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Baicalein Induction of Hydroxyl Radical Formation via 12-Lipoxygenase in Human Platelets: An ESR Study

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The pro-oxidant activities of baicalein, morin, myricetin, quercetin, and rutin were examined in various cell-containing systems including human platelets, rat vascular smooth muscle cells, human umbilical vein endothelial cells (HUVECs), human THP-1 cells, and fibroblast cells. Electron spin resonance (ESR) results showed that only baicalein generated hydroxyl radicals in a resting human platelet suspension, whereas the other flavonoids showed no effects on any of the resting cell systems. A low concentration of arachidonic acid (AA) increased the intensity of hydroxyl radicals, but a high concentration inhibited it. Collagen and thrombin, platelet aggregatory agents that can cause the release of AA by platelets, enhanced baicalein-induced hydroxyl radical formation, whereas ADP and U44619 showed no significant effects. Quinacrine and 5,8,11,14-eicosatetraenoic trifluoromethyl ketone, both PLA_2 inhibitors, significantly attenuated baicalein-induced hydroxyl radical formation. These results suggest that baicalein-induced hydroxyl radical formation is associated with AA metabolite enzymes in human platelets. The formation of hydroxyl radicals was significantly inhibited by lipoxygenase inhibitors including nordihydroguaiaretic acid, (−)-epicatechin, (−)-epicatechin gallate, and hinokitiol, but was not affected by desferroxamine or the heme protein inhibitors KCN and NaN_3. On the other hand, semiquinone free radicals were generated when baicalein was incubated with horseradish peroxidase/H_2O_2 or platelets/AA. The semiquinone radicals formed in the platelets/AA system could be extensively inhibited by desferroxamine, diethylenetriaminepentaacetic acid, KCN, and NaN_3, indicating that prostaglandin H synthase (PGHS)-peroxidase may be involved. The results of this study led to the proposal that baicalein induces hydroxyl radical formation via 12-lipoxygenase and induces semiquinone radical formation via PGHS-peroxidase in human platelets.

KEYWORDS: Flavonoids; baicalein; platelets; electron spin resonance; 12-lipoxygenase; hydroxyl radical; semiquinone radical

INTRODUCTION

Free radical formation has been implicated in a large number of disease states such as inflammation, immune injury, myocardial infarction, and certain forms of cancer. Various age-related diseases and the aging process itself have been assumed to be a result of the free radical mediated degeneration of cell components. Therefore, antioxidants may be of therapeutic use in such conditions. Many naturally occurring agents have shown antioxidant activities. Flavonoids, a group of low molecular weight polyphenolic compounds, are present in many plants and folk medicines. They have long been recognized to exhibit anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities [as reviewed by Yao et al. (*1*)]. In recent years, the antioxidant activities of flavonoids have been given much attention. Three mechanisms have been proposed to explain these antioxidant activities: a free radical scavenging effect, a metal-chelating effect, and a radical-forming enzyme-inhibitory effect. Of these possible mechanisms, the free radical scavenging effect of flavonoids occurs through hydrogen atom donation from the phenolic group on the flavonoid fragment (*1*). Flavonoids contain six major subgroups: chalcone, flavone, flavonol, flavanone, anthocyanins, and isoflavonoids. The basic structure of flavonoids allows for a large number of hydroxyl substitution patterns in benzene rings A, B, and C within each subgroup of flavonoids. Flavonoids can react with the superoxide anion through one-electron transfer, and it is converted to an aroxyl radical in the B ring. The stable aroxyl radical formation was proposed to be associated with the superoxide anion scavenging activity of flavonoids (*2*). In addition, Furuno et al. (*3*) demonstrated that the pyrogallol (benzene-1,2,3-triol) moiety in the B ring is an

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important component of flavonoids' ability to scavenge the superoxide anion.

In contrast, Miura et al. (*4*) reported that flavonoids which possess pyrogallol and catechol (benzene-1,2-diol) moieties in their structures showed strong H_2O_2-generating activities through the superoxide anion radical. Furthermore, Hanasaki et al. (*5*) showed that four flavonoids (baicalein, quercetin, morin, and myricetin) increase the hydroxyl radical production in the Fenton system. Quercetin and myricetin can undergo autoxidation when dissolved in an aqueous buffer; however, the rates of autoxidation of both quercetin and myricetin are highly pH dependent (*6*). Dietary phenolics may produce reactive oxygen species (ROS) and phenoxyl radicals in the presence of transition metals and O_2 (*7*). The ROS can further damage DNA, lipids, and other biological molecules. Some flavonoids induce H_2O_2 formation and cause oxidative damage to isolated and cellular DNA in the presence of transition metal ions. Flavonoids such as myricetin, baicalein, and quercetin have been shown to potently cleave plasmid pBR322 DNA and calf thymus DNA (*8*). Some flavonoids with phenol B rings, such as naringenin, naringin, hesperetin, and apigenin, form pro-oxidant metabolites that oxidized NADH upon oxidation by peroxidase/H_2O_2 (*7*). Peroxidase also catalyzes apigenin, naringin, and naringenin to produce phenoxyl radicals, which co-oxidize glutathione (GSH) to form thiyl radicals (*9*). It is reasoned that the same flavonoids can perform as both antioxidants and pro-oxidants, depending on the concentration and free radical source. However, there is no direct electron spin resonance (ESR) evidence of the same flavonoids acting as both antioxidants and pro-oxidants in the same cell-containing systems.

