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Activation of Eicosanoid Metabolism in Human Airway Epithelial Cells by Ozonolysis Products of Membrane Fatty Acids

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Includes the Commentary of the Institute's Health Review Committee

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Statement

Synopsis of Research Report Number 71

Biochemical Consequences of Ozone Reacting with Membrane Fatty Acids

BACKGROUND

Ozone, a major component of urban smog, forms in the air in the presence of sunlight as a result of complex photochemical reactions between oxides of nitrogen and volatile organic compounds, both of which are emitted from motor vehicles and other sources. Ozone is an oxidant gas regulated by the U.S. Environmental Protection Agency as a criteria pollutant. When ozone is inhaled at sufficiently high doses, it causes a number of functional and biochemical alterations in humans and animals. At the cellular level, ozone exposure results in injury to the airway epithelial cells; its precise mechanism of action is not well understood, however.

It has been suggested that because ozone is highly reactive, it is unlikely to penetrate beyond the fluid layer that lines the lungs or the epithelial cell membrane of the respiratory system. Its harmful effects are likely to be brought about by secondary products of its reactions with the fluids and the cell membrane. Such products include aldehydes, hydroxyhydroperoxides, and hydrogen peroxide. These products are more stable than ozone; they are also toxic and damage cells. This study, sponsored by the Health Effects Institute, examined the potential of such secondary products to cause biochemical effects in airway epithelial cells.

APPROACH

Dr. Leikauf and colleagues prepared aldehydes and hydroxyhydroperoxides of different carbon chain lengths. They tested these compounds and hydrogen peroxide in cultures of human airway epithelial cells grown from tissue explants. Cells were prelabeled with [^{3}H]–labeled arachidonic acid, which is a major, fatty acid component of the cell membrane attached to a radioactive form of hydrogen. The researchers measured the amount of radioactive metabolites of arachidonic acid released into the culture medium; these metabolites, called eicosanoids, are believed to play an important role in airway reactivity, cellular responses to inhaled pollutants, and asthma.

RESULTS AND IMPLICATIONS

The investigators found that aldehydes and hydroxyhydroperoxides with longer carbon chains produced a larger eicosanoid release from cultured human airway epithelial cells than compounds with shorter carbon chains; this response was dependent on dose and time. The hydroxyhydroperoxides caused much greater eicosanoid release than did the corresponding aldehydes. When eicosanoids released from the treated cells were analyzed, prostaglandin E_2 proved to be the major product of arachidonic acid metabolism; smaller amounts of other eicosanoids (such as prostaglandin $F_{2\alpha}$ and 15-hydroxyeicosatetraenoic acid) were found as well. The investigators also examined whether the hydroxyhydroperoxides' greater stimulatory effect resulted from their decomposition into aldehydes and hydrogen peroxide. They consider this unlikely because the response was related to the tested compound's carbon chain length and because exposure to hydrogen peroxide alone or hydrogen peroxide plus an aldehyde produced a response dissimilar to that produced by a hydroxyhydroperoxide.

The study results are consistent with the hypothesis that ozone's physiological effects are produced via secondary products formed when ozone reacts with cellular molecules, rather than by ozone itself. Elucidating the mechanisms of toxic effects of oxidant gases is important for understanding their implications for health effects in humans and for establishing relevant ambient air quality standards for these pollutants. The findings reported by Dr. Leikauf and colleagues add to our understanding of the mechanism of ozone toxicity and suggest several avenues for further exploration of such toxicity.

This Statement, prepared by the Health Effects Institute and approved by the Board of Directors, is a summary of a research project sponsored by HEI from 1990 to 1993. This study was conducted by Dr. George D. Leikauf of the University of Cincinnati Medical Center, Institute of Environmental Health, Cincinnati, OH. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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ABSTRACT

Inhaled ozone can react with a variety of cellular macromolecules within the lung. Recent analyses of the chemistry of ozone reactions with unsaturated fatty acids, which are present in all membranes and in mucus in the airways, indicate that ozonolysis yields one aldehyde and one hydroxyhydroperoxide molecule for each molecule of ozone. The hydroxyhydroperoxide molecule is unstable in aqueous environments, and subsequently yields a second aldehyde and hydrogen peroxide. The structure of common unsaturated fatty acids is such that attack by ozone at the carbon-carbon double bonds will yield 3-, 6-, and 9-carbon saturated and unsaturated aldehydes and hydroxyhydroperoxide.

This study examines the effects of ozonolysis products on eicosanoid metabolism in human airway epithelial cells. Eicosanoid biosynthesis is important in a wide array of pathophysiological responses in the airway, and the release of eicosanoids by the epithelial barrier is likely to be significant in diseases induced by environmental factors. Previously, we demonstrated that ozone can increase eicosanoid synthesis from airway epithelial cells exposed in vitro. Human exposures to concentrations of ozone below the current National Ambient Air Quality Standard (0.12 ppm, not to be exceeded for more than one hour once per year) also resulted in increased eicosanoids in bronchoalveolar lavage fluid. To determine whether ozonolysis products could activate eicosanoid release, we exposed human airway epithelial cells to 3-, 6-, and 9-carbon aldehydes, hydroxyhydroperoxides, and hydrogen peroxide. We measured (1) eicosanoid metabolism using high-performance liquid chromatography and radioimmunoassays, and (2) the effects of the aldehydes, hydroxyhydroperoxides, and hydrogen peroxide on cell lysis.

Eicosanoid release increased after exposure to aldehyde; release induced by 9-carbon (nonanal) aldehyde was greater than that induced by the 6-carbon (hexanal) or 3-carbon (propanal) aldehydes. Hydroxyhydroperoxides induced greater eicosanoid release than the corresponding aldehydes of equivalent chain length. Again, the longer the aliphatic chain length of the hydroxyhydroperoxide the greater the effect. These effects were noted at concentrations of hydroxyhydroperoxide below those that produce cell lysis, and the time course of the two responses was dissimilar.

Because hydroxyhydroperoxides can degrade into an aldehyde and hydrogen peroxide, it is conceivable that the effects observed were attributable to the formation of either hydrogen peroxide or hydrogen peroxide and aldehyde. This mechanism is unlikely, however, because the effects of hydroxyhydroperoxides on eicosanoid release were dependent on chain length, whereas each hydroxyhydroperoxide can produce only one hydrogen peroxide molecule. Although hydrogen peroxide alone also stimulated eicosanoid metabolism, this effect was not augmented when aldehyde and hydrogen peroxide were added together. In addition, the dose of hydroxyhydroperoxide needed to produce an effect (10 to 100 μM) was lower than that of hydrogen peroxide (300 μM).

We could not fully evaluate the effects of the unsaturated aldehydes and hydroxyhydroperoxides. Although the 6-carbon and 9-carbon *cis*-3-aldehydes could be synthesized from the *cis*-3-alcohols, the resulting aldehydes were not chemically stable. The *cis*-3-aldehydes were useful for producing the corresponding 1-hydroxy-alkenyl-hydroperoxides of high purity. These results support the method selected for chemical synthesis, but further studies are required to establish proper storage and handling methods before these compounds can be tested in assays of eicosanoid metabolism.

This Investigators' Report is one part of Health Effects Institute Research Report Number 71, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. George Leikauf, University of Cincinnati Medical Center, Institute of Environmental Health, Pulmonary Cell Biology Laboratory, 5251 Medical Sciences Building, 231 Bethesda Avenue, ML 182, Cincinnati, OH 45267-0056.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Agreement 816285 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement should be inferred. The contents of this document also have not been reviewed by private party institutions including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

In summary, products of ozonolysis exhibit biological activity in an important cellular target of ozone inhalation, the airway epithelial cells. Because secondary products (aldehydes, hydroxyhydroperoxides, and hydrogen peroxide) can have longer half-lives than ozone in biological systems, they may penetrate to areas not affected by ozone directly. These studies support a role for ozonolysis product formation as a chemical pathway by which ozone activates and damages constitutive cells of the lung.

INTRODUCTION

OZONE TOXICITY

Ozone is a common air pollutant produced in photochemical smog by reactions of nitrogen oxides, hydrocarbons, and oxygen in the presence of sunlight. Ozone concentrations at ground level can produce adverse health effects, primarily in pulmonary function. Previous clinical studies (Seltzer et al. 1986; Delvin et al. 1991) demonstrated that more than six hours of exposure to concentrations as low as 0.08 parts per million (ppm)* can increase pulmonary inflammation (as measured by leukocyte and elastase levels) and augment mediator release (as measured by prostaglandin levels) into bronchoalveolar lavage fluid.

Previously, we investigated the effects of two hours of exposure to ozone on eicosanoid metabolism in bovine airway epithelial cells (Leikauf et al. 1988). Eicosanoid release increased at concentrations as low as 0.1 ppm ozone. Eicosanoid biosynthesis is important in a wide array of pathophysiological responses in the airway, and the release of eicosanoids by the epithelial barrier is likely to be significant in diseases induced by environmental factors. Within the epithelium, endogenous eicosanoids of the prostaglandin family have a clear regulatory role in a number of functions including mucous secretion and ion transport (chloride secretion) across the airways. In addition, specific stimuli can produce a preferential release of prostaglandins toward the basolateral epithelial cell surface (almost 10-fold that directed to the apical cell surface). Because eicosanoids modulate a wide range of other pathophysiological processes in other cell types (for example, vascular and airway smooth-muscle contraction), eicosanoid release augmented by oxidant epithelial injury could serve to transduce environmental stimuli. Hence, the release of eicosanoids from airway epithelial cells is likely to cause several autocrine (within the epithelium) and paracrine (through cell-to-cell interactions) effects in the lung. A clearer understanding of how environmental stimuli can activate or inactivate specific cellular functions is worthy of additional study.

MECHANISMS OF OZONOLYSIS OF FATTY ACIDS

In biological systems, ozone, a strong oxidant, reacts with molecules that are susceptible to electrophilic attack. In the lung, these include the carbon-carbon double bonds of unsaturated fatty acids in phospholipid molecules contained in the airway lining fluid (mucus and surfactant) and in the apical membrane of the epithelial cell. Our understanding of the pathways of ozone's reactions with olefins is based largely on the work of Criegee (1957, 1959, 1975). In those studies, ozonolysis reactions were initiated in a nonreactive organic solvent, and the major products formed were ozonides. However, similar reactions in a protic, nucleophilic solvent such as water or alcohol yielded not ozonides, but hydroxyhydroperoxides and alkoxyhydroperoxides. This critical observation led Criegee to propose that a transient zwitterionic intermediate (carbonyl oxide) was formed during an early stage of ozonolysis, and that subsequent reactions of this compound determined the final products.

The mechanism proposed by Criegee is summarized in Figure 1. The first step is characterized as a 1,3-dipolar cycloaddition of ozone to the carbon-carbon double bond

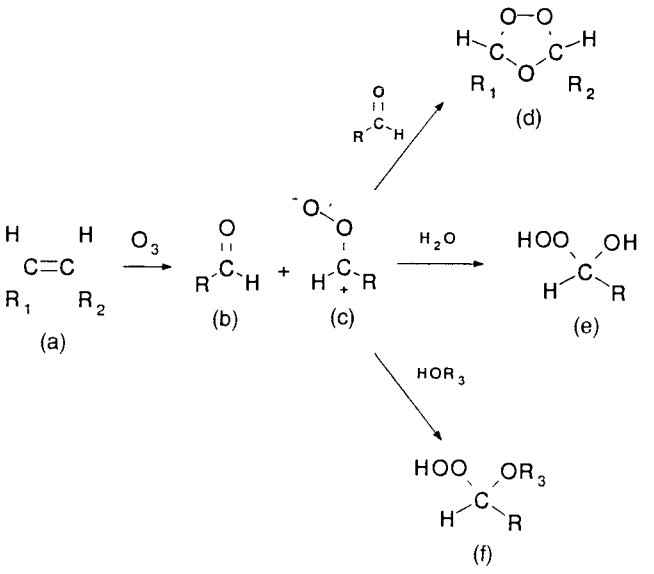

Figure 1. Mechanism proposed by Criegee for ozonolysis of unsaturated fatty acids.

* A list of abbreviations appears at the end of the Investigators' Report.

(a). The product of this reaction is a 1,2,3-trioxolane (not shown), also called a molozonide or primary ozonide. 1,2,3-Trioxolanes are stable only at low temperatures. At room temperature, they undergo rapid unimolecular decomposition to an aldehyde (b) and a carbonyl oxide (c). In the absence of protic compounds, even weak nucleophiles such as aldehydes react with the carbonyl oxide, yielding a 1,2,4-trioxolane (d), a secondary ozonide. Protic nucleophiles such as water and alcohols, however, react with the carbonyl oxide to give hydroxyhydroperoxide (e) and alkoxyhydroperoxide (f), respectively.

The carbonyl oxide is thus a branch point in this series of reactions. Because each product forms by a different reaction pathway, formation of one product prevents formation of the others. The rates of addition of protic nucleophiles to the carbonyl oxide are fast compared with the rate of reaction of the carbonyl oxide with an aldehyde; this means that even traces of water in an otherwise nonreactive solvent promote the formation of hydroxyhydroperoxides at the expense of ozonides. Thus, the products formed during ozonolysis of unsaturated lipids in biological systems, where water is ubiquitous, will depend on the availability of water to the reaction.

Tiege and associates (1974) addressed this problem, albeit indirectly, and found that ozonolysis of unsaturated phospholipids in liposomes yielded a mixture of hydrogen peroxide, aliphatic aldehydes, and carboxylic acids. Hydrogen peroxide and aldehydes were produced during the early stages of ozonolysis, and the carboxylic acids were produced only with the addition of more ozone. The chain lengths of the aldehydes and carboxylic acids were identical to the aliphatic fragments that would have been produced by scission of the carbon-carbon double bonds of the unsaturated fatty acids. These observations led Tiege and associates to propose the following: In a hydrated lipid membrane, ozonolysis of the unsaturated fatty acid groups of phospholipids yielded an aldehyde and a hydroxyhydroperoxide by the mechanism depicted in Figure 1. Hydroxyhydroperoxides were not detected because they decompose to hydrogen peroxide and aldehyde (g) as shown in Figure 2. The carboxylic acids (h) were produced by oxidation of aldehydes (b) and (g) by ozone (Figure 3). This suggests that the ubiquitous presence of water in the lipid membrane would prevent formation of ozonides in favor of hydroxyhydroperoxides.

