Defining Mechanisms of Toxicity for Linoleic Acid Monoepoxides and Diols in Sf-21 Cells

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Linoleic acid monoepoxides have been correlated with many pathological conditions. Studies using insect cells derived from *Spodoptera frugiperda* (Sf-21 cells) have suggested that conversion of the epoxides to the diols is required for toxicity. However, more recent studies using rabbit renal proximal tubules have suggested that linoleic acid monoepoxides are direct mitochondrial toxins. To better understand these discrepancies, we compared the toxicity of these linoleic acid metabolites in Sf-21 cells using mitochondrial respiration as an end point. Linoleic acid (100 μM) and 12,13-epoxy-9-octadecenoic acid (12,13-EOA, 100 μM) increased the rate of oligomycin-insensitive respiration by approximately 3.5- and 3-fold, respectively, decreased the rate of oligomycin-sensitive respiration by approximately 52 and 68%, respectively, and had no effect on the integrity of the electron transport chain. These effects were concentration-dependent, occurred within 1 min, and recovered to basal levels within 45 min. 12,13-Dihydroxy-9-octadecenoic acid (12,13-DHOA, 100 μM) had no effect on oligomycin-insensitive respiration but decreased the rate of oligomycin-sensitive respiration and uncoupled respiration in a concentration-dependent manner. Approximately 79 and 68% of oligomycin-sensitive respiration and uncoupled respiration was inhibited by 12,13-DHOA (100 μM), respectively. These effects occurred within 1 min and were not reversible in 6 h. Effects similar to those induced by 12,13-DHOA (100 μM) were observed using 12,13-EOA (100 μM) in Sf-21 cells expressing human soluble epoxide hydrolase. These data suggest that in this Sf-21 model linoleic acid and linoleic monoepoxides have transient uncoupling effects, whereas the primary mechanism of toxicity for linoleic acid diols in this model is inhibition of the electron transport chain.

Introduction

Linoleic acid is an essential fatty acid which is a major constituent in a Western diet (*1*, *2*). It is a major component of cellular membranes and, like other essential fatty acids, is involved in the oxidation and transport of cholesterol (*3*). Also, it is well established that linoleic acid uncouples oxidative phosphorylation in plant and rodent mitochondria (*4*−*6*). It is likely that uncoupling occurs by activation of uncoupling proteins (*4*, *5*). Uncoupling proteins are thought to be involved in many physiological processes. For example, uncoupling proteins in brown fat may be the major mechanism for heat production in hibernating animals (*7*). However, previous studies have shown that uncoupling by linoleic acid can lead to toxicity if unregulated (*8*).

A variety of human and rodent cytochrome P450 enzymes metabolize linoleic acid to form 9,10-EOA,[1] 12,13-EOA, and/or HODEs (*8*−*12*). Bylund et al. demonstrated that recombinant P450 1A2, 2C8, 2C9, 2C19, and 3A4 metabolize linoleic acid and form mixtures of EOA and HODE metabolites (*9*−*11*). Moran et al. showed also that recombinant P450 2E1 and 2J2 have high epoxygenase activity in the presence of linoleic acid (*8*). Draper and Hammock reported that P450 2C9 is the major human liver epoxygenase for linoleic acid (*12*).

Linoleic acid monoepoxides are thought to have many biological activities. For example, they may regulate arterial blood pressure by inducing vasodilation (*13*), protecting organisms from infectious diseases (*14*, *15*), and inducing multiple-organ failure associated with severe burns, acute trauma, and ARDS (*13*, *16*−*18*). Despite many in vivo and in vitro studies aimed at determining the mechanism of toxicity associated with linoleic acid monoepoxides, there remains controversy over the identity of the toxic intermediates. Ozawa et al. first showed 9,10-EOA to be toxic in rat and suggested that the toxicity is due to the uncoupling of liver mitochondria (*6*). Sakai et al. subsequently suggested that 9,10-EOA is also a rat lung toxin and inhibits mitochondrial respiration without uncoupling (*19*).

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[1] Abbreviations: 9,10-EOA, 9,10-*cis*-epoxyoctadecenoic acid; 12,13-EOA, 12,13-*cis*-epoxyoctadecenoic acid; HODEs, hydroxyoctadecadienoic acids; ARDS, adult respiratory distress syndrome; RPT, renal proximal tubules; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; hsEH, human soluble epoxide hydrolase; msEH, mouse soluble epoxide hydrolase; NP-HPLC, normal phase high-pressure liquid chromatography; MOI, multiplicity of infection; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; t-DPPO, [2-^{3}H]-*trans*-diphenylpropene oxide; BCA, bicinchoninic acid; ANOVA, analysis of variance.

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There are problems associated with comparing previous studies to determine a unifying mechanism. Both the methyl ester (*2*, *20*, *21*) and free acid derivatives (*2*, *8*, *22*, *23*) have been used, and although it is generally thought that the methyl ester moieties are rapidly hydrolyzed by intracellular esterases, Moran et al. showed that this is not always the case (*8*). They showed that methyl linoleate is not toxic to freshly isolated rabbit RPT (*20*), whereas linoleic acid uncouples mitochondria and induces cell death (*8*). Also, comparing studies using different end points (MTT vs mitochondrial respiration) to assay toxicity may lead to different conclusions. MTT is an indirect marker for mitochondrial function and only measures active succinate dehydrogenase activity (*24*). Mitochondrial respiration is a direct marker for mitochondrial function, but may not be a good marker for toxicity in glycolytic systems. Comparison of MTT staining with measurements of the rate of mitochondrial respiration may differ because MTT staining does not necessarily measure uncoupling of oxidative phosphorylation.

