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Differential Effects of Ganodermic Acid S on the Thromboxane A_2-Signaling Pathways in Human Platelets

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ABSTRACT. Ganodermic acid S (GAS) [lanosta-7,9(11),24-triene-3β,15α-diacetoxy-26-oic acid], isolated from the Chinese medicinal fungus *Ganoderma lucidum* (Fr.) Karst (Polyporaceae), exerted a concentration-dependent inhibition on the response of human gel-filtered platelets (GFP) to U46619 (9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha}$), a thromboxane (TX) A_2 mimetic. GAS at 2 μM inhibited 50% of cell aggregation. GAS at 7.5 μM inhibited 80% of Ca^{2+} mobilization, 40% of phosphorylation of myosin light chain and pleckstrin, 80% of α-granule secretion, and over 95% of aggregation. GAS also strongly inhibited U46619-induced diacylglycerol formation, arachidonic acid release, and TXB_2 formation. An immunoblotting study of protein-tyrosine phosphorylation showed that GAS inhibited the formation of phosphotyrosine proteins at the steps involving the engagement of integrin $\alpha_{IIb}\beta_3$ and aggregation. However, GAS did not inhibit U46619-induced platelet shape change or the inhibitory effect of U46619 on the prostaglandin E_1-evoked cyclic AMP level in GFP. It is concluded that GAS inhibits platelet response to TXA_2 on the receptor-G_q-phospholipase Cβ1 pathway, but not on the receptor-G_i pathway. BIOCHEM PHARMACOL **58**;4:587–595, 1999.

KEY WORDS. human platelet; ganodermic acid S; thromboxane A_2; cyclic AMP; Ca^{2+} mobilization

TXA_2§ is the metabolite of arachidonate formed through the sequential catalysis of cyclooxygenase and TXA_2 synthase [1]. TXA_2 is formed and released rapidly in response to platelet activation by diverse stimuli. In the immediate microenvironment of its formation, it acts as a positive feedback promoter on activated platelets and a strong agonist on resting platelets [2]. Platelets respond to TXA_2 and prostaglandin endoperoxide with shape change, Ca^{2+} mobilization, granule secretion, and aggregation [3]. These effects are transduced via the heterotrimeric GTP-binding protein (Gαβγ)-coupled receptors in the membrane [4]. There are two distinct receptor–effector systems in TXA_2-mediated platelet activation [5]. A gene for TXR has been cloned [6]. Two variants have been identified. Human platelets contain messenger RNA of both isoforms [7]. The TXRα isoform functionally couples to G_q [8] and the β1 isoenzyme of PLC [9]. The TXRα-G_q-PLCβ1 pathway causes an increase in $[Ca^{2+}]_i$ and cell aggregation, but it is not involved in inducing platelet shape change [10, 11]. The TXRβ isoform couples to G_i, and the pathway links to platelet shape change, PLA_2 activation, and inhibition of adenylate cyclase [7]. In addition, the TXA_2 mimetic U46619 (9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha}$) activates platelet protein-tyrosine phosphorylation [12, 13] in a manner similar to activation by collagen and thrombin [14]. The activation shows formation of three groups of phosphotyrosine proteins in accordance with the three sequential steps leading to platelet aggregation. The first step of protein-tyrosine phosphorylation (early activation) forms phosphorylated proteins including the tyrosine kinase syk [15], mitogen-activated protein kinases and several unidentified proteins [14]. The second step of protein-tyrosine phosphorylation is dependent upon fibrinogen binding to, and oligomerization of, the fibrinogen receptor $\alpha_{IIb}\beta_3$ integrin. The step brings about further phosphorylation of tyrosine kinase and several additional proteins [16, 17]. The third step, which is aggregation-dependent, involves tyrosine phosphorylation of an additional tyrosine kinase (focal adhesion kinase) [18] and other proteins [16, 19, 20].

GAS [lanosta-7,9 (11),24-triene-3β,15α-diacetoxy-26-oic acid] is the major oxygenated triterpenoid isolated from *Ganoderma lucidum* (Fr.) Karst (Polyporaceae) (Fig. 1). The fungus is used widely in a traditional Chinese remedy for improving blood circulation and liver and kidney functions [21]. GAS is an anionic amphiphile. Its uptake by platelets

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§ *Abbreviations*: TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; GAS, ganodermic acid S; GFP, gel-filtered platelets; PGE_1, prostaglandin E_1; TXR, TXA_2 receptor; PLC, phospholipase C; PLA_2, phospholipase A_2; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; IBMX, 3-isobutyl-1-methylxanthine; and Fura-2 AM, Fura-2 acetoxymethyl ester.

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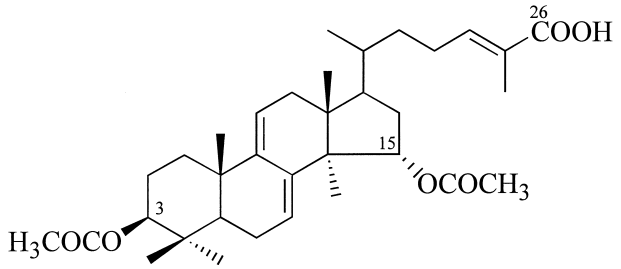

FIG. 1. Chemical structure of ganodermic acid S.

is saturable in 1 min, and it is highly soluble in platelet plasma membrane, with an estimated partition coefficient between membrane and aqueous solution of 10^5 [22]. GAS exerts multiple effects on platelet function in a concentration-dependent manner. The potency of GAS inhibition on platelet response to inducers is as follows: ADP-fibrinogen > collagen > thrombin [23]. The differential inhibitory potencies of GAS on platelet responses to these agonists may be due to the fact that the receptors exist in different membrane microdomains. Also, GAS potentiates the cyclic AMP level in platelet response to PGE_1.* It is important to investigate how GAS affects platelet response to the TXA_2 mimetic U46619.