The mechanism proposed by Tiege and associates raises a number of questions. Production of hydroxyhydroperoxides by the pathway illustrated in Figure 1 requires that carbonyl oxide have access to water. The concentration of water in a lipid bilayer membrane is low, however. Therefore, if the carbonyl oxide formed in the hydrophobic interior of the lipid bilayer, at the site of the carbon-carbon double bond, could there be sufficient water to produce hydroxyhydroperoxides? Were hydroxyhydroperoxides formed at all? Tiege and coworkers did not isolate or measure hydroxyhydroperoxides directly, but inferred their formation from the presence of aldehydes and hydrogen peroxide. Although others have made the same inference from similar observations (Mudd and Freeman 1977; Freeman et al. 1979; Pryor et al. 1991), the formation of hydroxyhydroperoxides has not been proved. If formed, were hydroxyhydroperoxides produced by the mechanism shown in Figure 1, or could they have been produced by another reaction pathway?

Recent work by Miura and coworkers indicates that acid-catalyzed hydrolysis of ozonides apparently can yield aldehydes and hydroxyhydroperoxides (Miura and Nojima 1980; Miura et al. 1980a,b, 1981, 1982, 1983a,b,c). *Ab initio* calculations indicate that the site of protonation is the ether oxygen, which yields the peroxy-linked carbonium ion shown in Figure 4 (Miura et al. 1983c). Adding water to the carbonium ion would yield an aldehyde and a protonated carbonyl oxide. However, the rate of acidolysis of ozonides is slow (Miura et al. 1980b, 1983a), especially for monocyclic ozonides with aliphatic substituents, such as fatty acid

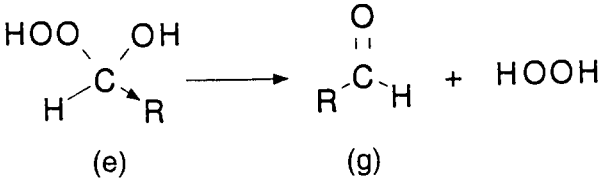

Figure 2. Decomposition of hydroxyhydroperoxide into aldehyde and hydrogen peroxide.

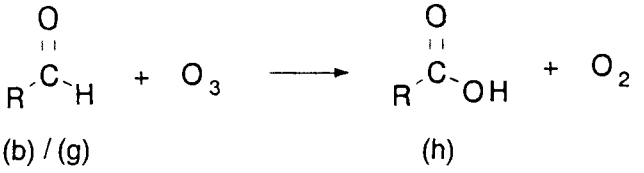

Figure 3. Oxidation of aldehydes by ozone.

ozonides (Miura et al. 1980b). The uncatalyzed hydrolysis of an ozonide would be even slower; this reaction requires displacement of one of the carbon-oxygen bonds by water. Depending on the mode of ring opening, either an ether-linked or a peroxy-linked intermediate would be formed (Figure 5). *Ab initio* calculations demonstrate that the ozonide ring carbons do not support a highly localized positive charge (Miura et al. 1983c). In fact, ozonides are relatively stable compounds. They can be isolated by liquid chromatography and analyzed by gas chromatography (GC) (Rubenstein 1967). In principle, hydrolysis of ozonides is an alternative pathway by which aldehydes and hydroxyhydroperoxides are produced. However, these reactions are generally too slow to explain the high yields of aldehydes and hydrogen peroxide, with no detectable ozonide, produced by the ozonolysis of unsaturated lipids in hydrated lipid bilayers.

In a recent study by Santrock and colleagues (1992), ozonolysis products of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine in a lipid bilayer membrane (unilamellar vesicles) were isolated and rigorously identified by mass spectrometry and nuclear magnetic resonance (NMR) spectrometry. In addition to hydrogen peroxide, the organic products included the expected aldehyde [1-palmitoyl-2-(9′-oxynonanoyl)-*sn*-glycero-3-phosphocholine] and carboxylic acid [1-palmitoyl-2-(9′-carboxynonanoyl)-*sn*-glycero-3-phosphocholine]. The hydroxyhydroperoxide [1-palmitoyl-2-(9′hydroxy-9′-hydroperoxynonanoyl)-*sn*-glycero-3-phosphocholine] proved to be an unstable product of this reaction. Hydrogen peroxide is rapidly eliminated from aliphatic hydroxyhydroperoxides in water (Sanders and Jenks 1968), and the observation of the unstable hydroxyhydroperoxide by Santrock and associates (1992) is consistent with the inability of Tiege and coworkers (1974) to detect hydroxyhydroperoxide. The pattern by which ^{18}O from $^{18}O_3$ is incorporated into these organic products showed that: (1) the aldehyde was an equimolar mixture of (b) and (g), (2) hydrogen peroxide was an ozonolysis product, with both oxygen atoms in the newly formed hydrogen peroxide molecules being derived from the same ozone molecule, and (3) the carboxylic acid was produced by ozonolysis of aldehydes (b) and (g). The sequence in which reactant disappeared and products appeared also indicated that the carboxylic acid resulted from ozonolysis of the aldehyde. These results confirm that ozonolysis of unsaturated lipids in a hydrated lipid bilayer membrane yields aldehydes and hydroxyhydroperoxides by the mechanism proposed by Tiege and colleagues (1974).

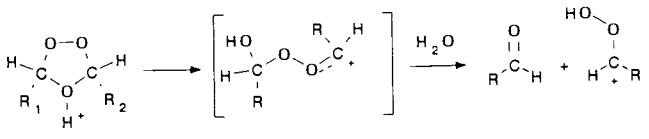

Figure 4. Acid-catalyzed hydrolysis of ozonides.

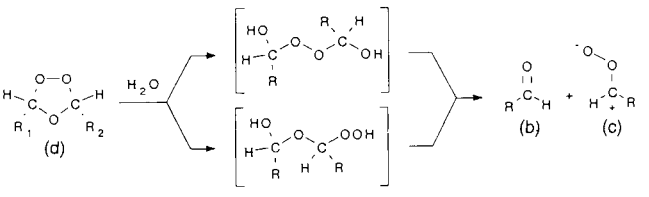

Figure 5. Alternate pathways for hydrolysis of ozonides.

We emphasize that ozonolysis is distinct from lipid peroxidation. In the latter, lipids are consumed in an autocatalytic free radical chain reaction in which molecular oxygen is the oxidant. This process may yield some of the same products as ozonolysis (for example, hydrogen peroxide, malonaldehyde, lipid aldehydes), but lipid hydroxides and lipid hydroperoxides are the major products of the central autoxidation reactions. The oxygen atoms in these products are derived from molecular oxygen (Hatch et al. 1989). Once the autocatalytic chain reactions are initiated by oxygen, lipid consumption proceeds even in the absence of the initiating agent. Moreover, the amount of lipid consumed is significantly greater that the amount of initiator. In contrast, ozonolysis of unsaturated lipids has a one-to-one stoichiometry (Santrock et al. 1992). That is, only one lipid unsaturated acyl moiety is consumed per molecule of ozone. Consumption of lipids ceases when ozone is no longer present. It is conceivable that hydrogen peroxide produced by the ozonolysis of unsaturated lipids can yield hydroxy radical (HO•) via decomposition catalyzed by heat or metal ions, thus initiating lipid peroxidation. However, these reactions must compete with detoxification reactions mediated by a number of highly efficient enzymes (such as catalase and glutathione peroxidase); thus, the amount of hydrogen peroxide formed by lipid peroxidation may be low compared with that formed by fatty acid ozonolysis. Validating ozonolysis reactions and identifying intermediates and products has two conceptual consequences. The first involves whether the reactive intermediates or stable products of ozonolysis exhibit biological activities that

explain some of ozone's effects on respiratory cells. This is a particularly interesting question because ozone is very reactive and unlikely to penetrate the apical surface of constitutive cells lining the respiratory tract (we estimate that, for all practical purposes, the limit of ozone's reaction path is less than 3 μm [Miller et al. 1985; Pryor 1992]). A second consequence of understanding ozone biochemistry is the potential for then developing valid biomarkers of ozone exposure. In this study we address the first question, by evaluating the biological impact of ozonolysis products.

SPECIFIC AIMS

Ozone exposure can augment eicosanoid metabolism in airway epithelial cells. Eicosanoids are important in regulating airway inflammation, smooth muscle contractility, ion transport, and cell growth. Reactions of ozone with membrane phospholipids, which are likely cellular targets, have yielded several possible ozonolysis products of unsaturated fatty acids. Exposure of lipid unsaturated fatty acids to ozone resulted in the formation of aldehydes, hydroxyhydroperoxides, and hydrogen peroxide. To provide a better understanding of the mechanisms of eicosanoid stimulation by ozone, we examined the relation of structure of the degradation products of ozonolysis to their effects on eicosanoid metabolism in human airway epithelial cells. Specifically, our objectives were:

- To synthesize and purify select ozonolysis products of unsaturated fatty acid moieties contained in membrane phospholipids (these compounds include saturated and unsaturated aldehydes and hydroxyhydroperoxides); and
- To determine the effects of these compounds on eicosanoid metabolism in human airway epithelial cells.

Ozonolysis product formation was evaluated by measuring the total ^3H released from cells incubated with radiolabeled arachidonic acid, by high-performance liquid chromatography (HPLC), and by radioimmunoassay (RIA) to confirm the results obtained by HPLC.

METHODS

EXPERIMENTAL DESIGN

To examine the relation of the structure of the fatty acid degradation products resulting from ozonolysis to their effects on eicosanoid metabolism in human airway epithelial cells, we prepared saturated aldehydes and hydroxyhydroperoxides, and then exposed cells in culture to these compounds. We evaluated eicosanoid metabolism by determining the total ^3H released from confluent cells previously incubated with [^3H]–arachidonic acid, and by identifying specific metabolites with HPLC and RIA.

SELECTION OF COMPOUNDS

Carbon-carbon double bonds are located in different places along fatty acid side chains in unsaturated phospholipids. For example, oleic acid is an 18-carbon (18:1) fatty acid with 1 carbon-carbon double bond between carbons 9 and 10. Cleavage of this bond would yield a 9-carbon aldehyde or hydroxyhydroperoxide. Other common fatty acids, linoleic (18:2) and linolenic (18:3) have 2 or 3 carbon-carbon double bonds. The spacing of these bonds is such that 3- and 6-carbon compounds, either saturated or unsaturated, can be formed.

Because ozone's reactions with membrane phospholipids can produce a wide array of fatty acid ozonolysis products, an efficient approach requires judicious selection of specific ozone reaction products to be tested. It is important that these compounds be chemically pure, which requires additional preparation. Thus, as an initial step to determine the effects, cells were treated with representative saturated and unsaturated 3-, 6-, and 9-carbon products (alkanals, 3-alkenals, 1-hydroxy-1-alkylhydroperoxides, and 1-hydroxy-1-alk-3-enehydroperoxides) and hydrogen peroxide.

To investigate the relative potency of specific molecules produced by ozonolysis, we examined the effects of selected compounds on eicosanoid metabolism. We chose three subclasses of compounds on the basis of the possible relation of structure and function. The first group, the alkanals, includes three saturated aldehydes (propanal, hexanal, and nonanal), permitting us to assess the effect of the length of the saturated carbon chain on enzyme activation. (Aldehydes with longer chains and greater lipid solubility may exhibit greater or lesser activity when added exogenously to cells.) The second group, 3-alkenals, includes two unsaturated aldehydes (3-hexenal and 3-nonenal). These compounds enabled us to assess the effect of unsaturated carbon chain aldehydes on enzyme activation. The third group, the corresponding hydroxyhydroperoxides, includes five compounds: 1-hydroxy-1-propanehydroperoxide, 1-hydroxy-1-hexanehydroperoxide, 1-hydroxy-1-nonanehydroperoxide (all three are saturated hydroxyhydroperoxides); and 1-hydroxy-1-hex-3-enehydroperoxide and 1-hydroxy-1-non-3-enehydroperoxide (both are unsaturated hydroxyhydroperoxides). This series permitted us to assess the effects of the hydroxyhydroperoxide group on enzyme activation.

Exposure concentrations were selected based on the limited literature on aldehydes (Leikauf 1992) and an estimate of possible ozonolysis product formation in vivo (see Appendix A).

PREPARATION OF CHEMICALS

Saturated aldehydes were purified by microdistillation to remove polymeric condensation products, carboxylic acids, water, and other contaminants; and by GC through silica gel using pentane-tetrahydrofuran (95:5) as an eluent. This synthetic strategy yielded stereoisomers of 3-hexenal and 3-nonenal, compounds likely to be produced by ozonolysis of unsaturated fatty acids in vivo.

The hydroxyhydroperoxides were synthesized by reacting the corresponding aldehyde with hydrogen peroxide and purifying the product again using GC. Hydrogen peroxide (more than 90% pure) was prepared by vacuum distillation of 30% hydrogen peroxide in water. Aldehydes (20 mmol) and hydrogen peroxide (10 g, 300 mmol) were combined in 20 mL dichloromethane (CH_2Cl_2). The mixture was stirred rapidly for 60 minutes at room temperature and then allowed to separate into two clear, colorless layers. After the excess hydrogen peroxide had settled to the bottom of the flask, the upper layer was carefully transferred into another flask and the solvent was removed by rotary evaporation. The crude product was placed under high vacuum at room temperature overnight to remove trace residual aldehyde and hydrogen peroxide.

Products were analyzed by ^1H NMR spectrometry (270 mHz) and by isobutane chemical ionization–mass spectrometry (CI–MS). Structural assignments of the signals in the ^1H NMR spectrum of 1-hydroxy-1-nonanehydroperoxide were confirmed from two-dimensional ^1H correlation spectroscopy, ^{13}C NMR, and ^1H-^{13}C cross-correlation spectra. The atomic formulae of the fragmentation ions of 1-hydroxy-1-nonanehydroperoxide were confirmed from the high-resolution chemical ionization mass spectrum. The ^1H NMR analysis revealed the starting aldehyde and hydrogen peroxide as the major impurities.

