Corticosteroid-induced MKP-1 represses pro-inflammatory cytokine secretion by enhancing activity of tristetraprolin (TTP) in ASM cells

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Abstract

Exaggerated cytokine secretion drives pathogenesis of a number of chronic inflammatory diseases, including asthma. Anti-inflammatory pharmacotherapies, including corticosteroids, are front-line therapies and although they have proven clinical utility, the molecular mechanisms responsible for their actions are not fully understood. The corticosteroid-inducible gene, mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1, *DUSP1*) has emerged as a key molecule responsible for the repressive effects of steroids. MKP-1 is known to deactivate p38 MAPK phosphorylation and can control the expression and activity of the mRNA destabilizing protein - tristetraprolin (TTP). But whether corticosteroid-induced MKP-1 acts via p38 MAPK-mediated modulation of TTP function in a pivotal airway cell type, airway smooth muscle (ASM), was unknown. While pretreatment of ASM cells with the corticosteroid dexamethasone (preventative protocol) is known to reduce ASM synthetic function *in vitro*, the impact of adding dexamethasone after stimulation (therapeutic protocol) had not been explored. Whether dexamethasone modulates TTP in a p38 MAPK-dependent manner in this cell type was also unknown. We address this herein and utilize an *in vitro* model of asthmatic inflammation where ASM cells were stimulated with the pro-asthmatic cytokine tumor necrosis factor (TNF) and the impact of adding dexamethasone 1 hr after stimulation assessed. IL-6 mRNA expression and protein secretion was significantly repressed by dexamethasone acting in a temporally distinct manner to increase MKP-1, deactivate p38 MAPK and modulate TTP phosphorylation status. In this way, dexamethasone-induced MKP-1 acts via p38 MAPK to switch on the mRNA destabilizing function of TTP to repress pro-inflammatory cytokine secretion from ASM cells.

Introduction

Tristetraprolin (TTP) is an anti-inflammatory protein that promotes mRNA decay of many proteins, including pro-inflammatory cytokines that drive respiratory disease progression. Due to its nature as an immediate-early gene (Carballo et al., 1998) and critical anti-inflammatory molecule, it is not surprising that the expression and function of TTP is highly regulated and amenable to rapid control in a mechanism akin to an on-off molecular switch. Critically, its function is controlled by p38 MAPK: its mRNA expression is p38 MAPK-dependent (Mahtani et al., 2001); and its mRNA destabilizing activity is controlled by p38 MAPK/MK2-mediated phosphorylation on two key serine residues (S52 and S178) preventing initiation of mRNA decay (Marchese et al., 2010).

We were interested in understanding the molecular mechanisms responsible for TTP regulation in order to develop strategies to enhance the anti-inflammatory capacity of TTP. Given the critical role of p38 MAPK in TTP regulation, it might seem logical to attempt to block the p38 MAPK pathway to enhance TTP anti-inflammatory function. However, this approach has been shown to be unsuccessful because it prevents expression of TTP mRNA in the first place, as transcription of TTP is p38-MAPK dependent (Mahtani et al., 2001). We suggest that a better approach is to gain an in depth understanding of the temporal regulation of TTP expression and control of its anti-inflammatory activity by p38 MAPK. Understanding cytokine regulatory networks in this way will allow future development of novel pharmacotherapeutic approaches that repress pro-inflammatory cytokines while ensuring that vital, anti-inflammatory proteins necessary for disease resolution remain operational (reviewed in (Prabhala and Ammit, 2015)).

