Endothelial Cells and Extracellular Calmodulin Inhibit Monocyte Tumor Necrosis Factor Release and Augment Neutrophil Elastase Release*  

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Cultured human umbilical vein endothelial cells inhibited tumor necrosis factor-α release from whole blood or isolated mononuclear cells exposed to endotoxin. In contrast, the endothelial cells augmented neutrophil elastase release in the same blood. A protein with these functional properties was isolated from endothelial cell-conditioned media and, surprisingly, was identified as calmodulin. Authentic calmodulin mimicked the effect of endothelium. 125I-Calmodulin bound to a high affinity site on monocyte cell lines (Kd ~ 30 nM, in agreement with its functional activity). Cross-linking of 125I-calmodulin to monocyctic cells identified a candidate calmodulin receptor. We conclude that calmodulin possesses an extracellular signaling role in addition to its intracellular regulatory functions. Calmodulin released at sites of tissue injury or possibly by specific mechanisms in the endothelium can bind to receptors, modulating the activities of inflammatory cells.

Tumor necrosis factor-α (TNF), a product chiefly of monocytes and their descendants, the tissue macrophages, is a multifunctional cytokine that enhances host immune responses but that is also implicated in diverse pathological processes such as septic shock, rheumatoid arthritis, tumor cachexia, multiple sclerosis, and graft-versus-host disease (1). A number of inflammatory stimuli can evoke its release from monocytes and macrophages, such as bacterial endotoxin (lipopolysaccharide; LPS), interferon-γ, and IL-1. Although several substances have been shown to inhibit the production of TNF in vitro, including adenosine (2), epinephrine (3), PGE₂ (4), PGI₂ (5), transforming growth factor-β (6, 7), IL-4 (8), IL-6 (9), IL-10 (10–12), and IL-13 (13, 14), the physiological circumstances under which monocytes would be exposed to these mediators remain undefined. The possibility that the endothelium, through these or other mediators, may control the activation of TNF-producing cells in blood has not been addressed directly.

The first objective of this study was to examine whether endothelial cells can influence the production of TNF from monocytes. For comparative purposes, elastase release was also measured as a marker of neutrophil activation in the same whole blood system.

The second objective was to identify the mediators of such effects. In this report we describe the purification of an active species from the conditioned media of human umbilical vein endothelial cells (HUVEC) and its unexpected identification as calmodulin. Furthermore, we provide preliminary evidence that there are cell-surface receptors for calmodulin on myeloid cells. The results imply that calmodulin released from endothelial cells serves the function of an extracellular signaling molecule, which regulates the activation of inflammatory cells.

MATERIALS AND METHODS

TNF and Elastase ELISAs—Monoclonal antibodies were raised against recombinant human TNF-α (a generous gift of Genentech, South San Francisco, CA) and a complex of human neutrophil elastase and human α₁-antitrypsin (Athens Research and Technology, Athens, GA) by standard techniques. (15) Polyclonal antibody to TNF was raised in a goat. TNF was assayed in plasma diluted 1:10 using mAb TNF1286 for capture and the goat polyclonal for detection or with mAb TNF1311 for capture and TNF1289 for detection. The lower limit of sensitivity for this assay was ~0.5 ng/mL Elastase-α₁-antitrypsin complexes were assayed in plasma diluted 1:50 using anti-elastase mAb HEL1076 for capture and anti-antitrypsin mAb HAT1099 for detection. Antibodies used for detection were biotinylated using NHS-LC-biotin (Pierce), and streptavidin-alkaline phosphatase and an amplified substrate system (Life Technologies, Inc.) were used for readout.

Endothelial Cell Culture—HUVEC were cultured in medium 199 (Mediatech) containing 15% v/v heat-inactivated bovine calf serum, 0.5% v/v endothelial cell growth supplement (prepared as described by Maciag et al. (16)), 10 μg/ml heparin (Sigma), 2 mM l-glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin (Mediatech) as described (17, 18) and used at first through fourth passage.