Sf-21 cells have been previously used for studying the mechanism of toxicity of linoleic acid monoepoxides. This model was initially chosen because it was used for baculovirus expression of hsEH and msEH, and these linoleic acid monoepoxides are excellent substrates for sEH, which converts linoleic acid monoepoxides to diols. To our knowledge, all of the studies using Sf-21 cells only use MTT staining as a marker of cell viability. On the basis of this end point, these studies suggest that linoleic acid and linoleic acid monoepoxides (methyl esters and free acids) are not toxic, and metabolism of linoleic acid monoepoxides via sEH to linoleic acid diols is an activation step which leads to toxicity (*2*, *21*−*23*). This finding is in disagreement with a recent study using a rabbit RPT model which suggested that linoleic acid, linoleic acid monoepoxides, and linoleic acid diols are all toxic (*8*). To better understand these discrepancies, we studied linoleic acid metabolites in Sf-21 cells using mitochondrial respiration as an end point. Assaying mitochondrial respiration directly is a logical end point for studying the toxicity of these compounds because all previous studies have suggested that this class of toxins specifically target mitochondria.

Experimental Procedures

Chemicals and Reagents. The rabbit anti-rhesus sEH IgG antibody was a gift from B. Hammock (Department of Entomology and Cancer Research Center, University of California, Davis, CA). Mitochondiral inhibitors were purchased from Calbiochem (San Diego, CA), and unless otherwise specified, all other chemicals were either synthesized and purified in our laboratory or purchased from Sigma-Aldrich (St. Louis, MO), Bio-Rad (Hercules, CA), or Fisher Scientific (Pittsburgh, PA). All purchased chemicals were reagent or HPLC grade.

Chemical Synthesis and Purity Analysis of EOA and DHOA. Linoleic acid monoepoxides were synthesized from linoleic acid using the method of Rudolph et al. (*25*) and purified as described by Moran et al. (*8*). In brief, linoleic acid was dissolved in dichloromethane and incubated for 30 min at room temperature in the presence of methyltrioxorhenium (0.5 mol %), pyridine (12 mol %), and hydrogen peroxide (1.5 molar equiv). The reaction was quenched by the addition of a catalytic amount of manganese(IV) oxide and the mixture extracted using dichloromethane. Linoleic acid monoepoxides were silica purified using a mobile phase consisting of dichloromethane (94.9%), ethyl acetate (5%), and acetic acid (0.1%). The silica-purified linoleic acid monoepoxides were either hydrolyzed to corresponding vicinal diols at room temperature for 1.5 h using $HClO_4$ (1.25%) or purified by NP-HPLC with a mobile phase consisting of hexane (98.9%), 2-propanol (1%), and acetic acid (0.1%). The vicinal diols were purified also by NP-HPLC utilizing a mobile phase containing hexane (95.9%), 2-propanol (4%), and acetic acid (0.1%). Retention times for linoleic acid monoepoxides and linoleic acid diols were approximately 7−10 and 10−14 min, respectively. A Dynamax Silica semipreparative column (Varian, Walnut Creek, CA) with a flow rate of 4.7 mL/min was used for all HPLC purifications. HPLC purity analysis of all products was carried out using a 5 μm, silica microsob-MV column (Varian), and the purities were routinely greater than 99%. The flow rate for purity analysis was 1 mL/min. Detection of all products was achieved at 202 nm using an SPD-10A UV/vis detector (Shimadzu Scientific Inc.). All products were stable and stored at −20 °C until they were assayed.

Cell Culture. Sf-21 cells (1×10^6 cells/mL) were regularly grown and infected in 25 mL of complete medium [96% EX-CELL 401 with L-glutamine from JRH Biosciences (Lenexa, KS), 3% heat-inactivated fetal bovine serum from JRH Biosciences (Purchase, NY), and 1% Pen/Strep antibiotics] in a 50 mL round-bottom flask. Aliquots were incubated for 1−180 min with diluent (ethanol), linoleic acid (5−100 μM), 12,13-EOA (5−100 μM), and 12,13-DHOA (5−100 μM) and assayed for mitochondrial function. For some experiments, we used Sf-21 cells, that were infected for 48 h with baculovirus (MOI = 0.1) containing the cDNA for hsEH or a control enzyme (β-galactosidase). This MOI has been shown previously to induce significant expression of hsEH in 48 h (*21*).

