Brief Definitive Report

Stimulation of Tumor Necrosis Factor α Production in Human Monocytes by Inhibitors of Protein Phosphatase 1 and 2A

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Summary

The protein phosphatase 1 and 2A inhibitor, okadaic acid, has been shown to stimulate many cellular functions by increasing the phosphorylation state of phosphoproteins. In human monocytes, okadaic acid by itself stimulates tumor necrosis factor α (TNF-α) mRNA accumulation and TNF-α synthesis. Calyculin A, a more potent inhibitor of phosphatase 1, has similar effects. TNF-α mRNA accumulation in okadaic acid–treated monocytes is due to increased TNF-α mRNA stability and transcription rate. The increase in TNF-α mRNA stability is more remarkable in okadaic acid–treated monocytes than the mRNA stability of other cytokines, such as interleukin 1α (IL-1α), IL-1β, and IL-6. Gel retardation studies show the stimulation of AP-1, AP-2, and NF-κB binding activities in okadaic acid–stimulated monocytes. This increase may correlate with the increase in TNF-α mRNA transcription rate. In addition to the stimulation of TNF-α secretion by monocytes, okadaic acid appears to modulate TNF-α precursor processing, as indicated by a marked increase in the cell-associated 26-kD precursor. These results suggest that active basal phosphorylation/dephosphorylation occurs in monocytes, and that protein phosphatase 1 or 2A is important in regulating TNF-α gene transcription, translation, and posttranslational modification.

TNF-α has been shown to play important roles in many inflammatory and pathological conditions, as well as in cellular activation and differentiation (reviewed in reference 1). The regulation of TNF-α production occurs at both translational and transcriptional levels. At the level of transcription, transcription factors NF-κB and AP-1 have been implicated in the activation of the TNF-α gene by LPS and PMA (2–5). Evidence has accumulated showing that the phosphorylation states of NFκB-associated proteins influence the activity of NFκB (6, 7). Similarly, the phosphorylation state of c-jun is related to the activation of AP-1 (8, 9). Activation of NF-κB and AP-1 involves serine/threonine phosphorylation. The investigation of the roles of protein serine/threonine phosphatases in cellular processes has been facilitated by the availability of inhibitors such as okadaic acid and calyculin A (10). Of relevance is the finding that okadaic acid has been shown to induce AP-1 and NF-κB in human T cells (11, 12). In the present study, these inhibitors are used to show that TNF-α gene transcription and production are induced in resting monocytes by the inhibition of protein phosphatases.

Materials and Methods

Reagents and Cells. Okadaic acid and calyculin A were from Moana Bioproducts (Honolulu, HI). The sources of other reagents have been described (5, 13). Rabbit anti-TNF-α antibody was a gift of Dr. Edward Jeffes III (University of California, Irvine, CA). Human peripheral blood monocytes were isolated from buffy coats (Richmond Metropolitan Blood Service, Richmond, VA) by Ficoll-Hypaque centrifugation, E-rosette cell depletion of T cells, and Percoll gradient centrifugation. Cell culture reagents were <0.002 ng/ml endotoxin (13). Monocyte preparations contained 90–95% monocytes, as shown by nonspecific esterase staining, and 95% of the cells were viable at the end of the experiments by trypan blue exclusion.

Northern Blots, Nuclear Run-on, and mRNA Stability Measurements. Isolation of total RNA by ultracentrifugation over a CsCl cushion and Northern blot hybridization procedures with cDNA of TNF-α, IL-1α, IL-1β, *c-jun*, β-actin, and the 26-mer 28S rRNA oligonucleotide probes have been described (13). The IL-6 cDNA probe was a kind gift of Dr. Pravin Sehgal (New York Medical College, Valhalla, NY). Blots were exposed multiple times and exposures within the linear range were scanned for absorbance. Nuclear run-on assays were performed essentially as described, using 15–25 × 10^6 nuclei per reaction (13). TNF-α nascent mRNA was detected with a 234-bp PvuII fragment. For mRNA stability studies, monocytes at 5 × 10^6/ml were preincubated in 500 ng/ml LPS or 100 ng/ml okadaic acid for 2 h. Actinomycin D was then added to make the final concentration 10 μg/ml. Samples were quickly cooled in three to four volumes ice-cold PBS at the indicated time points. RNA extraction and Northern blots were then performed.