Harnessing the power of the endogenous MAPK deactivator - mitogen-activated protein kinase phosphatase 1 (MKP-1) – is one such approach. The phosphatase MKP-1 dephosphorylates p38 MAPK (Manetsch et al., 2012a; Quante et al., 2008) and we recently demonstrated that the precise temporal regulation of p38 MAPK phosphorylation status by MKP-1 ensured that TTP

mRNA and protein was expressed and made functional (by increasing amounts of the unphosphorylated, active form of TTP) at an exact time to repress cytokine expression in a pivotal airway cell type implicated in respiratory disease - airway smooth muscle (ASM) cells (Prabhala et al., 2015). MKP-1 is a corticosteroid-inducible gene (Clark, 2003) and we have shown that dexamethasone (Manetsch et al., 2012a; Quante et al., 2008) and the clinically-used corticosteroid fluticasone propionate (Manetsch et al., 2013) upregulate MKP-1 in ASM cells. But to date, we have used a preventative protocol in our *in vitro* model of asthmatic inflammation, i.e. we added corticosteroids before stimulating cells with tumor necrosis factor (TNF), a pro-asthmatic cytokine found elevated in human disease (Broide et al., 1992) and widely utilized in *in vitro* studies of asthmatic inflammation. The repressive action of dexamethasone when it is added after stimulation (therapeutic protocol) had not been explored. Nor has the p38 MAPK-mediated modulation of TTP by dexamethasone in this cell type. These studies may ultimately uncover how inflammation can be targeted in a corticosteroid-sparing or a non-steroidal manner.

We address these gaps herein and show that TTP is a highly adaptable molecule that can exert anti-inflammatory effects even at low levels of expression. This is due to the fact that it is the activity, not overall amount of TTP, which is responsible for its anti-inflammatory mRNA destabilizing impact in ASM cells. We confirm that TTP protein exerts anti-inflammatory activity when it is in the unphosphorylated form and that p38 MAPK modulates the phosphorylation status of TTP. By regulating p38 MAPK activity, this switch is controlled by dexamethasone-induced MKP-1 in a temporally distinct manner

Material and Methods

ASM cell culture

Human bronchi were obtained from patients undergoing surgical resection for carcinoma or lung transplant donors in accordance with procedures approved by the Sydney South West Area Health Service and the Human Research Ethics Committee of the University of Sydney. ASM cells were dissected, purified and cultured as previously described by Johnson *et al.* (Johnson et al., 1995). A minimum of three different ASM primary cell lines were used for each experiment.

Chemicals

TNF was purchased from R&D Systems (Minneapolis, MN). Unless otherwise specified, all other chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

Real-time RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Doncaster, VIC, Australia) and reverse transcribed using the RevertAid First strand cDNA Synthesis Kit (Fermentas Life Sciences, Hanover, MD). Real-time RT-PCR was performed on an ABI Prism 7500 with IL-6 (Hs00174131_m1), TTP (Zfp36: Hs001856583_m1) and MKP-1 (DUSP1: Hs00610256_g1) TaqMan® Gene Expression Assays and the eukaryotic 18S rRNA endogenous control probe (Applied Biosystems) subjected to the following cycle parameters: 50°C for 2 min, 1 cycle; 95°C for 10 min, 1 cycle; 95°C for 15 s, 60°C for 1 min, 40 cycles and mRNA expression quantified by delta delta Ct calculations.

ELISAs

Cell supernatants were collected and stored at -20˚C for later analysis by ELISA. IL-6 ELISAs were performed according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA).

Western blotting

Western blotting was performed using rabbit monoclonal or polyclonal antibodies against phosphorylated (Thr^{180}/Tyr^{182}) and total p38 MAPK (from Cell Signaling Technology, Danvers, MA). TTP was measured by Western blotting using rabbit antisera against TTP (Sak21)(Mahtani et al., 2001)(generously provided by Professor Andrew R. Clark, University of Birmingham, UK). MKP-1 was measured using a rabbit polyclonal antibody (C19: Santa Cruz Biotechnology, Santa Cruz, CA), compared to α-tubulin as the loading control (mouse monoclonal IgG_1, clone DM 1A: Santa Cruz). Primary antibodies were detected with goat anti-mouse or anti-rabbit horse radish peroxidase–conjugated secondary antibodies (Cell Signaling Technology) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA). ImageJ 1.47v was used to perform densitometric analysis.