This study reports that GAS was more potent in inhibiting U46619-activated platelet aggregation than aggregations activated by ADP-fibrinogen or collagen. GAS strongly inhibited U46619-induced diacylglycerol formation, Ca^{2+} mobilization, granule secretion, and arachidonic acid release. An immunoblotting study of U46619-activated protein-tyrosine phosphorylation showed that GAS strongly inhibited phosphorylation dependent upon the engagement of fibrinogen-$\alpha_{IIb}\beta_3$ as well as aggregation. GAS did not inhibit U46619-induced platelet shape change or the U46619 inhibition of adenylate cyclase. These results suggested that GAS inhibits TXA_2 signaling of the receptor-G_q-PLCβ1 pathway.

MATERIALS AND METHODS

Materials

Chemicals, organic solvents, TLC plates (silica gel 60), EDTA (Triplex II), HEPES, Triton X-100, and Omniszintisol (scintillation fluid) were purchased from E. Merck. All organic solvents were redistilled before use. Bovine thrombin, Type I human placental collagen, U46619, PGE_1, apyrase, IBMX, Tween-20, and indomethacin were obtained from the Sigma Chemical Co. Fura-2 AM was obtained from Boehringer Mannheim, and Sepharose 2B, from Pharmacia. [^{3}H]Arachidonic acid (60–100 Ci/mmol) and EN [3]HANCE Spray were from DuPont-New England Nuclear. Monoclonal anti-phosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology Inc.; horseradish peroxidase-conjugated goat anti-mouse IgG (H+L), from Bio-Rad; an enhanced chemiluminescence system, from Pierce; skim milk, from Difco; and Immobilon-P membrane from Millipore. Carrier-free [^{32}P]P_i (10 mCi/mL), a [^{3}H]cyclic AMP radioimmunoassay kit, and a [^{3}H]TXB_2 radioimmunoassay kit were obtained from Amersham. The β-thromboglobulin immunoassay kit was from Stago, and X-ray film was purchased from the Fuji Photo Film Co. GAS was isolated from cultured mycelia of *Ganoderma lucidum* [21] and dissolved in ethanol prior to the study.

Preparation of Gel-Filtered [^{32}P]P_i-Labeled and [^{3}H]Arachidonic Acid-Labeled Platelets

Human blood was freshly drawn from healthy donors and anticoagulated with 10% (v/v) of 3.8% sodium citrate. The method of Lages *et al.* [24] was followed to obtain GFP, which were suspended in a modified Ca^{2+}-free HEPES (5 mM) Tyrode's buffer (pH 7.4) containing 0.1% dextrose, and adjusted to a concentration of 3×10^8 cells/mL. The cells were counted in a hemacytometer with a differential interference contrast microscope. Apyrase (0.5 U/mL) was added to platelet-rich plasma just before gel filtration. For preparing ^{32}P-labeled platelets, 5 mL of concentrated platelet-rich plasma was incubated with 1 mCi of carrier-free [^{32}P]P_i at 37° for 1 hr before gel filtration. Prior to further investigation, isolated platelets were warmed at 37° for 30 min to return the cells to a discoid shape [25].

For incorporation of [^{3}H]arachidonic acid into platelet phospholipids, platelets were concentrated from 20 mL of platelet-rich plasma and resuspended to approximately 3×10^9 cells/mL in HEPES Tyrode's buffer. After the addition of [^{3}H]arachidonic acid (1 μCi in 1 μL of DMSO) to a final radioactivity of 4 μCi/mL, the cell suspension was incubated at 37° for 1 hr. Unincorporated [^{3}H]arachidonic acid was removed by diluting the platelet suspension to 40 mL with HEPES Tyrode's buffer and centrifuging at 1000 *g* for 15 min. Radiolabeled platelets were washed twice with HEPES Tyrode's buffer and resuspended in the same buffer.

Analyses of Platelet Response to U46619

In the following experiments, platelets (1.5×10^8 cells/0.5 mL) were preincubated with various concentrations of GAS (in 1 μL ethanol), or 7.5 μM GAS at 37° for 5 min before stimulation with U46619 (1 μM). Platelet aggregation was estimated as the percent change of optical turbidity of platelet suspension observed in an aggrecorder (Daiichi Pharmaceuticals, model PA-3220, Japan). The absorbance at 650 nm was 0.38 for 3×10^8 cells/mL. Upon the addition of U46619, the change in absorbance of the cell suspension was followed for 10 min.

Fura-2 AM was employed as a probe to determine the change in $[Ca^{2+}]_i$. In brief, platelet-rich plasma was incubated with 1 μM Fura-2 AM at 37° for 45 min, and then was filtered through a Sepharose 2B column as described previously. Fluorescence intensity was measured in a fluorescence spectrophotometer (SLM, model 4800C) at an excitation wavelength of 339 nm and an emission wavelength of 500 nm. $[Ca^{2+}]_i$ was calibrated according to the method of Pollock *et al.* [26].

