Sustained Phosphorylation of Cytosolic Phospholipase A₂ Accompanies Cycloheximide- and Adenovirus-Induced Susceptibility to TNF

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Sustained Phosphorylation of Cytosolic Phospholipase A$_2$ Accompanies Cycloheximide- and Adenovirus-Induced Susceptibility to TNF$^1$

Jennifer B. O'Brien,* Debra L. Piddington,* Christina Voelkel-Johnson,† Debra J. Richards,* Leslie A. Hadley,* and Scott M. Laster$^{2*}$

In this report we examine the phosphorylation state of cytosolic phospholipase A$_2$ (cPLA$_2$) in C3HA fibroblasts that have been treated with TNF, cycloheximide (CHI), or a combination of both compounds. Our experiments show that TNF and CHI, when used independently, caused the rapid phosphorylation of cPLA$_2$ (within 10 min). In both cases, cPLA$_2$ was subsequently dephosphorylated to pretreatment levels by 40 min. In addition, under these conditions [$^3$H]arachidonic acid was not released, and we could not detect a change in the activity of cPLA$_2$ in vitro. In contrast, in cells treated with a combination of TNF and CHI, we found that the dephosphorylation of cPLA$_2$ was inhibited, and cPLA$_2$ remained phosphorylated for up to 2 h. In vitro we found that sustained phosphorylation of cPLA$_2$ was accompanied by a 60 to 80% increase in the activity of cPLA$_2$. The sustained phosphorylation of cPLA$_2$ also occurred in cells infected with the adenovirus mutant dl309, suggesting that sustained phosphorylation may be a general requirement for the activation of cPLA$_2$ in apoptotic cells. We also found that sustained phosphorylation of phosphoproteins is not a general consequence of apoptotic death, since the phosphorylation of p42 mitogen-activated protein kinase was not sustained. Finally, we show that the phosphatase inhibitor orthovanadate acts as does CHI to render cells susceptible to TNF, suggesting that resistance to TNF may depend on TNF’s ability to induce the expression of tyrosine or dual specificity phosphatase(s).


Tumor necrosis factor $\alpha$ is an inflammatory cytokine that can mediate a variety of different immune and inflammatory reactions. In this report we focus on the apoptosis-inducing activity of TNF. Most normal and transformed cells are resistant to TNF (1, 2), although their resistance can be overcome with inhibitors of transcription or translation, such as cycloheximide (CHI)$^1$ (3) or actinomycin D (4). Resistance to TNF is, therefore, dependent on the ability of TNF itself to induce the expression of one or more resistance gene products. A number of gene products have been implicated in resistance to TNF. For example, manganous superoxide dismutase (5) has been shown to provide resistance to TNF in certain cells, such as 293 human embryonic kidney cells. The expression of manganous superoxide dismutase does not, however, predict resistance in SV40-transformed fibroblasts (6), suggesting that it may not play a general role in resistance to TNF. Plasminogen activator inhibitor type 2 (7, 8) has also been shown to mediate protection against TNF-induced cytotoxicity in certain cells, although its general role has not been tested. Recently, NF-$\kappa$B has been shown to be responsible for the expression of resistance gene products (9, 10). At this time it is not known whether NF-$\kappa$B is responsible for the TNF-induced expression of manganous superoxide dismutase or plasminogen activator inhibitor type 2.

