Inhibitory Mechanisms of Metallothionein on Platelet Aggregation in *In Vitro* and Platelet Plug Formation in *In Vivo* Experiments

JOEN R. SHEU,*†[1] GEORGE HSIAO,* MING Y. SHEN,† YING WANG,† KUANG H. LIN,† CHIEN H. LIN,† AND DUEN S. CHOU*

*Department of Pharmacology and †Graduate Institute of Medical Sciences, Taipei Medical University, Taipei 110, Taiwan

Metallothionein (MT) is a low-molecular-weight, cysteine-rich protein that contains heavy metals such as cadmium and zinc. The biological function of MT in platelets is not yet understood. Therefore, the aim of this study was to systematically examine the inhibitory mechanisms of metallothionein in platelet aggregation. In this study, metallothionein concentration-dependently (1-8 μM) inhibited platelet aggregation in human platelets stimulated by agonists. Metallothionein (4 and 8 μM) inhibited phosphoinositide breakdown in [^{3}H]-inositol-labeled platelets, intracellular Ca^{+2} mobilization in Fura-2 AM-loaded platelets, and thromboxane A_2 formation stimulated by collagen. In addition, metallothionein (4 and 8 μM) significantly increased the formation of cyclic GMP but not cyclic AMP in human platelets. Rapid phosphorylation of a protein of *Mr* 47,000 (P47), a marker of protein kinase C activation, was triggered by PDBu (100 nM). This phosphorylation was markedly inhibited by metallothionein (4 and 8 μM) in phosphorus-32-labeled platelets. In an *in vivo* thrombotic study, platelet thrombus formation was induced by irradiation of mesenteric venules in mice pretreated with fluorescein sodium. Metallothionein (6 μg/g) significantly prolonged the latency period for inducing platelet plug formation in mesenteric venules. These results indicate that the antiplatelet activity of metallothionein may involve the following pathways: (1) metallothionein may inhibit the activation of phospholipase C, followed by inhibition of phosphoinositide breakdown and thromboxane A_2 formation, thereby leading to inhibition of intracellular Ca^{+2} mobilization; (ii) Metallothionein also activated the formation of cyclic GMP in human platelets, resulting in inhibition of platelet aggregation. The results strongly indicate that metallothionein provides protection against thromboembolism. Exp Biol Med 228:1321–1328, 2003

This work was supported by the National Science Council of Taiwan Grant NSC 90-2315-B-038-001-M61.

[1] To whom requests for reprints should be addressed at Graduate Institute of Medical Sciences, Taipei Medical University, No. 250, Wu-Hsing Street, Taipei 110, Taiwan. E-mail: sheujr@tmu.edu.tw

Received June 23, 2003.
Accepted September 19, 2003.

1535-3702/03/22811-1321$15.00

Key words: metallothionein; platelet aggregation; phospholipase C; cyclic GMP; protein kinase C

Metallothionein (MT) is a low-molecular-weight, cysteine-rich protein that contains heavy metals such as cadmium and zinc (1). This protein has been reported to be involved in the detoxification of heavy metals and metabolism of essential trace elements (1). MT also plays a role in the scavenging of free radicals, which are produced under various stress conditions (2). Therefore, MT can protect cells from cytotoxicity and DNA damage (3). The basal level of MT in biological systems is very low, although it may vary with age and type of tissue. However, this protein is induced to a significantly high level when a system is challenged by heavy metals, starvation, heat, inflammation, or other stress conditions (2).

It has been found that nonmetallic and physiologic factors such as thrombin and cytokines are able to induce MT expression in cultured aortic endothelial cells (4), suggesting that this protein may be involved in the process of hemostasis and/or thrombosis. Intravascular thrombosis is a factor in generating a wide variety of cardiovascular diseases. Initiation of intraluminal thrombosis is believed to involve platelet adherence and aggregation. Thus, platelet aggregation may play a crucial role in atherothrombotic processes. Sugiura and Nakamura (5) demonstrated the presence of MT in platelets. The average concentration of MT was quantitatively about 39.4 ± 4.2 $\mu g/10^{10}$ cells in human platelets (5). The biological function of MT in platelets is not yet understood, but it is possible that platelet MT serves to regulate thromboembolism. However, no data are available concerning the effect of MT in platelet aggregation. Therefore, the detailed inhibitory mechanisms of MT underlying the signaling pathways in platelets still remain obscure. We therefore systematically examined the influence of MT in washed human platelets, and utilized the findings to characterize the mechanisms involved in this influence. In addition, we previously reported that platelet

thrombi were induced by irradiation with filtered light in the microvasculature of mice pretreated with fluorescein sodium (6). Therefore, we used this model to further evaluate the inhibitory effect of platelet plug formation by MT *in vivo*.

Materials and Methods

Materials. Collagen (type I, bovine achilles tendon), MT (forms I and II, from rabbit liver containing approximately 7% metal as Cd + Zn), sodium citrate, luciferin-luciferase, indomethacin, fluorescein sodium, Dowex-1 (100–200 mesh; X_8, chloride form), myoinositol, PGE_1, arachidonic acid, phorbol-12, 13-dibutyrate (PDBu), aspirin, apyrase, heparin, and thrombin were purchased from Sigma Chem. (St. Louis, MO). Fura 2-AM and fluorescein iso-thiocyanate (FITC) were purchased from Molecular Probe (Eugene, OR). *Trimeresurus flavoviridis* venom was purchased from Latoxan (Rosans, France). Myo-2-[^{3}H] inositol was purchased from Amersham (Buckinghamshire, HP, UK). Thromboxane B_2, cyclic AMP, and cyclic GMP EIA kits were purchased from Cayman (Ann Arbor, MI).

Preparation of Human Platelet Suspensions. Human platelet suspensions were prepared as previously described (7). 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 (9:1, vol/vol). After centrifugation at 120 g for 10 min, the supernatant (platelet-rich plasma; PRP) was supplemented with prostaglandin E_1 (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 bovine serum albumin (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.

