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DEPRESSIVE EFFECTS OF LIPID PEROXIDES MEDIATED VIA A PURINE RECEPTOR

EFFECT OF LIPID PEROXIDE ON THE CORTICAL SYNAPTOSOMAL GTPase ACTIVITY

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Abstract—A circadian rhythm of lipid peroxides (LPO) in mouse whole brain was observed with a negative correlation between LPO levels in cerebral cortex and the locomotor activity of mice. Intraperitoneal administration of *t*-butyl hydroperoxide (BPO) to mice decreased spontaneous locomotor activity in a dose-dependent manner, and the effect was competitively antagonized by caffeine, suggesting a similar receptor. Cumene peroxide (CPO), BPO and known depressants such as phenobarbital and NaBr all stimulated GTPase activity in rat cerebral cortex synaptosomes. In contrast, stimulants, such as caffeine and amphetamine, inhibited GTPase activity. Using fresh and aged synaptosomes which differed markedly in endogenous LPO levels, it appeared as if the LPO competitively inhibited inosine-induced GTPase activity, suggesting that the receptor of LPO is the same as that of inosine. From these results, it is concluded that LPO in brain act as depressants whose activity can be competitively antagonized by caffeine, and that the effects of LPO are mediated through a purine receptor.

Exhaustive locomotion induces an increase in the levels of lipid peroxides (LPO) in various organs, especially in blood, liver, muscle and brain [1–7], but the physiological functions of these LPO in brain are still not known. It has been suggested that there may be some relationship between the enhancement of lipid peroxide levels in brain and a state of fatigue [4, 8]. In the present study, we show that the inhibitory effect of LPO on spontaneous locomotion is blocked competitively by caffeine, suggesting that LPO act at a purine receptor. We have studied the effects of LPO on GTPase activities since these may play a role in second-messenger systems that are involved in mediating neurotransmitter actions. Guanosine triphosphate-binding proteins (G-proteins), which are especially abundant in brain [9], couple many transmitter receptors to the key second-messenger generating enzymes. It is thought that continuous GTP hydrolysis at the G-proteins is an essential turn-off mechanism, terminating activation of G-protein [10]. Thus, we were interested in the effect of LPO on the GTPase activities of brain synaptosomes, and especially on adenosine-stimulated GTPase activities.

METHODS

Circadian rhythm of LPO and locomotor activities. The spontaneous locomotor activities were measured by placing five mice as a group into a plastic cage (16 × 28 × 12 cm) which was on an Animex instrument that detects the magnetic variance caused by the mouse locomotion and integrates the locomotor activities during every 90 min.

LPO levels in mouse brains were measured in brain homogenates. After killing the mice by cervical fracture, the whole brain was quickly removed and homogenized in 10 vol. of ice-cold 0.9% NaCl/8% perchloric acid. One milliliter of homogenate was added to the malondialdehyde (MDA) measuring medium containing 1 mL of 0.67% thiobarbituric acid (TBA) and 2 mL of 2% phosphoric acid, and the mixture was boiled for 15 min. After cooling, 1 mL of *n*-butanol was added to extract the TBA–MDA reaction compound. Ten microliters of the *n*-butanol layer was applied to an HPLC system consisting of a 15 cm CLC-ODS reverse column (Shimazu), and a mobile phase of 5 mM NaH_2PO_4 in 25% acetonitrile flowing at a speed of 0.8 mL/min. The retention time of the TBA–MDA compound was about 4 min. The reaction product of authentic MDA prepared from hydrolysis of 1,1,3,3-tetraethoxypropane and TBA had the same retention time. Lipid peroxide levels were described in terms of the MDA concentration.

Rat brain synaptosomes were isolated from the cerebral cortex according to the method of Abdel-Latif [11]. P-2 fractions, which were not sedimented by centrifuging at 10,000 *g* but were at 20,000 *g*, were put on 0.3 M sucrose, and synaptosomes were collected by the density gradient (between 0.8 M and 1.2 M sucrose) centrifugation method [11].