Oxygen Consumption. Oxygen consumption in the presence or absence of specific mitochondrial inhibitors was the end point used for assaying mitochondrial function. All oxygen consumption studies used a 2 mL aliquot of treated Sf-21 cells suspended at a density of 1×10^6 cells/mL and were conducted at 27 °C. Oxygen consumption of Sf-21 cells was assessed as previously described using a Clark-type oxygen electrode (*26*). After the basal rate of oxygen consumption was determined, oligomycin (1.2 μM), a specific inhibitor of F_0/F_1-ATPase, was added to the Sf-21 cell suspension to measure the rate of oligomycin-insensitive oxygen consumption. Oligomycin-insensitive oxygen consumption represents the oxygen being consumed that is not coupled to ATP synthesis. Oligomycin-sensitive oxygen consumption was assessed by subtracting the rate of oligomycin-insensitive oxygen consumption from that of basal oxygen consumption. Oligomycin-sensitive oxygen consumption represents the oxygen being consumed that is directly coupled to ATP synthesis. Function of the electron transport chain was determined by measuring the rate of uncoupled respiration. Uncoupled oxygen consumption was assayed by adding FCCP (1 μM) which is known to specifically uncouple oxidative phosphorylation. The concentration used for oligomycin and FCCP had an immediate effect on cellular respiration, suggesting that these inhibitors readily crossed the plasma membrane of Sf-21 cells, and these concentrations have also been shown to fully inhibit F_0/F_1-ATPase and uncouple oxidative phosphorylation, respectively (*26*).

Biochemical Analysis. For immunoblotting, whole cell lysates containing 100 μg of protein were separated using standard electrophoresis techniques in SDS−8% (w/v) polyacrylamide gels, and the resolved proteins were transferred electrophoretically onto a nitrocellulose membrane. The nitrocellulose membrane was immunoblotted using rabbit anti-rhesus sEH antiserum and an ECL Western blotting detection system (NEN Life Science Products, Boston, MA).

The specific sEH activity was determined by the method of Borhan et al. (*27*). In brief, the initial hydrolysis rate of t-DPPO was determined after a 5 min incubation at 37 °C using 1 μg of cellular protein.

The protein content for all experiments was precipitated with trichloroacetic acid (5%) from cells and media and assayed using the BCA method and BCA reagents supplied by Pierce (Rock-

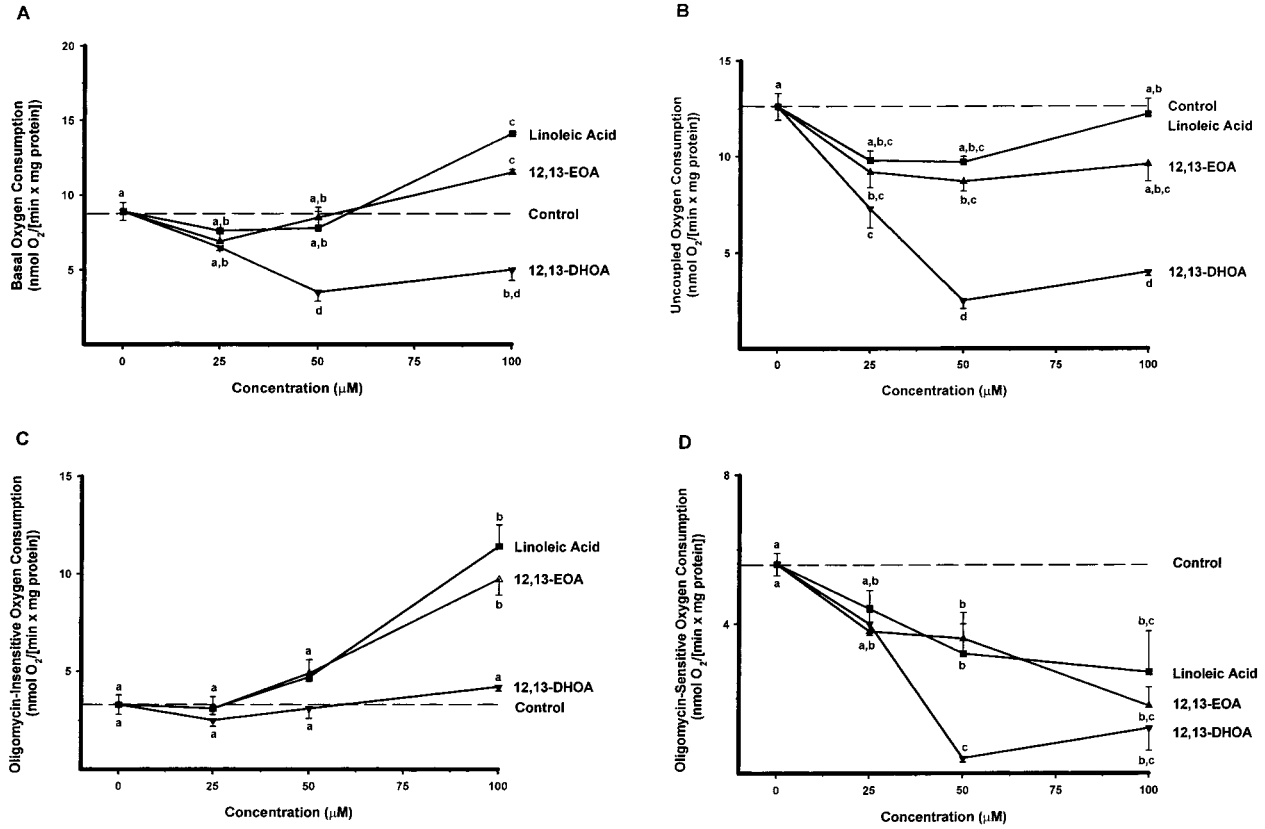

Figure 1. Concentration-dependent effects on basal (A), uncoupled (B), oligomycin-insensitive (C), and oligomycin-sensitive (D) oxygen consumption in Sf-21 cells exposed to diluent (0.05% final concentration), linoleic acid (5−100 μM), 12,13-EOA (5−100 μM), and 12,13-DHOA (5−100 μM) for 1 min. Data are means ± SE ($n = 3-11$). Means with different superscripts are statistically different ($p < 0.05$).

ford, IL). Bovine serum albumin was used as the standard for all protein determinations.

