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Original Contribution

ESR spin trapping of a carbon-centered free radical from agonist-stimulated human platelets

Duen-Suey Chou[a], George Hsiao[b], Ming-Yi Shen[a], Yan-Jyu Tsai[b], Tzeng-Fu Chen[b], Joen-Rong Sheu[a,b,*]

[a]*Graduate Institute of Medical Sciences, Taipei Medical University, Taipei 110, Taiwan*
[b]*Graduate Institute of Pharmacology, Taipei Medical University, Taipei 110, Taiwan*

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Abstract

Several free radical intermediates formed during synthesis of prostaglandin H synthase (PGHS) catalyze the biosynthesis of prostaglandins from arachidonic acid (AA). We attempted to directly detect free radical intermediates of PGHS in cells. Studies were carried out using human platelets, which possess significant PGHS activity. Electron spin resonance (ESR) spectra showed a $g = 2.005$ signal radical, which was formed by the incubation of collagen, thrombin, AA, and a variety of peroxides with human platelets. The ESR spectra obtained using 5,5-dimethyl-1 pyrroline *N*-oxide (DMPO) and α-phenyl *N-tert.*-butylnitron (PBN) were typical of an immobilized nitroxide. Extensive Pronase digestion of both the DMPO and PBN adducts allowed us to deduce that it was a carbon-centered radical. The formation of this radical was inhibited by potassium cyanide and by desferroxamine. Peroxides stimulated formation of the $g = 2.005$ signal radical and inhibited platelet aggregation induced by AA. PGHS cosubstrates increased the intensity of the radical signal but inhibited platelet aggregation induced by AA. Both *S*-nitro-L-glutathione and reduced glutathione quenched the $g = 2.005$ radical but could not restore platelet aggregatory activity. These results suggest that the carbon-centered radical is a self-destructing free radical formed during peroxide-mediated deactivation of PGHS in human platelets.

Keywords: platelets; electron spin resonance; carbon-centered free radical; prostaglandin H synthase; peroxides

Introduction

Prostaglandin H synthase (PGHS) is a key enzyme in the biosynthesis of prostaglandins and thromboxanes from arachidonic acid (AA). It has two distinct catalytic activities: as a cyclooxygenase that converts AA to PGG_2, and as a peroxidase that reduces a wide variety of peroxides to their corresponding alcohols [1]. Cyclooxygenase- and peroxidase-active sites are located on opposite sides of the protein, and both require heme as a cofactor. Cyclooxygenase activity depends on the oxidation of an active-site tyrosine by electron transfer to the oxidized ferriprotoporphyrin of the peroxidase [2]. Many nonsteroidal anti-inflammatory drugs that bind to the cyclooxygenase active site do not affect peroxidase function [2]. However, cyclooxygenase activity can be attenuated during damage either to the cyclooxygenase site or to the peroxidase site.

Several free radical intermediates formed during PGHS synthesis catalyze the biosynthesis of prostaglandins from

Abbreviations: AA, arachidonic acid; $cPLA_2$, cytosolic phospholipase A_2; $AACOCF_3$, 5,8,11,14-eicosatetraenoic trifluoromethyl ketone; DMPO, 5,5-dimethyl-1 pyrroline *N*-oxide; ESR, electron spin resonance; HPETE, hydroperoxyeicosatetraenoic acids; PBN, α-phenyl *N-tert.*-butylnitrone; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; PGHS, prostaglandin H synthase; GSNO, *S*-nitro-L-glutathione.

* Corresponding author. Graduate Institute of Medical Sciences, Taipei Medical University, No. 250 Wu-Hsing Street, Taipei 110, Taiwan. Fax: +886 2 27390450.
E-mail address: sheujr@tmu.edu.tw (J.-R. Sheu).

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AA. Karthein et al. proposed that resting PGHS reacts with peroxide to generate Intermediate I, a ferryl heme with a porphyrin radical, which is converted via an intramolecular electron transfer to Intermediate II, a ferryl heme with a Tyr-385 radical [3]. The latter species undergoes damage at the cyclooxygenase site to form a self-destructing free radical. The tyrosyl radical of Intermediate II extracts a hydrogen atom from AA to form a fatty acid radical and subsequently generates the PGG_2 radical.

The oxidation of xenobiotics by peroxidase has been proposed as a mechanism for activation of chemical carcinogens. It has been shown that different types of substrate-derived free radicals are generated by peroxidases, which can be phenylbutazone-derived carbon-centered radicals [4], phenidone-derived cation radicals [5], acetaminophen-derived phenoxy radicals [6], aminopyrine-derived nitrogen-centered radicals [7], or diethylstilbestrol-derived phenoxy (or phenoxy-derived) radicals [8]. These co-oxidation properties are shared by many other nonspecific heme-containing peroxidases such as horseradish peroxidase, lactoperoxidase, and myeloperoxidase, and the process requires only a highly active peroxidase and suitable peroxides. The co-oxidation of xenobiotics by PGHS was first reported by Marnett et al. [9]. PGG_2 and other peroxides are essential substrates, and xenobiotics are the cosubstrates in that system.

AA plays a significant role in platelet aggregation, as it is released from membrane phospholipids and subsequently converted to thromboxane by PGHS [10]. Collagen and thrombin, both platelet aggregatory agents, have been demonstrated to release AA in platelets [11,12]. Therefore, it is expected that PGHS-derived free radical intermediates should be generated during the blood platelet activation process. Recently, O'Donnell et al. [13] showed that PGHS consumes nitric oxide via its utilization as a reducing peroxidase substrate in both purified enzyme preparations and intact platelets. Thus platelets provide a good system for the AA/PGHS pathway in intact cells. However, direct demonstration of the existence of free radical intermediates of PGHS in intact cells has not been reported. The reason for this may be because of the inferior enzymatic activity of cells compared with purified enzyme preparations or ram seminal vesicle microsomes, as well as the presence of endogenous reducing substances or antioxidant enzymes in intact cells. We considered whether blood platelets can produce any free radical intermediates when reacting with platelet aggregatory agents or peroxidase cosubstrates.

In this article, we report the detection of a PGHS–peroxidase-derived carbon-centered radical induced by collagen, thrombin, AA, and peroxides in intact platelets by electron spin resonance (ESR) techniques. Furthermore, we propose that it is a self-destructing free radical that forms during the peroxide-mediated deactivation of PGHS in human platelets.

Materials and methods

Materials

Collagen (type I, bovine Achilles tendon), linoleic acid (LA), AA, prostaglandin E_1 (PGE_1), sodium citrate, thrombin, cumene hydroperoxide, deferoxamine mesylate, 5,5-dimethyl-1 pyrroline *N*-oxide (DMPO), diethylstilbestrol, α-phenyl *N-tert.*-butylnitron (PBN), salicylic acid, potassium cyanide (KCN), α-naphthol, bovine serum albumin (BSA), and heparin were purchased from Sigma Chemical (St. Louis, MO, USA). U44619, a prostaglandin endoproxide analog (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_2\alpha$), was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). $AACOCF_3$, *S*-nitro-L-glutathione (GSNO), PGG_2, PGH_2, 12-HPETE, and 15-HPETE were purchased from Cayman (Ann Arbor, MI, USA). The Sephadex PD-10 column was purchased from Pharmacia (Uppsala, Sweden).