The reaction for the determination of α-granule release from platelets was carried out at 5 min after the addition of U46619. It was terminated by adding a 50 μM concentration of ice-cold EDTA, and centrifuged at 12,000 *g* for 3 min in a microcentrifuge (Sigma, model CM 202). The supernatants were taken to assay the concentration of β-thromboglobulin by using a commercial immunoassay kit.

TXB_2 formation from platelets was determined at different time intervals after the addition of U46619. The reaction was stopped by adding 2 mM EDTA plus 50 μM indomethacin, and chilled in ice. Then the samples were centrifuged, and the TXB_2 level in the supernatants was determined by using a $[^3H]TXB_2$ radioimmunoassay kit.

Analyses of [^{3}H]Diacylglycerol Formation and [^{3}H]Arachidonic Acid Release

[^{3}H]Arachidonic acid-labeled platelets were preincubated with 7.5 μM GAS at 37° for 5 min before stimulation with U46619 (1 μM). At certain intervals, 1 mL of cell suspension was added with cooled methanol to stop the reaction. Arachidonic acid, its metabolites, and cellular lipids were extracted according to the method of Bligh and Dyer [27]. The bottom layer was collected and evaporated to dryness under nitrogen. The radioactive arachidonic acid and its metabolites were separated by TLC using the solvent system chloroform:methanol:acetic acid:water (90:8:1:0.8, by vol.) [28]. The TLC plate was sprayed with EN [3]HANCE Spray and autoradiographed at −70°. The visible bands included total phospholipids, TXB_2, 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT), 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), arachidonic acid, and diacylglycerol. The latter band was identified by comparison with 1,2-dimyristoylglycerol. Diacylglycerol, arachidonic acid, arachidonate metabolites, and phospholipids were scraped separately, and the radioactivity of each fraction was measured in a liquid scintillation counter (Packard, model 1600 TR). The radioactivity of diacylglycerol was employed as an index of diacylglycerol formation, with the value obtained from resting platelets set at 1.00. The percentage of arachidonic acid release is measured from the following equation: percentage of release = 100 × cpm of (arachidonic acid + TXB_2 + HHT + 12-HETE)/cpm of (phospholipids + diacylglycerol + arachidonic acid + TXB_2 + HHT + 12-HETE).

Assay of Cyclic AMP Level

Prewarmed GFP (0.5 mL) were incubated with various concentrations of GAS plus 0.2 mM IBMX for 5 min, and then either 2.4 μM PGE_1 alone or PGE_1 plus U46619 (1 μM) was added for another 2 min. The reaction was terminated by boiling for 5 min, and the mixture was centrifuged in a microcentrifuge [29]. The supernatant was taken to assay the cyclic AMP level by using a commercial [^{3}H]cyclic AMP radioimmunoassay kit.

Protein Phosphorylation

The study of protein phosphorylation was carried out by a modification of the method described by Sano *et al.* [30]. ^{32}P-Labeled platelets were preincubated with 7.5 μM GAS at 37° for 5 min in a Thermomixer (Eppendorf, model 5437), and then U46619 (1 μM) was added for another 2 min. A 45-μL aliquot was quenched with 10 μL of SDS sample buffer (8% SDS, 20% 2-mercaptoethanol, 40% glycerol, and 0.25 M Tris–HCl, pH 6.8). The mixture was boiled for 5 min and then separated by electrophoresis in 11% polyacrylamide gel containing 0.1% SDS as described by Laemmli [31]. The gel was stained with Coomassie brilliant blue and dried in a slab dryer (Bio-Rad, model 483). The dried gel was subjected to autoradiography (Fuji X-ray film). Intensities of the phosphoproteins were quantitated by using a Computing Densitometer (Molecular Dynamics, Inc.)

Tyrosine Phosphorylation

The effect of GAS on the protein-tyrosine phosphorylation in human platelets stimulated by U46619 was determined by a modification of the method described by Daniel *et al.* [32]. GFP (3×10^8 cells/mL) were preincubated with GAS at 37° for 5 min before stimulation with U46619 (1 μM). At the end of each reaction time point, the reaction was stopped with a one-tenth volume of 6.6 N perchloric acid on ice. The pellet obtained by centrifugation was washed twice with water, solubilized in the SDS sample buffer, boiled for 5 min, and subjected to SDS–PAGE on a 7.5% polyacrylamide gel as described by Laemmli [31]. Proteins in the gel then were electrophoretically transferred to Immobilon-P using 25 mM Tris, 192 mM glycine, and 10% (v/v) methanol for 4 hr at 400 mA. The Immobilon was treated overnight at 4° with 10% (w/v) skim milk in a buffer solution containing 150 mM NaCl, 0.1% NaN_3, 0.2% Tween-20, and 50 mM Tris–HCl, pH 7.4, to block nonspecific binding. The Immobilon was incubated at room temperature for 1 hr with an anti-phosphotyrosine antibody (4G10, 1 μg/mL of the buffer solution). The Immobilon was washed five times with the buffer solution. Bound primary antibody was detected by incubation at room temperature for 1 hr with horseradish peroxidase-conjugated anti-mouse IgG [diluted 1:5000 in the buffer solution containing 10% (w/v) skim milk]. The final treated Immobilon was washed five times with the buffer solution and exposed to enhanced chemiluminescence reagents for 2 min. The blot was exposed to Fuji X-ray film for 1–30 min for visualization, and phosphorylation levels were quantitated by using a Computing Densitometer (Molecular Dynamics, Inc.)

