Lipid Peroxidation in Adrenal and Testicular Microsomes

by Walter C. Brogan, III,* Philip R. Miles,** and Howard D. Colby*

Studies were carried out to determine the actions of and interactions between ascorbate, NADPH, Fe2+, and Fe3+ on lipid peroxidation in adrenal and testicular microsomes. Ascorbate-induced malonaldehyde production was maximal in adrenal and testicular microsomes at an ascorbate concentration of 1 x 10^-4 M. Fe2+, at levels between 10^-5 and 10^-3 M, produced concentration-dependent increases in lipid peroxidation in adrenal and testicular microsomes; Fe3+ had a far greater effect than Fe2+ in both tissues. In liver microsomes, by contrast, Fe2+ and Fe3+ had quantitatively similar effects on lipid peroxidation. NADPH alone had no effect on malonaldehyde production in adrenal or testicular microsomes. However, in the presence of low Fe3+ concentrations (10^-5 M), NADPH stimulated adrenal malonaldehyde production. The stimulation of lipid peroxidation by NADPH plus low Fe2+ was not demonstrable in testicular microsomes nor in adrenal microsomes which had been heat-treated to inactivate microsomal enzymes. Testicular malonaldehyde production was stimulated by NADPH if Fe3+ (5 x 10^-4 to 1 x 10^-3 M) was added to the incubation medium; the stimulation was not demonstrable in heat-treated microsomes. Fe3+ plus NADPH had little effect on adrenal lipid peroxidation. In the presence of high Fe3+ levels (10^-2 M), NADPH produced a concentration-dependent inhibition of adrenal lipid peroxidation; the inhibition was fully demonstrable in heat-treated microsomes. NADPH similarly inhibited ascorbate-induced lipid peroxidation in adrenal microsomes. In testicular microsomes, NADPH did not inhibit ascorbate or Fe2+-induced lipid peroxidation. The results indicate that various endogenous substances may be important in the control of adrenal and testicular lipid peroxidation and that the nature of the regulation differs from tissue to tissue.

Introduction

The process of lipid peroxidation has been implicated in the hepatotoxicity of ethanol and carbon tetrachloride as well as in the toxic effects of many other chemicals (1-3). Lipid peroxidation has been shown to occur in vitro in a wide variety of tissues (4-6) but has been most extensively studied in the microsomal fraction of liver cells. Among the endogenous substances known to stimulate lipid peroxidation in hepatic microsomes is NADPH (7). The microsomal enzyme, NADPH-cytochrome c reductase, is required for NADPH enhancement of lipid peroxidation in liver microsomes. Therefore, inactivation of microsomal enzymes blocks the stimulatory effect of NADPH on lipid peroxidation. Other substances, including ascorbate and ferrous or ferric ion, also initiate hepatic microsomal lipid peroxidation, but non-enzymatically, and their effects, therefore, are unaltered by inactivation of microsomal enzymes.

Among the endocrine tissues, lipid peroxidation has been demonstrated in adrenocortical and testicular subcellular fractions (5). However, relatively little is known about the role of endogenous substances in controlling lipid peroxidation in steroid-producing tissues. Many of the enzymes involved in the production of steroid hormones are membrane-bound cytochrome P-450-containing mixed function oxidases. Since lipid peroxidation is a membrane-damaging process, factors affecting lipid peroxidation in the adrenal cortex or testis may have significant effects on steroidogenesis in those...
tissues. The following studies were carried out, therefore, to determine the actions of and interactions between ascorbate, NADPH, and ferrous or ferric ions on lipid peroxidation in adrenal and testicular microsomes. Effects in the adrenal and testes were compared with those in hepatic microsomes to determine if the factors affecting lipid peroxidation in the three tissues were similar.

Methods

Male English short-hair guinea pigs weighing approximately 1000 g were obtained from Camm Research Institute, Wayne, N.J. Animals were maintained under standardized conditions of light (6:00 am-6:00 pm) and temperature (22°C) on a diet of Wayne Guinea Pig Diet and water ad libitum. Guinea pigs were killed by decapitation between 8:00 and 9:00 am. Adrenals, liver, and testes were quickly removed and placed in cold 1.15% KCl-0.05M Tris-HCl buffer (pH 7.4) on ice. Tissues were trimmed free of connective tissue, weighed, and homogenized in KCl-tris buffer. Microsomes were obtained by differential centrifugation as previously described (8) and resuspended in 1.15% KCl-0.05M tris buffer (pH 7.4). In some experiments, microsomal enzymes were inactivated by heating the microsomal suspension at 70°C for 2 min. Enzyme inactivation was confirmed by the absence of detectable NADPH-cytochrome c reductase activity.