Platelet Aggregation. The turbidimetric method was applied to measure platelet aggregation (8), using a Lumi-Aggregometer (Payton, Canada). Platelet suspensions (4.5×10^8 platelets/ml, 0.4 ml) were pre-warmed to 37°C for 2 min (stirring at 1200 rpm) in a silicone-treated glass cuvette. MT (1-8 μM) was added 3 min before the addition of platelet-aggregation inducers. The reaction was allowed to proceed for at least 6 min, and the extent of aggregation was expressed in light-transmission units. While measuring ATP release, 20 μl of a luciferin/luciferase mixture was added 1 min before the addition of agonists, and ATP release was compared with that of the control.

Analysis of the Platelet Surface Glycoprotein IIb/IIIa Complex by Flow Cytometry. Triflavin, a specific fibrinogen receptor (glycoprotein IIb/IIIa complex) antagonist, was prepared as previously described (9). Fluorescence-conjugated triflavin was also prepared as previously described (10). The final concentration of FITC-conjugated triflavin was adjusted to 1 mg/ml. Human platelet suspensions were prepared as described previously. Aliquots of platelet suspensions (4.5×10^8/ml) were preincubated with MT (4 and 8 μM) for 3 min, followed by the addition of 2 μl of FITC-triflavin. The suspensions were then incubated for another 5 min, and the volume was adjusted to 1 ml/tube with Tyrode's solution. The suspensions were then assayed for fluorescein-labeled platelets with a flow cytometer (Becton Dickinson, FACScan Syst., San Jose, CA). Data were collected from 50,000 platelets per experimental group. All experiments were repeated at least four times to ensure reproducibility.

Labeling of Membrane Phospholipids and Measurement of the Production of [^{3}H]-Inositol Phosphates. The method was carried out as previously described (11). Briefly, citrated human PRP was centrifuged, and the pellets were suspended in Tyrode's solution containing [^{3}H]-inositol (75 μCi/ml). Platelets were incubated for 2 hr followed by centrifugation, and were finally resuspended in Ca^{+2}-free Tyrode's solution (5×10^8/ml). MT (4 and 8 μM) was preincubated with 1 ml of loaded platelets at room temperature for 3 min, and collagen (1 μg/ml) was then added to trigger aggregation. The reaction was stopped after 6 min, and samples were centrifuged at 1000 *g* for 4 min. The inositol phosphates of the supernatants were separated in a Dowex-1 anion exchange column. Only [^{3}H]-inositol monophosphate (IP) was measured as an index of total inositol phosphate formation.

Measurement of Platelet [Ca^{+2}]i Mobilization by Fura 2-AM Fluorescence. Citrated whole blood was centrifuged at 120 *g* for 10 min. The supernatant was protected from light and incubated with Fura 2-AM (5 μM) at 37°C for 1 hr. Human platelets were then prepared as described previously. Finally, the external Ca^{+2} concentration of the platelet suspensions was adjusted to 1 mM. The rise in [Ca^{+2}]i was measured using a fluorescence spectrophotometer (CAF 110, Jasco, Japan) at excitation wavelengths of 340 and 380 nm, and an emission wavelength of 500 nm. [Ca^{+2}]i was calculated from the fluorescence, using 224 nM as the Ca^{+2}-Fura 2 dissociation constant (12).

Measurement of Thromboxane B_2 Formation. Washed human platelet suspensions (4.5×10^8/ml) were preincubated for 3 min in the presence or absence of MT (4 and 8 μM) before the addition of collagen (1 μg/ml). Six minutes after the addition of agonists, 2 mM EDTA and 50 μM indomethacin were added to the reaction suspensions. The vials were then centrifuged for 3 min at 15,000 g. The thromboxane B_2 (TxB_2) levels of the supernatants were measured using an EIA kit according to the instructions of the manufacturer.

Estimation of Platelet Cyclic AMP and Cyclic GMP Formations. The method of Karniguian *et al.* (13) was followed. In brief, platelet suspensions were warmed to 37°C for 1 min, then either PGE_1 (10 μM), nitroglycerin (10 μM), or MT (4 and 8 μM) was added and incubated for 6 min. The incubation was stopped, and the solution was immediately boiled for 5 min. After cooling to 4°C, the precipitated protein was collected as sediment after centrifu-

gation. Fifty microliters of supernatant was used to determine the cyclic AMP and cyclic GMP contents by EIA kits following acetylation of the samples as described by the manufacturer.

Measurement of Protein Kinase C Activity. Washed human platelets (2×10^9/ml) were incubated for 60 min at 37°C with phosphorus-32 (0.5 mCi/ml). Platelet suspensions were next washed twice with Tris-saline buffer. The [^{32}P]-labeled platelets were preincubated with MT (4 and 8 μM) in an aggregometer at 37°C for 3 min, then PDBu (100 nM) was added for 1 min to trigger protein kinase C activation. Activation was terminated by the addition of Laemmli sample buffer, and analyzed by electrophoresis (12.5%; wt/vol) as described previously (14). The gels were dried, and the relative intensities of the radioactive bands were analyzed using a Bio-imaging analyzer system (FAL2000, Fuji, Tokyo, Japan), and expressed as PSL/mm (PSL, photostimulated luminescence).

