Mechanisms Involved in the Antiplatelet Activity of Midazolam in Human Platelets

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Background: Midazolam is widely used as a sedative and anesthetic induction agent. The aim of this study was to systematically examine the inhibitory mechanisms of midazolam in platelet aggregation.

Methods: The inhibitory mechanisms of midazolam in platelet aggregation were explored by means of analysis of the platelet glycoprotein Ib-Ⅲa complex, phosphoinositide breakdown, intracellular Ca^{2+} mobilization, measurement of membrane fluidity, thromboxane B_{2} formation, and protein kinase C activity.

Results: In this study, midazolam dose-dependently (6–26 μM) inhibited platelet aggregation in human platelets stimulated by agonists. Midazolam also dose-dependently inhibited phosphoinositide breakdown and intracellular Ca^{2+} mobilization in human platelets stimulated by collagen. Midazolam (6–26 μM) significantly inhibited thromboxane A_{2} formation stimulated by collagen in human platelets. Moreover, midazolam (15 and 26 μM) dose-dependently decreased the fluorescence of platelet membranes tagged with diphenylhexatriene. Rapid phosphorylation of a platelet protein of Mr 47,000 (P47), a marker of protein kinase C activation, was triggered by collagen (2 μM/ml). This phosphorylation was markedly inhibited by midazolam (26 μM).

Conclusions: These results indicate that the antiplatelet activity of midazolam may be involved in the following pathways: the effects of midazolam may initially be caused by induction of conformational changes in platelet membrane, leading to a change in the activity of phospholipase C, and subsequent inhibition of phosphoinositide break down and thromboxane A_{2} formation, thereby leading to inhibition of both intracellular Ca^{2+} mobilization and phosphorylation of P47 protein.

Midazolam is widely used as a sedative and anesthetic induction agent. The pharmacologic actions of midazolam are identical to those of other benzodiazepines and include sleep induction, sedation, anxiolysis, and amnesia. Its clinical use is primarily reserved for conscious sedation and induction of general anesthesia.

Benzodiazepines exert many pharmacologic activities at the periphery. Among these activities, some benzodiazepines inhibit platelet aggregation. Triazolam and alprazolam inhibit platelet-activating factor-induced aggregation, and the inhibition of platelet-activating factor binding to platelets has been proposed to explain this antiaggregatory effect. However, this is controversial, for diazepam has been shown both to inhibit arachidonate-induced aggregation and to have no effect. In addition, Karaseva et al. reported that gidazepam and phenazepam exert inhibitory properties of platelet aggregation, possibly through inhibition of lipid peroxidation in rat platelets. Choppin and Berry found that clonazepam, diazepam, and flumazenil inhibit arachidonic acid-induced platelet aggregation, possibly by mediating the inhibition of thromboxane synthesis in rabbit platelets. These reports indicate that the effect of benzodiazepine derivatives on platelet aggregation in vitro remains unclear, controversial, and species-specific.

On the other hand, studies of midazolam in platelets have relative rarely been compared with other derivatives of benzodiazepines. Lingjaerde presented data indicating that midazolam inhibits serotonin uptake in human platelets. However, the detailed mechanisms underlying the midazolam signaling pathways still remain obscure. We therefore systematically examined the influence of midazolam on washed human platelets in this study and used the findings to characterize the mechanisms involved in this influence.

Materials and Methods

Materials
Collagen (type I, bovine achilles tendon), adenosine 5'-diphosphate, midazolam, arachidonic acid, EDTA, luciferin-luciferase, Dowex-1 (100–200 mesh; X 8 , chloride form), prostaglandin E_{1}, trichloroactic acid, EGTA, bovine serum albumin, acylamide, sodium pyruvate, β-NADH, diphenylhexatriene, apyrase, heparin, thrombin, and myo-inositol were purchased from Sigma Chemical Co. (St. Louis, MO). Fura 2-AM and fluorescein isothiocyanate (FITC) were purchased from Molecular Probe Inc. (Eugene, OR). Trimeresurus flavoviridis venom was purchased from Latoxan (Rosans, France). Myo-2-[^{3}H] inositol was purchased from Amersham (Buckinghamshire, United Kingdom). Thromboxane B_{2} enzyme immunoassay kits were purchased from Cayman Co. (Ann Arbor, MI).

