Formation of DNA–Protein Cross-Links in Mammalian Cells by Levuglandin E_2†

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ABSTRACT: Levuglandin E_2 (LGE_2), a rearrangement product derived from the prostaglandin endoperoxide, PGH_2, causes repair-resistant DNA–protein cross-links and cell death (LD_{50} = 230 nM) in V79 Chinese hamster lung fibroblasts. The half-life for sequestration of LGE_2 by covalent binding to cellular nucleophiles is at least an hour for 10 μM LG. This suggests that the in vivo production and distribution of free LGs should be measurable on this time scale. Following removal of the LGE_2 and the return of the cultures to normal growth medium, additional DNA–protein cross-links continued to form over the ensuing 6–24 h. The results suggest that LG adducts to DNA or protein are not repaired, but react further at sites on protein or DNA in close proximity to the initial adducts, forming cross-links in a slow phase of the process.

Covalent adduct formation between proteins (Anderson et al., 1979; Crutchley et al., 1979; Eling et al., 1977; Maclouf et al., 1980), peptides such as glutathione (Eling et al., 1977), or DNA (Vasdev & O'Brien, 1982) and unidentified electrophilic product(s) (UEP)[1] from the prostaglandin endoperoxide, PGH_2, is widely documented. Furthermore, of the well-known arachidonic acid (AA) metabolites, both malondialdehyde (MDA) and thromboxane A_2 (TXA_2) form covalent adducts with proteins (Fitzpatrick & Gorman, 1977; Tappel, 1980). However, neither of these electrophiles can account for the association of radioactivity with proteins or glutathione (GSH) which occurs upon incubation of [1-^{14}C]-AA with cyclooxygenase-containing microsomes in the presence of thromboxane synthetase inhibitors (Eling et al., 1977). The difficulty of characterizing the molecular structures of UEP–protein adducts is further complicated by the fact that binding of UEP with proteins results in cross-linking (Anderson et al., 1979). Unidentified oxidative metabolites of fatty acids have also been implicated in covalent modification of low-density lipoproteins (LDLs) to give an oLDL that is highly cytotoxic, triggering atherosclerosis by envelopment of tissue macrophages which take up oLDL to form lipid-laden foam cells (Haberland et al., 1984; Esterbauer et al., 1990; Kolberg, 1990).

We discovered that PGH_2 rearranges in the aqueous environment of its cyclooxygenase-catalyzed biosynthesis from AA to form two levulinaldehyde derivatives that were called levuglandin E_2 (LGE_2) and levuglandin D_2 (LGD_2) on the basis of their formal chemical relationship to prostaglandins PGE_2 and PGD_2 by hypothetical aldol condensations (Scheme I) (Salomon et al., 1984a; Zagorski & Salomon, 1982).

While exploring the hypothesis that LGs may account for one or more of the UEP, we observed that LGE_2 binds rapidly and covalently with proteins (Salomon et al., 1984b), resulting in intermolecular cross-linking (Iyer et al., 1989). While MDA and 4-hydroxynon-2-enal, both AA metabolites, can cause protein–protein cross-linking, LGE_2 is at least 2 orders of magnitude more effective. To explore the possible fate of LGs generated in vivo, we examined the effects of LGE_2 on cells. We showed that LGs can enter sea urchin eggs, bind with tubulin, and thereby inhibit microtubule formation and mitosis (Murthi et al., 1990). While the ID_{50} = 14 μM is, most likely, a higher concentration than occurs naturally, these experiments clearly demonstrated the ability of LGs to cross cell membranes, modify intracellular proteins, and interfere with their biological activities.

To provide further insight into the potential fate of LGs generated in mammalian cells in vivo, we now have examined the ability of LGE_2 to cause DNA–protein cross-links (DPCs) in V79 Chinese hamster lung fibroblasts in vitro. DPCs have been defined as a strong association between DNA and proteins within a cell (Oleinick et al., 1987). DPCs were first recognized as a form of cellular damage caused by ultraviolet radiation in bacteria (Smith, 1962). They are lesions formed upon the exposure of cells to a large variety of physical and chemical treatments. Besides ionizing or ultraviolet radiation, these treatments include visible light irradiation in the presence of a photosensitizer and oxygen, lipid-oxidizing agents, aldehydes such as formaldehyde or MDA, metals and metal complexes, DNA-alkylating agents, DNA intercalators, and agents which deplete the intracellular levels of thiols (Oleinick et al., 1987). DPCs are generally detected by assays which separate DNA and protein; resistance of the DNA–protein complexes to separation by organic solvents, alkaline conditions, detergents, filter-binding, or molecular sieving provides indirect evidence for the presence of tight, probably covalent, DPCs (Cress & Bowden, 1983). Further, treatment with a protease releases all of the DNA associated with protein (Chiu et al., 1984). This is additional evidence supporting the formation of covalent adducts between DNA and protein. The precise chemical linkage between DNA and protein has been defined in the case of the enzyme–DNA cleavable complex which accumulates in the presence of topoisomerase II inhibitors (e.g., *m*-AMSA); the enzyme is joined to each 5′-terminus by a

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[1] Abbreviations: AA, arachidonic acid; BD, botryodiplodin; DHP, dihydroxypyrrolidine; DPC, DNA–protein cross-links; GSH, glutathione; HBSS, Hank's balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; ID_{50}, dose that inhibits the observed response by 50%; LD_{50}, dose that reduces cell survival to 50%; LDL, low-density lipoprotein; LG, levuglandin; *m*-AMSA, 4-(9′-acridinylamino)methylsulfonyl-*m*-anisidide; MDA, malondialdehyde; MOPS, 4-morpholinepropanesulfonic acid; OA, ovalbumin; oLDL, oxidized low-density lipoprotein; PDT, photodynamic treatment; PG, prostaglandin; PIPES, 1,4-piperazinediethanesulfonic acid; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate; TXA_2, thromboxane A_2; UEP, unidentified electrophilic products.