GTPase activity was measured on synaptosomal preparations by incubating in 1 mL of a 0.15 M KCl, 50 mM Tris–HCl buffer (pH 7.5) at 37° for 10 min. Since Kent *et al.* reported that 100 μM GTP transformed the receptor to a low-affinity form [12], suggesting that the G-protein binds GTP exchanged from GDP [10], we used a high concentration of GTP (250 μM) in order to observe the intrinsic GTP hydrolysis step and to avoid agonist-stimulated exchange between GDP and GTP.

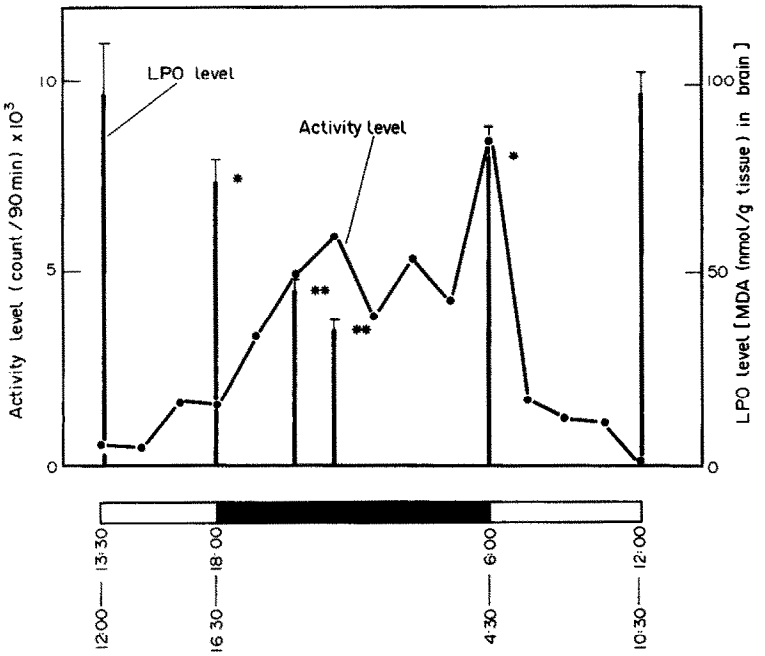


Fig. 1. Brain LPO levels and the spontaneous locomotor activity of mice as a function of time of day. Left axis and line show the spontaneous locomotor activity (integral counts/90 min), while the right axis and columns show the LPO levels in whole mouse brain. The x axis shows the time of day while the bar indicates the periods of darkness. Key: (*) and (**) indicate significant differences from LPO levels at 12:00–13:30: (*) $P < 0.02$ and (**) $P < 0.001$.

The phosphate liberated by GTP hydrolysis was measured according to the method of Martin and Doty [13]. Non-specific nucleotide triphosphatase was inhibited by the addition of 50 mM adenosine-5′-(β,γ-imino)-triphosphate (App(NH)p). In control experiments, the GTPase activities observed were linear for at least 10 min. Specific activities of GTPase in controls were 70 ± 15 pmol/mg protein/min (N = 6).

CPO and BPO used in GTPase assays were dissolved in ethanol (90%), and the concentrations of these lipid peroxides were measured by the method of Yagi *et al.* [14]. Stock solutions of various concentrations of CPO and BPO were prepared and were added to the reaction medium at a constant volume (10 μL). In the control group, 10 μL of ethanol was added. The effect of ethanol on GTPase activities was the same as that of phenobarbital, that is, when the concentration of ethanol in the reaction medium became high (2% final concentration), GTPase activities were stimulated.

Protein concentrations in the reaction medium were rather high (2–4 mg protein/mL), because we did not use the usual radioisotopic method with [^{32}P]GTP. However, the effect of protein concentration on the stimulative effect of LPO on GTPase activities was inhibitory; at high concentrations of protein, the stimulating effect of LPO on GTPase activities was difficult to observe. So, if we had used lower protein concentrations in the radioisotopic method, we should have observed a greater stimulating effect of LPO on GTPase activity. High protein concentrations also antagonize the stimulatory effects of phenobarbital, NaBr and caffeine.