Susceptibility to TNF can also be induced by infection with viruses, such as adenovirus (11, 12), hepatitis B virus (13), Newcastle’s disease virus (14), HIV (15), and herpes virus (16). The molecular mechanism of virus-induced susceptibility is not known, but it has been suggested that viruses may act by inhibiting the expression of the above-mentioned TNF-induced resistance gene products (17). The mechanism of virus-induced susceptibility to TNF has been studied most extensively in the adenovirus model system, where it has been shown that expression of the adenovirus E1A protein by infection (12) or transfection (11) causes susceptibility to TNF. E1A encodes two major proteins, 289R and 243R, that contain two conserved regions, CR1 and CR2, that are required for transfection (18). These same regions influence transcription of cellular genes by associating with cellular proteins, including p300 (19) and p105-Rb (20). By using deletion mutants (21) it has been shown that the binding of E1A via CR1 to either p300 or p105-Rb is sufficient to induce susceptibility to TNF. To overcome this effect, viruses have also evolved proteins that protect the infected cell against TNF-induced apoptosis. The adenovirus, for example, encodes four proteins, the E1B 19K protein (22), the E3 14.7K protein (23, 24), and the E3 10.4K/14.5K dimer (25), that function independently to inhibit TNF-induced apoptosis in specific cell types. Therefore, to cause susceptibility to TNF with adenovirus, infections must be performed with deletion mutants lacking one or more of these protective proteins (21–25). It should be noted that in addition to inducing susceptibility to TNF, expression of E1A has been found to induce susceptibility to lysis by NK cells and activated macrophages (26, 27).

The enzyme cytosolic phospholipase A$_2$ (cPLA$_2$) is an 85-kDa, Ca$^{2+}$-sensitive phospholipase that selectively catalyzes the hydrolysis of arachidonic acid from the sn-2 position of membrane phospholipids. Activation and translocation of...
cPLA₂ have been shown to require both phosphorylation by a kinase (28–30) and an increase in intracellular Ca²⁺ (29, 31–33). We have shown previously that the activity of cPLA₂ is necessary for the killing of 3T3-like fibroblasts and melanoma-derived tumor cells sensitized to TNF by inhibitors of transcription and translation (34). The activity of cPLA₂ has also been shown to be necessary for the killing of cells infected by human adenoviruses lacking the E3 14.7K gene product (35) and in the death of the TNF-sensitive cell line L929 (36). Current evidence suggests that the release of arachidonic acid by cPLA₂ is required for sphingomyelinase activation (37). In the L929 cell line, it was shown that cPLA₂ activation and the generation of arachidonic acid were necessary for the accumulation of ceramide in response to TNF (37). Finally, in the TNF-sensitive cell lines MCF-7/SI and WEHI-S, cPLA₂ activation was shown to be caspase dependent, indicating that cPLA₂ is downstream of caspase-3 in these cells (38).

As mentioned above, we have shown previously that the activity of cPLA₂ is required for the lysis of cells rendered sensitive to TNF by CHI. We found that the release of arachidonic acid occurred only from cells treated with TNF and CHI, not in those treated with TNF alone. We concluded, therefore, that CHI is inhibiting the expression of a negative regulatory factor upstream from cPLA₂ that normally prevents the apoptotic signal from reaching cPLA₂. In an attempt to gain some insight into the identity of this product, we have examined the phosphorylation state of cPLA₂ in cells treated with TNF, CHI, or a combination of both compounds. Our results show that the normal pattern of transient phosphorylation that is induced by TNF changes in the presence of CHI. Instead, in cells treated with TNF and CHI, cPLA₂ remains phosphorylated for an extended period of time (up to 2 h), suggesting that CHI may be inhibiting the expression of a phosphatase. Our experiments with phosphatase inhibitors suggest that a tyrosine phosphatase or dual specificity phosphatase may be necessary for resistance to TNF.

**Materials and Methods**

**Cell culture**

C3HA and NIH-3T3 cell lines are murine 3T3-like cell lines that were cultured in DMEM supplemented with 10% FCS and maintained at 37°C in 8% CO₂. Both cell lines were supplied by L. Gooding, Emory University (Atlanta, GA). L929 is a TNF-sensitive murine fibrosarcoma cell line that was obtained from the American Type Culture Collection (Rockville, MD) and was cultured in DMEM supplemented with 10% FCS and maintained at 37°C and 8% CO₂. WEHI 164 is a TNF-sensitive murine cell line that was obtained from the American Type Culture Collection and was cultured in RPMI 1640 medium supplemented with 10% FCS and maintained at 37°C and 8% CO₂.

