Clastogenic action of hydroperoxy-5,8,11,13-icosatetraenoic acids on the mouse embryo fibroblasts C3H/10T1/2

TAKAFUMI OCHI and PETER A. CERUTTI

Department of Carcinogenesis, Swiss Institute for Experimental Cancer Research, 1066 Epalinges s/ Lausanne, Switzerland

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ABSTRACT Phorbol 12-myristate 13-acetate induces the release of a low molecular weight clastogenic factor from monocytes. Hydroperoxy-5,8,11,13-icosatetraenoic acids represent major components of clastogenic factor. We report that several isomeric hydroperoxy-5,8,11,13-icosatetraenoic acids efficiently induce DNA strand breakage and/or alkali-labile sites in the mouse embryo fibroblasts C3H/10T1/2. Fe chelation by deferoxamine suppresses breakage by ~42% indicating the participation of Fe-catalyzed radical reactions. An additional 37% inhibition is observed upon addition of the Ca2+-chelators EGTA and quin-2. This result suggests that hydroxyperoxy-5,8,11,13-icosatetraenoic acid may activate a Ca2+-dependent nuclease. The addition of the antioxidant enzymes CuZn-superoxide dismutase and catalase had no effect, while glutathione peroxidase suppressed strand breakage by 90%. To our knowledge, our results yield a first insight into the mechanism of action of monocyte clastogenic factor and the role of inflammation in tumor promotion.

The infiltration of polymorphonuclear leukocytes and macrophages into the stroma is the hallmark of inflammation and may play a role in tumor promotion (1, 2). These cells are known to release a host of paracrine mediator molecules. The release of these substances can be stimulated or suppressed by a variety of xenobiotic and biotic molecules, such as particulates, lectins, certain peptides, lipids, and the phorbol ester tumor promoter phorbol 12-myristate 13-acetate (PMA) (3). It was of particular interest that the low molecular weight components (<10 kDa) released by PMA-stimulated human leukocytes were clastogenic, i.e., induced chromosomal aberrations in test cultures (4, 5). The observation that the clastogenic action of PMA on regular, monocyte-containing, lymphocyte cultures could be suppressed by inhibitors of the enzymes of the arachidonic acid (ΔAch) cascade (6) suggested the participation of ΔAch metabolites in this process. Detailed analysis of the ΔAch metabolites released by PMA-stimulated human monocytes led to the identification of the cyclooxygenase products thromboxanes B2, prostaglandins F2α, and E2, 12-hydroxy-5,8,10-heptadecatrienoic acid, the lipoygenase products 5,11- and 15-hydroxyicosatetraenoic acid, and free ΔAch in the clastogenic mixture (W. Kozumbo, D. Mühlematter, and P.A.C., unpublished data (7). It should be noted that these cyclooxygenase and lipoygenase products are formed by the cell via the intermediary of the hydroperoxy precursors 15-hydroperoxyprostaglandin E2, prostaglandin G2, and several hydroperoxy-5,8,11,13-icosatetraenoic acid isomers (HPETEs). These hydroperoxides are unstable and react to the corresponding hydroxyl derivatives that are then found on chromatograms of lipid extracts of the clastogenic mixture. However, in situ in the inflamed tissue the hydroperoxides that are released by the leukocytes are probably of sufficient stability to exert their effect on neighboring cells. In direct support of our model, Lewis et al. (8) have reported that stimulated macrophages induce oxidative thymine damage in cocultured fibroblasts because they release active oxygen plus ΔAch metabolites, in particular metabolites of the lipoygenase pathway. HPETE with the hydroperoxy group attached to C8 (8-HPETE) was found to be a major metabolite of ΔAch produced by hyperplastic mouse skin (9), whereas 15-lipoxygenase metabolites of ΔAch are produced predominantly by epithelial cells from human trachea (10). Hydroperoxy and hydroxy fatty acids stimulated DNA synthesis and induced ornithine decarboxylase in rat colon (11). Hydroperoxides stimulate the cyclo- and lipoxygenase of the ΔAch cascade and amplify their own biosynthesis (12, 13). Our strategy for the elucidation of the mechanism of action of “clastogenic factor” (CF) from PMA-stimulated monocytes (W. Kozumbo, D. Mühlematter, and P.A.C., unpublished data (14) was to examine the capacity of individual components of the clastogenic mixture to induce DNA strand breakage in cultured cells. In the present paper we report that HPETEs cause DNA breakage in the mouse embryo fibroblast C3H/10T1/2 and, therefore, contribute to the activity of CF from human monocytes. The clastogenic activity of HPETE depended on the presence of Ca2+ and Fe. The need for Ca2+ is intriguing in view of our finding that HPETEs efficiently mobilize mitochondrial Ca2+ (C. Richter, B. Frei, M. Graf, K. Winterhalter, and P.A.C., unpublished results).