Microsomal suspensions were incubated in 25 ml Erlenmeyer flasks in a Dubnoff metabolic incubator at 37°C for 60 min under air. Total volume in each flask was 2.5 ml. Adrenal microsomes were incubated at a concentration of approximately 0.25 mg protein/ml, testicular microsomes at approximately 0.50 mg protein/ml, and liver microsomes at approximately 0.75 mg protein/ml. The protein concentrations employed were found to be optimal for lipid peroxidation in each tissue. As indicated, the following agents were added to the reaction flasks prior to incubation: FeCl₃ (Fisher Scientific Company, Fairlawn, N.J.), FeSO₄, L-ascorbic acid, and NADPH-type I (Sigma Chemical Company, St. Louis, Mo.). All of the effects of ferrous ion presented in this report were obtained using FeSO₄ as the source of Fe²⁺. However, essentially identical results have also been obtained with FeCl₂.

Malonaldehyde production, as measured by the thiobarbituric acid test, served as an index of lipid peroxidation. Malonaldehyde was measured according to the method of Ottolenghi (9) as modified by Hunter et al. (10). Following incubation, 2.0 ml aliquots from each flask were transferred to centrifuge tubes containing 0.5 ml of 40% trichloroacetic acid and 0.25 ml of 5N HCl. After mixing, 0.5 ml of a 2% thiobarbituric acid solution was added and the samples were incubated for 20 min at 90°C. Following incubation the samples were cooled in an ice bath for 5 minutes and centrifuged at 30,000 g for 5 min in a Sorvall model SS-3 centrifuge. The amount of malonaldehyde in each sample was determined by measuring the optical density of the supernatants at 532 nm with a Gilford model 300-N spectrophotometer and by using a molar extinction coefficient of 1.56 × 10⁴ M⁻¹ cm⁻¹ (11).

Results

Lipid peroxidation in both adrenal and testicular microsomes was stimulated by the addition of either ascorbate of ferrous (Fe²⁺) ion to the incubation flasks (Table 1). The amount of lipid peroxidation was dependent upon the concentration of Fe²⁺ or ascorbate present in the reaction mixture. Malonaldehyde production, when expressed per ml of incubation medium, was similar in adrenal and testes at all concentrations of Fe²⁺ and ascorbate tested (Table 1). However, since the concentration of testicular microsomal protein incubated was twice that of adrenal microsomal protein, malonaldehyde production per mg of protein was greater in adrenal than testicular microsomes. Ascorbate stimulation was maximal in both tissues at a concentration of 1 × 10⁻⁴M and dropped off at higher or lower concentrations. Increasing concentrations of Fe²⁺ up to at least 1 × 10⁻³M caused increasing production of malonaldehyde. Solubility limitations in the reaction mixture prevented the use of higher concentrations of Fe²⁺. Heat treatment of adrenal or testicular microsomes did not alter the stimulatory effects of Fe²⁺ or ascorbate (data not shown), indicating the nonenzymatic nature of the stimulation by each.

The time courses for Fe²⁺- and ascorbate-induced lipid peroxidation were similar in adrenal and testicular microsomes. The time courses for adrenal lipid peroxidation are shown in Figure 1. Fe²⁺-induced lipid peroxidation continued to increase for at least 90 min, while ascorbate-induced malonaldehyde production reached a maximum after about 30 min. We have established that neither adrenal nor testicular microsomes metabolize malonaldehyde under the incubation conditions described. Therefore, the time courses reflect only malonaldehyde production.

It has been reported that Fe²⁺ and Fe³⁺ are equipotent stimuli of lipid peroxidation in rat liver microsomes (12). The data in Table 2 indicate that Fe²⁺ and Fe³⁺ also have similar effects on lipid peroxidation in guinea pig liver microsomes. However, in both adrenal and testicular microsomes, Fe²⁺ was a much more potent stimulus for lipid
Table 1. Effects of varying concentrations of ascorbate, Fe$^{2+}$, or NADPH on malonaldehyde production by adrenal and testicular microsomes.\(^a\)

