Chemiluminescent assay of lipid peroxide in plasma using cytochrome *c* heme peptide

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A convenient assay specific to lipid hydroperoxide in plasma is presented. Cytochrome *c* heme peptide obtained from *Saccharomyces* was found to emit a strong chemiluminescence with any hydroperoxide, but not with TBA-reactive substances. The benefit of measuring this luminescence using photon counting is discussed with respect to in vivo lipid peroxidation.

Chemiluminescent assay *Luminol sensitization* *Cytochrome c heme peptide* *Lipid hydroperoxide*
Thiobarbituric acid assay *In vivo lipid peroxidation*

1. INTRODUCTION

Lipid peroxidation has been of great interest to biochemists and biologists due to its association with a number of physiological processes. The arachidonic cascade, for example, includes many types of lipid peroxides such as prostaglandins and leucotriene precursors. The hydroperoxides of unsaturated fatty acids were recently suggested to cause some diseases such as arteriosclerosis, diabetes mellitus, and essential hypertension [1–3].

For the analysis of lipid peroxides, thiobarbituric acid (TBA) assay has been widely used to estimate the content of malondialdehyde (MDA) produced from the endoperoxide of lipids [4–8]. It is also well known that aldehydes, sugars, and sialic acid can also react with TBA [9–11]. These substances cause an overestimation of lipid peroxide in biological tissues and plasmas. Many alternative techniques for the assay of biological samples are susceptible to errors due to artifacts and side reactions [12–15].

We present here a method for evaluating lipid peroxide by decomposing it into active oxygen. The active oxygen is then quantitatively measured by sensitization with luminol using a highly sensitive photon counting system to observe the chemiluminescence directly. After a widespread search, cytochrome *c* heme peptide (CHP) was found to evolve active oxygen most efficiently.

2. MATERIALS AND METHODS

2.1. *Materials*

Cytochrome *c* heme peptide (CHP) was prepared from *Saccharomyces oviformis* M_2 with digestive actions of trypsin and pepsin [16,17]. Superoxide dismutase (Sigma) was used to confirm the participation of active oxygen in the chemiluminescence process. Horseradish peroxidase (HRP) was also obtained from Sigma. *t*-Butyl hydroperoxide (*t*-BuOOH) and di-*t*-butyl peroxide were purchased from Nakarai Chemicals (Japan) and were purified by distillation under reduced pressure. Luminol of guaranteed grade (Sigma) was used without further purification. Arachidonic hydroperoxide (15-hydroperoxyeicosatetraenoic acid, HPETE) was prepared from arachidonic acid (Sigma) in a method similar to that of [18].

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2.2. *Apparatus*

Photon counting was carried out with a 2-inch photomultiplier (R-1333) in combination with a photon counter (C-1250) from Hamamatsu Photonics. The photomultiplier was cooled to −20°C and was used under a fairly high potential (−1482 V). The counts were processed with an 8-bit microcomputer (IF 800, OKI Electric) and stored on a floppy disk for further data analysis. The apparatus was designed to mix automatically the analyte with a mixture of luminol and CHP in a quartz cuvette in the dark at a selected temperature.

2.3. *Assay of lipid hydroperoxide in plasma*

For the chemiluminescent assay of lipid hydroperoxide, plasma was added to a mixture of luminol and CHP (0.9 μg/ml luminol and 120 μg/ml CHP, pH 9.2 borate buffer) which had been previously saturated with an inert gas (e.g., N_2) and maintained at 37°C. Plasma was prepared in the usual manner by bleeding a 6-week-old mouse (BALB/cJ) or a 7-week-old rat (Wistar Imamichi). TBA assay in plasma was performed in a manner similar to [15].

3. RESULTS AND DISCUSSION

Table 1 shows the chemiluminescent responses to peroxides and other substances which could contaminate or coexist with lipid hydroperoxide in plasma. Di-*t*-butyl peroxide was used as a model compound for endoperoxide (-O-O-) groups to estimate the specificity of the reaction using CHP. It is worth noting that no luminescent response was observed by the luminol-CHP combination for NaCl, glucose and aldehyde, while HRP shows an undesirably large chemiluminescent response. In other words, HRP is not highly specific to hydroperoxide. Similar results were found with other peroxidases. On the other hand, CHP, after sensitization by luminol, generates a chemiluminescent reaction with any hydroperoxide.

As the first step in the chemiluminescent process is expected to be the encounter of the hydroperoxide with a CHP molecule, it is possible that luminescent efficiency could be lower for large hydroperoxide molecules. It is therefore necessary to examine the linearity of the luminescent signal against concentration using several lipid hydroperoxides. The signal for HPETE, an arachidonic hydroperoxide of biological impor-

Table 1

Luminescent responses (counts at peak) of peroxides and biologically fundamental substances

Substance		Luminescent intensity in cps[a]			
Name	Concentration	Luminol-CHP[b]		Luminol-HRP[b]	
		Air	N_2	Air	N_2
Background		181	163	174	186
H_2O		288	225	3453	537
Buffer (pH 9.2)	10 mM	575	282	6745	1402
NaCl	0.9%	400	294	4803	3726
NaCl	0.09%	258	226	3309	860
NaCl	0.009%	238	221	3540	998
EtOH	1 μmol/ml	274	447	5376	354
Glucose	1 μmol/ml	243	329	2873	298
MDA	1 μmol/ml	787	398	14708	6004
t-Bu-OO-*t*-Bu	0.02 μmol/ml	328	184	5307	1649
t-BuOOH	0.1 μmol/ml	99432	66268	19973	10289
15-HPETE	0.02 μmol/ml	19631	14435	18253	8076