Statistical Analysis

Data are presented as means ± SD of the indicated number of experiments (N). The differences between the effects of

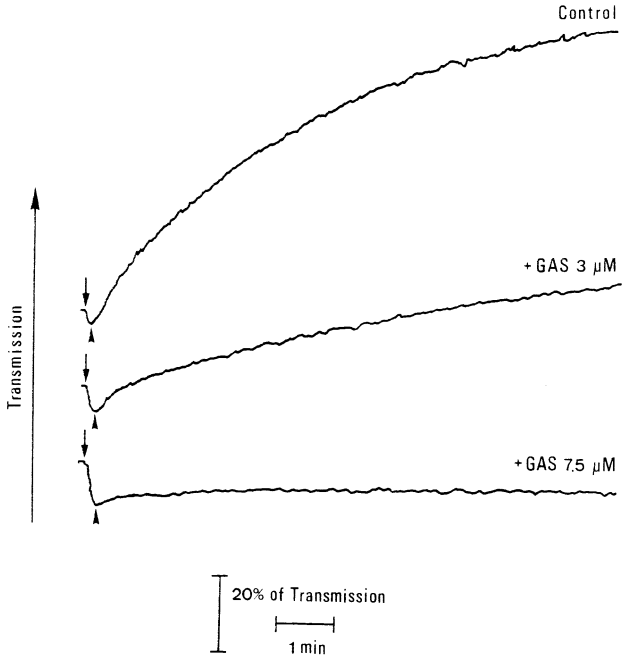

FIG. 2. Aggregation tracings showing the concentration-dependent inhibitory effect of GAS on platelet response to U46619. GFP (3×10^8 cells/mL) were preincubated with GAS at 37° for 5 min before the addition of U46619 (1 μM). The tracings are representative of five similar experiments. The arrows indicate the addition of U46619, and the arrowheads show the appearance of the minimum transmission observed in the aggrecorder. The shape change period represents the time between arrow and arrowhead. Control indicates the addition of U46619 alone.

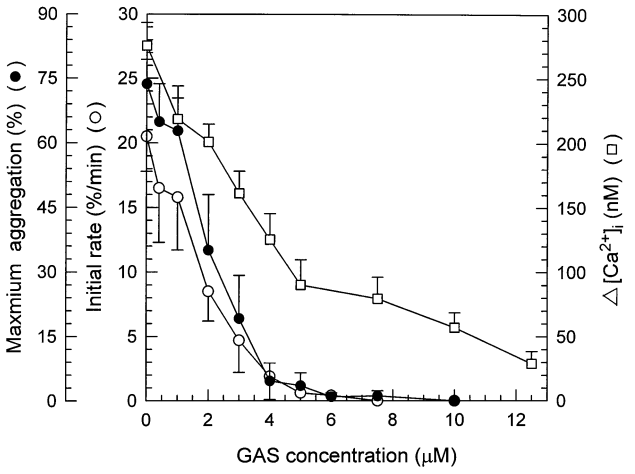

FIG. 3. Concentration–effect relationship of GAS on aggregation and Ca^{2+} mobilization in U46619-stimulated human platelets. GFP or Fura-2-loaded GFP (3×10^8 cells/mL) were preincubated with GAS at 37° for 5 min before the addition of U46619 (1 μM). The initial rate (○) was calculated from the initial slope of the aggregation curve and represented the percentage of platelet aggregation per minute. The maximum aggregation (●) was taken from the cell aggregation in 10 min. The fluorescence calibration for $[Ca^{2+}]_i$ was calculated as described in Materials and Methods. The change of $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) (□) was calculated from the difference between the maximum and basal levels of $[Ca^{2+}]_i$ obtained from each experiment. Data (means ± SD) were taken from five separate platelet preparations. All values are significantly different from their respective control value at $P < 0.05$.

GAS on each kind of assay were assessed by a paired *t*-test. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

The cell aggregation study showed that platelet response to U46619 included a short shape change period (about 5 sec), and then the cells aggregated with an initial aggregation rate of 20.5 ± 2.7% cells/min (N = 5) (Fig. 2). GAS exhibited a concentration-dependent inhibition of the aggregation of platelets in response to U46619. It prolonged the shape change period and reduced the initial cell aggregation rate and the percent of cell aggregation (Figs. 2 and 3). GAS at 2 μM exerted 50% inhibition of cell aggregation. GAS at 7.5 and 10 μM completely inhibited cell aggregation, but it did not abolish U46619-induced shape change as shown by the changes in optical transmission (Fig. 2).

Analysis of Ca^{2+} mobilization revealed that GAS exhibited a concentration-dependent inhibition of U46619-stimulated elevation of $[Ca^{2+}]_i$ (Fig. 3). Upon the addition of 1 μM U46619 to GFP, the maximum $[Ca^{2+}]_i$ level increased to 275.4 ± 18.0 nM (N = 5) within 5–12 sec, and dropped immediately to basal level at 30 sec. GAS strongly inhibited Ca^{2+} mobilization. The $[Ca^{2+}]_i$ level was decreased to one-third of control at 5 μM GAS, and 7.5 μM GAS reduced the $[Ca^{2+}]_i$ level to 79.4 ± 16.7 nM (N = 5). GAS at 7.5 μM also inhibited 40% of the phosphorylation of myosin light chain (p20) and pleckstrin (p47), and strongly inhibited α-granule secretion (Table 1).