**Virus and infections**

Construction of the mutant adenoviruses dl309 (39) and dl758 (40) have been described previously. The mutant dl309 is derived from an Ad5 virus and lacks the right-hand region of the E3 transcription unit, including the 10.4K, 14.5K, and 14.7K protein genes. The mutant dl758 is derived from an Ad5-Ad2-Ad5 recombinant and lacks the E3 14.7K protein gene. C3HA were plated overnight, washed, and incubated with 20 to 50 plaque-forming units/cell for 1.5 to 2 h in serum-free medium. Cells were then returned to medium with serum and incubated 24 h before treatment with TNF in [⁵¹Cr] release and [³H]arachidonic acid release assays. The dl309 was provided by L. Gooding (Emory University, Atlanta, GA), and dl758 was provided by W. S. M. Wold (St. Louis University, St. Louis, MO).

**Reagents**

Media and chemicals were purchased from Sigma (St. Louis, MO). TNF was obtained from Quality Control Biochemicals (Hopkinton, MA). SB 20358 was provided by Dr. J. Lee of SmithKline Beecham Pharmaceuticals (King of Prussia, PA). The radiolabeled compounds were purchased from DuPont-New England Nuclear (Boston, MA).

Cells were labeled with 100 μCi of Na₂[⁵¹Cr]O₄ overnight and harvested by trypsinization. Cells were then plated at 10⁶ cells/well into 96-well flat-bottom dishes that contained the appropriate concentrations of reagents for a total volume of 200 μl. At 16 h, 100 μl of the supernatant was counted with an autogamma counter (Packard, Downers Grove, IL). Maximum release was determined by adding 100 μl of 1 N HCl to untreated cells. The percent specific [⁵¹Cr] release was calculated by the following formula: [(experimental release – spontaneous release)/maximum release – spontaneous release] × 100. All treatments were performed in triplicate.

**[³H]Arachidonic acid release assays**

Cells were plated in 12-well tissue culture plates and labeled overnight with 0.1 μCi of [³H]arachidonic acid/ml. The following morning, cells were washed twice with HBSS, incubated for 2 h in medium, and washed two more times with HBSS before adding TNF and/or CHI for a final volume of 600 μl. Radio-label release in supernatants was determined by scintillation counting 200 μl of the supernatant and multiplying by a factor of 3 (Beckman, Fullerton, CA). All points were performed in triplicate, and maximal incorporation was determined by lysing untreated cells in 1% SDS.

**Cell lyses**

Whole cells were washed twice with cold PBS, solubilized in lysis buffer (30 mM HEPES (pH 7.4), 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate (OV), 1 mM PMSF, 0.2 mM leupeptin, and 0.5% SDS), and collected by scraping. The protein concentration for each sample was determined using the Bio-Rad D₅₆ Protein Assay kit (Bio-Rad, Hercules, CA), and volumes equivalent to 20 to 60 μg of protein were lyophilized. For cPLA₂ gel shift or enzyme assays, cells were incubated in serum-free medium overnight before addition of cytokines or reagents.

**SDS-PAGE and immunoblotting**

The lyophilized protein was resuspended in sample buffer and subjected to electrophoresis using the NOVEX system (San Diego, CA) with 10% Tris-glycine gels at 30 mA for 3 h for cPLA₂, gel-shift detection and for SDS-PAGE. Visualization of p38 and p42 mitogen-activated protein (MAP) kinases. Following transfer and blocking, the nitrocellulose was probed with either rabbit polyclonal antisera raised against human cPLA₂, phospho-specific p44/42 MAP kinase Ab, or phospho-specific p38 MAP kinase Ab. cPLA₂ Ab was provided by Jim Clark of Genetics Institute (Cambridge, MA), and p44/42 and p38 MAP kinase Abs were obtained from New England BioLabs (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit secondary Ab was obtained from Sigma. Bands were visualized using the SuperSignal Chemiluminescent system (Pierce, Rockford, IL).