Preparation of human platelet suspensions

Human platelet suspensions were prepared as previously described [5]. In this study, human volunteers gave informed consent. In brief, blood was collected from healthy human volunteers who had taken no medicine during the preceding 2 weeks, and was mixed with acid/citrate/glucose. After centrifugation at 120*g* for 10 min at room temperature, the supernatant (platelet-rich plasma, PRP) was supplemented with PGE_1 (0.5 μM) and heparin (6.4 IU/ml), then incubated for 10 min at 30°C and centrifuged at 500*g* for 10 min. The washed platelets were finally suspended in Tyrode's solution containing BSA (3.5 mg/ml) and adjusted to a concentration of 4.5×10^8 platelets/ml. The final concentration of Ca^{2+} in Tyrode's solution was 1 mM.

Measurement of free radicals in platelet suspensions by electron spin resonance (ESR) spectrometry

In ESR method, a Bruker EMX ESR spectrometer as described previously was used [14] but with some modifications. In brief, platelet suspensions (1×10^9 platelets/ml, 150 μl) were warmed to 37°C for 2 min, and then enzyme inhibitors were added for 10 min before the addition of the aggregatory agents or peroxides; cosubstrates of PGHS were added 10 s before the addition of the aggregatory agents or peroxides. AA and other hydrophobic agents were dissolved in ethanol, and their final concentration in platelet suspensions was 1%. ESR spectra were recorded at room temperature using a quartz flat cell designed for aqueous solutions. The dead time between sample preparation and ESR analysis was exactly 30 s after the last addition, except for the time-course studies of collagen- and thrombin-stimulated preparations, which was 1 min after the last addition. Conditions of ESR spectrom-

etry were as follows: 20 mW power at 9.78 GHz, with a scan range of 100 G and a receiver gain of 5×10^4. Modulation amplitudes, sweep times, and time constants are given in the legends to the figures and tables.

Platelet aggregation

The turbidimetric method was applied to measure platelet aggregation, using a Lumi-Aggregometer (Payton, Canada). Platelet suspensions (4.5×10^8 platelets/ml, 0.4 ml) were warmed to 37°C for 2 min (stirring at 1200 rpm) in a silicone-treated glass cuvette. PGHS–peroxidase cosubstrates, such as α-naphthol, acetaminophen, and diethylstilbestrol, or peroxides were added 3 min before the addition of AA. Either reduced glutathione (GSH) or GSNO was added 10 s before the addition of AA. The reaction was allowed to proceed for at least 6 min, and the extent of aggregation was expressed in light transmission units.

Reduced glutathione (GSH) assay

GSH was measured using a commercially available assay kit (ApoGSH glutathione colorimetric detection kit, BioVision, Mountain View, CA, USA). Platelets were incubated with a solution containing 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). DTNB and GSH react to generate 2-nitro-5-thiobenzoic acid and oxidized glutathione (GSSH). As 2-nitro-5-thiobenzoic acid is a yellow-colored product, the GSH concentration can be determined by measuring the absorbance at 412 nm as described by the kit manufacturer.

Results

ESR investigations of free radicals induced by aggregatory agents in human platelets

Stimulation of human platelets with collagen, thrombin, or AA at room temperature induced a $g = 2.005$ doublet signal radical (Fig. 1). No significant radical was detected when untreated platelets or platelets treated with ADP (Fig. 1B) or U44619 (Fig. 1C), a thromboxane A_2 (TXA_2) analog, were reacted with spin trapper DMPO. The intensity of the $g = 2.005$ radical induced by AA increased in a dose-dependent manner (Table 1), but was not dependent on the presence of DMPO, because the radical was also produced in the absence of DMPO (Fig. 5A). In each instance, the signals had a doublet peak, a linewidth of around 6 G, and no apparent hyperfine structure. When the modulation amplitude was changed to 3 G, the doublet became a singlet with a similar peak-to-trough width (Fig. 1G). Control incubations with either platelets alone or aggregatory agents alone produced no signals. Furthermore, the possibility of nonenzymatic processes causing the generation of such a radical species was excluded, as the system containing boiled platelets produced no signal (data not shown). These data suggest that the $g = 2.005$ radical may be involved in AA metabolism, as AA release is an early event when platelets are stimulated by thrombin [11] and collagen [12].

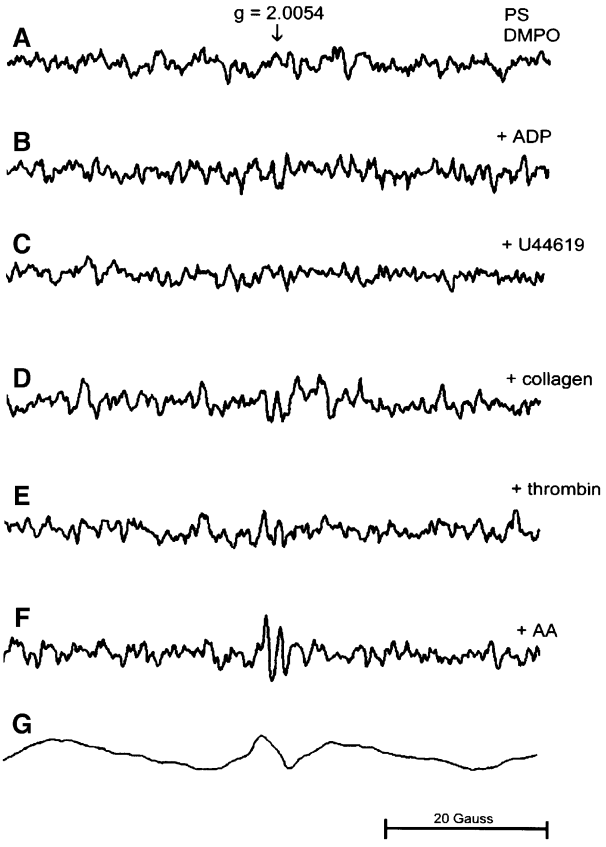

Fig. 1. ESR spectra obtained from the reaction of human platelet suspensions with aggregatory agents in the presence of DMPO. Human platelets (1×10^9 platelets/ml, 0.15 ml) preincubated with DMPO (100 mM), followed by the addition of (A) isovolumetric Tyrode's solution (control), (B) ADP (200 μM), (C) U44619 (10 μM), (D) collagen (10 μg/ml), (E) thrombin (0.5 U/ml), and (F) AA (1 mM) to trigger platelet activation. Instrumental parameters were as follows: modulation amplitude, 1 G; time constant, 164 ms; and scanning for 42 s with 3 scans accumulated. (G) Same as (F) except the instrumental parameters were as follows: modulation amplitude, 3 G; time constant, 328 ms; and scanning for 21 s with 10 scans accumulated.

