

## **Dexamethasone and Pentoxifylline Inhibit Endotoxin-induced Cachectin/Tumor Necrosis Factor Synthesis at Separate Points in the Signaling Pathway**

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### **Summary**

The induction of cachectin/tumor necrosis factor (TNF) synthesis by bacterial endotoxins is a process that entails activation at several levels. Cachectin/TNF gene transcription is accelerated, leading to rapid accumulation of mRNA within the macrophage cytosol. In addition, translational derepression occurs, leading to far more efficient message utilization. Through the use of post-transcriptional reporter constructs, we now demonstrate that certain agents capable of inhibiting cachectin/TNF biosynthesis operate through different mechanisms. In RAW 264.7 macrophages, pentoxifylline blocks cachectin/TNF mRNA accumulation but has no effect upon the efficiency of reporter mRNA translation. Dexamethasone, on the other hand, has only a modest effect on cachectin/TNF mRNA accumulation, but strongly impedes translational derepression. Combined application of dexamethasone and pentoxifylline to macrophages causes a greater suppression of cachectin/TNF biosynthesis than can be achieved by either agent alone. These findings suggest that the signaling pathway activated by endotoxin is branched, and that selective inhibition of different parts of the pathway may be achieved through the use of distinct agents.

It is widely accepted that bacterial endotoxins (LPS) provoke shock and tissue injury by eliciting the release of toxic factors from reticuloendothelial cells of the host (1). It also appears that cachectin/TNF is one of the principal endogenous factors involved in this process (2-4).

It is known that glucocorticoid agonists block the biosynthesis of cachectin/TNF (5). These agents cause a partial inhibition of cachectin/TNF mRNA accumulation; however, more important to their overall effect, they have been shown to markedly depress the translation of the mRNA that is produced (5). Among other agents that act to inhibit the release of cachectin/TNF, inhibitors of phosphodiesterase (most notably pentoxifylline), and cyclic AMP analogues seem to block the accumulation of cachectin/TNF mRNA (6). No studies of their effects at the translational level have been reported.

In this paper, we present evidence that the signaling pathway leading to affect the synthesis of cachectin/TNF is bifurcated, and that glucocorticoids and pentoxifylline affect different branches of this pathway.

### **Materials and Methods**

**Cell Culture.** Cells of the mouse macrophage line RAW 264.7, as well as primary mouse macrophages, were studied in these experiments. L-929 cells were used for cachectin/TNF assay.

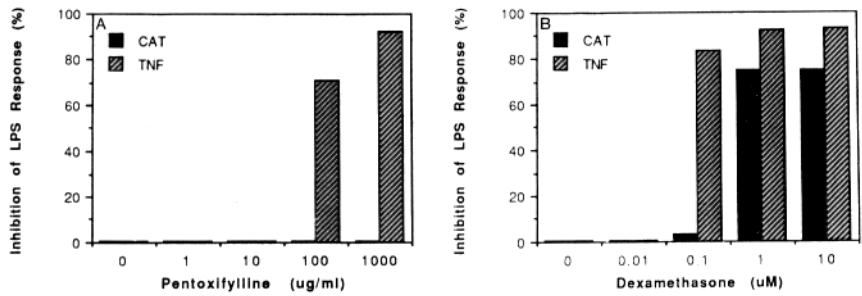
RAW 264.7 cells were transfected with two constructs designed

to assess posttranscriptional activation of cachectin/TNF synthesis. In each construct, CAT transcription is driven by the SV40 late promoter. In construct A, the CAT coding sequence is followed by most of the human cachectin/TNF 3'-untranslated region. In construct B (the control), no cachectin/TNF sequence is present. The RAW 264.7 cell lines transfected with constructs A and B have been described elsewhere (7); construct "A" is equivalent to construct "II" of that publication, and construct "B" is equivalent to construct "IX."

RAW 264.7 cells were maintained as previously described (7). Cells were plated at a density of  $\sim 10^5$  per well (1.6 cm diameter) for use in experiments involving LPS, pentoxifylline, and dexamethasone.

Mouse peritoneal macrophages were obtained as previously described (8). LPS (from *Escherichia coli*, strain 0127:B8; Difco Laboratories, Detroit, MI) was added to a final concentration of 1  $\mu\text{g}/\text{ml}$  in all experiments in which it was used. Varying concentrations of dexamethasone and pentoxifylline were used, as indicated. Dexamethasone (Fluka, Ronkonkoma, NY) and pentoxifylline (Hoechst-Roussel, Somerville, NJ) were added 1 h before addition of LPS in all instances. Quantitation of cachectin/TNF mRNA, CAT mRNA, and actin mRNA (control) was performed 4 h after activation by LPS. Cells and medium from identical cultures were harvested for measurement of CAT activity and cachectin/TNF activity 6 h after activation by LPS. L-929 (mouse fibrosarcoma) were grown and passaged in DME supplemented with 10% FCS.