**Cytosolic PLA₂ enzyme assay**

For lysis preparation cells were washed with PBS, scraped into homogenization buffer (20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, and 0.2 mM Na₃VO₄), and disrupted by sonication. The lysate was then cleared by ultracentrifugation (100,000 × g for 1 h at 4°C), and the supernatant was used as the source of cytosolic protein. The activity of cPLA₂ was measured in vitro in subcellular vesicles, as previously described (41). To prepare the subcellular vesicles, [¹⁴C]arachidonyl phosphatidylethanolamine and diolsglycerol were mixed, dried under nitrogen gas, and suspended in 50 mM HEPES, pH 7.4. The suspension was then sonicated for 10 s, frozen in liquid nitrogen, and sonicated again for 1 min. To initiate enzymatic reactions, 10 μl of substrate was combined with 90 μl of buffer (50 mM HEPES, 150 mM sodium chloride, 2 mM 2-ME, and 1 mM calcium chloride, pH 7.4, with 1 mg/ml BSA) and cytosolic protein. Reaction tubes were incubated for 1 h at 37°C, and the reaction was terminated by the addition of 50 μl of 100 mM EDTA. To extract free fatty acids, each reaction mixture was combined with 2 ml of Dole’s reagent and vortexed, 750 μl of ddH₂O and 1.2 ml heptane were added, and the tubes were vortexed again. The tubes were then allowed to sit on the bench for 5 min until the aqueous phase and heptane phase were completely separated, and then 1 ml of the heptane phase was transferred to another tube containing 1 ml of heptane and 100 mg of silicic acid. After vortexing, the tubes were allowed to sit for 1 h, and 1 ml of heptane was removed for scintillation counting. All points were performed in triplicate.
Results

The phenotype of C3HA fibroblasts

C3HA cells are 3T3-like fibroblasts that are resistant to TNF except when treated with CHI or infected with the adenovirus E3 deletion mutant dl309 (Fig. 1A). Figure 1B shows that the death of these sensitized cell populations is accompanied by the release of \(^{3}H\)arachidonic acid, which begins typically 2 to 4 h after treatment with TNF is initiated. We have shown previously that cPLA\(_2\) is responsible for the release of \(^{3}H\)arachidonic acid from these cell populations (34, 35).

TNF-induced phosphorylation of cPLA\(_2\) in sensitized cells

The requirement for CHI for the activation of cPLA\(_2\) suggests that CHI is inhibiting the expression of a negative regulatory protein upstream from cPLA\(_2\). To determine whether this protein controls the phosphorylation state of cPLA\(_2\), we used an electrophoretic mobility gel-shift assay (28). This assay has been used repeatedly to study phosphorylation of cPLA\(_2\) induced by a variety of agents, such as thrombin (28, 42), phorbol ester (28), and fibroblast growth factor (43). As shown in Figure 2A, cPLA\(_2\) in resting C3HA fibroblasts displays two electrophoretic mobilities, and treatment with TNF produced a very consistent shift to the slower migrating form. The shift upward to the slower migrating form was complete by 10 min and was sustained for up to 20 min (Fig. 2A). Also very consistent was the subsequent shift downward to the faster migrating form, which was complete by 40 min (Fig. 2A). We found that...
CHI itself caused some phosphorylation of cPLA₂ (Fig. 2B), although to a lesser extent than did TNF. Treatment with both CHI and TNF, however, changed this sequence of events. Treatment with both TNF and CHI (Fig. 2C) produced a shift upward by 10 min; however, dephosphorylation was inhibited, and the mobility of cPLA₂ failed to return to pretreatment levels. As shown in Figure 2D, the dephosphorylation of cPLA₂ was inhibited as long as 120 min in cells treated with TNF and CHI.

To determine whether this effect was unique to treatment with CHI, we also examined C3HA fibroblasts that had been rendered sensitive to TNF by infection with dl309. As shown in Figure 2E, although some dephosphorylation did occur, we also found that phosphorylation of cPLA₂ was sustained for up to 2 h.

Enzyme activity in TNF- and CHI-treated cells

Next, an in vitro enzyme assay was used to establish whether the change in mobility of cPLA₂ to the slower migrating form was associated with a change in enzyme activity. We have shown previously (34) that this vesicle-based assay measures selectively the activity of cPLA₂ in C3HA fibroblasts. As shown in Figure 3, we did not find any increase in enzyme activity in C3HA cells treated with either TNF or CHI alone. In contrast, we did find an increase in enzyme activity in cells treated with both TNF and CHI. Altered enzyme activity was evident as soon as 15 min post-treatment and was sustained for up to 1 h.

