Biochemical Pharmacology, Vol. 44, No. 3, pp. 505–508, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

PEROXIDE-DEPENDENT AMINO ACID OXIDATION AND CHEMILUMINESCENCE CATALYSED BY MAGNESIUM–PYRIDOXAL PHOSPHATE–GLUTAMATE COMPLEX

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(*Received* 17 *September* 1991; *accepted* 21 *April* 1992)

Abstract—Magnesium–pyridoxal-5′-phosphate–glutamate (MPPG) has been shown to ameliorate atherosclerotic symptoms in rabbits. *In vitro*, MPPG in the presence of peroxides such as cholesterolhydroperoxide or cumene hydroperoxide and Mn^{2+} ions produces "excited states" measurable as chemiluminescence or ethylene release from 1-aminocyclopropane-1-carboxylic acid (ACC). The reactions are stimulated synergistically by unsaturated fatty acids. Pyridoxal phosphate exhibits similar properties, but can be differentiated from the activities of MPPG or the sum of the components present in MPPG.

Genetic predisposition and different risk factors have been shown to advance atherosclerosis [1]. Modification of low density lipoproteins (LDL§) seems to represent an important factor in atherogenesis where fatty acid peroxidation within the LDL molecule appears to contribute to LDL modification [2–6]. Modified LDL is no longer taken up by macrophages via the normal B-/E-receptors but almost exclusively by the so-called scavenger receptors [6, 7]. LDL *in vivo* contains several antioxidants such as tocopherols and carotenoids, and the ratio between these antioxidants and polyunsaturated fatty acids in native LDL seems to be approximately 1:200 [5, 8].

Based on the above findings, it is not surprising that both natural and synthetic antioxidants have been found to inhibit LDL oxidation [9–11]. In addition, vitamin-rich nutrients containing supplements of antioxidants like certain flavonoids may contribute to the treatment of atherosclerosis in cooperation with calcium antagonists and hydroxymethylglutaryl-CoA-reductase inhibitors [12, 13].

Schneider [14] recently showed that MPPG, a magnesium – pyridoxal - 5′ - phosphate – glutamate complex, ameliorated atherosclerotic symptoms as shown with hypercholesterolemic rats and especially rabbits. In this communication, we report on experiments showing that MPPG interacts with peroxides detectable as increased chemiluminescence and fragmentation of the cyclic amino acid, 1-aminocyclopropane-1-carboxylic acid (ACC). The results indicate that MPPG might be involved in lipid hydroperoxide metabolism depending on certain amino compounds.

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§ Abbreviations: LDL, low density lipoprotein; MPPG, magnesium–pyridoxal-5′-phosphate–glutamate; PP, pyridoxal phosphate; CumOOH, cumene hydroperoxide; t-BuOOH, *t*-butyl hydroperoxide; ACC, aminocyclopropane-1-carboxylic acid; LA, linolenic acid; CholOOH, cholesterol hydroperoxide.

MATERIALS AND METHODS

Materials. D,L-Alanine, ACC, arachidonic acid, L-arginine, L-asparagine, L-aspartic acid, cumene hydroperoxide (CumOOH), L-cysteine, $FeSO_4$, glycine, D,L-leucine, linoleic acid (LA), α-linolenic acid, D-lysine, oleic acid, L-proline, D,L-valine and hydrogen peroxide were from Sigma-Chemie (Deisenhofen, F.R.G.). $CuCl_2$, $CuSO_4$, ethanol, $MnSO_4$, L-methionine, L-phenylalanine, L-serine, D,L-tryptophan and Tween 20 were obtained from Merck-Schuchardt (Darmstadt, F.R.G.). Pyridoxal-5′-phosphate (PP) was from Boehringer Mannheim GmbH (Mannheim, F.R.G.). Cholesterol (from lanoline) was obtained from Fluka Chemie (Buchs, Switzerland). MPPG was placed to our disposal from Steigerwald Arzneimittel GmbH (Darmstadt, F.R.G). *t*-Butyl hydroperoxide (t-BuOOH) was obtained from Aldrich-Chemie GmbH & Co. KG (Steinheim, F.R.G.).

Methods. Cholesterol hydroperoxide (CholOOH) was synthesized according to Schenck *et al.* [15].

ACC fragmentation yielding ethylene was followed by gas chromatography as described previously [16].

Ultra weak chemiluminescence was determined at 37° with a Berthold "Biolumat" model 9500 T with automatic dispenser-type LB 95-C-300 (injection volume 0.3 mL).