**CAT Assay.** CAT assay was performed according to the thin-layer chromatography method of Gorman et al. (9). Quantitation of enzyme activity was achieved by scraping the 3-acetylated form



**Figure 1.** The effect of pentoxifylline (A) and of dexamethasone (B) on the induction of CAT activity and TNF release by RAW 264.7 cells transfected with construct A. Cells that were not treated with pentoxifylline or dexamethasone served as controls; percent inhibition of the response to endotoxin by pentoxifylline or dexamethasone was calculated with reference to the amount of CAT or TNF that could be produced by LPS-activated control cells. Values for TNF represent the mean of quadruplicate assays; single CAT assays were performed.

of [<sup>14</sup>C]chloramphenicol from the plate, and counting it in scintillation fluid.

**mRNA Analysis.** Cytoplasmic mRNA was isolated from RAW 264.7 cells, or from peritoneal macrophages (7), and used for Northern blot analysis. RNA was resolved in formaldehyde gels, and transferred to nylon membranes by electroblotting. Probes for cachectin/TNF and actin mRNAs were produced by randomly primed DNA synthesis. An antisense RNA probe was used to detect CAT mRNA.

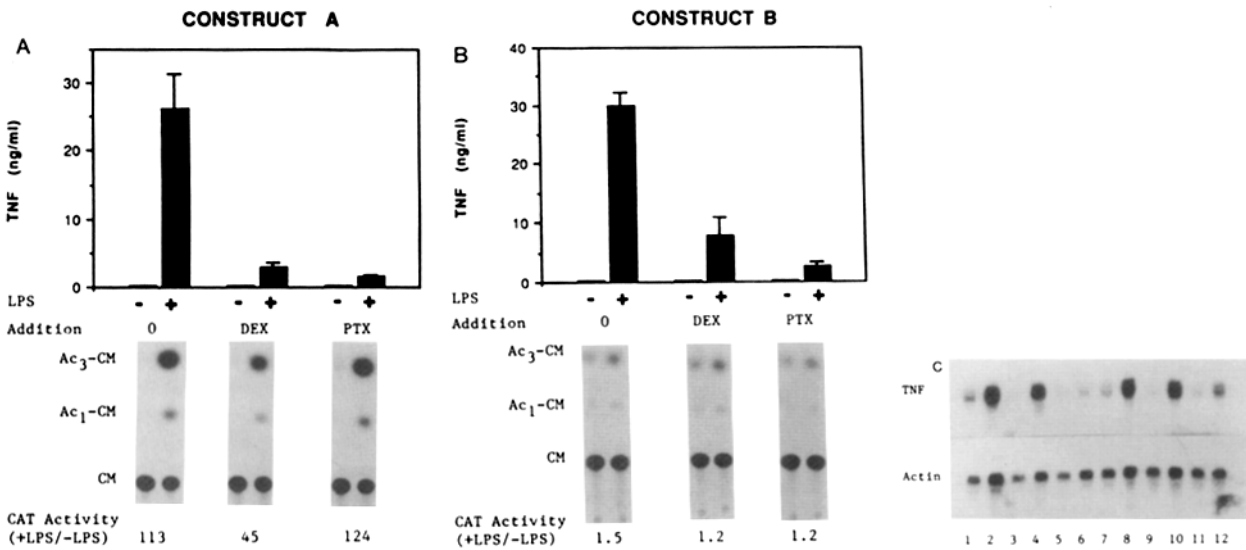
**Cachectin/TNF Assay.** Cachectin/TNF assay was carried out using cycloheximide-treated L-929 cells, as previously described (10). Results were expressed in terms of nanograms TNF per milliliter of culture medium, or in terms of percent inhibition of the response to LPS.

