Posttranscriptional Regulation of Macrophage Tissue Factor
Expression by Antioxidants


Tissue factor (TF) expression by cells of monocyte/macrophage lineage represents an important mechanism underlying the initiation of fibrin deposition at sites of extravascular inflammation. Recent evidence suggests a role for oxidant stress in the signalling pathway of various cell types by virtue of its ability to induce DNA binding of various transcription factors, including nuclear factor κB and AP-1. The effect of antioxidant treatment on lipopolysaccharide (LPS)-induced TF expression was examined in murine peritoneal macrophages and human monocytes. Both pyrrolidine dithiocarbamate, an oxidant scavenger, and N-acetyl-cysteine, a precursor of the endogenous antioxidant glutathione, inhibited stimulation of macrophage procoagulant activity by LPS. Northern blot analysis showed that neither of these agents reduced LPS-stimulated TF mRNA accumulation, thereby suggesting a posttranscriptional mechanism for the effect. Immunofluorescence studies of human monocytes using polyclonal anti-TF antibody showed that N-acetyl-cysteine treatment prevented the characteristic plasmalemmal localization of TF antigen that occurs in response to LPS. Western blot analysis showed that N-acetyl-cysteine reduced the accumulation of the 47-kD mature glycoprotein in LPS-treated cells, a finding consistent with the results of the immunofluorescence studies. Furthermore, these conditions did not result in an accumulation of the less mature forms of TF. When considered together, these data suggest that antioxidants exert their effects by impairing translation and/or by causing degradation of newly translated protein. The effect of antioxidants on tumor necrosis factor appeared to be species specific, with no effect on LPS-induced tumor necrosis factor in murine cells, but with inhibition in human monocytes. The posttranscriptional effect of antioxidants on TF expression data suggests a novel mechanism whereby these agents might modulate monocyte/macrophage activation.

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Local fibrin deposition mediated via the expression of cell-associated procoagulants appears to be important in the pathogenesis of several disease processes, including viral hepatitis, lupus glomerulonephritis, acute lung injury, and bacterial abscesses. Although cellular procoagulants represent a heterogeneous group of molecules, tissue factor (TF) appears to be the predominant procoagulant molecule synthesized in response to most proinflammatory stimuli. This 47-kD surface-expressed glycoprotein initiates the extrinsic pathway of the coagulation cascade by binding factor VII and augmenting its conversion to factor VIIa. The resulting factor VIIa complex activates both factor X and factor IX, leading to ultimate clot formation. The complete sequences for both the human and murine TF genes have been reported and both have a 56-bp LPS response element within their promoters. This region is characterized by the presence of two AP-1 and one NF-κB binding sites. Binding to both the distal AP-1 and the NF-κB sites is required for optimal transcription of the TF gene. Based on the demonstrated ability of oxidants to induce binding of these transcription factors to their respective DNA binding sites and initiate gene
transcription, we performed experiments to examine the effect of antioxidants on TF functional activity and gene expression in stimulated murine macrophages and human monocytes. These studies showed that both N-acetyl-cysteine (NAC) and pyrroglutamic acid, NAC, glutamine, and RPMI 1640 medium were not more than five animals per cage and were fed standard mouse chow and water ad libitum.

Pyroglutamic acid, NAC, glutamine, and RPMI 1640 medium were obtained from Sigma (St Louis, MO). Immobilon was from Millipore (Bedford, MA). Powdered Brewer's thioglycolate was obtained by peritoneal lavage, using 10 mL of thioglycolate medium. The antioxidant to be tested was added at t=0 hour and was present throughout the incubation period, except in the delayed addition experiments. At the end of the incubation period, cells were sedimented by centrifugation at 200g for 10 minutes. Supernatants were aspirated and frozen at -70°C for later measurement of TNF. Cell pellets were resuspended in RPMI 1640 and frozen at -70°C for measurement of procoagulant activity (PCA). For Western blot analysis, cells were rapidly sedimented and pellets were immediately solubilized in boiling Laemmli sample buffer.