Preparation of Human Platelet Suspensions
Human platelet suspensions were prepared as previously described. In this study, human volunteers gave

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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 120g for 10 min at room temperature, the supernatant (platelet-rich plasma) was supplemented with prostaglandin E1 (0.5 μM) and heparin (6.4 IU/ml), then incubated for 10 min at 37°C and centrifuged at 500g for 10 min. The washed platelets were finally suspended in Tyrode solution containing bovine serum albumin (3.5 mg/ml) and adjusted to approximately 4.5 × 10⁸ platelets/ml. The final concentration of Ca²⁺ in Tyrode solution was 1 mm.

**Platelet Aggregation**

The turbidimetric method used a Lumi-Aggregometer (Payton, Canada) as described previously. In brief, platelet suspensions (0.4 ml) were prewarmed at 37°C for 2 min, and then midazolam was added for 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 as a percentage of the control (in the absence of midazolam). The degree of aggregation was expressed in light-transmission units. While measuring adenosine triphosphate (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. Fluorescence-conjugated triflavin was also prepared as previously described. The final concentration of FITC-conjugated triflavin was adjusted to 1 mg/ml. Human platelet suspensions were prepared as described above. Aliquots of platelet suspensions (4.5 × 10⁹/ml) were preincubated with midazolam (15 and 26 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 solution. The suspensions were then assayed for fluorescence in human platelets with a flow cytometer (FACScan System; Becton Dickinson, San Jose, CA). Data were collected from 50,000 platelets per experimental group. All experiments were repeated at least five times to ensure reproducibility.

**Labeling of Membrane Phospholipids and Measurement of the Production of [³H]-Inositol Phosphates**

The method was conducted as previously described. Briefly, citrated human platelet-rich plasma was centrifuged, and the pellets were suspended in Tyrode solution containing [³H]-inositol (75 μCi/ml). Platelets were incubated for 2 h followed by centrifugation, and finally resuspended in Ca²⁺-free Tyrode solution (5 × 10⁸ platelets/ml). Midazolam was preincubated with 1 ml of loaded platelets at room temperature for 3 min, and collagen (2 μg/ml) was then added to trigger aggregation. The reaction was stopped after 6 min, and the samples were centrifuged at 1,000g for 4 min. The inositol phosphates of the supernatants were separated in a Dowex-1 anion exchange column. Only [³H]-inositol monophosphate (IP) was measured as an index of total inositol phosphate formation.

**Measurement of Platelet Intracellular Ca²⁺ Mobilization by Fura 2-AM Fluorescence**

Citrated whole blood was centrifuged at 120g for 10 min. The supernatant was protected from light and incubated with Fura 2-AM (5 μM) at 37°C for 1 h. Human platelet suspensions were then prepared as described above. Finally, the external Ca²⁺ concentration of the platelet suspensions was adjusted to 1 mm. The increase in intracellular Ca²⁺ concentration ([Ca²⁺]i) was measured using a fluorescence spectrophotometer (CAF 110; Jasco, Tokyo, Japan) with excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm, respectively. The [Ca²⁺]i was calculated from the fluorescence measured using 224 nm as the Ca²⁺-Fura 2 dissociation constant.

**Measurement of Membrane Fluidity by Fluorescent Probe**

The intensity of fluorescence in human platelets was measured as described previously. Briefly, platelets (4.5 × 10⁹/ml) were mixed with nine volumes of sodium-potassium-Tris medium. A solution of diphenylhexatriene in dimethyl formamide was added to the suspension at a final concentration of 0.5 μM. Platelets were preincubated with various concentrations of midazolam (15 and 26 μM) for 3 min followed by the addition of diphenylhexatriene at 37°C for another 6 min. The relative fluorescence intensity of platelets was measured in a fluorescence spectrophotometer (Hitachi F4500; Hitachi, Tokyo, Japan) at 37°C.

**Measurement of Thromboxane B₂ Formation**

Washed human platelet suspensions (4.5 × 10⁹/ml) were preincubated for 3 min in the presence or absence of midazolam (6–26 μM) before the addition of collagen (2 μg/ml). Six minutes after the addition of the agonist, 2 mM EDTA and 50 μM indomethacin were added to the reaction suspensions. The vials were then centrifuged for 3 min at 14,000 rpm. The thromboxane B₂ concentrations of the supernatants were measured using an enzyme immunoassay kit according to the instructions of the manufacturer.
Determination of Lactate Dehydrogenase

Lactate dehydrogenase was measured according to previously described methods. Platelets (4.5 × 10^8/ml) were preincubated with various concentrations of midazolam for either 10 or 30 min, followed by centrifugation at 14,000 rpm for 5 min. An aliquot of supernatant was incubated with phosphate buffer containing 0.2 mg β-NADH for 20 min at room temperature. Thereafter, 100 μl of pyruvate solution was added, and the absorbance wavelength was read at 334 nm using an ultraviolet-visible recording spectrophotometer (UV-160; Shimazu, Kyoto, Japan). A maximal value of lactate dehydrogenase was constructed from sonicated platelets.