When we estimated arachidonic acid release and diacylglycerol formation by using [^{3}H]arachidonic acid-labeled platelets, the results showed that 1 μM U46619 induced a 20% increase of diacylglycerol over the basal level in resting platelets (Fig. 4). The level increased within 5 sec and was maintained for up to 2 min. GAS at 7.5 μM strongly inhibited U46619-induced diacylglycerol formation (Fig. 4). The results of GAS inhibition on both diacylglycerol formation and Ca^{2+} mobilization indicated

TABLE 1. Inhibition of protein phosphorylations and granule release in U46619-stimulated human platelets by GAS

	Protein phosphorylation (%)		Granule release
	p47	p20	β-TG (μg/mL)
Control	100	100	1.13 ± 0.37
+GAS 7.5 μM	64.0 ± 10.8*	68.8 ± 6.6*	0.24 ± 0.12*

GFP (3×10^8 cells/mL) were preincubated with GAS at 37° for 5 min before the addition of U46619 (1 μM). Control indicates the addition of U46619 alone. The 100% phosphorylations of both p20 and p47 were those of GFP stimulated by 1 μM U46619 for 2 min. The concentration of β-thromboglobulin (β-TG) in each reaction was the difference between the value determined at 5 min, and the basal level of the resting GFP (0.5 ± 0.2 μg/mL). Data (means ± SD) were taken from five separate platelet preparations.

* $P < 0.01$ vs control.

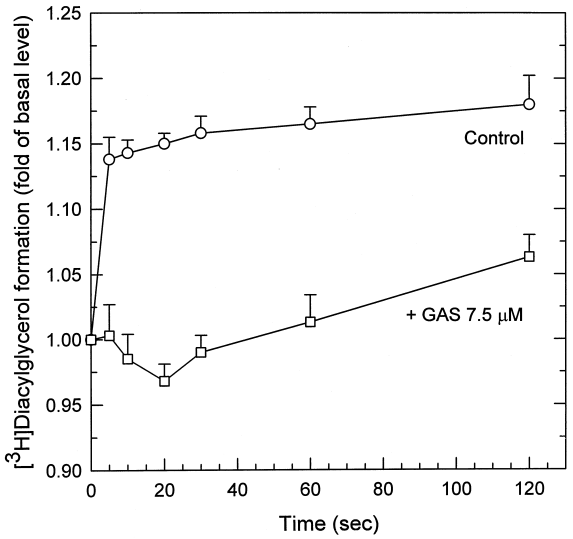

FIG. 4. Effect of GAS on the time course of diacylglycerol formation in U46619-stimulated human platelets. [^{3}H]Arachidonic acid-labeled platelets (3 × 10^8 cells/mL) were preincubated with GAS at 37° for 5 min before stimulation with U46619 (1 μM). Control indicates the addition of U46619 alone. The basal levels of [^{3}H]diacylglycerol radioactivity in resting cells and GAS-pretreated cells (594 ± 35 and 612 ± 42 cpm/10^9 cells) were set to 1.00 at zero time. The measurement of diacylglycerol formation is detailed in Materials and Methods. Data (means ± SD) were taken from four separate platelet preparations. All values are significantly different from the control at $P < 0.01$.

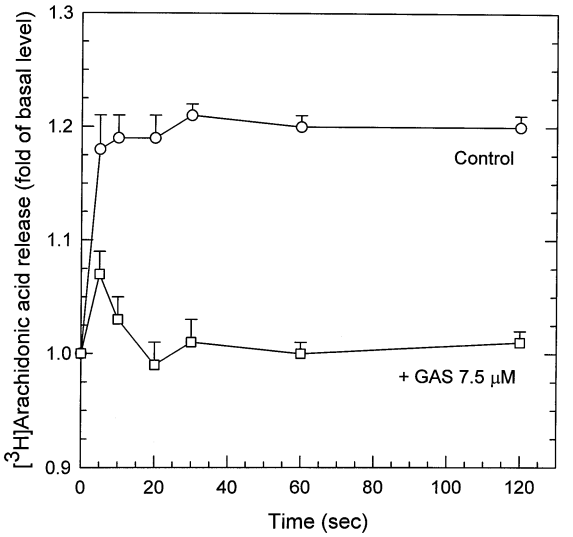

FIG. 5. Effect of GAS on the time course of arachidonic acid release in U46619-stimulated human platelets. [^{3}H]Arachidonic acid-labeled platelets (3 × 10^8 cells/mL) were preincubated with GAS at 37° for 5 min before stimulation with U46619 (1 μM). Control indicates the addition of U46619 alone. The basal levels of arachidonate metabolites in resting cells and GAS-pretreated cells (1.62 ± 0.06 and 1.92 ± 0.07% of total labeled radioactivity) were set to 1.00 at zero time. The measurement for arachidonic acid release is detailed in Materials and Methods. Data (means ± SD) were taken from four separate platelet preparations. All values are significantly different from the control at $P < 0.01$.

that GAS might inhibit U46619-induced activation of PLCβ1. GAS inhibited U46619-stimulated arachidonic acid release from [^{3}H]arachidonic acid-labeled platelets (Fig. 5). Measurement of TXB_2 formation showed that the formation induced by U46619 was 10.1 ± 3.1 ng/3 × 10^8 cells (N = 6) (Fig. 6). This level was only 2.5% of that induced by collagen (395.4 ± 7.9 ng/3 × 10^8 cells, N = 3). GAS inhibited U46619-induced TXB_2 formation by over 80%. The results indicated that GAS inhibited U46619-induced activation of PLA_2.

