromo-cAMPS. In macrophages treated for 1 h with LPS and 1 μM Sp-8-Bromo-cAMPS, TNF-α mRNA levels were significantly decreased to 70.9 +/- 15.1% of LPS-stimulated levels. Addition of 250 μM Sp-8-Bromo-cAMPS inhibited TNF-α mRNA levels to 41.5 +/- 20.6% of LPS-stimulated levels (Figure 3E). To test whether dissociation of PKA regulatory and catalytic subunits was necessary to reduce TNF-α mRNA levels, Rp-8-Bromo-cAMPS was used as a competitive antagonist of Sp-8-Bromo-cAMPS. Rp-8-Bromo-cAMPS binds to regulatory subunits but does not stimulate the dissociation of the regulatory and catalytic subunits, preventing activation of PKA [15]. Rp-8-Bromo-cAMPS fully reversed the inhibitory effect of Sp-8-Bromo-cAMPS on TNF-α mRNA levels in J774 macrophages (Figure 3E) indicating that dissociation of PKA regulatory and catalytic subunits was required for the inhibitory effect on TNF-α mRNA levels. Because Rp-8-Br-cAMPS and Sp-8-Br-cAMPS bind competitively to cAMP binding sites in the PKA regulatory subunits, use of 250 μM Rp-8-Br-cAMPS only partially alleviated the repression observed with 250 μM Sp-8-Br-cAMPS.

DISCUSSION

The generation of macrophage cytokine responses is important for coordinating the immune response to infection. Macrophage stimulation with LPS induces cytokines genes including TNF-α, concomitant with activation of cyclooxygenases and subseqent release of PGs.

Although the pro-inflammatory effects of PGs are well-appreciated, it is clear that PGE_2 and PGI_2 also have important endogenous roles to limit or direct inflammatory responses [4;6;7;16]. The limiting effects of PGE_2 on macrophage TNF-α secretion are partially responsible for increased susceptibility to lung infection after bone marrow transplantation [5]. PGE_2 or cAMP-mediated suppression of dendritic cell function may also be responsible for decreased tumor cell immunosurveilance and decreased dendritic cell cytotoxicity and TNF-α secretion [17;18]. Since autocrine PG signaling does not affect TNF-α secretion by Raw cells [6], and the cell line is frequently used to study inflammatory signaling, we characterized TNF-α regulation in Raw cells stimulated with LPS and PGE_2 or $cPGI_2$. We report that regulation of TNF-α mRNA levels is a dynamic process that happens within minutes after the addition of the PG to LPS-stimulated macrophages (Figure 1). The rapid inhibition of TNF-α mRNA levels by PGE_2 or $cPGI_2$ happens at early and late time points in the response of macrophages to LPS (Figure 1). Our results are consistent with previous reports that delayed addition of PGE_2 to LPS-stimulated macrophages inhibits TNF-α mRNA levels, when measured hours after the addition of the PG [7;8;16]. The biological half-lives of PGE_2 and PGI_2 are short (< 1.5 min in circulation) owing to rapid *in vivo* metabolism [19;20]. Consequently, it is physiologically important that regulation of TNF-α mRNA levels by PGs requires neither copious concentrations [7;8], nor pre-exposure to the inhibitory agent. Rather, PGE_2 and PGI_2 may regulate TNF-α mRNA levels at any point in the macrophage response to LPS, and on the same time scale as the PGs accumulate locally and are metabolized *in vivo*.

Our studies suggest key characteristics about the mechanism by which PGs and cAMP lower LPS-induced TNF-α secretion. PGE_2 and $cPGI_2$ rapidly alter TNF-α mRNA levels through a robust and abrupt halt of on-going TNF-α gene expression (Figure 2) leading to rapid reductions in TNF-α mRNA levels (Figure 1). The rapid kinetics of transcriptional inhibition by PGE_2 and $cPGI_2$ and the decrease in mRNA levels correspond temporally with observed increases in cAMP accumulation following $cPGI_2$ treatment of LPS-stimulated macrophages (Figures 3, S2). In addition, cAMP analogs recapitulate the effects of PGE_2 and $cPGI_2$ to inhibit LPS-induced TNF-α mRNA levels (Figure 3). These results indicate that rapid increases in intracellular cAMP levels are sufficient to inhibit TNF-α transcription in response to PGE_2 and $cPGI_2$. The timing of cAMP formation and rapid inhibition of transcription and mRNA stability, indicate that new protein synthesis is probably not necessary for PG-mediated regulation of TNF-α mRNA levels in macrophages.