Gel Retardation Assays. Nuclear extracts were prepared and gel

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retardation assays were performed with oligonucleotides for AP-1, AP-2, and κB-2 of TNF-α, and the myoD 48-bp oligonucleotide as described (5). The κB-3 oligonucleotide 5′-GAT CGA GCT CAT GGG TTT CTC CAC CAA GGA AG (4) and its complementary strand were used.

Immunoprecipitation. Labeling, immunoprecipitation, and gel analysis were performed according to the procedures described previously (13). Cysteine-free medium and [^{35}S]cysteine (50 μCi/ml, 1,000 Ci/mmol; NEN/DuPont, Wilmington, DE) were used instead because of the lack of methionine residues in the mature TNF-α. Monocytes were incubated in labeling solutions containing various stimulants at 5 × 10^6 cells/ml (15–20 × 10^6 cells/sample) for 6 h.

Results and Discussion

Induction of TNF-α mRNA by Phosphatase Inhibitors. Freshly isolated human peripheral blood monocytes were found to have little TNF-α mRNA. Okadaic acid was shown to be a potent inducer for TNF-α mRNA accumulation (Fig. 1). This induction was dose dependent in the range of 10–100 ng/ml, and occurred maximally 2–4 h after stimulation (data not shown). In some experiments, monocyte viability began to decline in 4-h cultures when 100 ng/ml okadaic acid was used. The okadaic acid effect was not due to endotoxin contamination. Polymyxin B at 100 μg/ml inhibited 80% of the LPS effect and did not affect okadaic acid–mediated mRNA accumulation. Okadaic acid was synergistic with a suboptimal dose of LPS in the induction of TNF-α mRNA accumulation (Fig. 1 *A*). Similar effects were observed regarding IL-1β mRNA accumulation. In contrast, minimal effects were observed in the case of IL-1α and IL-6.

Calyculin A is a phosphatase inhibitor with a similar spectrum of specificity as okadaic acid. However, it is 10–100-fold more potent than okadaic acid in inhibiting phosphatase 1, although it inhibits phosphatase 2A with similar potency (10). Calyculin A, at 10 and 30 ng/ml, induced TNF-α mRNA accumulation (Fig. 1 *B*). However, this inductive effect was not observed at a higher concentration of 100 ng/ml. Calyculin A at 10 ng/ml was markedly synergistic with LPS (Fig. 1 *B*). At 30 ng/ml, the synergism was less. At 100 ng/ml, calyculin A appeared to inhibit LPS-induced TNF-α mRNA accumulation. These results indicate that phosphatases are important in the regulation of TNF-α mRNA accumulation.

Previous studies show that elevation of cAMP inhibits TNF-α mRNA expression induced by PMA or LPS (14). This inhibition is postulated to be due to the interaction of cAMP-responsive element binding proteins with the cAMP-responsive element present at the −107 position of the TNF-α gene. Okadaic acid did not override this inhibitory effect (data not shown). Thus, serine/threonine phosphorylation regulated by phosphatase 1 and 2A and their regulatory phosphatase may play a minor role in this process. The addition of 1–10 μg/ml of cycloheximide enhanced the okadaic acid and LPS effects. This superinduction is similar to TNF-α mRNA induction by PMA in cell lines (reviewed in reference 1). Furthermore, de novo protein synthesis is not required.

Effects of Okadaic Acid on TNF-α mRNA Stability and Transcription Rate. The induction of TNF-α mRNA accumula-

