EFFECT OF OXYGEN CONCENTRATION ON PRODUCTION OF ETHANE AND THIOBARBITURIC ACID-REACTIVE SUBSTANCES BY PEROXIDIZING LUNG AND LIVER HOMOGENATES AND FORMATION OF ETHANOL BY PEROXIDIZING DOCOSAHEXAENOIC ACID PREPARATIONS UNDER HYPEROXIC CONDITIONS

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The oxygen dependence of ethane formation was investigated in rat lung and liver homogenates, incubated in sealed flasks, in which the peroxidation was stimulated by the addition of ferrous ions. For both tissues, the production of ethane was maximal under a 20% oxygenated gas phase, while hyperoxic conditions led to a decreased ethane in the gas phase. The formation of thiobarbituric acid-reactive substances (TBA-RS), another marker of the lipid peroxidation process, in the homogenates of lung and liver was strongly stimulated at 100% compared to 20% oxygen. Experiments were also carried out on iron-stimulated peroxidation of pure docosahexaenoic acid preparations, which under air led to a large production of ethane. As for tissue homogenates, the TBA-RS content was increased in the presence of 100% oxygen. Those conditions, however, did not induce an increase in ethane production but led to the formation of ethanol. Therefore, the quenching of ethyl radical by molecular oxygen seems to be a very attractive hypothesis to explain the lack of increased ethane production in favor of ethanol when iron-induced lipid peroxidation was stimulated by oxygen.

The measurement of ethane and pentane in exhaled air has been proposed as a noninvasive method for the detection of a lipid peroxidation process. So far, this technique has mainly been applied in vivo following exposure to hepatotoxic chemicals (Riely et al., 1974; Wendel et al., 1979; Younes et al., 1983). In a previous study (Schweich et al., 1994), we did not find any stimulation of ethane production in exhaled air of rats in which lung injury had been induced by intratracheal instillation of free radicals producing chemicals. In vitro, lung tissue has been shown to be more resistant than the liver to iron-stimulated lipid peroxidation (Schweich et al., 1994). Besides the relative resistance of lung tissue to an oxidative stress, we also hypothesized that ethane might not be an appropriate marker of a lipid peroxidation process in well-oxygenated tissues. The proposed mechanism for ethane formation involves the production of an alkoxyl radical by homolysis

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of the peroxide bond of an omega-3 peroxidized fatty acid. Beta-scission of the alkoxyl radical induces the formation of an ethyl radical. Among the possible reactions of the ethyl radical, the abstraction of a hydrogen atom leads to the formation of ethane (Dumelin et al., 1977; Donovan et al., 1978). Although direct reaction of molecular oxygen with singlet organic molecule is spin forbidden, the quenching by molecular oxygen of carbon-centered radicals, such as ethyl radical, can be easily achieved. However, in tissues with high oxygen tension, this reaction might become quantitatively more important than hydrogen abstraction and, instead of ethane formation, lead to the formation of oxygen-containing compounds. The present study was undertaken to investigate in vitro the oxygen dependence of ethane formation during iron-stimulated peroxidation of both lung and liver tissues. Experiments were also carried out to examine the possible formation of ethanol during peroxidation of fatty acid preparations under hyperoxia.

MATERIALS AND METHODS

Perfused lung and liver isolated from male Sprague-Dawley rats (300–350 g) were homogenized and diluted in Tris buffer (0.05 *M*, pH 7.5) to obtain a protein concentration of 1 mg/ml. Proteins were determined by the Coomassie blue staining method with bovine gamma-globulin as standard. Glass flasks containing 4 ml homogenate were first bubbled with nitrogen for 15 min, sealed, and then a fraction of the total volume of the gas phase (0–100%) was substituted with pure oxygen. After 10 min of preincubation at 37°C, to allow oxygen equilibration between the gas and aqueous phases, lipid peroxidation was stimulated by the addition of an aqueous solution of $FeSO_4$ (Merck, Darmstadt, Germany), prepared freshly (final concentration 1.5 m*M*). Ethane concentration in the gas phase was determined after an incubation period of 5 h as described in Schweich et al. (1994). The evolution of TBA-RS production was also monitored in the lung and liver homogenates exposed to air or to oxygen-saturated gas phase by the method of Okhawa et al. (1979).