RESULTS AND DISCUSSION

Circadian rhythm of LPO levels and locomotor activities. The circadian rhythms of the spontaneous locomotor activities and brain LPO levels in mice are shown in Fig. 1. Since mice are nocturnal animals,

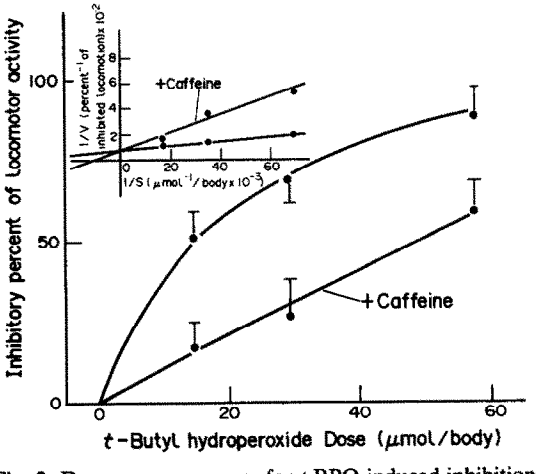


Fig. 2. Dose–response curve for *t*-BPO-induced inhibition of spontaneous locomotor activity. Male ddy mice (10 weeks old) received a single intraperitoneal injection of 14.4, 28.8, or 57.6 μmol/mouse of *t*-BPO dissolved in physiological saline buffered with Tris (pH 7.5). After administration of *t*-BPO, locomotor activity of each group of four mice was measured as described under methods. Caffeine (20 nmol/mouse) was administered intraperitoneally 15 min before administration of *t*-BPO. Values are means ± SD (N = 5 groups in each case). Inset: Lineweaver–Burk plots of data.

Table 1. Relationship between the LPO levels in various loci of brain and motor activities

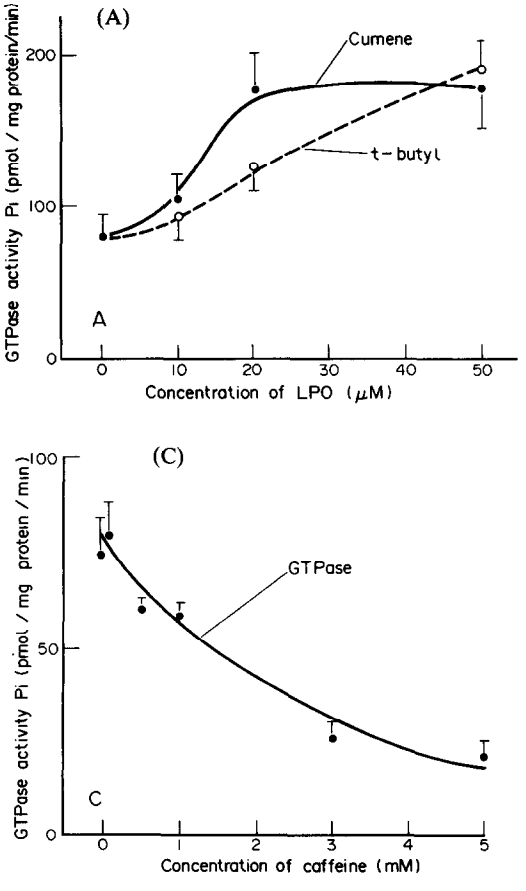
	Lipid peroxides (nmol MDA/g tissue)			Motor activities (counts/5 min)
	Midbrain + thalamus	Cerebral cortex	Cerebellum	
10:00 a.m.	110.1 ± 10	92.5 ± 7	76.9 ± 6	0
10:00 p.m.	90.4 ± 8	45.5 ± 4	59.5 ± 6	987
Forcefully awakened (10:00 a.m.)	82.9 ± 7	43.2 ± 1	54.1 ± 9	876
+Caffeine (30 nmol/mouse, i.p., at 10:00 p.m.)	111.7 ± 12	31.5 ± 5	69.7 ± 11	1205
Painful stress at 10:00 p.m.	71.9 ± 13	73.3 ± 8	56.8 ± 10	113