For the production of conditioned media, 2 m² of endothelial cells were grown to confluence in cell culture factories (Nunc, Denmark). After washing with Hanks’ balanced salt solution without phenol red (HBSS composition (in mM): CaCl₂, 1.26; KCl, 5.36; KH₂PO₄, 0.44; MgCl₂, 0.49; NaCl, 136.9; NaHCO₃, 4.17; NaH₂PO₄, 3.38; glucose, 5.56) (Life Technologies, Inc.), they were incubated in 1.5 liters of modified Eagle’s medium without phenol red (Mediatech) with the calcium ionophore A23187 (Sigma) at 3 × 10⁻⁶ M. After 4 h the conditioned media were collected and the cells placed in an additional 2.5 liters of medium without ionophore overnight. The conditioned media were pooled and concentrated to 150 ml by ultrafiltration with a 3,000 molecular weight cut-off membrane cartridge (S1Y3, Amicon, Beverly, MA).

Ex Vivo Culture of Whole Blood—24-Well culture plates (Costar)

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¶ The abbreviations used are: TNF, tumor necrosis factor-α; LPS, lipopolysaccharide (endotoxin); IL, interleukin; HUVEC, human umbilical vein endothelial cell; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; HBSS, Hanks’ balanced salt solution; PBMC, peripheral blood mononuclear cells; PAGE, polyacrylamide gel electrophoresis; PG, prostaglandin; HPLC, high performance liquid chromatography; HHA, Hanks/Hepees/albumin buffer; MOPS, 4-morpholinepropanesulfonic acid.
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were coated with 2% w/v gelatin (Sigma). For experiments involving endothelial cell monolayers, HUVEC were seeded onto gelatin-coated wells at 5 × 10⁵ cells per well 48 h prior to experimentation. All wells were washed with HBSS. Blood from healthy volunteers was drawn within 5 min of use into heparin (10 units/ml final; UpJohn, Kalamazoo, MI) or, where specified, hirudin (1 unit/ml final; Sigma) and centrifuged at 1000 g for 10 min. To minimize evaporation, only the center eight wells were used, and the outer wells were filled with sterile HBSS. Test reagents dissolved in HBSS (8–10 mM) were added to the wells immediately after the blood; calmodulin was diluted in HBSS supplemented with 0.1% sterile pyrogen-free gelatin (Sigma), which served as its buffer control. In some experiments, LPS (from *E. coli* strain 055:B5, Difco) was used to stimulate TNF release. Plates were incubated at 37 °C for 4 and 37 °C, as described above, except that after incubation for 37 °C, the calmodulin-binding sites were sensitive to proteolytic digestion. HL-60 cells were washed once in HBSS without Ca²⁺ or Mg²⁺ containing 20 mM HEPES, pH 7.5, supplemented with 1% human serum albumin and 1 mM EDTA (HHA/EDTA), and twice in HBSS with Ca²⁺ and Mg²⁺ containing 20 mM HEPES, pH 7.5, and 1% albumin (HHA/Ca²⁺/Mg²⁺). The cells were then resuspended in HHA/Ca²⁺/Mg²⁺ at a concentration of 1.25 × 10⁷ cells/ml. 80 µl (1 × 10⁶ cells) were aliquoted into 1.5 ml of siliconized Eppendorf tubes (GC Scientific, Gaithersburg, MD) and centrifuged at 200 × g for 2 min in siliconized 400-µl centrifuge tubes. The tip of the centrifuge tube containing the cell pellet was amputated and counted in a gamma counter (Isol-Data, Rolling Meadows, IL) for the determination of bound ¹²⁵I-calmodulin. To determine nonspecific binding, 10 µl HHA/EDTA (20 µl final) containing no unlabeled calmodulin were added to 10 µl of ¹²⁵I-calmodulin. The cells were then washed once in HHA/Ca²⁺/Mg²⁺, resuspended in HHA/Ca²⁺/Mg²⁺, and centrifuged at 200 × g for 2 min. The cell pellets were then washed twice in HH/Ca²⁺ or HH/Ca²⁺ and Mg²⁺, and washed once in HH, and then resuspended in HH/Ca²⁺. To determine calmodulin-binding sites were sensitive to proteolytic digestion, HL-60 cells were washed once in HBSS with 20 mM HEPES, pH 7.5, and 1 mM EDTA, without albumin (HHA/EDTA) and twice with HHA/Ca²⁺ and Mg²⁺ and resuspended in HHA/Ca²⁺ at 10⁷ cells per ml. 1-Tosyl-amido-2-phenylthylamino carboxylate-tetradecyltrypsin (Sigma) was then added to a final 41 units/ml and allowed to incubate at 37 °C for 30 min. The trypsin was then neutralized by the addition of diisopropyl fluorophosphate to 2 mM final. The cells were then washed once in HH/Ca²⁺ and resuspended in HHA/Ca²⁺ and binding studies performed as described above except that after the incubation period was complete, the cells were washed in HHA/EDTA and resuspended in HHA/EDTA before layering over dibutyl phthalate/apiezon oil.