Statistics. Data were analyzed by ANOVA or a paired *t* test ($P < 0.05$) and were represented as means ± standard error. Multiple means were determined for significance ($P < 0.05$) using the Student−Newman−Keuls test.

Results

To determine the toxicity of linoleic acid, 12,13-EOA, and 12,13-DHOA, Sf-21 cells were exposed to different concentrations (5−100 μM) for 1 min and the mitochondrial function was determined (Figure 1A−D). The basal rate of oxygen consumption for control was 8.9 ± 0.6 nmol of O_2 min^{-1} (mg of cellular protein)$^{-1}$ (Figure 1A). Interestingly, the basal rate of respiration increased for unknown reasons after 45 min. This small increase was not statistically different. The basal rate of oxygen consumption increased approximately 25% in the presence of linoleic acid (100 μM) and 12,13-EOA (100 μM). Lower concentrations (25−50 μM) of linoleic acid and 12,13-EOA had no significant effect. 12,13-DHOA (50 μM) significantly decreased the basal rate of oxygen consumption approximately 50%, with no additional decrease at higher concentrations (Figure 1A). 12,13-DHOA (25 μM) had no significant effect on the basal rate of oxygen consumption. The effects on basal oxygen consumption in the presence of linoleic acid (100 μM), 12,13-EOA (100 μM), and 12,13-DHOA (100 μM) were transient and were not statistically different from control after 45 min (Figure 2A). However, 12,13-DHOA (100 μM) numerically decreased the basal rate of respiration for 6 h.

FCCP (1 μM) increased the basal rate of oxygen consumption in Sf-21 cells approximately 30% (Figure 1A,B). The rate of uncoupled respiration in Sf-21 cells was 12.6 ± 0.7 nmol of O_2 min^{-1} (mg of cellular protein)$^{-1}$ (Figure 1B). 12,13-EOA and 12,13-DHOA decreased the rate of uncoupled oxygen consumption in a concentration-dependent manner (Figure 1B). Approximately 25% of the uncoupled oxygen consumption was inhibited in the presence of 12,13-EOA (50 μM), and higher concentrations (100 μM) had no additional effect (Figure 1B). 12,13-DHOA (25 μM) inhibited uncoupled oxygen consumption by approximately 40% at 25 μM, and approximately 75% at 50 μM (Figure 1B). No further decrease was observed at 100 μM (Figure 1B). The 20% inhibition of the uncoupled oxygen consumption induced by linoleic acid (100 μM) and 12,13-EOA (100 μM) was transient and recovered to control levels within 45 min (Figure 2B). However, 12,13-DHOA (100 μM) inhibition of uncoupled respiration did not recover within 6 h (Figure 2B).

Oligomycin is known to specifically inhibit the F_0 portion of the F_0/F_1-ATPase (*26*). Oligomycin-insensitive oxygen consumption represents the oxygen being consumed that is not coupled to ATP production. The rate of oligomycin-insensitive oxygen consumption for control Sf-21 cells is 3.3 ± 0.5 nmol of O_2 min^{-1} (mg of cellular protein)$^{-1}$. The rates of oligomycin-insensitive oxygen consumption increased approximately 3.5- and 3-fold in the presence of linoleic acid (100 μM) and 12,13-EOA (100 μM), respectively (Figure 1C). This increase was transient and fully recovered to basal levels within 45 min (Figure 2C). Lower concentrations (25−50 μM) of linoleic acid and