Determination of the cyclic AMP level was carried out to investigate how GAS affected the inhibition of U46619 on cyclic AMP formation in PGE_1-induced GFP (Fig. 7). In the control group, the cyclic AMP level in PGE_1-induced GFP was over the basal level by 34.2 ± 9.1 pmol/3 × 10^8 cells (N = 5). At concentrations up to 16 μM, GAS alone did not affect the cyclic AMP level in resting GFP. But GAS enhanced the cyclic AMP level in PGE_1-induced GFP in a linearly concentration-dependent manner (Fig. 7). U46619 was able to inhibit 40% of the pool size of cyclic AMP in either GFP stimulated by PGE_1 alone or cells evoked by PGE_1 plus GAS. PGE_1 alone inhibited U46619-induced platelet aggregation completely. The results indicated that GAS might not perturb the inhibition by U46619 of PGE_1-evoked cyclic AMP formation in platelets.

An immunoblotting study of protein-tyrosine phosphorylation in platelet response revealed that phosphorylation of proteins increased with time upon U46619 stimulation. Additional phosphotyrosine proteins appeared at different time points corresponding to the three sequential steps of tyrosine phosphorylation (Fig. 8, lanes 2–6). Early activa-

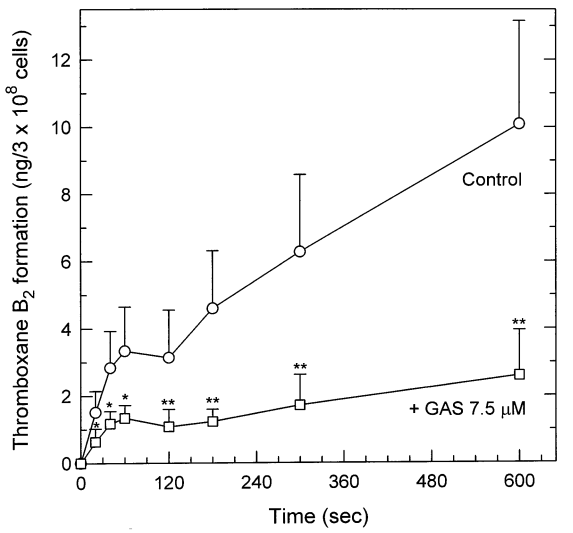

FIG. 6. Effect of GAS on the time course of TXB_2 formation in U46619-stimulated human platelets. GFP (3 × 10^8 cells/mL) were preincubated with GAS at 37° for 5 min before stimulation with U46619 (1 μM). Control indicates the addition of U46619 alone. The assay for TXB_2 was detailed in Materials and Methods. Data (means ± SD) were taken from six separate platelet preparations. Key: (*) $P < 0.05$; and (**) $P < 0.01$ versus control values.

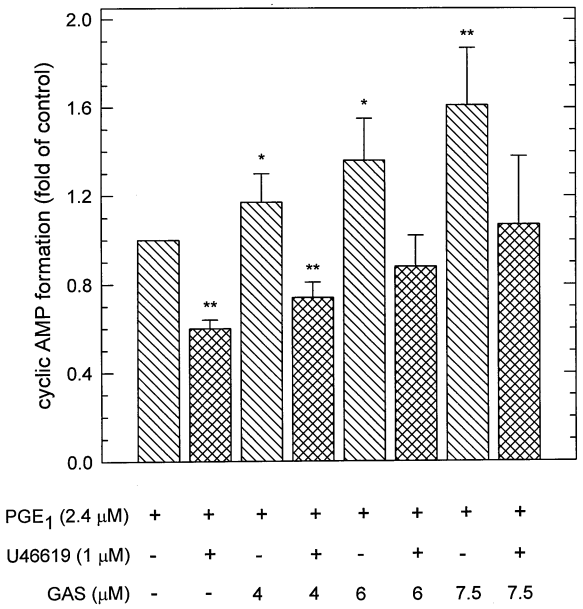

FIG. 7. Effect of GAS on the U46619 inhibition of cyclic AMP formation in PGE_1-induced human platelets. GFP (3×10^8 cells/mL) were preincubated with 0.2 mM IBMX and various concentrations of GAS at 37° for 5 min, and then either 2.4 μM PGE_1, or PGE_1 plus U46619 (1 μM) was added for another 5 min. Cyclic AMP assay was detailed in Materials and Methods. The cyclic AMP level was obtained by subtracting the basal level (4.7 ± 9.1 pmol/3×10^8 cells) in each condition. Control stands for the cyclic AMP level in GFP under the stimulation of PGE_1 alone. Data (means ± SD) were taken from five separate platelet preparations. Key: (*) $P < 0.05$; and (**) $P < 0.01$ versus control values.

