Tumour necrosis factor (cachectin) induces phospholipase A₂ activity and synthesis of a phospholipase A₂-activating protein in endothelial cells

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Tumour necrosis factor (TNF) is an important mediator of endotoxin-induced vascular collapse and other inflammatory reactions. Eicosanoids have been implicated in the pathogenesis of these responses. In order to explore further the potential interactions between TNF and eicosanoid metabolism in eliciting vascular responses, we studied the effects of TNF on the bovine endothelial cell line CPAE. TNF induced cellular retraction observed by light microscope. This morphological change was monitored by the passage of iodinated protein A between adjacent cells and by release of [³H]arachidonic acid metabolites from cells. Both the morphological and functional responses were abrogated by inhibition of eicosanoid synthesis with BW755c. The release of [³H]arachidonic acid metabolites appeared to be mediated by a transient increase in phospholipase A₂ activity. Phospholipase C activity was not affected by TNF. The maximal increase in phospholipase A₂ activity occurred at 5 min following the addition of TNF. Phospholipase A₂ activation, [³H]arachidonic acid-metabolite synthesis and passage of iodinated protein A, required both RNA and protein synthesis and were associated with an increase in the synthesis of a recently described phospholipase A₂-activating protein. The *Bordetella pertussis* toxin, islet-activating protein, also inhibited the increase in phospholipase A₂ activity, the release of [³H]arachidonic acid metabolites and the passage of iodinated protein A, suggesting that the TNF receptor–ligand interaction resulting in cellular retraction, phospholipase A₂ activation and eicosanoid synthesis, is coupled through the N_i guanine nucleotide regulatory protein in these cells.

INTRODUCTION

Tumour necrosis factor (TNF) is a polypeptide cytokine produced primarily by monocytes and macrophages in response to endotoxin and other immune and inflammatory stimuli (for review articles see [1,2]). TNF, also called cachectin, was originally described as an agent that induced haemorrhagic necrosis of experimental tumours [1], and is now also thought to mediate septic shock, cachexia and other inflammatory reactions. The pluripotent effects of TNF also include bone resorption, collagenase production and eicosanoid synthesis, as well as vasculitis and haemorrhagic necrosis of transplanted tumours [3] and other tissues [4]. Thus TNF exerts many of its effects through stimulation and disruption of the vasculature.

The endothelial cell lining of the vasculature plays an important role in the regulation of the permeability and integrity of the blood vessels, and alteration in endothelial cell function has been shown to modify the morphological and biological activities of the vasculature [5–8]. TNF may thus mediate at least some of its effects by interacting with the endothelial cell lining of the vasculature. TNF binds to specific cellular receptors [2,4], and this receptor–ligand interaction may result in activation of a number of intracellular processes, including phospholipase activation and eicosanoid synthesis. TNF has been

reported to increase the synthesis of eicosanoids [9–13], several of which are known to affect the vasculature. Thus, TNF may mediate at least some of its effects via stimulation of endothelial cells to increase eicosanoid synthesis. These eicosanoids could then affect adjacent endothelial cells or more distant target cells.

We have recently demonstrated that leukotriene D₄, another potent vasoactive substance, induces eicosanoid production by endothelial cells [14]. This action requires RNA and protein synthesis, and thus is similar to other inflammatory agents such as thrombin and phorbol esters which also require RNA and protein synthesis in order to increase eicosanoid synthesis [15–17]. Transduction of this leukotriene D₄ receptor–ligand interaction results in a transient increase in phospholipase A₂ activity [18]. Furthermore, the ability of leukotriene D₄ to increase phospholipase A₂ activity appears to be coupled through a guanine nucleotide-binding protein, possibly N_i, which is sensitive to the *Bordetella pertussis* toxin, islet-activating protein (IAP) [19].

Recently we have isolated and characterized a phospholipase A₂-activating protein (PLAP) that enhances phospholipase A₂ activity [20]. PLAP was originally discovered and isolated by its antigenic and functional relatedness to melittin, a bee venom peptide. Melittin has been shown to stimulate phospholipase A₂ activity [21–23] and has also been shown to affect

Abbreviations used: PLAP, phospholipase-activating protein; TNF, tumour necrosis factor; IAP, islet-activating protein; PBS, phosphate-buffered saline; e.l.i.s.a., enzyme-linked immunosorbent assay.