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 [32P]-labeled platelets were preincubated with midazolam (26 μM) in an aggregometer at 37°C for 3 min, then collagen (2 μg/ml) 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. The gels were dried, and the relative intensities of the radioactive bands were analyzed using a Bio-imaging analyzer system (FAL2000; Fuji, Tokyo, Japan), expressed as photostimulated luminescence per squared millimeters.

Statistical Analysis

Experimental results are expressed as the mean ± SD and are accompanied by the number of observations. Data were assessed by Student t test, and P < 0.05 was considered statistically significant.

Results

Effect of Midazolam on Platelet Aggregation in Human Platelet Suspensions

Midazolam dose-dependently inhibited platelet aggregation stimulated by collagen (2 μg/ml), arachidonic acid (100 μM), and thrombin (0.5 U/ml) in human platelet suspensions (figs. 1A and B). Furthermore, midazolam also inhibited the ATP-release reaction when stimulated by agonists (i.e., collagen; fig. 1A). The IC₅₀ values of midazolam for platelet aggregation induced by collagen, arachidonic acid, and thrombin were estimated to be approximately 16.1, 23, and 9.7 μM, respectively. In addition, midazolam (26 and 50 μM) did not significantly induce platelet aggregation in the absence of agonists (data not shown). Furthermore, platelets were preincubated with midazolam (26 μM) or normal saline for 10 min followed by two washes with Tyrode solution, and we found that there are no significant differences between the aggregation curves of both platelet preparations stimulated by collagen (2 μg/ml), indicating that the effect of midazolam on inhibition of platelet aggregation occurs in a reversible manner (data not shown). These results also indicate that midazolam significantly inhibits agonist-induced human platelet aggregation.

Effect of Midazolam on Collagen-induced Glycoprotein IIb-IIIa Complex Exposure in Human Platelets

Triflavin is an Arg-Gly-Asp-containing antiplatelet peptide, purified from Trimeresurus flavoviridis snake venom. Triflavin inhibits platelet aggregation through direct interference with fibrinogen binding to the glycoprotein IIb-IIIa complex. 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 ag-
midazolam (15 and 26 μM), the radioactivity of IP formation in collagen-stimulated human platelets markedly decreased. These results indicate that midazolam interferes with phosphoinositide breakdown in human platelets stimulated by collagen.

**Effect of Midazolam on Intracellular Ca$$^{2+}$$ Concentration Mobilization**

Free cytoplasmic Ca$$^{2+}$$ concentrations in human platelets were measured by the Fura 2-AM loading method. As shown in figure 4, collagen (2 μg/ml) evoked an increase of [Ca$$^{2+}$$], from 36.6 ± 5.0 to 201.6 ± 36.6 nM. This collagen-evoked increase in [Ca$$^{2+}$$] was markedly inhibited in the presence of midazolam (15 μM, 74.6 ± 8.4%; 26 μM, 89.5 ± 12.6%; n = 4). This suggests that midazolam exerts an inhibitory effect on [Ca$$^{2+}$$] mobilization in human platelets stimulated by collagen.

**Effect of Midazolam on Thromboxane B$$_2$$ Formation**

As shown in table 1, resting platelets produced relatively little thromboxane B$$_2$$ compared with collagen-activated platelets. Prostaglandin E1 (10 μM) inhibited thromboxane B$$_2$$ formation in collagen-activated platelets by 81% (data not shown). Furthermore, results obtained using various concentrations of midazolam indicated that midazolam (6–26 μM) dose-dependently inhibited thromboxane B$$_2$$ formation in platelets stimulated by collagen (2 μg/ml; table 1). These results suggest that midazolam exerts an inhibitory effect on thromboxane A$$_2$$ formation.