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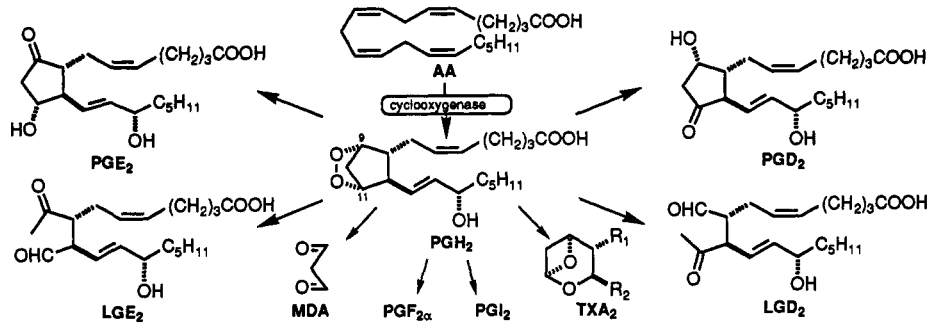
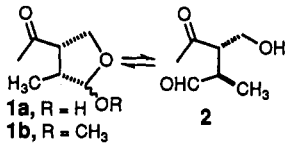
Scheme I



Scheme II



phosphotyrosine linkage (Nelson et al., 1984). A variety of base–amino acid cross-links (e.g., thymine–tyrosine) have been identified in nucleohistone or chromatin after exposure to ionizing radiation (Margolis et al., 1988) or H_2O_2 (Nackerdien et al., 1991). It is unlikely that the chemical nature of the DNA-to-protein linkage is identical for all of the DPC-inducing treatments.

When cells are treated with DNA-damaging agents, some of the damage can be repaired by cellular enzyme systems during incubation after the treatment (Friedberg, 1985). DPCs induced by ionizing radiation, thiol depletion, and intercalating agents are removed during a period of several hours post-treatment (Xue et al., 1988; Chiu et al., 1989). In the case of DPCs formed by exposure of cultured cells to ultraviolet radiation or to visible light in the presence of phthalocyanine or porphyrin photosensitizers, the majority of the DPCs appear to be stable, persisting in the cells for the remainder of the study (Chiu et al., 1984; Ramakrishnan et al., 1988).

The possibility that LGs might cause DPCs was suggested by the behavior of botryodiplodin (BD) (Gupta et al., 1966; Arsenault et al., 1969; Moreau et al., 1982), a mutagenic (Moulé et al., 1981a) mycotoxin isolated from the fungus *Botryodiplodia theobromae*. BD forms DPCs in eukaryotic cells (Moulé et al., 1982, 1984; Moulé & Darracq, 1984) and blocks DNA synthesis in a manner consistent with a DNA-damaging agent (Moulé et al., 1981b). BD exists as a hemiacetal (Scheme II, **1a**) in equilibrium with a levulinaldehyde structure **2**, which is the biologically active form. Thus, blocking the formation of the aldehyde form with a methyl group in **1b** abolishes the ability to cause DPCs (Moulé et al., 1982). The structure **2** contains the reactive electrophilic levulinaldehyde functionality found in the LGs (see Scheme I).

EXPERIMENTAL PROCEDURES

Cell Culture and Labeling. Chinese hamster V79-379 lung fibroblasts were grown in McCoy's 5A medium (Gibco Laboratories, Grand Island, NY) augmented with 10% calf serum and buffered with 20 mM HEPES (pH 7.4). Labeling of DNA was achieved by incubating exponentially growing cells for 20–24 h in medium containing [*methyl*-^{3}H]thymidine (0.1 μCi/mL) (ICN Radiochemicals, Irvine, CA).

Isolation of Nuclei. Monolayers from three 75-cm^2 flasks, each containing 2×10^7 cells, were washed and scraped into HEPES-buffered saline (10 mM HEPES, 0.154 M NaCl, pH 7.2). The cells were collected by centrifugation and resuspended in 10 mL of hypotonic solution (10 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 0.1% Triton X-100). The cell suspension was kept on ice for 15 min and then homogenized in a glass homogenizer (15 strokes). An equal volume of sucrose buffer (0.32 M sucrose, 10 mM Tris-HCl, pH 7.2, 3 mM $MgCl_2$, 0.3% Triton X-100) was added, and the mixture was kept on ice for another 15 min. The nuclei were then collected by centrifugation at 4000 rpm (SS-34 type rotor in a Sorvall RC-5C refrigerated centrifuge) at 4 °C and were ready to be treated with LGE_2.