Time-dependent incubation of collagen with human platelets

Iuliano et al. reported that collagen-induced hydroxyl radicals in platelets can be detected by ESR measurements with DMPO [15]. In this work, we demonstrated that incubation of human platelets with collagen produced a typical four-line hydroxyl radical signal ($a^N = a^H = 14.8$ G) and a long-lived $g = 2.005$ radical detectable by the spin trapper DMPO (Fig. 2). This four-line signal slowly disappeared, leading to the sequential generation of the $g = 2.005$ doublet signal with a maximum at 5 min (Fig. 2C). The half-life of the $g = 2.005$ signal was at least 20 min in this system. There was no radical adduct of DMPO in the control incubations of platelets (Fig. 2A). There are two

Table 1
Intensity of the $g = 2.005$ radical induced by hydroperoxides and fatty acids in washed human platelets[a]

Sample	Percentage of control value ± SEM ($n = 3$)
PS (blank)	10.4 ± 3.2
Arachidonic acid (300 μM) (control)	100.0 ± 0.0
Arachidonic acid (1000 μM)	173.6 ± 9.9
Arachidonic acid (2000 μM)	243.1 ± 17.6
Linoleic acid (300 μM)	12.3 ± 2.6
H_2O_2 (300 μM)	73.9 ± 7.9
Cumene hydroperoxide (300 μM)	220.6 ± 9.6
12-HPETE (30 μM)	358.7 ± 36.9
15-HPETE (30 μM)	477.4 ± 45.4
PGG_2 (30 μM)	339.4 ± 8.9
PGH_2 (300 μM)	14.7 ± 3.6

[a] The reaction conditions and techniques of ESR measurements are decribed under Materials and Methods. Substrates were added to the platelet suspension (PS) (1×10^9 platelets/ml, 150 μl). The instrumental parameters were exactly the same as those in Fig. 1. All values were normalized to 100% for 300 μM AA and represent the averages of three independent incubations. Data are presented as means ± SEM.

possible explanations for this finding. First, we used concentrations of collagen and platelets higher than those normally used in platelet aggregation experiments. Second, we used different time courses compared with prior studies. The $g = 2.005$ signal was not observed when the ESR system was identical to the platelet aggregation experiment (data not shown).

Effect of $AACOCF_3$ on $g = 2.005$ signal formation by thrombin-, collagen-, and AA-stimulated human platelets

$AACOCF_3$, a potent $cPLA_2$ inhibitor, has been shown to inhibit thrombin-induced endogenous AA release in platelets [11]. In this study, both the hydroxyl radical and the $g = 2.005$ signals generated by collagen were fully prevented by $AACOCF_3$ (Figs. 2G–H). This result is in agreement with the observation in a prior study [15] that activation by the hydroxyl radical depends on the concomitant release of AA and is blocked by $cPLA_2$ inhibitors. Furthermore, $AACOCF_3$ completely inhibited the $g = 2.005$ signal generated by thrombin (data not shown), but did not affect radical generation by AA (Table 2) or other lipid peroxides (data not shown). This indicates that the $g = 2.005$ signal generated by collagen and thrombin occurs through endogenous AA release.

Characterization of the $g = 2.005$ signal induced by AA in human platelets

The DMPO/platelet-derived adduct persisted following size exclusion chromatography (Sephadex GD-10 column), which identified the $g = 2.005$ radical adduct to be a macromolecule (data not shown). The intensities of this radical induced by AA (2 mM) after 80 min in the presence or absence of DMPO were about 50 and 25%, respectively (Fig. 3). These results indicate that the $g = 2.005$ radical can be trapped by DMPO. When the DMPO/platelet-derived adduct was subjected to nonspecific proteolysis, a six-line paramagnetic signal ($a^N = 15.9$ G, $a^H = 22.8$ G) was detected (Fig. 3B). Control reactions, without AA, without DMPO, without platelets, or without Pronase produced no six-line signal (Figs. 3C–E). The identity of the radical species was deduced to be a carbon-centered radical adduct based on the close similarity of the hyperfine coupling constants of the observed signal to those of published data [16–18]. This signal was also obtained when AA was substituted by 12-HPETE, 15-HPETE, or PGG_2 (Fig. 3F) in the reaction system.

An immobilized nitroxide was also detected when the reaction was performed in the presence of PBN (Fig. 4A). When this sample was subjected to nonspecific proteolysis for 60 min, a double-triplet signal overlapping with the $g =$

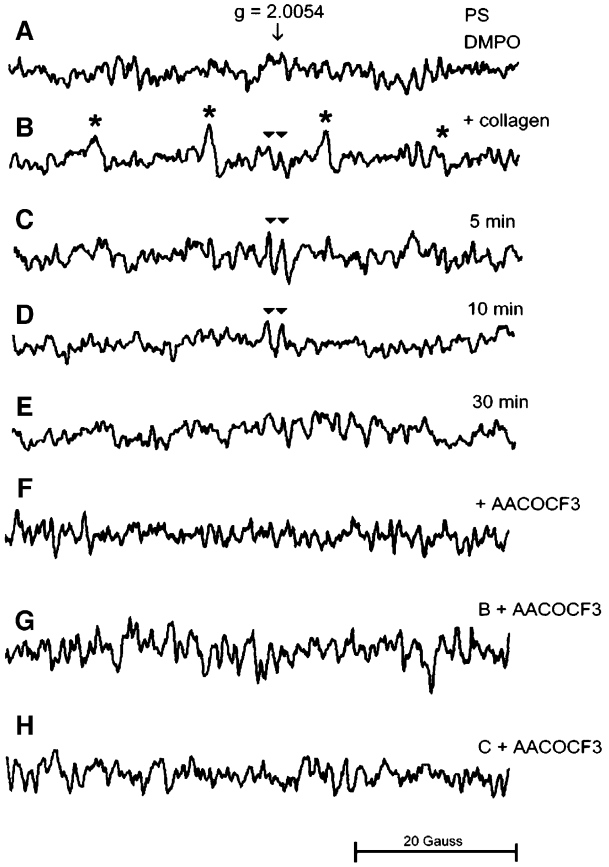

Fig. 2. ESR spectra detected from the reaction of human platelet suspensions with collagen in the presence of DMPO. Human platelets (1×10^9 platelets/ml, 0.15 ml) preincubated with DMPO (100 mM), followed by the addition of (A) isovolumetric Tyrode's solution (control) and collagen (10 μg/ml) to trigger platelet activation for (B) 1 min, (C) 5 min, (D) 10 min, and (E) 30 min. (F) Same as (A) except that 30 μM $AACOCF_3$ was added. (G) Same as (B) except that 30 μM $AACOCF_3$ was added. (H) Same as (C) except that 30 μM $AACOCF_3$ was added. The instrumental parameters were exactly the same as those in Fig. 1A. The ESR spectra are labeled to show their components: DMPO-hydroxyl radical adduct (∗) and $g = 2.005$ signal (▼).

