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EVIDENCE THAT LIPID PEROXIDATION IN MICROSOMAL MEMBRANES OF EPIDERMIS IS ASSOCIATED WITH GENERATION OF HYDROGEN PEROXIDE AND SINGLET OXYGEN

Rakesh Dixit, Hasan Mukhtar and David R. Bickers

Department of Dermatology, Case Western Reserve University,
University Hospitals of Cleveland and
Veterans Administration Medical Center
Cleveland, Ohio 44106

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The role of superoxide, singlet oxygen and hydrogen peroxide in the augumentation of enzymic lipid peroxidation in epidermal microsomes was assessed. Incubation of epidermal microsomes with NADPH or xanthine oxidase-system resulted in significant generation of lipid peroxides as measured by malondialdehyde formation. Epidermal lipid peroxidation was inhibited by catalase and singlet oxygen quenchers, 2,5-dimethylfuran, histidine and β carotene whereas OH^- scavengers, benzoate, mannitol and ethyl alcohol had no effect. Superoxide dismutase had no protective effect on lipid peroxidation. Vitamin E and cytochrome c^{3+} substantially inhibited the epidermal lipid peroxidation. These studies demonstrate that polyunsaturated lipids of skin microsomes are susceptible to peroxidation. Chemical agents known to protect against the toxic effects of singlet oxygen and hydrogen peroxide inhibited microsomal lipid peroxidation suggesting that these moieties may play a significant role in mediating the reaction.

INTRODUCTION

The biological importance of unsaturated lipids for maintenance of structural and functional integrity of biomembranes has generated a surge of interest in the peroxidation of lipids. Skin is the largest body organ and serves as a major protective barrier between the body and its environment. Cutaneous tissue therefore assumes an important role in regulating the entry of toxic xenobiotics into the body and in maintaining homeostasis. Recent studies indicate that membrane lipids of skin can undergo active peroxidation on exposure to environmental agents which produce oxygenated free radical species (1-3). Photosensitization of skin on exposure to certain drugs and light as well as the aging of human skin (4,5) are thought to be related to increased peroxidation of membrane lipids (3,6). Since the early observation of Hochstein et al. (7) of an enzymatic NADPH-dependent oxidation of microsomal lipids, a number of mechanisms have been proposed for lipid peroxidation.

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Lipid peroxidation in microsomal membranes is induced by either superoxide radicals (O_2^-) generated by 1 electron reduction of molecular oxygen and/or hydroxyl radical or hydrogen peroxide and singlet oxygen (8-10). Despite the possibility that lipid peroxidation of skin membranes may be a mechanism whereby photosensitization, cellular aging, UV-induced damage to cells and certain dermatological disorders are mediated (1-6), no prior studies have attempted to understand the mechanism of lipid peroxide formation in skin. Evidence presented in this study shows that hydrogen peroxide and singlet oxygen may mediate peroxidation of microsomal lipids of the epidermis.

MATERIALS AND METHODS

Thiobarbituric acid, mannitol, β-carotene, sodium benzoate, xanthine, L-ascorbic acid, superoxide dismutase, xanthine oxidase, and catalase were purchased from Sigma Chemical Company, St. Louis, MO. All other chemicals were purchased in the highest purity commerically available.

Animals: Sperm-positive pregnant Sprague-Dawley rats were obtained from Holtzman Rat Farm, Madison, Wisconsin and shipped during the last trimester. Neonatal rats born in situ were allowed to suckle until the 4th day after birth.

Preparation of Tissue: The neonatal rats were sacrificed by decapitation with surgical scissors. The head, and extremities of the animal were removed and whole skin from the remaining body, which constitutes 80-90% of total skin, was excised and immediately placed in ice cold 0.15 M KCl. Each skin was placed epidermal side down on a covered glass petri dish containing crushed ice. The skin was scraped with a sharp scalpel blade (Bard-Parker No. 20) to remove subcutaneous fat and muscle. For the separation of epidermis and dermis the method of Epstein et al. (11), was adapted. Epidermal microsomes were prepared essentially as described earlier (12,13). The microsomal pellet was washed once using 0.1 M phosphate buffer, pH 7.4, containing 10^{-4}M $MgCl_2$, and was finally suspended and homogenized in the same buffer.

Assay of lipid peroxidation:

Malondialdehyde levels as a measure of lipid peroxide formation were estimated by the method described by Wright et al. (14). NADPH-mediated lipid peroxidation was carried out by incubating 1.4 - 1.8 mg microsomal protein, 1 mM NADPH in 0.6 ml of Ca^{+2} free 0.1 M phosphate buffer pH 7.4 containing 10^{-4}M $MgCl_2$ for 60 minutes at 37°C. Blanks were prepared using microsomes only. The reaction was terminated by the addition of 0.6 ml of 10% (w/v) trichloroacetic acid, 1.2 ml of 0.5% (w/v) 2-thiobarbituric acid was added to the mixture and this was heated for 10 minutes in a boiling water bath. After cooling under running tap water the contents of the tubes were centrifuged and levels of malondialdehyde were measured by recording the absorbance of color formed at 535 nm. Final concentration of malondialdehyde was calculated using a molar extinction coefficient of $1.56 \times 10^5 M^{-1} cm^{-1}$.