To show whether bound calmodulin was internalized, binding studies were performed at 4 and 37 °C. As described above except that after the incubation period was complete, the cells were washed in HHA/EDTA and resuspended in HHA/EDTA before layering over dibutyl phthalate/apiezon oil.

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Incubation of whole blood with LPS for 4 h evoked a dose-dependent release of TNF (Fig. 1). In control wells not treated with LPS, TNF levels were usually below the limit of detection by our ELISA. When blood treated with LPS was incubated over an endothelial monolayer for 4 h, TNF release was reduced by about half at all concentrations of LPS (Fig. 1). Donors varied in the extent of inhibition by endothelial cells, and in 8 of 70 total experiments, no inhibition of TNF release was observed.

In contrast to the effect on TNF, blood incubated over a HUVEC monolayer exhibited increased elastase release at all concentrations of LPS (Fig. 2). In some subjects (8 of 35 total experiments), elastase release was not augmented by endothelium; five subjects appeared to show inhibition.

One contribution to these cellular responses might be the release of active peptides from the endothelium. To test this possibility, conditioned media from endothelial cells were assayed for TNF inhibiting activity. After concentrating 66-fold by ultrafiltration, conditioned media (collected without A23187 stimulation) were able to inhibit TNF release by 43% (n = 2). Empirically, it was observed that stimulation of the endothelial cells with the calcium ionophore A23187 yielded increased inhibitory activity in the conditioned media, and therefore, purification was attempted from ionophore conditioned media. The purification was performed as detailed under “Materials and Methods.” At each chromatographic step a single peak of activity was identified. The activity eluted from a Mono Q anion exchange column at 0.35 mM NaCl at pH 7.5. The isoelectric point cusing, the sample was essentially pure as judged by 12% SDS-PAGE. Silver staining demonstrated a single negative staining (ghost) band with an apparent mass of 21 kDa (Fig. 3).

No N-terminal sequence was generated from the purified protein, suggesting that the N terminus was blocked. To obtain internal sequence information, a trypsin digest was performed and the resulting fragments separated by HPLC. The eluted fragments were analyzed in-line with electro-spray mass spectrometry. Sequence was obtained from two fractions; one (eluate peak 3) contained clearly distinguishable major (VFDRDGNGYISAAELR) and minor (MKDTDSEEEIREAFR) sequences, and the other (eluate peak 4) contained a faint sequence (XXMTNLXEXLTDDXXDDXX). In total 40 amino acid residues were identified in the three fragments. This sequence information was compared with known sequences in the SwissProt data base using the GCG software package (Version 7; Genetics Computer Group, Madison, WI), which revealed complete identity with three contiguous sequences of calmodulin, corresponding to amino acids 76–126. The sequences of the three fragments and their measured masses (as well as the masses of the other HPLC eluate peaks, which were not sequenced) corresponded to known tryptic fragments of calmod-
ulin (28–30) whose masses were predicted by the GCG software program (Table I).

