Biochemical and Molecular Roles of Nutrients

Oleic Acid Incorporation Protects Cultured Hamster Fibroblasts from Oxygen-Induced Cytotoxicity\textsuperscript{1,2,3}

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ABSTRACT The effects of oleic, linoleic and arachidonic acid on oxygen toxicity were evaluated in cultured hamster fibroblasts. Each fatty acid was incorporated separately using a protocol that resulted in dose-dependent increases in the respective cellular fatty acid content to as much as 20-fold greater than unsupplemented controls. Linoleic acid produced no changes in cell survival after 48 h treatment with 95\% oxygen, regardless of fatty acid content of the cells. Oleic acid incorporation resulted in a dose-dependent increase in cell survival at 48 h in 95\% oxygen, whereas arachidonic acid incorporation resulted in a dose-dependent decrease in cell survival at 48 h in 95\% oxygen. No significant differences in amounts of linoleic or arachidonic acids were detected in control, oleic acid- enriched or linoleic acid-enriched cells during oxygen exposure. In cells enriched with arachidonic acid, exposure to oxygen significantly reduced the amounts of linoleic and arachidonic acid to 79 and 84\%, respectively, of the amounts found in air-exposed cells. The results indicate that oleic acid incorporation into cells provides protection against 95\% oxygen-induced cytotoxicity. In contrast, arachidonic acid incorporation led to sensitization of cells to 95\% oxygen-induced cytotoxicity that was accompanied by a loss of polyunsaturated fatty acids. As a result, it would appear that in situations of increased oxidative stress, high monounsaturated fatty acid diets that increase cellular oleic acid content may provide a protective environment compared with high polyunsaturated fatty acid diets that increase cellular arachidonic acid content. J. Nutr. 126: 2952–2959, 1996.

INDEXING KEY WORDS:

- hamster ovary fibroblasts
- oleic acid
- linoleic acid
- arachidonic acid
- oxidant injury

Patterns of lung injury in neonates following treatment with elevated levels of oxygen have been described clinically and in a number of experimental models [Bader et al. 1987, Bucher and Roberts 1981, Northway et al. 1967, Roberts et al. 1983, Yam et al. 1978]. The causes of these injuries are not fully understood, but they are generally believed to result from deleterious reactions of reactive oxygen species with the various classes of biomolecules in cells including membrane lipids [Jamieson 1989, Spitz et al. 1990, Sullivan et al. 1991]. Oxidation of membrane polyunsaturated fatty acids is an intriguing possible component of oxygen injury because the chain-reaction mechanism of autoxidation allows for an amplification of the injury [Porter 1990].

Researchers have utilized a number of intervention strategies to reduce or eliminate oxygen-induced lung injury. Treatment with vitamin E has produced mixed results but is generally viewed as clinically ineffective under conditions of pre-existing vitamin E sufficiency [Roberts and Matthew 1987]. Recently, a beneficial effect of the antioxidant activity of bilirubin has been proposed [Neuzil and Stocker 1994]. The most promising protective results have been achieved with manipulations of the antioxidant enzyme systems. Frank and co-workers [Frank and Roberts 1979, Frank et al. 1978] demonstrated that rats treated with endotoxin were protected against oxygen-induced mortality and lung damage in association with increased superoxide dis-


\textsuperscript{2} Supported by grants HL42057 and HL51469 from the National Institutes of Health.

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mutase [SOD], catalase and glutathione peroxidase activities in the lung. Wispe et al. (1992) and Warner et al. (1993) have achieved similar protective effects in mice transfected with additional copies of the MnSOD gene. Clinically, however, no single pharmacological agent is available that effectively reduces oxygen-induced lung injury in neonates.

Because cellular fatty acid composition can be altered by manipulating the fatty acid composition of the diet, the component of oxygen injury due to fatty acid oxidation should also be susceptible to manipulation. In the neonatal intensive care unit, lipid emulsion infusions are a vital component of the nutritional management of premature infants, especially very low birthweight infants. As a result, it would appear feasible to reduce oxygen-induced lung injury in these patients by manipulating the fatty acid composition of lung tissues through changes in the fatty acid composition of these lipid emulsions.