Fluorescein Sodium-Induced Platelet Thrombi in Mesenteric Microvessels of Mice. As we previously described (6), mice were anesthetized with sodium pentobarbital (50 mg/kg, ip). After a tracheotomy was performed, an external jugular vein was cannulated with polyethylene tubing (PE-10) for administration of the dye and drug (by an iv bolus), while additional tubing was cannulated through the femoral artery to monitor blood pressure. A segment of the small intestine with its mesentery attached was loosely exteriorized through a midline incision in the abdominal wall and was placed onto a transparent culture dish for microscopic observation. Microvessels in the mesentery were observed under transillumination from a halogen lamp. Venules with diameters of 30 to 40 μm were selected for irradiation to produce a microthrombus. In the epi-illumination system, light from a 100-W mercury lamp was passed through a filter (B-2A, Nikon, Tokyo, Japan) with a dichromic mirror (DM 510, Nikon). Filtering the light eliminated wavelengths below 520 nm, and this light was used to irradiate a microvessel through an objective lens (20×). The area of irradiation was about 100 μm in diameter on the focal plane. The dose of fluorescein sodium used was 10 μg/kg. Various concentrations of MT (3 and 6 μg/g), aspirin (150 and 250 μg/g), or normal saline (control) were administered 1 min after fluorescein sodium addition. The injected volume of the MT, aspirin, or normal saline (control) was smaller than 50 μl. Five minutes after administration of the dye, a timer and irradiation with filtered light were simultaneously started, and platelet aggregation was observed on a monitor. The time lapse for inducing thrombus formation leading to cessation of blood flow was measured. The elapsed time for inducing platelet plug formation was repeatedly measured every 5 min during irradiation of the venules.

Statistical Analysis. The experimental results are expressed as the means ± SEM and are accompanied by the number of observations. Data were assessed using analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, each group was compared using the Newman-Keuls method. A P value < 0.05 was considered statistically significant.

Results

Effect of Metallothionein on Platelet Aggregation in Human Platelet Suspensions. Metallothionein (1–8 μM) concentration dependently inhibited platelet aggregation stimulated by collagen (1 μg/ml), thrombin (0.1 U/ml), and arachidonic acid (60 μM) in human platelets (Fig. 1A, 1B). Furthermore, MT inhibited the ATP-release reaction when stimulated by agonists (i.e., collagen) (Fig. 1A). The IC_{50} values of MT for platelet aggregation induced by collagen, thrombin, and arachidonic acid were estimated to be approximately 4.2, 6.5, and 3.7 μM, respectively. In

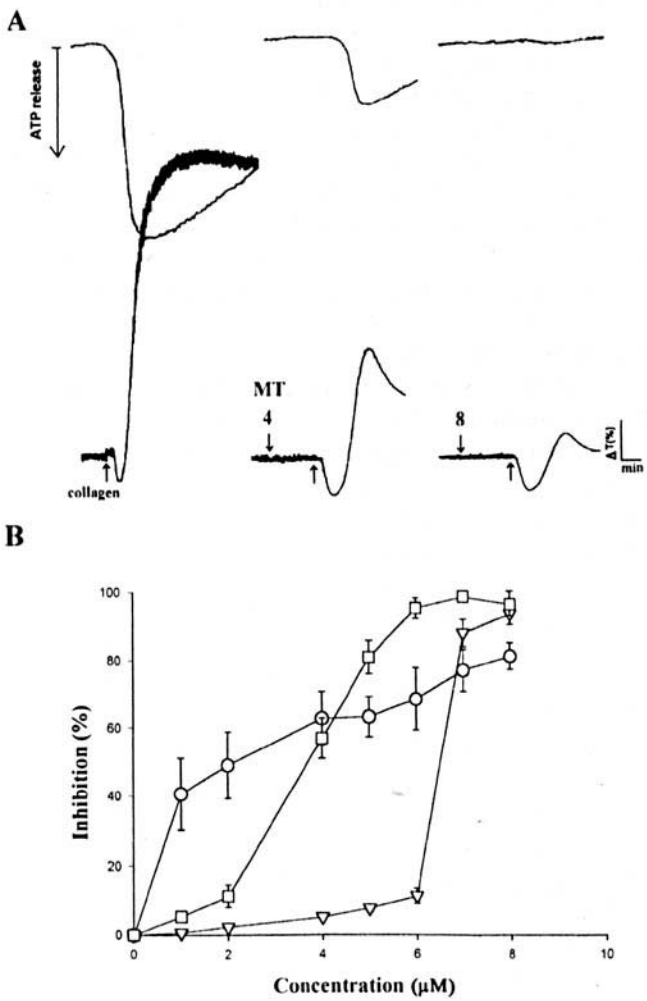

Figure 1. (A) Tracing curves of metallothionein on collagen (1 μg/ml)-induced aggregation and ATP release, and (B) concentration-inhibition curves of metallothionein on collagen (1 μg/ml, ○)-, thrombin (0.1 U/ml, ▽)-, and arachidonic acid (60 μM, □)-induced platelet aggregation in washed human platelets. Platelets were preincubated with metallothionein (1-8 μM) for 3 min, then agonists were added to trigger aggregation (lower tracings) and ATP release (upper tracings) (A). A luciferin-luciferase mixture (20 μl) was added 1 min before the agonist addition to measure the ATP-release reactions. Data are presented as a percentage of the control (means ± SEM, $n = 5$).

the following experiments, we used collagen as an agonist to explore the inhibitory mechanisms of MT in platelet aggregation.

Effect of Metallothionein on Collagen-Induced Conformational Change of the Glycoprotein IIb/IIIa Complex in Human Platelets. Triflavin is an Arg-Gly-Asp-containing antiplatelet peptide purified from *Trimeresurus flavoviridis* snake venom (9, 15). Triflavin inhibits platelet aggregation through direct interference with fibrinogen binding to the glycoprotein IIb/IIIa complex ($\alpha_{IIb}\beta_3$ integrin) (11). There is now a multitude of evidence suggesting that the binding of fibrinogen to the glycoprotein IIb/IIIa complex is the final common pathway for agonist-induced platelet aggregation. Therefore, we decided to further evaluate whether MT binds directly to the platelet glycoprotein IIb/IIIa complex, leading to inhibition of platelet aggregation induced by agonists.

