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Mechanisms of antiplatelet and antithrombotic activity of midazolam in in vitro and in vivo studies

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Abstract

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. In this study, midazolam concentration-dependently (15 and 30 μM) inhibited platelet aggregation in washed human platelets stimulated by thrombin (0.05 U/ml). Midazolam (15 and 30 μM) also inhibited phosphoinositide breakdown and intracellular Ca^{+2} mobilization in platelets stimulated by thrombin (0.05 U/ml). In addition, midazolam (15 and 30 μM) increased the formation of cyclic AMP but not cyclic GMP or nitric oxide. The thrombin-evoked increase in pHi was markedly inhibited in the presence of midazolam (15 and 30 μM). Rapid phosphorylation of a platelet protein of molecular weight (Mr.) 47,000 (P47), a marker of protein kinase C activation, was triggered by thrombin (0.05 U/ml). This phosphorylation was markedly inhibited by midazolam (15 and 30 μM). Midazolam (30 μM) did not significantly reduce the electron spin resonance signal intensity of hydroxyl radicals in activated platelets. In the vivo study, intravenous injection of midazolam (10 μg/g) significantly prolonged the latent period of inducing platelet plug formation in mesenteric venules. These results indicate that midazolam can significantly prevent thrombus formation in vivo. Its antiplatelet activity may be involved in the inhibition of the activation of phospholipase C and the Na^+/H^+ exchanger and increased cyclic AMP formation. These lead to lower intracellular Ca^{+2} mobilization and phosphorylation of P47.

Keywords: Midazolam; Platelet aggregation; Phospholipase C; Cyclic AMP; Na^+/H^+ exchanger; Thrombosis

1. Introduction

Midazolam is widely used as a sedative and anesthetic agent (Claassen et al., 2002). It is also effective in the treatment of generalized seizures and refractory status epilepticus (Claassen et al., 2002). Although midazolam has anticonvulsant and muscle relaxant properties, its clinical use is primarily reserved for consciousness sedation and induction of general anesthesia (Claassen et al., 2002).

Benzodiazepines exert many pharmacological activities at the periphery. Among these activities, some benzodiazepines inhibit platelet aggregation. Triazolam and alprazolam inhibit platelet-activating factor (PAF)-induced aggregation (Kornecki et al., 1984; Chesney et al., 1987). This is, however, controversial since diazepam has been shown both to inhibit arachidonate-induced aggregation and to have no effect (Kornecki et al., 1984; Romstedt and Huzoor, 1985). In addition, Karaseva et al. (1998) also reported that gidazepam and phenazepam exert inhibitory properties of platelet aggregation possibly through inhibition of lipid peroxidation in rat platelets. Choppin and Berry (1995) found that clonazepam and diazepam inhibited arachidonate-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's effect on platelets have relatively rarely been compared with those of other derivatives of benzodiazepines. Lingjaerde (1986) presented data indicating that midazolam inhibits serotonin

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uptake in human platelets. Recently, we found that midazolam (6–26 μM) concentration-dependently inhibited platelet aggregation through interference of the membrane fluidity, phospholipase C activation, and thromboxane A_2 formation in human platelets (Sheu et al., 2002). However, the detailed antiplatelet mechanisms of midazolam and its in vivo antithrombotic activity still remain obscure. We therefore further examined the signaling pathways of midazolam on washed human platelets in this study. In addition, we previously reported that platelet thrombi were induced by irradiation with filtered light in the microvasculature of mice pretreated with fluorescein sodium (Sheu et al., 1994). Therefore, we used this model to evaluate the inhibitory effect of thrombus formation by midazolam in vivo.