Values are means ± SD (N = 4) for the biochemical data in each case. Motor activities were measured as described in Fig. 1, and the mice were killed for LPO assays at the conclusion of the activity measurements. Painful stress was imposed by pinching the tail with a clip.

they show greater spontaneous locomotion in the dark than in the light. On the other hand, LPO levels in brain were generally high in light and low in dark periods. There appeared to be a general negative correlation between LPO levels and spontaneous locomotor activity. However, with the small number (N = 6) of time periods studied and the unusual occurrence of both high activity and high LPO levels at the end of the dark period (Fig. 1), the negative correlation was not significant for whole brain LPO levels and activity ($r = -0.64$, $P = 0.17$).

As shown in Table 1, LPO in the cerebral cortex showed more dramatic changes than in other brain regions, both with time of day and following various treatments, and it is noticeable that stress seemed to induce an increment in LPO levels. In Table 1, there also appeared to be a negative correlation between LPO levels in the cerebral cortex and motor activities, and this was significant ($r = -0.97$, $P < 0.005$). We therefore studied the direct effect of peroxides on mouse locomotor activity.

As shown in Fig. 2, the administration of *t*-BPO



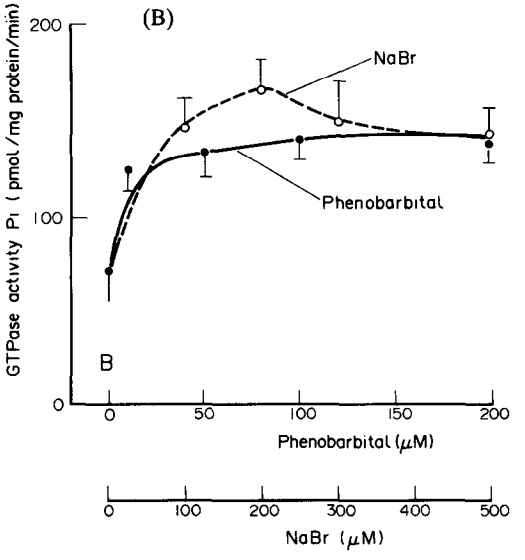


Fig. 3. Effects of various depressants and stimulants on GTPase activity of rat cortical synaptosomes. Each data point is the mean ± SD of four separate determinations. (A) Effects of CPO (cumene hydroperoxide) and BPO (*t*-butyl hydroperoxide). (B) Effects of phenobarbital and NaBr. (C) Effect of caffeine.

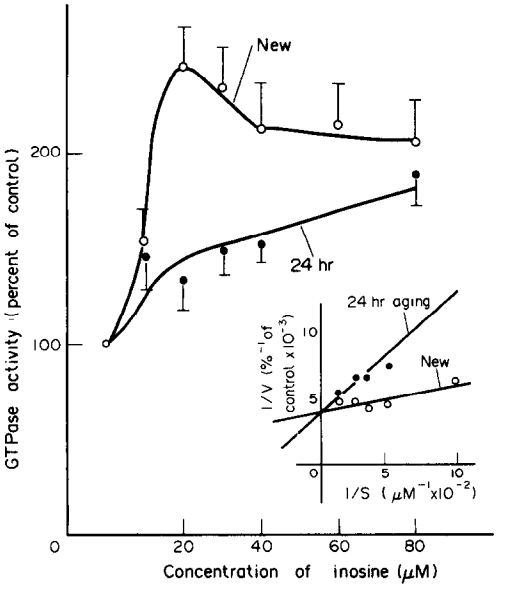


Fig. 4. Effect of inosine on synaptosomal GTPase activity in fresh preparations and in preparations aged for 24 hr at 0°. Activities in the absence of inosine were 120 ± 40 pmol/mg protein/min (N = 6) in the fresh samples and 216 ± 65 pmol/mg protein/min (N = 5) in the aged samples. LPO levels, in MDA equivalents, were 5 ± 1 nmol/mg protein (N = 6) in the fresh synaptosomal preparation and 10 ± 0.8 nmol/mg protein (N = 5) in the aged samples. The GTPase activities in the figure are shown as percent of their respective control, and each data point represents the mean ± SD (N = 4). Inset: Lineweaver–Burk plots of data.