Table 2
Effects of inhibitors on the intensity of the $g = 2.005$ radical induced by 1 mM arachidonic acid (AA) in washed human platelets[a]

Sample	Percentage of control value ± SEM ($n = 3$)
PS (blank)	5.6 ± 1.2
PS + AA (1 mM) (control)	100.0 ± 0.0
PS + AA + KCN (100 μM)	53.6 ± 5.6
PS + AA + KCN (300 μM)	28.4 ± 4.9
PS + AA + desferroxamine (1 mM)	13.3 ± 2.3
PS + AA + $AACOCF_3$ (100 μM)	97.3 ± 8.9

[a] The reaction conditions and techniques of ESR measurements are decribed under Materials and Methods. Inhibitors were added to the platelet suspension (PS) (1×10^9 platelets/ml, 150 μl) in the presence or absence of AA. The instrumental parameters were exactly the same as those in Fig. 1. All values were normalized to 100% for 1 mM AA and represent the averages of three independent incubations. Data are presented as means ± SEM.

2.005 signal was detected (Fig. 4B). After 80 min, only the double-triplet signal was present. Control reactions, without AA, without PBN, without platelets, or without Pronase produced no double-triplet signal (Fig. 4D). This spectrum consisted primarily of six lines with hyperfine coupling constants of $a^N = 16.1$ G and $a^H = 3.4$ G, which were deduced to be a carbon-centered radical adduct (PBN/$^{\cdot}CH(OH)CH_3$) (data from the spin trap database of NIEHS at http://epr.niehs.nih.gov). Neither the DMPO/platelet-derived nor the PBN/platelet-derived adduct was dependent on the presence of the solvent (ethanol), as control experiments using dimethyl sulfoxide or benzene also produced similar signals (data not shown).

Formation of radicals during peroxide reduction in human platelets

The formation of an ESR signal was monitored following the addition of a variety of peroxides or fatty acids to 150 μl of human platelet suspensions. Table 1 lists these substrates along with their concentrations. It has been demonstrated that PGHS–peroxidase activity prefers alkyl

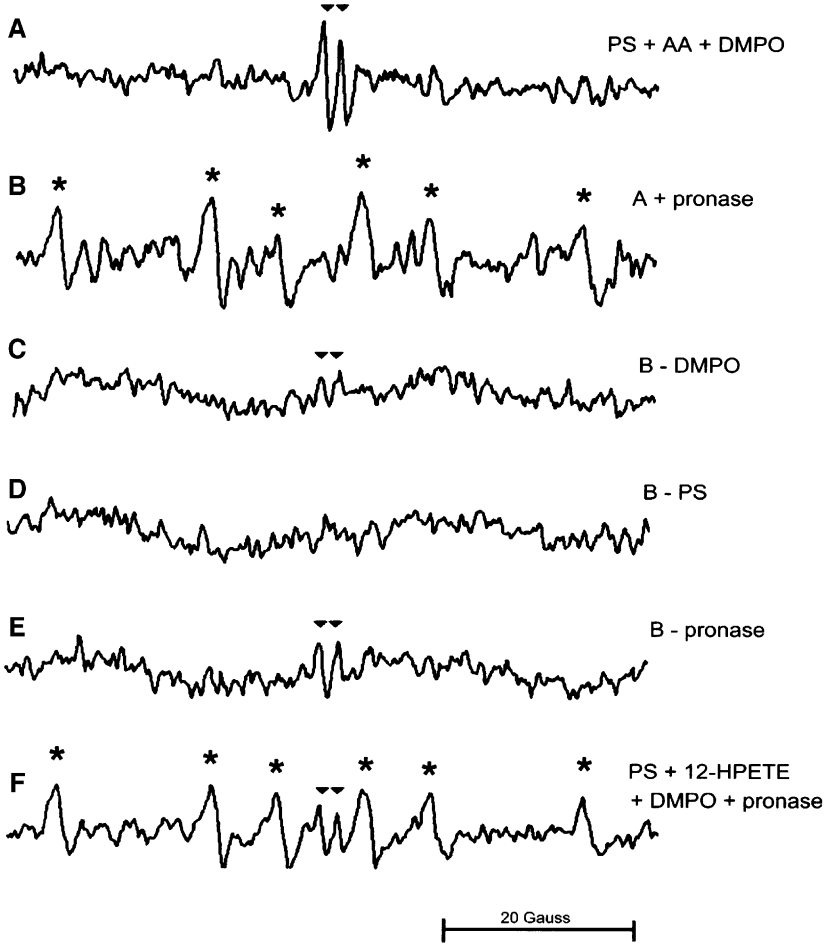

Fig. 3. ESR spectra obtained from the reaction of human platelets with arachidonic acid in the presence of DMPO. (A) Initial reaction mixture containing human platelet suspensions (1×10^9 platelets/ml, 0.15 ml), 2 mM arachidonic acid (AA), and 100 mM DMPO. (B) Same as (A) except that 7 mg/ml Pronase was added. The reaction was allowed to proceed for 80 min. (C) Same as (B) without DMPO. (D) Same as (B) without human platelets. Similar spectra were seen when AA was omitted. (E) Same as (B) without Pronase. (F) Same as (B) except that the AA was replaced by 30 μM 12-HPETE. Similar spectra were seen when 12-HPETE was respectively replaced by 15-HPETE and PGG_2. The instrumental parameters were exactly the same as those in Fig. 1A. The ESR spectra are labeled to show their components: DMPO-carbon-centered radical adduct (*) and $g = 2.005$ signal (▼).