Because the unsaturated aldehydes are not commercially available, *cis*-3-hexenal and *cis*-3-nonenal were prepared by oxidation of the alcohols *cis*-3-hexen-1-ol and *cis*-3-nonen-1-ol, respectively, with pyridium chlorochromate (PCC). In each case, the alcohol (0.035 mol) was added slowly to 150 mL of CH_2Cl_2 containing 2 molar equivalents (0.07 mol) of PCC, and the mixture was stirred for 1 hour. Another 2 molar equivalents of PCC was added and the mixture was stirred for an additional hour, after which it was filtered through silica gel (40 to 140 mesh). Silica gel was added to the filtrate, the mixture was swirled, and the silica gel was removed by filtration. This procedure was repeated until a pale yellow solution was obtained. Solvent was removed by rotary evaporation, yielding the crude product.

The aldehydes were purified using column chromatography on silica gel. The eluent was pentane-tetrahydrofuran (95:5), and absorbance was monitored at 297 nm. Fractions corresponding to 90% of the peak area were collected and pooled, and the solvent was removed by rotary evaporation. This produced a mixture of compounds, and a second chromatographic step was necessary to obtain a purity greater than 90%. All products were analyzed by ^1H NMR spectrometry (270 mHz) and by gas chromatography–mass spectrometry (GC–MS) (electroluminescence, 70 eV).

CHEMICAL SYNTHESIS OF SATURATED ALDEHYDES, HYDROXYHYDROPEROXIDES, AND HYDROGEN PEROXIDE

Analysis by ^1H NMR spectrometry (270 mHz) and by isobutane CI–MS confirmed the structure of each saturated compound. Propanal, hexanal, and nonanal were obtained in high (at least 98%) purity after minimal preparation (microdistillation).

For the hydroxyhydroperoxides, structural assignments of the signals in the ^1H NMR spectrum also were confirmed from the two-dimensional ^1H correlation spectroscopy, ^{13}C NMR, and ^1H-^{13}C cross-correlation spectra. The atomic formulae of the fragmentation ions of 1-hydroxy-1-nonanehydroperoxide were confirmed from the high-resolution chemical ionization mass spectrum. The ^1H NMR spectrometry revealed that the starting aldehyde and hydrogen peroxide were the major impurities. Results for the hydroxyhydroperoxide were as follows:

1. 1-Hydroxy-1-propanehydroperoxide: a clear, colorless, viscous liquid. ^1H NMR (270 mHz, CD_2Cl_2): δ 1.15 ppm (t, 3H), 2.38 (q, 2H), 5.12 (t, 0.8H); 5.19 (t, 0.1H); 5.20 (t, 0.1H). CI–MS (C_4H_{10}): 59 (51); 75 (98); 91 (100).
2. 1-Hydroxy-1-hexanehydroperoxide: a clear, colorless, viscous liquid. ^1H NMR (270 mHz, CD_2Cl_2): δ 0.88 (t, 3H); 1.35 (m, 4H); 1.60 (m, 2H); 2.35 (dt, 0.8H); 3.9 (bs); 5.12 (t, 0.8H); 5.19 (t, 0.1H); 5.20 (t, 0.1H); 9.1 (bs); 9.75 (t, 0.2H). CI–MS (C_4H_{10}): 73 (13); 83 (21); 101 (100); 117 (55); 135 (0.2).
3. 1-Hydroxy-1-nonanehydroperoxide: a white, waxy solid. ^1H NMR (270 mHz, CD_2Cl_2): δ 8.50 (s, 1H), 5.20 (t, 0.1H), 5.19 (t, 0.1H), 5.11 (q, 0.8H), 3.94 (s, 0.1H), 3.50 (s, 0.1H), 3.30 (d, 0.8H), 1.57 (d, t, 2H), 1.25 – 1.40 (m, 12H), 0.88 (3.3H). ^{13}C NMR (270 mHz, CD_2Cl_2): δ 102.14 (0.5C), 101.45 (0.25C), 101.07 (0.12C), 33.32 (1C), 32.25 (1C), 29.50, 29.53, 29.61, 29.76, 29.82 (3C), 24.90 (1C), 23.05

(1C), 14.25 (1C). CI–MS (C_4H_{10}): 159.1361 (53, $C_9H_{19}O_2$, $[M+H-H_2O]^+$); 143.1416 (100, $C_9H_{19}O$, $[M+H-H_2O_2]^+$); 141.1264 (72, $C_9H_{17}O$, $[M+H-2H_2O]^+$); 125.1292 (74, C_9H_{17}, $[M+H-H_2O-H_2O_2]^+$); 123.1156 (38, C_9H_{15}, $[M+H-3H_2O]^+$).

CELL CULTURE

We grew human airway epithelial cells from tissue explants in a serum-free medium as described previously (Leikauf et al. 1990). Cells were seeded (5000 cells/cm^2) in 6-well, 35-mm cluster dishes or 60-mm dishes coated with fibronectin and collagen and grown to confluency in supplemented MCDB-153 medium (see Materials section). Cell preparations obtained by this method are free of fibroblasts, retain a human karyotype, express epithelial keratin and blood group antigens, possess a plasma membrane with specialized characteristics, and can differentiate into a complex ciliated epithelium (Leikauf and Driscoll 1993). Donors were free from respiratory disease (10 male, 8 female, 7 unknown; 59 to 76 years of age; 2 to 22 hours postmortem; 14 Caucasian, 6 African-American, 5 unknown).

EICOSANOID ANALYSIS

To evaluate total ^3H release, we incubated airway epithelial cells in [^3H]-labeled arachidonic acid (1.0 μCi/well) in supplement MCDB-153 with 10% fetal calf serum. After 24 hours, each dish was washed three times with Dulbecco's phosphate-buffered saline (DPBS). In an initial group of 18 wells, medium and washes were sampled to determine incorporation of the [^3H]–arachidonic acid (92.6% ± 0.3%, $n = 18$). Cells then were equilibrated with DPBS solution for 1 hour. The DPBS solution was removed and 3 mL fresh DPBS solution containing 3 μL DMSO (control), 3 to 300 μM aldehyde, or 3 to 100 μM hydroxyhydroperoxide was added to each well. Samples (2 × 50-μL aliquots) were removed after 15, 30, 60, 120, or 240 minutes and mixed with scintillation fluid; total ^3H release was determined by beta-scintillation spectrometry.

To identify specific eicosanoids in separate tests, we grew cells in 60-mm dishes and exposed them to 1-hydroxy-1-hexanehydroperoxide. Cells were incubated in either [^3H]–arachidonic acid (2.0 μCi/dish) as described above or supplemented MCDB-153 alone. After 24 hours, cells were washed three times with DPBS solution and incubated for 60 minutes in 3 mL DPBS solution. The solution was collected and then cells were exposed to control vehicle (DMSO) or vehicle plus 100 μM 1-hydroxy-1-hexanehydroperoxide for 30 minutes. Each supernatant was centrifuged (150 × g for 10 minutes at 4°C) and then frozen (−70°C). [^3H]-Labeled eicosanoids were extracted from the recovered samples containing ^3H activity by first acidifying the sample with 8.8% formic acid (53 μL/mL), then adding ethyl acetate at twice the sample volume and mixing vigorously. After the mixture settled into two phases, the upper phase, ethyl acetate, was removed; this procedure was repeated twice with fresh ethyl acetate. The ethyl acetate fractions were pooled and dried with 100% nitrogen at 30°C. The dried residue was resuspended in 100 μL of solvent A:solvent B (described below). Before extraction, 125 ng of prostaglandin B_2 (PGB_2) was added to each sample to monitor the recovery efficiency of the extraction procedure. The recovery efficiency was calculated as the ratio of the integrated area of the PGB_2 UV absorbance (280 nm) peak measured in the sample to the integrated area measured from a 125-ng PGB_2 standard. Extraction efficiencies were greater than 90% in all samples analyzed. In dishes of supplemented MCDB-153 alone (not incubated with [^3H]–arachidonic acid), cell-free supernatants were analyzed by RIA.

REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Eicosanoids were separated by HPLC using a modification of a method described previously (Doupnik and Leikauf 1990). We injected 90 μL of the 100-μL sample containing extracted [^3H]-labeled eicosanoids onto a 3-μm, ultrasphere, octadecyl silane, 75- × 4.6-mm column (Altex Division, Beckman, San Ramon, CA). Elution of each eicosanoid was achieved using a programmed gradient from solvent A (70:30 water:acetonitrile containing 2.5 mM H_3PO_4) to solvent B (60:40 methanol:acetonitrile containing 2.5 mM H_3PO_4). The gradient for the first 10 minutes was held constant at 100% solvent A. From 10 to 16 minutes, solvent A was reduced linearly to 44% while solvent B was increased to 56%. From 16 to 24 minutes, the gradient was held steady at 44% A:56% B; then from 24 to 35 minutes, solvent B was increased linearly to 100%, at which it was held for the remainder of separation. Before each analysis, retention times for authentic standards of selected eicosanoids were determined by UV absorbance (Model 490 multiwavelength detector, Waters Division, Millipore Corp., Boston, MA). For the first 13 minutes, UV absorbance was monitored at 205 nm, during which retention times of 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$), thromboxane (Tx)B_2, $PGF_{2\alpha}$, PGE_2, and PGD_2 were 3.0, 6.3, 8.0, 11.0, and 12.6 minutes, respectively. From 13 to 27 minutes, UV absorbance was monitored at 280 nm; retention time of PGB_2 was 17 minutes, of 8*R*,15*S*-dihydroxyeicosatetraenoic acid (diHETE)/8*S*,15*S*-LT was 17.6 minutes, of 8*S*,15*S*-diHETE/8*R*,15*S*-LT was 17.6 minutes, and of 5*S*,12*S*-diHETE/LTB_4 was 18.6 minutes. Finally, from 27 minutes onward, the UV absorbance was monitored at 240 nm; retention times of 12-hydroxyheptadecatrienoic acid

(12-HHT), 15-hydroxyeicosatetraenoic acid (15-HETE), 12-HETE, 5-HETE, and arachidonic acid were 20.0, 26.3, 28.0, 29.3, and 34.0 minutes, respectively. Eluate and scintillation fluid were mixed, and radioactivity (disintegrations per minute [dpm]) was determined with an HPLC-scintillation detector (Model β-RAM, IN-US Systems, Inc., Tampa, FL). Areas under peaks with coeluting retention times were integrated and corrected for collection efficiency determined using the PGB_2 standard.

RADIOIMMUNOASSAY

Previous studies (Leikauf et al. 1988, 1990; Doupnik and Leikauf 1990) and initial HPLC analysis have indicated that PGE_2 is a major product of eicosanoid metabolism in epithelial cells. In supernatants from unlabeled cells, we measured PGE_2 using a commercially obtained radioimmunoassay. In each test, two dishes containing cells from each of four tissue sources were exposed to the control vehicle (0.1% DMSO), or to 1, 3, 10, 30, or 100 μM 1-hydroxy-1-hexanehydroperoxide for 30 minutes. After treatment, recovered DPBS solution samples were centrifuged (150 × *g* for 10 minutes at 4°C) and the supernatant was frozen (–70°C). Radioimmunoassays were performed on unextracted aliquots of each sample.

CHROMIUM RELEASE ASSAY

We tested the cytolytic effects of hexanal, 1-hydroxy-1-hexanehydroperoxide, and hydrogen peroxide. Airway epithelial cells were grown to confluency in 12-well cluster dishes in MCDB-153 medium supplemented with growth factors. Cells were washed three times, and incubated in MCDB-153 medium containing radiolabeled sodium chromate ($Na_2[^{51}Cr]O_4$) (50 μCi/well; specific activity = 521 Ci/g) for two hours (Cohen et al. 1991). The medium containing unincorporated ^{51}Cr was removed and the cell monolayer was washed three times with DPBS. Each compound or the DMSO control vehicle was added to its respective well, duplicate samples (50 μL) were collected, and radioactivity was determined with a gamma counter. Spontaneous release was measured in wells containing cells treated with the DMSO vehicle. The percentage of specific ^{51}Cr release was defined as: [(experimental release – spontaneous release)/total ^{51}Cr incorporation] × 100. Total ^{51}Cr incorporation was determined by treating cells with 2% Triton X-100 for 10 minutes. The spontaneous release at 15, 30, 60, 120, and 240 minutes, respectively, after treatment with DPBS medium alone was 7.8% ± 0.6%, 8.9% ± 1.1%, 8.0% ± 1.0%, 9.8% ± 1.0%, and 12.9% ± 0.7%; after treatment with DPBS medium plus 0.1% DMSO, it was 8.4% ± 0.6%, 8.2% ± 0.7%, 8.4% ± 0.6%, 9.5% ± 0.4%, and 12.4% ± 0.6%. These values were not statistically different; therefore, 0.1% DMSO values were used in the calculation of specific ^{51}Cr release.