To assess the possible formation of ethanol, docosahexaenoic acid (Sigma Chemical Co., St Louis, Mo.) was mixed with Tris buffer (0.05 *M*, pH 7.5), and this preparation was used as substrate for lipid peroxidation (final concentration 12.5 mg/ml Tris). The preparations were bubbled for 15 min with either air or pure oxygen. The fatty acid preparations (volume 4 ml) were then submitted to peroxidation as described earlier. In order to avoid alcoholic fermentation by possible microorganism contamination, all the materials used were sterilized by autoclaving, all the solutions were filtered through a 0.22-μm filter (Sterivex, Millipore, Bedford, Mass.), and 70 μl amphotericin B (250 μg/ml, Gibco, Paisley, UK) and 30 μl gentamicin (50 mg/ml, Gibco) were added to the fatty acid preparations. Peroxidation was stimulated by addition of ferrous sulfate (1.5 m*M* final concentration). Ethane and ethanol were determined in the gas and aqueous phases, respectively, at

time 0 and after 24 h of incubation at 37°C. Ethanol was determined by removing 0.2 ml of the aqueous phase. The sample was filtered (Chromafill filter, 0.22 μm, Macherey-Nagel, Düren, Germany) before injection into a gas chromatograph (Carlo Erba 6000) equipped with a 25-m capillary column (CP-WAX-52-CB, Chrompack, Antwerp, Belgium). The temperature of the column was held at 95°C and the carrier gas used was helium. The detector was a mass spectrometer (Finnigan MAT ion trap detector 700). The method was optimized for ethanol determination by using the multiple ion detection program with selection of the main fragments (m/z = 43 and m/z = 45). Calibration was achieved with serial dilutions of absolute ethanol (UCB, Leuven, Belgium) in Tris buffer. The detection limit was 43.5 nmol ethanol/ml. In some experiments, the antioxidant butylated hydroxytoluene (BHT, Sigma Chemical Co., St. Louis, Mo.) was added to the fatty acid preparations (final concentration of 1.4% w/v).

In a separate experiment, the production of TBA-RS was measured in fatty acid preparations (6.25 mg docosahexaenoic acid/ml Tris) incubated for 24 h under air or pure oxygen in the presence of ferrous ions (1.5 mM).

RESULTS

In order to assess the effect of oxygen on ethane production, lung and liver homogenates were incubated with ferrous ions under various oxygen concentrations in the gas phase. Preliminary experiments showed that ethane progressively accumulated in the gas phase, with a maximal concentration reached after 5 h. Figure 1 shows the ethane production from liver

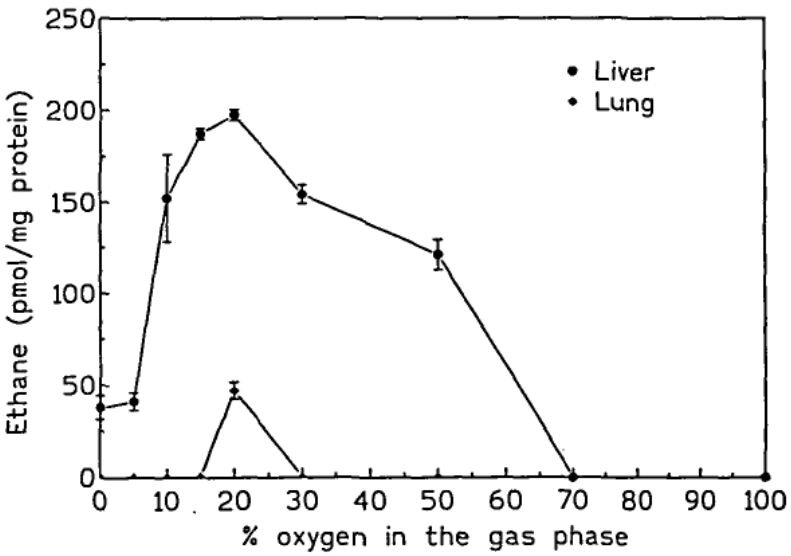

FIGURE 1. Ethane production by liver and lung homogenates (1 mg protein/ml) incubated for 5 h with $FeSO_4$ (1.5 mM) under various oxygen concentrations. Values are means ± SE of three animals.