RESULTS

Lipid peroxide formation in epidermal microsomes

NADPH-stimulated lipid peroxidation in microsomal membranes of epidermis is summarized in Table 1. Significant formation of malondialdehyde occurred when microsomes were incubated with NADPH in the presence of oxygen. Formation of

Table 1
Lipid peroxide formation in microsomal membranes of epidermis.

INCUBATION SYSTEM	LIPID PEROXIDE (n mole MDA/mg protein/60 minutes)
Microsomes	0.39 ± 0.02
+ NADPH (0.6 μmole)	1.30 ± 0.02
+ NADPH (1.2 μmole)	1.82 ± 0.21
+ NADPH (1.8 μmole)	1.90 ± 0.25
+ NADH (1.5 μmole)	0.41 ± 0.03
+ Fe^{+3} (1 mM) + ADP (5 mM)	3.31 ± 0.41
+ Xanthine oxidase* system	6.49 ± 0.72
Boiled Microsomes	0.19 ± 0.02
+ NADPH (1.8 μmole)	0.20 ± 0.03

*Xanthine oxidase system consisted of 1.5 mM xanthine, 200 μg/ml xanthine oxidase and Fe^{+3}-ADP (1 mM - 5 mM), in 0.6 ml incubation mixture.

Data represent Mean ± S.D. of 3 experiments.

MDA = malondialdehyde

Assay mixture consisted of 0.1 M phosphate buffer pH 7.4, 1.2 - 1.8 mg microsomal protein and following contents. Details of malondialdehyde estimation are provided in the text.

malondialdehyde in epidermal microsomes was NADPH-dependent and was linear up to 1.5 - 2.0 μmole. NADPH-supported lipid peroxidation showed a critical requirement for oxygen and was inhibited in the presence of nitrogen or carbon monoxide (data not shown). Microsomal lipid peroxidation was linear up to 70 minutes of incubation (data not shown). NADH was ineffective in supporting lipid peroxidation in epidermis. Boiling the microsomes destroyed the NADPH-supported lipid peroxidation. Xanthine oxidase added to an aerobic reaction with xanthine produces reactive oxygen species including O_2^- and H_2O_2 which can cause lipid peroxidation (15,16). Incubation of epidermal microsomes with a xanthine - xanthine oxidase system resulted in a enhanced formation of malondialdehyde (Table 1).

Effect of superoxide dismutase and catalase on epidermal lipid peroxidation:

Superoxide dismutase is an active scavenger of O_2^- ($2H^+ + 2O_2^- \dashrightarrow H_2O_2 + O_2$) (8). Catalase inactivates H_2O_2 at a rapid rate (8-10). To assess the role of O_2^- and H_2O_2 in the initiation of lipid peroxidation in epidermal microsomes, the effect of these scavenger

Table 2

Effect of superoxide dismutase and catalase on NADPH and Xanthine oxidase supported lipid peroxidation in epidermal microsomes.

INCUBATION SYSTEM	LIPID PEROXIDE (n mole MDA/mg protein/60 minutes)	
	NADPH-supported	Xanthine* Oxidase-supported
Complete	1.61 ± 0.02	6.49 ± 0.72
+ SOD (25 μg/ml)	1.62 ± 0.03	6.58 ± 0.51
+ SOD (50 μg/ml)	1.72 ± 0.01	6.60 ± 0.61
+ Catalase (200 μg/ml)	1.21 ± 0.01	4.50 ± 0.42
+ Catalase (500 μg/ml)	1.03 ± 0.01	4.40 ± 0.32
+ Boiled SOD (50 μg/ml)	1.56 ± 0.04	6.30 ± 0.41
+ Boiled Catalase (500 μg/ml)	1.58 ± 0.05	6.40 ± 0.53

*Xanthine oxidase system consisted of 1.5 mM xanthine, -xanthine oxidase (200 μg/ml), Fe^{+3} (1 mM) and ADP (5 mM) in 0.6 ml incubation mixture.

Data represent Mean ± S.D. of 3 experiments.

SOD = superoxide dismutase

Complete system consisted of 0.1 M phosphate buffer pH 7.4, 1.2 - 1.8 mg microsomal protein and 1 mM NADPH. Other details are provided in text.

enzymes on lipid peroxidation was studied. Superoxide dismutase had no inhibitory effect on peroxidation whereas catalase significantly inhibited the formation of lipid peroxides. Heat-inactivated enzymes were ineffective in this respect. In the xanthine -xanthine oxidase stimulated peroxidation system, superoxide dismutase was ineffective, while addition of catalase protected the microsomal membranes from peroxidation (Table 2).