tion of protein-tyrosine phosphorylation (detected at 15 sec after U46619 stimulation) appeared to show phosphorylation of the additional proteins p96, p91, p85, p78, and p71 (Fig. 8, lane 2). The second step of tyrosine phosphorylation was observed at 1 min of stimulation (Fig. 8, lane 4). The third step of aggregation-regulated tyrosine phosphorylation was detected at 2 min after U46619 stimulation. This step showed phosphorylation of the additional proteins p112 and p105 (Fig. 8, lane 5). GAS alone at 7.5 μM decreased the intensities of all endogenous phosphotyrosine proteins (Fig. 8, lane 7). GAS inhibited protein-tyrosine phosphorylation of U46619-stimulated platelets in two ways (Fig. 8, lanes 7–12) ($N = 4$, $P < 0.05$). First, GAS did not inhibit the early activation of protein-tyrosine phosphorylation significantly, except for the phosphorylations of p122/127, which were inhibited by 30%. Second, GAS prominently inhibited the second and third steps of tyrosine phosphorylation. At the third step, GAS reduced 30% of the phosphorylation of p53–58 and p51, while it inhibited that of the remaining phosphotyrosine proteins by over 70%. The results indicated that GAS inhibited protein-tyrosine phosphorylation at the steps involving the engagement of integrin $\alpha_{IIb}\beta_3$ and aggregation.

In summary, these results suggest that GAS may perturb the TXA_2 signaling by inhibiting the receptor-G_q-PLC pathway. However, it did not affect the pathway leading to shape change and TXA_2 inhibition of adenylate cyclase.

DISCUSSION

The study shows that the insertion of GAS into human platelet plasma membrane exerts more potent inhibition on platelet aggregation in response to U46619 than in response to other agonists such as ADP-fibrinogen, collagen, and thrombin [23]. The integrity of the membrane microdomain surrounding TXA_2 receptors may be more important than that of the microdomains surrounding the receptors of other agonists. GAS did not inhibit the U46619-induced cell shape change. However, the agent strongly inhibited the responses in diacylglycerol formation, Ca^{2+} mobilization, granule secretion, and the fibrin-

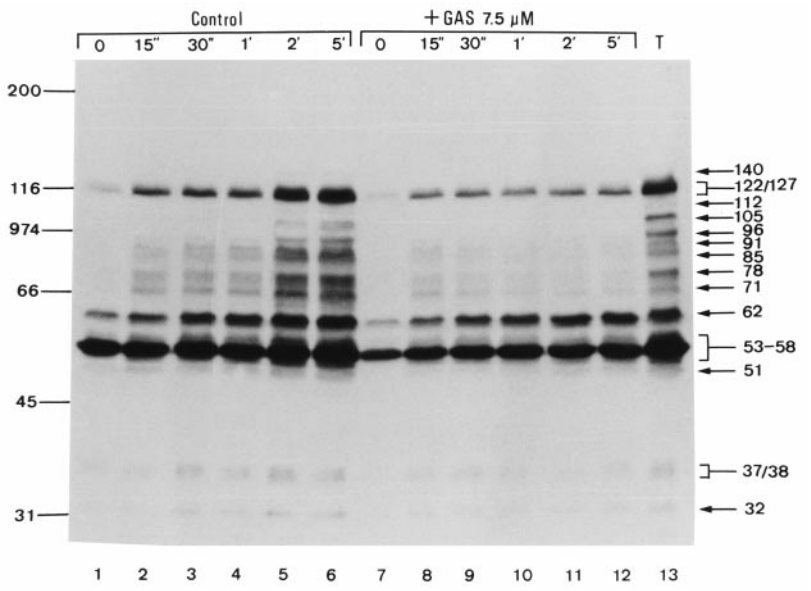

FIG. 8. Effect of GAS on protein-tyrosine phosphorylation in human platelets stimulated by U46619. GFP (3×10^8 cells/mL) were preincubated with GAS at 37° for 5 min before stimulation with U46619 (1 μM). At the end of each reaction time point, proteins were solubilized and separated on a 7.5% polyacrylamide gel, transferred, and immunoblotted with anti-phosphotyrosine antibody as described in Materials and Methods. Control (lanes 1–6) stands for the time course of protein-tyrosine phosphorylation in the presence of U46619, and that of U46619 plus GAS 7.5 μM is shown in lanes 7–12. Lane 13 shows the pattern of resting platelets stimulated by thrombin (T, 0.1 U/mL) for 2 min. Molecular mass markers and estimated molecular masses of major tyrosine-phosphorylated protein bands are indicated in kDa on the *left* and *right* of each blot, respectively. Immunoblots are representative of four separate platelet preparations.

ogen- and aggregation-dependent protein-tyrosine phosphorylations. These results indicated that GAS inhibits the TXRα-G_q-PLCβ1 pathway of TXA_2 signaling [8, 9]. It has been suggested that the TXRβ-G_i pathway links to TXA_2-induced platelet shape change and TXA_2-induced inhibition of adenylate cyclase [7]. The fact that GAS did not inhibit platelet shape change or the TXA_2 inhibition of adenylate cyclase suggests that GAS may not perturb TXA_2 signaling via G_i [11].

There exists a hierarchy of platelet responses, i.e. shape change, secretion, and aggregation. The biochemical events related to agonist-induced platelet shape change are the phosphorylation of myosin light chain [33], the activation of protein tyrosine kinase through a Ca^{2+}- and integrin-independent mechanism, and tyrosine phosphorylation of novel protein substrates [34]. Reports have indicated that agonist-stimulated protein-tyrosine phosphorylations are involved in the regulation of the cytoskeleton in platelets [35–37]. The study shows that GAS strongly inhibits Ca^{2+} mobilization in U46619-activated platelets, but it does not perturb the U46619-induced shape change. The $[Ca^{2+}]_i$ level required for TXA_2-induced platelet shape change is less than that for aggregation [38]. In addition, GAS does not inhibit protein-tyrosine phosphorylation at the early step of U46619 activation, including p71, p85/p78, and p62. The result suggests that protein-tyrosine phosphorylation at the early activation of platelets may be essential for platelet shape change.