In this study, the relative intensity of fluorescence of FITC-triflavin (2 μg/ml) bound directly to collagen (1 μg/ml)-activated platelets was 325.8 ± 22.3 (n = 4), and it was markedly reduced in the presence of 5 mM EDTA (negative control, 47.2 ± 6.3, $P < 0.001$, n = 4) (data not shown). MT (4 and 8 μM) did not significantly inhibit FITC-triflavin binding to the glycoprotein IIb/IIIa complex in platelet suspensions (312.4 ± 30.3 and 296.5 ± 23.7, n = 4) (data not shown), indicating that the mechanism of MT's inhibitory effect on platelet aggregation does not involve binding to the glycoprotein IIb/IIIa complex.

Effect of Metallothionein on Phosphoinositide Breakdown in Human Platelets. Phosphoinositide breakdown occurs in platelets activated by many different agonists (16). In this study, we found that collagen (1 μg/ml) induced the rapid formation of radioactive IP, IP_2, and IP_3 in human platelets loaded with [^{3}H]-inositol. We only measured [^{3}H]-IP formation as an index of total inositol phosphate formation. As shown in Figure 2A, the addition of collagen (1 μg/ml) resulted in a rise in IP formation of about 2.6-fold compared with that in resting platelets ([4.8 ± 0.5 vs 12.5 ± 1.3] × 10^3 cpm). In the presence of MT (4 and 8 μM), the radioactivity of IP formation in collagen-stimulated human platelets markedly decreased. Furthermore, free cytoplasmic Ca^{+2} concentrations in human platelets were measured by the Fura 2-AM loading method. As shown in Figure 2B, collagen (1 μg/ml) evoked an increase in [Ca^{+2}]i of from 36.4 ± 4.5 to 378.6 ± 25.3 nM. This collagen-evoked increase in [Ca^{+2}]i was markedly inhibited in the presence of MT (4 μM, 76.5 ± 9.6%; 8 μM, 85.8 ± 6.1%) (Fig. 2B). This suggests that MT exerts an inhibitory effect on phosphoinositide breakdown and [Ca^{+2}]i mobilization in human platelets stimulated by collagen.

Effects of Metallothionein on Thromboxane B_2, Cyclic AMP, and Cyclic GMP Formations in Human Platelets. As shown in Table I, resting platelets produced relatively little TxB_2 compared with collagen-activated platelets. PGE_1 (10 μM) inhibited TxB_2 formation in collagen-activated platelets by 81% (data not shown). Furthermore, results obtained using various concentrations of MT indicated that MT (4 and 8 μM) significantly inhibited TxB_2 formation in platelets stimulated by collagen (1 μg/ml). In addition, the level of cyclic AMP in unstimulated platelets was about 35.8 ± 2.9 pmol/ml. The addition of PGE_1 (10 μM) increased the cyclic AMP level to 94.3 ± 5.1 pmol/ml (Table II). MT (4 and 8 μM) did not significantly increase cyclic AMP levels in human platelets (Table II). We also performed similar studies measuring the cyclic GMP response. The level of cyclic GMP in unstimulated platelets was very low, but when nitroglycerin (10 μM) was added to the platelet suspensions, the cyclic GMP level increased from the resting level to 14.3 ± 0.8 pmol/ml (Table II). The addition of MT (4 and 8 μM) resulted in

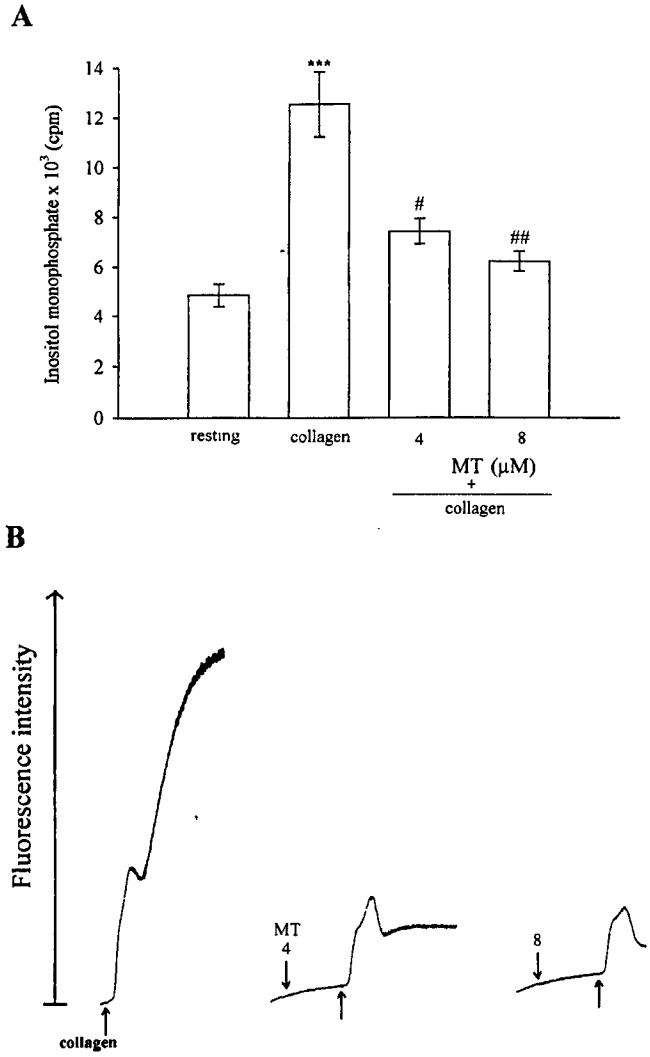

Figure 2. Effect of metallothionein on collagen-induced (A) inositol monophosphate formation and (B) intracellular Ca^{+2} mobilization in human platelet suspensions. Platelets were preincubated with [^{3}H]-inositol or Fura 2-AM (5 μM) followed by the addition of collagen (1 μg/ml) in the absence or presence of metallothionein (4 and 8 μM), which was added 3 min before the addition of collagen. (A) Data are presented as the means ± SEM (n = 4). $^{***}P < 0.001$ as compared with the resting group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ as compared with the collagen group. (B) The profiles are representative of examples of four similar experiments.