Details concerning the individual reaction mixtures are shown in the figures. The data are means of at least three parallel experiments showing standard deviations (σ_{n-1}).

RESULTS

Fragmentation of ACC yielding ethylene and other products

ACC is the natural precursor of the plant growth regulator ethylene. It is oxidatively converted into ethylene and other products such as formic acid and cyanide during fruit ripening and after wounding of plant tissues. PP or its derivative, MPPG, in the

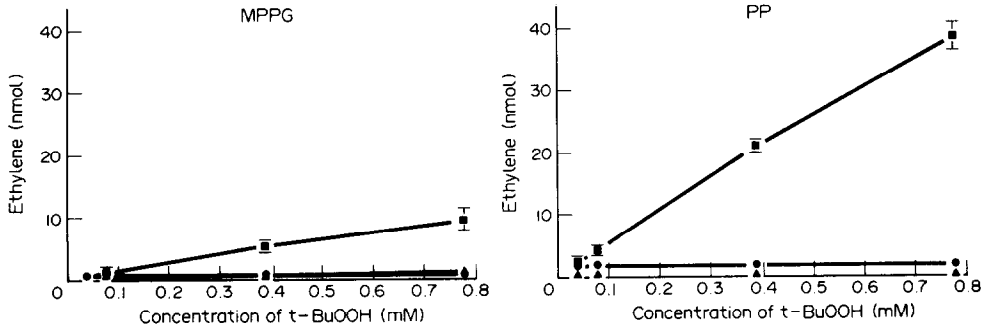


Fig. 1. Fragmentation of ACC in the presence of t-BuOOH. (●) LA alone, (▲) t-BuOOH alone, (■) LA + t-BuOOH. The reaction mixture contained in 2.0 mL: phosphate buffer (0.1 M, pH 7.4); Mn^{2+} (0.1 mM); MPPG or PP (0.5 mM); LA (0.44 mM); ACC (1.0 mM); t-BuOOH (0.0389–0.777 mM). Incubation time was 30 min at 37°. Ethylene was determined gas chromatographically.

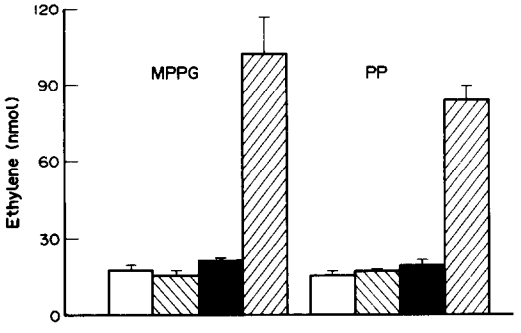


Fig. 2. Fragmentation of ACC in the presence of CholOOH. (□) LA alone, (▨) solvent + cholesterol, (■) cholesterol + LA, (▨) CholOOH + LA. The reaction mixture contained in 2.0 mL: phosphate buffer (0.1 M, pH 7.4); Mn^{2+} (0.1 mM); MPPG or PP (0.5 mM); LA (0.88 mM); CholOOH (1.0 mM) or solvent (0.04% Tween 20 in 96.6% ethanol) or cholesterol (1.0 mM). Incubation time was 60 min at 37°. Ethylene was determined gas chromatographically. Controls with solvent alone, cholesterol alone or CholOOH alone produce no ethylene from ACC.

presence of manganese ions and LA cause ethylene formation from ACC, dependent on the concentrations of all three factors, where Mn^{2+} ions and PP exhibit saturation-type kinetics at *ca.* 0.1 mM and LA stimulates linearly without showing saturation up to 2 mM (data not shown).

In the presence of the above "quasi"-optimal concentrations of Mn^{2+}, LA and MPPG (or PP), time-dependent ethylene formation is terminated after 2 hr (data not shown).

ACC-dependent ethylene formation can also be shown at the expense of an organic hydroperoxide or H_2O_2, where LA can be substituted by t-BuOOH. In this reaction, PP has higher activities than MPPG (data not shown). In the presence of both t-BuOOH and LA, ethylene formation from ACC in the presence of Mn^{2+} shows synergism between t-BuOOH and LA (Fig. 1).

If t-BuOOH is replaced by CumOOH MPPG exhibits higher converting activities as compared to PP (data not shown).

In a similar manner, CumOOH can be substituted by CholOOH. In this experiment MPPG is slightly more active than PP. Cholesterol, instead of its hydroperoxide, is inactive in this respect (Fig. 2).