Phosphorylation of MAP kinases in cells treated with TNF and CHI

Does treatment with CHI or infection by adenovirus alter the TNF-dependent phosphorylation of all cellular proteins? To test this hypothesis, we examined the phosphorylation state of the p42 MAP kinase (p42) and the p38 MAP kinase (p38) in C3HA treated with TNF, CHI, and a combination of TNF and CHI. Both p42 (44) and p38 (45) are activated by dual phosphorylation of threonine and tyrosine residues, and Abs that recognize the phosphorylated form of p42 and p38 are available commercially. As shown in Figure 4A, under all three treatment conditions, p42 is phosphorylated rapidly (within 5 min) and is dephosphorylated by 20 min,
Ca\textsuperscript{2+} and its role in enzyme activation

As shown above, although phosphorylation of cPLA\textsubscript{2} is seen as early as 10 min in dying cells (Fig. 2C), the release of \textsuperscript{[3]H}arachidonic acid is not detected until 2 to 4 h, suggesting that a second signal is also required for enzyme activation in situ (Fig. 1B). Ca\textsuperscript{2+} has been reported to be necessary for the translocation of cPLA\textsubscript{2} (46), and recently, Kong et al. (47) have shown that Ca\textsuperscript{2+} is required for the lysis of L929 fibroblasts. These authors used verapamil to inhibit the import of extracellular Ca\textsuperscript{2+} and prevent cell death (47). As shown in Figure 7A, in agreement with their findings, we found that the death of L929 fibroblasts can be inhibited by pretreatment with 100 \textmu M verapamil. We also found that the death of C3HA cells sensitized by CHI was inhibited by verapamil, although not to the same extent as with L929 fibroblasts. We could not, however, use verapamil to block the death of cells infected with dl758, an adenovirus mutant lacking only the E3 14.7K gene (Fig. 7A). Similarly, we could not block the TNF-induced release of \textsuperscript{[3]H}arachidonic acid from cells infected with dl758 (Fig. 7B), while verapamil prevented the release of \textsuperscript{[3]H}arachidonic acid from L929 fibroblasts treated with TNF or from C3HA cells treated with TNF and CHI.

Discussion

In this report we have examined the phosphorylation state of cPLA\textsubscript{2}. We found that treatment of C3HA cells with either TNF or CHI produced a pattern of transient phosphorylation. Peak phosphorylation occurred at 10 min, and dephosphorylation to pretreatment levels was complete by 40 min. In contrast, treatment of C3HA cells with a combination of TNF and CHI caused sustained phosphorylation of cPLA\textsubscript{2} for up to 120 min. We also determined that the combined treatment of TNF and CHI enhanced the activity of cPLA\textsubscript{2} in vitro, suggesting a direct link between phosphorylation and enzyme activity. Finally, we observed a similar pattern of sustained phosphorylation of cPLA\textsubscript{2} in C3HA cells infected with
the adenovirus deletion mutant dl309, which suggests that this may be an effect common to agents that induce susceptibility to TNF.

Taken together, these results can be used to construct a model for the TNF-dependent regulation of cPLA₂ in C3HA fibroblasts. The data suggest that TNF initiates a signal leading to the activation of a kinase that phosphorylates cPLA₂ within 10 to 15 min. This signal is attenuated quickly (by 30–40 min), suggesting that either serine/threonine phosphatases (by OA) or tyrosine phosphatases (by sodium OV) could shift the balance in a cell, leading to the sustained phosphorylation of cPLA₂. Alternatively, OV could be acting to inhibit a tyrosine phosphatase that dephosphorylates an unrecognized site on cPLA₂. Finally, OV could be acting like pervanadate, another inhibitor of tyrosine phosphatases, which has been shown to block the TNF-induced activation of NF-κB (55, 56), which could, in turn, prevent the induction of resistance gene products.