Treatment of V79 Cells and Nuclei with LGE_2. LGE_2 was synthesized by a procedure described previously (Salomon et al., 1984b). For exposure of cells to LGE_2, cultures contained $(2–5) \times 10^6$ cells in each of a series of 9-cm Petri dishes. The growth medium was removed, and the cell monolayer was washed twice with 5 mL of Hank's balanced salt solution (HBSS) to remove traces of serum and then overlaid with 5 mL of HBSS containing various concentrations (0–10^{-5} M) of LGE_2. After incubation at 37 °C, the LGE_2-containing buffer was removed, and the monolayer was washed once with fresh HBSS. Then, either the cells were collected immediately for assay of DPC or the monolayer was overlaid with 10 mL of fresh warm growth medium, and the cultures were returned to the 37 °C incubator for various intervals before collection and assay. For exposure of nuclei to LGE_2, suspensions of 5×10^5 nuclei/mL were incubated in PBS containing various concentrations (0–10^{-5} M) of LGE_2, collected by centrifugation, and prepared for the assay of DPC (see below).

Clonogenic Cell Survival. Cell survival was determined by the ability of cells to form colonies containing at least 50 cells. V79 cells were plated on 9-cm Petri dishes (10^2–10^5 cells/dish) and allowed to attach for 3 h prior to treatment. The cells were washed once with HBSS and incubated for 2 h at 37 °C in HBSS containing various concentrations of LGE_2 ($(0–3) \times 10^{-6}$ M). The cultures were washed with HBSS to remove the LGE_2 and were incubated in growth medium for 8 days to allow colony formation. The medium was removed, and the colonies were stained with crystal violet and counted by eye.

Nitrocellulose Filter-Binding Assay for DNA–Protein Cross-Links. For the study of DPC formation in cells, monolayer cultures were prepared in 9-cm Petri dishes. Following treatment with LGE_2, cell monolayers were washed twice and scraped into ice cold HEPES-buffered saline. The cells were collected by centrifugation (4000*g*, 5 min) and resuspended at a cell density of $(2–5) \times 10^5$ cells/mL in cold SSC (0.15 M NaCl, 15 mM sodium citrate) solution containing 5 mM EDTA. The cells were lysed by addition of $^1/_{10}$ vol of 5 M sodium perchlorate and, after thorough vortexing, $^1/_{10}$ vol of 10% Sarkosyl (sodium lauroyl sarcosinate) and vortexed

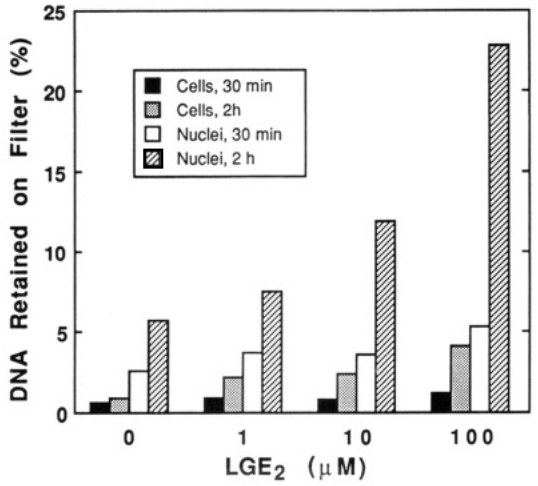


FIGURE 1: Formation of DPC in V79 cells and nuclei treated with LGE_2. V79 cells (5×10^6 cells/culture) or nuclei (5×10^5 nuclei/mL) were treated with the indicated concentrations of LGE_2 for 30 or 120 min at 37 °C. Immediately following the incubation, cell and nuclear lysates were prepared and assayed for DPC as described in the Experimental Procedures.

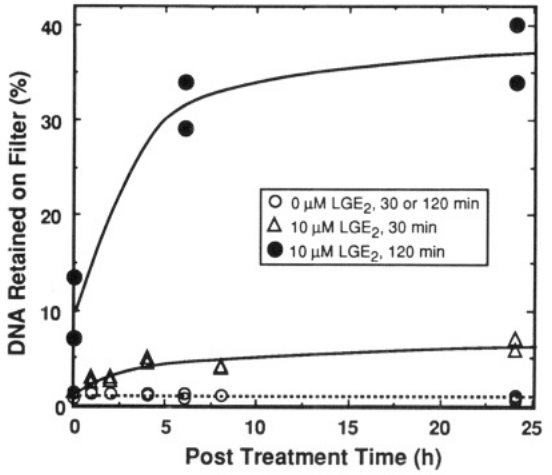


FIGURE 2: DPC formation during incubation following exposure of V79 cells to LGE_2. V79 cells (4.8×10^6 cells/culture) were treated with 0 (○) or 10 μM (△ and ●) LGE_2 solution in HBSS for 30 min (△) or 2 h (●) at 37 °C. The cells were then washed to remove nonbound LGE_2 and incubated at 37 °C in fresh growth medium for various time periods, as indicated on the abscissa, before they were collected for the assay of DPC. Duplicate determinations were made for each incubation condition. The precision and accuracy of the measurements were estimated by comparing the data from three experiments (i.e., those presented in Figures 2–4) corresponding to a 2-h treatment with 10 μM LGE_2 and 24-h post-treatment time. The average value for DNA retained on the filter was 34.7%. The accuracy of this value is indicated by a standard deviation of ±8.8% for all of the determinations, whereas the precision of each paired determination is indicated by the average of the differences observed for each data pair, 3.5%.

vigorously again. After incubation at 60 °C for 20 min and cooling to room temperature, aliquots of 1 mL were used for the filter-binding assay. In some cases, aliquots (1 mL) of the lysates were incubated with Proteinase K (100 μg/mL) at 25 °C for 24 h before filtration.