We attempted to detect and identify the free radicals formed from some flavonoids in several intact cell systems by using ESR and spin-trapping methods. Our results show that only baicalein generated hydroxyl radicals in human platelet suspensions.

MATERIALS AND METHODS

Materials. Arachidonic acid (AA), bovine serum albumin (BSA), collagen (type I, bovine achilles tendon), desferroxamine mesylate, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), diethylenetriaminepentaacetic acid (DTPA). glutathione (GSH), heparin, indomethacin, nordihydroguaiaretic acid (NDGA), linoleic acid (LA), morin, myricetin, potassium cyanide (KCN), prostaglandin E_1 (PGE_1), quinacrine, quercetin, rutin, superoxide dismutase (SOD), catalase (CAT), sodium azide (NaN_3), sodium citrate, and thrombin were purchased from Sigma Chemical Co. (St. Louis, MO). Baicalein was purchased from Aldrich Chemical Co. (Milwaukee, WI). (−)-Epicatechin, (−)-epicatechin gallate, and hinokitiol were purchased from Wako Pure Chemical Industries (Osaka, Japan). U44619 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). 5,8,11,14-Eicosatetraenoic trifluoromethyl ketone ($AACOCF_3$) was purchased from Cayman (Ann Arbor, MI).

Preparation of Human Platelet Suspensions. Human platelet suspensions were prepared as previously described (*10*). 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.

Cell Cultures. *Vascular smooth muscle cells (VSMCs)* were enzymatically dispersed from male Wistar rats (250−300 g). Thoracic aortas from Wistar rats were removed and stripped of endothelium and adventitia. VSMCs were obtained by a modification of the combined collagenase and elastase digestion method (*11*). These cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) containing 10% fetal bovine serum (PAA Laboratories GmbH, Linz, Austria) and antibiotics. The growth medium was changed every 2−3 days until cells reached confluency. The growth medium was removed, and the monolayer was rinsed with phosphate-buffered saline. Trypsin−EDTA solution (Gibco) was added, and the monolayer was incubated at 37 °C for 2 min. The culture dishes were observed under a phase-contrast microscope until the cells were detached. Cells were removed with 10 mL of DMEM and centrifuged at 900*g* for 7 min. The pellet was resuspended in DMEM on a culture dish, and the VSMCs were grown on this.

THP-1. The THP-1 human monocytic cell line [American Type Culture Collection (ATCC), Rockville, MD] was grown in RPMI 1640 medium (Gibco) containing 10% fetal calf serum (FCS; Gibco), 100 units/mL penicillin G, and 100 μg/mL streptomycin (Gibco). Cells were maintained in culture at 37 °C and 5% CO_2.

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical veins and harvested by enzymatic treatment with chymotrypsin. HUVECs were grown in medium 199 (Gibco), 20% FCS (Gibco), 20 mg/mL endothelial cell growth supplement (ECGS; Upstate Biotechnologies, Lake Placid, NY), 0.1% heparin, 100 ng/mL gentamicin (Gibco), and 2% 1 mol/L HEPES-buffer (J. T. Baker Chemical Co., Phillipsburg, NJ). At confluence after 2 days of culturing, cells were incubated with fresh culture medium. After up to 4 days of incubation at 37 °C, the culture medium was collected and centrifuged, and the supernatant was harvested for each experiment.

Fibroblast. A normal human lung fibroblast cell line consisting of WI-38 cells was purchased from Bioresource Collection and Research Center (BCRC, Taiwan) as original CCL-75 obtained from ATCC and grown in DMEM supplemented with 20 mM HEPES, 10% FCS, 100 units/mL penicillin G, 100 mg/mL streptomycin, and 2 mM glutamine at 37 °C in a humidified incubator of 5% CO_2.

Measurement of Free Radicals in Platelet Suspensions by Electron Spin Resonance (ESR) Spectrometry. The ESR method used a Bruker EMX ESR spectrometer as described previously (*12*) but with some modifications. The culture medium was changed to Tyrode's solution before each experiment. Platelet suspensions (4.5×10^8 platelets/mL, 150 μL) were prewarmed to 37 °C for 2 min, and then enzyme inhibitors or other reagents were added before the addition of baicalein. ESR spectra were recorded at room temperature using a quartz flat cell designed for aqueous solutions. The dead time of sample preparation and ESR analysis was exactly 30 s after the last addition. Conditions of ESR spectrometry 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. The modulation amplitude, sweep time, and time constant are given in the figure captions and table footnotes.

Statistical Analysis. Experimental results are expressed as the mean ± SEM and are accompanied by the number (*n*) of observations. Data were assessed using analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared using the Newman−Keuls method. A *p* value of <0.05 was considered to be statistically significant.