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mammalian cell functions. For example, melittin can increase eicosanoid synthesis in fibroblasts, smooth muscle and endothelial cells [22,24,25]. Thus, we have postulated that eicosanoid synthesis and phospholipase A₂ activity may be regulated in mammalian cells by the synthesis of PLAP. The purpose of these experiments is to determine if TNF also increases phospholipase A₂ activity and arachidonic acid-metabolite release in endothelial cells in the same manner as leukotriene D₄, and to determine if TNF affects the synthesis of PLAP.

MATERIALS AND METHODS

Materials

The recombinant TNF- α used in these experiments was cloned, expressed in *Escherichia coli* and purified as previously described from normal human peripheral-blood monocytes [26]. The specific activity of purified TNF- α was $(2-4) \times 10^7$ units/mg of protein in the L929 mouse fibroblast assay. Thus 1 unit of activity/ml was $\sim 10^{-10}$ M. IAP was purchased from List Laboratories, Campbell, CA, U.S.A. All radioactive materials were obtained from New England Nuclear (Boston, MA, U.S.A.) except ¹²⁵I-protein A which was obtained from Amersham (Arlington Heights, IL, U.S.A.). Peroxidase-conjugated goat anti-rabbit IgG antibodies were obtained from Cappel (Malvern, PA, U.S.A.). The anti-actin antibody was purchased from Amersham. HpB Scintillation fluid was from Beckman (Fullerton, CA, U.S.A.). BW755c was from Burroughs-Wellcome (Research Triangle Park, NC, U.S.A.). Cycloheximidine and actinomycin D were from Sigma (St. Louis, MO, U.S.A.).

Cell culture

The bovine endothelial cell line, CPAE, was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and was used within 10 passages of receipt. These cells were grown as previously described [14].

Microscopy

Cells were grown on glass coverslips (11 mm $\times$ 24 mm) for 24 h before being treated with TNF (1000 units/ml) for 20 min. The coverslips were then quickly rinsed three times in phosphate-buffered saline (PBS) before being fixed with 3% formalin in PBS. The coverslips were examined using phase contrast optics.

Permeability assays

These assays were performed as described [32] except for the following modifications. Cells were grown to confluency in 24 mm Costar Transwell apparatus. The upper and lower chambers each contained 2 ml of medium. ¹²⁵I-Protein A (40 Ci/mmol) (10^6 c.p.m./well) was added at the same time as TNF to the upper chamber. Portions (50 μ l) were removed from the lower chamber at the indicated times and counted in a gamma counter.

[³H]Arachidonic acid release and phospholipase assays

These experiments were performed as we have previously described [14,18,27,28].

E.l.i.s.a. assays

Antibodies used in these experiments were those prepared using melittin as an antigen [20]. This antibody

has been shown to cross-react with the mammalian protein PLAP [20] and was used to quantify PLAP because antibodies to the mammalian protein are not yet available. However, in these cells, this antibody immunoprecipitated a unique protein of *M_r* 28 000 and had PLAP activity and appeared identical with the protein we have previously termed PLAP [16]. Pre-immune sera did not react with these cells (results not shown). Cells were grown in 96-well Linbro plates for 24 h and were in the log phase of growth. Cells were treated with TNF (1000 units/ml) for the indicated periods of time, quickly rinsed twice with PBS and then fixed for 20–30 min using a formalin/PBS solution (3% v/v). The cells were then permeabilized with 50 μ l of PBS containing 0.05% (v/v) Tween 20 for 3 min before being rinsed with PBS. The plates were then filled to capacity with PBS containing 20% fetal calf serum and placed at 4 °C overnight. Anti-melittin antibody was diluted 1:1000 in PBS and 50 μ l was added to each well. In control experiments the anti-actin antibody was diluted 1:1000 and used exactly as the anti-melittin antibody. The plates were then incubated at room temperature for 1 h before being rinsed three times (5 min each time) with PBS. The goat anti-rabbit antibody (peroxidase-conjugated) was diluted 1:5000 in PBS and 50 μ l was added to each well. The plate was again incubated for 1 h at room temperature before being rinsed three times (5 min each time) with PBS. The peroxidase substrate *o*-phenylenediamine was added (1 mg/ml in 0.1 M-citrate buffer, pH 4.5, containing 0.12% H₂O₂) in a final vol. of 100 μ l and allowed to react for approx. 10 min. The reaction was stopped by the addition of 25 μ l of NaF (0.1 M) to each well. The absorbance of each well was determined using an automatic plate reader, Titertek Multiskan model MC (Helsinki, Finland). Therefore, in each experiment, each time point was determined in eight wells. Thus the data shown were obtained from three separate experiments which represent the means for 24 wells.