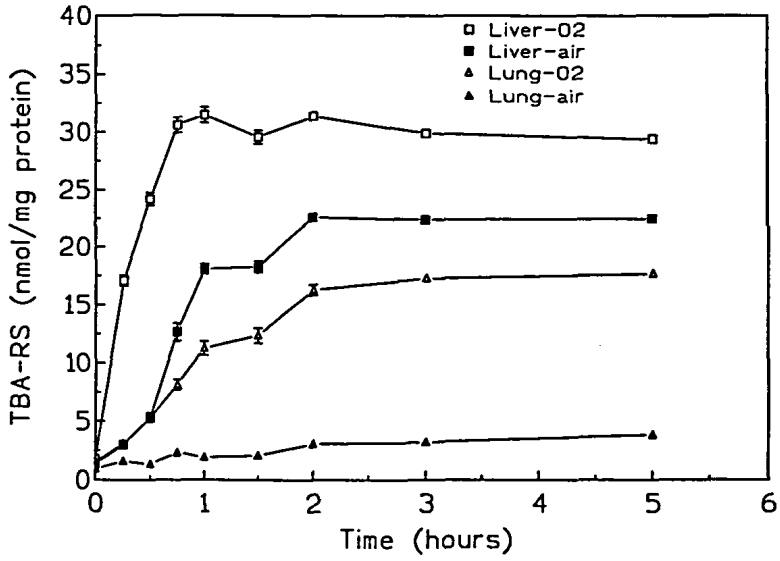

FIGURE 2. Time course of TBA-RS production from lung (n = 3) and liver (n = 3) homogenates (1 mg protein/ml) incubated in the presence of $FeSO_4$ (1.5 m*M*) under air or oxygen-saturated conditions. Values are means ± SE.

tissue after a 5-h incubation period. Maximal production was observed under a 20% oxygenated gas phase. Below or above this concentration, the production of ethane was lower and even became undetectable above 70% oxygen. In lung homogenates, ethane was only detected when incubated under 20% oxygen. While ethane formation was totally inhibited under hyperoxic conditions for both tissues, the production of TBA-RS in the homogenates of lung and liver was strongly stimulated at 100% compared to 20% oxygen (Figure 2). Due to the low sensitivity of the method used for ethanol quantification (43 nmol/ml aqueous phase, compared to 1.5 pmol/ml gas phase for ethane), we were not able to demonstrate ethanol formation from lung or liver homogenates under hyperoxia. Therefore, the following experiments were performed with a pure preparation of docosahexaenoic acid, a fatty acid precursor of ethane. As for homogenates, the TBA-RS production resulting from iron-stimulated peroxidation of docosahexaenoic acid was increased in the presence of 100% oxygen (746 nmol/ml vs. 343 nmol/ml in air-bubbled preparations). Before starting the incubation, ethane and ethanol were not detected either in aerated or in oxygenated fatty acid preparations. The incubation of docosahexaenoic acid preparations under air in the presence of ferrous ions led to a large accumulation of ethane in the gas phase (Table 1). Hyperoxic conditions did not induce a significant increase in ethane production (Table 1, $p < .05$, modified Student's t-test for unequal variances). However, ethanol was only detected in the aqueous phase of preparations flushed with 100% oxygen (Table 1). Ethane production induced by iron-stimulated peroxidation of docosahexaenoic acid was nearly totally

prevented by BHT in both air- (0.13 nmol/ml) and oxygen-bubbled (0.2 nmol/ml) preparations. Likewise, ethanol could not be detected in any preparations when incubations were performed with the antioxidant.