Our laboratory has shown that cultured cells enriched in the polyunsaturated fatty acids linoleic [18:2(n-6)] and eicosapentaenoic acid [20:5(n-3)] are sensitized to the toxicity of 95% oxygen, whereas cells that are enriched in the monounsaturated fatty acid oleic acid [18:1(n-11)] are protected (Spitz et al. 1992). The alteration of oxygen toxicity observed in these studies is consistent with the participation of polyunsaturated fatty acids in autodioxidation chemistry. This is in contrast to the relative inability of monounsaturated fatty acids to participate in this chemistry. There was, however, no correlation between the effects of these fatty acids on oxygen toxicity and the degree of unsaturation introduced into the cells as quantified by the double bond index or the polyunsaturated fatty acid: saturated fatty acid ratio. Our interpretation of these results was that individual fatty acids must be treated as unique nutrients with critical properties other than the number of chemical double bonds.

The purpose of this investigation was to specifically determine the relative merits of oleic acid vs. linoleic acid feedings in situations of high oxidative stress. As seen in Table 1, lipid emulsions used for the nutritional management of premature infants are composed primarily of linoleic acid. Linoleic acid is an essential fatty acid that is converted by most mammalian cells to arachidonic acid [20:4(n-6)], another essential fatty acid. The doses of lipid emulsion used are typically five- to sixfold greater than the essential fatty acid requirements of premature infants. Cultured cells that receive an excess of lipid in the medium have fatty acid compositions that reflect those treatments (Hart et al. 1992, Spitz et al. 1992). Similarly, animal and human experiments have shown that individuals fed high poly-

 unsaturated fatty acid diets have tissue fatty acid compositions that reflect those diets (Sosenko et al. 1989, van Schacky et al. 1985). One would therefore expect that lung cells and other cells of premature infants receiving this type of lipid emulsion could become enriched in both linoleic and arachidonic acids. However, if these emulsions were formulated such that they were predominantly oleic acid, the cells of infants receiving lipid emulsion infusions would instead become enriched in oleic acid. We hypothesize that, given the lesser ability of oleic acid to participate in lipid peroxidation chemistry relative to linoleic and arachidonic acids, preparations high in oleic acid would be preferred lipid sources in situations of high oxidative stress.

**MATERIALS AND METHODS**

**Cells and culture conditions.** Chinese hamster fibroblasts were maintained in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 1 × 10^5 U/L penicillin and 0.1 g/L streptomycin (MEM + 10% FCS) and grown at 37°C in a humidified tri-gas incubator. Cell cultures were routinely screened for mycoplasma contamination. For each experiment, cells were plated into 60-mm culture dishes at a density of 1 × 10^3 cells/dish and grown for 72 h in air containing 5% CO_2 to the starting densities used for the toxicity experiments. Cells were enriched in different fatty acids by incubation in MEM + 10% FCS with the specific fatty acids added as described below for this 72-h growth period. At the end of this period, the fatty acid-supplemented medium was removed and replaced with MEM + 10% FCS without added fatty acid for all groups of cells and the dishes placed in air/5% CO_2 or 95% O_2/5% CO_2 in humidified tri-gas incubators. The CO_2 and O_2 concentrations were verified using medical gas analyzers. Cytotoxicity was quantified by clonogenic cell survival as described below.

**Preparation of fatty acid enriched medium.** Oleic, linoleic and arachidonic acids were purchased at >99%.

### Table 1

<table>
<thead>
<tr>
<th>Fatty acid composition of common lipid emulsions for parenteral nutrition as described by the manufacturers</th>
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<tbody>
<tr>
<td>Fatty acid</td>
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<tr>
<td>Linoleic [18:2(n-6)]</td>
</tr>
<tr>
<td>Oleic [18:1(n-11)]</td>
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<tr>
<td>Palmitic [16:0]</td>
</tr>
<tr>
<td>Linolenic [18:3(n-3)]</td>
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<tr>
<td>Searic [18:0]</td>
</tr>
</tbody>
</table>