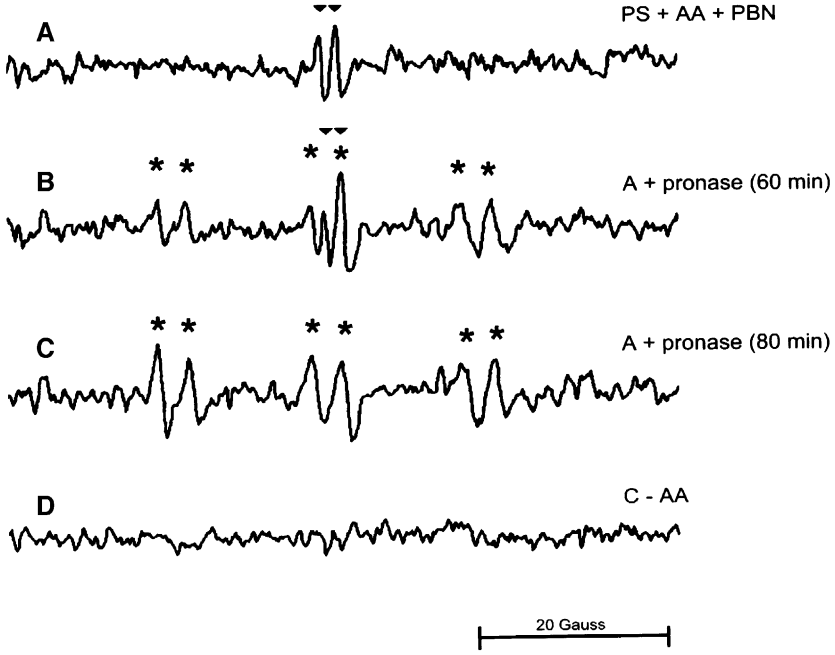

Fig. 4. ESR spectra obtained from the reaction of human platelets with arachidonic acid in the presence of PBN. (A) Initial reaction mixture containing human platelet suspensions (1×10^9 platelets/ml, 0.15 ml), 1 mM arachidonic acid (AA), and 100 mM PBN. (B) Same as (A) except that 7 mg/ml Pronase was added for 60 min. (C) After further reaction for 80 min. (D) Same as (C) without AA. Similar spectra were seen when platelets, PBN, or Pronase was omitted. The instrumental parameters were exactly the same as those in Fig. 1A. The ESR spectra are labeled to show their components: PBN-carbon-centered radical adduct (∗) and $g = 2.005$ signal (▼).

peroxides, although it is much less substrate specific than cyclooxygenase [19,20]. Our results are consistent with those observations, indicating that the $g = 2.005$ signal radical is generated during PGHS–peroxidase-mediated oxidation.

Inhibition studies on the formation of the g = 2.005 signal radical in AA-stimulated human platelets

Formation of the $g = 2.005$ signal radical was monitored following the addition of several inhibitors to 150 μl of platelet suspensions. The inhibitors were added 10 min before the addition of AA. Table 2 shows the effect of various inhibitors on AA (1 mM)-stimulated human platelets. These data suggested that the formation of the $g = 2.005$ radical is associated with the peroxidatic activity of PGHS–peroxidase in intact platelets.

Effects of cosubstrates of PGHS on the g = 2.005 radical generated by AA in human platelets

α-Naphthol [21], acetaminophen [6,22], diethylstilbestrol [8], GSNO [13], and GSH [23] have been proposed to function as cosubstrates of PGHS–peroxidase. The effects of these materials on $g = 2.005$ radical formation were examined as shown in Table 3. GSNO (1 mM) and GSH (1 mM) significantly inhibited the formation of the $g = 2.005$ signal to 35.9 and 32.9% of the control, respectively. Similar results were obtained when GSNO and GSH were added after the formation of the $g = 2.005$ signal (data not shown).

The hypothesis that the $g = 2.005$ signal is a PGHS–peroxidase-derived free radical is based on previous experimental results: (1) AA and peroxides, but not PGH_2 or linoleic acid induced the $g = 2.005$ signal (Table 1); (2) the $g = 2.005$ signal was inhibited by the hemoprotein inhibitor KCN and the iron chelator desferroxamine (Table 2); and (3) a variety of PGHS–peroxidase cosubstrates did not induce the $g = 2.005$ signal alone, but stimulated or inhibited it in the presence of peroxides (Table 3).

Table 3
Effects of peroxidase cosubstrates on the intensity of the $g = 2.005$ radical induced by 1 mM arachidonic acid (AA) in washed human platelets[a]

Sample	Percentage of control value ± SEM ($n = 3$)
PS (blank)	5.9 ± 3.2
PS + AA (1 mM) (control)	100.0 ± 0.0
PS + AA (1 mM) + α-naphthol (1 mM)	168.3 ± 16.9
PS + AA (1 mM) + acetaminophen (1 mM)	135.4 ± 8.9
PS + AA (1 mM) + diethylstilbestrol (1 mM)	258.3 ± 29.3
PS + AA (1 mM) + GSNO (1 mM)	35.9 ± 5.6
PS + AA (1 mM)+ GSH (1 mM)	32.9 ± 6.5
PS + AA (60 μM)	7.2 ± 2.6
PS + AA (60 μM) + α-naphthol (1 mM)	30.3 ± 5.5
PS + AA (60 μM) + acetaminophen (1 mM)	23.3 ± 6.9
PS + AA (60 μM) + diethylstilbestrol (1 mM)	36.2 ± 8.9

[a] The reaction conditions and techniques of ESR measurements are described under Materials and Methods. Cosubstrates were added to the platelet suspension (PS) (1×10^9 platelets/ml, 150 μl) in the presence or absence of AA. The instrumental parameters were exactly the same as those in Fig. 1. All values were normalized to 100% for 1 mM AA and represent the averages of three independent incubations. Data are presented as means ± SEM.

Formation of the thiyl radical in human platelets

A previous study reported that free radicals formed by PGHS–peroxidase oxidize GSH to a thiyl radical, which is easily trapped by DMPO [24]. This technique may be useful for indirectly determining free radical formation by PGHS–peroxidase in cells [24]. When human platelets were incubated with AA, the $g = 2.005$ signal radical was directly detected by the ESR technique (Fig. 5A) and was inhibited by GSH (Fig. 5B), following a four-line DMPO/GS$^{\bullet}$thiyl radical ESR spectrum (Fig. 5C) with hyperfine splitting constants of $a^{N} = 15.3$ G and $a^{H} = 16.2$ G being detected, which were identical to those demonstrated by Schreiber et al. [24]. Replacement of AA by 12-HPETE and PGG_2 also supported that the thiyl radical adduct signal indicated the involvement of PGHS–peroxidase (Figs. 5D,E).

Effects of peroxidase cosubstrates and peroxides on platelet aggregation in human platelet suspensions

The peroxidase cosubstrates were added 3 min before the addition of AA, and we found that α-naphthol, acetamino-