MATERIALS

The materials used in this study (with catalog numbers in parentheses if available) were obtained from the following sources. Airway tissue was placed in CMRL-1066 (330-1540, GIBCO/BRL, Grand Island, NY) containing 0.8 μM insulin (I-5500, Sigma Chemical Co., St. Louis, MO), 0.1 μM hydrocortisone (H-0888, Sigma), 0.32 μM β-retinyl acetate (R-3000, Sigma), 1.17 mM glutamine (G-6392, Sigma), 50 U/mL penicillin (Sigma), 50 μg/mL streptomycin (P-3539, Sigma), 50 μg/mL gentamicin (600-5750AD, GIBCO/BRL), 1.0 μg/mL amphotericin B (600-5295AE, GIBCO/BRL), and 1.0% fetal calf serum (6102, Biocell Inc., Rancho Dominguez, CA). Medium was changed daily and the tissue cultures were incubated in an atmosphere of 50% oxygen, 45% nitrogen, and 5% carbon dioxide. After 10 to 14 days on a rocker platform (Bellco Inc., Vineland, NJ), explants were placed on 100-mm tissue culture plates (Corning Co., Corning, NY) coated with 10 μg/mL fibronectin (40008, Collaborative Research, Lexington, MA), 30 μg/mL collagen (Vitrogen 100, Collagen Corp., Palo Alto, CA), and 100 μg/mL bovine serum albumin (fatty acid–free, A0281, Sigma), in a solution of MCDB-153 medium (M-7403, Sigma), with 110 μM Ca^{2+} (C-79, Fisher Scientific, Cincinnati, OH) containing 10 U/mL penicillin, 10 μg/mL streptomycin, 50 μg/mL gentamicin, and 1.0 μg/mL amphotericin B. After coating, dishes were washed once with DPBS (Ca^{2+}- and Mg^{2+}-free, 310-4190, GIBCO/BRL). Cells were grown in MCDB-153 supplemented with 0.2 μM hydrocortisone (Sigma), 0.9 μM insulin (Sigma), 0.5 μM ethanolamine (A-5629, Sigma), 0.5 μM phosphoethanolamine (P-6386, Sigma), 10 nM 3,3′,5-triiodo-L-thyronine (T-6397, Sigma), 0.8 nM epidermal growth factor (40001, Collaborative Research), 0.1 μM transferrin (4024, Collaborative Research), 25 μg/mL bovine pituitary extract prepared in our laboratory from bovine pituitaries (57133-2B4, Pel Freeze, Rogars, AR), 0.16 mM histidine (BP-383, Fisher), 0.5 mM isoleucine (BP-384, Fisher), 60 μM methionine (BP-388, Fisher), 60 μM phenylalanine (BP391, Fisher), 30 μM tryptophan (BP-395, Fisher), and 50 μM tyrosine (BP-396, Fisher), and antibiotics (penicillin, streptomycin, amphotericin B, and gentamicin at the same concentrations as in the MCDB-153 medium above). After growth from explants, cells were removed in 0.025% trypsin (T-5650, Sigma), 0.02% ethylene glycol-bis(β-aminoethylether) N,N,N′,N′-tetraacetic acid (E-4378, Sigma), and 1.0% polyvinyl pyrrolidone (PVP K-30, 20611, United States Biochemical Co., Cleveland,

OH) in Ca^{2+}- and Mg^{2+}-free DPBS. Each test was conducted in DPBS with Ca^{2+}, Mg^{2+}, and glucose (310-4287PJ, GIBCO-BRL). We used analytical grade propionaldehyde (propanal P5,145-1, Aldrich Chemical Co., Milwaukee, WI), hexanal (11,560-6, Aldrich), nonanal (N3,080-3, Aldrich), dinitrophenylhydrazine (D19,930-3, Aldrich), and anhydrous dimethyl sulfoxide (27685-5, Aldrich), acetonitrile (0154-4, Burdick and Jackson, Baxter Scientific, Columbus, OH), methanol (230-4, Burdick and Jackson), and ethyl acetate (E1954, Fisher Scientific). Liquid scintillation fluid (Aquasol-2, NEF-952) and $Na[^{51}Cr]O_4$ (NEZ-030S, specific activity = 521 Ci/g) were obtained from NEN Dupont, (Boston, MA). The RIAs for [^{3}H]-PGE_2 (8-6001) and $PGF_{2\alpha}$ (8-6002) came from Advanced Magnetics, Inc. (Cambridge, MA). Authentic eicosanoid standards (2003) were obtained from Caymen Chemical Co. (Ann Arbor, MI), except LTB_4 and 15-diHETE, which were a kind gift from Merck Frosst Canada, Dorval, Quebec. All other materials were obtained from Fisher Scientific.

STATISTICAL ANALYSIS

All values are reported as means ± standard errors. To test for statistical significance, we performed analysis of variance (Number Cruncher Statistical System 5.03), comparing with each other the four or five different treatment groups in an experiment. If the analysis of variance showed statistical significance at $p \leq 0.05$, we compared each treatment group to the control by t test (Sigma Plot 4.1) and reported the mean as significantly different from control when $p \leq 0.05$. The t tests were performed repeatedly and separately for each treatment group.

RESULTS

TOTAL [^{3}H]-LABELED EICOSANOID RELEASE INDUCED BY SATURATED ALDEHYDES

The [^{3}H]-labeled material released from cells incubated with 3 to 300 μM aldehyde for 240 minutes is presented in Figure 6. The results are expressed as a percentage of the mean control value, which equalled 23,125 ± 2,112 dpm/well (mean ± SE, n = 17). The observed values for concentrations at or below 300 μM propanal and hexanal did not differ significantly from the mean control value. The 9-carbon aldehyde, nonanal, in contrast to the 3- or 6-carbon aldehydes (propanal and hexanal), stimulated ^{3}H release at concentrations at or above 100 μM, indicating that the stimulatory effect of saturated aliphatic aldehydes increased with increasing chain length.

TOTAL [^{3}H]-LABELED EICOSANOID RELEASE INDUCED BY SATURATED HYDROXYHYDROPEROXIDES

When tested under identical conditions with cells from the same donors used in the previous tests, the 3-carbon hydroxyhydroperoxide, 1-hydroxy-1-propane-hydroperoxide (Figure 7), caused more release of radiolabeled material than its comparable 3-carbon aldehyde (propanal) (Figure 6). The 3-carbon hydroxyhydroperoxide was also more potent than the 6- or 9-carbon aldehyde. Again, compounds of longer chain length, 6-carbon (1-hydroxy-1-hexanehydroperoxide) or 9-carbon (1-hydroxy-1-nonanehydroperoxide) hydroxyhydroperoxides, produced a greater response than an equivalent exposure to the 3-carbon hydroxyhydroperoxide with a shorter chain. Thus, hydroxyhydroperoxides are more potent than aldehydes of equivalent chain length, and increasing the carbon chain length of these molecules increases their effect on eicosanoid release.

To examine the time course of this response, the effects of the 6-carbon hydroxyhydroperoxide, the corresponding aldehyde, and the control vehicle were compared. As shown in Figure 8, ^{3}H release increased within 30 minutes

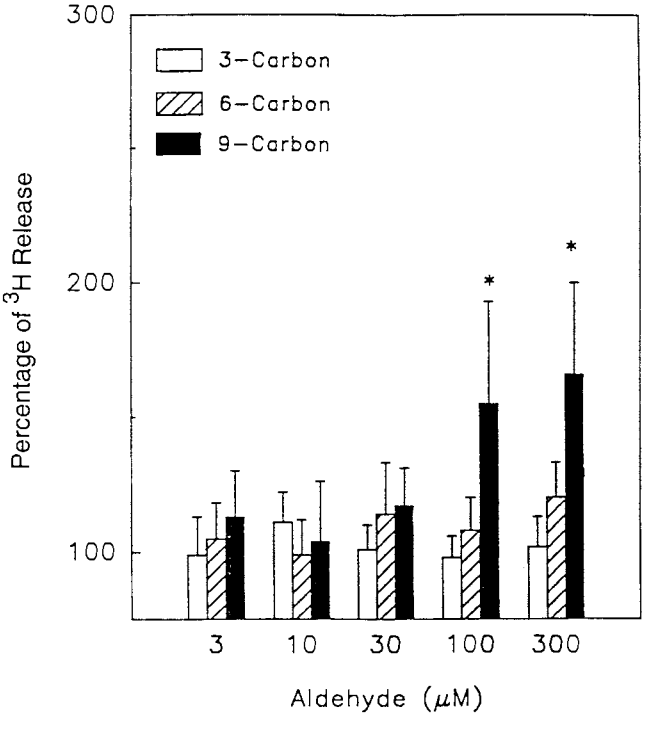

Figure 6. Release of [^{3}H]-labeled eicosanoids from human airway epithelial cells after 240 minutes of exposure to propanal (3-carbon) (n = 6), hexanal (6-carbon) (n = 6), or nonanal (9-carbon) (n = 5). Treatment with either propanal or hexanal was without effect, whereas nonanal increased ^{3}H release. Control values varied by 18% (2 × SE) so that the mean ± 2SE = 118 (n = 17). Values are means ± SE (n = 6). An asterisk (*) indicates a statistically significant value ($p \leq 0.05$).

after initiation of exposure to the hydroxyhydroperoxide. Again, the 6-carbon 1-hydroxy-1-hydroperoxide stimulated more ^3H release than did the 6-carbon aldehyde.

TOTAL [^{3}H]-LABELED EICOSANOID RELEASE INDUCED BY HYDROGEN PEROXIDE

The effectiveness of the hydroxyhydroperoxides could be due to degradation of the hydroxyhydroperoxide into hydrogen peroxide and the corresponding aldehyde, with hydrogen peroxide being the chemical species responsible for the effect. To address this question, we exposed cells to 0 to 300 μM hexanal, hydrogen peroxide, or hydrogen peroxide plus hexanal. The lattermost would mimic the degradation products of 1-hydroxy-1-hexanehydroperoxide. The results depicted in Figure 9 indicate that a concentration at or above 300 μM hydrogen peroxide caused an increase in ^3H release. This release did not demonstrate a dose-effect relation similar to that noted with the 1-hydroxy-1-hexanehydroperoxide (Figure 7). The magnitude of the response at 300 μM hydrogen peroxide was similar to that at 100 μM 1-hydroxy-1-hexanehydroperoxide. When

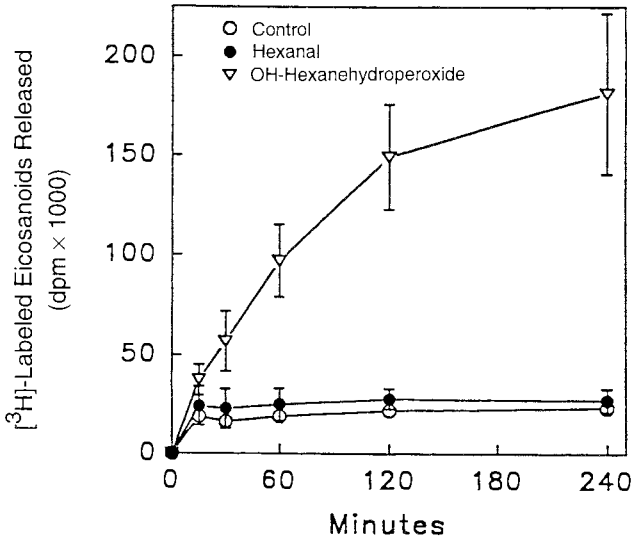

Figure 8. Release of [^{3}H]-labeled eicosanoids from human airway epithelial cells exposed to control, 300 μM hexanal, or 300 μM 1-hydroxy-1-hexanehydroperoxide. The response to the 6-carbon hydroxyhydroperoxide was markedly greater than to control. The corresponding 6-carbon aldehyde stimulated no more ^3H release than the control. Values are means ± SE (n = 6).

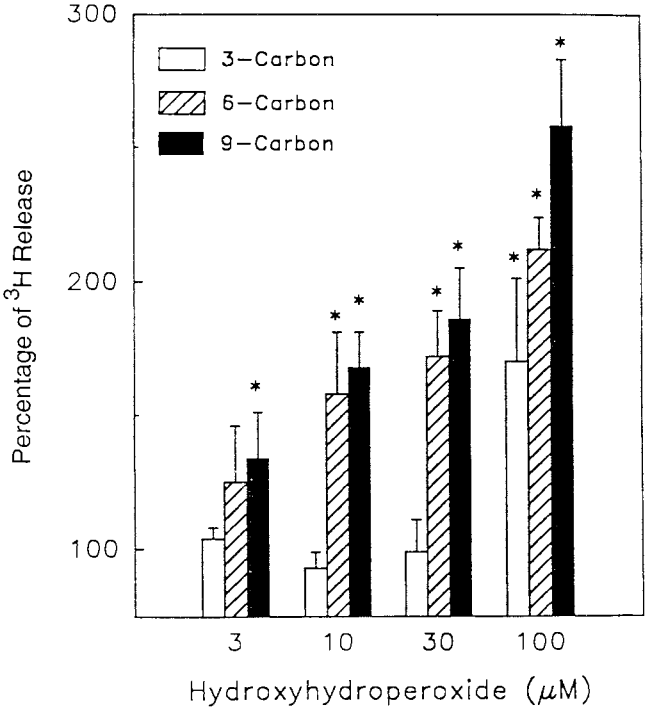

Figure 7. Comparison of the response of three saturated hydroxyhydroperoxides after 240 minutes of exposure. The release of [^{3}H]-labeled eicosanoid was greater for the hydroxyhydroperoxides than for the corresponding aldehyde of the same chain length (Figure 6). In addition, compounds of longer chain length produced larger increases in ^3H release. Values are means ± SE (n = 6). An asterisk (*) indicates a statistically significant value ($^*p \leq 0.05$).

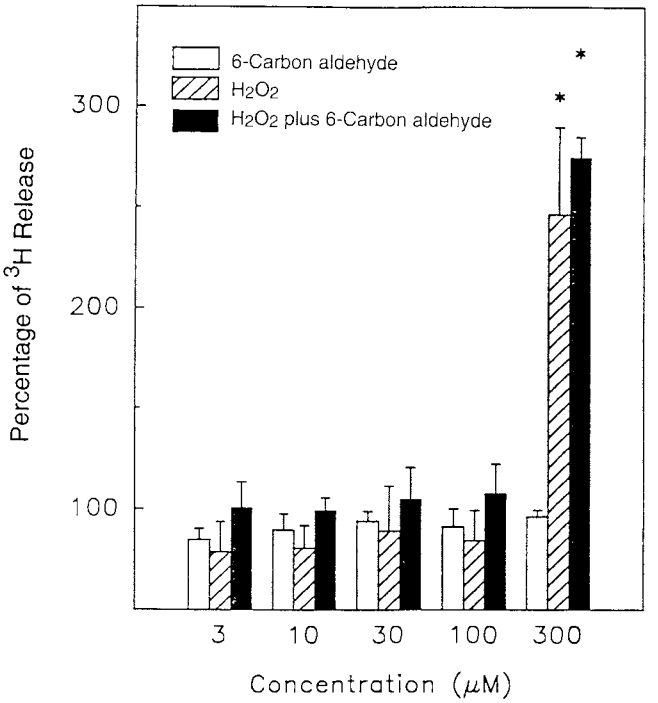

Figure 9. Release of [^{3}H]-labeled eicosanoid from human airway epithelial cells after 240 minutes of exposure to hexanal (6-carbon aldehyde), hydrogen peroxide (H_2O_2) or hydrogen peroxide plus hexanal (H_2O_2 plus 6-carbon aldehyde). Compare with findings presented in Figures 6 and 7. Values are means ± SE (n = 6). An asterisk (*) indicates a statistically significant value ($^*p \leq 0.05$).

hydrogen peroxide and hexanal were added together, no difference was noted between that combination or hydrogen peroxide alone.