1 Soybean oil, Pharmacia, Columbus, OH.
2 Safflower and soybean oil, Abbott, Abbott Park, IL.
3 Soybean oil, Abbott.

1 Abbreviations used: 20:4(n-6), arachidonic acid; 20:5(n-3), eicosapentaenoic acid; FCS, fetal calf serum; 17:0, heptadecanoic acid; 18:2(n-6), linoleic acid; MEM, minimal essential medium; 18:1(n-11), oleic acid; 16:0, palmitic acid; SOD, superoxide dismutase.
purity from Nu-Chek-Prep (Elysian, MN). NaOH (1 mol/L) was made fresh in sterile water. The respective fatty acids (20 µL) were added to 200 µL of 1 mol/L NaOH in a plastic tube and the formation of the sodium salt of the fatty acid observed immediately. A 10-mL volume of warm sterile water was added, the solution gently mixed and sterile filtered through a two-stage 0.6-µm filter followed by a 0.2-µm filter into a sterile tube. Duplicate 200-µL aliquots were taken for analysis by gas chromatography to determine the fatty acid concentration. Typical fatty acid concentrations of these stock solutions were ~4 mmol/L. Small aliquots of the fatty acid stock solutions were added to appropriate volumes of MEM + 10% FCS to give the desired final fatty acid concentrations.

Quantitation of cytotoxicity. Cells were harvested by treating the plates with trypsin and were serially diluted in MEM + 5% FCS to ~4 × 10^6 cells/L. Volumes of this solution containing ~200 cells were transferred to a 60-mm dish containing 4 mL of MEM + 10% FCS. The cells were grown for 7 d, fixed in 70% ethanol and stained with Coomassie blue. Colonies containing >50 cells were counted as survivors. Cell survival was calculated as the number of colonies counted, divided by the number of cells originally placed in the dish.

Fatty acid analysis. For the analyses of cells, the media were discarded, the cells washed with ice-cold PBS and scraped into a second volume of PBS. The cells were pelleted by centrifugation, extracted and analyzed as described below. For the analyses of fatty acid stocks, 200 µL aliquots were extracted and analyzed as described below. For the analyses of the entire contents of a culture dish (combined cells and media) the cells were scraped into the media and collected in a glass tube. The dish was washed with 2 mL of PBS, combining the PBS washes in the glass tube. The samples were freeze-dried, the residue reconstituted in 200 µL of 0.1 mol/L acetic acid, and extracted and analyzed as described below.

For the fatty acid extraction and analyses, all samples were mixed with 2 mL of methanol and 4 mL of methylene chloride. A 50-µL volume of 50 mmol/L butylated hydroxytoluene in methanol and 100 µL of 100 mmol/L diethylenetriamine pentaacetic acid in 0.1 mol/L HCl were added as antioxidants along with 100 µL of an internal standard solution containing heptadecanoic acid (17:0). Dilute acetic acid (1 mL of 0.1 mol/L) was added, the samples mixed with a vortex and the phases separated by centrifugation for 1 min at 1000 × g. The top phase was discarded and the bottom phase fully evaporated. The fatty acids were converted to their methyl esters by reaction with 14% BF₃ in methanol at 70°C for 15 min and at room temperature overnight. The fatty acid methyl esters were extracted into petroleum ether, evaporated, and reconstituted in methanol for analyses by gas chromatography. The respective fatty acids were quantified using the ratio of the chromatographic peak areas of the fatty acid relative to the chromatographic peak area of the internal standard.

Statistical analyses. All assays were performed on three independent populations of cells. Significant differences were determined by either a one-way ANOVA using a Tukey-Kramer multiple comparisons test or a paired t test comparing 95% oxygen-treated cells with air-treated controls (Zar 1984).

**RESULTS**

Fatty acid incorporation into cellular lipids. Addition of oleic acid to the growth media increased cellular oleic acid content linearly from 12 ± 1 nmol/10^6 cells in unsupplemented cells to 135 ± 2 nmol/10^6 cells in cells treated with media containing 50 µmol/L oleic acid (Fig. 1). These same cells showed no alterations of the content of any other fatty acid measured with the exception of palmitic acid (16:0). Most notably, no significant differences were seen in cellular polyunsaturated fatty acid contents over this oleic acid range. Addition of linoleic acid to the growth media increased cel-
FIGURE 2 Fatty acid contents of hamster fibroblasts with increasing levels of linoleic acid in the growth media. All values were determined in triplicate and are plotted as the mean ± SD. Significant differences between a given treatment level and 0 μmol/L treated controls (*P < 0.05) are designated by an asterisk (*).