RESULTS

ESR Investigations of Free Radicals Induced by Flavonoids in Human Platelets, Rat Vascular Smooth Muscle Cells, HUVECs, Human THP-1 Cells, and Fibroblast Cells. We demonstrated that baicalein dose-dependently produced a typical four-line hydroxyl radical signal ($a^N = a^H = 14.8$ G) detectable by spin-trapping DMPO in human platelet suspensions (**Figure 2a−c**) but not in rat vascular smooth muscle cells, HUVECs, human THP-1 cells, or fibroblast cells (**Figure 1b−e**). No significant radical was detected when all of these cell preparations were treated with morin (300 μM), myricetin (300 μM), quercetin (300 μM), or rutin (300 μM) (**Figure 1 b−e**). This indicates that baicalein-induced hydroxyl radical formation is associated with particular molecules or enzymes which are

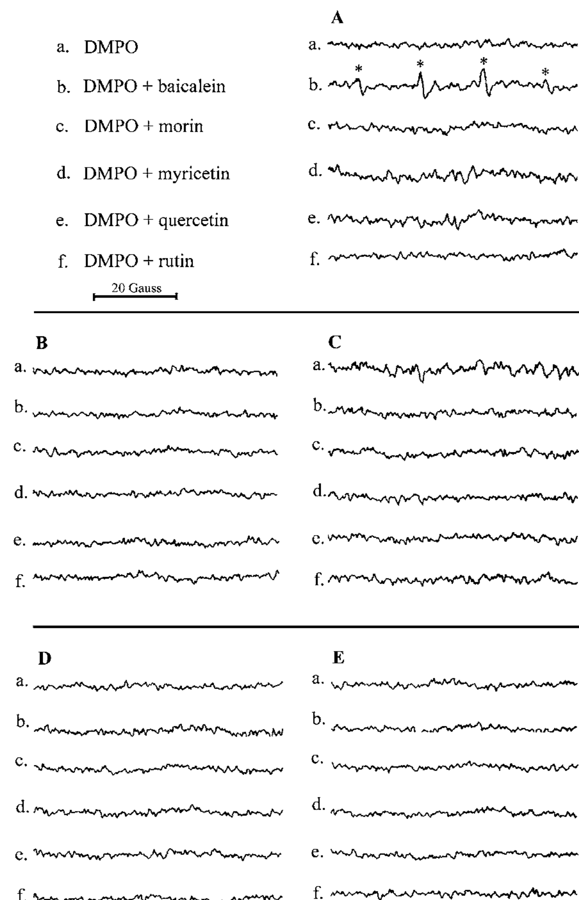

Figure 1. ESR spectra obtained from the reaction of (**A**) human platelet suspensions, (**B**) rat vascular smooth muscle cells, (**C**) HUVECs, (**D**) human THP-1 cells, and (**E**) fibroblast cells with (**b**) baicalein (300 μM), (**c**) morin (300 μM), (**d**) myricetin (300 μM), (**e**) quercetin (300 μM), and (**f**) rutin (300 μM) in the presence of (**a**) DMPO (100 mM). Instrument parameters were as follows: modulation amplitude, 1 G; time constant, 164 ms; scanning for 42 s with 3 scans accumulated.

present only in human platelets but not in rat vascular smooth muscle cells, HUVECs, human THP-1 cells, or fibroblast cells. Treatment with a higher concentration (300 μM) of baicalein observed more marked free radical signals than a lower concentration. In subsequent experiments, we used the concentration of 300 μM of baicalein to study the detailed mechanism of baicalein-stimulated free radical formation.

Effects of 12-Lipoxygenase (LOX) Inhibitors on the Hydroxyl Radical Signal Induced by Baicalein in Human Platelets. Baicalein was shown to inhibit 12-LOX activity in human platelets (*13*). 12-LOX is the predominant route by which AA is metabolized in human platelets. Because of the importance of this enzyme in the AA cascade, we chose to use four inhibitors to investigate the role of 12-LOX in baicalein-treated platelets. Platelets incubated with NDGA (300 μM), (−)-epicatechin (300 μM), (−)-epicatechin gallate (300 μM), or hinokitiol (300 μM) showed a decrease (49−60%) in hydroxyl radical generation (**Table 1**). These observations suggest a role for 12-LOX in the generation of baicalein-induced hydroxyl radicals in human platelets.

Effects of PLA_2 Inhibitors on the Hydroxyl Radical Signals Induced by Baicalein in Human Platelets. The main product of PLA_2 activation in platelets is AA. AA may be further metabolized by cyclo-oxygenase (COX) to yield prostaglandin or by 12-LOX to yield leukotriene B_4 (LTB_4). We therefore investigated the roles of AA in the activation of 12-LOX.

Table 1. Effects of Antioxidants on the Intensity of Hydroxyl Radicals Induced by 300 μM Baicalein in Washed Human Platelets[a]

sample	% of control value ± SEM (n = 4)
PS + baicalein (300 μM) (control)	100.0 ± 0.0
PS + baicalein + NDGA (300 μM)	42.7 ± 3.2**
PS + baicalein + (−)-epicatechin (300 μM)	47.6 ± 2.7**
PS + baicalein + (−)-epicatechin gallate (300 μM)	51.2 ± 2.2**
PS + baicalein + hinokitiol (300 μM)	50.0 ± 1.5**
PS + baicalein + rutin (300 μM)	65.4 ± 2.2**
PS + baicalein + quercetin (300 μM)	78.3 ± 5.2*
PS + baicalein + myricetin (300 μM)	69.9 ± 12.0*
PS + baicalein + morin (300 μM)	72.6 ± 6.9*

[a] The reaction conditions and techniques or ESR measurements are described under Materials and Methods. Inhibitors were added to platelet suspensions (PS) (4.5×10^8 platelets/mL, 150 μL) in the presence or absence of baicalein. The instrument parameters were exactly the same as those in **Figure 1**. All values were normalized to 100 for 300 μM baicalein and represent the average of four independent incubations. Data are presented as the mean ± SEM (n = 4). *, $p < 0.05$, and **, $p < 0.005$, as compared with the control groups.

