# INHIBITION OF THE TPNH-LINKED LIPID PEROXIDATION OF LIVER MICROSOMES BY DRUGS UNDERGOING OXIDATIVE DEMETHYLATION

Sten Orrenius, Gustav Dallner, and Lars Ernster

Wenner-Gren Institute, University of Stockholm, and Dept. of Pathology at Sabbatsberg Hospital, Karolinska Institutet, Stockholm, Sweden.

Received December 2, 1963

The demonstration of a TPNH-linked peroxidation of lipids in microsomes (Hochstein and Ernster, 1963a,b) has raised the question as to the possible relationship of this process to other microsomal TPNH-dependent reactions. Liver microsomes are known to catalyze a number of TPNH-dependent hydroxylation reactions, among them the oxidative demethylation of various drugs (cf. Brodie et al., 1958). In this paper it is shown that drugs undergoing oxidative demethylation strongly inhibit the TPNH-linked peroxidation of lipids in microsomes and that the inhibition probably is the result of a competition between the two processes for a common TPNH-oxidizing enzyme.

Experimental. Rat-liver microsomes were prepared as described by Ernster et al. (1962). The microsomal pellets were throughly rinsed with 0.15 M KCl in order to remove the bulk of adhering sucrose, which may interfere with the determination of malonaldehyde (Hochstein and Ernster, 1963a); washing with KCl was avoided since it diminished the oxidative demethylation activity.

Lipid peroxidation activity was measured by determining the amount of malonaldehyde formed with the thiobarbituric acid reaction (Bernheim et al., 1948). The incubation system included microsomes containing 3 mg protein, 0.05 M tris-buffer, pH 7.5, 0.1 ml ADP-$Fe^{2+}$ solution (0.03 µmole $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ + 8 µmoles ADP), 50 mM nicotinamide, 0.5 mM TPN, and a TPNH-generating system consisting of 5 mM DL-isocitrate, 0.01 mM $MnCl_2$, and 0.1 ml dialyzed 105,000 g liver supernatant (corresponding to 10 mg fresh weight liver), in a

final volume of 2 ml. The temperature was 37°C and the time of incubation 6 min. When ascorbate was used to induce lipid peroxidation, it was added in a final concentration of 1 mM, and TPN and the TPNH-generating system were omitted.

Oxidative demethylation activity was assayed with aminopyrine or codeine as substrates and the amount of formaldehyde formed was measured by the Nash (1953) reaction. The composition of incubation system was similar to that employed for lipid peroxidation, except that ADP-$Fe^{2+}$ was omitted, and 5 mM aminopyrine or codeine was added. The temperature was 37°C and the time of incubation 40 min.

Oxygen consumption and TPNH disappearance were measured with a Clark oxygen electrode and an Eppendorf fluorometer, respectively. Measurements were started with 0.025 M tris-buffer, pH 7.5, 0.15 M KCl, 0.2 mM TPNH, and microsomes containing 4 mg protein, in a final volume of 3 ml. After recording the TPNH oxidase activity for 2 minutes, 30 µl 0.5 M aminopyrine or codeine in ethanol, or 30 µl ethanol alone, were added. After further 5.5 min., 50 µl ADP-$Fe^{2+}$ (36 mµmoles $Fe^{2+}$ and 3 µmoles ADP) were added, and recording was continued for another 2-4 min. The temperature was 28°C.

Results. Fig. 1 shows the validity of the conditions used for the measurement of the TPNH-linked oxidative demethylation activity with aminopyrine as substrate. The formation of formaldehyde was linear with time and with the amount of microsomes used, and it was maximal at 5 mM aminopyrine. The reaction was strongly inhibited by carbon monoxide. Similar results were obtained with codeine.

Data in Table I illustrate the effect of codeine and aminopyrine on the TPNH-linked peroxidation of lipids. The latter was activated by ADP-$Fe^{2+}$, in accordance with previous results (Hochstein, Nordenbrand and Ernster, 1963). Codeine and aminopyrine, 5 mM, greatly inhibited the ADP-$Fe^{2+}$-activated lipid peroxidation. On the contrary, ADP-$Fe^{2+}$ had no effect on the oxidative demethylation activity. Codeine and aminopyrine also greatly depressed the ADP-$Fe^{2+}$--induced $O_2$ uptake and TPNH disappearance, as shown in Table II.