[a] Average of 3 experiments

[b] Gas used for saturating the samples and purging the apparatus

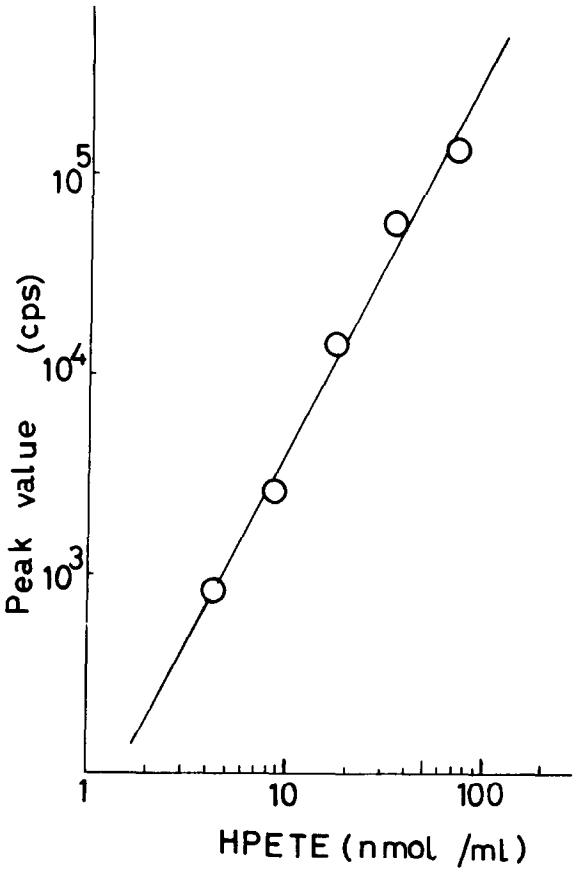

Fig.1. Chemiluminescent intensity (counts at peak) against concentration in the assay of HPETE with luminol-CHP. The concentrations of luminol and CHP were as indicated in section 2. The concentration of HPETE was determined independently by iodometries.

tance, shows the same linearity (fig.1) with concentration as was observed using *t*-BuOOH. This result strongly supports our belief that the chemiluminescent signal is dependent solely on the concentration of hydroperoxyl group (-OOH) and is not a function of the structure of the lipid hydroperoxide.

The minimum sample volume for the assay was held at 50 μl to avoid data fluctuation due to the inhomogeneity of the plasma sample. The optimum concentration was found to be 10 μg/ml in CHP. Under these conditions, the detection limit was estimated to be 2 nmol/ml lipid hydroperoxide to yield a signal-to-noise ratio of 5.

As mentioned in section 1, this chemiluminescent method using CHP as a luminescence catalyst is a very convenient procedure, and permits a good estimate of the lipid hydroperoxide level to be obtained. The lipid hydroperoxide level in rats (Wistar Imamichi) and mice (BALB/cJ) was determined to permit a comparison with TBA assay. The lipid hydroperoxide level in plasma has been found to be very high in mice and lower in rats, as shown in table 2. The lipid hydroperoxide level in plasma kept for several days at -2°C showed that there was a significant decomposition of the lipid hydroperoxide in storage. On the other hand, MDA values determined by TBA assay showed constant values on the same samples. These facts substantiate that TBA-reactive substances in plasma show no response to this chemiluminescent assay. Many substances produced metabolically from lipids, e.g., PG G_2 and PG H_2 and other biological constituents, react positively to TBA assay. On the other hand, this chemiluminescent

Table 2

Comparisons of the hydroperoxide levels with MDA values in the same plasmas of mice and rats

Mouse (BALB/cJ)			Rat (Wistar Imamichi)		
Individual	LOOH value (nmol/ml)	MDA value (nmol/ml)	Individual	LOOH value (nmol/ml)	MDA value (nmol/ml)
1	124	20	1	32	4.2
2	100	34	2	26	2.5
3	96	28	3	30	1.9
4	90	21	4	12	7.0
5	106	15	5	20	5.9

assay appears to evaluate the content of only the lipid hydroperoxide.

In our preliminary experiments on mice fed with alloxan (75 mg/kg body wt per day), the luminescent assay showed no increase in the lipid hydroperoxide level, while TBA assay apparently showed an explosive increase in the concentration of TBA-reactive substances. These results could be interpreted by the increase of lipid endoperoxide, but not of lipid hydroperoxide.

Therefore, in the investigation of physiological effect of a drug on in vivo peroxidation, this chemiluminescent assay can be a powerful method to evaluate precisely the mechanism of peroxidation.

In conclusion, the method presented here gives definite quantitative information about the level of lipid hydroperoxide in plasma. The application of this method to a biochemical system such as microsomal lipid peroxidation was found more practical. Although the apparatus was designed to obtain high sensitivity and easy procedure, any commercial luminometer can be used for this purpose at the expense of somewhat high detection limit.

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