**Results**

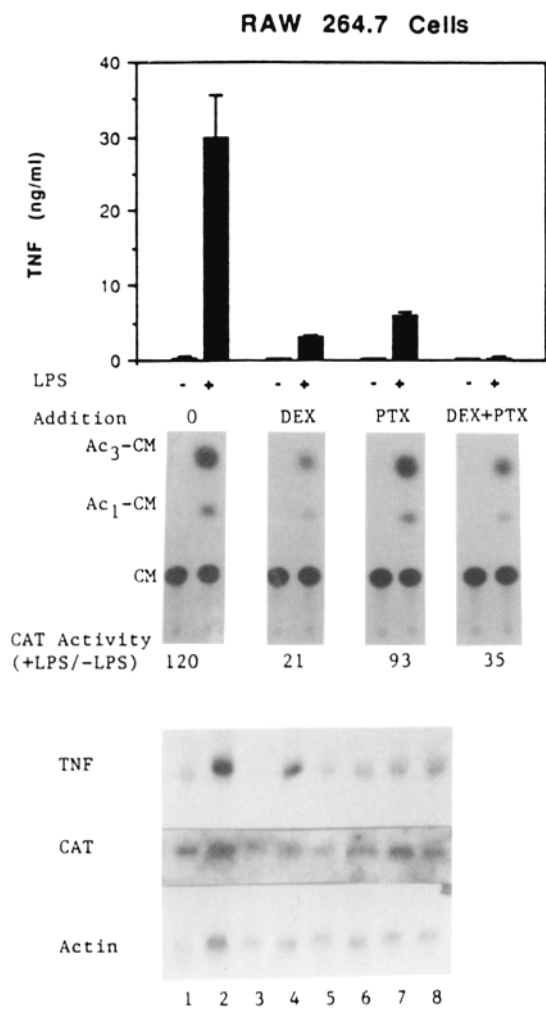
The presence of the cachectin/TNF 3'-untranslated region permits translational derepression of CAT synthesis to occur

in cells containing construct A (Fig. 1, A and B). While dexamethasone and pentoxifylline each caused strong inhibition of cachectin/TNF release from these cells, only dexamethasone caused a detectable inhibition of CAT expression. Inhibition of CAT biosynthesis required the application of slightly higher concentrations of dexamethasone than were required to inhibit cachectin/TNF synthesis. Moreover, the maximum inhibition of CAT synthesis fell slightly short of the inhibition of cachectin/TNF synthesis.

The effects of dexamethasone and pentoxifylline upon expression of CAT in RAW 264.7 cells transfected with construct A were then compared with the effects upon cells transfected with construct B (Fig. 2, A and B). As previously described, an endotoxin strongly induces CAT expression in cells bearing construct A, but has a negligible effect upon CAT expression in cells bearing construct B. Both dexamethasone and pentoxifylline inhibited the release of cachectin/TNF from LPS-induced RAW 264.7 cells, and as previously, only



**Figure 2.** The effects of dexamethasone and pentoxifylline on the induction of CAT activity and TNF release by RAW 264.7 cells transfected with construct A (A), or construct B (B). TNF release is presented in terms of nanograms/milliliter culture medium. Assays were performed in quadruplicate. Cultures were left unstimulated (-) or were stimulated (+) by LPS. Either no addition (0), or the addition of dexamethasone (DEX; 10 µM) or pentoxifylline (PTX; 100 µg/ml) was made to the culture 1 h before the time of LPS stimulation. Autoradiograms of CAT assays performed on cultures are displayed below the corresponding TNF assays; the CAT induction ratio measured for each sample is displayed below each autoradiogram. (C) TNF mRNA and actin mRNA (control) were examined in cultures displayed in (A) and (B) by Northern blotting. (Lanes 1-6) cells containing construct A. (Lanes 7-12) Cells containing construct B. Order of samples is the same as that shown in A and B.

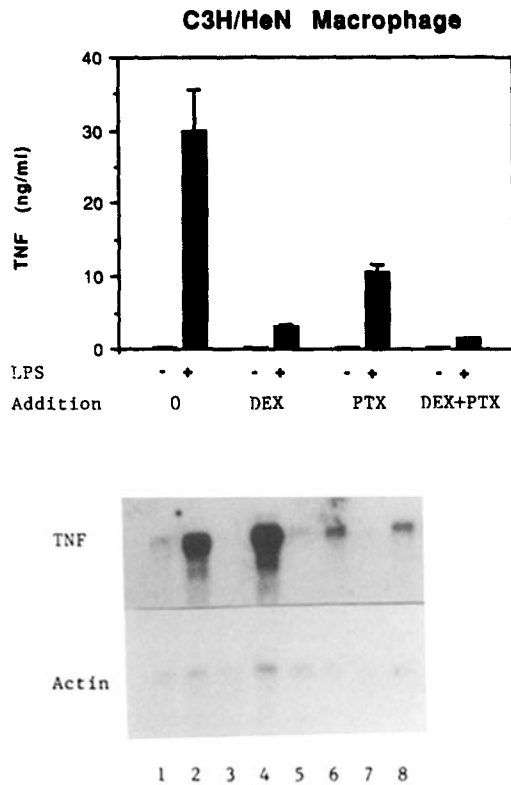


**Figure 3.** The effect of dexamethasone (10  $\mu$ M) and pentoxifylline (100  $\mu$ g/ml), alone or in combination, on the induction of CAT and TNF activities, and on the accumulation of CAT mRNA and TNF mRNA in RAW 264.7 cells transfected with construct A. Data are presented according to the same format as that described in Fig. 2. CAT mRNA, as well as TNF mRNA, was examined by Northern blotting. The effect of combined treatment with dexamethasone and pentoxifylline (DEX+PTX) is also assessed. Lanes 1-8 of the Northern blot correspond, sequentially, to the order of the TNF and CAT assays.