2. Materials and methods

2.1. Materials

Midazolam, HEPES, nigericin, Dowex-1 (100–200 mesh; X_8, chloride form), myoinositol, β-mercaptoethanol, prostaglandin E_1 (PGE_1), bovine serum albumin, acrylamide, Tris–HCl, apyrase, heparin, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), fluorescein sodium, VCl_3, and thrombin were purchased from Sigma (St. Louis, MO). Fura 2-acetomethylester (Fura 2-AM) and 2′,7′-bis (2-carboxyethyl)-5(6)-carboxyfluorescein-acetomethylester (BCECF-AM) were purchased from Molecular Probe (Eugene, OR). Phosphorus-32 and *myo*-2-[^{3}H] inositol were purchased from Amersham (Buckinghamshire, HP, UK). Cyclic AMP and cyclic GMP enzyme immunoassay (EIA) kits were purchased from Cayman (Ann Arbor, MI).

2.2. Preparation of washed human platelets

Human platelet suspensions were prepared as previously described (Sheu et al., 1998). 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, v/v). The washed platelets were finally suspended in Tyrode's solution containing bovine serum albumin (3.5 mg/ml) and adjusted to about 4.5×10^8 platelets/ml.

2.3. Platelet aggregation

The turbidimetric method was applied to measure platelet aggregation using a Lumi-Aggregometer (Sheu et al., 1998). Platelet suspensions (0.4 ml) were preincubated with midazolam for 3 min before the addition of thrombin (0.05 U/ml). 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.

2.4. Labeling of membrane phospholipids and measurement of the production of [^{3}H] inositol phosphates

The method was carried out as previously described (Sheu et al., 1998). Briefly, citrated human platelet-rich plasma was centrifuged and pellets were suspended in Tyrode's solution containing [^{3}H] inositol (75 μCi/ml). Platelet pellets were incubated for 2 h followed by centrifugation and, finally, were resuspended in Ca^{2+}-free Tyrode's solution (5×10^8 platelets/ml). Midazolam was preincubated with 1 ml loaded platelets for 3 min, and thrombin (0.05 U/ml) was then added to trigger aggregation. The reaction was stopped after 6 min and the samples were centrifuged at $1000 \times g$ 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 the total inositol phosphate formation.

2.5. Measurement of platelet $[Ca^{2+}]_i$ mobilization by Fura 2-AM fluorescence

Citrated whole blood was centrifuged at $120 \times g$ for 10 min. The supernatant was incubated with Fura 2-AM (5 μM) at 37 °C for 1 h. The human platelet suspensions were then prepared as described above. Finally, the external Ca^{2+} concentration of the platelet suspensions was adjusted to 1 mM. The $[Ca^{2+}]_i$ rise 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. The $[Ca^{+2}]_i$ was calculated from the fluorescence measured using 224 nM as the Ca^{+2}-Fura 2 dissociation constant (Grynkiewicz et al., 1985).

2.6. Estimation of platelet cyclic AMP and cyclic GMP formations

The method of Karniguian et al. (1982) was followed. In brief, platelet suspensions were warmed at 37 °C for 1 min, then either PGE_1 (10 μM), nitroglycerin (10 μM), or midazolam (15 and 30 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 centrifugation. 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.

2.7. Estimation of nitrate in human platelet suspensions

The method was carried out as previously described (Sheu et al., 1999). In brief, platelet suspensions (1×10^9/ml) were preincubated with thrombin (0.05 U/ml), midazolam (15 and 30 μM), or thrombin combined with midazolam (30 μM) for 6 min, respectively, followed by centrifugation.

The supernatants were deproteinized by incubation with 95% ethanol at 4 °C for 30 min. The nitric oxide (NO) of the supernatants was then drawn into a Sievers Nitric Oxide Analyzer (Sievers 280 NOA, Sievers, Boulder, CO). Nitrate concentrations were calculated by comparison with standard solutions of sodium nitrate.