MATERIALS AND METHODS

Mouse embryo fibroblasts C3H/10T1/2 C19 were grown as monolayers on plastic Petri dishes as described (15). They were labeled with [14C]thymidine for 24 hr and chased in fresh medium for 4 hr. The cells were removed from the plastic dishes by treatment with trypsin/EDTA and washed with complete medium and phosphate-buffered saline (PBS: KH2PO4, 0.2 g/liter; Na2HPO4, 1.15 g/liter; NaCl, 8 g/liter; KCl, 0.2 g/liter, pH 7.4). It should be noted that prolonged exposure of the cells to trypsin resulted in some DNA breakage in controls. In some experiments the cells were scraped from the plastic surface with a rubber policeman. Aliquots of 1.5–2 ml of the cell suspension containing 5 × 105 cells were added to filter funnels of an alkaline elution apparatus that contained 5 ml of PBS. The solutions were drained, and the cells that were adsorbed to the polyvinyl chloride filters were washed twice with PBS. Treatment with HPETEs was in 2.5 ml of PBS at 37°C for 15 min in a CO2 incubator. In the experiments with antioxidants, the cells were preincubated with the enzymes or butylated hydroxytoluene for 5 min prior to the addition of HPETE with the hydroperoxy group on C15 (15-HPETE). Cell lysis and alkaline elution were carried as described (16, 17). Fractions of 4.5 ml of the tetrapropylammonium hydroxide/EDTA

Abbreviations: HPETE, hydroperoxy-5,8,11,13-icosatetraenoic acid; HETE, hydroxy-5,8,11,13-icosatetraenoic acid; ΔAch, arachidonic acid; PMA, phorbol 12-myristate 13-acetate; GSH, glutathione; CF, clastogenic factor.
buffer, pH 11.9, were collected directly into scintillation vials. The amounts of radioactivity contained in the eluates and retained on the filters were determined. Elution curves relating the fraction number to the percent radioactivity retained on the filter were linear to about 20% residual retention. When comparing the effect of different incubation conditions on the efficiency of strand break formation, we present directly the alkaline elution curves. We have normalized the data for dose–response curves by calculating C20 = 100 log (D/D₀) (18) where D and D₀ are the percentages of DNA retained on the filter after five fractions of 4.5 ml of elution buffer have been collected for the treated sample and the untreated control, respectively. For an estimation of the number of strand breaks from the elution curves, 10T½ cells were irradiated with 100–400 rad (1 rad = 1×10⁻² Gy) of γ-rays under aerobic conditions at 0°C. A value of 4.05 × 10⁻¹₀ dalton⁻¹ for the efficiency of strand breakage by 150 rad (19) was used for the computation. For comparison we also determined DNA strand breakage and/or alkali-labile site formation by 15-HPETE and by γ-rays with the alkaline unwinding assay developed by Birnboim (20, 21). As in the alkaline elution experiments, freshly trypsinized cells were treated in suspension at 37°C or irradiated with γ-rays under aerobic conditions at 0°C. The data is evaluated as described by Birnboim and Kanabus-Kaminska (22).

The HPETEs were the generous gift of L. Marnett (Wayne State University, Detroit) and R. Schmidt (Deutsches Krebsforschungs-Zentrum, Heidelberg). They were synthesized enzymatically or photochemically and purified by HPLC. The purity of these compounds as well as that of β₂-ACh was monitored chromatographically during the course of our experiments. Stock solutions were in ethanol or methanol and the final concentrations of the alcohol in the treatment buffer varied between 0.2 and 0.4%. Desferrioxamine was a gift of Ciba-Geigy (Basel, Switzerland). All other chemicals were from Sigma, and radiochemicals were from New England Nuclear.