Inhibitor studies

IAP (100 ng/ml) was added to the cells 1 h before the addition of TNF as previously described [19]. Cycloheximide and actinomycin D (100 μ g/ml and 10 μ g/ml respectively) were added 30 min before TNF treatment as previously described [14]. None of these treatments were cytotoxic during the course of these experiments (as determined by Trypan Blue dye exclusion).

H.p.l.c. of [³H]arachidonic acid metabolites

Cells were incubated overnight in [³H]arachidonic acid (10 μ Ci/ml). The next day the monolayers were rinsed twice with saline and treated with TNF (1000 units/ml) for 10 min. The supernatants were then collected, acidified with phosphoric acid (final concn. 0.1%) and extracted with two volumes of ethyl acetate. The organic layer was removed and reduced in volume to 200 μ l under nitrogen before being analysed by h.p.l.c.

H.p.l.c. analysis was performed using a 4 μ m C-18 reverse-phase column (Waters, Boston, MA, U.S.A.). The eluant from the column was analysed using an on-line radioactivity detector (IN/US, Fairfield, NJ, U.S.A.). The mobile phase consisted of acetonitrile and acidified water (0.01% phosphoric acid). A multi-step gradient was used to elute the column. The initial conditions were 28% acetonitrile for the first 4 min, then the acetonitrile concentration was increased to 35%

Fig. 1. Induction of endothelial-cell retraction by TNF

CPAE cells were grown to confluency on glass coverslips, and were then treated for 20 min with medium alone (a), or for 10 min with TNF (1000 units/ml) (b), or for 20 min with TNF (1000 units/ml) (c). Note increased cellular retraction (arrows) induced by TNF. The bar in each micrograph represents 10 μ m.

during the next 20 min of the run. Finally the acetonitrile concentration was increased to 100% over the next 15 min. The identities of 6-oxoprostaglandin F_{1 α} , thromboxane B₂ and prostaglandin E₂ was confirmed by radioimmunoassays (New England Nuclear). Identifications of the other peaks were based on co-migration with standards obtained from BioMol (Philadelphia, PA, U.S.A.) and u.v. spectral analysis.

Analysis of mammalian proteins recognized by anti-melittin antibodies

In order to characterize the endothelial cell protein which cross-reacted with the antibodies made using melittin as antigen, 10⁴ cells were sonicated and the total cellular proteins were radiolabelled using ¹²⁵I-Bolton-Hunter reagent as suggested by the manufacturer (New England Nuclear). The resulting radioactive material was next diluted to a final volume of 5 ml in PBS containing 0.05% Tween 20 and 2% gelatin. Next, 100 μ l of undiluted serum was added to the mixture and incubated at 4 °C for 30 min, after which 100 μ l of a protein A-Sephadex slurry was added and the mixture

Fig. 2. Effect of TNF on the permeability of ¹²⁵I-protein A

Cells were grown to confluency in 24 mm Coster Transwell apparatus. ¹²⁵I-Protein A was added to the upper chamber at the same time as TNF (1000 units/ml) (●), TNF plus BW755c (10 μ M) (□), or control (medium only) (○). At the indicated times, 50 μ l portions were removed from the lower chamber and counted in a gamma counter. The results are the means $\pm$ S.D. of three experiments performed in triplicate.

incubated for 15 min at 4 °C (with constant agitation). The Sephadex particles were collected by centrifugation and washed three times using 5 ml of PBS containing 0.05% Tween 20. Next, 100 μ l of sample buffer [29] was added to the particles and the samples were boiled for 5 min. A volume of 50 μ l was analysed using a 15% denaturing acrylamide gel [29].