2.8. Platelet pHi measurement

Platelet pHi was measured with the fluorescent probe, BCECF-AM, according to a previously described method (Touyz and Schiffrin, 1993). Washed platelets were incubated with BCECF-AM (5 μM) for 30 min in HEPES-buffered solution (HBS) and then centrifuged at 450 × *g* for 8 min. The washed pellets were finally suspended in buffer and adjusted to 4.5×10^8/ml. Leukocyte contamination was less than 0.01%. Aliquots of this platelet suspension (50 μl) were transferred into cuvettes containing 2 ml HBS (pH 7.4, 37 °C) in a dual-excitation wavelength spectrofluorometer (CAF 110, Jasco). Fluorescence signals for BCECF-AM were recorded at 430- and 490-nm excitation wavelengths with an emission wavelength of 530 nm (5-nm slit). The background fluorescence of platelets was subtracted from each reading. Calibration was carried out after diluting the BCECF-loaded platelets in a high-K^+ buffer in the presence of nigericin (0.2 mg/ml), as described by Horne et al. (1981). In all experiments, platelets were stimulated by thrombin (0.05 U/ml) to trigger the Na^+/H^+ exchanger.

2.9. Measurement of protein kinase C activity

Washed platelets (2×10^9/ml) were suspended in Tris-saline buffer containing 2 mM EDTA and then were incubated for 60 min with phosphorus-32 (0.5 mCi/ml). [^{32}P]-labeled platelets were preincubated with midazolam (15 and 30 μM) at 37 °C for 3 min, and then thrombin (0.05 U/ml) was added for 1 min to trigger protein kinase C activation. Activation was terminated by the addition of an equal volume of 2 × concentrated Laemmli sample buffer, as described previously (Sheu et al., 2000). Samples were boiled at 95 °C for 5 min in a reduced condition and analyzed by electrophoresis on 12.5% (w/v) polyacrylamide gels. 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 were expressed as PSL/mm^2 (PSL: photostimulated luminescence).

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

The ESR method used a Bruker EMX ESR spectrometer as described previously (Hsiao et al., 2003). In brief, platelet suspensions (1×10^9/ml) were preincubated with midazolam (30 μM) for 3 min before the addition of thrombin (0.05 U/ml). The reaction was allowed to proceed for 5 min, followed by the addition of 100 mM DMPO for the ESR study. ESR spectra were recorded on a Bruker EMX ESR spectrometer using a quartz flat cell designed for aqueous solutions. Conditions of ESR spectrometry were as follows: 20-mW power at 9.78 GHz, 1-G modulation, and 100-G scanning in 42 s, with 10 scans accumulated.

2.11. Fluorescein sodium-induced platelet thrombi in mesenteric microvessels of mice

As we previously described (Sheu et al., 1994), mice were anesthetized, and an external jugular vein was cannulated with polyethylene tubing (PE-10) for administration of the dye and drug (by an i.v. bolus), while additional tubing was cannulated through the femoral artery for monitoring blood pressure. A segment of the small intestine with its mesentery attached was loosely exteriorized through a midline incision on the abdominal wall and was placed onto a transparent culture dish for microscopic observation. Microvessels in the mesentery were observed under trans-illumination from a halogen lamp. Venules with diameters of 30–40 μm were selected for irradiation to produce a microthrombus. A dose of 15 μg/kg fluorescein sodium was used. The injected volume of the midazolam or normal saline (control) was smaller than 50 μl. The time lapse for inducing thrombus formation leading to cessation of blood flow was measured. The elapsed time for inducing platelet plug formation was measured repeatedly, every 5 min with irradiation of venules.

2.12. Statistical analysis

Experimental results are expressed as the means ± S.E.M. and are accompanied by the number of observations. Data were assessed by Student's *t*-test and the method of analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared by the Newman–Keuls method. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of midazolam on platelet aggregation and $[Ca^{2+}]_i$ mobilization

In our previous report (Sheu et al., 2002), midazolam (6–26 μM) concentration-dependently inhibited platelet aggregation stimulated by collagen (2 μg/ml), arachidonic acid (100 μM), and thrombin (0.5 U/ml) in washed human platelets. In this study, midazolam (30 μM) almost completely inhibited platelet aggregation stimulated by a lower concentration of thrombin (0.05 U/ml) (Fig. 1A). The IC_{50} value of midazolam for platelet aggregation induced by thrombin (0.05 U/ml) was about 14.7 μM. In the following