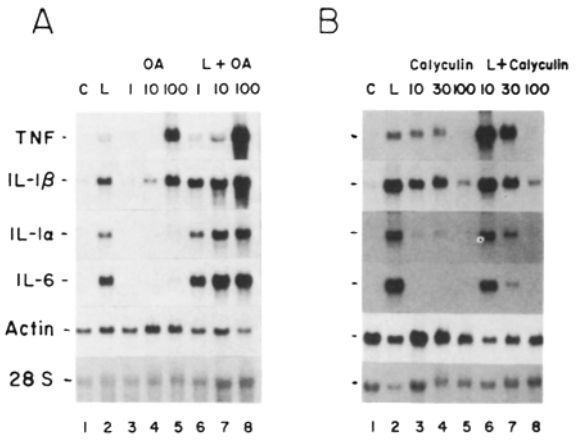

Figure 1. Okadaic acid (*A*) and calyculin A (*B*) stimulation of cytokine mRNA expression in human monocytes. Freshly isolated monocytes were incubated with control medium (*C*) or medium containing the indicated stimulants for 2 h. Northern blot assays were carried out. LPS (L) was used at 100 ng/ml. The concentrations of okadaic acid (OA) were from 1 to 100 ng/ml and calyculin A from 10 to 100 ng/ml as indicated. Monocytes in *A* and *B* were from different individuals, and 15 × 10^6 cells were used in each sample. The result is representative of three experiments.

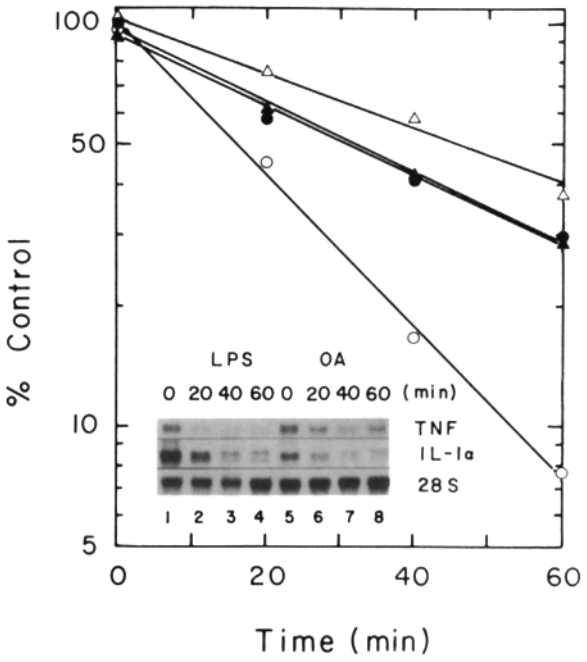

Figure 2. Comparison of TNF-α and IL-1α mRNA decay in monocytes stimulated with LPS and okadaic acid. Monocytes were preincubated in LPS (lanes *1–4*) or 100 ng/ml okadaic acid (OA; lanes *5–8*) for 2 h. At the indicated time points after the addition of 10 μg/ml actinomycin D, RNA were extracted and analyzed by Northern blot. The absorbance of TNF-α or IL-1α bands were measured by densitometry and normalized against that of 28S rRNA bands. The normalized absorbance values were plotted in a semilogarithmic plot as percent of the zero-time absorbance. The curves were fitted by linear regression by the computer software program SigmaPlot. mRNA decay curves in LPS are in open symbols for LPS and in closed symbols for okadaic acid. Decays for TNF-α mRNA are in circles, and the decay for IL-1α mRNA is shown in triangles.

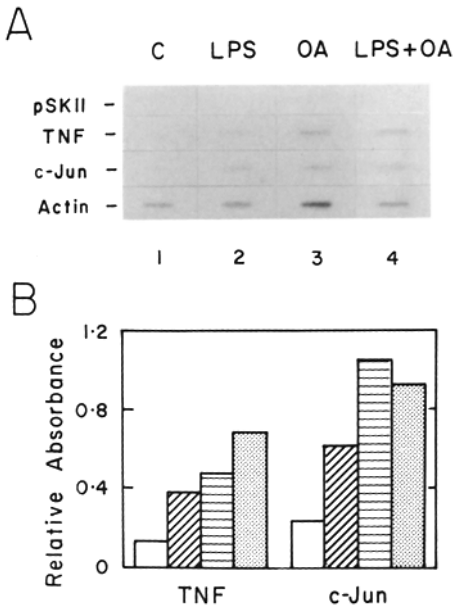

Figure 3. Nuclear run-on assay of monocytes stimulated with okadaic acid. Monocytes (25 × 10^6/sample) were treated with control medium, or medium containing 100 ng/ml LPS, 100 ng/ml okadaic acid (OA), or 100 ng/ml each of LPS and okadaic acid for 2 h at 5 × 10^6/ml. Nuclei were isolated and labelled. Nascent mRNA was run off the template using 250 μCi/sample α-[^{32}P]UTP (3,000 Ci/mmol). The absorbance of TNF-α and c-*jun* mRNA signals in *A* were scanned by densitometry and normalized against that of β-actin. This relative absorbance is plotted in *B*. (□) Control; (▨) LPS; (▤) okadaic acid; and (▩) LPS plus okadaic acid.