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**Effect of Midazolam on Phosphoinositide Breakdown in Human Platelet Suspensions**

Phosphoinositide breakdown occurs in platelets activated by many different agonists. In this study, we found that collagen (2 μg/ml) induces the rapid formation of radioactive IP, IP$$_2$$, and IP$$_3$$ in human platelets loaded with [3H]-inositol. We only measured [3H]-IP formation as an index of total inositol phosphate formation. As shown in figure 3, the addition of collagen (2 μg/ml) resulted in an increase of IP formation of approximately 2.1-fold compared with that in resting platelets (11.6 ± 0.6 vs. 3.4 ± 0.4) × 10$$^{-3}$$ cpm). In the presence of

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Fig. 2. Flow cytometric analysis of fluorescein isothiocyanate (FITC)–trilavan binding to human platelets in the absence or presence of various concentrations of midazolam (15 and 26 μM). (A) The solid line represents the fluorescence profiles of FITC–trilavan (2 μg/ml) in the absence of midazolam as a positive control; (B) in the presence of EDTA (5 mM) as the negative control; or in the presence of (C) 15 μM and (D) 26 μM midazolam. The profiles are representative examples of five similar experiments.

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Collagen (*P* (resting platelets, 35.6 ± 3.5 platelets for 10 min did not significantly increase collagen-induced intracellular 
Ca**2**+ mobilization in Fura 2-AM-loaded human platelets. Platelet suspensions were preincubated with Fura 2-AM (5 μM), followed by the addition of collagen (2 μg/ml) in the absence or presence of midazolam (15 and 26 μM), which was added 3 min before the addition of collagen. The profiles are representative examples of four similar experiments.

### Effect of Midazolam on Platelet Membrane Fluidity

Platelet membrane fluidity was measured in diphenylhexatriene-labeled human platelets. Measurements using the fluorescent probe technique demonstrated that midazolam is capable of direct interaction with platelet membranes (fig. 5). Addition of midazolam (15 and 26 μM) to platelet preparations for 3 min resulted in a dose-dependent decrease in the diphenylhexatriene-related fluorescence intensity. This result implies that the inhibitory effect of midazolam on platelet aggregation may be a result, at least in part, of the results of its effects on platelet membrane fluidity.

### Effect of Midazolam on Lactate Dehydrogenase Released from Platelet Cytosol

In this study, midazolam (26 μM) treatment of human platelets for 10 min did not significantly increase lactate dehydrogenase activity compared with resting platelets (resting platelets, 35.6 ± 5.6 units vs. midazolam-treated platelets, 37.5 ± 9.8 units; n = 4), even when the incubation time of midazolam with platelets was prolonged to 30 min (34.6 ± 8.6 units; n = 4; data not shown). This result indicates that although midazolam (15 and 26 μM) significantly changes the fluidity of platelet membranes, by itself, it does not affect platelet permeabilization or induce platelet cytolysis under this range of concentrations.

### Effect of Midazolam on Collagen-stimulated Phosphorylation of the 47-kd Protein

Stimulation of platelets with a number of different agonists induces activation of protein kinase C, which then phosphorylates proteins of Mr 40,000–47,000 in addition to other proteins.20 In this study, phosphorylation experiments were conducted to examine the role of midazolam in activation of protein kinase C in human platelets. When collagen (2 μg/ml) was added to human platelets prelabeled with 32PO4 for 2 min, a protein with an apparent Mr of 47,000 (P47) was predominately phosphorylated as compared with resting platelets (figs. 6A and B). On the other hand, midazolam (26 μM) markedly inhibited the phosphorylation of P47 in human platelets stimulated by collagen (2 μg/ml) when preincubated with midazolam for 3 min. In this study, the extent of radioactivity in P47 was expressed as a relative detection density (photostimulated luminescence per squared millimeters) of the radioactive bands.

### Discussion

The principal objective of this study was to describe the detailed mechanisms involved in the inhibition of agonist-induced human platelet aggregation by midazolam. Midazolam is a familiar agent commonly used to provide sedation and for behavioral emergencies.1 The results demonstrate that midazolam exerts inhibitory activity on human platelet aggregation, a finding that has not been described previously. This inhibitory effect of midazolam was demonstrable with the use of various agonists: collagen, arachidonic acid, and thrombin. The inhibition was directly proportional to the concentration of midazolam used. Ramoska et al.21 demonstrated adequate sedation with effective intravenous doses of 1–3 mg midazolam. Harper et al.22 reported that intravenous doses of 0.3 mg/kg may be required when midazolam is used for sedation in surgical patients. In this study, midazolam was used at concentrations (approximately 6–26 μM) that inhibited platelet aggregation induced by agonists. These results indicate that the concentrations of midazolam used to inhibit platelet aggregation in vitro are reasonably close to those of blood concentrations obtained during midazolam-induced sedation in vivo.23 In this study, both platelet aggregation and the ATP-release reaction induced by these agonists (i.e., collagen) appeared to be affected by the presence of midazolam. Therefore, this partly infers that midazolam may affect 
Ca**2**+ release from intracellular...
lar Ca\(^{2+}\)-storage sites (i.e., dense tubular systems or dense bodies; fig. 4), and this is in accord with the concept that intracellular Ca\(^{2+}\) release is responsible for the ATP-release reaction.\(^{24}\)