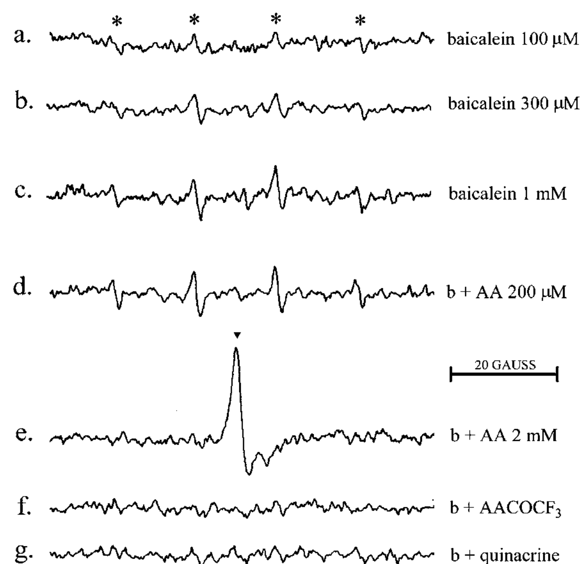

Figure 2. ESR spectra detected from the reaction of human platelet suspensions with baicalein in the presence of DMPO. Human platelets were preincubated with DMPO (100 mM) followed by the addition of baicalein at (**a**) 100 μM, (**b**) 300 μM, and (**c**) 1 mM. (**d**) Same as (**b**), but with AA (200 μM); (**e**) same as (**b**), but with AA (2 mM); (**f**) same as (**b**), but with $AACOCF_3$ (50 μM); (**g**) same as (**b**), but with quinacrine (20 μM). The instrument parameters were exactly the same as those in **Figure 1**.

$AACOCF_3$, a potent $cPLA_2$ inhibitor, has been shown to inhibit thrombin-induced endogenous AA release by platelets (*14*). Quinacrine, a widely used nonselective PLA_2 inhibitor, inhibited the release of AA from fMLP-stimulated PMN. The inhibitors were added 10 min before the addition of baicalein. Our results showed that baicalein-induced hydroxyl radical formation was significantly prevented by $AACOCF_3$ and quinacrine (**Figure 2f**,**g**; **Table 2**). These results imply a role for AA in the activation of 12-LOX.

Effects of Aggregatory Agents on the Hydroxyl Radical Signals Induced by Baicalein in Human Platelets. Collagen and thrombin, both platelet aggregatory agents, have been demonstrated to release AA in platelets (*14*, *15*). Thus, we wanted to determine whether collagen and thrombin can enhance

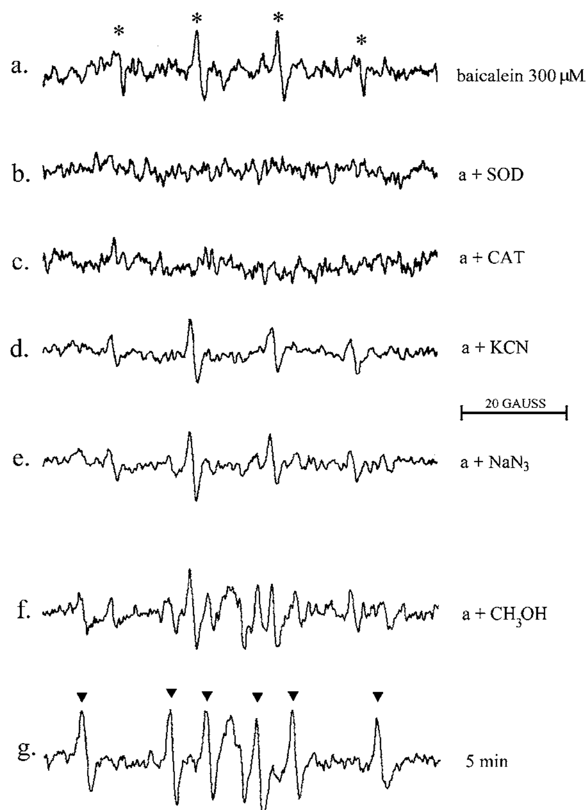

Figure 3. ESR spectra detected from the reaction of human platelet suspensions with baicalein in the presence of DMPO. Human platelets were preincubated with DMPO (100 mM) followed by the addition of (**a**) 300 μM baicalein. (**b**) Same as (**a**), but with SOD (120 units/mL); (**c**) same as (**a**), but with CAT (1000 units/mL); (**d**) same as (**a**), but with KCN (300 μM); (**e**) same as (**a**), but with NaN_3 (1 mM); (**f**) same as (**a**), but with CH_3OH (15 μL); (**g**) after further reaction for 5 min. The instrument parameters were exactly the same as those in **Figure 1**.