Amino acid analysis was performed in our institution and by a reference laboratory (Harvard Micro Chem, Cambridge, MA). Similar results were obtained from both analyses (Table II), which compare well to the known amino acid composition of calmodulin (28).

To exclude the possibility that the endothelial factor was a modified form of calmodulin, both authentic calmodulin from hog brain (which shares amino acid sequence identity with human calmodulin) and the purified endothelial factor were subjected to ion-spray mass spectrometry for the determination of molecular mass. Both species of calmodulin had a similar spectral pattern. The value obtained for the endothelial factor (16,785.77 ± 1.75 daltons) agrees well with the predicted molecular mass of calmodulin of 16,790 daltons (16,706 as calculated by the GCG software for the amino acid sequence, plus 84 daltons for the known trimethylation of lysine 115 and the acetylation of the N terminus) and with the value obtained for hog brain calmodulin (16,788.65 ± 1.11 daltons).

Calmodulin was obtained from human erythrocytes, hog brain, and bovine testes, and recombinant epitope-tagged calmodulin was expressed in E. coli. Each of these calmodulins demonstrated inhibition of TNF production in the whole blood assay system (Fig. 4). Maximum inhibition was observed at 1 × 10⁻⁷ M and the half-maximal effect occurred at approximately 3 × 10⁻⁸ M (Fig. 4 and Fig. 5). Calmodulin and the material purified from endothelial cell-conditioned media, like HUVEC monolayers, also augmented elastase release from whole blood (Fig. 5). The concentration of calmodulin evoking half-maximal augmentation of elastase was similar to that required for inhibition of TNF release.

The responses to calmodulin were more consistent than the responses to the HUVEC monolayers, although individual variability was still evident. Failure to inhibit TNF release with 10⁻⁷ M calmodulin was seen in only 3 of 62 total experiments, and failure to augment elastase release was seen in 12 of 56 total experiments, with just one of the 56 demonstrating inhibition of elastase release by >20%.

The amount of calmodulin present in the active fraction from the Mono P column was estimated by comparing the activity of authentic hog brain calmodulin with dilutions of the active chromatographic fraction. The resulting estimate, 134 mg/ml, agreed well with a functional phosphodiesterase assay, which yielded an estimate of 128 mg/ml.

On SDS-PAGE gels, the purified endothelial factor co-migrated with authentic calmodulin and demonstrated the same negative staining with silver. It also demonstrated the same shift in mobility when electrophoresis was performed in the presence of calcium as compared with EDTA (not shown), which occurs because calmodulin retains the ability to bind calcium and undergo conformational change in the presence of SDS (31, 32).

The binding of Ca²⁺ to calmodulin induces the exposure of a hydrophobic patch at either end of the molecule allowing calcium-
dependent binding to media such as phenyl-Sepharose (33). TNF-inhibiting activity was selectively removed from the purified endothelial factor preparation by passage over a phenyl-
Superose column in the presence of calcium, and TNF-inhibiting activity could subsequently be eluted from this column by EDTA (data not shown). Another unusual property of calmodulin is its resistance to thermal denaturation. Neither authentic calmodulin nor the purified endothelial cell factor was inactivated by heat treatment (data not shown).

To exclude the possibility that calmodulin may act by accelerating the degradation of TNF in blood or by interfering with the ELISA for TNF, rather than by inhibiting TNF release from monocytes, experiments were performed in which blood samples were spiked with exogenous recombinant human TNF-α (9 or 36 ng/ml final) with or without authentic calmodulin (10^{-10} M final), and the recovery, as a percentage of the added concentration, was determined by the ELISA. After 10 min incubation at 37 °C, the recovery averaged 87.1 ± 4.1% in control and 82.3 ± 4.0% in calmodulin-supplemented samples; after 4 h incubation, recovery was 72.2 ± 3.4 and 71.1 ± 2.5%, respectively (p = not significant for difference between control and calmodulin-supplemented samples at either time).