Nitrocellulose filters (HAWP, 02500, pore size 0.45 μm, Millipore) were soaked in 2× SSC for at least 15 min before filtration. The filters were placed on a Millipore manifold filtration chamber and washed once with 0.5 mL of 2× SSC solution. The vacuum was adjusted to give a flow rate of about 1–2 mL/min. One mL of lysate was loaded along with 1 mL of the lysis solution (SSC, 1 M $NaClO_4$, 1% Sarkosyl) onto each filter. The two solutions were mixed well and the vacuum was turned on. When filtration was complete, the filters were washed twice with 5 mL of high-salt buffer (3 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.8) and once with 5 mL of low-salt buffer (1 mM Tris-HCl, 1 mM EDTA, pH 7.8). Each filter was cut and placed in a scintillation vial containing 1 mL of an extraction solution (1 mM Tris-HCl, 1 mM EDTA, pH 7.4, 0.5% SDS). The vials were placed in a water bath at 95 °C for 2 h to release the radiolabeled DNA from the filters, cooled to room temperature, and vortexed vigorously. Scintillation fluid (Ecolite, ICN Biochemicals) was added, and the radioactivity was determined using a Beckmann Model 3801 liquid scintillation spectrometer.

RESULTS

In order to examine the ability of LGE_2 to cause the formation of DPC, intact V79 cells or nuclei isolated from these cells were incubated with increasing concentrations of LGE_2 for 30 min or 2 h, and the cells or nuclei were recovered immediately, lysed, and assayed for DPC by nitrocellulose filter-binding. As shown in Figure 1, under all of the conditions studied the untreated cells were observed to contain a low level of DPC. This level was greater for nuclei than for cells and increased further during the incubation in buffer. A 30-min incubation in the presence of LGE_2 at concentrations ranging from 1 to 100 μM caused up to a doubling of the level of DPC observed in cells, while treatment of cells with LGE_2 for 2 h caused an increase in DPC up to 4-fold greater than that observed in untreated cells. The levels of DPC found in LGE_2-treated nuclei were markedly greater than for cells, consistent with a greater fraction of the added levuglandin reaching nuclear constituents. The smaller effect in the intact cells is probably due to sequestration of some of the LGE_2 by extracellular and intracellular but extranuclear proteins and other nucleophiles.

Effect of Post-Treatment Incubation Time on DPC Formation after Treatment in HBSS. DPCs induced by agents such as botryodiplodin (Moulé & Darracq, 1984), formaldehyde (Craft et al., 1987; Wilkins & Macleod, 1976; Schwencke & Ekert, 1978), and ionizing radiation (Chiu et al., 1986a) are removed completely during post-treatment incubation of exposed cells. In contrast, DPCs induced by ultraviolet light (Chiu et al., 1986a,b; Smith, 1968) and by photodynamic therapy (PDT) (Ramakrishnan et al., 1988, 1989) appear not to be removed post-treatment but to increase in frequency during post-treatment incubation. To establish the fate of LGE_2-induced DPCs, V79 cells were treated with LGE_2 in HBSS for 30 min or 2 h at 37 °C, following which they were incubated in growth medium for various time intervals and the resultant level of DPCs was determined. The results (Figure 2) demonstrated that LGE_2-induced DPCs were not removed from V79 cells during a 24-h incubation in the absence of LGE_2. On the contrary, the level of DPCs continued to rise, reaching a maximum level after 6–24 h.

Both the initial level of DPCs and the level attained during post-treatment incubation were dependent upon the initial treatment time. The amount of DPCs formed after a 2-h treatment was much higher than the amount formed after the shorter treatment, both initially and during all post-treatment incubation periods. The continual increase in DPCs after removal of extracellular LGE_2 shows that LGE_2 not only enters the cells and causes DPCs but once it is sequestered within the cells, cross-linking reactions continue for at least several hours. The fact that the 2-h treatment results in more DPCs than the 30-min treatment means that the amount of LGE_2 sequestered by covalent binding to protein or DNA after 2 h

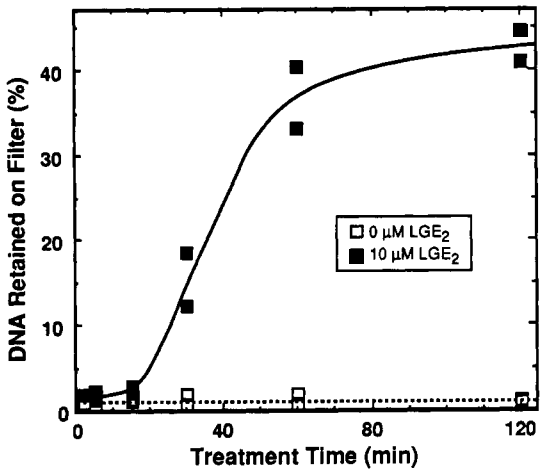


FIGURE 3: Effect of time of exposure to LGE_2 on DPC formation in V79 cells. V79 cells (4.8×10^6 cells/culture) were incubated at 37 °C with HBSS containing either 0 (□) or 10^{-5} (■) M LGE_2 for different lengths of time (2 min to 2 h). The LGE_2 solution was removed. The cultures were washed and then further incubated in fresh growth medium for 24 h at 37 °C, after which time the cells were collected for DPC assay. For error analysis, see the legend for Figure 2.