dexamethasone suppressed CAT biosynthesis in cells transfected with construct A. No such suppressive effect was noted in cells transfected with construct B. Thus, the inhibitory effect of dexamethasone, like the translational activating effect of LPS, is dependent upon sequences present in the 3'-untranslated region of cachectin/TNF.

It was further observed that dexamethasone had a rather modest inhibitory effect on cachectin/TNF mRNA accumulation in both cell lines (Fig. 2 C). Pentoxifylline had a far stronger effect: cachectin/TNF mRNA levels were depressed by 90% or more in each line. Thus, dexamethasone and pentoxifylline caused inhibition of cachectin/TNF synthesis by different mechanisms.

Combined application of dexamethasone and pentoxifyl-



**Figure 4.** The effect of dexamethasone (10  $\mu$ M) and pentoxifylline (100  $\mu$ g/ml), alone or in combination, on the induction of TNF and on the accumulation of TNF mRNA in primary macrophages derived from C3H/HeN mice. Lanes 1-8 of the Northern blot correspond, sequentially, to the order of the TNF assays.

line caused more pronounced inhibition of cachectin/TNF synthesis than either agent alone (Fig. 3). Neither agent substantially affected the levels of CAT mRNA expressed within the same cells. CAT mRNA, unlike cachectin/TNF mRNA, was not subject to induction by LPS in this experiment, although on some occasions (not shown) a comparatively weak inducing effect was noted.

As demonstrated in Fig. 4, macrophages derived from C3H/HeN mice are also affected by both dexamethasone and pentoxifylline, which act to suppress the production of cachectin/TNF. As in RAW 264.7 cells, dexamethasone has little or no effect upon cachectin/TNF mRNA accumulation, whereas pentoxifylline markedly diminishes the accumulation of this mRNA. When applied to primary macrophages in combination, dexamethasone and pentoxifylline achieve a higher degree of suppression than either agent is capable of causing by itself.

## Discussion

Recently, we demonstrated that cachectin/TNF synthesis largely depends upon translational derepression (7). In resting RAW 264.7 cells, cachectin/TNF translation is extremely inefficient; however, following activation by LPS, the rate of translation per unit mRNA increases some 200-fold. Thus, one part of LPS signaling elicits translational activation.

In the present study we have demonstrated that pentoxifylline and dexamethasone, which both inhibit the induction of cachectin/TNF synthesis by LPS, achieve their inhibitory effects through different mechanisms. Dexamethasone exerts a strong inhibitory effect upon translational derepression, and weakly inhibits the accumulation of cachectin/TNF mRNA. Pentoxifylline, by contrast, strongly inhibits the accumulation of cachectin/TNF mRNA, but has no effect upon the translational derepression process. At present, little is known about the molecular events that follow stimulation of the macrophage by LPS. We may now conclude, however, that the signaling pathway bifurcates at least once, so as to allow activation of TNF synthesis at pretranslational and translational levels. These features of the activation pathway are independently inhibitable.

Since dexamethasone blocks both cachectin/TNF mRNA accumulation and, to a greater extent, the effective translation of this mRNA, we consider it likely that this agent acts at a very proximal point in the chain of events that follow contact between LPS and the cell. Conceivably, dexamethasone might influence the behavior of the LPS receptor itself,

or the actions of the early transducing protein involved in the response. The comparatively selective effect of pentoxifylline may reflect inhibition at a level well removed from the LPS receptor. As one possible interpretation, it might be suggested that pentoxifylline diminishes LPS-induced transcription of the cachectin/TNF gene. However, we are reluctant to draw this conclusion since no measurements of transcription have yet been performed.

Since dexamethasone and pentoxifylline prevent cachectin/TNF synthesis by exercising inhibition at separable points in the LPS signaling pathway, it is not surprising to note that the two agents together block cachectin/TNF synthesis more effectively than either alone. This effect is demonstrable in primary macrophage cultures, just as in RAW 264.7 cells, and presumably has a similar molecular basis. It is probable that the biosynthesis of other cytokines is similarly affected by pentoxifylline, alone or in combination with dexamethasone, although we have yet to investigate this issue. It is also possible that the combined use of pentoxifylline and dexamethasone in vivo would attenuate the lethal effect of endotoxin more effectively than either agent administered by itself.

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