RESULTS

Microscopy

The addition of TNF (100–1000 units/ml) to the CPAE endothelial cell line resulted in maximal cellular retraction, initially observed after 10 min of treatment (Fig. 1b) and becoming more pronounced after 20 min of treatment (Fig. 1c). Maximal retraction was obtained with 1000 units/ml of TNF, therefore all subsequent experiments were performed using this dose. Similar morphologic results were observed by other investigators using endothelial cells obtained from different sources [30]. These results indicate that the CPAE cells respond to TNF and suggest that cellular retraction is a possible mechanism by which TNF increases the permeability of the vasculature. It was also observed that pretreatment of the cells with the eicosanoid-biosynthesis inhibitor, BW755c [31], blocked these morphological changes (results not shown), suggesting that they were dependent on eicosanoid biosynthesis. In order to attempt to quantify this morphological phenomena, permeability assays were performed.

Permeability assays

The recently described permeability assay [32] was used in order to attempt to quantify the retraction phenomenon. Cells were grown to confluency in the upper chamber of a disposable Costar Transwell apparatus on a porous (0.4 μ m) membrane and a radioactive macromolecule (¹²⁵I-labelled protein A; 10⁶ c.p.m.) was

Fig. 3. Effect of TNF on the release of [^3H]arachidonic acid metabolites

Endothelial cells were grown to confluency, incubated with [^3H]arachidonic acid ($10\ \mu\text{Ci}$ for 12 h), rinsed with PBS, overlaid with medium, and treated with medium only (○) or TNF (1000 units/ml) (●). At the indicated times, $50\ \mu\text{l}$ portions were collected in triplicate and the amount of radioactivity determined. Results are expressed as the means $\pm$ S.D. of two experiments performed in triplicate.

added to the upper chamber. At the indicated times, portions ($50\ \mu\text{l}$) were removed from the lower chamber. It was reasoned that, as the cells retracted, more pores in the membrane separating the upper and lower chamber would be uncovered, and this would allow more iodinated protein A to pass from the upper to the lower chamber. Results obtained by this technique are shown in Fig. 2. TNF treatment resulted in more radioactive protein passing through the membrane. This was found to be time-dependent with the rate of diffusion increasing significantly by 5 min and appearing to be greatest after ~ 10 min of treatment (the time at which the retraction shown in Fig. 1 was observed). Pretreatment of the cells with BW755c ($10\ \mu\text{M}$ for 30 min) inhibited not only the morphological retraction observed microscopically but also the diffusion of radioactive material from the upper to the lower chamber (Fig. 2). These results suggest that the cellular retraction observed was dependent on TNF inducing eicosanoid biosynthesis.

[^3H]-Arachidonic acid release

To determine if TNF treatment of these endothelial cells also increases eicosanoid synthesis, as monitored by the release of [^3H]arachidonic acid [19], cells were incubated with [^3H]arachidonic acid ($10\ \mu\text{Ci}$ for 12 h), rinsed with PBS, overlaid with medium, and treated with TNF (1000 units/ml). Portions ($50\ \mu\text{l}$) were collected at the indicated times and the amount of radioactivity was determined in a scintillation counter (Fig. 3). TNF stimulated significant release of [^3H]arachidonic acid following 5 min of treatment. H.p.l.c. analysis of the released material demonstrated that approx. 40% of the [^3H]arachidonic acid had been metabolized into a variety of both cyclo-oxygenase and lipoxygenase metabolites (Fig. 4).