RESULTS

To elucidate the clastogenic mechanism of the low molecular weight components that are released by PMA-stimulated human monocytes, we examined DNA strand breakage by HPETEs in mouse embryo fibroblasts C3H/10T½. An experimental protocol was developed that avoids a time delay between treatment and strand-break determination during which resealing of breaks could take place. Cells were removed from the monolayers by trypsin/EDTA, suspended in PBS, and treated directly in the filter funnels of an alkaline elution apparatus (16). All experiments were carried out in PBS without the addition of Ca²⁺ and Mg²⁺. As shown in Fig. 1, 8-, 9-, 12-, and 15-HPETE (9- and 12-HPETE, the hydroperoxy group is attached to C9 and C12, respectively) induced DNA single-strand breaks and/or alkali-labile sites (pH 11.9) with comparable efficiencies regardless of the position of the hydroperoxy group. Most experiments were carried out with the allylic 15-HPETE, and a dose–response curve is given in Fig. 2A that also includes data for ⁶⁰Co γ-rays for comparison. The curve for 15-HPETE is steep and nonlinear at low doses (0–10 μM) and then continues linearly with a diminished slope. Using the value of 4.05 × 10⁻¹₀ dalton⁻¹ for the efficiency of strand breakage by 150 rad of γ-rays under aerobic conditions, we estimate the rate of strand breakage by 15-HPETE in the high-dose region above 10 μM at 0.75 ± 0.1 × 10⁻¹₀ dalton⁻¹-μM⁻¹ (15 min treatment at 37°C).

We measured strand breakage and alkali-labile damage by a second, independent method, the alkaline unwinding assay developed by Birnboim (21). Fig. 2B shows dose–response curves for 15-HPETE and γ-rays that were obtained under

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**Fig. 1.** Alkaline elution of DNA from control C3H/10T½ cells and cells that had been treated with 20 μM of isomeric HPETEs for 15 min at 37°C. •, Control; ○, 12-HPETE; ▼, 8-HPETE; ◊, 9-HPETE; ◨, 15-HPETE.

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**Fig. 2.** Dose–response curves for the formation of DNA strand breaks and alkali-labile sites by 15-HPETE and γ-rays under aerobic conditions. Treatment with 15-HPETE was for 15 min at 37°C, while the irradiation with γ-rays was at 0°C. The data are expressed in Qₙ units as described in ref. 18. ◇, 15-HPETE; ×, γ-rays under aerobic conditions. (A) Alkaline elution assay (16, 17). (B) Alkaline unwinding assay (21).
was also the presence of certain ionophores, which induced chelation of Ca^2+ produced DNA breaks. Therefore, we studied the mechanisms involved in this process. Our laboratory showed that HPETEs efficiently mobilize Ca^2+ from mitochondria (Richter, C., Frei, B., Graf, M., Winterhalter, K. and P.A.C., unpublished data). An increase in cytosolic Ca^2+ may trigger a cascade of reactions including the activation of certain proteases and nucleases. Therefore, we predicted the potential of HPETEs to induce DNA breakage by 15-HPETE in intact cells.

Data from our experiments show that HPETEs efficiently induce DNA breakage in intact cells. As shown in Fig. 3, HPETEs were most effective when combined with EGTA and quin 2, most efficiently when combined, suppressed 15-HPETE-induced DNA breakage by 37%. No suppression was observed with the Ca^2+ ionophores ionomycin and A23187. The combination of Fe and Ca^2+ chelators maximally suppressed 15-HPETE-induced DNA breakage (Table 2). Because the inhibitory effect of Fe and Ca^2+ chelators was additive, it is concluded that the clastogenic action of HPETEs occurs by two independent mechanisms: one mediated by Fe, the other by Ca^2+ (Mg^2+). Of the antioxidant enzymes glutathione (GSH) peroxidase in the presence of GSH almost completely suppressed strand breakage by 15-HPETE while bovine Cu/Zn superoxide dismutase and catalase were inactive. The diffusible radical scavenger butylated hydroxytoluene that penetrates the cell was also slightly active in some experiments but inactive in others. The data on the effect of DNA breakage are summarized in Table 2.

**DISCUSSION**

An early effect of the application of PMA on mouse skin is the infiltration of inflammatory cells, i.e., polymorphonuclear cells and monocytes/macrophages (1, 2, 23). Upon stimulation they react with an oxidative burst and the release of a complex mixture of phospholipids, free Δ4Ach, and Δ4Ach metabolites. This mixture of active oxygen and lipids exerts a paracrine and clastogenic effect on neighboring epidermal cells (24, 25). Our aim is to elucidate the mechanism of DNA damage formation by the low molecular weight components that are contained in CF from PMA-stimulated human monocytes.