To determine whether the effects observed with calmodulin were exerted directly on monocytes and neutrophils, or mediated by an intermediary cell type, PBMCs and neutrophils were isolated from whole blood. Plasma was added back to the washed cells as a source of LPS-binding protein (to restore responsiveness to LPS) and of α1-antitrypsin (to permit detection of complexes with elastase in our ELISA). In response to 100 ng/ml LPS, PBMCs in 10% plasma produced quantities of TNF at the threshold of detection by our ELISA, but secreted markedly more when suspended in 100% plasma. Neutrophils released similar amounts of elastase under either condition. The release of TNF by PBMCs was significantly and dose-dependently inhibited by calmodulin, whereas the release of elastase by neutrophils was augmented (Fig. 6). Heparin, used as an anticoagulant in most of these experiments, may have nonspecific effects; for example, it can bind TNF directly and induce the release of TNF-binding proteins in vivo (34). Therefore, experiments were done using the specific thrombin antagonist, hirudin. The inhibitory effect of calmodulin was similar in blood anticoagulated with hirudin as with heparin (27 ± 3 and 40 ± 2% inhibition, respectively; quadruplicate determinations in one subject). Likewise, the augmentation of elastase release was similar (52 ± 7 versus 46 ± 5%).

The inhibition of monocyte TNF production by extracellular calmodulin would most likely involve a membrane receptor. To investigate this possibility, binding studies were performed using 125I-calmodulin. A time course showed that binding was maximal by 40 min. Two classes of binding sites were identified...
on several, but not on all, monocytic cell lines investigated (Table III). A high affinity site on THP-1 cells was identified with a $K_d$ of approximately 30 nM and 11,000 sites per cell (Fig. 7). This affinity matches the half-maximal biological activity for both TNF and elastase release, suggesting that this binding site may represent the calmodulin receptor that mediates these effects. A second, low affinity site of $K_d$ 51.8 $\mu$M and 420,000 sites per cell was also identified.

A binding study was performed at 37 °C to determine whether calmodulin is internalized by THP-1 cells. Although nonspecific binding was increased at 37 °C compared with 4 °C, specific binding after 40 min incubation was the same. Furthermore, if the cells were washed with EDTA at the end of the period of incubation at either temperature, essentially all of the counts were removed. Only 5.8 ± 1.1% of specifically bound counts remained after washing with cells incubated at 4 °C, compared with 4.9 ± 0.4% with cells incubated at 37 °C, indicating that active internalization of the ligand had not occurred.

Trypsin treatment of HL-60 cells prior to binding caused a 90% reduction in high affinity binding sites and 75% reduction in low affinity sites, suggesting that the receptor is a trypsin-sensitive protein.

To further characterize the cell-surface calmodulin-binding proteins, we performed cross-linking studies. Autoradiograms of SDS-PAGE gels of $^{125}$I-calmodulin cross-linked to monocytic cell membrane proteins showed two bands, one with an apparent molecular mass of 110 kDa and a second, less distinct complex of 44 kDa. No complexes were observed when cross-linking was done in the presence of EDTA, confirming that the interaction of calmodulin with its membrane receptor is calcium-dependent (Fig. 8). The fact that an excess concentration of unlabeled calmodulin blocked the appearance of the complexes indicates that they form as a result of specific binding. The calmodulin antagonist trifluoperazine also blocked binding, suggesting that the interaction of calmodulin with its receptor involves the hydrophobic patch characteristically involved in calmodulin binding to its intracellular targets.

**DISCUSSION**

These studies demonstrate that calmodulin derived from endothelial cells can interact with receptors on leukocytes, dampening the elaboration of TNF from monocytes while enhancing neutrophil elastase release. Based on the chromatographic results, the major protein obtained from endothelial cultures that is able to inhibit LPS-stimulated TNF production appears to be calmodulin. The inhibitor was identified as calmodulin based on chromatographic properties, electrophoretic characteristics, thermal stability, peptide sequence, and mass identities with calmodulin. Furthermore, calmodulin purified from diverse tissue sources, and recombinant calmodulin expressed in *E. coli*, possessed the
same potency and functions as the protein preparation from the endothelial cell supernatants. Thus, it is extremely unlikely that the activities observed are due to a co-purifying contaminant in these preparations.