|                | Malonaldehyde, nmole/ml |
|----------------|------------------------|
|                | 1 \times 10^{-6}M  | 5 \times 10^{-6}M  | 1 \times 10^{-5}M  | 5 \times 10^{-5}M  | 1 \times 10^{-4}M  |
| Ascorbate      |                        |                      |                    |                      |                      |
| Adrenal        | 1.1 \pm 0.3           | 3.0 \pm 0.2         | 4.0 \pm 0.3       | 6.7 \pm 0.2         | 7.4 \pm 1.5         |
| Testes         | 0.9 \pm 0.1           | 1.7 \pm 0.2         | 2.8 \pm 0.6       | 4.0 \pm 0.9         | 7.5 \pm 2.0         |
| Fe$^{2+}$      |                        |                      |                    |                      |                      |
| Adrenal        | 0.8 \pm 0.3           | 1.4 \pm 0.1         | 1.7 \pm 0.1       | 1.5 \pm 0.2         | 2.8 \pm 0.1         |
| Testes         | 0.8 \pm 0.1           | 1.8 \pm 0.1         | 2.3 \pm 0.2       | 3.0 \pm 0.3         | 3.6 \pm 0.6         |
| NADPH          |                        |                      |                    |                      |                      |
| Adrenal        | ND\(^b\)              | ND                   | ND                 | ND                   | ND                   |
| Testes         | ND                    | ND                   | ND                 | ND                   | ND                   |

\(^a\) Malonaldehyde was measured by the thiobarbituric acid test following a 60 min. aerobic incubation at 37°C. Values are the means \pm SE of 4-6 determinations at various concentrations of ascorbate, Fe$^{2+}$, or NADPH.

\(^b\) ND = not detectable.

NADPH alone did not stimulate lipid peroxidation in adrenal or testicular microsomes (Table 1). However, when low levels (1 \times 10^{-6}) of Fe$^{2+}$ were added to the reaction mixture, NADPH produced concentration-dependent increases in lipid peroxidation in adrenal microsomes (Fig. 2). This stimulatory effect of NADPH in the presence of low levels of Fe$^{2+}$ was not demonstrable in testicular microsomes, nor was it demonstrable in heat-treated adrenal microsomes (Fig. 2), suggesting an enzymatic process. Testicular malonaldehyde production was stimulated by NADPH if Fe$^{3+}$ (5 \times 10^{-5} to 1 \times 10^{-3}M) was added to the incubation medium (Table 3). In adrenal microsomes, by contrast, Fe$^{3+}$ (at concentrations from 1 \times 10^{-4} to 1 \times 10^{-3}M) only slightly enhanced NADPH-induced lipid peroxidation (data not shown). The interaction between Fe$^{2+}$ and NADPH in testicular microsomes, like that between Fe$^{2+}$ and NADPH in adrenal microsomes, was not demonstrable in heat-treated microsomes (Table 3 and Fig. 2, respectively), suggesting that enzymatic reactions were required.

Table 2. Effects of varying concentrations of Fe$^{2+}$, or Fe$^{3+}$ on malonaldehyde production by liver, adrenal and testicular microsomes.\(^a\)

|                | Malonaldehyde, nmole/ml |
|----------------|------------------------|
|                | 1 \times 10^{-6}M  | 1 \times 10^{-5}M  | 5 \times 10^{-5}M  |
| Liver          |                        |                      |                    |
| Fe$^{2+}$      | 1.7 \pm 0.2           | 1.5 \pm 0.2         | 1.8 \pm 0.2       |
| Fe$^{3+}$      | 1.6 \pm 0.2           | 1.8 \pm 0.2         | 2.2 \pm 0.4       |
| Adrenal        |                        |                      |                    |
| Fe$^{2+}$      | 1.8 \pm 0.2           | 2.8 \pm 0.2         | 5.9 \pm 0.8       |
| Fe$^{3+}$      | 0.6 \pm 0.1           | 0.9 \pm 0.1         | 1.0 \pm 0.1       |
| Testes         |                        |                      |                    |
| Fe$^{2+}$      | 2.3 \pm 0.2           | 3.6 \pm 0.6         | 7.8 \pm 0.4       |
| Fe$^{3+}$      | 0.8 \pm 0.1           | 0.9 \pm 0.1         | 1.9 \pm 0.1       |

\(^a\) Microsomes were incubated aerobically at 37°C for 60 min at optimal protein concentrations for lipid peroxidation (liver at approximately 0.75 mg/ml; adrenal at 0.25 mg/ml; testes at 0.50 mg/ml). Values are the means \pm SE of 4-6 determinations.