We investigated the downstream signaling of PGE_2 and cAMP to determine the target of cAMP signaling in macrophages. Using synergistic pairs of cAMP analogs that preferentially activate PKA type I or PKA type II, we demonstrate that inhibition of TNF-α mRNA levels by cAMP occurs selectively through the activation of PKA type I in both Raw 264.7 and J774 macrophages (Figure 3). Inhibition of TNF-α mRNA levels by PKA type I was not altered by the co-activation of Epac (Figure 3). These results agree with recently published results in alveolar macrophages demonstrating a lack of Epac involvement and PKA-dependent inhibition of TNF-α secretion from alveolar macrophages [11]. Thus, selective activation of PKA type I with cAMP analogs recapitulated the effects of PGE_2 and $cPGI_2$ on TNF-α mRNA levels in two macrophage cell lines. These results provide an important link between the known mechanisms that stimulate TNF-α gene transcription in J774 macrophages [13] and the pathway for cAMP-dependent inhibition of TNF-α biosyntheses in Raw and J774 macrophages. Collectively, our results indicate that PGE_2 and $cPGI_2$ inhibit LPS-induced TNF-α transcription in macrophages through an immediate increase of intracellular cAMP levels that selectively activates PKA type I to inhibit TNF-α gene transcription.

Differences in the cellular effects of PKA type I and PKA type II also occur in other immune cells [18;21]. For example, activation of PKA type I, but not PKA type II inhibits cytotoxic activity and cytokine secretion from lymphokine-activated killer cells [18]. Also, the most upstream mechanism for cAMP-mediated inhibition of T cell activation by T cell receptor stimuli requires the activation of PKA type I leading to the inhibition of Lck [22]. Signaling specificity occurs because PKA type I specifically co-localizes in lipid rafts with the T cell receptor and Lck. The LPS receptor complex, TLR4, may also reside within lipid raft membrane microdomains [23]. As with T cells, PKA type I could also be specifically targeted to the lipid raft domains in macrophages, and similar targeting may be responsible for the specific involvement of PKA type I in the inhibitory action of PG on TNF-α biosynthesis. However, we have observed that PGE_2 does not globally or specifically inhibit LPS stimulated phosphorylation of MAP kinases, p38, or JNK (JBS, unpublished findings), suggesting that, unlike T cell receptor activation, inhibition of LPS-stimulated TNF-α transcription by PKA type I may not occur at the level of TLR4 receptor activation. In addition, recently reported dual ligand screening studies of cytokine secretion from Raw cells co-treated with LPS and PGE_2 indicate that PGE_2 does not globally inhibit cytokine secretion from Raw cells. Rather, PGE_2 inhibits only LPS-induced TNF-α and MIP1α secretion, while stimulating LPS-induced GCSF and IL-10 secretion [24]. These results suggest that the mechanism by which PKA type I activation inhibits TNF-α transcription may not occur through upstream inhibition of TLR4 signaling.