Effect of hydroxyl radical and singlet oxygen scavengers on epidermal lipid peroxidation:

In order to understand the role of hydroxyl radicals and singlet oxygen in the initiation of lipid peroxidation in epidermal microsomes the effect of scavengers of these agents on NADPH-supported lipid peroxidation was studied. Data in Table 3 indicate that none of the hydroxyl radical scavengers including benzoate and mannitol (10) provided any protection against peroxidation of microsomal lipids. Singlet oxygen quenchers such as 2,5-dimethyl-furan, histidine and β-carotene (10) afforded significant protection against peroxidation of

Table 3

Effect of hydroxy radical and singlet oxygen quenchers on NADPH supported lipid peroxidation in epidermis.

INCUBATION SYSTEM	LIPID PEROXIDE (n mole MDA/mg protein/60 minutes)
Complete	1.66 ± 0.12
+ Benzoate (5 mM)	1.61 ± 0.15
+ Mannitol (5 mM)	1.58 ± 0.15
+ ETOH (5 mM)	1.45 ± 0.11
+ Histidine (5 mM)	0.69 ± 0.05
+ 2, 5-Dimethylfuran (5 mM)	0.79 ± 0.06
+ β-Carotene (10 μM)	0.53 ± 0.04
+ α-Tocopherol (10 μM)	0.59 ± 0.03
+ Cytochrome c^{+3} (0.1 mM)	0.55 ± 0.03

Data represent Mean ± S.D. of three experiments.

ETOH = ethyl alcohol

Complete system consisted of 0.1 M phosphate buffer pH 7.4, 1.4 - 1.8 mg microsomal protein and 1 mM NADPH. Details of the method are provided in text.

microsomal lipids. Cytochrome c^{+} which competes with cytochrome P-450 for reducing equivalents from flavin reductase resulted in a strong inhibition of lipid peroxidation. Vitamin E(α-tocopherol), a potent antioxidant offered significant protection against peroxidation of microsomal lipids in epidermis.

DISCUSSION

Membrane lipids of epidermal microsomes undergo active peroxidation in the presence of NADPH and oxygen, or in a xanthine oxidase-stimulated system. Several mechanisms have been proposed to explain the initiation of NADPH-supported enzymic lipid peroxidation in liver and lung microsomes. One proposed mechanism centers on the generation of O_2^- resulting from microsomal oxidation of endogenous substrates or autooxidation of xenobiotics (8-10). Our data indicate that the catalytic scavenger of O_2^-, superoxide dismutase, was ineffective in providing any protection against epidermal lipid peroxidation. This indicates either that O_2^- is not produced in the epidermal microsomes or if it is produced it

is not responsible for lipid peroxidation. Inhibition of NADPH-supported or xanthine oxidase-stimulated lipid peroxidation by catalase, a scavenger of H_2O_2 suggests that H_2O_2 may be involved in the peroxidation of microsomal lipids. H_2O_2 is produced in significant amounts during incomplete oxidation of certain substrates by microsomal monooxygenases and NADPH cytochrome c reductase (17,18). The reaction of O_2^- with H_2O_2 produced in microsomes can also result in the generation of hydroxyl radical and singlet oxygen ($H_2O_2+O_2^- \longrightarrow OH^-+OH^{\cdot}+O_2^*$) which are effective initiators of lipid peroxidation (8). Since hydroxyl radical scavengers were ineffective in inhibiting lipid peroxidation in epidermis, it is unlikely that hydroxyl radicals are involved in peroxidation of epidermal lipids. Singlet oxygen (O_2^*) quenchers such as 2,5-dimethylfuran, histidine and β carotene inhibited the peroxidation of epidermal lipids. This indicates the possibility that singlet oxygen produced during the reaction of O_2^- and H_2O_2 is capable of initiating lipid peroxidation in epidermal microsomes. Reduction of oxygen to superoxide by flavin reductase and the subsequent production of H_2O_2 and hydroxyl radicals or singlet oxygen is important as a side reaction in microsomes (17,18). Since epidermal microsomes contain substantial activity of flavin reductase (12,13), it is possible that lipid peroxidation in microsomal membranes of skin, as a consequence of H_2O_2 and singlet oxygen production is an ongoing process. A primary defense is provided by catalase which effectively removes H_2O_2 before it triggers membrane lipid peroxidation. Secondary defense would be offered by singlet oxygen quenchers against singlet oxygen which is more potent than H_2O_2 in causing membrane damage. Since vitamin E (α-tocopherol) inhibited lipid peroxidation in epidermis it may therefore offer another secondary defense against singlet oxygen associated lipid peroxidation in epidermal microsomes.

In conclusion our results demonstrate that enzymic (flavin reductase and cytochrome P-450) reduction of oxygen in the presence of NADPH in epidermal microsomes results in the formation of reactive oxygen species including H_2O_2 and singlet oxygen which initiate peroxidative deterioration of membrane lipids in epidermis. This membrane damage in epidermis can be offset by a number of homeostatic cellular defense mechanisms including that of catalase and singlet oxygen quenchers.

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