Phospholipase assays

TNF was seen to increase the release of [^3H]arachidonic acid, the precursor for eicosanoids, there-

Fig. 4. H.p.l.c. analysis of [^3H]arachidonic acid metabolites produced in response to TNF treatment

Cells labelled with [^3H]arachidonic acid were treated with TNF 1000 units/ml for 10 min. The supernatants were then collected and analysed by reverse-phase h.p.l.c. using an on-line radioactivity detector. The results from a representative experiment are described. Analysis of the data above indicated the following distribution of label: 6-oxoprostaglandin $\text{F}_{1\alpha}$ (6-oxo $\text{PGF}_{1\alpha}$), 4%; thromboxane B_2 (TxB_2), 5.2%; prostaglandin F_2 (PGF_2), 4%; prostaglandin E_2 (PGE_2), 2.4%; prostaglandin D_2 (PGD_2), 6.2%; 5,15-dihydroxyeicosatetraenoic acid (5,15-dihydroxyHETE), 1.4%; 5-hydroxyeicosatetraenoic acid (5-HETE), 13.7%; and arachidonic acid, 57.2%.

fore phospholipase assays were performed. Cells were treated for 5 min, sonicated, and the phospholipase activities quantified (Table 1). Using both phosphatidylinositol and phosphatidylcholine as substrate phospholipids we observed that phospholipase C activity in the sonicate was not affected. However phospholipase A_2 activity using phosphatidylcholine as a substrate was increased ~ 4 -fold following 5 min of TNF treatment, a time which appeared to coincide with the release of [^3H]arachidonic acid (Table 1 and Fig. 5). This increase in phospholipase A_2 activity was transient (Fig. 5), reaching maximal activity by 5 min, and returning to baseline by 10 min. Furthermore, the increase in phospholipase A_2 activity was not observed if phosphatidylethanolamine was used as substrate phospholipid in the phospholipase A_2 reactions (Table 1).

Effect of metabolic inhibitors on phospholipase A_2 activation

In order to examine the roles of RNA and protein synthesis and receptor-associated proteins, cells were

Table 1. Phospholipase activation by TNF

The previous experiments indicated that TNF induced the release of [³H]arachidonic acid-labelled material from these cells. In order to determine which phospholipase was being activated at the time of [³H]arachidonic acid release, cells were treated with TNF (1000 units/ml), a dose which induced maximal release of [³H]arachidonic acid metabolites, for 5 min (the time of onset of [³H]arachidonic acid-metabolite release). The cells were then sonicated and the cell-free sonicate was assayed for phospholipase activity as previously described [18]. The values shown represent the means $\pm$ s.d. of at least three experiments following treatment of cells with 1000 units/ml of TNF for 5 min. Abbreviations: ND, not detected; PLC, phospholipase C; PLA₂, phospholipase A₂; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

Phospholipase	Phospholipid substrate	Treatment ...	Activity (pmol of product/min per mg of protein)	
			Control	+ TNF (1000 units/ml)
PLC	PC		0.9 $\pm$ 0.1	1.1 $\pm$ 0.1
	PI		1.6 $\pm$ 0.4	1.1 $\pm$ 0.2
	PE		ND	ND
PLA ₂	PC		1.2 $\pm$ 0.4	5.2 $\pm$ 0.5
	PE		0.4 $\pm$ 0.2	0.2 $\pm$ 0.3
	PI		ND	ND

Fig. 5. Time course of phospholipase A₂ activation by TNF

Endothelial cells were treated with TNF (1000 units/ml) for 4 min. Cells were then scraped from the dish and sonicated. Phospholipase A₂ activity was determined using radiolabelled phospholipids as described in the Materials and methods section. The results shown represent the average for three separate experiments, each performed in triplicate. The s.d. was approx. 10% for each data point shown.

treated with a variety of inhibitors. The increase in phospholipase A₂ activity was abrogated by pretreating the cells with cycloheximide, actinomycin D or IAP (Table 2). These results suggest that the TNF receptor may be coupled through an IAP substrate (possibly N₁) and that both transcription and translation are necessary for TNF to increase phospholipase A₂ activity. These inhibitors not only blocked the increase in phospholipase A₂ activity but also blocked both the release of [³H]arachidonic acid metabolites, and the increased rate of diffusion observed with iodinated protein A (Table 3).