Ultra weak chemiluminescence

During lipid peroxidation intermediately formed excited states can be detected by their light emission [17, 18]. In the test system containing LA, manganese ions, MPPG/PP and hydrogen peroxide, light emission is measurable where PP as catalyst is only active if ACC is additionally present (Fig. 3a).

In the absence of ACC, the sum of the components in MPPG, namely PP with stoichiometric amounts of added glutamic acid and magnesium ions show different activities: dependent on the amount of H_2O_2 reasonable light emission is only obtained with MPPG as catalyst whereas PP in the presence of glutamate and magnesium is almost inactive (Fig. 3b).

Since ACC is a very unusual amino acid in animals as compared to plants, other amino acids have been tested in this respect. We observed that the MPPG chemiluminescence system is also very active in the presence of phenylalanine and especially tryptophan while the system with PP exhibits high activity only in the presence of the non-animal-type amino acid, ACC. The carboxyl group of the amino acid does not seem to be necessary for the catalytic function in the MPPG system. This is shown with tyramine which is only active in the MPPG system and inactive in the PP system (Fig. 4).

Manganese, as an active ionic cofactor, can partly be substituted by iron(II) (*ca.* 25% activity) but not by copper salts (data not shown).

In the absence of ACC, MPPG also exhibits high rates of light emission in the presence of oleic acid, LA and to a lesser extent, arachidonic acid. PP is only active with oleic acid and linoleic acid in the presence of ACC whereas in its absence only arachidonic acid exhibits reasonable rates (data not shown).

DISCUSSION

Oxidative modification of LDL has been shown to involve unsaturated fatty acids, oxygen free radicals and/or hydroperoxides where oxidation of LDL starts as soon as endogenous antioxidants have been consumed [3].