DETAILED ANALYSIS OF EICOSANOIDS RELEASED AFTER EXPOSURE TO HEXANAL OR 1-HYDROXY-1-HEXANEHYDROPEROXIDE

Because 6-carbon compounds produced a significant increase in ^3H release, we used them as agonists to analyze the specific eicosanoids released from human airway epithelial cells. Ethyl acetate extracts of the supernatants from cells treated with 0.1% DMSO (control vehicle), 100 μM hexanal, or 100 μM 1-hydroxy-1-hexanehydroperoxide were separated by HPLC, and the ^3H activity coeluting with known standards was integrated. Results are presented in Table 1. Stimulation with 1-hydroxy-1-hexanehydroperoxide produced increases in ^3H activity coeluting with PGE_2 and $PGF_{2\alpha}$, with a smaller increase in ^3H activity coeluting with 15-HETE and related products (e.g., 8*S*,15*R*-diHETE). In addition, the release of material coeluting with unmetabolized arachidonic acid was greater in cells treated with the hydroxyhydroperoxide. Hexanal (100 μM), the corresponding aldehyde, was without discernible effect.

To support these findings, radioimmunoassay was performed with supernatants from cells not preincubated with [^3H]-labeled arachidonic acid. The results are presented in Figure 10. Again, PGE_2 release from airway epithelial cells induced by 1-hydroxy-1-hexane-hydroperoxide was dose-dependent, with concentrations of 30 μM and 100 μM producing significant increases. In separate tests with $PGF_{2\alpha}$ antiserum, reactant products also were increased (values in pmol $PGF_{2\alpha}/10^6$ cells:control = 0.24 ± 0.03 [n = 8], 10 μM = 0.75 [n = 2], 30 μM = 1.28 [n = 2], 100 μM = 1.76 [n = 2]).

CYTOLYTIC ASSAY OF SATURATED 6-CARBON COMPOUNDS AND HYDROGEN PEROXIDE

^{51}Cr release increased in cells treated with hexanal, 1-hydroxy-1-hexanehydroperoxide, or hydrogen peroxide (Figure 11). The concentrations used here overlapped those used in assays of eicosanoid metabolism (3 to 300 μM), and included higher concentrations. Although 100 and 300 μM hexanal produced no significant increase in [^3H]-labeled eicosanoid release (Figure 6), these concentrations of hexanal were sufficient to increase specific ^{51}Cr release (Figure 11). In contrast, 10 and 30 μM 1-hydroxy-1-hexanehydroperoxide produced significant increases in [^3H]-labeled eicosanoid release (Figure 7), but had no effect on specific ^{51}Cr release (Figure 11). Concentrations of 100 and 300 μM 1-hydroxy-1-hexanehydroperoxide increased ^{51}Cr release by 5% to 10%, whereas 1000 μM 1-hydroxy-1-hexanehy-

Table 1. [^3H]-Labeled Arachidonic Acid Metabolites from Human Airway Epithelial Cells Treated with Phosphate-Buffered Saline (Control), 100-μM Hexanal, or 100-μM 1-Hydroxy-1-hexanehydroperoxide

Coeluting Standard	Retention Time (minutes)	Radioactivity[a] (dpm[^3H]/dish/hr)		
		Control	6-Carbon Aldehyde	6-Carbon-Hydroxy-hydroperoxide
$PGF_{1\alpha}$	3.0	132 ± 30	109 ± 21	774 ± 145[b]
$PGF_{2\alpha}$	8.0	251 ± 68	316 ± 76	1,247 ± 236[b]
PGE_2	11.0	1,745 ± 258	1,940 ± 430	6,088 ± 534[b]
PGD_2	12.6	63 ± 23	123 ± 33	117 ± 43
8*R*,15*S*diHETE/8*S*,15*S*-LT	17.0	226 ± 60	166 ± 38	905 ± 189[b]
8*S*,15*S*diHETE/8*R*,15*S*-LT	17.6	214 ± 35	103 ± 44	1,211 ± 240[b]
5*S*,12*S*diHETE/LTB_4	18.6	276 ± 69	126 ± 32	1,324 ± 298[b]
12-HHT	20.0	127 ± 47	138 ± 56	739 ± 161[b]
15-HETE	26.3	473 ± 76	307 ± 82	1,097 ± 316[b]
12-HETE	28.0	586 ± 116	308 ± 83	800 ± 184
5-HETE	29.3	574 ± 125	483 ± 159	610 ± 192
Arachidonic acid	34.0	559 ± 121	459 ± 153	1,151 ± 256[b]

[a] Values are presented as means ± SE.

[b] Significantly different from control values at $p = 0.05$ (analysis of variance followed by t test).

droperoxide increased specific ^{51}Cr release by approximately 30% (Figure 11). Similarly, a concentration of hydrogen peroxide (300 μM) that increased [^{3}H]-labeled eicosanoid release (Figure 9) had no effect on specific ^{51}Cr release, whereas a higher concentration (1000 μM) produced a large increase in specific ^{51}Cr release (Figure 11). The time course of radioactivity recovered in cell culture medium also differed; maximum [^{3}H]-labeled eicosanoid release occurred between 120 and 240 minutes (Figure 8), whereas maximum ^{51}Cr release occurred in the first 15 minutes (Figure 12). It is noteworthy that a small amount of [^{3}H]-labeled eicosanoid was released (up to approximately 25,000 dpm) at 15 minutes after treatment with 300 μM hexanal (Figure 8) and that this concentration of hexanal was sufficient to produce an increase in specific ^{51}Cr release (Figure 11). However, this release did not differ statistically from that produced by the control vehicle.

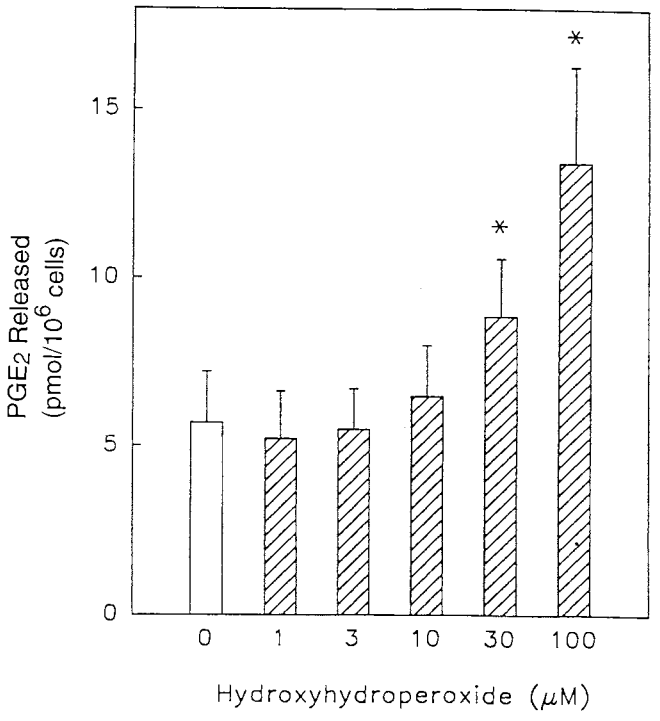

Figure 10. Release of immunoreactive PGE_2 from human airway epithelial cells exposed to hydroxyhydroperoxide (1-hydroxy-1-hexanehydroperoxides for 30 minutes. Cells were grown to confluency in MCDB-153 supplemented with growth factors, washed three times, and equilibrated with DPBS. After one hour, medium was replaced and 0.1% DMSO or 1 to 100 μM 1-hydroxy-1-hexanehydroperoxide was added. Cell-free supernatants were frozen and later analyzed by radioimmunoassay. Values are means ± SE of eight dishes obtained from four donors. An asterisk (*) indicates a statistically significant value ($*p \leq 0.05$).

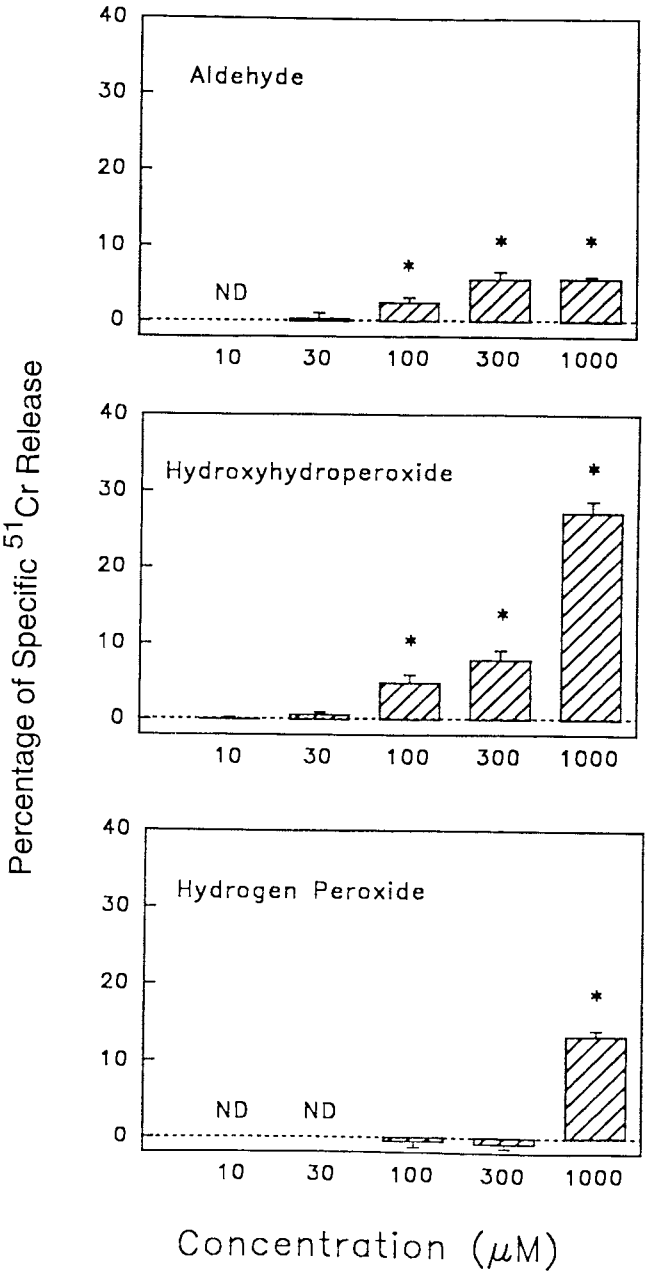

Figure 11. Release of ^{51}Cr from human airway epithelial cells exposed to aldehyde (hexanal), hydroxyhydroperoxide (1-hydroxy-1-hexanehydroperoxide), or hydrogen peroxide. Cells were grown to confluency in 48-well plates, exposed to labeled $Na_2[^{51}Cr]O_4$ (50 μCi/well) for two hours, and washed three times with DPBS. One of the three treatment solutions was added for 240 minutes and samples were collected. Values (specific ^{51}Cr release was obtained by dividing the release produced by treatment by that produced by control vehicle alone × 100) are means ± SE of six dishes. An asterisk (*) indicates a statistically significant value ($*p \leq 0.05$).

UNSATURATED ALDEHYDES AND HYDROXYHYDROPEROXIDES

Chemical Synthesis of Unsaturated Aldehydes

Because the unsaturated aldehydes are not commercially available, *cis*-3-hexenal and *cis*-3-nonenal were prepared by oxidation of *cis*-3-hexenol and *cis*-3-nonenol. The resulting aldehydes were purified by column chromatography and absorbance was monitored at 297 nm. Fractions corresponding to 90% of the peak area were collected and pooled, and solvent was removed by rotary evaporation. This produced a mixture of compounds necessitating a second chromatographic step to obtain better than 90% purity. All products were analyzed by ^{1}H NMR spectrometry (270 mHz) and by GC–MS (electroluminescence, 70 eV). The major impurities in both products were residual pentane and tetrahydrofuran. Traces of the corresponding *trans* isomers were also present. The products were dissolved in pentane and refrigerated until used.

1. *cis*-3-Hexenal: ^{1}H NMR (270 mHz, CD_2Cl_2): δ 0.88 (t, 2H); 2.03 (q, 2H); 3.15 (dd, 2H); 5.45 (m, 1H); 5.67 (m, 1H); 9.61 (t, 1H). GC–MS (electroluminescence, 70 eV): 27 (18); 41 (100); 55 (33); 69 (39); 83 (14); 98 (M^+, 5.6). GC–MS and ^{1}H NMR analysis showed that 3-hexenal was more than 93% pure in initial analyses.

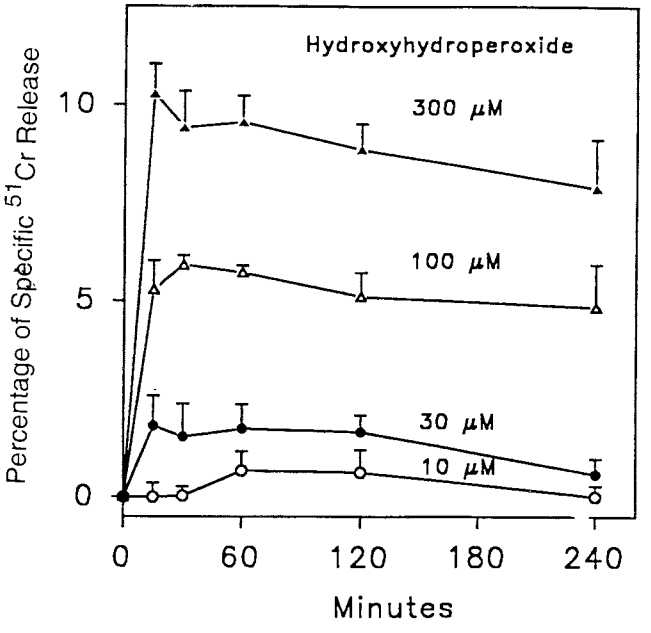

Figure 12. Release of ^{51}Cr from human airway epithelial cells exposed to hydroxyhydroperoxide (1-hydroxy-1-hexanehydroperoxide). Cells were grown to confluency in MCDB-153 supplemented with growth factors, washed three times, and labeled with $Na_2[^{51}Cr]O_4$ in MCDB-153 medium. After two hours, cells were washed three times with DPBS, the treatment solution was added, and duplicate samples were collected for gamma irradiation determination. Values (normalized to release produced by control vehicle alone) are means ± SE of six dishes.