Linar linoleic acid content from 4 ± 1 nmol/10⁶ cells in unsupplemented cells to 169 ± 3 nmol/10⁶ cells in cells treated with media containing 50 μmol/L linoleic acid [Fig. 2]. Linoleic acid incorporation reduced cellular 18:1(n-9) content from 14 ± 1 nmol/10⁶ cells in unsupplemented cells to 5 ± 1 nmol/10⁶ cells in cells treated with media containing 50 μmol/L linoleic acid. Addition of arachidonic acid to the growth media increased cellular arachidonic acid content from 7 ± 2 nmol/10⁶ cells in unsupplemented cells to 138 ± 13 nmol/10⁶ cells in cells treated with media containing 50 μmol/L arachidonic acid but did not significantly alter cellular monounsaturated fatty acid content [Fig. 3].

Effects of fatty acid incorporation on oxygen-induced cytotoxicity. Increasing cellular oleic acid was associated with a significant increase in cell survival in 95% oxygen from 46 ± 3% in unsupplemented cells to 88 ± 10% in cells treated with 50 μmol/L oleic acid [Fig. 4]. In contrast, increasing cellular arachidonic acid was associated with a significant decrease in cell survival in 95% oxygen from 46 ± 3% in unsupplemented cells to 18 ± 1% in cells treated with 50 μmol/L arachidonic acid. No effects on cell survival in 95% oxygen were seen with increasing concentrations of linoleic acid in the growth media from 0 to 50 μmol/L. For all fatty acid

FIGURE 3 Fatty acid contents of hamster fibroblasts with increasing levels of arachidonic acid in the growth media. All values were determined in triplicate and are plotted as the mean ± SD. Significant differences between a given treatment level and 0 μmol/L treated controls (*P < 0.05) are designated by an asterisk (*).

Oleic acid
Linoleic acid
Arachidonic acid

FIGURE 4 Cell survival of hamster fibroblasts at 48 h in 95% oxygen with increasing levels of oleic, linoleic and arachidonic acid in the growth media. Cell survival was determined in triplicate at each concentration and is plotted as the mean ± SD. Significant differences between a given treatment level of a particular fatty acid and corresponding 0 μmol/L treated controls (*P < 0.05) are designated by an asterisk (*).
supplementation levels, cell survival at the onset of the experiment was greater that 85%, and cell counts were within experimental error limits of cells grown in unsupplemented media. These data indicate that the increases in cytotoxicity observed with arachidonic acid supplementation are not due to toxic oxidation products such as hydroperoxides in the fatty acid preparation.

**Changes in fatty acid content during oxygen exposure.** Results of quantitation of fatty acids in the cell culture systems during the time course of 95% oxygen treatment are shown in Figures 5 and 6. The data presented in these figures are for unsupplemented cells and cells treated with media supplemented with 5 μmol/L arachidonic acid. The data presented for unsupplemented cells in Figure 5 are representative of data obtained in similar experiments for cells treated with media supplemented with either 25 μmol/L oleic acid or 25 μmol/L linoleic acid; consequently, those data are not shown. Data are summarized for amounts of 18:1(n-11), 18:2(n-6) and 20:4(n-6) for each treatment group. The measurements were made by harvesting and extracting the entire contents of the culture dish,
specifically both cells and media containing 10% FCS. Therefore, these values represent the sum of cellular and media fatty acids. This design was necessary because as cells become injured by oxygen they often either lyse or detach from the dish, making difficult the complete recovery of intact cells for quantitative comparisons with air-treated cells. In addition, because essential fatty acids cannot be synthesized by the cell, the amounts of linoleic and arachidonic acids present in the combined cells and media represent all that is available to this system. Therefore, this design allows detection of damage to linoleic and arachidonic acids even if those fatty acids are replaced in the cell membrane because replacement of fatty acids in the cell membranes must utilize amounts of fatty acids present in other pools such as triacylglycerides.