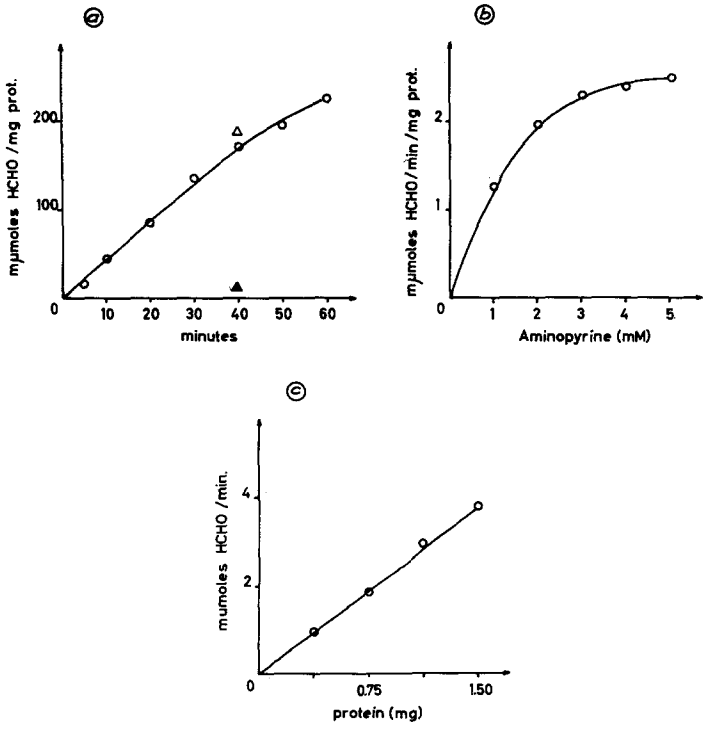


Fig. 1. Assay conditions for oxidative demethylation of aminopyrine.

For additions, see text. All assays were performed with air as the gas phase, except those marked with △ (4 % $O_2$, 96 % $N_2$), and ▲ (40 % $CO$, 4 % $O_2$, 56 % $N_2$).

Table I. Effect of codeine and aminopyrine on the TPNH-linked peroxidation of lipids in rat liver microsomes.

| Additions | Malonaldehyde mμmoles/min./mg protein | Formaldehyde mμmoles/min./mg protein |
|---|---|---|
| None | 0.16 | |
| ADP-$Fe^{2+}$ | 5.21 | |
| Codeine | | 2.32 |
| Codeine, ADP-$Fe^{2+}$ | 0.72 | 2.02 |
| Aminopyrine | | 3.62 |
| Aminopyrine, ADP-$Fe^{2+}$ | 0.42 | 3.92 |

Table II. Effect of codeine and aminopyrine on the ADP-$Fe^{2+}$-induced stimulation of $O_2$ uptake and TPNH oxidation of rat-liver microsomes.

| Addition | $O_2$ uptake | | | TPNH oxidation | | |
|---|---|---|---|---|---|---|
| | -ADP-$Fe^{2+}$ | +ADP-$Fe^{2+}$ | stim. | -ADP-$Fe^{2+}$ | +ADP-$Fe^{2+}$ | stim. |
| | (mμmoles/min./mg prot.) | | (times) | (mμmoles/min./mg prot.) | | (times) |
| None | 2.7 | 148 | 55 | 2.4 | 12.0 | 5.0 |
| Codeine | 5.1 | 44 | 9 | 5.1 | 6.4 | 1.3 |
| Aminopyrine | 5.2 | 56 | 11 | 4.8 | 8.3 | 1.7 |

The possibility that codeine and aminopyrine per se might inhibit lipid peroxidation, without the involvement of the TPNH-linked oxidative demethylation, could be eliminated by the following three types of experiments:

1) When the microsomes were aged in the cold (0-2°C), the capacity for oxidative demethylation decreased to a large extent, whereas that for TPNH--linked lipid peroxidation remained unchanged (Fig. 2a). In the 24-hour-aged microsomes, lipid peroxidation was only slightly inhibited by codeine and aminopyrine (Fig. 2b).

2) Ascorbate, which is a poor electron donor for oxidative demethylation in liver microsomes (Mitoma et al., 1956), but a good inducer of ADP-$Fe^{2+}$-activated lipid peroxidation (Hochstein, Nordenbrand and Ernster, 1963), gave rise to a malonaldehyde formation, that was only slightly inhibited by codeine and aminopyrine (Table III).

3) In microsomes from rat brain, which do not catalyze the oxidative demethylation of codeine and aminopyrine but do exhibit TPNH-linked lipid peroxidation (Hochstein and Ernster, 1963b), the latter was not inhibited by the drugs.

In complementary experiments it could also be shown that added formaldehyde, in the concentration range here in question, did not interfere with the formation or determination of malonaldehyde.

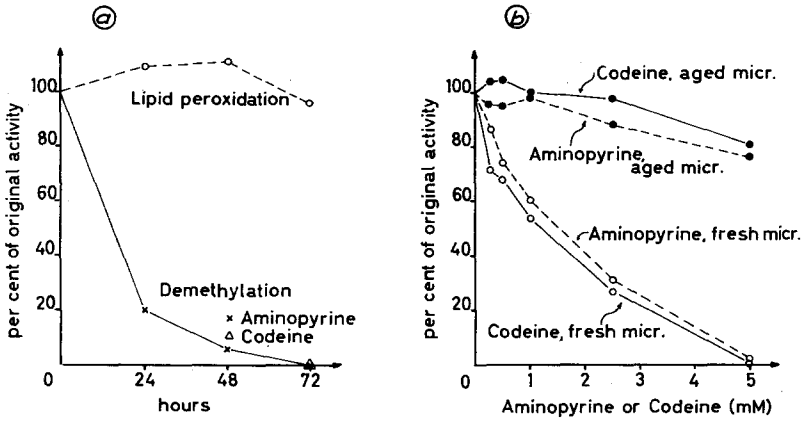


Fig. 2. Effect of aging on TPNH-linked lipid peroxidation and oxidative demethylation.