Table I. Effect of Metallothionein on Thromboxane B_2 Formation Induced by Collagen in Washed Human Platelets

	Dose	Thromboxane B_2 (ng/ml)
Resting	—	267.5 ± 9.6
Collagen (μg/ml)	1	510.6 ± 29.1[a]
+ Metallothionein (μM)	4	406.1 ± 19.8[b]
	8	288.0 ± 24.0[c]

Note. Platelet suspensions were preincubated with metallothionein (4 and 8 μM) for 3 min at 37°C, and then collagen (1 μg/ml) was added to trigger thromboxane B_2 formation. Data are presented as the means ± SEM (n = 4). [a]$P < 0.001$ as compared with the resting group; [b]$P < 0.05$ and [c]$P < 0.001$ as compared with the collagen group.

Table II. Effect of Metallothionein, Prostaglandin E_1, and Nitroglycerin on Cyclic AMP and Cyclic GMP Formation in Washed Human Platelets

	Concentration (μM)	Cyclic AMP (pmol/ml)	Cyclic GMP
Resting		35.8 ± 2.9	4.6 ± 0.1
Prostaglandin E_1	10	94.3 ± 5.1[b]	—
Nitroglycerin	10	—	14.3 ± 0.8[b]
Metallothionein	4	39.6 ± 0.7	8.3 ± 1.0[a]
	8	42.1 ± 3.9	9.2 ± 0.5[b]

Note. Platelet suspensions were preincubated with metallothionein (4 and 8 μM) for 3 min at 37°C. Addition of prostaglandin E_1 and nitroglycerin into the platelet suspensions served as positive controls. Data are presented as the means ± SEM (n = 4). [a]$P < 0.01$ and [b]$P < 0.001$ as compared with the resting groups.

significant increases in platelet cyclic GMP levels (8.3 ± 1.0 and 9.2 ± 0.5 pmol/ml, respectively) (Table II).

Effect of Metallothionein on PDBu-Stimulated Phosphorylation of the 47-kDa Protein. Stimulation of platelets with a number of different agonists, and PDBu in particular, induces activation of protein kinase C, which then phosphorylates proteins of *Mr* 40,000 to 47,000 in addition to other proteins (17). In this study, phosphorylation experiments were performed to examine the role of MT in the activation of protein kinase C in human platelets. When PDBu (100 nM) was added to human platelets prelabeled with $^{32}PO_4$ for 2 min, a protein with an apparent *Mr* of 47,000 (P47) was predominately phosphorylated as compared with resting platelets (Fig. 3A, 3B). On the other hand, MT (4 and 8 μM) concentration dependently inhibited the phosphorylation of P47 in human platelets stimulated by PDBu. In this study, the extent of radioactivity in P47 was expressed as a relative detection density (PSL/mm^2; PSL, photostimulated luminescence) of the radioactive bands. Moreover, MT (4 and 8 μM) also significantly inhibited collagen (1 μg/ml)-induced phosphorylation of P47 in human platelets (data not shown).

Effect of Metallothionein on Thrombus Formation in Microvessels of Fluorescein Sodium-Pretreated Mice. When 10 μg/kg of fluorescein sodium

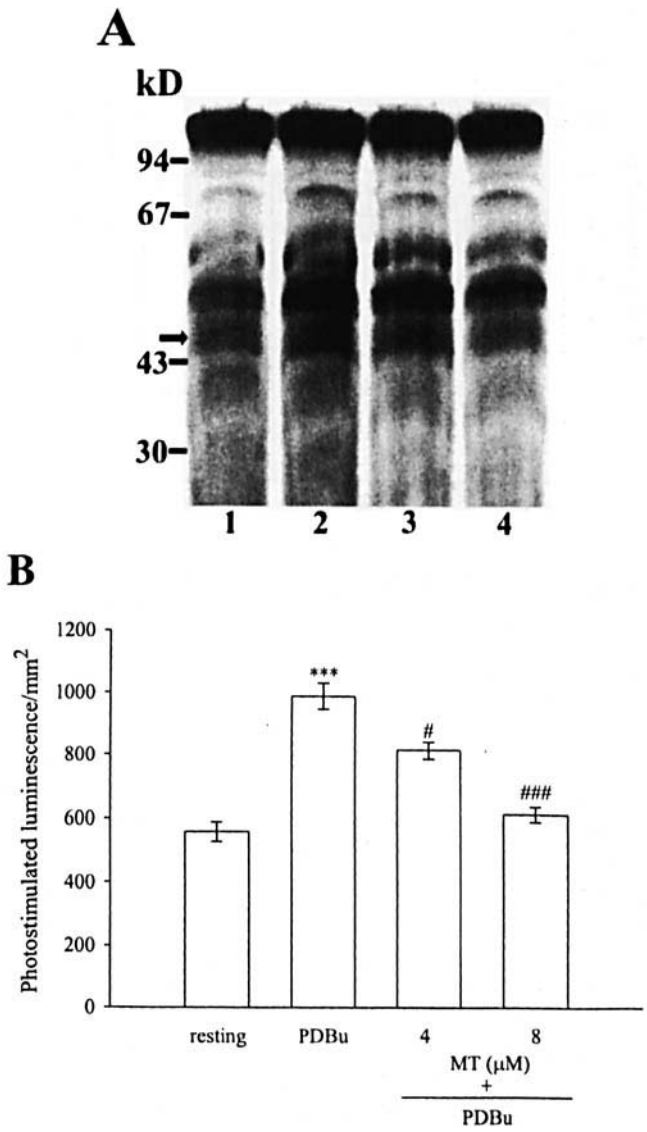

Figure 3. (A) Effect of metallothionein on phosphorylation of a protein of *Mr* 47,000 (P47) in human platelets challenged with PDBu. Platelets were preincubated with metallothionein (4 and 8 μM) before challenge with PDBu (100 nM). Lane 1, platelets with Tyrode's solution only (resting group); lane 2, platelets with PDBu (100 nM); lane 3, platelets with metallothionein (4 μM); and lane 4, platelets with metallothionein (8 μM) for 3 min followed by the addition of PDBu (100 nM). (A) The profiles are representative examples of four similar experiments. The arrow indicates a protein of *Mr* 47,000 (P47). (B) The relative detection densities of the radioactive bands are expressed as PSL/mm^2 (PSL, photostimulated luminescence). Data are presented as the means ± SEM ($n = 4$). ***$P < 0.001$ as compared with the resting group; #$P < 0.05$ and ###$P < 0.001$ as compared with the PDBu group.