Increasing quantities of 18:1(n-11) in the cell culture systems, seen in Figures 5A and 6A, were found over the time course of both air and 95% oxygen exposure in all fatty acid treatment groups. Similar increases were seen in the amounts of 16:1, 16:0 and 18:0 in the cell culture systems (data not shown). The quantities of 18:2(n-6) and 20:4(n-6) in the cell culture systems, illustrated in Figures 5B,C and 6B, and C, were constant in cells exposed to air in all fatty acid treatment groups. This trend was expected because 18:2(n-6) and 20:4(n-6) are essential fatty acids that cannot be synthesized by mammalian cells. No differences were seen in the quantities of 18:2(n-6) and 20:4(n-6) in systems exposed to 95% oxygen relative to systems exposed to air for unsupplemented, oleic acid–supplemented or linoleic acid–supplemented groups, as exemplified in Figure 5 for unsupplemented cells. However, as illustrated in Figure 6, in the arachidonic acid–supplemented cells, the quantities of 18:2(n-6) and 20:4(n-6) in the cell culture systems were significantly lower in those exposed to 95% oxygen relative to those exposed to air for the same period of time.

**DISCUSSION**

The purpose of these experiments was to determine the relative effects of different dietary fatty acids on oxygen-induced tissue injury. The effects of different fatty acid feedings were modeled in vitro by supplementing the medium in which cultured cells are grown with specific fatty acids. The data presented in Figure 4 show a significant dose-dependent sensitization of such cells to 95% oxygen-induced cytotoxicity by arachidonic acid incorporation and a dose-dependent protection of such cells from 95% oxygen-induced cytotoxicity by oleic acid incorporation. These data are consistent with the hypothesis that polyunsaturated fatty acid oxidation plays a role in the tissue injury associated with hyperoxia. The contribution of polyunsaturated fatty acid oxidation to oxygen toxicity was also tested by measuring the fatty acid levels in the culture systems during oxygen treatment. Because 18:2(n-6) and 20:4(n-6) are essential fatty acids, consumption of these fatty acids can be detected without compensating for synthesis. No detectable consumption of 18:2(n-6) or 20:4(n-6) in cells exposed to 95% oxygen was seen in the control, oleic acid– or linoleic acid–enriched cells. In contrast, Figure 6 shows the consumption of polyunsaturated fatty acid observed in Figure 6 with the lack of polyunsaturated fatty acid consumption seen in Figure 5, it is important to realize that the enrichment medium containing elevated concentrations of arachidonic acid was removed prior to oxygen exposure and replaced with standard MEM + FCS. Therefore, the only difference between the two systems illustrated in these figures is the cellular fatty acid content of the arachidonic acid–enriched cells. The lack of polyunsaturated fatty acid consumption in Figure 5 supports the interpretation that the consumption of polyunsaturated fatty acids seen in Figure 6 is a result of a cell-mediated interaction of oxygen with the cellular fatty acids. These data indicate that incorporation of arachidonic acid into cells creates a situation of enhanced polyunsaturated fatty acid oxidation that results in enhanced cytotoxicity.

The incorporation of the respective fatty acids from supplemented media into the cells is clearly seen in Figures 1, 2 and 3. In general, the incorporation is specific to the supplemented fatty acid with minimal changes in the quantities of other fatty acids in the cells. As an example, cells cultured in media enriched with 50 μmol/L oleic acid added 120 nmol of oleic acid/10^6 cells with no reduction in the linoleic or arachidonic acid content. Therefore, the incorporation data strongly support the interpretation that the effects of the different fatty acid supplementations on cytotoxicity are due to increases in the specific supplemented fatty acid rather than losses of other fatty acids. These data also show that the degree of enrichment achieved was comparable for each fatty acid treatment. The 50 μmol/L treatments yielded amounts of the incorporated fatty acid of 135 ± 2, 169 ± 1 and 138 ± 6 nmol/10^6 cells for oleic, linoleic and arachidonic acid, respectively. Therefore, it is unlikely that any effects of the fatty acid supplementations on oxygen-induced cytotoxicity can be attributed to a variation in the extent of incorporation of the different fatty acids into the cells.