2. *cis*-3-Nonenal: ^{1}H NMR (270 mHz, CD_2Cl_2): δ 0.88 (t, 3H); 1.25 (m, 6H); 2.02 (q, 2H); 3.15 (dd, 2H); 5.52 (m, 1H); 5.68 (m, 1H); 9.63 (t, 1H). GC–MS (electroluminescence, 70 eV): 27 (42); 29 (68); 41 (100); 55 (79); 69 (50); 84 (36); 96 (13); 111 (2); 122 (2); 140 (M^+, 0.2). GC–MS and ^{1}H NMR analysis showed that 3-nonenal was more than 95% pure in initial analyses.

These compounds were prepared in Dr. Santrock's laboratory (General Motors, Warren, MI), and then transported to Dr. Leikauf's laboratory (University of Cincinnati Medical Center, Cincinnati, OH) and stored at 4°C for several months. Preliminary tests showed little effect of these stored compounds on release of [^{3}H]-labeled eicosanoids from airway epithelial cells. When Dr. Santrock reexamined compound purity it was approximately 30% for *cis*-3-hexenal and approximately 50% for *cis*-3-nonenal. In contrast, saturated aldehydes handled in the same manner maintained better than or equal to 98% purity during the same period. Because of this difficulty, the tests of eicosanoid release upon exposure to unsaturated aldehydes were not repeated. Further experimentation with storage and handling methods (perhaps in ampules under argon) may be necessary to address this problem.

Synthesis of Unsaturated Hydroxyhydroperoxides

We combined the initially purified unsaturated aldehydes with hydrogen peroxide to determine whether they could be used to synthesize the corresponding hydroxyhydroperoxides. The resulting compounds were examined by ^{1}H NMR spectrometry (270 mHz) and by isobutane CI–MS. The ^{1}H NMR showed that the starting unsaturated aldehyde and hydrogen peroxide were the major impurities and indicated the presence of:

1. 1-Hydroxy-1-hex-3-enehydroperoxide: a clear, colorless, viscous liquid. ^{1}H NMR (270 mHz, CD_2Cl_2): δ 0.88 (t, 3H); 2.05 (dt, 1.8H); 2.40 (m, 1.6H); 5.12 (t, 0.8H); 5.19 (t, 0.1H); 5.20 (t, 0.1H); 5.35 (m, 0.8H); 5.55 (m, 0.8H); 9.1 (bs); 9.70 (t, 0.2H).
2. 1-Hydroxy-1-non-3-enehydroperoxide: a clear, colorless, viscous liquid. ^{1}H NMR (270 mHz, CD_2Cl_2): δ 0.88 (t, 3H); 1.30 (m, 6.6H); 2.05 (m, 1.8H); 2.40 (m, 1.8H); 3.9 (bs); 5.12 (t, 0.8H); 5.19 (t, 0.1H); 5.20 (t, 0.1H); 5.35 (m, 1.8H); 5.55 (m, 1.8H); 9.0 (bs); 9.65 (t, 0.1H).

DISCUSSION

Ozonolysis of the common fatty acids (oleic, linoleic, and linolenic acids) yields a mixture of aldehydes (propanal, hexanal, 3-hexenal, nonanal, 3-nonenal, and 3,6-nonadienal); the corresponding hydroxyhydroperoxides [1-hydroxy-1-

propanehydroperoxide, 1-hydroxy-1-hexanehydroperoxide, 1-hydroxy-1-(3-hexene)-hydroperoxide, 1-hydroxy-1-nonanehydroperoxide, 1-hydroxy-1-(3-nonene)-hydroperoxide, and 1-hydroxy-1-(3,6-nonadiene)-hydroperoxide]; and hydrogen peroxide. In this study we examined the effects of aliphatic compounds on airway epithelial cells. While 3-, 6-, or 9-carbon saturated aldehydes had little or no effect at the concentrations studied, exposure to hydroxyhydroperoxides increased eicosanoid release from human airway epithelial cells. These findings imply that ozone can initiate biological activity through an indirect mechanism involving the formation of hydroxyhydroperoxide from the fatty acids in phospholipids.

The chemical analyses performed indicate that certain of these products can be prepared in sufficient purity for biological tests using the methods described. The hydroxyhydroperoxides were prepared easily from the aldehydes and were stable over several months. However, the unsaturated *cis*-3-alkenyl aldehydes were unstable. Further tests to determine why these compounds degraded during storage at 4°C need to be conducted. Nonetheless, several tests were performed with the chemically pure saturated compound.

We found that the activity of each saturated hydroxyhydroperoxide depended on the carbon chain length of the molecule, with longer molecules producing a greater response. In addition, neither hydrogen peroxide alone nor hydrogen peroxide plus hexanal was as effective as 1-hydroxy-1-hexanehydroperoxide in stimulating eicosanoid release.

One explanation of the effect of chain length on the observed response is that the effective dose to the cells is controlled by the partitioning of the hydroxyhydroperoxide between the medium and the plasma membrane. Ampholyte molecules, such as the aliphatic hydroxyhydroperoxides, are soluble in both aqueous and nonaqueous solvents (Nelson and Hoff 1968). The partition coefficient is the thermodynamic property most frequently used to predict biological activities including transmembrane mobility, protein binding, receptor affinity, or accumulation in adipose tissue. The trend in partition coefficients for homologous series of *n*-alcohols or *n*-aldehydes in both water and *n*-octanol and water and oil systems (Tewari et al. 1982) demonstrates that molecules with longer aliphatic chains are more soluble in the nonaqueous phases of such systems. Hydroxyhydroperoxides added to the medium above cells in culture will partition between the aqueous medium and the hydrophobic membrane. Being more lipophilic, molecules with longer aliphatic chains will move into the membrane more effectively. Hydroxyhydroperoxides also are likely to be more stable in the membrane than in the aqueous medium because there is less water in the membrane (Hanke et al. 1987). Thus, thermodynamic driving forces favor the partition of hydroxyhydroperoxides with longer aliphatic chains into the membrane, where their rate of decomposition is probably slower.

Hydrogen peroxide alone has been shown to induce eicosanoid metabolism in other lung cell types (e.g., alveolar macrophages, and type II pneumocytes). It can form in airways both exogenously (through ozone inhalation [Pryor et al. 1991; Santrock et al. 1992]) and endogenously (through reactive oxygen metabolite formation during inflammation and injury [Freeman and Crapo 1982]). The dose-response relation noted in at least one study was similar to that observed here, in that application of exogenous 100 or 250 μM hydrogen peroxide was without effect while hydrogen peroxide at concentrations above 500 μM produced significant arachidonic acid release in rat alveolar macrophages (Sporn et al. 1988). Likewise, high hydrogen peroxide doses were required to stimulate eicosanoid metabolism in alveolar type II cells (Van Overveld et al. 1992). Also noteworthy in the study of alveolar macrophages was that TxB_2 release increased at hydrogen peroxide doses that had no effect on PGE_2 release. In the present study, hydroxyhydroperoxide exposure stimulated release of PGE_2 by human airway epithelial cells, suggesting that the profile of eicosanoid produced by oxidant injury is likely to be specific to cell type.

Hydrogen peroxide produced by the degradation of hydroxyhydroperoxides does not appear to be the chemical compound that is responsible for stimulating eicosanoid metabolism. Neither hydrogen peroxide alone nor hydrogen peroxide plus hexanal stimulated eicosanoid release as effectively as 1-hydroxy-1-hexanehydroperoxide. In addition, the activity of each hydroxyhydroperoxide depended on its chain length, which is not consistent with the stoichiometry of hydroxyhydroperoxide degradation. Hydrogen peroxide elimination from a hydroxyhydroperoxide molecule in aqueous systems is fairly rapid (Santrock et al. 1992), and could occur either in the aqueous culture medium outside the cell, in the plasma membrane, or in the cytoplasm of the cell. The stoichiometry of this reaction, $R\text{-}CH(OH)(OOH) \rightarrow R\text{-}CHO + H_2O_2$, indicates that decomposition of hydroxyhydroperoxide yields an equimolar amount of hydrogen peroxide regardless of the chain length. Thus, degradation of equal concentrations of hydroxyhydroperoxides with different chain lengths would produce equivalent doses of hydrogen peroxide. In our experiment, however, the hydroxyhydroperoxides with longer aliphatic chains released greater amounts of $[^3H]$-labeled eicosanoid into the medium than did equal concentrations of hydroxyhydroperoxides with shorter aliphatic

chains. This additional ^{3}H release suggests that these longer-chain hydroxyhydroperoxides are probably acting directly on the plasma membrane.

Previously, Cohen and associates (1991) demonstrated that a one-hour exposure to ozone (0.05 or 0.2 ppm) or hydrogen peroxide (at or beyond a concentration of 1000 μM) increased specific ^{51}Cr release in guinea pig tracheal epithelial cells and alveolar macrophages. In this study with human airway epithelial cells, we also found that 1000 μM hydrogen peroxide increased specific ^{51}Cr release, which could possibly be attributed to cell lysis. However, this appears unlikely because increases in [^{3}H]-labeled eicosanoid release were noted at doses of hydroxyhydroperoxide lower than those producing cytolysis, and the time course of the two responses was dissimilar. These results suggest that ozonolysis products have a role in cell activation (eicosanoid biosynthesis) at doses lower than those necessary to produce cell death (as indicated by ^{51}Cr release). This is in agreement with our previous finding that acrolein stimulated increased eicosanoid metabolism at doses below those that are cytotoxic (as indicated by lactate dehydrogenase release) in bovine airway epithelial cells (Doupnik and Leikauf 1990).

In humans, inhalation of ozone at low levels can lead to increases in PGE_2, $PGF_{2\alpha}$, TxB_2, neutrophils, neutrophil elastase, and protein levels in bronchoalveolar lavage fluid (Seltzer et al. 1986; Koren et al. 1989; Devlin et al. 1991). Exposure of bovine airway epithelial cells to ozone in similarly low concentrations augmented eicosanoid metabolism, indicated by increased PGE_2 and $PGF_{2\alpha}$ release (Leikauf et al. 1988). Mediators derived from epithelial cells have been implicated in neutrophil recruitment into the airways (Koyama et al. 1991a,b; Nakamura et al. 1992). Thus, epithelial activation with mediator release is likely to be an initial event in integrated responses to ozone injury. This study supports the concept that the reaction of ozone with phospholipids in the airway fluid layer or the apical membrane of epithelial cells (and other cells resident in the airways) affects subsequent mediator release important to an acute airway response.

The molecular sites reacting with ozone in the lung are unknown. A recent paper hypothesizes that unsaturated fatty acids in surfactant lipids are major sinks for inhaled ozone (Pryor 1992). Lipids containing unsaturated fatty acids in the apical membrane of the airway epithelium are other potential reaction sites of ozone. In this study, hydroxyhydroperoxides added to the culture medium produced a measurable increase in eicosanoid release by human airway epithelial cells. Thus, hydroxyhydroperoxides produced by ozonolysis of surface lipids, which are in close proximity to the apical membrane of the airway epithelium, could produce a similar effect. Moreover, hydroxyhydroperoxides produced by ozonolysis of membrane lipids would already be in the membrane. In either case, hydroxyhydroperoxides would have access to airway epithelial cells.

In summary, structure and activity studies of 3-, 6-, and 9-carbon aldehydes and hydroxyhydroperoxides demonstrate that longer-chain compounds can increase total [^{3}H] release from human airway epithelial cells incubated with [^{3}H]-radiolabeled arachidonic acid. Each hydroxyhydroperoxide was more potent than its corresponding aldehyde. Hydroxyhydroperoxides have been identified as ozonolysis products of phospholipids that contain unsaturated fatty acids in liposomes. Inasmuch as hydroxyhydroperoxides are produced in vivo, this study suggests a mechanism that links ozone chemistry to the activation of human epithelial airway cells and their subsequent release of eicosanoids, pathophysiological agonists with wide-ranging effects in the airways.

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APPENDIX A. Estimate of Ozonolysis in Vivo

To estimate the concentration of hydroxyhydroperoxide or aldehyde that can result from ozone inhalation, a number of assumptions must be made about the deposition and reactions of ozone in the lung. Nonetheless, it appears that micromolar concentrations of aldehydes and hydroxyhydroperoxides could result from exposures to 0.12 ppm of ozone (the current National Ambient Air Quality Standard and a concentration that can induce eicosanoid release in human subjects [Seltzer et al. 1986; Koren et al. 1989; Delvin 1991]). It is important to recognize that these uncertainties make it difficult to estimate the possible concentrations in vivo.

Applying the calculations of the current Miller-Overton model (Miller et al. 1985), the ozone dose to the airway epithelium could be in the vicinity of $(2 \times 10^{-6}\ \mu g\ O_3/cm^2 \times min)/(ambient\ O_3/m^3)$; this value can be higher or lower, depending on ventilatory pattern and site of deposition in the lung and airway. Ozone's reaction path may be as deep as 10 μm. (Given that Miller predicted that less than 0.1% of the inhaled ozone would penetrate only as far as 2.2 μm, the 10 μm figure used for these calculations is likely to be conservative). The volume of a 1.0-cm^2 surface area that is 10 μm (0.001 cm) deep would be $1.0\ cm^2 \times 0.001\ cm = 0.001\ cm^3 = 0.001\ mL = 1.0\ \mu L$. At an ambient ozone concentration of 0.12 ppm (240 $\mu g/m^3$), the current ambient air quality standard), the resulting ozone concentration in the fluid epithelial layer would equal:

$$\frac{(2 \times 10^{-6}\ \mu g/cm^2) \times min}{(\mu g\ ambient\ O_3)/m^3} \times \frac{240\ \mu g\ O_3}{m^3} \times \frac{1.0\ cm^2}{1.0\ \mu L} \times \frac{1\ mol\ O_3}{48\ \mu g/\mu L}$$

$$= 1 \times 10^{-5}\ mol/minute\ or\ 10\ \mu mol/minute.$$

This could be integrated over a longer period, e.g., 60 minutes, and the exposure could be as high as 600 μmol/hour).

One mol of ozone reacts with 1 mol of unsaturated fatty acids to produce 1 mol of aldehyde plus 1 mol of hydroxyhydroperoxide. The hydroxyhydroperoxide decomposes to an additional 1 mol of aldehyde plus 1 mol of hydrogen peroxide.