April 1981
peroxidation in hepatic microsomes also influence lipid peroxidation in steroidogenic tissues, but that the specific effects vary from one tissue to another. An absolute dependence on non-heme iron for lipid peroxidation in hepatic microsomes was first suggested by Wills (12) and recently confirmed by Kornbrust and Mavis (13). In our studies, ferrous (Fe$^{2+}$) ion stimulated malonaldehyde production in guinea pig liver, adrenal, and testicular microsomes in vitro. The extent of lipid peroxidation was directly proportional to the Fe$^{2+}$ concentration in all three tissues, but the amount of malonaldehyde produced varied with the source of the microsomes. Malonaldehyde production, when expressed per milligram of microsomal protein, was greatest in the adrenal, perhaps as a result of the high concentration of unsaturated fatty acids in the adrenal cortex (14). The extent of Fe$^{2+}$-induced testicular microsomal lipid peroxidation was greater than that in hepatic microsomes but less than adrenal lipid peroxidation.

In both adrenal and testicular microsomes, ascorbate-induced malonaldehyde production was maximal at an ascorbate concentration of 1 × 10^{-3} M. Higher concentrations resulted in a rapid decline in lipid peroxidation in the adrenal and a more gradual decline in testicular lipid peroxidation, probably as a result of the antioxidant properties of ascorbate being manifested. The normal level of ascorbate in the guinea pig adrenal is nearly 1 × 10^{-2} M, a concentration at which ascorbate may exert primarily antioxidant effects (15,16). However, upon stimulation by ACTH, the ascorbate concentration of the adrenal cortex decreases to levels which may promote lipid peroxidation (17). The normal level of ascorbate in the guinea pig testis is approximately 1 × 10^{-3} M (15), a concentration which we have found to stimulate lipid peroxidation in testicular microsomes.

As previously reported for rat liver microsomes, Fe$^{3+}$ and Fe$^{2+}$ had similar effects on lipid peroxida-

Table 3. Effects of varying concentrations of NADPH on malonaldehyde production by normal or heat-treated testicular microsomes in the presence or absence of Fe$^{3+}$ (1 × 10^{-3} M). *

| Malonaldehyde, nmole/ml | Normal microsomes | Heat-treated microsomes |
|-------------------------|-------------------|------------------------|
| 0                       | 1 × 10^{-6} M NADPH | 5 × 10^{-6} M NADPH | 1 × 10^{-4} M NADPH | 5 × 10^{-4} M NADPH | 1 × 10^{-3} M NADPH |
| Fe$^{3+}$               | 0.4 ± 0.1         | 0.5 ± 0.1              | 0.5 ± 0.1            | 0.4 ± 0.1            | 0.9 ± 0.1            | 0.9 ± 0.1 |
| + Fe$^{2+}$             | 1.6 ± 0.2         | 1.6 ± 0.1              | 4.2 ± 0.2            | 6.9 ± 0.3            | 11.0 ± 0.7           | 12.7 ± 0.2 |
| Fe$^{3+}$               | 1.6 ± 0.1         | 1.5 ± 0.1              | 1.7 ± 0.1            | 1.8 ± 0.1            | 1.8 ± 0.2            | 2.0 ± 0.1 |

* Malonaldehyde was determined following a 60-min aerobic incubation at 37°C. Values are the means ± SE of 4-6 determinations.

Discussion

The results demonstrate that a number of endogenous substances previously found to affect lipid

![Figure 2](image-url). Concentration-dependent stimulation of malonaldehyde (MDA) production by NADPH in the presence of 10^{-3} M ferrous ion in normal or heat-treated adrenal microsomes (0.25 mg protein/ml). Malonaldehyde was determined following a 60 min aerobic incubation at 37°C. Values are the means ± SE of 4-6 determinations.
lipid peroxidation was initiated by high levels ($1 \times 10^{-3} M$) of Fe$^{2+}$ or by ascorbate ($1 \times 10^{-4} M$). This inhibitory effect of NADPH, which was not seen in hepatic or testicular microsomes, was fully demonstrable in heat-inactivated adrenal microsomes, suggesting a non-enzymatic mechanism. We previously found that NADPH could inhibit the Fe$^{2+}$-induced oxidation of linoleic acid, indicating that NADPH may act as a direct antioxidant. Thus, NADPH appears to exert opposing actions on adrenal lipid peroxidation, the net effect depending on the level of iron present. It is not clear why the actions of NADPH on adrenal lipid peroxidation differ in the presence of high and low iron concentrations. However, since the stimulatory effect of NADPH requires microsomal enzyme activity, it is possible that high iron concentrations inhibit microsomal enzymes, shifting the balance in favor of the antioxidant effects of NADPH. Further studies are also necessary to determine why the inhibitory effects of NADPH on adrenal lipid peroxidation are not demonstrable in testicular microsomes under the same experimental conditions.