Our present report concentrates on the clastogenic action of HPETEs. It is demonstrated that HPETEs, regardless of the location of the hydroperoxy substituent, cause extensive DNA strand breakage and alkaline-labile sites induced by 20 μM 15-HPETE in 15 min at 37°C. Extracellular Ca^2+ chelation with 1 mM EGTA; intracellular Ca^2+ chelation was with 25 μM quin 2. Untreated control; quin 2; quin 2 + EGTA; quin 2 + 15-HPETE; quin 2 + EGTA; quin 2 + 15-HPETE/EGTA.

**Table 1.** Comparison of breakage of free DNA and DNA in intact cells by 15-HPETE

<table>
<thead>
<tr>
<th>% DNA eluted</th>
<th>Free DNA</th>
<th>Intact cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.2 ± 4.6 (3)</td>
<td>10.5 ± 3.1 (3)</td>
</tr>
<tr>
<td>Desferrioxamine (1 μM)</td>
<td>12.1 ± 3.3 (2)</td>
<td>8.5 ± 3.6 (2)</td>
</tr>
<tr>
<td>15-HPETE (20 μM)</td>
<td>14.2 ± 5.1 (3)</td>
<td>78.3 ± 7.2 (3)</td>
</tr>
<tr>
<td>15-HPETE (20 μM)/desferrioxamine (1 mM)</td>
<td>10.8 ± 4.1 (3)</td>
<td>47.0 ± 6.2 (3)</td>
</tr>
</tbody>
</table>

The cells were lysed on polyvinylchloride filters, and the lysates washed according to the standard procedures of the alkaline elution assay; the high molecular weight filter-adsorbed DNA was treated for 15 min in PBS pH 7.4, at 37°C before alkaline elution; means ± SD are listed; the number of experiments is given in parentheses. Treatment of filter-adsorbed intact cells and alkaline elution analysis was under standard conditions.

**Table 2.** Effect of antioxidants and calcium- and iron-chelators on DNA breakage by 15-HPETE in C3H/10T^{1/2} cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% DNA eluted</th>
<th>% inhibition</th>
</tr>
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<tbody>
<tr>
<td>15-HPETE</td>
<td>63.7 ± 6.7 (14)</td>
<td>0</td>
</tr>
<tr>
<td>Antioxidant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15-HPETE/SOD (50 μg/ml)</td>
<td>70.2 ± 2.6 (3)</td>
<td>(−10)</td>
</tr>
<tr>
<td>15-HPETE/CAT (50 μg/ml)</td>
<td>72.6 ± 2.6 (3)</td>
<td>(−14)</td>
</tr>
<tr>
<td>15-HPETE/EGTA (1 mM)</td>
<td>37.1 ± 5.4 (5)</td>
<td>42</td>
</tr>
<tr>
<td>Ca chelator and ionophores</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15-HPETE/EGTA (1 mM)</td>
<td>49.7 ± 5.9 (3)</td>
<td>22</td>
</tr>
<tr>
<td>15-HPETE/quin 2 (25 μM)</td>
<td>55.4 ± 9.7 (7)</td>
<td>13</td>
</tr>
<tr>
<td>15-HPETE/EGTA (1 mM)/quin 2 (25 μM)</td>
<td>40.1 ± 8.9 (4)</td>
<td>37</td>
</tr>
<tr>
<td>15-HPETE/ionomycin (0.7 μM)</td>
<td>75.7 ± 2.6 (3)</td>
<td>(−19)</td>
</tr>
<tr>
<td>15-HPETE/A23187 (3 μM)</td>
<td>67.1 ± 5.8 (3)</td>
<td>(−5)</td>
</tr>
<tr>
<td>Ca and Fe chelators</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15-HPETE/EGTA (1 mM)/quin 2 (25 μM)/desferrioxamine (1 mM)</td>
<td>18.3 ± 7.2 (3)</td>
<td>71</td>
</tr>
</tbody>
</table>

Fraction of DNA eluted in 7.5 hr; following treatment with 20 μM 15-HPETE for 15 min in PBS, pH 7.4, at 37°C; mean ± SEM are listed; the number of independent experiments is given in parentheses. Inhibition of DNA elution is relative to that of 15-HPETE alone.