Several other macromolecular targets (for example, thiols contained in proteins) can react with ozone. Miller and associates (1985) have estimated that a principal other reactant for ozone is histidine. Histidine accounts for 5.8 molar equivalents/cm3 × 107, and unsaturated fatty acids account for 12 molar equivalents/$cm^3 \times 10^7$, in airway mucus. (In the plasma membrane, unsaturated fatty acids are more abundant than in the airway lining fluids.) If we conservatively estimate that 20% of ozone reacts with fatty acids, this could yield as much as 200 μM/hour of ozonolysis products. This estimate is based on a study by Freeman and colleagues (Mudd and Freeman 1977; Freeman et al. 1979), who found that phospholipid vesicles and erythrocytes exposed to ozone consumed about 20% of the ozone in reactions producing hydrogen peroxide.

Notwithstanding the many physiological and physicochemical caveats associated with these estimates, concentrations of hydroxyhydroperoxides in the range of 30 to 100 μM appear to be achievable in vivo. Because effects were noted in this range, we believe that the doses used in this study certainly have toxicological significance to in vivo exposure. Please note that this is a theoretical and specula-

tive explanation, however, owing to the difficulty in predicting several experimental details (for example, completion of reaction sites).

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ABBREVIATIONS

^{13}C	carbon-13
CH_2Cl_2	dichloromethane
CI–MS	chemical ionization–mass spectrometry
^{51}Cr	chromium-51
5-diHETE	5-dihydroxyeicosatetraenoic acid
12-diHETE	12-dihydroxyeicosatetraenoic acid
15-diHETE	15-dihydroxyeicosatetraenoic acid
DMSO	dimethylsulfoxide
DPBS	Dulbecco's phosphate buffered saline
dpm	disintegrations per minute
eV	electronvolt
GC–MS	gas chromatography–mass spectrometry
HO•	hydroxy radical
H_2O_2	hydrogen peroxide
H_3PO_4	phosphoric acid
5-HETE	5-(*S*)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid
12-HETE	12-(*S*)-hydroxy-5,8,14-*cis*-10-*trans*-eicosatetraenoic acid
15-HETE	15-(*S*)-hydroxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid
12-HHT	12-hydroxy-5,8,10-heptadecatrienoic acid
HPLC	high-performance liquid chromatography
LTB_4	leukotriene B_4
MCDB-153	Molecular and Cellular Developmental Biology (a cell line)
NAAQS	National Ambient Air Quality Standard
$Na_2[^{51}Cr]O_4$	radiolabeled sodium chromate
NMR	nuclear magnetic resonance
^{18}O	oxygen-18
PCC	pyridium chlorochromate
PG	prostaglandin (B_2, D_2, E_2, $F_{2\alpha}$)
PGF1α	6-keto prostaglandin F1α
ppm	parts per million
RIA	radioimmunoassay
TxB2	thromboxane B_2

INTRODUCTION

Ozone, a highly reactive oxidant gas, is a major component of urban air pollution. When inhaled at sufficiently high concentrations, ozone can induce a wide range of effects in biological systems, many of which are of public health concern. One of the major unanswered scientific questions is the mechanism by which ozone causes its toxic effects.

The airway epithelial lining is an important site of action of ozone and other inhaled pollutants. In 1992, HEI published the report from a study performed by Dr. George Leikauf on the mechanisms of aldehyde-induced bronchial reactivity in airway epithelial cells; this study focused on the in vivo effects of low-molecular-weight aldehydes on eicosanoid metabolism and on other end points in guinea pigs (Leikauf 1992). Previously, Dr. Leikauf had demonstrated that ozone and low-molecular-weight aldehydes (formaldehyde, acetaldehyde, and acrolein) augment eicosanoid metabolism in canine, bovine, and guinea pig airway epithelial cells, and had proposed that compounds such as ozone and aldehydes induce hyperreactivity in part through injury to epithelial cells and alteration of the normal eicosanoid metabolism (Leikauf et al. 1988, 1989).

After completing the guinea pig studies, Dr. Leikauf requested funds from HEI for additional studies to investigate the effect of ozonolysis products on airway epithelial cells in vitro. He proposed to study the effects of products formed when ozone interacted with cell membranes in airway epithelial cells; the end point to be studied was eicosanoid metabolism.

The HEI Research Committee, having high regard for the work Dr. Leikauf had performed under the previous contract, felt that an extension of the studies to ozonolysis products would make a valuable contribution to the field. After peer review of the initial proposal, at the Research Committee's urging, Dr. Leikauf added an unpaid consultant to his research team, Dr. Jeffrey Santrock of the General Motors Research Laboratories, who had extensive experience in the chemistry of ozonolysis products. With this change, the Research Committee approved the project funding.

Total expenditures for the three-year project were $468,143. The Investigators' Report was received at the HEI in July 1993, and a revised report was received in April 1994. The revised report was accepted by the Review Committee in July 1994. During the review of the Investigators' Report, the Review Committee and the investigator had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary. The Health Review Committee's Commentary is intended to place the Investigators' Report in perspective, as an aid to the sponsors of HEI and to the public.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA) sets standards for oxidants (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1990. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Sections 202(a), (b)(1), (g), and (h), and Sections 207(c)(4) through (6) impose specific requirements for reductions in motor vehicle emissions of certain oxidants (and other pollutants) and, in some cases, provide the EPA with limited discretion to modify those requirements.

Section 109 of the Clean Air Act provides for the establishment of National Ambient Air Quality Standards (NAAQS)* to protect the public health. Ozone's potentially harmful effects on respiratory function led the EPA to promulgate the NAAQS for ozone of 0.12 parts per million (ppm), a level not to be exceeded for more than one hour once per year. Section 181 of the Act classifies the 1989 nonattainment areas according to the degree that they exceed the NAAQS and assigns a primary standard attainment date for each classification.

The current ozone standard relies heavily on data derived from controlled human exposure studies that have demonstrated lung dysfunction following acute exposures to ozone at concentrations similar to those of polluted urban air. However, the scientific knowledge regarding how ozone produces the adverse health effects is rudimentary. Studies to elucidate ozone's mechanism(s) of action are important to informed decision making as required under the Clean Air Act.

SCIENTIFIC BACKGROUND

FORMATION OF OZONE

Ozone, a highly reactive gas, is a major constituent of photochemical smog. Although ozone in the upper atmos-

* A list of abbreviations appears at the end of the Investigators' Report for your reference.

phere shields the earth from harmful ultraviolet solar radiation, ozone in the lower atmosphere is a major health concern. Ozone is not emitted directly into the atmosphere from human activities, but forms in the air in the presence of sunlight as a result of complex photochemical reactions between oxides of nitrogen and volatile organic compounds. Both volatile organic compounds and nitrogen oxides are emitted from motor vehicles and other sources. The current NAAQS for ozone is 0.12 ppm, a level not to be exceeded for more than one hour once per year. However, summertime peak hourly ozone levels in some urban areas often can reach levels as high as 0.3 ppm. The EPA has estimated that, in 1991, more than 69 million people lived in counties in which the ozone level exceeded the NAAQS (U.S. Environmental Protection Agency 1992).

HEALTH EFFECTS OF OZONE

Ozone is a powerful airway irritant, and data from a variety of studies have documented functional and biochemical responses to ozone exposure in animals and in humans (Lippmann 1989, 1993; U.S. Environmental Protection Agency 1993). Acute ozone exposure in humans can cause a reversible decrease in lung function and athletic performance, and an increase in respiratory symptoms (cough, shortness of breath, nose and throat irritation, difficulty with deep breathing). Ozone exposure also can cause transient airway hyperreactivity (increased responsiveness to inhaled bronchoconstrictors). It should be noted that the health effects of ozone depend on a number of factors (such as the dose, exposure conditions, and level of physical activity), and that there is a wide range of responses in the general population to ozone exposure.

The functional responses to ozone exposure noted above are accompanied by cellular and biochemical changes characteristic of both inflammation and injury in lungs (Menzel 1984; Mustafa 1990; U.S. Environmental Protection Agency 1993). Ozone exposure leads to an influx of polymorphonuclear leukocytes in the lung, increased airway permeability, and increased pulmonary (mucociliary and alveolobronchiolar) clearance. The bronchoalveolar lavage fluid from ozone-exposed individuals contains increased amounts of a number of biologically active compounds, including eicosanoids, cytokines, and fibronectin (Seltzer et al. 1986; Koren et al. 1989; Devlin et al. 1991; McDonnell 1991).

Although the functional, cellular, and biochemical changes caused by ozone have been the subject of intense study, ozone's precise mechanism of action is not understood. The cellular and biochemical effects described above suggest that, among other actions, ozone exposure injures the epithelial cells that line the airways. Several studies have documented the injurious effects of ozone on pulmonary epithelium (Plopper et al. 1978; Castleman et al. 1980; Chang et al. 1992). Epithelial cells in the centriacinar region, the junction between conductive airways and the respiratory region, are among the most vulnerable to ozone effects (Barry et al. 1985). Epithelial degenerative changes are observed very soon after ozone exposure; if exposure continues, simultaneous degenerative and repair-related changes may be seen. In addition to the pulmonary epithelium, other cell types (such as nasal epithelial cells and lung macrophages) also are affected by ozone exposure. Note that these effects appear to be sensitive to experimental conditions; factors such as the species under investigation, the health status of subjects, and the level of physical activity during exposure appear to influence the observed effects.

OZONE'S MECHANISM OF ACTION

A major question regarding ozone's toxicity is to what extent the pollutant exerts its effects directly and to what extent effects are mediated via secondary reaction products. It has been suggested that ozone, because of its reactivity, is unlikely to penetrate the fluid layer lining the lung or the epithelial cell membrane of the respiratory system, and that its physiological effects are likely to be mediated via the secondary products of its reactions with the fluid layer and the cell membrane (Pryor 1992). In vitro, ozone reacts with a wide range of chemical groups including carbon-carbon double bonds, sulfur in thiols, and amine groups in various cellular molecules (see Pryor 1992). Consequently, ozone reaction products formed in the body are a complex array of compounds, and ozone may exert its action through one or a combination of these compounds.

Ozone has been shown to react with unsaturated fatty acyl moieties in the lipid bilayer of the cell membrane (reviewed comprehensively in the Introduction section of the Investigators' Report). Ozonides, aldehydes, hydroxyhydroperoxides, and hydrogen peroxides are the major secondary and tertiary products formed from ozone's interaction with the cell membrane. Dr. Leikauf and his colleagues discuss the chemical reactions by which unsaturated fatty acid components of the cell membrane phospholipids may be degraded by ozone to form 3-, 6-, and 9-carbon saturated and unsaturated aldehydes and hydroxyhydroperoxides. These compounds are more stable than ozone and exhibit cytotoxicity.

Biochemical and cellular factors that lead to airway inflammation and hyperreactivity in response to inhaled toxicants (such as ozone) and in diseases (such as asthma) are currently under intense study. The epithelial cells are among the prime targets of ozone. In addition to forming a protective barrier, they appear to have certain metabolic roles, including the release of mediators, such as ei-

cosanoids and cytokines, and other substances, such as fibronectin (Shoji et al. 1990; Campbell et al. 1993; McKinnon et al. 1993).

Eicosanoids, the subject of the current study, are increasingly recognized as having an important role in airway reactivity, and in cell influxes observed in response to inhaled toxicants and in asthma (Holtzman 1991). Eicosanoids are metabolites of arachidonic acid, a major, unsaturated fatty acid component of the phospholipids. Insult to the cell membrane releases arachidonic acid from the membrane phospholipids via the action of the enzyme phospholipase A_2. An ensuing complex sequence of arachidonic acid reactions, catalyzed by the enzymes cyclooxygenase and lipoxygenase, results in the formation of eicosanoids (Holtzman 1991). Three broad groups of compounds within the eicosanoid family are the prostaglandins (PG) and thromboxanes (Tx), which arise from the cyclooxygenase pathway, and the leukotrienes (LT), which originate from the lipoxygenase pathway.

Experiments in vivo and in vitro suggest that eicosanoids can produce a wide spectrum of changes in the lungs. However, the different eicosanoid compounds generally exert their own distinct physiological effects, which are in some instances antagonistic to the effects produced by other eicosanoid family members; therefore, as a group, eicosanoids enable both selective stimulation and differential inhibition of cellular functions (Holtzman 1991). Some examples of the physiological effects of eicosanoids in the respiratory system are the bronchodilatory effect of PGE_2 and the bronchoconstrictive effects of $PGF_{2\alpha}$ and TxA_2; LTB_4 has a strong chemotactic effect on polymorphonuclear leukocytes and is a lymphocyte activator. Prostaglandin E_2 also appears to be a growth modulator and affects epithelial permeability as well. Some eicosanoids exert their action by stimulating nerve endings in the respiratory system (for example, $PGF_{2\alpha}$ may intensify reflex bronchoconstriction by sensitizing airway nerve endings).

The lung is a major site of eicosanoid synthesis and inactivation. The bronchodilatory and protective effect of PGE_2 has been inferred from a number of studies in human volunteers (Pavord et al. 1993; Melillo et al. 1994); however, therapeutic use of PGE_2 has been hampered by its side effects, which include transient cough and retrosternal soreness upon inhalation.

A number of studies report that ozone exposure increases the levels of several eicosanoids; the results of these experiments have not always been in agreement, probably in large part because of differences in study designs. In vitro experiments have demonstrated eicosanoid release from airway epithelial cells, alveolar macrophages, and polymorphonuclear leukocytes in response to ozone (Driscoll et al. 1988; Driscoll and Schlesinger 1988; Gunnison et al. 1990; Madden et al. 1991; McKinnon et al. 1993). In some studies, these effects were seen at relatively high levels (around 1 ppm) but not at low levels (around 0.1 ppm) of ozone.

Alterations in the levels of eicosanoids and other mediators in the bronchoalveolar lavage fluid in response to ozone exposure have been the subject of several studies. Elevated levels of several eicosanoids (for example, PGE_2 and $PGF_{2\alpha}$, thromboxane, and leukotrienes such as LTB_4) have been detected in bronchoalveolar lavage fluids obtained from volunteers exposed to low or modest levels of ozone, compared to those exposed to clean air. Most of these studies enrolled healthy human volunteers, who exercised intermittently at moderate levels during exposure to ozone (Seltzer et al. 1986; Koren et al. 1989; Devlin et al. 1991). The ozone-induced decrement in pulmonary function appeared to be partially prevented by treating subjects with cyclooxygenase inhibitors before exposure to ozone (Chatham et al. 1987; Schlegle et al. 1987; Eschenbacher et al. 1989; Hazucha et al. 1991). Similar observations have been reported in a number of animal studies (U.S. Environmental Protection Agency 1993).