Collectively, these studies indicate that regulation of the TNF-α cytokine levels happens in part through rapid and dynamic alterations in mRNA levels. Owing to the short TNF-α mRNA half-life and the sensitivity of TNF-α gene transcription to repression, TNF-α mRNA levels are regulated rapidly in response to PG signaling. The proximal inhibitory mechanism involves the specific activation of PKA type I. Mediators that increase cAMP may serve to direct, modulate, or turn off TNF-α biosynthesis in response to inflammatory stimuli, or may be necessary for normal regulation of inflammatory pathways under physiologic conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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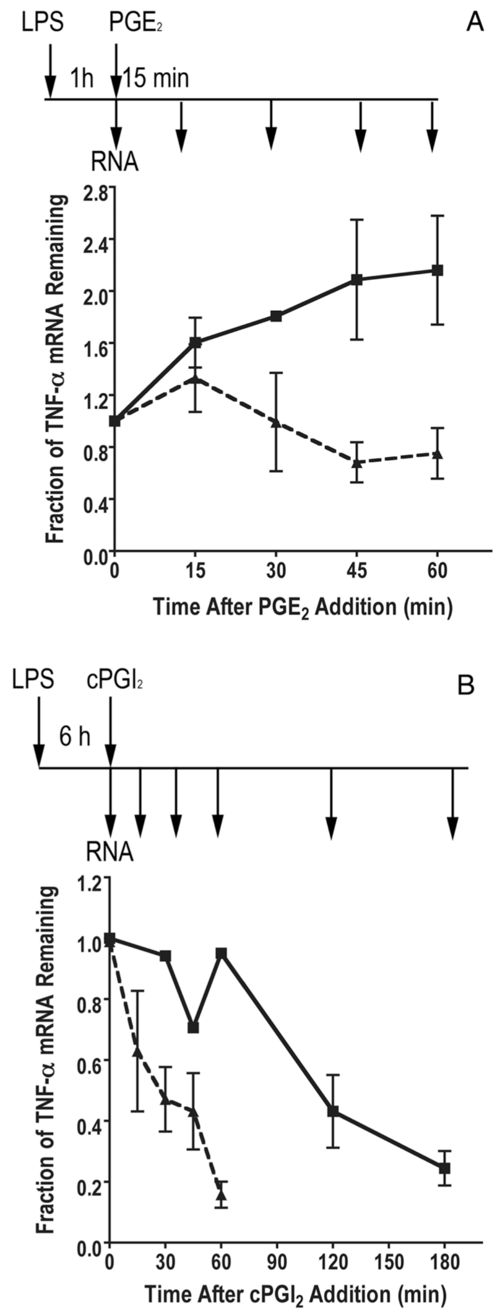

Figure 1. PGE_2 and $cPGI_2$ rapidly decrease TNF-α mRNA levels

Raw cells were pretreated with 1 μg/mL LPS for 1 (A) or 6 (B) hours. PGE_2 (A, σ) or $cPGI_2$ (B, σ) or DMSO (ν) was added and TNF-α mRNA levels were measured at 15 minute intervals following PG addition and normalized to GAPDH.

