Interaction of electrophilic lipid oxidation products with mitochondria in endothelial cells and formation of reactive oxygen species

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LIPID PEROXIDATION can generate a broad range of reactive compounds that modify cell function through the activation of signaling pathways. This has been demonstrated most clearly with specific lipid oxidation products, particularly those containing an electrophilic carbon center (13, 14, 17, 18, 23, 31). The mechanisms entail the direct modification of nucleophilic protein residues by electrophilic lipids and/or the secondary formation of reactive oxygen species (ROS) in the cell (11, 19, 23, 29, 31, 38). Of particular interest are compounds formed by lipid peroxidation, such as 4-hydroxyxenononal (HNE), which possess both an aldehydic function and an electrophilic carbon. In contrast, the cyclopentenones, which can be generated through both specific enzymatic pathways and nonspecific lipid peroxidation, possess only an electrophilic carbon. Various lines of evidence support the concept that both these classes of compounds are formed during the inflammatory process (27, 30, 32, 37).

With the use of biotin tagging techniques, a number of protein targets for the electrophilic cyclopentenones, such as 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), have been identified that participate in redox cell signaling (23, 33, 38, 39). This electrophile-responsive proteome includes important signaling molecules such as ras, thioredoxin, and the regulator of key antioxidant defenses in the cell, Kelch-like erythroid cell-derived protein 1 (Keap-1) (5, 12). For example, modification of thiols in Keap-1 by reactive electrophiles leads to release of the transcription factor NF-E2-related factor 2 (Nrf2) and subsequent increase in transcription of genes under the control of the electrophile response element (23). The fact that electrophilic lipids target a number of redox-sensitive proteins suggests that these lipids induce a coordinated cellular response through several mechanisms (17). Furthermore, the overall effect of lipid electrophiles is also determined by their concentration. At low concentrations, the cyclopentenones and similar oxidized lipids present in oxidized low-density lipoprotein (oxLDL) induce the synthesis of cytoprotective antioxidant enzymes, including those that control glutathione (GSH) synthesis and heme oxygenase-1 (HO-1) (1, 13, 28). At high concentrations, lipid electrophiles can induce apoptosis through a number of mechanisms, including both the extrinsic and intrinsic pathways (10, 19, 22, 36).

Many of the biological responses induced by oxLDL are also found with purified individual lipid electrophiles (16, 23). This is of particular interest in understanding the response of vascular endothelial cells to exposure to oxidized lipids during the atherosclerotic process. In a recent study, we demonstrated that the generation of ROS and reactive nitrogen species (RNS) from the endothelial cell on exposure to oxLDL has a substantial mitochondrial contribution (42). This finding leads to the hypothesis that electrophilic lipid oxidation products may have a direct interaction with the mitochondrion. A role for the mitochondrion as a target for reactive lipid electrophiles has not been examined in a cellular context. This is important because it is now becoming evident that the organelle can transduce a number of oxidative and nitrosative signals and

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Cyclopentenone Electrophilic Lipids and Mitochondria

The generation of mitochondrial ROS and the mitochondrial response to electrophilic lipids.

In this study, we selected for investigation the cyclopentenone electrophilic lipids 15d-PGJ2 and 15-J2-isoprostane (15-J2-IsoPs) and the structurally distinct electrophilic aldehyde HNE (Fig. 1). These compounds then represent products derived from the enzymatic reactions of cyclooxygenase (15d-PGJ2) and two examples of products from nonspecific lipid peroxidation, HNE and a member of the isoprostane family (15-J2-IsoPs). Evidence for the formation of these lipid oxidation products in vivo has been presented in a number of studies, although the free concentrations are not known due to rapid metabolism and propensity to form protein adducts.

Materials and Methods

Materials. MitoTracker Deep Red 633 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3-propionyl ethylenediamine, hydrochloride (BODIPY FL EDA), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), 5-(and 6) carboxy-2′,7′-dichlorofluorescein diacetate (carboxy DCF-DA), and anti-BD antibody were purchased from Molecular Probes (Eugene, OR). Glucose oxidase was obtained from Sigma (St. Louis, MO). All other reagents used were of analytical grade.