E.I.i.s.a. assay for PLAP

The previous experiments suggest that a protein was synthesized in response to TNF treatment that resulted in an increase in phospholipase A₂ activity. To demonstrate that these antibodies recognized a single protein in the cells, cell sonicates were radiolabelled using ¹²⁵I-Bolton-Hunter reagent which was then reacted with anti-melittin antibodies. The immunoprecipitants were analysed by gel electrophoresis (Fig. 6). We observed that the predominant protein isolated by this technique had an M_r of 28000, similar in size to the PLAP previously isolated by us from mouse smooth-muscle

Table 2. Inhibition of PLA₂ activation

The previous experiment indicated that TNF increased phospholipase A₂ activity after 5 min of treatment. In this experiment cells were pretreated with inhibitors of protein and RNA synthesis. The concentrations and time of pretreatment were sufficient to block > 95% of the incorporation of [³H]leucine or [³H]uridine into acid-precipitable material, yet they were not sufficient to increase the cells' permeability to Trypan Blue. We have previously shown that the concentration and time of IAP pretreatment were sufficient to ribosylate essentially all of the M_r 41 000 protein (N₁) that is in these cells [19]. The values shown represent the means $\pm$ s.d. of at least three experiments. Cycloheximide (CX) (100 μ g/ml) and actinomycin D (AD) (10 μ g/ml) were added 30 min before TNF, and IAP (100 ng/ml) 60 min before.

Treatment	Inhibitor ...	Activity (pmol of reaction product produced/min per mg of protein)			
		None	CX	AD	IAP
None		0.8 $\pm$ 0.2	1.1 $\pm$ 0.5	1.7 $\pm$ 0.4	0.4 $\pm$ 0.6
	TNF (1000 units/ml)	4.6 $\pm$ 0.5	0.9 $\pm$ 0.3	1.4 $\pm$ 0.5	0.9 $\pm$ 0.4

Table 3. Effects of cycloheximide, actinomycin D and IAP on the release of [³H]arachidonic acid and the diffusion of ¹²⁵I-labelled protein

Cells were pretreated with cycloheximide (100 µg/ml) or actinomycin D (10 µg/ml) for 30 min, or with IAP (100 ng/ml) for 60 min, before the addition of TNF (1000 units/ml) for 10 min. The [³H]arachidonic acid-metabolite release data were obtained as in Fig. 4. The diffusion data were obtained as in Fig. 3. Values shown are the means of representative experiments, two of which were performed. The s.d. in each case was less than 10%. Abbreviation: AA, arachidonic acid.

Stimulus	Treatment							
	None		Cycloheximide		Actinomycin		IAP	
	[³ H]AA release (c.p.m.)	¹²⁵ I diffusion (c.p.m.)	[³ H]AA release (c.p.m.)	¹²⁵ I diffusion (c.p.m.)	[³ H]AA release (c.p.m.)	¹²⁵ I diffusion (c.p.m.)	[³ H]AA release (c.p.m.)	¹²⁵ I diffusion (c.p.m.)
None	2428	987	2132	1120	2243	1026	2531	932
TNF (1000 units/ml)	4720	2200	2010	1097	2119	977	2146	986

Fig. 6. Characterization of endothelial cell proteins which cross-react with anti-melittin antibodies

Total cell sonicates were radiolabelled using ¹²⁵I-Bolton-Hunter reagent immunoprecipitants with anti-melittin antibody and then analysed by gel electrophoresis. The results shown above indicate that the major protein recognized by the antibodies has an M_r of 28000 and agrees with the molecular mass of PLAP [20].

cells [25]. An e.l.i.s.a. assay using antibodies which recognize PLAP was performed (Fig. 7). PLAP induction was maximal by 2–5 min following treatment with TNF, and returned to baseline levels by 15–30 min. In order to determine if other proteins were increased by TNF, antibodies to actin were used in control experiments. However, TNF did not appear to increase the synthesis of actin in these cells (results not shown).

Fig. 7. Induction of PLAP by TNF

An e.l.i.s.a. assay for PLAP using anti-melittin antibody and peroxidase-conjugated goat anti-rabbit antibody was performed as described in the Materials and methods section. Each time point in this assay was performed on eight wells in a 96-well dish and these experiments were performed three times separately. This resulted in the standard error of each time point being smaller than the symbols used.