Fig. 3. Effect of chelation of Ca^2+ on DNA strand breakage and alkaline-labile sites induced by 20 μM 15-HPETE in 15 min at 37°C. Extracellular Ca^2+ chelation was with 1 mM EGTA; intracellular Ca^2+ chelation was with 25 μM quin 2. Untreated control; quin 2; quin 2 + EGTA; quin 2 + 15-HPETE; quin 2 + 15-HPETE/EGTA; quin 2 + 15-HPETE/quin 2/EGTA.
Breakage is induced by two independent mechanisms of approximately equal importance: an Fe- and a Ca\(^{2+}\) (Mg\(^{2+}\)) dependent reaction. Dependence on Fe is demonstrated by the inhibitory action of the chelator desferrioxamine. In contrast, the lipophilic iron chelator o-phenanthroline, which penetrates the cell, was inactive or even caused some strand breakage. This result was unexpected because o-phenanthroline completely protected mammalian cells from superoxide produced extracellularly by xanthine oxidase and acetaldehyde (26, 27). Our results are reminiscent of those of Birnboim and Kanabus-Kaminska (22) who found that o-phenanthroline caused an increase in DNA breakage in PMA-stimulated neutrophils. It appears that simple Fenton reactions in the proximity of the nuclear DNA are not responsible for HPETE-induced DNA breakage. Rather alkoyx radicals formed in Fe\(^{2+}\)-catalyzed reactions may be intermediates in desferrioxamine-inhibitable strand breakage. HPETE-derived alkoyx radicals are lipophilic and expected to penetrate readily the plasma membrane. They possess a half-life in the neighborhood of 10\(^{-3}\) sec (28).

Alternatively, desferrioxamine-inhibitable DNA breakage by HPETE might be due to cooxygenation reactions. The ΔAch-hydroperoxide prostaglandin G\(_2\) and the allylic 15′-HPETE have been shown to serve as peroxidase substrates in the oxidation of benzo[a]pyrene to quinones (29) and in the epoxidation of 7,8-dihydroxy-7,8-dihydrosbenzo[a]pyrene (30-31) by the hemoprotein containing prostaglandin H synthetase. Hemoprotein, alone, in the absence of protein, catalyzed the epoxidation by fatty acid hydroperoxides (32). In these reactions peroxy radicals can serve as the ultimate oxygen species. This reaction entails a consecutive increase in cytosolic and nuclear Ca\(^{2+}\) and/or the inactivation of a Ca\(^{2+}\)-ATPase then results in the release of mitochondrial Ca\(^{2+}\). A consecutive increase in cytosolic and nuclear Ca\(^{2+}\) may result in the activation of certain lysosomal and nuclear enzymes (34). DNA breakage determinations by alkaline elution and alkaline unwinding do not yield information about the chemical structure of the ends of the broken strands or the DNA damage that may lead to breaks under the alkaline conditions of these assays. It appears likely that the Fe- and Ca\(^{2+}\)-dependent mechanisms induce structurally different forms of damage.

Our results show that HPETEs that are contained in PMA-induced CF from human monocytes contribute to its clastogenic activity. Besides HPETEs, PMA also stimulates the formation of prostaglandins, and it is expected that the hydroperoxide precursors prostaglandin G\(_2\) and 15′-hydroperoxy-prostaglandin E\(_2\) induce DNA damage by analogous mechanisms. In addition to these hydroperoxy-ΔAch derivatives CF contains other components that are potentially clastogenic. We have proposed that CF consists of a complex mixture of H\(_2\)O\(_2\), free ΔAch, ΔAch metabolites, and other lipids that possess the capacity to start radical chain reactions and to disturb Ca\(^{2+}\) homeostasis in the target cell (7, 24). Organic hydroperoxides with promotional activity (35, 36) may act in a similar fashion.

The role of CF in carcinogenesis remains unclear. CF may function as an intercellular signal that acts over considerable distances. CF-induced DNA breaks might stimulate the transcription of neighboring sequences, facilitate DNA rearrangements, or affect chromatin structure because they stimulate poly(ADP-ribosylation) of chromosomal proteins. CF has the potential to induce gross chromosomal aberrations that may be manifested in late stages of tumor promotion and progression. Finally, the toxicity of CF may play a role in the selection and clonal expansion of initiated cells (7, 24).

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