Cell culture and preparation of pseudo rho0 cells in BAEC. BAEC were prepared as described previously and used between passages. BAEC or rho0 cells were grown in four-well chambers (Nalge, Naperville, IL) until confluent, followed by incubation in media containing 0.5% serum for 24 h. Cells were then treated with electrophilic lipids for 16 h at the concentrations indicated, lysed, and resolved on SDS/PAGE, followed by Western blotting using anti-HO-1 antibody (StressGen). Total glutathione (GSH + GSSG) was determined by using the same lysate (28).

Measurement of HO-1 and GSH. BAEC or rho0 cells were grown on six-well plates until confluent, followed by incubation in media containing 0.5% serum for 24 h. Cells were then treated with electrophilic lipids for 16 h at the concentrations indicated, lysed, and resolved on SDS/PAGE, followed by Western blotting using anti-HO-1 antibody (StressGen). Total glutathione (GSH + GSSG) was determined by using the same lysate (28).

Cytochemistry and detection of BD-15d-PGJ2 protein adducts. BAEC or rho0 cells were grown in four-well chambers (Nalge, Naperville, IL) until confluent, followed by incubation in media (0.5% serum) for 16 h. Cells were then treated with electrophilic lipids for 30 min with MitoTracker Deep Red 633 (0.5 μM) added 15 min before being imaged. The subcellular localization of BD and BD-tagged lipids was assessed by four bidirectional scans of live cells with the use of a Leica DMIRBE inverted epifluorescence/Nomarski microscope outfitted with Leica TCS NT laser confocal optics (filters absorption and emission: BD FL 505/513 nm; MitoTracker 644/665 nm). Images were merged, and colocalization efficiency of BD, BD-lipids, and MitoTracker was estimated by using SimplePCI software (Compix, Inc., Pittsburgh, PA).

Cranberry Township, PA). Digital processing of the images was also undertaken by using Adobe Photoshop (Adobe System). For the competition experiment (unlabeled 15d-PGJ2 with BD-15d-PGJ2), treated cells were washed twice with PBS and fixed with paraformaldehyde (2%) for 15 min and images acquired as described above.

For detection of BD-15d-PGJ2 protein adducts, BAEC were incubated as described in Cell culture and preparation of pseudo rho0 cells in BAEC and then washed twice (PBS) and lysed (50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% (vol/vol) Triton X-100, 1 mM EDTA, 1 mM Na3VO4, 50 mM NaF, 0.5 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, and 2 μg/ml aprotinin). After separation by SDS-PAGE, samples were transferred to nitrocellulose and protein adducts detected with an anti-BD antibody.

Detection of dichlorodihydrofluorescein fluorescence. Cells were grown in four-well chambered coverslips as described in Cytokinesis and detection of BD-15d-PGJ2 protein adducts and then treated with electrophiles for 4 h. Cells were loaded with DCFH-DA (5 μM) for 60 min and incubated with MitoTracker Deep Red 633 (0.5 μM) for 15 min before being imaged. Furthermore, cells were washed twice with culture medium and images acquired by using epifluorescence or confocal microscopy. In some studies, a structural analog of DCF, carboxy DCF-DA (5 μM), was added to cells after preincubation with MitoTracker for 10 min, followed by image acquisition at 0, 2.5, and 15 min.

**RESULTS**

Electrophilic lipids induce cytoprotective pathways in BAEC. It has been shown that electrophilic lipids can induce cytoprotective pathways by binding to Keap-1, leading to the translocation of Nrf2 to the nucleus and activation of the electrophile response element (23, 26). This results in the upregulation of a number of cytoprotective genes, including glutathione synthetic enzymes and HO-1 (21). In the first series of experiments, we examined three distinct electrophilic lipids, HNE, 15-J2-IsoP, and 15d-PGJ2, for their ability to increase GSH and HO-1 levels (Fig. 2). The cyclopentenone lipids 15-J2-IsoP and 15d-PGJ2 were most potent, with a significant induction in both GSH and HO-1 levels achieved with ~1–2.5 μM, whereas 10 μM were required for HNE to exert similar effects. We have previously shown that lipids lacking an electrophile carbon center, such as PGE2, do not induce GSH (23). The fact that HNE exerts similar effects on GSH induction as the electrophilic cyclopentenone lipids, whereas the nonelectrophilic lipid (PGE2) does not, is consistent with a requirement for an electrophilic carbon for these biological responses.