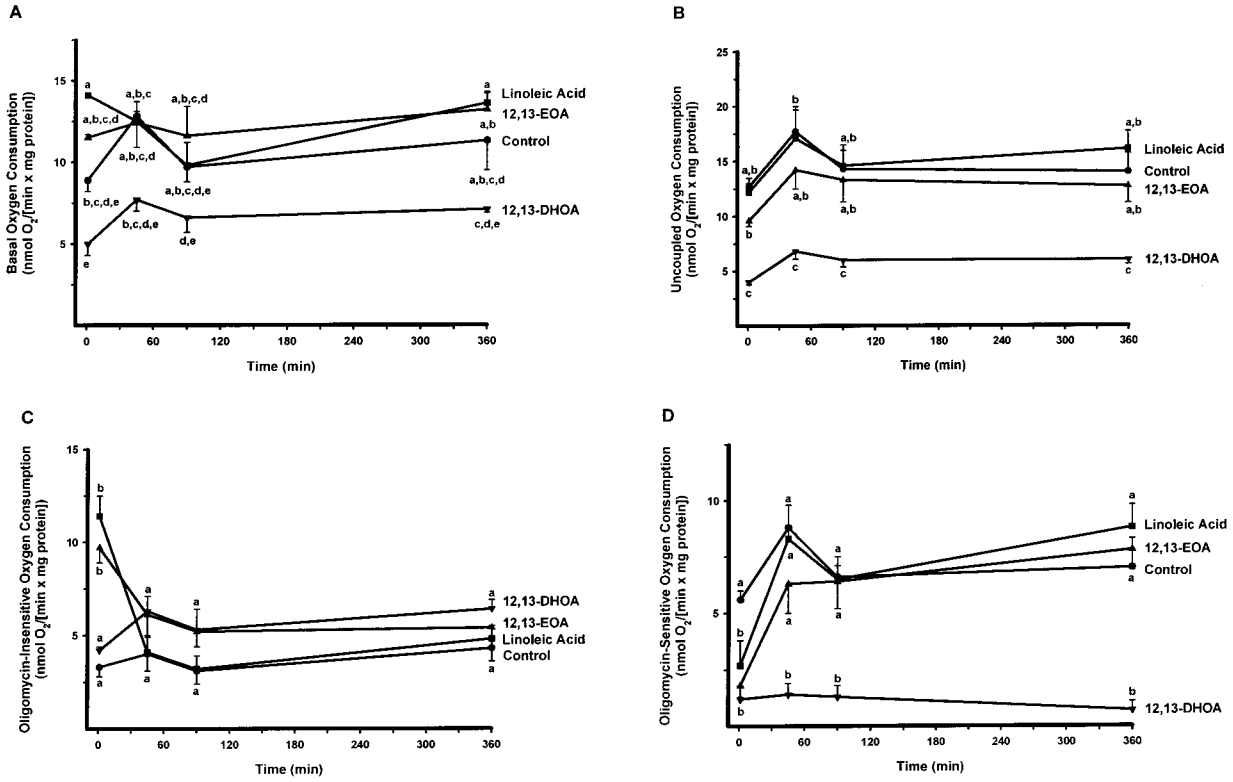

Figure 2. Time-dependent effects on basal (A), uncoupled (B), oligomycin-insensitive (C), and oligomycin-sensitive (D) oxygen consumption in Sf-21 cells exposed to diluent (0.05% final concentration), linoleic acid (100 μM), 12,13-EOA (100 μM), and 12,13-DHOA (100 μM) for 1−360 min. Data are means ± SE (n = 3−11). Means with different superscripts are statistically different (p < 0.05).

12,13-EOA had no effect on oligomycin-insensitive oxygen consumption (Figure 1C). 12,13-DHOA (5−100 μM) also had no effect on oligomycin-insensitive oxygen consumption (Figure 1C).

Oligomycin-sensitive oxygen consumption represents the amount of oxygen used for ATP production. Linoleic acid, 12,13-EOA, and 12,13-DHOA significantly decreased the rate of oligomycin-sensitive oxygen consumption in a concentration-dependent manner (Figure 1D). The rate of oligomycin-sensitive oxygen consumption in control Sf-21 cells was 5.6 ± 0.3 nmol of O_2 min^{-1} (mg of cellular protein)$^{-1}$. Linoleic acid (50 μM) and 12,13-EOA (50 μM) inhibited approximately 33% of the oligomycin-sensitive oxygen consumption (Figure 1D). No further decreases in the rates of oligomycin-sensitive oxygen consumption were observed with linoleic acid (100 μM); whereas, 12,13-EOA (100 μM) and 12,13-DHOA (50−100 μM) decreased the rate of oligomycin-sensitive oxygen consumption by approximately 80% (Figure 1D). 12,13-DHOA (50 μM) also decreased the rate of oligomycin-sensitive oxygen consumption approximately 80%, and no further decreases were observed at 100 μM (Figure 1D). Decreases in the rates of oligomycin-sensitive oxygen consumption in the presence of linoleic acid (100 μM) and 12,13-EOA (100 μM) were transient and returned to basal levels within 45 min (Figure 2D). However, oligomycin-sensitive oxygen consumption in the presence of 12,13-DHOA (100 μM) did not recover within 6 h (Figure 2D).

To compare the relative potencies of the 9,10-EOA with linoleic acid and 12,13-EOA, oligomycin-insensitive respiration was chosen as the end point, and to compare the relative potencies of 9,10-DHOA with 12,13-DHOA, uncoupled respiration was chosen as the end point. These end points were chosen because these markers showed the greatest effect (Figures 1 and 2). Linoleic acid (100 μM), 9,10-EOA (100 μM), and 12,13-EOA (100 μM) increased the rate of oligomycin-insensitive oxygen consumption approximately 2.3-fold, whereas lower concentrations (25−50 μM) had no effect (Figure 3A). 9,10-EOA (50 μM) produced a slight numerical, but not statistical, increase in the rate of oligomycin-insensitive oxygen consumption (Figure 3A). 12,13-DHOA appears to be more potent than 9,10-DHOA at inhibiting uncoupled respiration (Figure 3B). 9,10-DHOA (100 μM) and 12,13-DHOA (25 μM) inhibited approximately 50% of the uncoupled respiration, representing a 4-fold difference in potencies (Figure 3B). 12,13-DHOA (50−100 μM) decreased the rate of uncoupled oxygen consumption approximately 80% in Sf-21 cells (Figure 3B).