DISCUSSION

TNF is an important mediator of inflammation that may cause haemorrhage, vascular collapse and ultimately death of an organism [1,2,4]. Several investigators have implicated pathophysiological roles for endothelial cells and eicosanoid production in the transduction of these disease processes [1,7,12,25,33].

The CPAE endothelial cell line was used as a model system for examining the effects of TNF *in vitro*. Cells were treated with TNF and observed for morphological changes by microscopy. This response was maximal following 10 min of TNF treatment, and was abrogated by pretreatment with the eicosanoid synthesis inhibitor,

BW755c. The cells responded in a physiologically relevant manner (i.e. retracted) which may be a mechanism by which the vasculature loses its integrity in response to TNF [3,8]. The simple permeability assay used here demonstrated further that treatment of these cells with TNF increased the diffusion of macromolecules through confluent monolayers of these cells. This endothelial response *in vitro*, appears to be similar to the increased plasma exudation observed *in vivo*, and supports the concept that the endothelial cells may regulate vascular permeability.

The rate-limiting step in eicosanoid production is phospholipase hydrolysis of eicosanoid precursor fatty acid from membrane phospholipids [34]. TNF increased the amount of phospholipase A₂ activity observed when phosphatidylcholine was used as a substrate phospholipid, but not when phosphatidylethanolamine was used. Furthermore, specificity of TNF was observed as phospholipase C activity remained unchanged when assayed by this technique. The time course of phospholipase A₂ activation also coincided with the increased release of [³H]arachidonic acid metabolites. In other experiments we observed that the increase in phospholipase A₂ activity was dose dependent with the maximal increase obtained with 100–1000 units of TNF/ml (results not shown). Similar concentrations induce eicosanoid synthesis by human umbilical vein endothelial cells [12], human synovial cells [10], dermal fibroblasts [10], and rodent macrophages [9]. These same concentrations also stimulate bone resorption and inhibition of bone formation, processes mediated, at least in part, by eicosanoids [35].

We had previously observed a similar transient increase in phospholipase A₂ activity in these endothelial cells in response to leukotriene D₄, an event which was dependent on RNA and protein synthesis [18]. To determine if the increase in phospholipase A₂ activity in response to TNF was under similar control, cells were pretreated with cycloheximide and actinomycin D before TNF treatment. Both of these reagents inhibited the increase in phospholipase A₂ activity in response to TNF. We have recently identified a protein which is able to increase phospholipase A₂ activity that we have termed PLAP [20]. This protein is antigenically related to melittin, a well described bee venom peptide that stimulates phospholipase A₂ activity [21–23], and e.l.i.s.a. assays for this protein using anti-melittin antibody indicated that TNF increases the synthesis of PLAP. Melittin is a peptide isolated from bee venom that stimulates not only phospholipase A₂ activity but also eicosanoid synthesis [12,21,22,25] in mouse and human fibroblasts, rabbit aorta endothelial cells, rat pneumocytes, rabbit smooth-muscle cells, peripheral blood after intraperitoneal injection in mice [24], and human endothelial arterial and venous cells [25]. These arachidonic acid metabolites have previously been implicated in endothelium-dependent vasodilation induced by melittin [33]. Similar phospholipase stimulatory proteins have been found in numerous other cell types from various species [21,22,31]. Thus there is precedence for our findings of PLAP in these cells and its potential role in endothelial cell retraction and eicosanoid synthesis.

In summary we propose that the CPAE cells used in these experiments may be a useful model system for studying the effects of TNF on the endothelium. The results described here indicate that eicosanoids, which

are produced in response to TNF, may regulate, at least in part, some of the responses of the endothelium. We found that phospholipase A₂ appeared to be activated by a process that required RNA and protein synthesis. We have also demonstrated an increase in the amount of PLAP detected in these cells following TNF treatment. The time course of the induction of PLAP correlates with the induction of phospholipase A₂ activity. However, in order to conclusively prove the role of PLAP in this response, genetic manipulation of the PLAP gene will be required.

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Received 4 June 1987/10 September 1987; accepted 15 October 1987