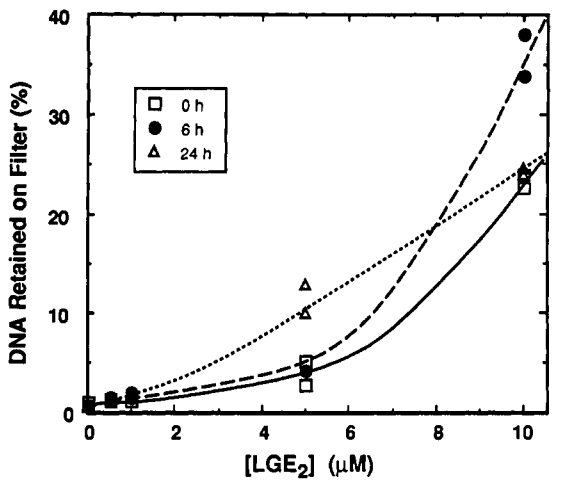


FIGURE 4: Effect of dose of LGE_2 and post-exposure time on DPC formation in V79 cells. V79 cells (4.8×10^6 cells/culture) were incubated with several concentrations of LGE_2 (0–10^{-5} M) in HBSS for 2 h at 37 °C. The cells were then washed, incubated at 37 °C in fresh growth medium for 0 (□), 6 (●), or 24 h (△), and collected for DPC assay. For error analysis, see the legend for Figure 2.

is much more than after the shorter treatment. Also, the fact that the amount of DPCs formed increases with time upon post-treatment incubation suggests that the initial adducts formed by LGE_2 with DNA and proteins are reactive. They continue reacting for many hours with other cellular constituents causing further damage to the cell.

Effect of Time of Exposure on DPC Formation. To explore the time course for the initial cellular uptake and sequestration of LGE_2, V79 cells were treated with 10 μM LGE_2 for periods ranging from 2 min to 2 h, and then the cells were allowed a maximum expression time of 24 h in growth medium before being collected for DPC measurement. The cellular uptake of LGE_2 increases with time of treatment initially and then approaches a maximum after 1 h (Figure 3).

Dose Response for the Induction of DPC by LGE_2. V79 cells were treated with varying concentrations of LGE_2 (0–10 μM) for 2 h and then were allowed to incubate in growth medium for 0, 6, or 24 h prior to the recovery of the cells for the assay of DPCs by the filter-binding assay. As seen in Figure 4, the amount of DNA retained on the filter increased above the background level when the cells were treated with LGE_2 concentrations between 1 and 5 μM. When the cells were treated with 5 or 10 μM LGE_2, there were large amounts of DPCs formed both initially and after a 6- or a 24-h post-treatment incubation period. In the case of 10 μM LGE_2, the amount of DNA retained on the filter after a 6-h post-treatment incubation was more than that retained after a 24-h post-treatment incubation. Although such a result was not always observed (cf. Figure 2), when the recovery of DPC decreases with post-treatment incubation time, as in Figure 4, the loss of DPC is likely due to cell death and the detachment of heavily damaged cells from the monolayer during the long post-treatment incubation, leaving the relatively less damaged cells.

Enzymatic Hydrolysis of the DPCs Induced by LGE_2. To confirm that LGE_2 causes the formation of covalent adducts of protein with DNA within the cell, V79 cells were treated with LGE_2, and aliquots of the lysates were digested with Proteinase K treatment before the nitrocellulose filter assay. As shown in Table I, filtration of lysate aliquots without Proteinase K treatment resulted in a high percentage of DNA being retained on the filter, whereas for aliquots filtered after

Table I: Enzymatic Hydrolysis of DPC Formed in LGE_2-Exposed V79 Cells[a]

$[LGE_2]$ (M)	%DPC w/o Proteinase K treatment	%DPC with Proteinase K treatment
0	0.8	0.2
0	0.6	0.4
5×10^{-6}	14.8	0.5
5×10^{-6}	11.0	0.3
10^{-5}	25.1	0.9
10^{-5}	19.6	0.4

[a] V79 cells were treated with varying concentrations of LGE_2 (0, 5×10^{-6}, 10^{-5} M) for 2 h followed by a 24-h post-treatment incubation in complete growth medium. Lysates were prepared as described in the Experimental Procedures. Duplicate 1-mL aliquots of the lysate were incubated with a solution of Proteinase K (100 μL of 1 mg/mL stock solution) at 25 °C for 24 h and then were subjected to the filter-binding assay. Additional 1-mL aliquots of the lysate were also subjected to the DPC assay but without Proteinase K treatment.

Proteinase K treatment, virtually no DNA was retained on the filter. The dependence of DNA retention on the intact protein is generally taken as evidence of the presence of DNA–protein cross-links.

Effect of pH on DPC Formation. Numerous cytotoxic treatments are more effective when the cells are treated in a medium or buffer at slightly acid pH. Likewise for the induction of DPCs by LGE_2, the yield of DPCs was greater when LGE_2 treatment was carried out in HBSS at pH 6.6 than at pH 7.0 or 7.3 (Figure 5). This result was obtained for DPCs determined immediately after the LGE_2 treatment as well as for DPCs determined after a 24-h post-treatment incubation. In the latter case, the DPC level was high at all three values of pH, although it was demonstrably higher at the most acidic pH. The acidic pH may increase the amount of LGE_2 taken up by the cells or promote one or more of the reactions leading to DPC formation.