Statistical analysis

Statistical analysis was performed using Student's unpaired *t* test. *P* values < 0.05 were sufficient to reject the null hypothesis for all analyses.

Results

Temporal kinetics of TNF-induced IL-6 mRNA expression and protein secretion: impact of treating with dexamethasone 1 hr after TNF stimulation

We (Ammit et al., 2002; Quante et al., 2008) and others (McKay et al., 2000) have established that the corticosteroid dexamethasone significantly represses cytokine IL-6 secretion from ASM cells. But these publications have used a preventative protocol where ASM cells have been pretreated with corticosteroids 1 hr *before* cell stimulation with TNF. Whether corticosteroids repress IL-6 when added *after* cell stimulation was unknown. To address this, we treated ASM under the following conditions: vehicle; dexamethasone at 1 hr; TNF; TNF then dex at 1 hr (time of TNF stimulation designated as 0 hr), and examined the impact on IL-6 mRNA expression and protein secretion (Figure 1). Under unstimulated conditions (vehicle), there is no induction of IL-6 mRNA and adding dexamethasone in the absence of TNF (dex at 1 hr) had no effect on IL-6 mRNA expression. In accordance with our previous publications (Prabhala et al., 2015; Quante et al., 2008), TNF rapidly induces IL-6 mRNA expression with a peak at 1 hr. Notably, dexamethasone added after TNF stimulation (TNF then dex at 1 hr) did inhibit TNF-induced IL-6 expression, with significantly less IL-6 mRNA expression demonstrated at 2, 4 and 8 hr (Figure 1A: $P<0.05$). Accordingly, the resultant IL-6 protein secretion was reduced, with significantly less IL-6 protein secretion demonstrated at 8 and 24 hr in cells where dexamethasone was added 1 hr after TNF, compared to TNF alone (Figure 1B: $P<0.05$)

Temporal kinetics of TNF-induced MKP-1 mRNA expression: impact of treating with dexamethasone 1 hr after TNF stimulation

Dexamethasone induces MKP-1 expression in ASM cells (Che et al., 2014a; Manetsch et al., 2012b; Quante et al., 2008). Thus, it was of interest to examine the temporal kinetics of MKP-

1 mRNA in this context. ASM cells were treated under the four experimental conditions as outlined above and the temporal kinetics of MKP-1 mRNA expression measured. As shown in Figure 2, adding dexamethasone at 1 hr induced a significant 2.9±0.6-fold increase in MKP-1 mRNA expression at the 2 hr time point that was sustained for up to 24 hr (3.2±0.5-fold) (P<0.05). Vehicle alone was without effect. When cells were stimulated with TNF, MKP-1 mRNA expression peaked at 1 hr and then declined; confirming the temporal kinetics of MKP-1 mRNA expression induced by TNF in this cell type (Prabhala et al., 2015). The impact of treating cells with dexamethasone 1 hr after TNF stimulation was characterized by a sustained expression of MKP-1 mRNA at 4-24 hr (P<0.05); that is, while TNF-induced MKP-1 mRNA expression is transient, adding dexamethasone at 1 h ensures that MKP-1 mRNA expression exists at the later time points. We were intrigued to examine the consequence of this sustained expression of MKP-1 mRNA expression.

Treatment with dexamethasone 1 hr after TNF stimulation results in sustained MKP-1 protein upregulation: effects on p38 MAPK phosphorylation

MKP-1 is an immediate early gene that can be rapidly translated into protein (Sun et al., 1993). MKP-1 dephosphorylates members of the MAPK superfamily and in ASM cells we have shown that the extent and duration of p38 MAPK phosphorylation is controlled by MKP-1 (Manetsch et al., 2012a; Prabhala et al., 2015). Thus it is likely that MKP-1 mRNA expressed as a result of adding dexamethasone after TNF stimulation may exert similar repressive effects, although the impact of this regulatory network after the peak of cytokine expression has been established was unknown. To address this, we compared the temporal kinetics of MKP-1 protein upregulation and impact on p38 MAPK phosphorylation in cells treated with TNF, compared to TNF then dexamethasone at 1 hr (Figure 3). Western blotting was performed and results expressed as representative blots (Figure 3A (TNF) & Figure 3B (TNF then dex at 1