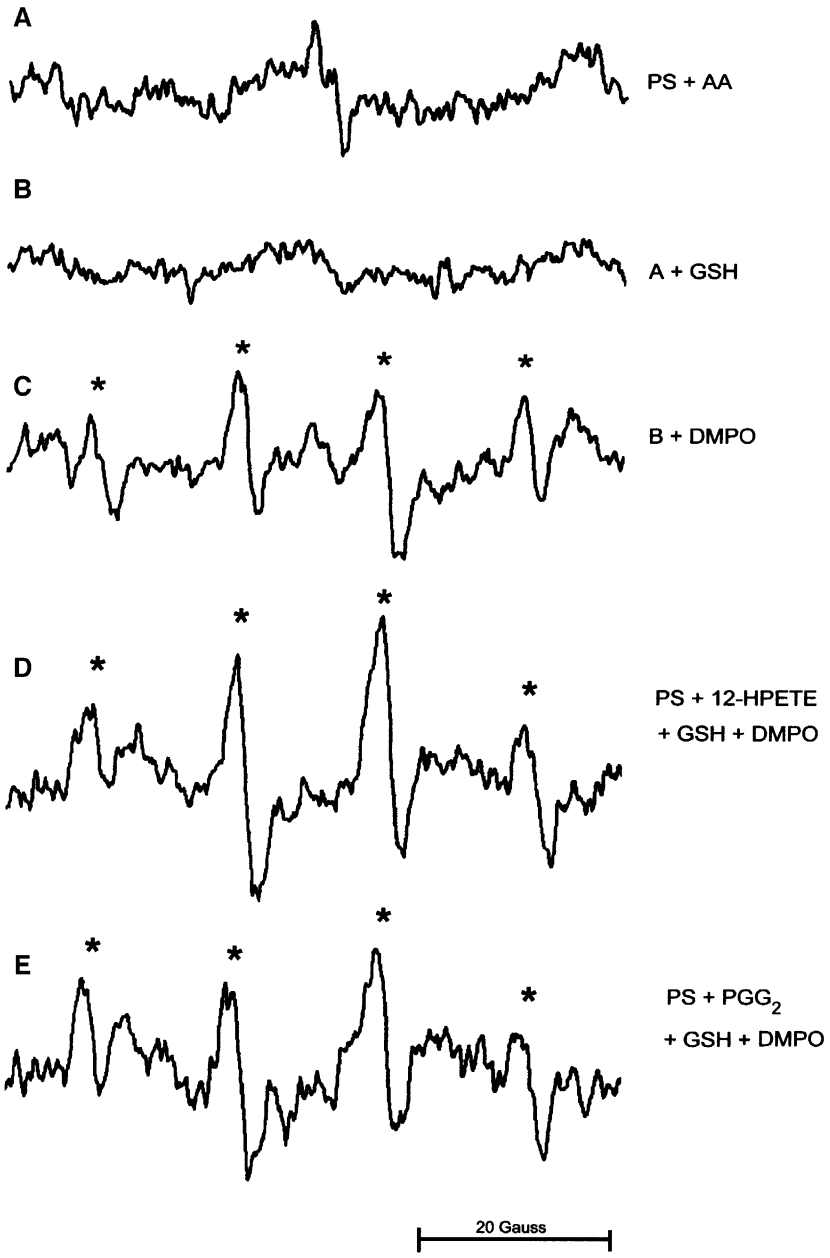

Fig. 5. DMPO spin trapping of the thiyl radical in the arachidonic acid/platelet system. Human platelets (1×10^9 platelets/ml, 0.15 ml) preincubated with (A) arachidonic acid (2 mM), followed by the addition of (B) GSH (10 mM) for 2 min; then (C) DMPO (100 mM) was added. (D, E) Same as (C) except that the AA was replaced by 60 μM 12-HPETE and PGG_2, respectively. The instrumental parameters were exactly the same as those in Fig. 1A, except that the modulation amplitude was now 3 G. The ESR spectra are labeled to show their components: DMPO-thiyl radical adduct (∗).

phen, and diethylstilbestrol concentration-dependently inhibited platelet aggregation stimulated by AA (60 μM) in human platelet suspensions (Fig. 6A). The IC_{50} values of α-naphthol, acetaminophen, and diethylstilbestrol for platelet aggregation induced by collagen were estimated to be 0.5 ± 0.1, 456.3 ± 11.1, and 355.2 ± 9.8 μM, respectively. The lower IC_{50} values of α-naphthol may have resulted from its antioxidation and nonspecific inhibition of both prostaglandin synthetase and soybean lipoxidase [25]. GSH showed activity for quenching the g = 2.005 signal in the AA/platelet system (Fig. 5B). Thus, we wanted to determine whether it could reverse the inhibitory activity of the above peroxidase cosubstrates on platelet aggregation. Pretreatment of diethylstilbestrol was carried out for 3 min, and then GSH (1 mM) was added 10 s before the addition of AA. We detected no aggregation in this study (Fig. 6A). Similar results were found when concentrations of GSH were increased or decreased (from 10 μM to 10 mM) or when diethylstilbestrol was replaced by α-naphthol or acetaminophen (data not shown). GSNO did not reverse the inhibitory activity of these peroxidase cosubstrates on AA-induced platelet aggregation (data not shown). However, the direct inhibition of platelet aggregation by GSNO [26,27] cannot be excluded.

These data indicate that the g = 2.005 radical may be an irreversibly inactivated intermediate of PGHS. The inability of AA to induce aggregation in peroxide-pretreated platelets is probably due to peroxide-induced destruction of PGHS [19], as increasing the concentration of AA (200–300 μM) reversed the inhibition (Fig. 6B). Similar results were obtained with 15-HPETE (3 μM), cumene hydroperoxide (30 μM), and H_2O_2 (300 μM) (data not shown).

AA-induced decrease in GSH levels

We measured platelet GSH concentrations to test the hypothesis that suprathreshold concentrations of AA result in GSH depletion. In this study, we found that resting platelets contained a GSH concentration of about 8.0 ± 0.7 nM/5 × 10^8 platelets (n = 4, data not shown). Suprathreshold concentrations of AA (300 and 600 μM) markedly inhibited GSH concentrations by about 28.4 ± 3.4 and 47.9 ± 3.9% as compared with resting platelets, respectively (n = 4, data not shown). Platelets treated with the threshold concentration of AA (60 μM) inhibited GSH concentration by only about 14.0 ± 2.4% as compared with the resting platelets (n = 4, data not shown).