There are two pools of cyclic AMP in PGE_1-induced platelets [39–41]. A small pool is important for inhibition of platelet aggregation and takes part in cyclic AMP-dependent phosphorylation processes in the membrane [42, 43]. About 10% of the PGE_1-induced cyclic AMP level is involved in inhibiting the exposed integrin $\alpha_{IIb}\beta_3$ [44]. The second cytoplasmic pool is unimportant for aggregation. U46619 inhibited the PGE_1-induced cyclic AMP level in platelets via TXRβ-G_i signaling [7, 11]. The present study demonstrated that U46619 reduced 40% of the cyclic AMP level in intact cells when the level was elevated by either PGE_1 or PGE_1 plus GAS. Since PGE_1 completely inhibited TXA_2-induced platelet aggregation, the 40% of pool size inhibited by U46619 may be a portion of the cyclic AMP in the second cytoplasmic pool. In GAS potentiation of PGE_1-induced cyclic AMP formation, we have observed that collagen does not inhibit cyclic AMP formation.* This is due to the fact that the inhibition of collagen on PGE_1-induced cyclic AMP formation is caused by TXA_2 released from the secondary collagen response [45]. The concomitant presence of GAS plus PGE_1 almost abolishes TXA_2 formation in the platelet response to collagen. Therefore, in the presence of GAS, collagen is unable to inhibit PGE_1-induced cyclic AMP formation.

GAS inhibits the activation of PLA_2 by U46619. There is a suggestion that TXA_2 activation of PLA_2 is through the TXRβ-G_i pathway [7]. Also, a study of platelets in bleeding disorder patients has reported impaired PLC activation but preserved platelet shape change and PLA_2 activation after TXA_2 receptor signaling [46]. The activation of platelet cytosolic PLA_2 is by the synergistic action of elevated $[Ca^{2+}]_i$ level and phosphorylation of serine residues of the enzyme [47]. p38 mitogen-activated protein kinase and other kinases participate in the regulation of cytosolic PLA_2 activity [48, 49]. The present study demonstrated that GAS strongly inhibits U46619-stimulated protein-tyrosine phosphorylation. This may result in the inhibition of PLA_2 activity. Therefore, the inhibition of TXA_2-induced PLA_2 activation by GAS may result from the inhibition of GAS on both Ca^{2+} mobilization and protein-tyrosine phosphorylation.

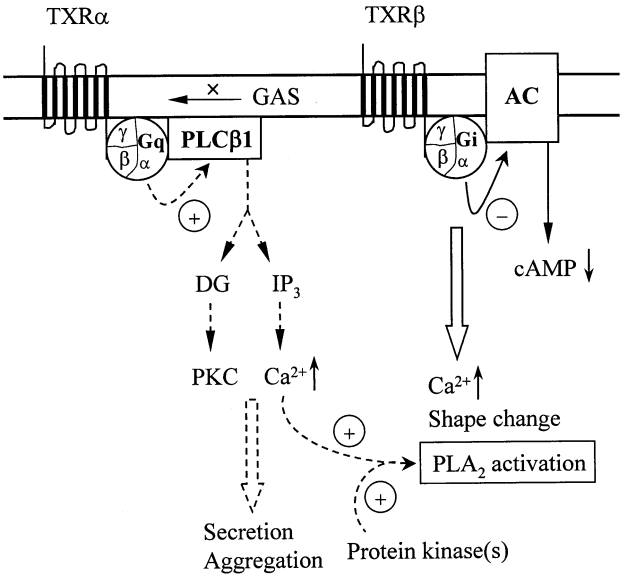

FIG. 9. Effects of GAS on the separate TXA_2 receptor-mediated pathways in human platelets. The TXRα-G_q-mediated pathway leads to activation of PLCβ1 and subsequently induces diacylglycerol (DG) formation, Ca^{2+} mobilization, secretion, and aggregation [8–11, 46]. The TXRβ-G_i-mediated pathway is involved in platelet shape change, PLA_2 activation, and the inhibition of adenylate cyclase [7, 11, 46]. Broken arrows indicate the events inhibited by GAS; solid arrows indicate the events that are not affected by GAS. The plus (+) and minus (−) symbols represent the activation and inhibition of the specific enzyme, respectively. AC = adenylate cyclase, and IP_3 = inositol 1,4,5-trisphosphate.

In conclusion, the present study demonstrated that GAS inhibits the TXRα-G_q-PLCβ1 pathway in the platelet-TXA_2 response without perturbing the TXRβ-G_i pathway (Fig. 9). It is conceivable that the TXRα-G_q-PLCβ1 pathway is more sensitive than the TXRβ-G_i pathway to perturbation by GAS. Alternatively, there may be a difference in lipid environments between the TXRα-G_q-PLCβ1 system and the TXRβ-G_i system. It has been suggested that lipid domains exist in the platelet plasma membrane [50]. GAS may preferentially insert into the microdomain of TXRα-G_q-PLCβ1 and inhibit the signaling pathway.

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