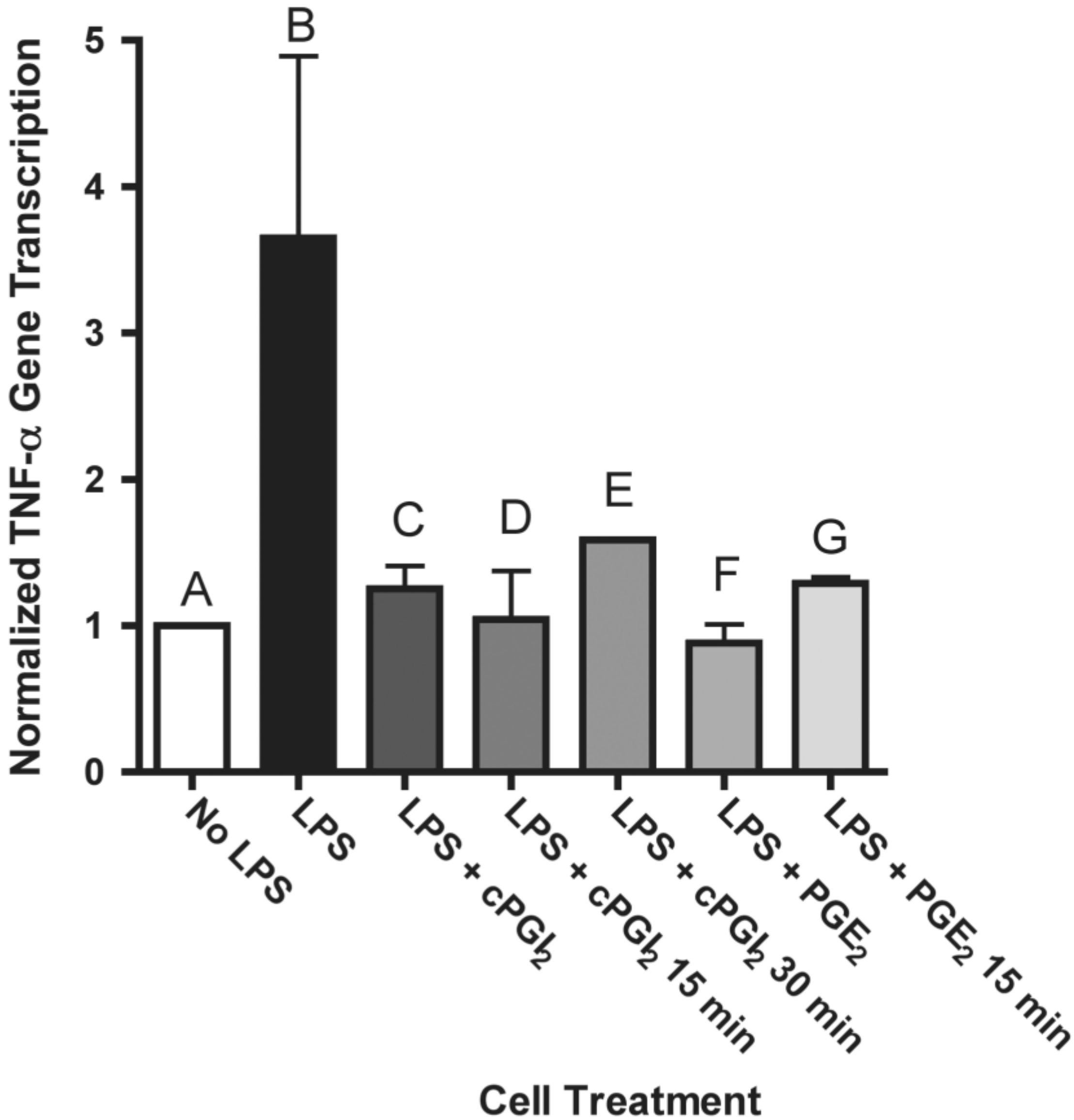

Figure 2. PGE_2 and $cPGI_2$ prevent TNF-α transcription and halt ongoing TNF-α transcription
Nuclear run-on transcription was measured in Raw cells treated with 1 μg/mL LPS in the presence of DMSO/vehicle (B), 1 μM $cPGI_2$ (C) or 1 μM PGE_2 (F) for 6 h. Bars D, E, G: Raw cells were pretreated with1 μg/mL LPS for 6 h followed by addition of 1 μM $cPGI_2$ (D, E) or 1 μM PGE_2 (G) for 15 or 30 min.