Table 2. Effects of Inhibitors on the Intensity of Hydroxyl Radicals Induced by 300 μM Baicalein in Washed Human Platelets[a]

sample	% of control value ± SEM ($n = 4$)
PS + baicalein (300 μM) (control)	100.0 ± 0.0
PS + baicalein + NaN_3 (1 mM)	95.7 ± 3.1
PS + baicalein + KCN (300 μM)	99.0 ± 6.1
PS + baicalein + desferroxamine (1 mM)	112.0 ± 4.9
PS + baicalein + indomethacin (30 μM)	133.1 ± 9.7*
PS + baicalein + quinacrine (20 μM)	48.4 ± 3.3*
PS + baicalein + $AACOCF_3$ (50 μM)	58.3 ± 2.9*

[a] The reaction conditions and techniques or ESR measurements are described under Materials and Methods. Inhibitors were added to platelet suspensions (PS) (4.5×10^8 platelets/mL, 150 μL) in the presence or absence of baicalein. The instrument parameters were exactly the same as those in **Figure 1**. All values were normalized to 100 for 300 μM baicalein and represent the average of four independent incubations. Data are presented as the mean ± SEM ($n = 4$). *, $p < 0.05$, as compared with the control group.

the formation of baicalein-induced hydroxyl radicals. Our results are consistent with this expectation (**Table 3**).

Effects of Fatty Acids on the Hydroxyl Radical Signals Induced by Baicalein in Human Platelets. Platelet 12-LOX has a narrower substrate specificity in that it reacts with only eicosapolyenoic acids but not with linoleic acid or linolenic acids (*16*). We therefore investigated the roles of fatty acids in the activation of 12-LOX. Our results showed that the formation

Table 3. Effects of Aggregatory Agents on the Intensity of Hydroxyl Radicals Induced by 300 μM Baicalein in Washed Human Platelets[a]

sample	% of control value ± SEM ($n = 4$)
PS + baicalein (300 μM) (control)	100.0 ± 0.0
PS + baicalein + U44619 (10 μM)	104.1 ± 10.1
PS + baicalein + ADP (5 μM)	95.3 ± 3.6
PS + baicalein + thrombin (0.5 unit/mL)	133.6 ± 7.6*
PS + baicalein + collagen (20 μg/mL)	124.4 ± 7.3*
PS + baicalein + AA (0.2 mM)	132.3 ± 3.9**
PS + baicalein + AA (2.0 mM)	0.0 ± 0.0**
PS + baicalein + LA (1.2 mM)	142.8 ± 11.6*
PS + baicalein + LA (12 mM)	65.8 ± 7.3**

[a] The reaction conditions and techniques or ESR measurements are described under Materials and Methods. Aggregatory agents were added to platelet suspensions (PS) (4.5×10^8 platelets/mL, 150 μL) in the presence or absence of AA. The instrument parameters were exactly the same as those in **Figure 1**. All values were normalized to 100 for 300 μM baicalein and represent the average of four independent incubations. Data are presented as the mean ± SEM ($n = 4$). *, $p < 0.05$, and **, $p < 0.005$, as compared with the control groups.

of baicalein-induced hydroxyl radicals was enhanced by lower concentrations (200 μM) but was completely inhibited by higher concentrations (2 mM) of AA (**Figure 2d**,**e**; **Table 3**); however, linoleic acid showed less sensitivity to this response (**Table 3**). These results indicate that the activity of 12-LOX can be abolished by 2 mM AA. It has been shown that baicalein can form stable semiquinone radicals (*17*) which can be further oxidized into *o*-quinone-6,7-dehydrobaicalein (*18*). We demonstrated that baicalein generated semiquinone radicals in a dose-dependent manner (**Figure 4a−c**) when 12-LOX was inhibited by 2 mM AA. This semiquinone radical is not an AA-induced carbon-centered free radical in platelets, which we had reported previously (*12*), because Pronase could not further digest this adduct (data not shown). Similar ESR spectra were obtained when baicalein was incubated with horseradish peroxidase (HRP) and H_2O_2 (**Figure 4f**,**g**).

Effects of Several Enzymes and Enzyme Inhibitors on the Hydroxyl Radical Signals Induced by Baicalein in Human Platelets. Formation of hydroxyl radicals was monitored following the addition of several inhibitors to 150 μL of platelet suspensions. The inhibitors were added 3 min before the addition of baicalein. Our results showed that formation of the baicalein-induced hydroxyl radicals was abolished by SOD and CAT (**Figure 3b**,**c**). This reveals that the superoxide anion may be the original free radical produced by incubation of platelets with baicalein. To ensure the kind of four-line ESR signal, CH_3OH, a specific scavenger for hydroxyl radical, was added in the experimental system with DMPO. After 5 min, a double-triplet signal was detected (**Figure 3g**). This spectrum consisted primarily of six lines with hyperfine coupling constants of $a^N = 15.7$ G and $a^H = 22.5$ G, which could be deduced to be a carbon-centered radical adduct (DMPO/$^\bullet CH_2OH$) (data from the spin-trap database of NIEHS at http://epr.niehs.nih.gov). This confirms that the four-line ESR signal is a DMPO−hydroxyl radical adduct. **Table 2** and **Figure 3** show the effect of various inhibitors on baicalein (300 μM)-stimulated human platelets. Baicalein-induced hydroxyl radical formation was not affected by the iron chelator desferroxamine, or the heme protein inhibitors KCN and NaN_3. Indomethacin, a COX inhibitor, increased the formation of the hydroxyl radicals induced by baicalein. Possible explanations for these findings is that indomethacin inhibits endogenous AA metabolism by COX, therefore increasing the endogenous AA concentration, which may in turn activate 12-LOX.