hr)) and densitometric analysis comparing MKP-1 upregulation and p38 MAPK phosphorylation under these conditions demonstrated as Figures 3C & 3D, respectively. TNF rapidly induces p38 MAPK phosphorylation at 0.25 and 0.5 hr (Figure 3A); this was similar under both treatment conditions, since in Figure 3B dexamethasone was not added until 1 hr. At 1 hr, TNF induced a 12.1±2.4-fold increase in MKP-1 protein upregulation that declined to 2.8±0.7-fold by 8 hr (Figures 3A & 3C). In accordance with the sustained expression pattern for MKP-1 mRNA, TNF-induced MKP-1 protein upregulation was also sustained when cells were treated with dexamethasone at 1 hr (Figures 3B & 3C); MKP-1 protein peaked at 2 hr (19.8±2.6-fold) and this significantly increased when compared to TNF at the same time point (11.9±1.3-fold) (Figure 3C: $P<0.05$). While the impact of dexamethasone on TNF-induced p38 MAPK phosphorylation (Figures 3B & 3D) was less pronounced than that observed for MKP-1, the extent of TNF-induced p38 MAPK phosphorylation appeared reduced (from 2-24 hr) in cells treated with dexamethasone and this difference was significant at 2 hr (Figure 3D: $P<0.05$).

Treatment with dexamethasone 1 hr after TNF stimulation results in reduced TTP mRNA expression at 2 hr

In Prabhala *et al.* (Prabhala et al., 2015) we showed that IL-6 cytokine expression in ASM cells is temporally controlled by regulatory network between MKP-1, p38 MAPK and TTP. By controlling p38 MAPK phosphorylation, MKP-1 ensures that TTP mRNA and protein is expressed and made functional in temporally-specific manner. Dexamethasone-induced MKP-1 will likely contribute in a similar, TTP-dependent way. To confirm this assertion, we examined the impact of dexamethasone (at 1 hr) on the temporal kinetics of TTP mRNA expression stimulated by TNF. As shown in Figure 4, TNF increased TTP mRNA expression in ASM cells with a peak at 1 hr. TTP is a p38 MAPK-responsive gene (Mahtani et al., 2001;

Prabhala et al., 2015), accordingly, it is interesting to note that at 2 hr, the same time point where dexamethasone treatment increases MKP-1 protein upregulation (Figures 3B & 3C), mirrored by repressed p38 MAPK phosphorylation (Figures 3B & 3D), TTP mRNA expression is significantly repressed (Figure 4: $P<0.05$). Vehicle and dexamethasone-only controls had no significant effect on TTP mRNA expression (Figure 4).

Treatment with dexamethasone 1 hr after TNF stimulation increases abundance of the unphosphorylated (active form) of TTP, not TTP protein upregulation

Finally, we examined TTP protein upregulation and activity by Western blotting using the rabbit antisera against TTP - Sak21. TTP protein stability and activity is controlled by phosphorylation. TTP phospho-forms appear with different electrophoretic mobility on SDS-PAGE and can be detected by Sak21. When TTP is phosphorylated by MK2 the protein is stabilized (Brook et al., 2006; Hitti et al., 2006; Tchen et al., 2004), but this phosphorylated form of TTP is inactive as an anti-inflammatory RNA destabilizing factor (Marchese et al., 2010). In the unphosphorylated state, TTP is active and can induce mRNA decay however; this form of TTP is also subject to degradation by the ubiquitin-proteasome system (Brook et al., 2006). Previous studies utilizing Sak21 (King et al., 2009b; Mahtani et al., 2001; Prabhala et al., 2015; Rahman et al., 2015) reported that bands at higher molecular weight indicate phosphorylated TTP (inactive), while lower bands are unphosphorylated (active). Thus, it is critical to note that it is not just the amount of TTP protein present that is important (because this may be the inactive, but stable, phosphorylated form), but the activity (estimated by densitometric analysis of unphosphorylated immunoreactive bands detected by Sak21). To explore this further in our experimental context we have compared the temporal kinetics of TTP protein upregulation (and phosphorylation status) in cells treated with TNF or TNF then dexamethasone at 1 h (Figure 5). Results are expressed as representative blots (Figure 5A