Loss of Clonogenicity in LGE_2-Treated Cells. A standard method to study the survival of mammalian cells in culture after a cytotoxic treatment involves the exposure of a large number of cells and the measurement of colony-forming ability by removing the treated cells from the monolayer with trypsin and then replating them in aliquots sufficient to yield 100–200 cells per dish. However, when this method was attempted, it was found that the LGE_2-treated cells were difficult to

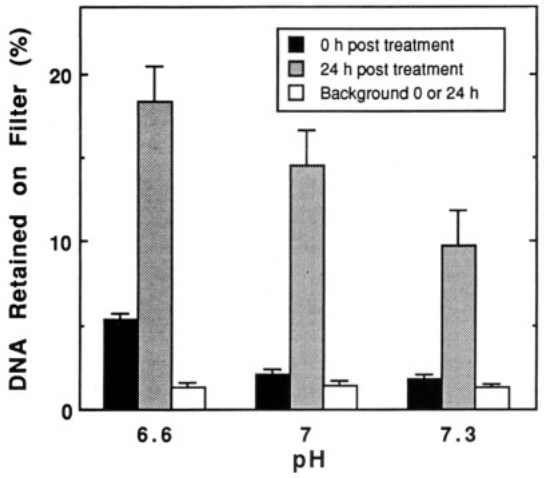


FIGURE 5: Effect of pH on DPC formation by LGE_2 in V79 cells. V79 cells were treated with LGE_2 (5×10^{-6} or 0 M = "background") for 2 h in HBSS buffered with PIPES, pH 6.6; HBSS buffered with HEPES, pH 7.0; or HBSS buffered with MOPS, pH 7.3. Following a 0- or 24-h post-treatment incubation in fresh complete growth medium, cell lysates were prepared, and the DPCs were determined as described above. Duplicate determinations were made for each incubation condition. Error bars indicate the largest difference among the three data pairs for each post-treatment condition or among the six "background" data pairs.

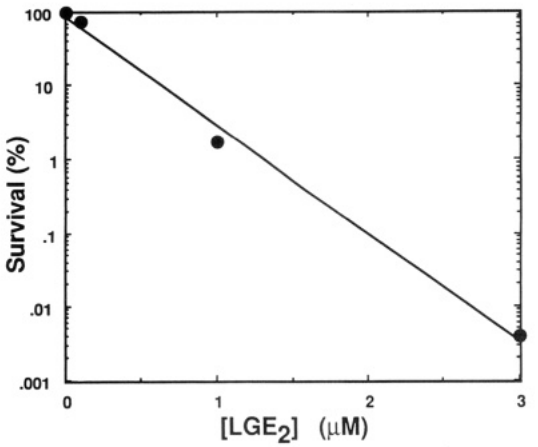


FIGURE 6: Survival of LGE_2-treated V79 cells. Cultures were set up containing 10^2–10^5 cells in 9-cm Petri dishes. After 3 h were allowed for cell attachment, the cultures were treated with various concentrations of LGE_2 for 2 h and then refed with fresh complete growth medium and returned to the incubator for 10 days to allow surviving cells to form colonies of >50 cells. Stained colonies were counted by eye. Three plates were prepared for each experimental point. The plating efficiency for the control was 62%.

remove from the Petri dish upon trypsinization, suggesting that cross-linking of the extracellular proteins by LGE_2 had occurred. Hence an alternative method was used to study the survival of the cells treated with LGE_2. Cells were plated at a range of cell densities, treated with LGE_2, and then given fresh growth medium and returned to the incubator to permit colony formation.

As seen in Figure 6, when cells were treated for 2 h, there was a dramatic increase in cell killing between 0.1 and 3 μM LGE_2. On the basis of this protocol, the LD_{50} dose, i.e., the dose resulting in 50% loss of clonogenicity, was 0.23 μM LGE_2. Thus, very low concentrations of LGE_2 seem to cause cellular damage sufficient to kill a large number of cells. However, it should be noted that the number of LGE_2 molecules per cell would be much greater for treatment of the small numbers of cells in the clonogenicity assay shown in Figure 6 than for the treatment of the larger numbers of cells for the DPC assay (Figures 1–5), and it is possible that the level of cell killing would be less in the latter case than that revealed in Figure 6.

DISCUSSION

Assay of Cross-Linking. Several methods, such as alkaline elution, extraction with organic solvents, equilibrium density gradient centrifugation in CsCl, gel filtration, and nitrocellulose filter-binding, have been used to measure DPCs (Oleinick et al., 1987; Smith, 1962; Cress & Bowden, 1983; Chiu et al., 1984; Strniste & Rall, 1976). The nitrocellulose filter-binding assay is a simple way to quantitate DPCs, and it also allows recovery of the cross-links from the filter for further analysis. Under the conditions of the filtration, the nitrocellulose filters have an affinity only for proteins, and free DNA, either single- or double-stranded, elutes during filtration. Thus, any DNA retained on the filter is tightly bound to protein. There is usually some DNA associated with protein in the untreated cells, and this shows up as the background level of DPCs (~1%).

Cross-Linking Mechanisms. A number of chemical as well as physical agents have been widely studied for their ability to cross-link proteins as well as DNA. Many of the chemical agents possess an electrophilic aldehyde functionality which is believed to be responsible for their cross-linking abilities (Auerbach et al., 1977). The reactions of formaldehyde, the simplest aldehyde, with biological macromolecules (proteins and DNA) have been extensively studied to understand the chemistry involved in the cross-linking reaction. Formaldehyde is known to react with the amino groups of proteins and nucleic acids to give methanol derivatives (eq 1). It is believed to cause intermolecular cross-linking by the formation of methylene bridges (eq 2) (French & Edsall, 1945). Five such formaldehyde-induced cross-links have been isolated involving adenine, guanine, and cytidine, with the 9 and 7 positions of the purine heterocycle being the reactive sites (Figure 7) (Shapiro, 1968).