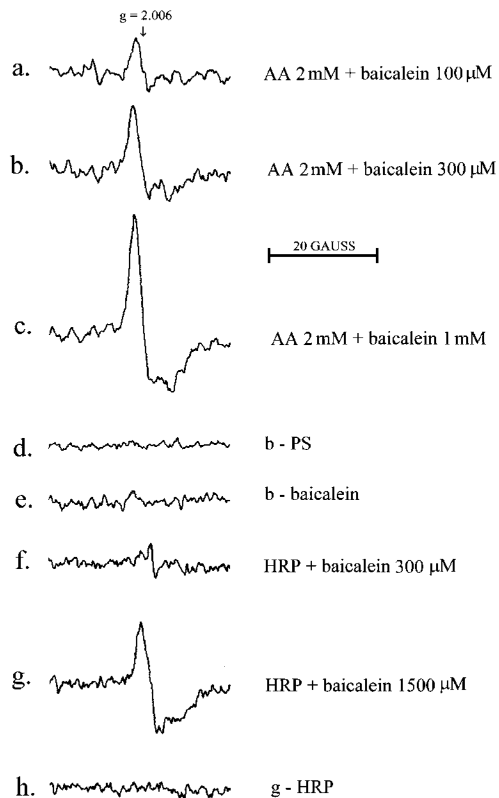

Figure 4. ESR spectra obtained from the reaction of human platelets with baicalein in the presence of arachidonic acid. The reaction mixture contained human platelet suspensions, 2 mM arachidonic acid (AA), 100 mM DMPO, and baicalein at (**a**) 100 μM, (**b**) 300 μM, and (**c**) 1 mM. (**d**) Same as (**b**), but no platelets; (**e**) same as (**b**), but no baicalein. Similar ESR spectra were obtained from the reaction mixture of 1000 units/mL HRP, 1 mM H_2O_2, and baicalein at (**f**) 300 μM and (**g**) 1500 μM in Tyrode's solution. (**h**) Same as (**g**), but no HRP. The instrument parameters were exactly the same as those in **Figure 1**.

Inhibition Studies on the Formation of the Semiquinone Radical Signals Induced by Baicalein in Human Platelets. Formation of the semiquinone radicals was monitored following the addition of several inhibitors to 150 μL of platelet suspensions in the presence of 2 mM AA. The inhibitors were added 3 min before the addition of baicalein. **Table 4** shows the effect of various inhibitors on the formation of the semiquinone radical signals induced by baicalein in human platelets. These data suggest that the formation of the semiquinone radicals is associated with the peroxidatic activity of prostaglandin H synthases (PGHS) in intact platelets.

Effects of Baicalein on the Thiyl Radical Signals Induced by AA and GSH in Human Platelets. Our previous study reported that free radicals formed by PGHS-peroxidase oxidize GSH to a thiyl radical in human platelets (*12*). Baicalein is a well-known antioxidant; therefore, we wanted to determine whether it can scavenge this thiyl radical. When human platelets were incubated with AA, GSH, and DMPO, a four-line DMPO/GS$^•$ thiyl radical ESR spectrum (**Figure 5a**) with hyperfine splitting constants of $a^N = 15.3$ G and $a^H = 16.2$ G was detected, which was identical to those demonstrated by our previous study (*12*). Baicalein dose-dependently inhibited the thiyl radical signals (**Figure 5b**,**c**), indicating that it can perform as an antioxidant in such a condition.

Effects of Antioxidant Flavonoids on the Hydroxyl Radical Signals Induced by Baicalein in Human Platelets. The antioxidant flavonoids, including morin (300 μM), myricetin

Table 4. Effects of Various Inhibitors on the Intensity of Semiquinone Radicals Induced by 300 μM Baicalein in the Presence of 2 mM AA[a]

sample	% of control value ± SEM ($n = 4$)
PS +AA (2 mM) + baicalein (300 μM) (control)	100.0 ± 0.0
control + NDGA (300 μM)	100.1 ± 4.1
control + (−)-epicatechin (300 μM)	79.1 ± 3.3**
control + (−)-epicatechin gallate (300 μM)	86.7 ± 3.2**
control + hinokitiol (300 μM)	98.0 ± 3.4
control + NaN_3 (300 μM)	54.7 ± 5.6**
control + KCN (300 μM)	54.7 ± 3.9**
control + desferroxamine (1 mM)	45.1 ± 3.5**
control + DTPA (100 μM)	45.5 ± 4.1**

[a] The reaction conditions and techniques or ESR measurements are described under Materials and Methods. Inhibitors were added to platelet suspensions (PS) (4.5×10^8 platelets/mL, 150 μL) in the presence or absence of AA. The instrument parameters were exactly the same as those in **Figure 1**. All values were normalized to 100 for the control and represent the average of four independent incubations. Data are presented as the mean ± SEM ($n = 4$). **, $p < 0.005$, as compared with the control groups.