Electrophilic lipids stimulate ROS production in BAEC. To test for the formation of ROS in BAEC, cells were treated with 15d-PGJ2, HNE, or 15-J2-IsoP at concentrations selected from the previous experiments which caused a similar degree of GSH induction. Treatment with 15d-PGJ2, HNE, and 15-J2-IsoP increased DCF fluorescence in BAEC, consistent with increased ROS formation (Fig. 3). HNE increased DCF fluorescence but to a lesser extent than the cyclopentenone lipids 15d-PGJ2 or 15-J2-IsoP. Treatment with the nonelectrophilic lipid PGE2 (10 μM) did not increase DCF fluorescence, consistent with the hypothesis that this effect is specific for lipids containing electrophilic carbon centers.

**Lipid electrophiles induce formation of ROS in mitochondria.** To test for a mitochondrial contribution to ROS generation induced by oxidized lipids, we used DCF fluorescence in conjunction with a mitochondrion-specific marker and confocal microscopy. BAEC were exposed to 15d-PGJ2 (4 μM), HNE (5 μM), or 15-J2-IsoP (2.5 μM) for 4 h. Cells were loaded with DCFH-DA 60 min before being imaged. To assess colocalization with mitochondria, cells were also treated with MitoTracker. As shown in the left column in Fig. 4, all electrophiles caused an increase in the level of DCF fluorescence when compared with control. Merging of the DCF signal with the MitoTracker image indicates that DCF fluorescence colocalizes with the mitochondria (yellow in Fig. 4, right column). However, less intense DCF fluorescence was also present in nonmitochondrial compartments.

The association of DCF fluorescence with mitochondria could arise from a number of indirect mechanisms. One possibility is that the electrophile-dependent formation of DCF occurs at some other location in the cell and is then accumulated by the mitochondria. To test for this possibility, cells were exposed to 15d-PGJ2 (4 μM) for 4 h and then loaded with...
carboxy DCF-DA (5 μM). As carboxy DCF-DA enters the
cell, fluorescence increases with time over 2–15 min. At 15
min, a substantial amount of carboxy DCF has diffused from
the cell, but at neither time point is a pattern resembling the
MitoTracker staining observed (Fig. 5A). This is in marked
contrast to the pattern of DCF fluorescence evident after
exposure to electrophiles (Fig. 4). The yellow color in the
merge of the red and green fluorescence images in Fig. 5 is
solely due to the nonspecific overlap of each stain. As an
additional control, an extracellular source of hydrogen perox-
ide derived from the enzyme glucose oxidase was used and
DCF colocalization with mitochondria assessed. As shown in
Fig. 5B, glucose oxidase induces DCF fluorescence, but this
shows no mitochondrial association at 15 min. We have pre-
viously demonstrated that the DCF fluorescence induced by the
mitochondria-specific generator of ROS, myxothiazol, is
clearly mitochondrialy associated (42).

**Fig. 3. Effects of electrophilic lipids on reactive oxygen species (ROS) formation in BAEC.** Confluent BAEC were treated with 15d-PGJ₂ (4 μM), HNE (5 μM), 15-J₂-IsoP (2.5 μM), and PGE₂ (10 μM) for 4 h. Live cells were loaded with 2',7'-dichlorodihy-
drofluorescein diacetate (DCFH-DA, 2 μM) 60 min before being imaged. A: cells were then washed and imaged on an inverted fluores-
cence microscope. CTL, control. B: average of dichlorofluorescein (DCF) fluorescence intensity of cells treated as indicated in A. Data are
means ± SE; n = 3, *P < 0.01 compared with CTL.
PGE₂ (10 μM) and MitoTracker (0.5 μM). The confocal microscopy revealed a relatively diffuse pattern for the BODIPY dye alone interspersed with a punctate pattern that was not significantly associated with MitoTracker. In contrast, BD-15d-PGJ₂ showed a discreet filamentous pattern of staining that predominantly colocalized with the mitochondrial reticular structure characteristic of these cells (Fig. 6A). A similar mitochondrial localization was observed with concentrations of BD-15d-PGJ₂ as low as 1 μM (data not shown). The structurally similar lipid BD-PGE₂, which lacks electrophilic carbons, exhibited a diffuse fluorescence and did not show accumulation in the organelle.