Table III. Effect of codeine and aminopyrine on the TPNH- and ascorbate-linked peroxidation of lipids in rat liver microsomes.

(MA = malonaldehyde; FA = formaldehyde)

| Addition | None | | TPNH | | Ascorbate | |
|---|---|---|---|---|---|---|
| | MA | FA | MA | FA | MA | FA |
| | mμmoles/min./mg protein | | | | | |
| None | 0.25 | - | 5.30 | - | 4.44 | - |
| Codeine | 0.22 | 0 | 0.14 | 2.78 | 4.18 | 0.36 |
| Aminopyrine | 0.11 | 0 | 0.15 | 4.47 | 3.56 | 0.40 |

Discussion. The results reported above demonstrate that oxidative demethylation inhibits the TPNH-linked lipid peroxidation of microsomes. The simplest interpretation of this inhibition seems to be that the two processes compete for a common TPNH-oxidizing enzyme. This competition is schematically illustrated in Fig. 3. The TPNH-oxidizing enzyme probably is identical with Horecker's (1950) TPNH-cytochrome c reductase, which flavoprotein (Fp) has recently been implicated in microsomal hydroxylations (Gaudette and Brodie, 1959; Krisch and Staudinger, 1961). The "Fe" in the scheme stands for a hypothetic iron-containing catalyst, which is thought to be involved in microsomal hydroxylations and which,

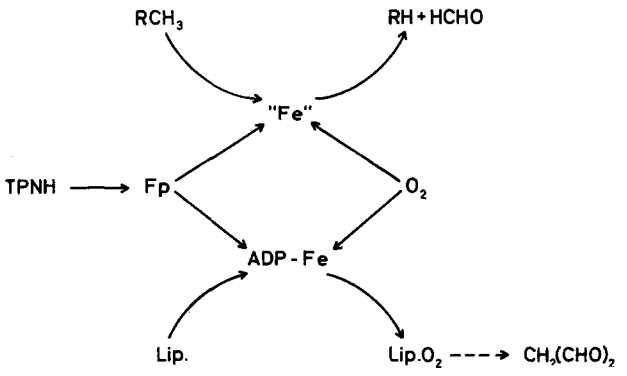


Fig. 3. Schematic representation of competition between TPNH-linked lipid peroxidation and oxidative demethylation.

as indicated both by the present data and by those recently reported by Estabrook et al. (1963) on adrenal cortex microsomes, appears to be identical with Klingenberg's (1958) "CO-binding pigment". The difference in sensitivity between the TPNH- and ascorbate-linked lipid peroxidation to drugs undergoing oxidative demethylation may be explained by assuming that the TPNH-oxidizing enzyme reacts more readily with "Fe" than with ADP-Fe, whereas the converse relationship holds for ascorbate.

This work has been supported by a grant from the Swedish Cancer Society. We thank Miss Margareta Sparthan for skilful technical assistance.

REFERENCES

Bernheim, F., Bernheim, M.L. and Wilbur, K.M. (1948) J.Biol.Chem. 174, 257.
Brodie, B.B., Gillette, J.R. and La Du, B.N. (1958) Ann.Rev.Biochem. 27, 427.
Ernster, L., Siekevitz, P. and Palade, G.E. (1962) J.Cell Biol. 15, 541.
Estabrook, R.W., Cooper, D.Y. and Rosenthal, O. (1963) Biochem.Z. 338, 741.
Gaudette, L.E. and Brodie, B.B. (1959) Biochem.Pharmacol. 2, 89.
Hochstein, P. and Ernster, L. (1963a) Biochem.Biophys.Res.Communs. 12, 388.
Hochstein, P. and Ernster, L. (1963b) in Ciba Foundation Symp. on Cellular Injury. In press.
Hochstein, P., Nordenbrand, K. and Ernster, L. (1964) Biochem.Biophys.Res. Communs. This issue.
Horecker, B.L. (1950) J.Biol.Chem. 183, 593.
Klingenberg, M. (1958) Arch.Biochem.Biophys. 75, 376.
Krisch, K. and Staudinger, H.J. (1961) Biochem.Z. 334, 312.
Mitoma, C., Posner, H.S., Reitz, H.C. and Udenfriend, S. (1956) Arch.Biochem. Biophys. 61, 431.
Nash, T. (1953) Biochem.J. 55, 416.