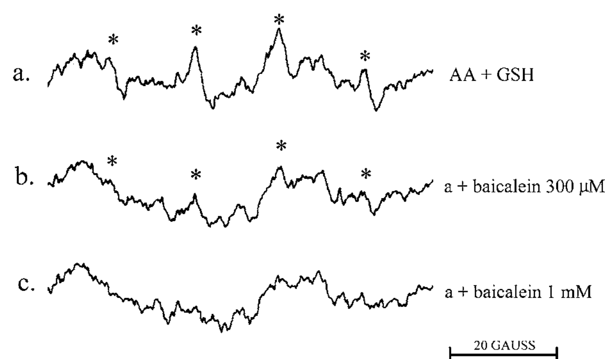

Figure 5. DMPO spin trapping of the thiyl radical in the arachidonic acid/platelet system. Human platelets were preincubated with (**a**) arachidonic acid (2 mM) followed by the addition of 10 mM GSH for 2 min, and then 100 mM DMPO was added. (**b**) Same as (**a**), but with baicalein (300 μM); (**c**) same as (**a**), but with baicalein (1 mM). The instrument parameters were exactly the same as those in **Figure 1**, except that the modulation amplitude was 3 G.

(300 μM), quercetin (300 μM), and rutin (300 μM), inhibited hydroxyl radical generation in response to baicalein but were less effective than 12-LOX inhibitors (**Table 1**). Surprisingly, a new free radical signal was shown when myricetin was incubated with baicalein in human platelets (**Figure 6b**). This spectrum consisted primarily of six lines with hyperfine coupling constants of $a^N = 16.5$ G and $a^H = 23.7$ G, which was deduced to be a methyl radical adduct (DMPO/$^•CH_3$) (data from the spin-trap database of NIEHS at http://epr.niehs.nih.gov).

DISCUSSION

Baicalein, a major flavonoid extracted from the traditional Chinese herb *Scutellaria baicalensis* Georgi (Huangqin), is commonly used for treating chronic hepatitis in China and Japan. Previous studies have shown that baicalein possesses potent anti-inflammatory and antioxidant properties (*17*). The antithrombotic, antiproliferative, and antimitogenic effects of this flavonoid have also been reported [as reviewed by Huang et al. (*19*)]. Several lines of evidence indicated that baicalein can scavenge the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (*24*), superoxide radical, hydroxyl radical (*19*), and alkyl radical (*24*) in cell-free systems. On the other hand, baicalein protects

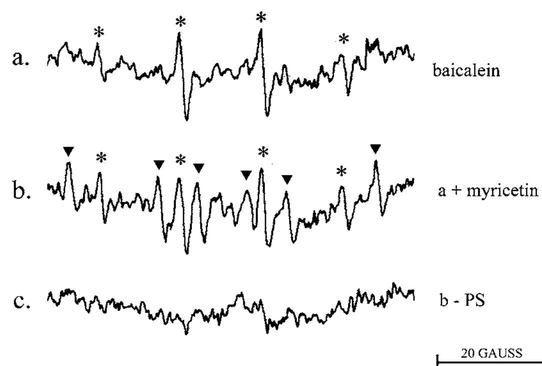

Figure 6. DMPO spin trapping of the methyl radical in the baicalein/myricetin/platelet system. Human platelets were preincubated with (**a**) 300 μM baicalein and 100 mM DMPO, (**b**) in presence of 300 μM myricetin; (**c**) same as (**b**) without platelet suspensions. The instrument parameters were exactly the same as those in **Figure 1**.

cardiomyocytes, liver microsomes, hippocampus, and neuroblastoma HS-SY5Y cells against oxidative damage (*19*). These studies showed that the high effectiveness of baicalein on scavenging free radicals contributes to its cell-protective effect. Moreover, Shen et al. demonstrated that baicalein diminished fMLP- or PMA-induced reactive oxygen intermediate production in neutrophils and monocytes (*20*). However, there is no direct ESR evidence about the free radical scavenging ability of baicalein in cell-containing systems. In human platelets, our earlier study demonstrated an AA-induced free radical, which was scavenged by GSH and the ultimately generated thiyl radical. In the present study, we showed that baicalein scavenges the thiyl radical in human platelets using ESR techniques.

It has been proposed that the autoxidation of flavonoids generates semiquinone radicals and superoxide radicals (*21*). Baicalein induced the apoptosis of Jurkat cells, which was accompanied by intracellular ROS generation, mitochondrial cytochrome *c* release, and disruption of the mitochondrial transmembrane potential prior to the activation of caspase-3. This suggests that baicalein acts as a pro-oxidant and induces caspase-3 activation and apoptosis via a mitochondrial pathway (*22*). On the other hand, Woo et al. reported that baicalein protects rat cardiomyocytes from hypoxia/reoxygenation damage via a pro-oxidant mechanism. They suggested that hydrogen peroxide is produced during the autoxidation of baicalein (*19*). In fact, flavonoids, particularly those possessing pyrogallol and catechol moieties in their structures, are prone to oxidation by oxygen with concomitant production of superoxide radicals, hydrogen peroxide, semiquinone radicals, and quinines (*21*). However, our results showed that only baicalein but not morin, myricetin, quercetin, or rutin produced hydroxyl radicals in a human platelet suspension (**Figure 1**). In addition, baicalein could not produce hydroxyl radicals in other cultured cell preparations including rat vascular smooth muscle cells, HUVECs, human THP-1 cells, and fibroblast cells (**Figure 1**). These results indicate that baicalein-induced hydroxyl radical formation in human platelets is independent of autoxidation reactions. It has been shown that baicalein inhibits 12-LOX activity without affecting COX in human platelets (*13*). Therefore, we hypothesized that baicalein produces hydroxyl radicals via 12-LOX in human platelets.