appeared to inhibit spontaneous locomotor activity in a dose-dependent manner. A Lineweaver–Burk plot (inset) of these data indicates a K_i value for *t*-BPO of about 22 nmol/body weight. Caffeine inhibited the effect of *t*-BPO in a competitive manner (inset), suggesting that the receptor for BPO is the same as that for caffeine, which reportedly acts at a purine receptor [15, 16].

GTPase activity. As clearly shown in Fig. 3A, CPO and BPO each stimulated GTPase activity by about 2-fold. Representative depressants, phenobarbital and NaBr, also stimulated GTPase activity by 2- and 2.5-fold respectively (Fig. 3B). On the other hand, caffeine, which is known as a stimulant in the central nervous system (CNS), inhibited synaptosomal GTPase activity by about 80% (Fig. 3C). Similar results were obtained with amphetamine and theobromine (data not shown).

Inosine also stimulated GTPase activity, as expected from its depressant effect in the CNS [17] (Fig. 4). The stimulation of GTPase activity induced by inosine was much less in aged synaptosomal samples than in fresh ones, a phenomenon that may be related to the higher LPO levels in the aged samples. In accord with these higher endogenous LPO levels, the control GTPase activity in the aged samples was almost 2-fold higher than in the fresh samples. A competitive inhibition between endogenous LPO and inosine is indicated by Lineweaver–Burk analysis of the data (Fig. 4, inset).

Adenosine is also known as a depressant in the CNS [17], and thus was expected to stimulate synaptosomal GTPase activity. Such stimulation was found at 10, 20 and 50 μM adenosine, but not at 200 μM (Fig. 5). When 68 μM CPO was added, the stimulation of GTPase activity induced by 10–50 μM adenosine was still observed, and greater stimulation was seen at 200 μM. As shown in the inset of Fig. 5, there appears to be partial competition between CPO and adenosine. The effect of endogenous LPO appeared similar to that of CPO (data not shown).

The results on GTPase activity are consistent with the hypothesis that depressants commonly stimulate GTPase activity, indicating probable induction of the inactive form of the G-protein, whereas stimulants have opposite effects. LPO appears to act pharmacologically on the CNS as a depressant, and its receptor seems to be that of a purine. The depressive effect of LPO was specifically antagonized by caffeine.

It is proposed that endogenous LPO in the cerebral cortex may have depressant effects unrelated to the non-specific lipid peroxidation of membrane.

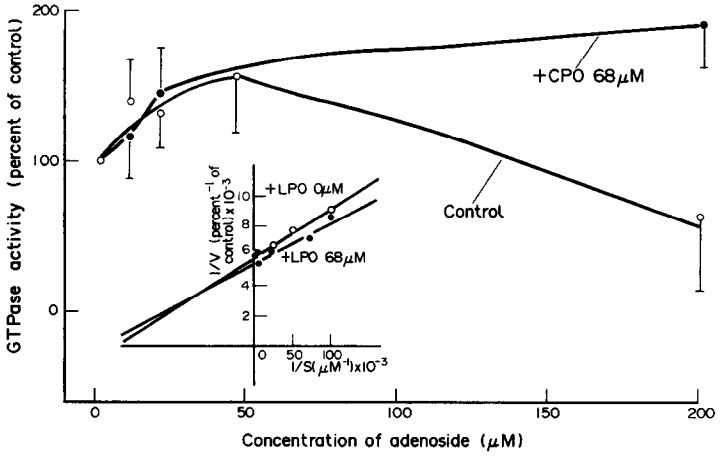


Fig. 5. Effect of adenosine in the presence and absence of CPO on fresh synaptosomal preparations. Activities are expressed as percent of that in the preparation without adenosine. Each data point shows the mean ± SD (N = 4). Inset: Lineweaver–Burk plots of data.

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