(TNF) & Figure 5B (TNF then dex at 1 hr)) and densitometric analysis; where Figure 5C is total TTP protein upregulation (normalised to α-tubulin) and Figure 5D is TTP (% active) (i.e. % unphosphorylated TTP/total TTP) over time. TNF upregulates TTP protein in a temporally-distinct manner (Figure 5C) and interestingly, it appears that the total amount of TTP is reduced by dexamethasone (albeit non-significantly); this aligns with the mRNA data. But it is the activity, not the amount of TTP that dictates its anti-inflammatory function. Notably, at 1 hr after TNF stimulation, TTP is observed in both phosphorylated and unphosphorylated immunoreactive forms (Figures 5A & 5B). Densitometric analysis revealed that TTP (% active) was 43.3±2.3% (Figure 5D); these data concur with our earlier study (Prabhala et al., 2015). Notably, the impact of dexamethasone aligned with its ability to increase MKP-1 and repress p38 MAPK phosphorylation at 2 hr; as shown in Figure 5B, there is a region of immunoreactivity indicative of unphosphorylated phospho-forms at 2 hr. This is shown in Figure 5D, where TTP (% active) was significantly greater in cells treated with dexamethasone (41.0±2.4%), compared to TNF alone (24.9±5.4%) at the same time point ($P<0.05$). Taken together our study suggests that by increasing MKP-1, dexamethasone represses p38 MAPK to control TTP phosphorylation status (and therefore TTP activity) in a temporally-specific manner to regulate IL-6 cytokine secretion in ASM cells.

Discussion

This study underscores the importance of the MKP-1/p38/TTP regulatory network in the control of cytokine expression in ASM cells. *In vivo* in MKP-1 and TTP transgenic mice {Smallie, 2015 #4217;Ross, 2015 #4219} and *in vitro* in cell types apart from ASM , MKP-1 has been shown to deactivate p38 MAPK and co-operate with TTP to orchestrate cytokine expression. Our previous study in ASM cells (Prabhala et al., 2015) showed that TTP acts in a negative feedback manner to temporally regulate cytokine expression via MKP-1/p38 MAPK dynamic control. Although corticosteroids have been shown to increase MKP-1 and deactivate MAPKs in ASM cells (Issa et al., 2007; Quante et al., 2008), a direct examination of the expression and activity of TTP, and its regulation by dexamethasone-induced MKP-1 had not been explored.

We address this herein and confirm that dexamethasone induces MKP-1 mRNA expression and protein upregulation in ASM cells. We commonly pretreat ASM cells with corticosteroids for 1 hr (Che et al., 2014b; Quante et al., 2008; Rahman et al., 2014), so in this study, treating cells with dexamethasone 1 hr after stimulation with TNF shifted the time-course of MKP-1 expression. In this way, dexamethasone-induced MKP-1 protein and TNF-induced MKP-1 repressed p38 MAPK phosphorylation at 2 hr. Accordingly, as the phosphorylation status of TTP is regulated by p38 MAPK, there was a shift from phosphorylated (inactive) to unphosphorylated (active) at this time point. We propose that this active TTP then represses IL-6 mRNA expression and protein secretion.