DISCUSSION

The oxygen dependence of ethane formation was investigated in lung and liver tissues in which peroxidation was stimulated by the addition of ferrous ions. In vitro, Thom and Elbuken (1991) described an antagonistic effect of oxygen on the rate of peroxidation of fatty acid preparations assessed by the measurement of conjugated dienes and TBA-RS products, two other markers of lipid peroxidation. However, this effect was shown to be dependent on pH and on the presence of monounsaturated fatty acids. In our experiments, an inhibitory effect of oxygen on the process of peroxidation is excluded since the production of TBA-RS was greatly stimulated by oxygen in both lung and liver homogenates. Maximal amounts of ethane were detected in both tissues at 20% oxygenated gas phase, while hyperoxic conditions led to a decreased ethane production. However, those in vitro conditions probably do not perfectly reproduce the in vivo situation. Oxygen is only poorly soluble in aqueous solution and, in our experiments, the liver and lung homogenates were diluted in Tris buffer, on the average, 25- and 15-fold, respectively. Thom and Elbuken (1991) reported that, using fatty acid preparations, oxygen partial pressures in the gas phase above the physiological range were required to achieve dissolved oxygen concentrations similar to those expected in vivo. In the lung, due to its role in gaseous exchange and its large surface exposed to air, oxygen concentrations in cell membranes are probably higher than in diluted lung homogenates. The slight production of ethane detected in liver preparations bubbled with nitrogen could be explained by the persistence of traces of oxygen sufficient to induce a lipid peroxidation process; since the lung tissue is more resistant to peroxidation than the liver, a similar phenom-

TABLE 1. Ethane and Ethanol Production Induced by $FeSO_4$-Stimulated Peroxidation of Docosahexaenoic Acid Preparations Incubated Under Air or Pure Oxygen

	Ethane (nmol/ml suspension, gas phase)	Ethanol (nmol/ml suspension, liquid phase)
Air	123 ± 4.5	ND
100% O_2	159 ± 25.3	119 ± 18

Note. Values are means ± SE for four experiments. ND, not detectable. Preparations (12.5 mg/ml Tris) were incubated at 37°C for 24 h. Difference in ethane production following incubation under air or 100% O_2 was not statistically significant.

enon was not observed in this tissue. We performed experiments on the whole lung and liver homogenates and therefore have to consider a possible stimulatory effect of oxygen on ethane metabolism. The in vitro evaluation of ethane metabolism is precluded by its gaseous nature and insolubility in aqueous solution. However, in vitro experiments carried out on the metabolism of pentane, a more soluble gas, have shown that under a 100% oxygenated gas phase less than 10% of pentane added to a liver microsomal preparation was metabolized in the presence of NADPH (Reiter et al., 1987).

Smith and Reilly (1989) demonstrated the inhibition of pentane formation in favor of 1-pentanol when peroxidation of hydroperoxyeicosatetraenoic acid was achieved in oxygen-saturated buffer. We have indeed found that iron-stimulated peroxidation of a pure docosahexaenoic acid preparation produced ethanol when the preparations were bubbled with pure oxygen before starting the incubation. Contrary to experiments performed on homogenates, we did not find inhibition of ethane formation in oxygenated pure fatty acid preparations. However, peroxidation of docosahexaenoic acid was only assayed after a 24-h incubation period and the oxygen consumed in the course of the process was not replaced. Progressive deoxygenation of the system might have allowed ethane production. Moreover, as in homogenates, the peroxidation of docosahexaenoic acid when evaluated by the TBA-RS production increased in a 100% oxygen-bubbled preparation. Therefore, the quenching of ethyl radical by molecular oxygen seems to be a very attractive hypothesis to explain the lack of increased ethane production in favor of ethanol formation when iron-induced lipid peroxidation was stimulated by oxygen. However, before assessing ethanol formation in peroxidizing lung and liver homogenates, improvement of the method used for quantitative determination of ethanol is required.

In conclusion, we have shown in vitro that oxygen could inhibit ethane formation from both peroxidizing lung and liver tissues and lead to ethanol formation in peroxidizing pure docosahexaenoic acid preparations. Degradation of hydroperoxide lipids involves a very complex set of reaction pathways and can lead to a large variety of products such as alkanes, aldehydes, and other oxygenated products (Frankel, 1982). Among these, ethane is generally presented as a marker of choice for detecting a lipid peroxidation process in vivo, since its determination can be achieved by a rapid and sensitive gas chromatographic analysis of the expired air. However, owing to the inhibitory effect of oxygen on its formation, ethane might not be an appropriate marker of a lipid peroxidation process in some well-oxygenated tissues (i.e., lung, heart) or under hyperoxic conditions.

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