Measurement of macrophage PCA. PCA in freeze-thawed macrophages was determined by measuring their capacity to shorten the spontaneous clotting time of normal citrated human plasma in a one-stage clotting assay. An 80 mL sample of macrophage cell lysates obtained by freeze-thawing was added to 80 mL of citrated normal human platelet-poor plasma, and then 80 mL of 25 mmol/L CaCl2 was added to initiate the reaction. The time taken for the appearance of a fibrin gel at 37°C with gentle agitation was recorded. Previous studies by Lando and Edgington indicated that LPS-induced murine macrophage PCA is only slightly less effective at shortening the clotting time of human plasma than at shortening the clotting time of mouse plasma. Clotting times were converted to milliliters of PCA by comparison with clotting times of a rabbit brain thromboplastin standard in which 36 mg (dry weight) per milliliter was assigned a value of 100,000 mU of PCA. The induction of PCA from a baseline
of 150 mU/l × 10⁶ macrophages to 1,500 mU/10⁶ macrophages in cells stimulated by LPS alone represented a shortening of the clotting time from 85 to 57 seconds. The assay was used over the range of 10 to 10,000 mU of PCA, this range being linear with normal plasma substrate. Previous studies have shown that PCA induced with Ecoli LPS has TF-like activities, making comparison with a thromboplastin standard valid.²⁷

TF detection by immunochemistry. PBM cells were incubated on poly-L-lysine–coated glass coverslips for 20 minutes at 37°C in 5% CO₂ and nonadherent cells were washed off. Adherent cells were then fixed and permeabilized in cold acetone. Cells were then incubated with the polyclonal antihuman TF antibody in 1% bovine serum albumin (BSA) for 30 minutes, washed, and then stained with fluorescein isothiocyanate (FITC)-labeled secondary antibody for a further 30 minutes. Cells were viewed with a Nikon Diaphot-TMD epiﬂuorescence microscope (Nikon Canada, Inc, Toronto, Canada).

TF detection by Western blotting. TF expression in human PBM was determined by immunoblotting with polyclonal antihuman TF antibodies.²⁴ After electrophoresis on 10% polyacrylamide gels, the samples were blotted onto Immobilon using the Bio-Rad Mini Trans-Blot system (Bio-Rad, Richmond, CA) for 1 hour at 100 V. The blot was incubated with 10 mL of blocking solution and then exposed to a 1/1,000 dilution of the primary antibody for 2 hours while shaking at room temperature. The blot was then washed three times with antibody buffer solution and incubated with a 1/5,000 dilution of anti-IgG antibody conjugated to horseradish peroxidase. The blots were washed, dried, and quantitated using an enhanced chemiluminescence detection system (ECL; Amersham, Arlington Heights, IL).²⁸

Measurement of TNF. TNF in supernatants was measured by enzyme-linked immunosorbent assay (ELISA). The antibody sandwich was detected by fluorescence.²⁹ Microtiter plates were coated with a monoclonal antimurine TNF antibody, incubated with samples, washed, and then exposed to the polyclonal rabbit antimurine TNF antibody. The enzyme sandwich was then incubated with goat antirabbit IgG alkaline phosphatase for 1 hour at room temperature before the addition of the substrate solution, 5-fluorosalicyl phosphate, and the developing reagent, Terbium-EDTA. The ﬂuorescence was measured with a time-resolved fluorometer and the calibration curve and the data reduction were performed by automatic immunanalyzer (Cyber Fluor 615; Scintrex, Concord, Canada).

Measurement of ROS. ROS were determined by luminol-dependent chemiluminescence according to Allen.³⁰ Briefly, 10⁶ macrophages in a luminol-balanced salt solution were added to polystyrene tubes and placed in an automated luminometer (Axxis; ExO/Emis, San Antonio, TX) at 37°C for 45 minutes. Cells were then treated with LPS or zymosan in the presence or absence of the antioxidants.
PDTC or NAC. These antioxidants were present during the 45-minute preincubation period and remained present during the subsequent incubation. Addition of these agents at the time of stimulation with either LPS or zymosan gave similar results. Changes in chemiluminescence were recorded over a 4-hour period. Relative total oxidant production was obtained by integrating the area under the curve of three separate experiments.