was given, the occlusion time required was 257.4 ± 19.2 s (Fig. 4). MT inhibited platelet aggregation induced by agonists *in vitro*; therefore, we further examined its effect on the formation of platelet-rich thrombi in this *in vivo* model. When MT was administered at 3 μg/g, the occlusion time was not significantly prolonged (295.2 ± 19.0 s) until it was administered at 6 μg/g in mice pretreated with fluorescein sodium (10 μg/kg) (342.4 ± 32.5 s) (Fig. 4). The occlusion time was also significantly prolonged within 20 min (326.2

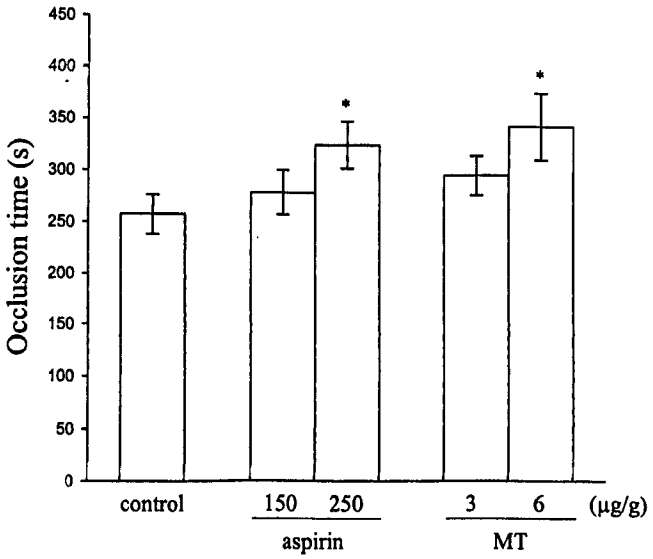

Figure 4. Effect of metallothionein (3 and 6 μg/g) and aspirin (150 and 250 μg/g) on occlusion times for inducing thrombus formation upon light irradiation of mesenteric venules of mice pretreated with fluorescein sodium (10 μg/kg). Data are presented as the means ± SEM of occlusion time (s) of platelet plug formation ($n = 8$). *$P < 0.05$ as compared with the control group.

± 21.5 s, $P < 0.05$, $n = 6$) and returned to the control value within 40 min (298.5 ± 17.1 s, $n = 6$) after MT (6 μg/g) administration (data not shown). MT also exhibited an antithrombotic effect in arterioles (data not shown). However, arterioles sometimes showed slight vasoconstriction when fluorescein sodium was irradiated, thus venules were chosen for induction of platelet plug formation in this study.

On the other hand, aspirin also exhibited antithrombotic activity in this experiment. When 250 μg/g of aspirin was administered, the occlusion time was prolonged (324.6 ± 22.5 s) (Fig. 4). The occlusion time was significantly prolonged within 40 min (313.8 ± 17.5 s, $P < 0.05$, $n = 6$) and returned to the control value within 60 min (284.3 ± 15.6 s, $n = 6$) after aspirin (250 μg/g) administration (data not shown). MT was over approximately 500-fold more potent than aspirin at prolongation of occlusion time in microvessels on a molar basis.

Discussion

The results obtained from this study demonstrate for the first time that MT provides antiplatelet activity. The principal objective of this study was to describe the detailed mechanisms involved in the inhibition of agonist-induced human platelet aggregation by MT. This inhibitory effect of MT was demonstrable with the use of various agonists: collagen, thrombin, and arachidonic acid. In this study, platelet aggregation induced by these agonists (i.e., collagen) appeared to be affected in the presence of MT. Therefore, this partly infers that MT may affect Ca^{+2} release from intracellular Ca^{+2}-storage sites (i.e., dense tubular systems or dense bodies) (Fig. 2), and this is in accord with the concept that intracellular Ca^{+2} release is responsible for platelet aggregation (18).

Although the action mechanisms of various platelet aggregation agonists, such as collagen, thrombin, and arachidonic acid, differ, MT significantly inhibited platelet aggregation stimulated by all of them. This implies that MT may block a common step shared by these inducers. These results also indicate that the site of action of MT is not at the receptor level of individual agonists. Triflavin acts by binding to the glycoprotein IIb/IIIa complex on the platelet surface membrane, resulting in interference with the interaction of fibrinogen with its specific receptor (9, 10, 15). In this study, we found that MT did not significantly affect FITC-triflavin binding to the glycoprotein IIb/IIIa complex, indicating that the antiplatelet activity of MT is possibly not directly due to interference with the binding of fibrinogen to its specific receptor on the platelet membrane.

Stimulation of platelets by agonists (i.e., collagen) results in phospholipase C-catalyzed hydrolysis of the minor plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, with concomitant formation of inositol 1,4,5-trisphosphate and diacylglycerol (Fig. 5) (19). There is strong evidence that inositol 1,4,5-trisphosphate induces the release of Ca^{+2} from intracellular stores (Fig. 5) (20). Diacylglycerol activates protein kinase C, inducing protein phosphorylation and a release reaction. In this study, phosphoinositide breakdown of collagen-activated platelets was inhibited by MT, suggesting that inhibition of platelet aggregation by MT is related to inhibition of phospholipase C activation. Moreover, TxA_2 is an important mediator of the release reaction and aggregation of platelets (Fig. 5) (21). Collagen-induced TxB_2 formation, a stable metabolite of TxA_2, was markedly inhibited by MT (4 and 8 μM) (Table I). It has been demonstrated that phosphoinositide breakdown can induce TxA_2 formation via free arachidonic acid release by diglyceride lipase or by endogenous phospholipase A_2 from membrane phospholipids (Fig. 5) (22). Thus, it seems likely that TxB_2 formation plays a role in mediating the inhibitory effect of MT on human platelets.