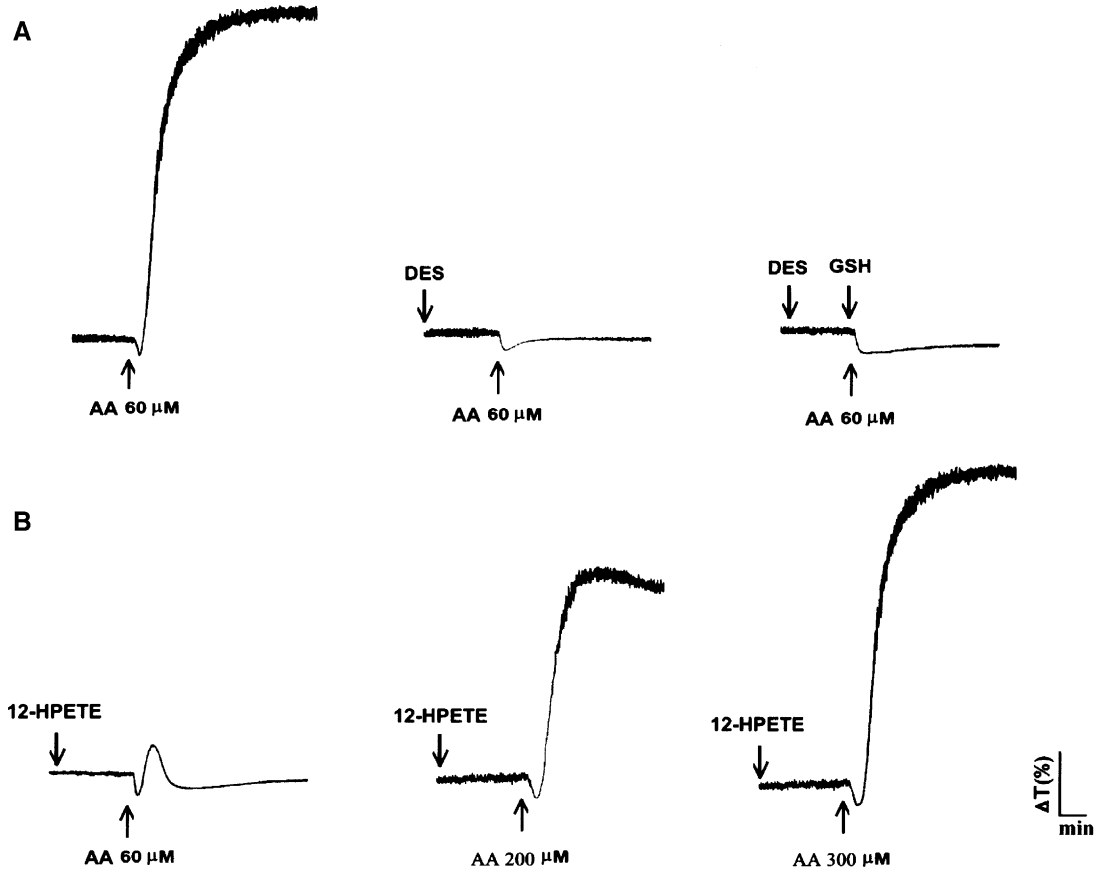

Fig. 6. Tracing curves of PGHS-peroxidase cosubstrates or peroxides on arachidonic acid-induced aggregation in washed human platelets. Platelets were preincubated with (A) diethylstilbestrol (DES) (500 μM) or (B) 12-HPETE (3 μM) for 3 min; arachidonic acid (AA) was then added to trigger platelet aggregation. Reduced glutathione (GSH) (1 mM) was added 10 s before the addition of AA.

Discussion

Using the direct ESR technique, we detected a $g = 2.005$ signal radical induced by collagen, thrombin, AA, 12-HPETE, 15HPETE, PGG_2, H_2O_2, and cumene hydroperoxide in human platelets. We suggest that the $g = 2.005$ signal formed is derived from the reduction of peroxide by PGHS–peroxidase in intact platelets. To the best of our knowledge, this is the first report demonstrating a peroxidase-derived free radical in isolated human platelets.

The first ESR evidence concerning PGHS–peroxidase oxidation was reported by Egan et al. [28]. They detected a free radical signal during AA and PGG_2 metabolism in ram seminal vesicles and suggested that it was an oxygen-centered radical. Similar spectral features have been described in the reduction of a variety of eicosahydroperoxides to their corresponding alcohols by ram seminal vesicle microsomes [19]. This group of investigators suggested that the oxidant was a hydroxy radical. However, Kalyanaraman et al. provided evidence that the free radical formed during PGHS–peroxidase reduction is neither a hydroxyl nor any known oxygen-centered radical [29]. They proposed that the free radical generated by the reaction of PGG_2 with ram seminal vesicles is a hemoprotein-derived radical, which may be formed by the oxidation of an amino acid located near the iron of the heme. In the present study, an immobilized nitroxide was detected when either DMPO or PBN was added to the reaction mixture containing human platelets and AA. Extensive Pronase digestion of either the DMPO/platelet- or PBN/platelet-derived adducts definitively indicated that the radical was a carbon-centered radical. Our results are in agreement with the hypothesis of Kalyanaraman et al. [29]. In addition, we show direct evidence that the free radical formed by the reaction of peroxides with PGHS present in human platelets is a carbon-centered radical.

A branched-chain radical mechanism has been proposed to integrate cyclooxygenase and peroxidase catalytic activities [3]. Resting PGHS (FeIII) reacts with a peroxide (ROOH) to generate Intermediate I . Activation of cyclooxygenase tyrosine to the radical state (Intermediate II) is generated by an internal electron transfer in peroxidase Intermediate I. Resting enzymes can be regenerated by reducing Intermediate I with endogenous or exogenous reductants [13,30,31]. Peroxides are an important stimulus for activation of cyclooxygenase and their functioning as substrates for peroxidase activity [32]. Both peroxidase and cyclooxygenase activities can undergo a limited number of catalytic turnovers before they become irreversibly inactivated during reaction with peroxides [20,33]. Wu et al. reported that the self-inactivation process of cyclooxygenase and peroxidase originates with Intermediate II [34,35]. Cyclooxygenase has a much greater response to peroxide-induced inactivation than peroxidase [36]. Based on these observations, one may speculate that relatively high concentrations of peroxides amplify accumulation of Intermediate II, thus facilitating the formation of inactivated species.

Previous studies have shown that low concentrations of peroxides, for example, micromolar concentrations of 12-HPETE, 15-HPETE (1–2 μM) [37], and H_2O_2 (0.5–5 μM) [38], can trigger aggregation of platelets co-incubated with subthreshold concentrations of AA. Furthermore, H_2O_2 (1–200 μM) was also reported to potentiate AA-induced platelet aggregation [39]. In contrast, inhibition of platelet aggregation occurs at relatively high concentrations of peroxides. For example, 12-HPETE (IC_{50} of 2–3 μM) [40] and 15-HPETE (IC_{50} of 4–10 μM) [41] have been demonstrated to inhibit AA-induced platelet aggregation. Similar results were observed in this study (Fig. 6B). In addition, inhibitory activities of cumene hydroperoxide (30 μM) and H_2O_2 (300 μM) on AA-induced platelet aggregation were also observed (data not shown). Taken together, these data indicate that PGHS partially loses its activity after incubation with relatively high concentrations of peroxides; thus, much higher concentrations of AA are required to induce platelet aggregation.