Effect of antioxidants on PCA. Having defined their antioxidant properties, these molecules were tested for their effect on LPS-induced PCA. PDTC caused a dose-dependent inhibition of PCA in cells stimulated with LPS (Fig 2A), without affecting cell viability (>95% viability by trypan blue exclusion). The IC_{50} for PDTC was approximately 77 μmol/L and reached almost complete inhibition at 5 mmol/L. PDTC alone had no effect on basal levels of PCA. Neither pyrrolidine nor pyrogallol acid, a cyclic molecule resembling PDTC but without radical scavenging properties, was able to mimic the effect. PDTC added to the functional PCA assay also had no effect on the clotting time reductions induced by rabbit brain thromboplastin or cell-associated TF induced by 4 hours of preincubation with LPS (data not shown). A time course of PCA induction showed that PDTC exhibited inhibitory effects starting at 2 hours, a time point at which functional PCA activity first became evident (Fig 2B). Figure 2C illustrates the effect of delayed addition of PDTC on PCA expression by cells exposed to LPS for 4 hours. The addition of PDTC more than 2 hours after treatment with LPS resulted in a reversal of the inhibitory effect of PDTC. The inhibitory effect was not unique for LPS-induced PCA, because PDTC also inhibited the stimulation of PCA by the fungal cell wall component, zymosan (data not shown). Disulfiram, which has antioxidant properties similar to those of PDTC, effected a comparable reduction in LPS-induced PCA (data not shown).

As noted in the previous section, NAC exerts its antioxidant effect by increasing levels of the endogenous antioxidant, reduced glutathione. Figure 3 illustrates the effect of NAC on PCA induction by LPS. NAC inhibited PCA at 30 mmol/L, a concentration shown to cause a fivefold increase in intracellular glutathione levels over 4 hours of incubation (data not shown). Despite the reduced absolute level of PCA in NAC-treated LPS cells, the fold increase after stimulation with LPS was comparable in the presence or absence of NAC (10.6-fold v 9.3-fold, respectively). Viability by trypan blue exclusion was greater than 94%.

Effect of antioxidants on TF mRNA levels. Previous studies have shown that LPS-induced PCA in macrophages was caused by the expression of TF on the cell surface.\textsuperscript{8} This increase required new RNA and protein synthesis.\textsuperscript{9} Because antioxidants have been reported to prevent agonist-induced gene transcription by virtue of their ability to inhibit translocation of the transcriptional activator NF-κB into the nucleus,\textsuperscript{11} it was hypothesized that PDTC and NAC might inhibit TF expression at the transcriptional level. Northern analysis was performed to assess TF mRNA levels in LPS-treated cells in the presence or absence of the antioxidants. Similar to findings reported for human monocytes,\textsuperscript{9} LPS induced an increase in TF mRNA in murine macrophages.
Effect of antioxidants on TF expression. Immunofluorescence and Western blot analysis with polyclonal antihuman TF antibody were used to evaluate posttranscriptional alterations in TF. These studies were performed in human monocytes because of the availability of the antihuman TF antibody and the fact that others have documented poor cross-reactivity with rodent TF. Figure 5A shows the effect of NAC (30 mmol/L) on PCA in human monocytes after exposure to LPS. As shown for murine cells, NAC markedly reduced LPS-induced PCA in these cells. Similarly, PDTC (1 mmol/L) inhibited LPS-stimulated PCA in human monocytes (Fig 5B). Human monocytes exposed to either NAC or PDTC for 4 hours retained their ability to exclude trypan blue (>95%), suggesting that the effect was not caused by cytotoxicity. The fold increase in PCA after stimulation with LPS was also higher in the presence of NAC (62-fold) than in the absence of NAC (36-fold), further showing the lack of cytotoxicity. Finally, the level of TF mRNA transcripts in human monocytes was similar in cells treated with LPS in the presence or absence of NAC (Fig 5C). Thus, the effects of antioxidants on human cells appear to recapitulate those observed in murine cells, thereby justifying further study in these cells.
increased at the plasma membrane in LPS-treated cells, whereas in controls no distinct reactivity was observed (Fig 6). These findings are consistent with previous observations. NAC completely prevented the plasmalemmal localization of antigen in cells exposed to LPS and cells appeared similar to controls. To investigate this effect in further detail, Western blot analysis was used (Fig 7). LPS-treated cells exhibited a large increase in a band with approximate molecular mass of 47 kD, consistent with an increase in the mature fully glycosylated TF, as well as a slight increase in a band at 33 kD. This result likely represents the unglycosylated form of the protein. The addition of NAC during stimulation with LPS completely prevented the increase in the high molecular mass band and had little effect on the band at 33 kD. There was no evidence of degraded immunoreactive TF in NAC-treated cells. Incubation of preformed TF with NAC also had no effect on the reactivity of the antibody (data not shown).