Native double-stranded DNA does not react with formaldehyde. Treatments which destroy the hydrogen bonds in DNA increase the reactivity of formaldehyde toward DNA. Furthermore, during studies of the reactions of formaldehyde with nucleohistones, it was found that the rate of reaction with DNA was enhanced in the presence of lysine-rich proteins (Kolmark & Westergaard, 1953). Further studies demonstrated that the methanol derivatives of glycine- or lysine-rich histones were formed very rapidly (within several seconds) and that the methanol derivatives react secondarily with nucleotides or DNA (Kondo et al., 1970). The stability of the final product formed depends on the nature of the amino acid and the nucleotides (Kolmark & Westergaard, 1953). Furthermore, the reaction of formaldehyde with DNA in this manner resulted in the breakage of the sugar phosphate moiety of the double helix in a rapid manner (Kolmark & Westergaard, 1953; Kondo et al., 1970). Thus, a major activity of formaldehyde is the ability to induce DNA–protein cross-links.

MDA, a bifunctional agent that can be generated from PGH_2 (see Scheme I), causes intermolecular cross-linking in human serum albumin and in bovine serum albumin (De-

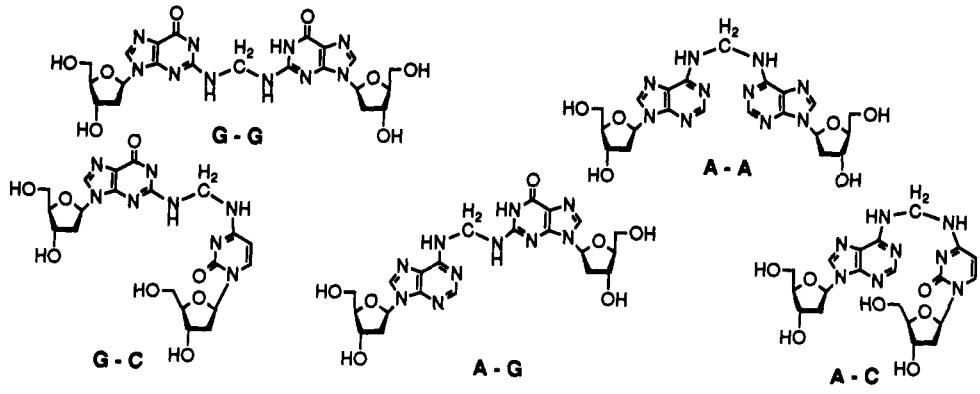
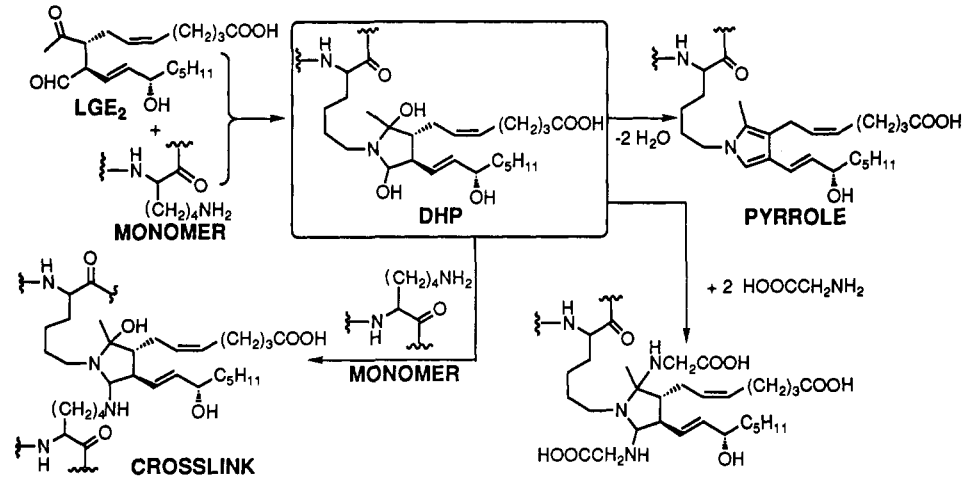
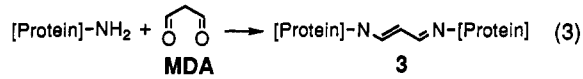


FIGURE 7: Aminal cross-links between DNA bases induced by formaldehyde.

Scheme III



Caprio, 1986). This cross-linking is believed to be due to the formation of 1-amino-3-iminopropene adducts such as **3** (eq 3) (Chio & Tappel, 1969). MDA generated in vivo by oxidation in the liver and brain of rats fed 1,3-propanediol causes interstrand cross-linking of DNA as well as DNA–protein cross-links (Summerfield & Tappel, 1984a). It was found that MDA binds to the guanine and cytosine residues of DNA, and these two bases are believed to be involved in the interstrand cross-linking (Summerfield & Tappel, 1984b). DPCs induced by MDA are believed to consist of covalent linkages between the lysine groups in protein and the cytosine, guanine, and adenine residues in DNA (Summerfield & Tappel, 1984c). However, the exact nature of the cross-link is not known.



(3)

Although the rate of binding of LGE_2 to protein is very rapid, cross-linking is a slower process which can be completely prevented by addition of excess glycine to the reaction mixture 5 min after incubation of LGE_2 with the protein (Iyer et al., 1989). One possible cross-linking mechanism involves the rapid formation of a reactive electrophilic dihydroxypyrrolidine (DHP) adduct between LGE_2 and a protein monomer. This DHP intermediate is an activated monomer that reacts further with the primary amino groups of other protein molecules to form cross-links (Scheme III).