Surprisingly, the serine/threonine phosphatase inhibitor, OA, had no effect on TNF-induced killing. These results contrast with those previously reported by Wright et al. (57). These authors found that the serine/threonine phosphatase inhibitors, OA and calyculin A, synergized with TNF in the killing of several TNF-sensitive tumor cell lines, including the U937 histiocytic lymphoma, the BT-20 mammary carcinoma, and the LNCap prostatic tumor cell line, as well as the TNF-resistant cell line U9-TR, derived from U937. The difference between our two studies may arise from biochemical differences in the apoptotic pathways of the cell lines tested. For example, we have found that TNF-induced apoptosis in U937 cells is cPLA₂ independent (data not shown), indicating that C3HA and U937 cell lines use different apoptotic pathways. In addition, Wright et al. (57) reported that OA was toxic to U937 cells at doses >25 nM, while we did not observe any toxicity toward any of the cells tested here at concentrations as high as 200 nM.

To determine whether the sustained phosphorylation of phosphoproteins is a general consequence of apoptotic death we also examined the phosphorylation state of p42 MAP kinase and p38 MAP kinase. We found that p42 MAP kinase was rapidly dephosphorylated, even in the presence of CHI, indicating that sustained phosphorylation of phosphoproteins is not an obligate consequence of apoptosis. We did find some enhanced phosphorylation of p38 MAP kinase in CHI-treated cells, suggesting that p38 might be the kinase responsible for the activation of cPLA₂. Since p38 MAP kinase has been implicated in the activation of cPLA₂ (49, 58), we tested the compound SB 203580, which has been shown to be an effective inhibitor of p38 (58, 59). We found that SB 203580 did not inhibit cell death or the release of [³H]arachidonic acid (data not shown). In fact, it enhanced the release of [³H]arachidonic acid, suggesting that p38 is not the kinase responsible for phosphorylation of cPLA₂. Another kinase that has been shown to become activated in response to TNF through the noncysteotoxic pathway is the Jun N-terminal kinase (60, 61). The Jun N-terminal kinase, however, has not been implicated in the activation of cPLA₂ (30).

We also investigated that nature of the second signal that is necessary for the activation of cPLA₂ in the apoptotic cell. In
certain situations Ca\(^{2+}\) has been shown to be necessary for the translocation of cPLA\(_2\) to its membrane phospholipid substrate (32, 33, 46, 62). It has been demonstrated previously by Kong et al. (47) that extracellular Ca\(^{2+}\) is necessary for the TNF-induced death of L929 cells. These authors showed a slow, steady rise in intracellular Ca\(^{2+}\) levels that could be blocked by verapamil. Our experiments with verapamil suggest that extracellular Ca\(^{2+}\) is also required for the activation of cPLA\(_2\) in CHI-sensitized C3HA fibroblasts. Verapamil was not effective in adenovirus-infected cells, suggesting that these cells may rely entirely on internal stores of Ca\(^{2+}\). Alternatively, virus infection itself (via E1A) may elevate basal levels of intracellular Ca\(^{2+}\), so that a ligand-dependent rise in intracellular Ca\(^{2+}\) is not necessary. It is also possible that a signal other than Ca\(^{2+}\) acts as the second signal in virus-infected cells. For example, Wissing et al. (16) have shown that a specific tetrapeptide inhibitor of caspase-3, Ac-DEVD-CHO, can inhibit the activation of cPLA\(_2\), suggesting that at least one caspase is upstream of cPLA\(_2\). In fact, a sequence on cPLA\(_2\) has been identified (38) that is similar to the caspase-3 recognition sequence on poly-(A)DP ribose polymerase, and it has been suggested that cleavage of cPLA\(_2\) itself may be an activating signal. This hypothesis remains unproven, however, since enhanced cPLA\(_2\) activity following cleavage was not demonstrated (16). In fact, we have shown (63) that cleaved cPLA\(_2\), which does appear in C3HA fibroblasts at later time points, is less active than the intact molecule. Alternatively, exteriorization of annexin V, which has been shown to be an inhibitor of cPLA\(_2\) (64), could be the second signal for cPLA\(_2\) activation in adenovirus-infected cells. These hypotheses are under investigation at the present time.

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