This is the first study to demonstrate that dexamethasone alone has no effect on TTP mRNA expression and protein upregulation in ASM cells and indicates that cell type differences exist. In A549 pulmonary epithelial cells, Smoak & Cidlowski (Smoak and Cidlowski, 2006) showed that 100 nM dexamethasone induced TTP expression by 4-5-fold from 2-8 hr. This was confirmed by King *et al.,* where dexamethasone (albeit at 10-fold higher concentration, i.e. 1

μM) induced a 3-4-fold induction in TTP mRNA expression after 1 hr of treatment that was sustained for up to 18 hr (King et al., 2009b). Why differences exist in ASM cells is unclear at present, although perhaps the relative expression of MKP-1 *vs.* TTP in each cell type may play a compensatory role (Rahman and Ammit, unpublished data).

Regardless of the relative expression of each of the players in this regulatory network, the emerging view is that MKP-1/p38 and TTP co-operate in a coordinated fashion to orchestrate cytokine expression. These molecules are amenable to manipulation and represent legitimate targets for pharmacotherapeutic intervention. But perhaps most notably, our study has revealed that the timing of the intervention is important. As outlined in our recent review (Prabhala and Ammit, 2015), TTP can be directly regulated by p38 MAPK in a number of ways, but as its mRNA expression and protein stability is p38 MAPK-mediated, pretreatment with p38 MAPK inhibitors would result in reduced expression of this critical anti-inflammatory protein. This is not ideal. Instead, we have shown in the current study that by adding dexamethasone after stimulation by TNF had commenced (therapeutic protocol), MKP-1 induction is allowed to occur and protein is expressed at 2 hr. This then acts to reduce p38 MAPK phosphorylation and consequently repress TTP mRNA expression at this time point. However, TTP is a highly adaptable molecule that can exert anti-inflammatory power even at very low levels of expression. This is because p38 MAPK also controls the switch between active and inactive TTP phospho-forms. Put simply, the anti-inflammatory function of TTP is dictated by the phosphorylation status of TTP at S52 and S178 (phosphorylated – OFF; unphosphorylated – ON) (Mahtani et al., 2001). This is detected in our study as an increase in the TTP (% active) at 2 hr. Also notable from our study is the predominance of the higher molecular weight, immunoreactive band for TTP; this equates to the inactive phosphorylated form of TTP. By confirming that TTP exerts anti-inflammatory activity when in the unphosphorylated form, our

results in ASM cells are in accord with recent publication that states “that TTP is most evident when it is least active and most active when it is least evident” (Smallie et al., 2015).

Collectively, our study suggests that corticosteroids increase TTP activity, not expression, via MKP-1 in a p38 MAPK-mediated manner. Excitingly, as the phospho-states of TTP are amenable to pharmacological manipulation, our study reveals a number of potential sites for intervention and demonstrates that switching TTP on to repress cytokines in airway inflammation is a feasible strategy towards combating respiratory disease.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Figure Legends

Figure 1. Temporal kinetics of TNF-induced IL-6 mRNA expression and protein secretion: impact of treating with dexamethasone 1 hr after TNF stimulation. Growth-arrested ASM cells were stimulated with TNF (10 ng/ml) or vehicle for 0, 1, 2, 4, 8, and 24 hr (time of TNF stimulation designated as 0 hr). To demonstrate the impact of adding dexamethasone 1 hr after stimulation with TNF, cells were treated with dexamethasone (100 nM), compared to vehicle controls. (A) IL-6 mRNA expression was quantified by real-time RT-PCR expression (results expressed as the percentage of TNF-induced IL-6 mRNA at 1 hr (peak of expression)); (B) IL-6 protein secretion measured by ELISA (results are expressed as the percentage of TNF-induced IL-6 protein at 24 hr (peak of secretion)). Statistical analysis was performed using Student's unpaired *t* test, where * denotes a significant effect of dexamethasone on TNF-induced IL-6 ($P<0.05$)). Data are mean+SEM values from n=6 primary ASM cell cultures.