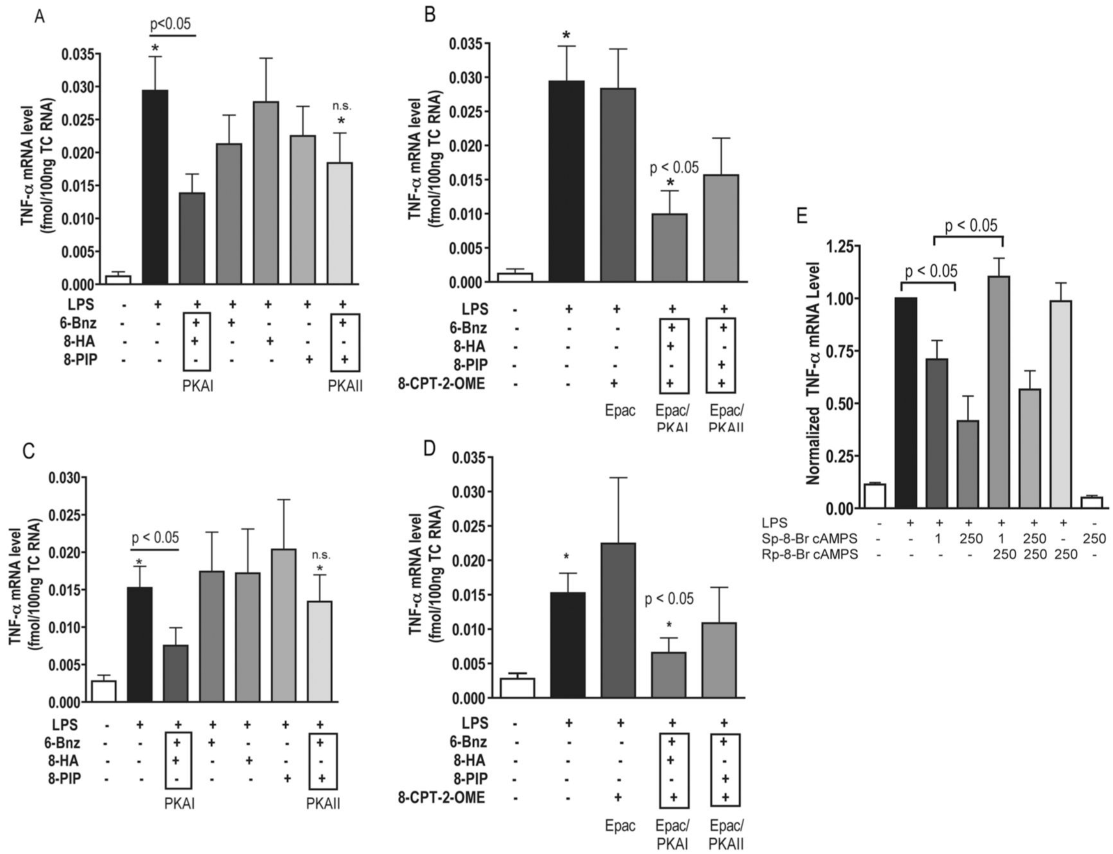

Figure 3. Selective activation of PKA Type I inhibits TNF-α mRNA levels in both J774 and Raw 264.7 macrophages

A, C) Synergistic combinations of cAMP analogs (250 μM) were used to activate PKA type I or PKA type II in Raw (A) and J774 (C) macrophages. B, D) The addition of the Epac activator 8-CPT-2-OME did not alter inhibition of TNF-α mRNA levels by PKA type I or PKA type II activator combinations in Raw (B) or J774 (D) cells. E) J774 macrophages were induced with LPS (1 μg/mL) for 1 h with increasing concentrations of Sp-8-Br-cAMPS and/or Rp-8-Br-cAMPS. Macrophages were pretreated for 30 min with cAMP analogs prior to the addition of LPS (1 μg/mL).

Table 1

Binding selectivity of cAMP analogs used in cell culture treatments.

cAMP Analog	Abbreviation	PKA Type Selectivity	cAMP Binding Site Selectivity	
Sp-8-Bromoadenosine 3',5'-cyclic monophosphorothioate	Sp-8-Br-cAMPS	Nonselective Agonist	Nonselective	
Rp-8-Bromoadenosine 3',5'-cyclic monophosphorothioate	Rp-8-Br-cAMPS	Type I > Type II Antagonist		
8-Hexylaminoadenosine-3',5'-cyclic monophosphate	8-HA	Type I	Site B	Type I Activator
N^6-Benzoyladenosine-3',5'-cyclic monophosphate	6-Bnz	Type I and II	Site A	Type II Activator
8-Piperidinoadenosine-3',5'-cyclic monophosphate	8-PIP	Type I Type II	Site A Site B	
8-(4-Chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate	8-CPT-2-OME	Epac		

Synergistic PKA type-selective cAMP analog combinations are indicated with a vertical bar.