It has been reported that long-lived protein radicals that are stable at room temperature in the range of $g = 2.004$ to 2.005 can be tyrosyl [42–44] or trytophanyl radicals [45]. Goodwin and co-workers [46] demonstrated that $NO^{\bullet}$ terminates the tyrosyl radical generated by the reaction of purified ram seminal vesicle PGHS with AA. Recently, O'Donnell et al. showed that PGHS-dependent consumption of $NO^{\bullet}$ occurred in AA-, thrombin-, and collagen-stimulated platelets but not in ADP- or U44619-stimulated ones, and suggested that $NO^{\bullet}$ reacts with Intermediate II of PGHS in platelets [13]. These observations indicate that only AA-, thrombin-, and collagen-induced platelet activations are involved in the AA/PGHS metabolic pathway. Notably, suprathreshold concentrations of aggregatory agents were also applied in the former study, although they were less than those used in this study. Consistent with this, we demonstrate the formation of PGHS–peroxidase-mediated free radicals in AA-, thrombin-, and collagen-stimulated platelets, but not in ADP- or U44619-stimulated ones. In addition, we show that GSNO quenches the $g = 2.005$ signal generated by reaction of platelets with AA (Table 3). This means that the $g = 2.005$ radical may be a tyrosyl radical from cyclooxygenase. However, the spectrum of our DMPO/carbon-centered radical adduct differed from that of the DMPO/tyrosyl radical adduct reported by Witting et al. [47]; thus, the $g = 2.005$ carbon-centered radical is not a tyrosyl radical. This suggests that the $g = 2.005$ carbon-centered radical may be an inactivated species that originates after formation of Intermediate II. In this study, we directly detected a PGHS-derived inactivated species generated by relatively high concentrations of endogenous or exogenous peroxides in intact platelets.

PGHS–peroxidase catalyzes the oxidation of a wide variety of xenobiotics. The effects of cosubstrates on PGHS

catalytic activity have intensively been investigated. It is well established that PGHS cosubstrates stimulate cyclooxygenase at low concentrations and inhibit it at higher concentrations. The stimulation effect may be due to their ability to scavenge the peroxide-mediated self-destructing radical [28] or the original radical [48,49]. The inhibitory effect has been proposed to be associated with the scavenging of radicals that are necessary for PGHS' catalytic activity [48,49] or by specific binding at the two sites of the enzyme [50]. Recently, Wu et al. reported that reducing cosubstrates protected peroxidase activity more than it protected cyclooxygenase activity [36]. In this work, we used AA at the threshold concentration (60 μM) as the PGHS substrate to induce platelet aggregation. Acetaminophen significantly increased the intensity of the $g = 2.005$ signal (Table 3) and inhibited platelet aggregation induced by AA (data not shown). In addition to acetaminophen, other reducing cosubstrates, such as α-naphthol and diethylstilbestrol, produced similar effects in the ESR study (Table 3) and platelet aggregation experiments (Fig. 6). Based on the present data and other findings, we propose a new hypothesis: the inhibition of cyclooxygenase activity at higher concentrations of peroxidase cosubstrates possibly occurs due to the facilitation of PGHS–peroxidase turnover induced by peroxides, thus enhancing accumulation of Intermediates I and II, leading to formation of inactivated species.

Platelets contain a large fraction of GSH [51]. Bosia et al. suggested that endogenous platelet GSH acts as a reducing cofactor in the PGHS–peroxidase-mediated pathway [23]. Free radicals formed by PGHS–peroxidase can oxidize GSH to a thiyl radical, which is trapped by DMPO [24]. Our study using this technique for the AA/platelet system demonstrated the formation of a thiyl radical (Fig. 5C) identical to that of a previous study, but it has not yet been described in intact platelets. In addition to that previous study, we directly detected free radicals derived from PGHS–peroxidase. The generation of a thiyl radical after the addition of GSH is further evidence of one electron mechanism operating in these reactions and indicates that the $g = 2.005$ signal radical can interact with other endogenous reducing substances or antioxidant enzymes. This mechanism may explain the observation that the $g = 2.005$ signal was not obtained using threshold concentrations of aggregatory agents applied in the platelet aggregation experiment (Table 3). Based on our results, we propose that suprathreshold concentrations of aggregatory agents may induce much greater quantities of free radical intermediates leading to depletion of endogenous GSH in platelets. Thus, the remaining free radicals make ESR detection achievable. The hypothesis that the $g = 2.005$ radical is an irreversibly deactivated species was further strengthened by the observation that both GSH and GSNO quenched the $g = 2.005$ radical, but neither could restore platelet aggregation activity. A model that accounts for our present results and other reports of PGHS catalysis and inactivation is presented in Fig. 7. The species Fe(IV)C* is the free radical described in this study.

In summary, we have detected a free radical species that is generated by the reaction of thrombin, collagen, AA, and several peroxides with human platelets. Under these conditions, free radical generation subsequent to the attack on PGHS may be the event that results in its irreversible deactivation. Several peroxidase cosubstrates are presumed to stimulate the enzyme and the enhanced accumulation of intermediate free radicals, leading to formation of a self-destructing species.

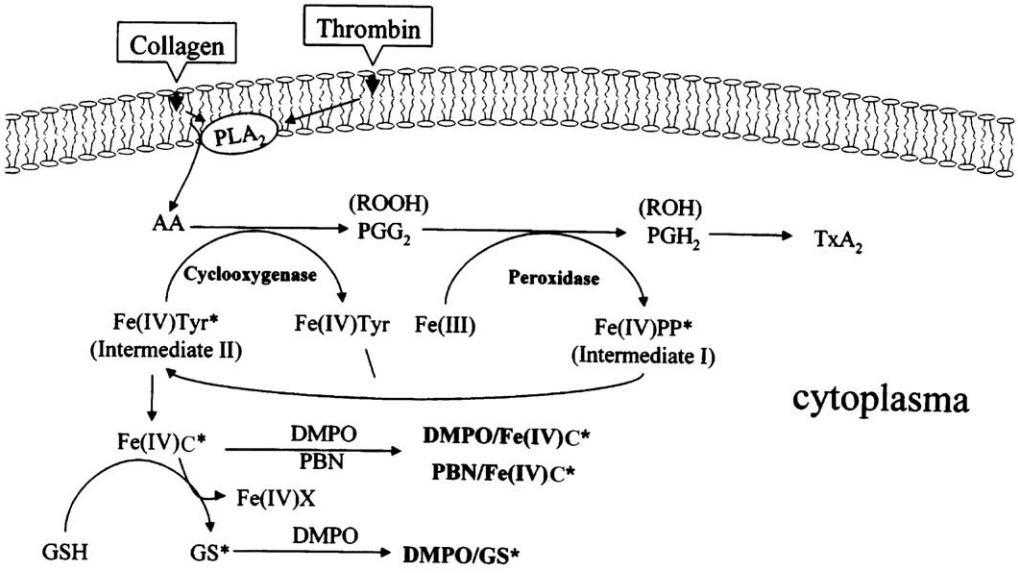

Fig. 7. Proposed pathway for the branched-chain radical mechanism of PGHS in intact human platelets and subsequent spin trapping of the carbon-centered free radical and the thiyl radical. Fe(III), resting enzyme with ferric heme; Fe(IV)PP*, intermediate I with ferryl heme and porphyrin radical; Fe(IV)Tyr*, intermediate II with ferryl heme and Tyr-385 radical; Fe(IV)C*, intermediate II with ferryl heme and a carbon-centered free radical.

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