Two classes of binding sites for calmodulin were detected on monocytic cell lines. The affinity of the first site matches the biological activity of calmodulin, suggesting that this may be the transducing receptor. Cross-linking studies also demonstrate two species. The absence of an increase in cell-associated 125I-calmodulin at 37 °C compared with 4 °C also suggests that calmodulin signals by binding to a receptor, rather than by being internalized and supplementing the cytoplasmic calmodulin pool.

It is possible that the inhibitory effect of calmodulin is not exerted directly upon the monocytes but mediated instead by an indirect mechanism involving other blood cells. For example, it has been reported that elastase and cathepsin-G released by neutrophils can degrade TNF (35). This possibility was examined by experiments using isolated peripheral blood mononuclear cells. These cells seemed, in general, to be less sensitive to inhibitory stimuli, as demonstrated by their decreased responsiveness to FGF2, perhaps due to partial activation during the process of isolation. Nonetheless, they also demonstrated inhibition of TNF release when exposed to calmodulin, supporting a direct mechanism of inhibition. Likewise, elastase release from isolated neutrophils was enhanced by calmodulin. The possibility that an interaction with plasma proteins is required to support these effects is not excluded by these experiments.

Although the identification of calmodulin as an extracellular signaling molecule was unexpected (after all, calmodulin is best known as an intracellular protein important in transducing cytoplasmic calcium signals (36, 37)), it is not implausible. Calmodulin is an abundant protein found in almost all cells and may be up to 0.4% of the total cell protein content of endothelial cells (38). Calmodulin has been found to accumulate in the conditioned media of endothelial cells over 24 h (39). It is not known whether calmodulin is released constitutively or through regulated secretion in response to inflammatory stimuli. Calmodulin release might also occur nonspecifically in response to cell injury or death. In this regard, calmodulin may be analogous to adenosine, which is released with cell injury and inhibits TNF release by binding to adenosine receptors on monocytes (2). Release of calmodulin from activated or injured cells may constitute a feedback mechanism modulating self-directed inflammation, preventing further cytotoxicity by inhibiting TNF release, and enhancing cleanup of debris by augmenting elastase release. By having calmodulin, a ubiquitous and abundant intracellular protein, as a modulator of the inflammatory response ensures rapid dampening of the local response whenever cell death or injury ensues and bypasses the need for a delayed biosynthetic response to cell injury. In this regard, it is interesting that calmodulin is found in all eukaryotic cells but is absent from prokaryotes (40) and therefore could constitute a primitive self-injury recognition system.

Other activities of extracellular calmodulin have been reported intermittently. It may stimulate the proliferation of cultured hepatocytes, keratinocytes, melanoma cells, K562 leukemia cells, HUVECs, and fibroblasts (39, 41–45). However, effects of calmodulin on inflammatory responses have not previously been described, and the effects of calmodulin on other monocyte and neutrophil functions, and on other cell types, are not known. The apparent contrasting activity of calmodulin on monocyte TNF release and on neutrophil elastase release suggests that calmodulin may have effects on multiple inflammatory cell functions.

Although the original observations were made with endothelium, it is unclear from these studies whether the calmodulin was released in a specific fashion or emerged as a result of limited cell disruption. Questions regarding the specificity of the effect for endothelial cells, the influence of other endothelial cell products on the TNF and elastase release, and other signals that might work synergistically with calmodulin are being examined.

In summary, these experiments have shown that calmodulin can modulate inflammatory responses of two different leukocyte populations. These observations argue for the likely physiological importance of this new inflammation-modulating mechanism. They further suggest that some forms of cell injury or dysfunction may lead to altered calmodulin signaling, consequently changing TNF- and elastase-mediated inflammatory activity, which could play a role in vascular and inflammatory diseases.

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