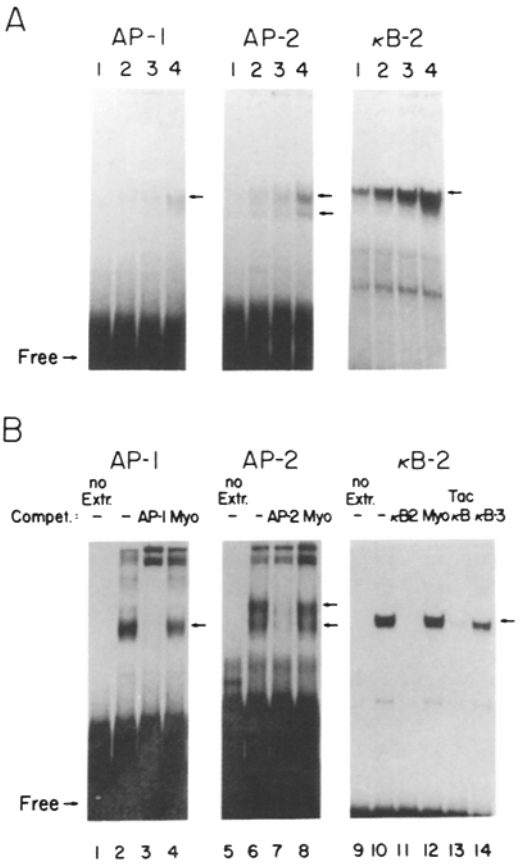

Figure 4. Stimulation of AP-1, AP-2, and NF-κB binding activities by okadaic acid. Gel mobility shift assay was performed as described in Materials and Methods using 10 μg of nuclear extract per assay. In *A*, the extracts were from monocytes in control medium (lanes *1*), in medium containing 100 ng/ml LPS (lanes *2*), 100 ng/ml okadaic acid (lanes *3*), or LPS plus okadaic acid (lanes *4*). Similar results were obtained in two additional experiments. In *B*, the specificity of the retarded bands were examined by a binding competition assay. Nuclear extracts were incubated with 100-fold excess of the cold competitors for 10 min before the addition of AP-1, AP-2, and κB-2 probes. No extract was present in samples depicted in lanes *1*, *5*, and *9*. MyoD (48 bp) and IL-2R κB (50 bp) (Tac κB) have been described (5). In the case of the cold κB-3 probe. The inhibition was ~70% by scanning. Arrows in both panels indicate specific retarded bands. Similar results were obtained in one additional experiment.

tion by okadaic acid may be due to increases in mRNA stability and/or increased transcription rate. TNF-α mRNA stabilization is particularly pertinent in this case, in view of the short half-life of TNF-α mRNA and the increase of mRNA stability by protein kinase C (PKC) activation (5, 15).

Since freshly isolated monocytes have very little TNF-α mRNA, the half-life of TNF-α mRNA in LPS-stimulated monocytes is used as a reference point. Thus, monocytes were incubated with okadaic acid or LPS. Actinomycin D was added to inhibit transcription and mRNA decay was then measured. As shown in Fig. 2, TNF-α mRNA decayed rapidly in LPS-treated cells, with a $t_{1/2}$ of 17 min. In the okadaic acid–treated cells, the $t_{1/2}$ of TNF-α mRNA was lengthened to 35 min. In contrast to TNF-α mRNA, IL-1α mRNA has a slightly longer $t_{1/2}$ in LPS-treated monocytes. The occurrence of doublets in IL-1α mRNA in actinomycin D–treated cultures may be due to processing of the poly(A) tail (16). Although not shown, the stability of the mRNA of other cytokines such as IL-1β and IL-6 was not substantially different in the LPS- and okadaic acid–treated cells. Similar results were obtained in two additional experiments. Recently, a 32-kD protein was described to bind the AU-rich 3′-untranslated region of certain cytokine and oncogene mRNA, and this binding was thought to be related to mRNA stability (17). Of more relevance is the work by Bohjanen et al. (18), describing a PMA-regulated 32-kD protein binding to the AU-rich domain of TNF-α, but not c-*myc* mRNA. Whether this protein is involved in the preferential stabilization of TNF-α mRNA by okadaic acid remains to be determined.