Figure 2. Temporal kinetics of TNF-induced MKP-1 mRNA expression: impact of treating with dexamethasone 1 hr after TNF stimulation. Growth-arrested ASM cells were stimulated with TNF (10 ng/ml) or vehicle for 0, 1, 2, 4, 8, and 24 hr (time of TNF stimulation designated as 0 hr). To demonstrate the impact of adding dexamethasone 1 hr after stimulation with TNF, cells were treated with dexamethasone (100 nM), compared to vehicle controls. MKP-1 mRNA expression was quantified by real-time RT-PCR expression (results expressed as fold increase compared to 0 hr). Statistical analysis was performed using Student's unpaired *t* test, where § denotes a significant effect of dexamethasone on MKP-1 mRNA expression, and * denotes a significant effect of dexamethasone on TNF-induced MKP-1 mRNA expression ($P<0.05$)). Data are mean+SEM values from n=6 primary ASM cell cultures.

Figure 3. Treatment with dexamethasone 1 hr after TNF stimulation results in sustained MKP-1 protein upregulation: effects on p38 MAPK phosphorylation. Growth-arrested ASM cells were stimulated with TNF (10 ng/ml) or vehicle for 0, 0.25, 0.5, 1, 2, 4, 8, and 24 hr (time of TNF stimulation designated as 0 hr). Dexamethasone (100 nM), or vehicle, was added 1 hr after stimulation with TNF and the temporal kinetics of MKP-1 upregulation and impact on p38 MAPK phosphorylation compared by Western blotting (with α-tubulin as the loading control). (A, B) Results are representative Western blots when cells were treated with: (A) TNF, (B) TNF then dexamethasone at 1 hr. (C, D) demonstrates densitometric analysis of (C) MKP-1 protein upregulation (normalized to α-tubulin and expressed as fold increase compared to 0 hr) and (D) p38 MAPK phosphorylation (normalized to total p38 MAPK and expressed as fold increase compared to 0 hr) over time. Statistical analysis was performed using Student's unpaired *t* test, where * denotes a significant effect of dexamethasone on (C) TNF-induced MKP-1 protein upregulation or (D) p38 MAPK phosphorylation ($P<0.05$). Data are mean±SEM values from n=4 primary ASM cell cultures.

Figure 4. Treatment with dexamethasone 1 hr after TNF stimulation results in reduced TTP mRNA expression at 2 hr. Growth-arrested ASM cells were stimulated with TNF (10 ng/ml) or vehicle for 0, 1, 2, 4, 8, and 24 hr (time of TNF stimulation designated as 0 hr). To demonstrate the impact of adding dexamethasone 1 hr after stimulation with TNF, cells were treated with dexamethasone (100 nM), compared to vehicle controls. TTP mRNA expression was quantified by real-time RT-PCR expression (results expressed as fold increase compared to 0 hr). Statistical analysis was performed using Student's unpaired *t* test, where * denotes a significant effect of dexamethasone on TNF-induced TTP mRNA expression ($P<0.05$)). Data are mean+SEM values from n=6 primary ASM cell cultures.

Figure 5. Treatment with dexamethasone 1 hr after TNF stimulation increases abundance of the unphosphorylated (active form) of TTP, not TTP protein upregulation. Growth-arrested ASM cells were stimulated with TNF (10 ng/ml) or vehicle for 0, 0.25, 0.5, 1, 2, 4, 8, and 24 hr (time of TNF stimulation designated as 0 hr). Dexamethasone (100 nM), or vehicle, was added 1 hr after stimulation with TNF and the temporal kinetics of TTP upregulation compared by Western blotting (with α-tubulin as the loading control). Please note the two bands of immunoreactivity for TTP indicated by brackets: bands at higher molecular weight indicate phosphorylated TTP (inactive), while lower bands are unphosphorylated (active). (A, B) Results are representative Western blots when cells were treated with: (A) TNF, (B) TNF then dexamethasone at 1 hr. (C, D) demonstrates densitometric analysis performed by ImageJ of: (C) TTP protein upregulation (normalized to α-tubulin and expressed as fold increase compared to 0 hr) and (D) TTP (% active) (i.e. % unphosphorylated TTP/total TTP) over time. Data are mean±SEM values from n=4 primary ASM cell cultures).