Lipoxygenases are non-heme iron proteins that incorporate a molecular oxygen into various positions of AA and other polyunsaturated lipids. There are many LOX isoforms such as p12-LOX in platelets, 5-LOX in neutrophils, and 1-LOX in soybeans. Yamamoto et al. found two isoforms of 12-LOX: leukocyte- and platelet-type enzymes [as reviewed by Kulkarni (*23*)]. Incubation of AA with homogenized human platelets produced 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12*S*-HPETE) (*23*). Lipoxygenases require activation by lower but inactivation by higher concentrations of peroxides (*23*). The resting platelets contain a small amount of free AA (*15*) and lipid peroxides (*24*), indicating that some of the 12-LOX may be in a catalytically active form. In this work, we show that baicalein produced hydroxyl radicals in resting human platelets without the addition of AA. This reaction was enhanced by the addition of lower concentrations of AA but was completely inhibited by higher concentrations of AA, suggesting that 12-LOX catalyzes baicalein to produce hydroxyl radicals in human platelets. However, peroxides are also an important stimulus for activation of COX and its functioning as substrates for peroxidase activity (*25*). Prostaglandin H synthases are heme-containing enzymes that catalyze the biosynthesis of prostaglandins from AA and also catalyze a LOX-like reaction (*26*). PGHS has two distinct catalytic activities: a COX, which converts AA to prostaglandin G_2 (PGG_2), and a peroxidase, which reduces a wide variety of peroxides to their corresponding alcohols. Our results show that PGHS-peroxidase was not involved in this reaction (**Table 2**).

Human platelet 12-LOX was inhibited by (−)-epicatechin, (−)-epicatechin gallate (*27*), and hinokitiol (*28*) with IC_{50} values of about 2.12, 0.25, and 0.1 μM, respectively. The concentrations of 12-LOX inhibitors we used in this study were much higher than the IC_{50} values reported by previous studies. It was reasoned that the enzymatic inhibition by 12-LOX inhibitors, such as catechins, is reversible (*27*). All of the 12-LOX inhibitors, including baicalein, are substrates of the enzyme. The high concentrations of baicalein and other 12-LOX inhibitors we applied in this study were due to the competitive nature of the substrate. The contribution of the scavenging activity of 12-LOX inhibitors to their antioxidative effects must also be considered. However, other antioxidative flavonoids such as morin, myricetin, quercetin, and rutin showed smaller inhibition potencies toward baicalein-induced hydroxyl radical formation (**Table 3**). Therefore, this result provides still further evidence that the pro-oxidative action of baicalein on human platelets may be the result of 12-LOX inhibition.

The antioxidant properties of some flavonoids are often linked to their ability to form stable radicals after they take effect. Those flavonoids usually produce semiquinone free radicals in an alkaline solution but not in physiological pH solutions (*6*, *17*). Semiquinone free radicals were also generated when polyphenols were incubated with HRP/H_2O_2 (*29*). Similarly, in this study we demonstrated that semiquinone free radicals were generated when baicalein was incubated with HRP/H_2O_2 (**Figure 4f**,**g**) or platelets/AA (**Figure 4a**). Furthermore, the semiquinone free radicals generated in the platelet/AA system could be inhibited by the heme protein inhibitors KCN and NaN_3 (**Table 4**). These results suggest that PGHS-peroxidase may catalyze baicalein to form semiquinone radicals in human platelets.

A previous study showed that 1-LOX inhibitors, such as phenidone and NDGA, reduce the catalytically active ferric LOX to its inactive ferrous form (*23*). In such conditions, the inhibitors are oxidized to free radical metabolites (*30*). Therefore, we propose that platelet 12-LOX catalyzes baicalein, NDGA, (−)-epicatechin, (−)-epicatechin gallate, and hinokitiol to generate free radical metabolites, which are difficult to detect in this system. However, baicalein radicals may be further converted to hydroxyl radicals and can be trapped by DMPO. In our

system, baicalein was identified as a pro-oxidant independent of autoxidation reactions.

In conclusion, the present study demonstrated that baicalein induces hydroxyl radical formation via 12-LOX and induces semiquinone radical formation via PGHS-peroxidase in human platelets. Our data suggest that baicalein can act either as an antioxidant or as a pro-oxidant in human platelet suspension, depending on the activity of platelet 12-LOX.

ABBREVIATIONS USED

AA, arachidonic acid; PLA_2, phospholipase A_2; $AACOCF_3$, 5,8,11,14-eicosatetraenoic trifluoromethyl ketone; CAT, catalase; COX, cyclooxygenase; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DTPA, diethylenetriaminepentaacetic acid; ESR, electron spin resonance; HRP, horseradish peroxidase; HUVEC, human umbilical vein endothelial cells; HPETE, hydroperoxyeicosatetraenoic acids; LA, linoleic acid; LOX, lipoxygenase; NDGA, nordihydroguaiaretic acid; PGG_2, prostaglandin G_2; PGHS, prostaglandin H synthase; ROS, reactive oxygen species; SOD, superoxide dismutase; NaN_3, sodium azide.

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