Furthermore, MT significantly inhibited PDBu-induced activation of protein kinase C. PDBu is known to intercalate with membrane phospholipids and form a complex with protein kinase C translocated to the membrane (23). Moreover, increased cyclic GMP formation can negatively affect agonist-induced protein kinase C activation (Fig. 5) (24). Signaling by cyclic GMP somehow interferes with the agonist-stimulated phosphoinositide turnover that creates Ca^{2+}-mobilizing second messengers (25). MT increased the level of cyclic GMP in human platelets; therefore, the inhibitory effect of MT in PDBu-induced activation of protein kinase C may be due, at least partly, to mediating the increase in cyclic GMP formation.

Platelet aggregation plays a pathophysiologic role in a variety of thromboembolic disorders. Therefore, inhibition of platelet aggregation by drugs may represent an increased therapeutic possibility for such diseases. In this study, we evaluated the inhibition of thrombus formation by MT *in vivo*, and found that MT significantly inhibited platelet plug

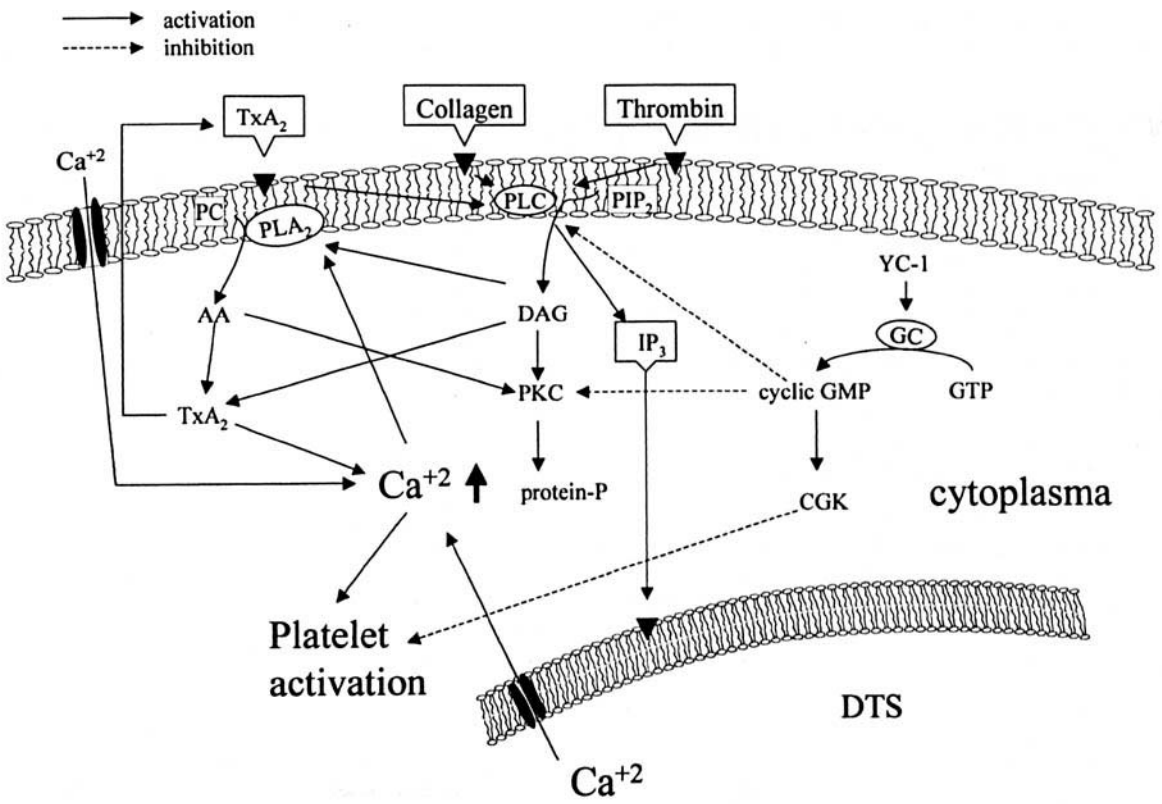

Figure 5. Signal transductions of platelet aggregation. Agonists can activate several phospholipases, including phospholipase C (PLC) and phospholipase A_2 (PLA_2). The products of phospholipase C's action on phosphatidylinositol 4,5-bisphosphate (PIP_2) include 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG stimulates protein kinase C (PKC), followed by phosphorylation of a 47-kDa protein. IP_3 induces the release of Ca^{+2} from the dense tubular system (DTS). The major metabolite of arachidonic acid (AA) in platelets is thromboxane A_2 (TxA_2). Cyclic GMP, cyclic 3′-5′-guanosine monophosphate; PC, phosphatidylcholine; GC, guanylate cyclase; ATP, adenosine triphosphate; CGK, cyclic GMP activated cyclic GMP-dependent protein kinase; YC-1, a soluble guanylyl cyclase activator.

formation. It shortened the occlusion time of thrombus formation induced by irradiation of fluorescein sodium in venules or arterioles. Since the light beam covered the entire microscopic field, a simultaneous observation of arterioles and venules was made. Our data reveal that platelet aggregation usually occurred first in venules rather than in arterioles. This may be explained by the higher-flow velocities found in arterioles, resulting in delayed adhesion of platelets to arteriolar endothelial cells. In this system, the occlusion time was related to the blood flow rate, the diameter of the microvessel, and the dose of fluorescein dye. In this study, MT caused occlusion times to be significantly prolonged in mice pretreated with fluorescein sodium mainly through its inhibition of platelet aggregation.