Although these studies indicate that interactions between a number of substances normally found in the adrenal cortex and testes can have substantial effects on lipid peroxidation in those tissues, the significance of lipid peroxidation in steroid-producing cells has yet to be established. Many of the enzymes required for steroidogenesis are membrane-bound mixed function oxidases, and the membrane destruction resulting from excessive lipid peroxidation is known to compromise the activities of such enzymes. It is possible, therefore, that lipid peroxidation could contribute to the control of steroid hormone synthesis and release. Further investigations are now needed into the regulation of lipid peroxidation in steroidogenic tissues and its relationship to hormone production and secretion.

The technical assistance of Marlene Pope, Peggy Johnson and Jo Zulkoski is gratefully acknowledged. These investigations were supported by grants from the USPHS (CA 22182) and the Department of Energy (DE-AT 21-79-MC-11264).

REFERENCES

1. Diluzio, N. R., and Stege, T. E. In: Alcohol and the Liver, M. M. Fisher and J. G. Rankin, Eds. Plenum Press, New York, 1977, pp. 45-62.
2. Recknagel, R. O., and Glende, E. A. Carbon tetrachloride hepatotoxicity: An example of lethal cleavage. CRC Crit. Rev. Toxicol. 2: 263 (1973).
3. Haugaard, N., Cellular mechanisms of oxygen toxicity. Physiol. Rev. 48: 311 (1968).
4. Zalkin, H., and Tappel, A. L. Studies of the mechanism of vitamin E action. IV. Lipid peroxidation in the vitamin E
deficient rabbit. Arch. Biochem. Biophys. 88: 113 (1960).
5. Bieri, J. G., and Anderson, A. A. Peroxidation of lipids in tissue homogenates as related to vitamin E. Arch. Biochem. Biophys. 90: 105 (1960).
6. Pritchard, E. T., and Singh, H. Lipid peroxidation in tissues of vitamin E deficient rats. Biochem. Biophys. Res. Commun. 2: 184 (1960).
7. Hochstein, P., and Ernster, L. ADP-activated lipid peroxidation coupled to the TPNH oxidase systems of microsomes. Biochem. Biophys. Res. Commun. 12: 388 (1963).
8. Pitrolo, D. A., Rumbaugh, R. C., and Colby, H. D. Maturation changes in adrenal xenobiotic metabolism in male and female guinea pigs. Drug Metab. Dispos. 7: 52 (1979).
9. Ottolenghi, A. Interaction of ascorbic acid and mitochondrial lipides. Arch. Biochem. Biophys. 79: 355 (1959).
10. Hunter, F. E., Gebicki, J. M., Hoffstein, P. E., Weinstein, J., and Scott, A. Swelling and lysis of rat liver mitochondria induced by ferrous ions. J. Biol. Chem. 238: 829 (1963).
11. Sinnhuber, R. D., and Lu, T. C. 2-Thiobarbituric acid method for the measurement of rancidity in fishery products. Food Technol. 12: 9 (1958).
12. Wills, E. D. Lipid peroxide formation in microsomes. Biochem. J. 113: 315 (1969).
13. Kornbrust, D. J. and Mavis, R. D. Microsomal lipid peroxidation. I. Characterization of the role of iron and NADPH. Mol. Pharmacol. 17: 400 (1980).
14. Kitabchi, A. E. Inhibition of steroid C-21 hydroxylase by ascorbate: Alteration of microsomal lipids in beef adrenal cortex. Steroids 10: 567 (1967).
15. Ginter, E., Bobek, P., and Vargova, D. Tissue levels and optimum dosage of vitamin C in guinea pigs. Nutr. Metab. 23: 217 (1979).
16. Reid, M. E. Ascorbic acid metabolism in guinea pigs in relation to growth. J. Nutr. 42: 347 (1950).
17. Sayers, M. A., Sayers, G., and Woodbury, L. A. The assay of adrenocorticotropic hormone by the adrenal ascorbic acid-depletion method. Endocrinology 42: 379 (1948).
18. Pederson, T. C., and Aust, S. D. The mechanism of liver microsomal lipid peroxidation. Biochim. Biophys. Acta 386: 232 (1975).