To determine whether these mitochondrial effects could be mediated by hsEH, Sf-21 cells were infected with the human sEH (hsEH) or control (expressing β-galatosidase) baculoviruses for 48 h and exposed to diluent, 12,13-EOA (100 μM), or 12,13-DHOA (100 μM) for 45 min. Immunoblotting (Figure 4A) and sEH specific activity measurements (Figure 4B) of whole cell extracts showed expression of active hsEH, as shown previously (*21*). The specific sEH activity was approximately 50-fold higher in Sf-21 cells infected with baculovirus containing hsEH cDNA than in Sf-21 cells infected with the control baculovirus (Figure 4B). 12,13-EOA (100 μM) and 12,13-DHOA (100 μM) were chosen for these studies because they appear to be the most active (Figure 3A,B), and a concentration of 100 μM gives a maximum response within 45 min (Figure 2B). The 45 min time point was chosen to ensure significant metabolism of 12,13-EOA to 12,13-DHOA.

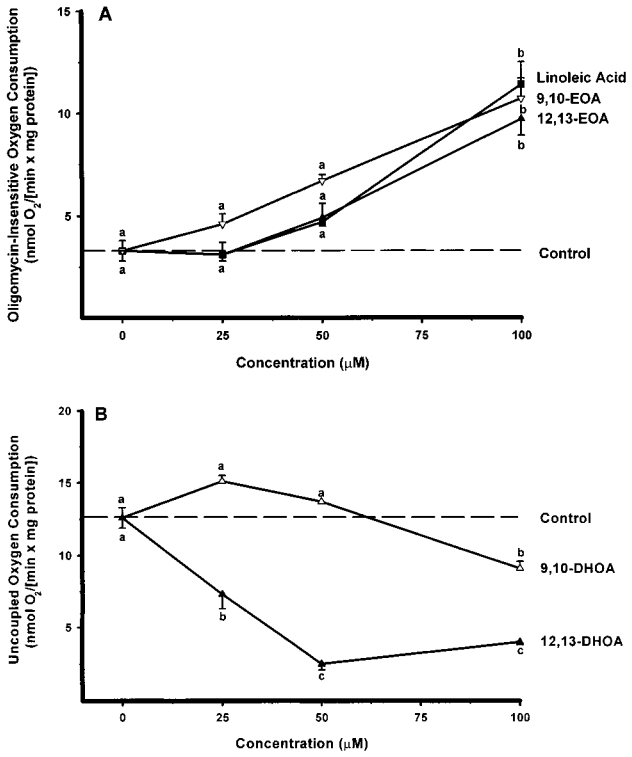

Figure 3. (A) Concentration-dependent effects on oligomycin-insensitive oxygen consumption in Sf-21 cells exposed to diluent (0.05% final concentration), linoleic acid (5−100 μM), 12,13-EOA (5−100 μM), and 9,10-EOA (5−100 μM) for 1 min. (B) Concentration-dependent effects on uncoupled oxygen consumption in Sf-21 cells exposed to diluent (0.05%), 12,13-DHOA (5−100 μM), and 9,10-DHOA (5−100 μM) for 1 min. Data are means ± SE (n = 3−11). Means with different superscripts are statistically different ($p < 0.05$).

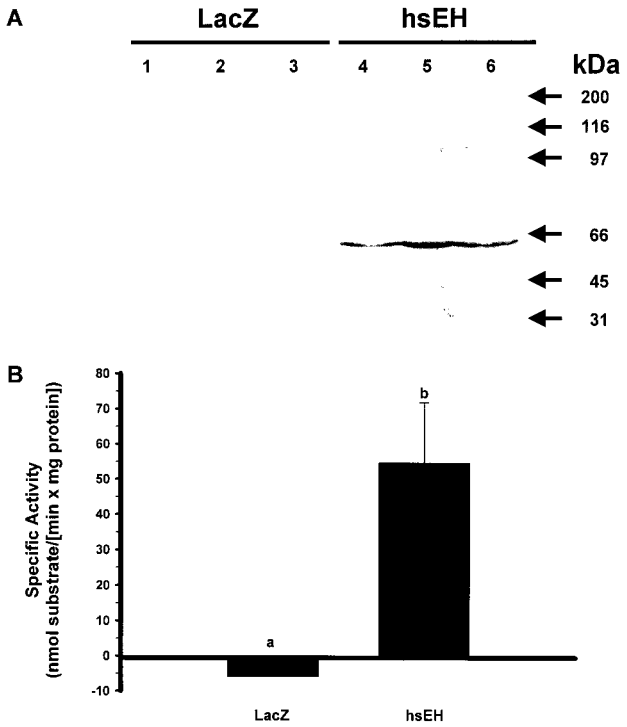

Figure 4. (A) Western blot analysis of Sf-21 cells expressing β-galactosidase (lanes 1−3) or hsEH (lanes 4−6). Approximately 100 μg of whole cell lysates was immunoblotted using a rabbit anti-rhesus sEH IgG antibody. (B) Specific sEH activity in the samples immunoblotted above. Data are means ± SE (n = 3). Means with different superscripts are statistically different ($p < 0.05$).

Infection with hsEH or control baculoviruses did not significantly affect basal (Figure 5A), uncoupled (Figure 5B), and oligomycin-insensitive (Figure 5D) oxygen consumption when compared to uninfected cells. However, baculovirus infection caused numerical decreases in the basal (Figure 5A), uncoupled (Figure 5B), and oligomycin-sensitive (Figure 5D) rates of oxygen consumption.