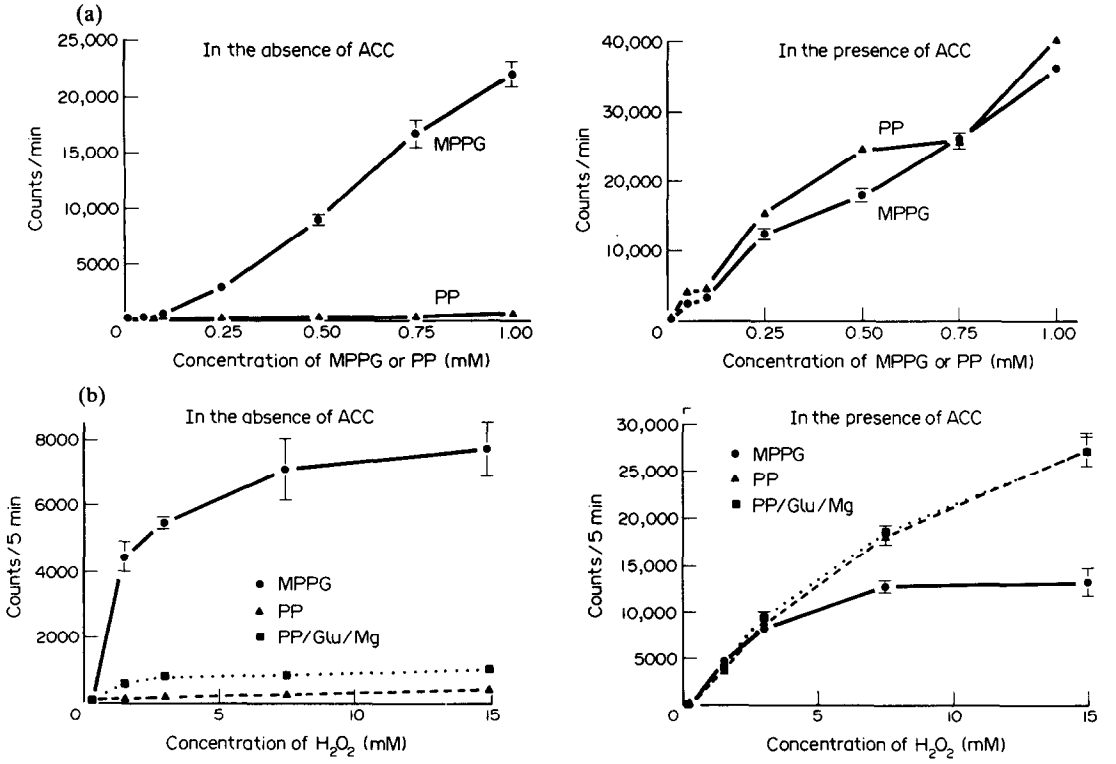


Fig. 3. (a) Chemiluminescence dependent on the MPPG or PP concentration. The reaction mixture contained in 2.0 mL: phosphate buffer (0.1 M, pH 7.4); ±ACC (1.0 mM); LA (0.44 mM); Mn^{2+} (0.1 mM); MPPG or PP (0.01–1.0 mM); H_2O_2 (15 mM). (b) Chemiluminescence independent of the hydrogen peroxide concentration: different effects of MPPG and PP. The reaction mixture contained in 2.0 mL: phosphate buffer (0.1 M, pH 7.4); ±ACC (1.0 mM); LA (0.44 mM); Mn^{2+} (0.1 mM); MPPG or PP (0.5 mM); H_2O_2 (0.15–15 mM).

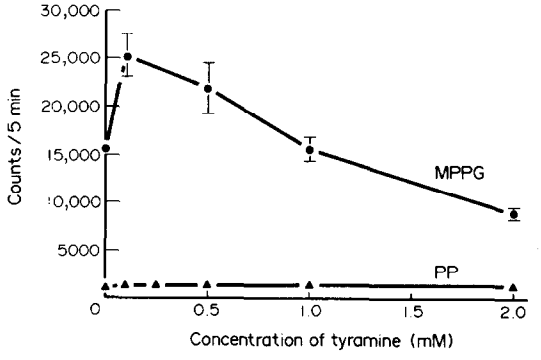
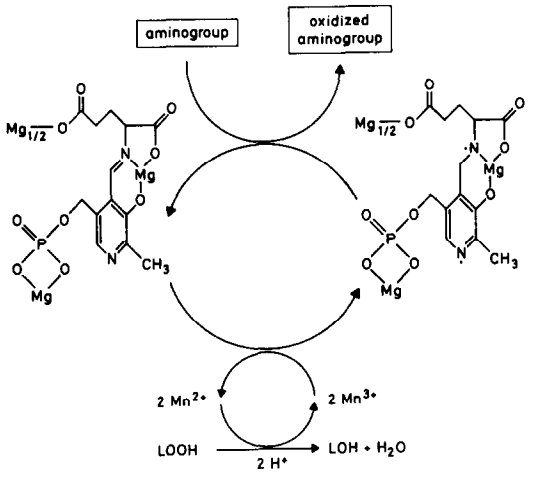


Fig. 4. Chemiluminescence with tyramine. The reaction mixture contained in 2.0 mL: phosphate buffer (0.1 M, pH 7.4); tyramine (0–2.0 mM); LA (0.44 mM); Mn^{2+} (0.1 mM); MPPG or PP (0.5 mM); H_2O_2 (15 mM).

Ethylene formation from ACC and chemiluminescence have frequently been used to document the involvement of reactive oxygen species in biological systems [17–20]. We show in this communication that the MPPG complex can substitute for PP. However, in the presence of MPPG the mechanism of the formation of reactive oxygen species or "excited states" seems to be different. MPPG-dependent light emission is independent of the addition of ACC (Fig. 3b) and, in contrast to PP or stoichiometric mixtures of PP, glutamate and magnesium, is stimulated by tyramine (Fig. 4). The combination of the individual components of MPPG apparently do not produce the reactive complex as present in MPPG. Both systems (MPPG and PP) are dependent on the presence of manganese ions. These ions have been shown to be accumulated at sites of inflammation [21]. Since at the same sites peroxides are produced by activated macrophages and unsaturated fatty acids are present in the lipoprotein fraction, the preconditions for the above reactions are given as soon as the PP derivative is available. Although it is not clear how MPPG interferes with plaque formation or facilitates plaque decomposition, several possibilities for an interaction can theoretically be discussed: at the expense of a preformed peroxide (which is also present in commercially available LA preparations) a manganese-dependent radical formation at the nitrogen position(s) of the cofactor is possible. The nitrogen-centered free radical(s) seem(s) to interact with certain amino groups. The nitrogen-centered free radical, on the other hand, may interact with carbon atoms of unsaturated fatty acids, forming alkyl radicals which spontaneously add oxygen forming hydroperoxyl radicals which decompose under light emission. During this process peroxides are consumed and amino acid residues are oxidized thus eventually facilitating proteolysis [22]. This sequence is shown in Scheme 1.



Scheme 1. Proposed mechanism of the MPPG–Mn^{2+}-catalysed metabolism of hydroperoxides and amino compounds.

Acknowledgement—This work was supported by "Fonds der chem. Industrie".

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