The effect of okadaic acid on TNF-α transcription was examined by nuclear run-on. A representative result of four experiments is depicted in Fig. 3. In this experiment, both LPS and okadaic acid increased the transcription rate of the TNF-α gene. In addition, some additive effect was observed when both reagents were used. Although not shown, okadaic acid induced the accumulation of c-*jun* mRNA. Thus, the transcription rate of c-*jun* was included in Fig. 3. Indeed, the transcription rate for the c-*jun* gene was enhanced by both LPS and okadaic acid. However, there was no additive effect when both were included in the same culture. In experiments by gel retardation assays, there was an increase in NFκB, AP-1, and AP-2 binding activities in nuclear extracts of monocytes

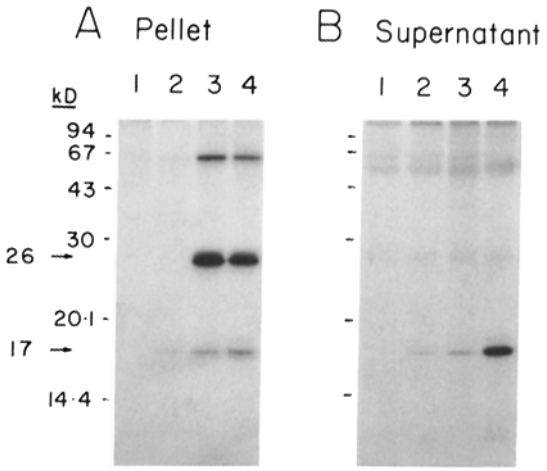

Figure 5. Production of TNF-α from monocytes stimulated with okadaic acid. TNF-α was immunoprecipitated from monocyte pellets (*A*) and cell supernatants (*B*). The monocytes (20 × 10^6/ml) were incubated in control medium (lane *1*), 100 ng/ml LPS (lane *2*), 50 ng/ml okadaic acid (lane *3*), or 100 ng/ml LPS plus 50 ng/ml okadaic acid (lane *4*) for 6 h. 26- and 17-kD markers indicate precursor and mature TNF-α. The 60-kD band of uncertain significance was markedly increased in okadaic acid cultures. A representative of three experiments is described.

treated with either LPS or okadaic acid (Fig. 4 *A*). These binding activities were specific as they could only be inhibited by specific probes (Fig. 4 *B*). Similar results were obtained for both κB-2 and κB-3 binding sites (4), although only the data for the κB-2 binding site were presented. Higher binding activities were observed consistently in the nuclear extracts of monocytes treated with both LPS and okadaic acid. The increase in these DNA binding activities may in part account for the observed increase in transcription rate for TNF-α mRNA. Thus, both increase in mRNA stability and increased transcription rate contribute to the accumulation of TNF-α mRNA induced by okadaic acid.

Increase in TNF-α Production Induced by Okadaic Acid. TNF-α production has been shown to be under translational control (1). The possibility for the involvement of phosphatases 1 and 2A in this process was investigated. Okadaic acid–stimulated monocytes were metabolically labeled with [^{35}S]cysteine. Because of the length of incubation of 6 h and the toxicity of okadaic acid, a reduced dose of 50 ng/ml was used. Both the supernatant and the cell pellet were examined for TNF-α production by immunoprecipitation. As shown in Fig. 5, there was little TNF-α production in the control culture. LPS stimulated TNF-α production, as evidenced by the precipitated bands in both the supernatant and cell pellet. Okadaic acid was more potent in its induction of TNF-α secretion and it was synergistic with LPS in this stimulation. It is of interest to note that the 26-kD TNF-α precursor form was more prominent in okadaic acid–stimulated, but not in LPS-stimulated, cells. This 26-kD precursor form has been detected to be cell associated (19). In this regard, monocytes stimulated with okadaic acid were stained with two blocking anti-TNF-α mAbs. No surface staining was detected. Thus, the majority of precursor TNF-α may not be expressed on the surface of these stimulated cells. These results would implicate phosphatase 1 and 2A in playing a role in translational and posttranslational modification of TNF-α.

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