The basal rate of oxygen consumption in noninfected Sf-21 cells and Sf-21 cells expressing β-galactosidase or hsEH exposed to 12,13-DHOA (100 μM) decreased approximately 40% (Figure 5A). The basal rate of oxygen consumption was also decreased in Sf-21 cells expressing hsEH when exposed to 12,13-EOA (100 μM) (Figure 5A). Uncoupled oxygen consumption in noninfected Sf-21 cells and Sf-21 cells infected with β-galactosidase and hsEH exposed to 12,13-DHOA (100 μM) was inhibited approximately 65% (Figure 5B). Also, 65% inhibition of the uncoupled respiration was observed using 12,13-EOA (100 μM) in Sf-21 cells expressing hsEH (Figure 5B). Oligomycin-insensitive oxygen consumption in noninfected Sf-21 cells or Sf-21 cells expressing β-galactosidase or hsEH exposed to 12,13-DHOA (100 μM) or 12,13-EOA (100 μM) was not significantly affected (Figure 5C). However, oligomycin-sensitive oxygen consumption in noninfected Sf-21 cells or Sf-21 cells expressing β-galactosidase or hsEH exposed to 12,13-DHOA (100 μM) was inhibited approximately 80% (Figure 5D). Sf-21 cells expressing hsEH and exposed to 12,13-EOA (100 μM) for 45 min showed significant decreases in the rates of oligomycin-sensitive oxygen consumption (Figure 5D).

Discussion

Linoleic acid and its metabolites are important biological molecules that may be involved in many physiological and pathological processes (*3*, *7*, *13*, *16*−*18*). Linoleic acid monoepoxides have been correlated with an increased mortality of severely burned patients (*17*) and ARDS patients (*16*). Also, there have been many in vitro and in vivo studies demonstrating the toxicity of linoleic acid monoepoxides and diols (*2*, *8*, *23*−*26*, *31*). Use of methyl ester and free acid derivatives and the use of multiple end points with mammalian, rodent, and insect cell models have made it difficult to determine a unifying mechanism of toxicity. Previous studies using Sf-21 cells suggest that conversion of the epoxide to the corresponding diol is required for toxicity (*2*, *21*−*23*). Rodent and rabbit studies, however, suggest that linoleic acid and linoleic acid monoepoxides are direct mitochondrial toxicants and act by uncoupling mitochondrial respiration (*8*, *19*). The current study suggests also that linoleic acid and linoleic acid monoepoxides have transient uncoupling effects in Sf-21 cells. However, these transient effects do not lead to cellular death in this Sf-21 cell model. This is consistent with previous Sf-21 studies showing that linoleic acid and linoleic acid monoepoxides are not toxic (*2*, *21*−*23*). In contrast, the uncoupling effects of these compounds in rabbit (*8*) and rodent models (*19*) are not reversible and lead to toxicity.

The mechanism of toxicity, as assayed by the inhibition of uncoupled respiration and oligomycin-sensitive oxygen consumption, for linoleic acid diols appears to be inhibition of the electron transport chain which leads to a decrease in the rate of ATP synthesis in Sf-21 cells (Figure 1B). This mechanism is different from the known