In conclusion, the most important observations of this study suggest that MT inhibits agonist-induced human platelet aggregation. This inhibitory effect may possibly involve the following two mechanisms: (i) MT may inhibit the activation of phospholipase C, followed by inhibition of phosphoinositide breakdown and thromboxane A_2 formation, thereby leading to inhibition of intracellular Ca^{+2} mobilization; (ii) on the other hand, MT increases cyclic GMP formation and subsequently inhibits phosphoinositide breakdown and protein kinase C activity, ultimately resulting in inhibition of the phosphorylation of P47 and intracellular Ca^{+2} mobilization. Results of this study clearly indicate that MT may exhibit physiological functions during thromboembolism formation. We therefore believe that our data represent a physiologically relevant function for MT as a negative feedback regulator during platelet activation. In effect, when MT accumulates in the microenvironment of a generating thrombus, the recruitment and activation of nearly platelets is then prevented. However, the physiological relevance of a direct anti-aggregatory effect of MT still remains to be further studied.

1. Hamer DH. Metallothionein. Annu Rev Biochem **55:**913–951, 1986.
2. Kagi JHR. Overview of metallothionein. Methods Enzymol **205:**613–626, 1991.
3. Cai L, Koropatnick J, Cherian MG. Metallothioneins protects DNA from copper-induced but not iron-induced cleavage in vitro. Chem Biol Interact **96:**143–155, 1995.
4. Kaji T, Yamamoto C, Suzuki M, Miyajima S, Fujiwara Y, Sakamoto M, Koizumi F. Induction of metallothionein by thrombin in cultured vascular endothelial and smooth muscle cells. Biol Pharm Bull **18:**1272–1274, 1995.
5. Sugiura T, Nakamura H. Metallothionein in platelets. Int Arch Allergy Immunol **103:**341–348, 1994.
6. Sheu JR, Chao SH, Yen MH, Huang TF. In vivo antithrombotic effect of triflavin, an Arg-Gly-Asp containing peptide on platelet plug formation in mesenteric microvessels of mice. Thromb Haemostas **72:**617–621, 1994.

7. Sheu JR, Lee CR, Lin CC, Kan YC, Lin CH, Hung WC, Lee YM, Yen MH. The antiplatelet activity of PMC, a potent α-tocopherol analogue, is mediated through inhibition of cyclo-oxygenase. Br J Pharmacol **127**:1206–1212, 1999.
8. Born GVR, Cross MJ. The aggregation of blood platelets. J Physiol **168**:178–195, 1963.
9. Sheu JR, Teng CM, Huang TF. Triflavin, an RGD-containing antiplatelet peptide, binds to GP IIIa of ADP-stimulated platelets. Biochem Biophys Res Commun **189**:1236–1242, 1992.
10. Sheu JR, Lin CH, Peng CH, Huang TF. Triflavin, an Arg-Gly-Asp-containing peptide, inhibits the adhesion of tumor cells to matrix protein via binding to multiple integrin receptors expressed on human hepatoma cells. Proc Soc Exp Biol Med **213**:71–79, 1996.
11. Huang TF, Sheu JR, Teng CM. Mechanism of action of a potent antiplatelet peptide, triflavin, from *Trimeresurus flavoviridis* snake venom. Thromb Haemostas **66**:489–493, 1991.
12. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{+2} indicator with greatly improved fluorescence properties. J Biol Chem **260**:3440–3450, 1985.
13. Karniguian A, Legrand YJ, Caen JP. Prostaglandins: specific inhibition of platelet adhesion to collagen and relationship with cyclic AMP level. Prostaglandins **23**:437–457, 1982.
14. Grabarek J, Raychowdhury M, Ravid K, Kent KC, Newman PJ, Ware JA. Identification and functional characterization of protein kinase C isozymes in platelets and HEL cells. J Biol Chem **267**:10011–10017, 1992.
15. Sheu JR, Hung WC, Wu CH, Ma MC, Kan YC, Lin CH, Lin MS, Luk HN, Yen MH. Reduction in lipopolysaccharide-induced thrombocytopenia by triflavin in a rat model of septicemia. Circulation **99**:3056–3062, 1999.
16. Broekman MJ, Ward JW, Marcus AJ. Phospholipid metabolism in phosphatidyl inositol, phosphatic acid and lysophospholipids. J Clin Invest **66**:275–283, 1980.
17. Siess W, Lapetina EG. Platelet aggregation induced by alpha 2-adrenoceptor and protein kinase C activation. A novel synergism. Biochem J **263**:377–385, 1989.
18. Charo IF, Feinman RD, Detwiler TC. Inhibition of platelet secretion by an antagonist of intracellular calcium. Biochem Biophys Res Commun **72**:1462–1467, 1976.
19. Kirk CJ, Creba JA, Downes CP, Michell RH. Hormone-stimulated metabolism of inositol lipids and its relationship to hepatic receptor function. Biochem Soc Transact **9**:377–379, 1981.
20. Berridge MJ. Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. Biochem J **212**:249–258, 1983.
21. Hornby EJ. Evidence that prostaglandin endoperoxides can induce platelet aggregation in the absence of thromboxane A_2 production. Biochem Pharmacol **31**:1158–1160, 1982.
22. McKean ML, Smith JB, Silver WJ. Formation of lysophosphatidylcholine in human platelets in response to thrombin. J Biol Chem **256**:1522–1524, 1981.
23. Kraft AS, Anderson WB. Phorbol esters increase the amount of Ca^{+2}, phospholipid-dependent protein kinase associated with plasma membrane. Nature **301**:621–623, 1983.
24. Murohara T, Parkinson SJ, Waldman SA, Lefer AM. Inhibition of nitric oxide biosynthesis promotes P-selectin expression in platelets. Role of protein kinase C. Arterioscler Thromb Vasc Biol **15**:2068–2075, 1995.
25. McDonald LJ, Murad F. Nitric oxide and cyclic GMP signaling. Proc Soc Exp Biol Med **211**:1–6, 1996.