Ozone inhalation provokes several responses, including airway hyperreactivity and influxes of inflammatory cells. The information regarding cellular and molecular targets of ozone's action and the physiological properties of eicosanoid mediators summarized above raises several questions regarding the underlying mechanisms of ozone's effects. Is direct damage to the epithelial or other cells in the airways the primary event in airway reactivity? What is the role of receptors in the response? What is the role of inflammatory cell influxes in airway reactivity and tissue injury? How important is the contribution of eicosanoids to airway reactivity, and what is their origin and mechanism of action? These and related questions continue to stimulate an active area of pulmonary research. The research described in Dr. Leikauf's report adds important new information toward the resolution of some of these questions.

RATIONALE FOR THE STUDY

Ozone studies are an important part of HEI's overall research program. The Institute has funded a number of studies on ozone dosimetry and on various aspects of the health effects of ozone exposure, including the mechanisms of action of ozone.

Because ozone is a very reactive oxidant gas, Dr. Leikauf and others have hypothesized that it rapidly reacts with unsaturated lipids present on or in the cell membrane to form aldehydes and hydroxyhydroperoxides, and that these compounds, rather than ozone itself, are the actual

chemical signals that produce the various cellular responses including alteration in eicosanoid metabolism. After a successful investigation of the effect of low-molecular-weight aldehydes on the guinea pig airway epithelium, Dr. Leikauf proposed to extend his studies to a detailed examination of the effects of ozone exposure on human airway epithelial cells.

The originally proposed studies had three main components:

- chemical synthesis and purification of selected products of ozone's reactions with unsaturated fatty acyl moieties of cell membrane phospholipids;
- examination of the effects of these compounds on the response of human airway epithelial cell cultures in vitro by determination of eicosanoid metabolism; and
- investigation of gene expression of the key enzymes involved in eicosanoid metabolism in response to exposure to ozone reaction products.

It was proposed that the synthesis and purification of ozone reaction products would be performed in the laboratory of Dr. Santrock who had extensive experience in this area. The biochemical studies would be conducted in Dr. Leikauf's laboratory. The HEI Research Committee considered these studies to be of value in providing a better understanding of the mechanism of action of ozone. However, the Committee questioned the need for the mechanistic gene expression studies at this stage of scientific understanding, and recommended deleting this portion of the work. The Committee then funded the two-year study reported here.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The investigators' long-term goal was to obtain a better understanding of the mechanism of ozone-initiated alterations in cellular functions. In the present study, they focused on the action of selected products of ozone's reaction with cell membrane constituents. Given the location of the double bonds in the most common unsaturated fatty acyl moities in membrane phospholipids, reaction with ozone is expected to yield 3-, 6-, and 9-carbon saturated and unsaturated aldehydes and hydroxyhydroperoxides. Therefore, the investigators synthesized and purified 3-, 6-, and 9-carbon aldehydes and hydroxyhydroperoxides, and studied the effects of these compounds on eicosanoid metabolism and on cell lysis in cultured human airway epithelial cells.

Overall, the investigators accomplished their objectives and demonstrated the importance of understanding the mechanism by which ozone exerts its effect. The results of this study highlight the potential role of secondary products generated from reactions between ozone and unsaturated fatty acids in epithelial cells and in the fluid lining the airways for producing the cellular injury associated with ozone exposure.

ASSESSMENT OF METHODS AND STUDY DESIGN

To examine the structure and activity relationships of ozone–fatty acid degradation products on eicosanoid metabolism in human airway epithelial cells, three subclasses of aldehydes and hydroxyhydroperoxides were synthesized by Dr. Santrock. The first group, the alkanals, comprised three saturated aldehydes: propanal, hexanal, and nonanal. The second group comprised the unsaturated aldehydes 3-hexenal and 3-nonenal. The third group comprised the five corresponding hydroxyhydroperoxides.

Chemical synthesis of the specific aldehydes and hydroxyhydroperoxides was verified by product analysis with proton nuclear magnetic resonance spectrometry and chemical ionization–mass spectrometry. The starting aldehyde and hydrogen peroxide were the major impurities present in the hydroxyhydroperoxides, although specific compound purity information was not provided. The investigators encountered difficulty in the preparation of the *cis*-alkenyl aldehydes, due to the low stability of these compounds, and thus did not them. In addition, the 9-carbon compounds were very hydrophobic and proved difficult to work with in the aqueous cell culture medium; the 6-carbon compounds were, therefore, preferentially used for many of the experiments even though the 9-carbon compounds produced a stronger response.

Dr. Leikauf and colleagues ed the compounds in cultured human airway epithelial cells grown from tissue explants in serum-free medium. Cells were incubated with [^{3}H]-labeled arachidonic acid, a major cell membrane component, in medium containing fetal calf serum. After 24 hours, the dishes were washed and exposed to the appropriate aldehyde, hydroxyhydroperoxide, or hydrogen peroxide. Total [^{3}H]-labeled eicosanoid release was determined by beta-scintillation counting.

The eicosanoids released by the cells were extracted from the culture medium and identified with radioimmunoassay or reverse-phase high-performance liquid chromatography. Cytolytic effects of the aldehydes and hydroxyhydroperoxides under study were determined by the use of a [^{51}Cr]-release assay. For statistical analysis, all values were recorded as means ± standard errors. The control values in any given experiment were pooled, and experimental and control values were compared using standard analysis of variance followed by *t* test.

The design and experimental methods used were appropriate for meeting the study goals, and the data are clearly presented. Overall, the investigators achieved their objectives with appropriate study design and methods.

STATISTICAL METHODS

The investigators ed for statistical significance by conducting analysis of variance to compare the various experimental treatment groups. If the analysis of variance showed statistical significance ($p \leq 0.05$), each treatment group was compared to the control group by the Student t test.

The statistical procedures used are appropriate, although the analyses could have been improved in several ways. In comparing more than one experimental group with a control group, the critical value of t should have been made stricter by using the Dunnett rather than the simple Student t test. Also, rather than using repetitive point-by-point comparisons, a two-factor analysis of variance in the data in Figures 1 and 2, or a curve-fitting approach for the data in Figures 3 through 7, would have provided more statistical power; curve-fitting is also preferable because it represents a more systematic approach for analysis of dose-response data.

RESULTS AND INTERPRETATION

Among the 3-, 6- and 9-carbon aldehydes and hydroxyhydroperoxides ed, the longer-chain-length compounds produced a stronger effect on eicosanoid release from cultured human airway epithelial cells than the shorter-chain-length compounds; this response was dose- and time-dependent. These results are consistent with the investigators' hypothesis that products of cell membrane ozonolysis stimulate eicosanoid release from airway epithelial cells.

In addition, the hydroxyhydroperoxide caused much greater eicosanoid release than did the corresponding aldehyde. Because hydroxyhydroperoxides can decompose to form hydrogen peroxide and the corresponding aldehyde, the question arises whether hydrogen peroxide mediates the greater cellular eicosanoid release exhibited in response to the hydroxyhydroperoxides. The investigators examined the effect of hydrogen peroxide alone and with hexanal and reported no difference in the dose-response relation. Therefore, they concluded that the eicosanoid release could not be attributed to hydrogen peroxide.

It is conceivable, however, that the hydroxyhydroperoxides penetrate or intercalate the cell membrane and release hydrogen peroxide in proximity to the target molecule(s). Released in the vicinity of the target site(s), hydrogen peroxide would presumably be more harmful than if it is released farther from the target site(s). Because longer-chain compounds are expected to penetrate the lipid bilayer to a greater extent than shorter-chain compounds, this hypothesis may also explain why the longer-chain compounds are more potent at inducing eicosanoid release than the shorter-chain compounds. This could be tested by measuring the formation of lipid hydroperoxides in the membrane of epithelial cells (see Implications for Future Research section). If the hypothesis is correct, more lipid hydroperoxides would be formed when cells are incubated with longer-chain-length hydroxyhydroperoxides than with shorter-chain-length hydroxyhydroperoxides.

The investigators used a [^{51}Cr]-release assay, an indicator of cell death, to the possibility that the increased [^{3}H]-labeled eicosanoid release after treatment with the compound was due to cytotoxicity. They report that [^{3}H]-labeled eicosanoid release was observed in the absence of any significant ^{51}Cr release at low concentrations of both 1-hydroxy-1-hexanehydroperoxide and hydrogen peroxide; at higher concentrations of these compounds, both [^{3}H]-labeled eicosanoids and ^{51}Cr were released. However, observations with hexanal showed the opposite pattern. The authors also investigated the time course of release of [^{3}H]-labeled eicosanoids and of ^{51}Cr. They report that maximum [^{3}H]-labeled eicosanoid release was reached some 120 to 240 minutes after exposure, whereas maximum ^{51}Cr release was observed after only 15 minutes. The reason for the differences in ^{51}Cr release, as well as the differences between the time course of release of [^{3}H]-labeled eicosanoids and ^{51}Cr, were not investigated further.

Detailed analysis of the [^{3}H]-labeled eicosanoids released after exposure to hexanal and to 1-hydroxy-1-hexanehydroperoxide permitted the chromatographic characterization and quantification of [^{3}H]-labeled arachidonic acid metabolites. The 11 metabolites identified after exposure represented a three-fold increase in ^{3}H release over control values. Prostaglandin E_2 represented approximately 38% of the released ^{3}H compared with 33% in the controls; therefore, the relative increase in PGE_2 synthesis, if any, was very modest. Other metabolites of arachidonic acid (such as hydroxyeicosatetraenoic acid derivatives, 6-keto $PGF_{1\alpha}$, and $PGF_{2\alpha}$) represented a much smaller percentage of the released radioactivity. Furthermore, with the exception of some of the hydroxyeicosatetraenoic acid derivatives, which exhibited up to a two-fold relative increase, most metabolites appeared to be released from the treated cells in roughly the same proportion as from control cells. It would therefore appear that, under the conditions of these experiments, the increased eicosanoid release following treatment with 1-hydroxy-1-hexanehydroperoxide is likely to be related to the greater amounts of arachidonic acid or eicosanoid metabolites available in the treated cells as compared to the control cells, and the effects are probably not due to the increased synthesis of any specific eicosanoid.

Prostaglandin E_2 was the major eicosanoid released in the medium, representing approximately one-third of the total ^3H released. Several other investigators have also reported that PGE_2 is a major product of eicosanoid metabolism in a variety of cell types (Becker et al. 1991; Holtzman 1991; Madden et al. 1991; Friedman et al. 1992; McKinnon et al. 1993). However, it should be noted that the pattern of arachidonic acid metabolism appears to be sensitive to experimental conditions. The studies on synthesis of prostaglandin and other eicosanoids have varied greatly in design: The stimulus type and the biochemical or immunological probes used to characterize the eicosanoids have differed, as have the animal species and the cell culture conditions. It is, therefore, difficult to draw any general conclusions regarding the physiological significance of the predominance of PGE_2 production observed in this study.

The mechanism of action of the aldehydes and hydroxyhydroperoxides on epithelial cells was not a part of this study. However, several possible mechanisms may explain Dr. Leikauf's observations. For example, the added hydroxyhydroperoxide may activate phospholipase or cyclooxygenases. Both of these enzymes have been shown to be susceptible to such activation (see Holtzman 1991). Another mechanism may involve the interaction of the aldehydes and hydroxyhydroperoxide with other proteins on the cell membrane, causing chemical changes in those proteins; the unsaturated aldehyde 4-hydroxy-2-nonenal has been shown to inactivate 2-glyceraldehyde-3-phosphate dehydrogenase in vitro (Uchida and Stadtman 1993). Those authors found that 4-hydroxy-2-nonenal preferentially reacted with cysteine and lysine residues in a Michael's addition reaction; the products of this reaction underwent secondary reactions with the lysine amino acid groups to yield inter- and intra-unit cross-links. No data are currently available to distinguish among the mechanisms outlined above.

IMPLICATIONS FOR FUTURE RESEARCH

The exact mechanism of action of the aldehydes and hydroxyhydroperoxides on the cell deserves further study. As discussed above, the compounds may directly or indirectly stimulate or inactivate some of the enzymes responsible for arachidonic acid metabolism, and future studies to investigate the mechanism of the observed effects would be useful.

One of the issues not fully resolved by this study is whether the hydroxyhydroperoxides exert their effect by decomposing into hydrogen peroxide and the corresponding aldehyde. As noted above, based on experiments with hydrogen peroxide alone and hydrogen peroxide plus hexanal, the investigators concluded that the effect could not be explained by the formation of hydrogen peroxide alone or in combination with aldehyde. However, another mechanism for the action of hydroxyhydroperoxide is that these compounds penetrate the cell membrane and release hydrogen peroxide in proximity to the target molecule(s). This hypothesis can explain several aspects of the observations reported in this study (discussed in the last section), and can be ed by measuring the formation of lipid hydroperoxides in the epithelial cell membrane. If the hypothesis is correct, more lipid hydroperoxides would be formed when cells are incubated with longer-chain-length hydroxyhydroperoxides than with shorter-chain-length hydroxyhydroperoxides.

Finally, the physiological relevance of eicosanoid synthesis needs to be explored to understand better whether the release of PGE_2 or some other eicosanoid is a specific and valid marker for the early effects of ozone.

CONCLUSIONS

Elucidating the mechanisms of toxic effects of oxidant gases is important for understanding their implications for health effects in humans and for establishing relevant ambient air quality standards for these pollutants. The study by Dr. Leikauf and colleagues reported here presents new and important findings related to ozone's mechanism of action.

Because ozone is highly reactive, it has been hypothesized that molecular ozone does not last long in the respiratory system; that, after inhalation, it reacts with various biological components to form more stable products that produce the toxic effects attributed to ozone. In the present study, the investigators chemically synthesized 3-, 6- and 9-carbon aldehydes and hydroxyhydroperoxides and ed them in vitro in human airway epithelial cells. The hydroxyhydroperoxides stimulated greater eicosanoid release than the aldehydes in a fashion that was dose- and time-dependent, and, most interestingly, dependent upon chain length. Prostaglandin E_2 was the major eicosanoid released.

The results of this study are consistent with the hypothesis that the physiological effects of ozone are produced through the somewhat more stable and less reactive products of ozonolysis of cellular molecules rather than by ozone itself. These findings provide a better understanding of the mechanism of ozone toxicity and suggest several avenues for further exploration of such toxicity.

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