Formation of stable electrophilic lipid-protein adducts in endothelial cells. If the reactive electrophilic carbon center in the lipid is participating in the localization to the mitochondrion, then formation of stable adducts with proteins is predicted (23). To test for this, cells were treated with BD and BD-tagged lipids (10 μM) for 30 min, and a cell lysate was subjected to SDS-PAGE followed by Western blotting analysis with an anti-BD antibody. As seen in Fig. 6, B and C, significant labeling of proteins was only found after treatment with BD-15d-PGJ₂. The low level of labeling observed with BD-PGE₂ may be due to some conversion of BD-PGE₂ to small amounts of the electrophilic lipid BD-PGA₂ by nonenzymatic dehydration within the cells. If specific protein targets for electrophilic lipids are present in the mitochondrion, it suggests that preincubation of the cells with untagged lipid would block these sites and prevent localization to the organelle. To test for this possibility, cells were preincubated with 15d-PGJ₂ (20 μM, 2 h) followed by exposure to BD-15d-PGJ₂ (2.5 μM, 30 min). As can be seen in Fig. 7, pretreatment with 15d-PGJ₂ resulted...
in a more disperse intracellular pattern of BD-15d-PGJ2 and less intense yellow color when the green BD-15d-PGJ2 and the red MitoTracker images are overlaid, indicating less mitochondrial localization of the BD-15d-PGJ2.

Effects of respiratory chain on mitochondrial localization of electrophilic lipids. In the next series of experiments, we prepared BAEC that have been depleted of a functional mitochondrial respiratory chain by treatment with ethidium bro-
mide (pseudo rho0 cells). These cells are characterized by having decreased mitochondrial ΔΨ and low levels of mitochondrially encoded respiratory chain subunits as compared with normal BAEC (42). Normal BAEC and pseudo rho0 cells were exposed to BD-15d-PGJ2, and colocalization to the mitochondria was assessed. The tagged electrophilic lipid colocalized to the mitochondria in normal BAEC, as observed in previous experiments (Fig. 8A). However, in pseudo rho0 cells,
Fig. 6. Subcellular localization of BD-tagged 15d-PGJ2 and PGE2. BAEC were treated with BD (10 μM), BD-15d-PGJ2 (10 μM), or BD-PGE2 (10 μM) for 30 min. Cells were also incubated with MitoTracker (0.5 μM) for 15 min before being imaged. A, top to bottom shows confocal images of live cells treated with BD, BD-15d-PGJ2, or BD-PGE2: BD (green), MitoTracker (red), and merged BD and MitoTracker images. B, cells treated as described in A were lysed and the proteins were analyzed by Western blotting with anti-BD antibody. C, quantitative analysis where results are expressed as percentage of BD-15d-PGJ2 labeling and represent means ± SE. *P < 0.01 indicates statistically significant differences compared with CTL BAEC.
colocalization was substantially decreased (Fig. 8B), suggesting a requirement for a functional respiratory chain for electrophilic lipids to target the organelle. In addition, measurement of total cell DCF fluorescence was substantially decreased in the pseudo rho0 cells on exposure to 15d-PGJ2 (Fig. 9).

DISCUSSION

Although it has been known for some time that lipid oxidation products can stimulate ROS formation in cells, the sources and mechanisms involved still remain unclear (15, 39, 41, 42). In this respect, the mitochondrion is of particular interest because it is known that the organelle can produce ROS in response to stress (2). Furthermore, it has recently become evident that mitochondria may be a regulated source of ROS and so contribute to redox cell signaling (4). It is therefore important to investigate the signals leading to mitochondrial ROS formation. In this respect, lipid oxidation products are of particular interest because they contain families of molecules that are hydrophobic and are highly reactive with the nucleophilic centers present on proteins, particularly thiols. The mitochondrion is a likely site of reaction of electrophiles because of its extensive membranous network and abundance of proteins containing reactive thiol residues (24). Whether electrophilic lipids can penetrate the cytosolic milieu and localize to mitochondria in intact cells has not been previously addressed. This is potentially important because the literature describing experiments with isolated mitochondria suggests that lipid electrophiles, such as HNE, can modify mitochondrial function (2, 41).