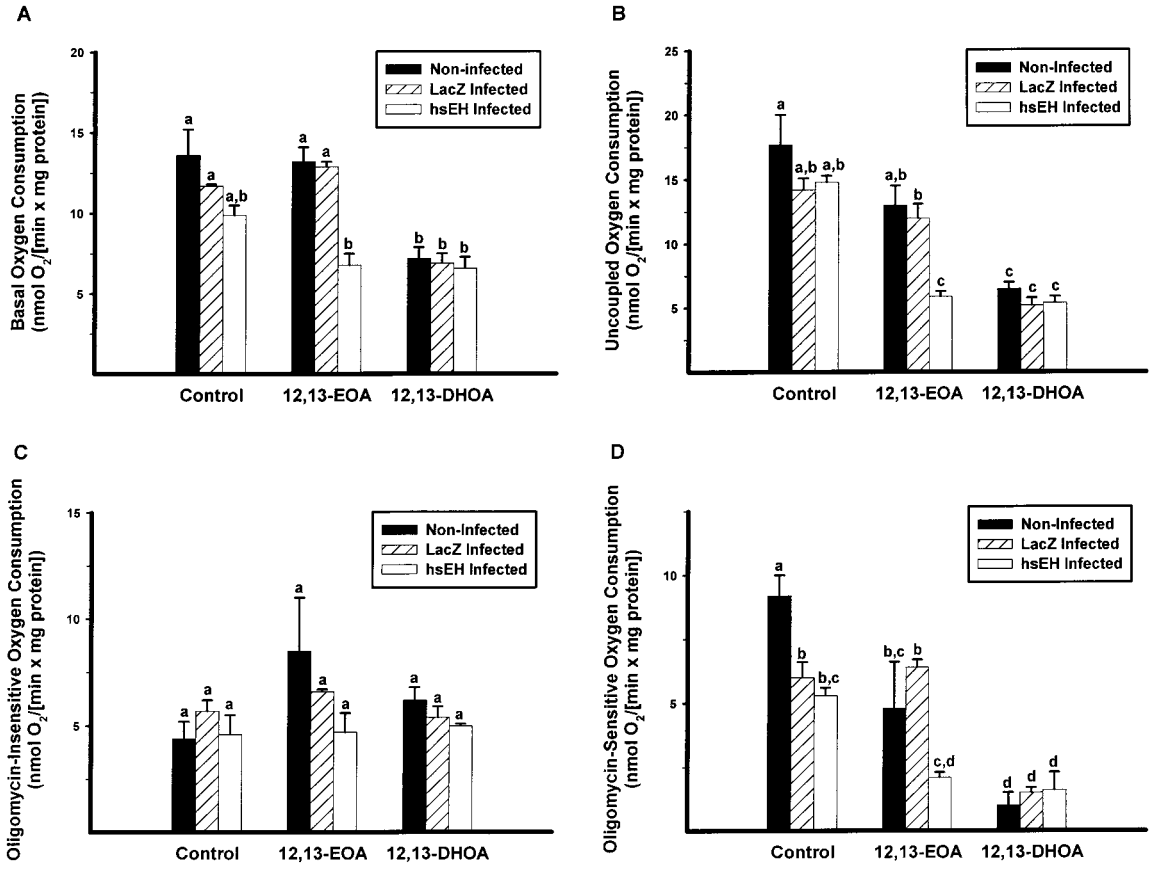

Figure 5. Effect on basal (A), uncoupled (B), oligomycin-insensitive (C), and oligomycin-sensitive (D) oxygen consumption in noninfected Sf-21 cells or Sf-21 cells expressing hsEH or β-galactosidase exposed to diluent (0.05% final concentration), 12,13-EOA (100 μM), or 12,13-DHOA (100 μM) for 45 min. Data are means ± SE ($n = 3$). Means with different superscripts are statistically different ($p < 0.05$).

uncoupling effects of linoleic acid and linoleic acid monoepoxides (*4*, *5*, *8*). It is not known whether linoleic acid diols inhibit the electron transport chain in mammalian or rodent models. However, Sakai et al. suggested that 9,10-EOA inhibits mitochondrial respiration without uncoupling (*19*). In their study, it is possible that the 9,10-EOA was converted in vivo to the vicinal diol which specifically inhibited the electron transport chain, resulting in a decreased rate of mitochondrial respiration.

Examination of relative potencies shows no difference in the uncoupling potential of linoleic acid and linoleic acid monoepoxides. However, 12,13-DHOA is approximately 4-fold more potent than 9,10-DHOA in inhibiting the electron transport chain. The diols have been shown to be substrates for human UDP-glucuronosyltransferases which glucuronidate 9,10- and 12,13-DHOA on either hydroxyl functional group (*29*). These glucuronide conjugates are found in high levels in the urine of patients with peroxisomal diseases (*30*). This is interesting because 9,10-glucuronic acid metabolites are excreted in the urine of healthy humans, whereas in patients with peroxisomal disorders, high levels of 12,13-glucuronic acid metabolites are excreted in urine.[2] These observations suggest that the 12,13-metabolites of linoleic acid might be associated with the toxicity observed in these patients. Interestingly, non-heme-dependent epoxygenases in plants have been shown to specifically produce 12,13-EOA (*31*).

Metabolism of linoleic acid monoepoxides via hsEH may also play a key role in mediating toxicity of these compounds (*2*, *20*, *22*, *23*). Sf-21 cells expressing hsEH are more susceptible to toxicity induced by 12,13-EOA. Analysis of the mechanism of toxicity suggests that the epoxide is being hydrolyzed to the corresponding vicinal diol which irreversibly inhibits the electron transport chain. These results are consistent with what has been reported previously using MTT as a marker for cellular viability (*2*, *21*−*23*).

In summary, linoleic acid and linoleic acid monoepoxides transiently uncouple oxidative phosphorylation in Sf-21 cells, whereas 12,13-DHOA irreversibly inhibits the electron transport chain in Sf-21 cells. These effects can be mediated by hsEH in Sf-21 cells, and metabolism of linoleic acid monoepoxides to linoleic acid diols may be an important metabolic step in preventing uncoupling of oxidative phosphorylation. There appears to be no difference in the uncoupling potential of linoleic acid and linoleic acid monoepoxides, but 12,13-DHOA is 4-fold more potent at inhibiting the electron transport chain than 9,10-DHOA in Sf-21 cells. These results agree with all previous reports using free acids in mammalian (*8*) and rodent models (*19*), except that the uncoupling effects induced by linoleic acid and linoleic acid monoepoxides are not toxic in this model. Therefore, it is also consistent with studies from Greene et al. that reported that linoleic acid and linoleic acid monoepoxides are not toxic at 24 h in Sf-21 cells (*2*, *22*, *23*). The mechanism by which Sf-21 cells are protected from the uncoupling effects of linoleic

[2] Unpublished observations.

acid and linoleic acid monoepoxides is unknown, but the ability of different models to recover from the uncoupling effects of linoleic acid and linoleic acid monoepoxides shows the importance of fully understanding the toxic effects of these linoleic acid metabolites. Future in vivo and in vitro studies determining if linoleic acid diols inhibit the electron transport chain in mammalian models will significantly help our understanding of how these compounds relate to human health.

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