**Release of TNF by LPS-treated cells.** To discern whether the inhibitory effect of PDTC and NAC on TF expression was caused by a global effect on protein synthesis, the effect of these antioxidant molecules on release of TNF was studied. As shown in Fig 8A, PDTC augmented LPS-stimulated TNF release from murine macrophages, whereas PDTC alone had no effect on basal TNF levels. By contrast, NAC did not alter either basal or LPS-stimulated TNF release (Fig 8B). Northern blot analysis showed that PDTC augmented TNF mRNA accumulation in LPS-treated murine cells, over the time course of augmented TNF release (Fig 9A). However, this effect was not observed in NAC-treated cells.
Fig 6. Immunofluorescence of human monocytes using antihuman TF antibody. Cells were adhered to glass coverslips and treated for 4 hours with (A) medium alone, (B) NAC alone, (C) LPS alone, or (D) LPS plus NAC (added at t = 0 hour). Cells were then fixed, permeabilized, stained, and viewed as outlined in the Materials and Methods. A representative study of two independent studies is shown.

However, PDTC alone had no effect. Figure 9B shows a Northern blot analysis of the effect of PDTC on LPS-induced TNF mRNA in human monocytes. In contrast to murine cells, PDTC reduced levels of TNF mRNA in human cells exposed to LPS.

DISCUSSION
Antioxidants acting by two distinct mechanisms inhibited the functional activity of TF in LPS- and zymosan-treated murine macrophages. In human monocytes, one of these agents, NAC, was shown to inhibit both the functional and antigenic expression of mature TF normally induced by exposure to LPS. Inhibition did not appear to be caused by cytotoxicity or a nonspecific effect related to interference with the assay systems. The observation that both compounds exhibited potent antioxidant activity coupled with the finding that PDTC was ineffective when added at a time point after the decline of LPS-induced chemiluminescence.
was either comparable or increased in the presence of NAC and PDTC, respectively.

Inhibition of TF expression appeared to be unrelated to alterations in gene transcription, because neither agent reduced the level of TF gene transcripts compared with cells treated with LPS alone in either the murine or human system. Rather, the effect appeared to be posttranscriptional. Several precedent exist whereby the redox state of the cell might modulate posttranscriptional events. The redox state has been shown to alter mRNA stability, to modulate translation, and to affect posttranslational modification of newly synthesized proteins. Finally, the redox potential might directly affect the functional activity of the completely processed mature protein. The observation that the accumulation of mRNA for TF in LPS-treated cells was equivalent in either the presence and absence of antioxidants suggests that an effect on mRNA stability is unlikely. Furthermore, the addition of NAC to commercially acquired rabbit brain thromboplastin or to presynthesized cell-associated TF had no effect on its procoagulant function, indicating that direct reduction of the active glycoprotein did not alter function. Thus, it is unlikely that the antioxidants directly altered the activity of membrane-associated TF. The findings are more consistent with either reduced translation of the mRNA or some posttranslational alteration of the protein. The observation that TNF release was normal or increased in LPS-treated cells exposed to both antioxidants indicates that the translational machinery remained intact despite exposure to these molecules. However, this does not rule out the possibility that these agents might have a specific effect on the translation of the mRNA for TF.

Although Western blot analysis of cells treated with LPS plus NAC showed no evidence of the fully glycosylated mature form of TF, there was also no accumulation of the less mature forms. This observation is consistent with reduced translation and/or degradation of the newly translated protein, a phenomenon that likely occurred within the endoplasmic reticulum (ER). Recent studies have reported that the stability of proteins retained in the ER was sensitive to redox conditions. Specifically, induction of reducing conditions within the ER promoted protein degradation. These investigators showed that the addition of reduced glutathione to permeabilized CHO cells caused a marked reduction in the half-life of IL-2 receptor in the ER. The mechanism behind this reduction is unclear, although reduction of disulfide bonds formed in the normally oxidizing conditions in the ER may lead to unfolding of proteins, retention in the ER, and enhanced degradation. In this regard, two disulfide bonds (between Cys-49 and Cys-57 and between Cys-186 and Cys-209) exist within the extracellular domain of TF. Prevention of disulfide linkages by altering the redox state of the cell may underlie the effect of antioxidants on TF expression. In contrast to TF, TNF secretion was unaffected by NAC. This observation may be accounted for by the absolute or relative resistance of some proteins to proteolytic degradation by ER proteases, even under reducing conditions. Alternatively, the fact that TNF contains only one disulfide bond may account for its resistance compared with TF. This differential effect may be similar to that reported