One of the compounds we have used as a model electrophile in this study is the cyclopentenone prostaglandin 15d-PGJ2. Several lines of evidence suggest a mitochondrial interaction with exogenously added 15d-PGJ2. For example, high proapoptotic concentrations of 15d-PGJ2 increase ROS formation in a transformed breast cancer cell line, and this effect can be inhibited by the mitochondrial complex I inhibitor rotenone (34). Similar increases in ROS formation have also been reported in isolated mitochondria treated with 15d-PGJ2, and an electrophile responsive proteome in the mitochondria is composed of proteins with reactive thiols (20, 25). We have also shown that exposure of cells to low, nontoxic concentrations of 15d-PGJ2 induces the activity of complex I in the mitochondrion (6). However, because of the lack of availability of specific tags to monitor the fate of electrophiles in the cell, mitochondrial localization has not been previously investigated.

To test for a mitochondrial contribution to the increased ROS production after exposure to electrophilic lipids, endothelial cells were treated in conjunction with the mitochondrial label, MitoTracker. The concept that the electrophilic carbon center is required for mitochondrial ROS formation is supported by the fact that the electrophilic lipids 15d-PGJ2, 15-J2-IsoP, and HNE showed a strong mitochondrially localized formation of DCF fluorescence (Fig. 4). In contrast, the non-electrophilic lipid (PGE2) did not induce DCF fluorescence (Fig. 3). Carboxy DCF was not accumulated in the mitochondrion after formation, and an extracellular source of hydrogen peroxide did not show a mitochondrial localization at early time points of exposure (Fig. 5). Nevertheless, the interpretation of DCF fluorescence is complicated by the fact that it requires the action of intracellular esterases, peroxidases, as well as extracellular iron (40). It is then possible that an indirect effect of the electrophilic lipids in the cytosol could lead to iron release or GSH depletion in the organelle and the observed mitochondrial association of DCF. Despite these limitations, it can still be used to effectively detect ROS/RNS such as hydrogen peroxide and peroxynitrite in cells (40). One important advantage of this technique is that it offers the possibility of localizing the source of ROS production within the cell.

Fig. 7. 15d-PGJ2 competes with BD-15d-PGJ2 for localization to mitochondrion. BAEC were treated with or without unlabeled 15d-PGJ2 (20 μM) for 2 h, followed by 30 min exposure to BD-15d-PGJ2 (2.5 μM). Cells were washed and fixed, and images were acquired by using confocal microscopy. Top: mitochondrial localized BD-15d-PGJ2 (green), MitoTracker (red), and merged images in CTL cells. Bottom: effects of preincubation with unlabeled 15d-PGJ2 on mitochondrial localization of BD-15d-PGJ2.
To address whether exogenous electrophilic lipids can impact on mitochondrial proteins through a direct association, we designed a novel series of fluorescently tagged electrophilic and nonelectrophilic lipids. These compounds were used in combination with MitoTracker, and the results support the hypothesis that an electrophilic carbon center leads to the association of these reactive lipids with the organelle (Fig. 6). As expected, protein adducts were formed only with the electrophilic lipid (Fig. 6). The fact that mitochondrial localization could be blocked by preincubation with untagged 15d-PGJ2 suggests that there is a saturable component involved in mitochondrial localization (Fig. 7). Interestingly, localization of the electrophilic lipid to the mitochondrion and induction of ROS were dependent on the presence of a functional respiratory chain (Figs. 8 and 9). The reasons for this association are not clear at present but may be related to the fact that the mitochondrion contains a large concentration of proteins with reactive nucleophilic centers in a range of metabolic and regulatory enzymes.

In summary, we have shown that specific electrophilic cyclopentenones and HNE are capable of inducing mitochondrial ROS formation. It appears that the electrophilic lipids can diffuse to the organelle from an exogenous source and accumulate within the mitochondrion in a manner that requires a functional respiratory chain. This may be of biological significance under conditions of chronic inflammation, such as...
atherosclerosis, which are associated with the production of exogenous electrophilic lipid oxidation products due to the oxidation of low-density lipoprotein.

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