The binding of glycine to the LGE_2–ovalbumin (OA) adduct was studied as a model for cross-linking (Anderson et al., 1979; Iyer et al., 1989). In fact, the electrophilic LGE_2–OA adducts bind nearly 2 equiv of glycine (per equivalent of bound LGE_2). This is the ratio anticipated if both hemiaminal hydroxyls in DHP are replaced with glycyl amino groups. The DHP intermediate could react with other protein monomers to form aminal cross-links or dehydrate to form a virtually nonelectrophilic pyrrole. LG-derived DPCs could involve mixed aminals incorporating one protein and one DNA amino group. Such a structure is analogous to the aminal cross-links, i.e., the methylene-bridged products of eq 2, between DNA bases induced by formaldehyde (see Figure 7). As seen previously, the amount of damage to the mammalian cells caused by LGE_2 through DPC formation increases with treatment time as well as post-treatment incubation time. On the basis of these facts and previous studies, it seems that LGE_2 reacts rapidly with protein and DNA monomers to form a reactive electrophilic adduct. The amount of LGE_2 sequestered by the cellular proteins and DNA apparently increases with time of exposure of the cells to LGE_2, resulting in the formation of more adducts. Initially, LGE_2 probably forms adducts primarily with proteins as it has to pass through the extracellular as well as intracellular proteins on its way to the nucleus. During post-treatment incubation, these electrophilic protein–LGE_2 adducts continue to react with other proteins and DNA forming various cross-links, e.g., DNA–protein and protein–protein, resulting in serious damage to the cell.

Biological Significance. We have proposed that LGs are byproducts of the cyclooxygenase-promoted bioconversion of AA into PGs in vivo. Because of their high reactivity, LGs may mediate some of the toxic effects of treatments of cells with agents that induce the biosynthesis of PGs. However, their high reactivity complicates detection and quantification of LGs in vivo. The present study demonstrates not only that

LGs are able to cross cell membranes and modify intracellular proteins but also that sequestration by intracellular proteins does not prevent passage of at least some LGs through the cytoplasm and into the nucleus. Furthermore, these experiments provide a detailed picture of the probable fate of LGs in vivo. Thus, the fact that a 120-min preincubation results in many more DPCs than a 15-min preincubation means that the amount of LGE_2 sequestered by covalent binding to protein or DNA after 120 min is much more than after 15 min. In other words, the half-life for sequestration of LGE_2 by covalent binding to cellular nucleophiles must be at least an hour for 10 μM LG. At lower concentrations, the half-life for sequestration would be even longer unless high-affinity binding sites (receptors) exist for LGs. This important finding establishes conditions under which the in vivo production and distribution of free LGs should be measurable.

The present study shows that LGE_2 causes extensive lesions in mammalian cells in the form of DPCs. The formation of DNA–protein cross-links is probably relevant to the mechanism of cytotoxicity of LGE_2. The largest increase in DPCs observed in these experiments occurred when the cells were exposed to supralethal levels of LGE_2. However, measurable amounts of DPCs were formed during the post-treatment incubation following exposure of cells to more modest levels of LGE_2. Although other types of DNA damage may also occur, e.g., single base adduction or strand breaks, the DPCs appear to be nonreparable, which supports the notion that the damage is relevant to cell killing. Not only was there an absence of observable repair of the LGE_2-induced DPCs but the level continued to increase during post-treatment incubation of the cells in growth medium. In this respect, the DPCs induced by LGE_2 appear to be similar to DPCs induced by ultraviolet irradiation (Chiu et al., 1984) and by photodynamic action sensitized by an aluminum phthalocyanine (Ramakrishnan et al., 1988). Interestingly, the photodynamic treatment can cause the release from cells of cyclooxygenase AA metabolites (Henderson & Donovan, 1989; Henderson & Dougherty, 1992) as well as the formation of DPCs (Chiu et al., 1984; Ramakrishnan et al., 1988; Ben-Hur et al., 1992). It has not yet been demonstrated definitively that LGs mediate the cellular damage attributable to AA metabolites through the induction of DPCs. However, if LGs are produced in vivo in cells, the present study shows that LG-derived DPCs would be generated in those cells and that such DPCs could be important forms of cellular damage responsible for cell killing.

DNA–protein cross-linking by LGE_2 in cells may have other profound biological implications. For example, with in vitro perfused heart and in vivo myocardial and brain models, a torrent of unesterified AA is released by lipolysis of phospholipids during myocardial and cerebral ischemia. In the ischemic area of dog hearts, AA concentrations rise from a normal level of 2 μM to a concentration as high as 40 μM (Chien et al., 1984; van der Vusse et al., 1982). Similarly, in an ischemic brain, up to 0.4 mM concentrations of AA are formed (Fishman & Chan, 1981; Siesjo & Wieloch, 1983). Generation of abnormally high concentrations of PGH_2 during a postischemic burst of cyclooxygenase activity provides an excellent opportunity for the formation of LGs in vivo. Furthermore, since LG-induced DPCs are repair resistant, their levels and the consequent cytotoxicity are expected to be cumulative. Thus, for example, LGs may contribute to the tissue damage associated with heart attacks and stroke not only by inducing protein–protein cross-links but also by causing DPCs in these tissues.

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