Although the action mechanisms of various platelet aggregation agonists, such as collagen, arachidonic acid, and thrombin, differ (fig. 7), midazolam significantly inhibited platelet aggregation stimulated by all of them. This implies that midazolam may block a common step shared by these inducers. These results also indicate that the site of action of midazolam is not at the receptor level of individual agonists. Platelet membrane plays a dominant role in regulating and executing platelet functions, particularly platelet aggregation. Triflavin acts by

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Fig. 5. Fluorescence emission spectra of platelet membranes in the (A) absence or (B) presence of diphenylhexatriene (1 \( \mu \)M). (C and D) Curves are the emission spectra of membranes labeled with diphenylhexatriene in the presence of 15 \( \mu \)M (C) and 26 \( \mu \)M (D) midazolam for 3 min. The profiles are representative examples of four similar experiments.

Fig. 6. (A) Effect of midazolam on phosphorylation of a protein of Mr 47,000 (P47) in human platelets challenged with collagen. Platelets were preincubated with midazolam (26 \( \mu \)M) before challenge with collagen (2 \( \mu \)g/ml). Lane 1, platelets with Tyrode solution; lane 2, platelets with collagen (2 \( \mu \)g/ml); lane 3, platelets with midazolam (26 \( \mu \)M) and collagen (2 \( \mu \)g/ml). The arrow indicates a protein of Mr 47,000 (P47). Data are representative examples of four similar experiments. (B) The relative detection densities of the radioactive bands are expressed as photostimulated luminescence (PSL) per squared millimeter. Data are presented as mean ± SD (n = 4).

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Fig. 7. Signal transductions of platelet aggregation. Agonists can activate several phospholipase, including phospholipase C (PLC) and phospholipase A₂ (PLA₂). The products of the action of phospholipase C on phosphatidylinositol 4,5-bisphosphate (PIP₂) include 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). DAG stimulates protein kinase C (PKC), followed by phosphorylation of a 47-kd protein. IP₃ induces the release of Ca²⁺ from dense tubular systems (DTS). The major metabolite of arachidonic acid (AA) in platelets is thromboxane A₂ (TxA₂). Cyclic AMP = cyclic 3'-5'-adenosine monophosphate; AC = adenylate cyclase; ATP = adenosine triphosphate; IP₃ = inositol 1,4,5-trisphosphate; CAK = cyclic AMP-dependent protein kinase.

In conclusion, the observations of this study suggest that midazolam inhibits agonist-induced human platelet aggregation. This inhibitory effect may involve the following mechanisms: the antiplatelet activity of midazolam may be caused by the induction of conformational changes in platelet membrane, with a resulting influence of the release reaction and aggregation of platelets. Collagen-induced formation of thromboxane B₂, a stable metabolite of thromboxane A₂, was markedly inhibited by midazolam (6–26 μM; table 1). It has been demonstrated that phosphoinositide breakdown can induce thromboxane A₂ formation via free arachidonic acid release by diglyceride lipase or by endogenous phospholipase A₂ from membrane phospholipids (fig. 7). Thus, it seems likely that thromboxane B₂ formation plays a role in the mediation of the inhibitory effect of midazolam on human platelets.

Furthermore, in this study, activation of protein kinase C in human platelets occurred in response to collagen. The important finding in our studies is that midazolam inhibits collagen-induced activation of protein kinase C. In addition, it has been reported that increased nitric oxide–cyclic guanosine monophosphate formation negatively affects agonist-induced protein kinase C activation. However, midazolam (15 and 26 μM) did not significantly increase the concentration of nitric oxide or cyclic guanosine monophosphate in human platelets (data not shown). Therefore, based on the aforementioned observations, we suggest that the inhibitory effect of midazolam in collagen-induced activation of protein kinase C may be a result, at least in part, of its involvement in changing platelet membrane fluidity. This change in membrane fluidity influences the activation of phospholipase C, which is incorporated into plasma membranes, resulting in inhibition of phosphoinositide breakdown and subsequent inhibition of the activation of protein kinase C.
on membrane fluidity followed by inhibition of the activation of phospholipase C and subsequent inhibition of phosphoinositide breakdown and thromboxane A2 formation, thereby leading to inhibition of both intracellular Ca\(^{2+}\) mobilization and phosphorylation of P47.

References


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