(t = 3 hours, see Fig 1) is consistent with the notion that the agents exerted their effects via their antioxidant properties. In addition, structural analogues of PDTC without antioxidant activity had no effect on TF. Finally, the antioxidants had a differential effect on TNF secretion compared with TF expression in murine macrophages. Although both PDTC and NAC inhibited LPS-induced TF, stimulated TNF release

\[ \text{Time (hr)} \]

\[ \text{TNF (ng/ml)} \]

\[ \text{Medium} \]

\[ \text{LPS} \]

\[ \text{Stimulus} \]
Fig 9. Effect of PDTC on levels of TNF mRNA in murine macrophages (A) or in human monocytes (B). Murine macrophages or human monocytes were unstimulated or treated with LPS (10 μg/mL) in the presence or absence of PDTC (1 mmol/L added at t = 0 hour) for 4 hours. Levels of TNF and α-tubulin mRNA were evaluated by Northern analysis using the cDNA probe for murine TNF or human TNF and rat α-tubulin, respectively, as described in the Materials and Methods. A representative blot of three (A) or six (B) independent studies is illustrated.

by Lentz and Sadler showing that homocysteine caused retention of the immature form of von Willebrand factor within the ER of endothelial cells with subsequent degradation, whereas plasminogen activator inhibitor-1 was normally secreted. Further studies are required to define the precise mechanism of the effect observed in the present studies.

In human monocytes, NAC reduced LPS-induced expression of the 47-kDa form of TF almost back to control levels (Fig 7), whereas the mean reduction in functional activity by the addition of NAC was approximately 65% (Fig 5A). Based on neutralizing studies using antihuman TF antibody, the residual procoagulant activity was almost entirely caused by TF activity. The difference between the mean functional activity from several experiments and the TF antigen expression shown in the single Western blot study NAC-treated cells is caused by day-to-day variability in the inhibitory effect of NAC. The Western blot shown in Fig 7 corresponds to an experiment in which there was an 89% reduction in functional PCA after NAC treatment.

ROS have been implicated as second messengers during cell activation. Direct application of oxidant molecules or other oxidant stresses induces translocation of transcriptional activating factors such as AP-1 and NF-κB into the nucleus and the initiation of gene transcription. Incubation with antioxidants during cell stimulation has been shown to prevent DNA binding and transcriptional activation by NF-κB for some genes, but not for others. Furthermore, in a somewhat paradoxical fashion, antioxidants such as PDTC and NAC have been reported to have a stimulatory effect on transactivation by AP-1. In the present studies, both PDTC and NAC increased the level of TNF mRNA and the secretion of TNF in murine macrophages.
of immunoreactive TNF by murine macrophages stimulated with LPS. These observations concur with those recently reported by Schmalbach et al.\(^5\) showing the ability of diethyl-dithiocarbamate to augment levels of TNF mRNA in HL-60 cells and human peripheral blood monocytes. The mechanism underlying this effect remains undefined, although antioxidant-induced AP-1 binding may be contributory.\(^5,4\) given that TNF gene transcription is at least partly dependent on an AP-1 site in the promoter.\(^3,5\)\(^\text{The results in murine cells differ from those recently reported by Ziegler-Heitbrock et al.}\(^1\) and confirmed in the present studies, showing the ability of PDTC to inhibit TNF gene expression in LPS-stimulated human monocytes. Species differences as well as differences in the level of activation of the cell (thioglycolate-elicited macrophages versus peripheral blood monocytes or monocytic cell line) may account for these disparate findings.

In summary, the ability of antioxidants to modulate macrophage TF expression at a posttranscriptional level represents a novel mechanism whereby these agents might exert their effects on cell activation in response to microbial products. The studies also suggest alternative clinical uses for antioxidants. Cell-mediated initiation of the coagulation cascade is an important mechanism underlying the fibrin deposition associated with multiple pathologic processes, including intraabdominal abscess formation, lung injury during sepsis, autoimmune renal disease, and various forms of arthritis. Furthermore, because TF may also be expressed by macrophages in atherosclerotic plaques,\(^3,5,6,7\) the data also point to a mechanism whereby antioxidants might abrogate acute thrombosis caused by ulceration of a plaque. Studies using in vivo models of these disease processes are required to explore these possibilities.

**REFERENCES**

19. Diamondi AP, Christopoulos TK, Diamandis EP Ultrasensitive thyrotropin immunoassay based on enzymatically amplified
43. Maller JS, Hong Y: A redox switch and phosphorylation are involved in the post-translational up-regulation of the adiponecin binding factor by phorbol ester and ionophore. J Biol Chem 266:3167, 1991
Posttranscriptional regulation of macrophage tissue factor expression by antioxidants

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