At this time, possible molecular mechanisms of the protective effect of oleic acid incorporation are unknown. One postulated mechanism for this protective effect is that oleic acid inhibits lipid peroxidation. An alternative mechanism for this protective effect is that elevated concentrations of oleic acid may reduce damage to other biomolecules in a presently unknown manner. For example, oleic acid may be capable of partici-
pating in the reactions that lead to oxidative modification of proteins or DNA in a manner that lessens the degree of modification. Because oxidative modifications of both protein and DNA have been linked to the damaging effects of oxidative stress, a reduction of such modifications would be protective.

The lack of sensitization by linoleic acid is also intriguing. Because of bond dissociation energies, bis-allylic hydrogens in polyunsaturated fatty acids favor hydrogen abstraction reactions that initiate and propagate autoxidation chemistry (Koppenol 1990). Other investigators have shown that increasing bis-allylic hydrogen content in a fatty acid in solutions increases oxidizability in a manner that can be predicted by the reaction kinetics (Buettner 1993, Cosgrove et al. 1987). A recent report by Wagner et al. (1994) demonstrates that increasing bis-allylic hydrogens in cells increases the oxidizability of those cell by the iron(II)/ascorbate hydroxyl radical generating system. These data would predict that, if lipid peroxidation does contribute to oxygen toxicity, linoleic acid at 50 μmol/L should sensitize cells to the same extent as 17 μmol/L arachidonic acid, and 100 μmol/L linoleic acid should sensitize cells to the same extent as 33 μmol/L arachidonic acid. As seen in Figure 4, linoleic acid does not sensitize cells at concentrations up to 50 μmol/L. Additional experiments performed in our laboratory showed that concentrations up to ~100 μmol/L linoleic acid did not generate the degree of injury seen in Figure 4 with low amounts of arachidonic acid (data not shown). Therefore, our results indicate that some aspect of polyunsaturated fatty acid oxidation chemistry not modeled by these systems is contributing to the observed cytotoxicity. One possible explanation is that oxidation of different polyunsaturated fatty acids can generate different types and quantities of secondary products, such as aldehydes, that contribute to the cytotoxicity (Estebauer 1991). It is also possible that increased arachidonic acid content provides additional substrate for enzymatic activities associated with the arachidonic acid cascade. Cyclooxygenase, lipoxygenase and cytochrome P450 activities contribute to this cascade and have been associated with oxidative stresses (Dai et al. 1993, Rankin et al. 1991, Sporn et al. 1990). Therefore, increasing availability of substrate for these reactions could modulate the activities of these enzymes in a manner that enhances cytotoxicity.

In summary, the purpose of the studies described in this report was to test the effects of specific fatty acid supplantations on 95% oxygen-induced cytotoxicity. The rationale for these experiments was that if polyunsaturated fatty acid oxidation contributes to oxygen toxicity then alterations of the quantities of substrate for these reactions should alter the degree of injury. Oleic acid incorporation protected cells from oxygen toxicity, whereas arachidonic acid incorporation sensitized cells. Although these data would support a role for polyunsaturated fatty acid oxidation in the mechanism of oxygen toxicity, the lack of sensitization by linoleic acid suggests a more complicated role for polyunsaturated and monounsaturated fatty acids in oxygen toxicity. In particular, these data suggest that the relationship between fatty acid structure and oxidant injury goes beyond the number of bis-allylic hydrogens and additional factors must be considered to predict the effects of any given fatty acid on oxidant injury.

These data also indicate that lipid emulsion preparations with high oleic acid content would be a preferred agent for parenteral nutrition in situations of high oxidative stress. In the use of hyperoxia in the management of respiratory distress syndrome in premature infants, emulsions with oleic acid content would be preferred because they would lead to enrichment of tissues with oleic acid rather than linoleic and arachidonic acids. A practical advantage of using emulsions high in oleic rather than linoleic acid may also be a reduction in formation of potentially toxic oxidation by-products during storage or administration of the emulsions (Helbock et al. 1993, Marshall and Roberts 1990, Neuzil et al. 1995).

ACKNOWLEDGMENT

The authors thank Donna Adams for her expert technical support.

LITERATURE CITED