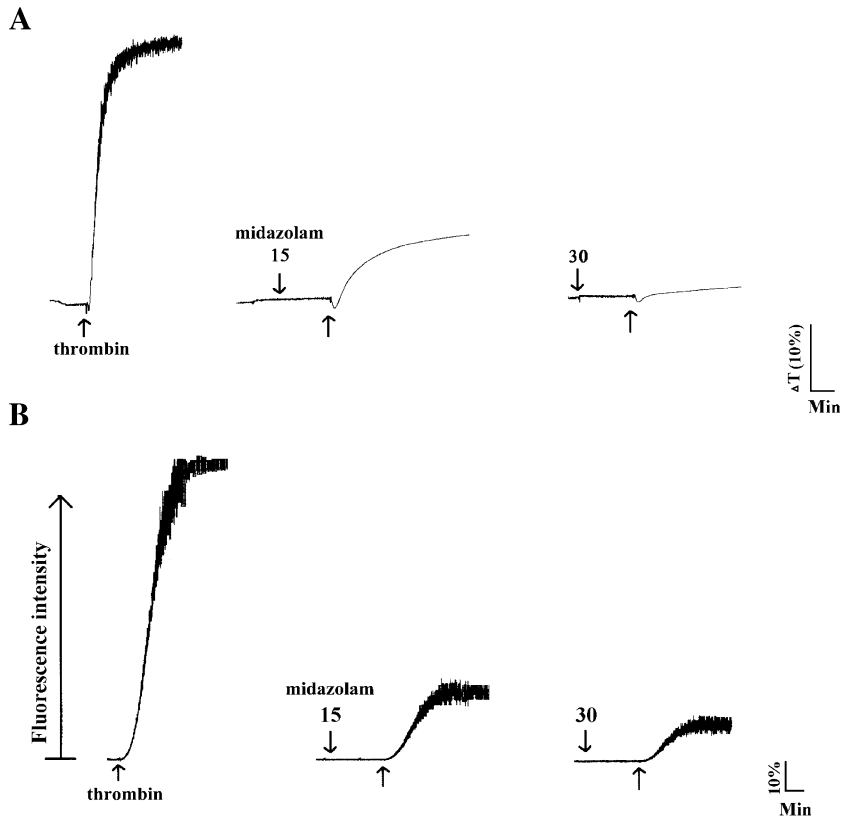

Fig. 1. Tracing curves of midazolam on thrombin (0.05 U/ml)-induced (A) platelet aggregation and (B) intracellular Ca^{2+} mobilization in human platelets. Platelets and Fura 2-AM-loaded platelets were preincubated with midazolam (15 and 30 μM), respectively, followed by the addition of thrombin (0.05 U/ml). The profiles are representative examples of five similar experiments. ΔT indicates the changes of transmission.

experiments, we used this concentration (0.05 U/ml) of thrombin as stimulator to further explore the inhibitory mechanisms of midazolam in platelet aggregation.

Free cytoplasmic Ca^{2+} concentrations in human platelets were measured by the Fura 2-AM loading method. As shown in Fig. 1B, thrombin (0.05 U/ml) evoked an increase of $[Ca^{2+}]_i$ from 31.5 ± 2.8 to 298.6 ± 26.5 nM ($n = 5$, $P < 0.001$). The thrombin-evoked increase of $[Ca^{2+}]_i$ was markedly inhibited in the presence of midazolam (15 μM, 72.9%; 30 μM, 86.5%; $n = 5$).

3.2. Effect of midazolam on phosphoinositide breakdown in washed human platelets

Phosphoinositide breakdown occurs in platelets activated by many different agonists (Broekman et al., 1980). In this study, we found that thrombin (0.05 U/ml) induced rapid formation of radioactive inositol monophosphate (IP), inositol-4,5-biphosphate (IP_2), and inositol-1,4,5-trisphosphate (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. Addition of thrombin (0.05 U/ml) resulted in an increase in IP formation of about 2.6-fold compared to that in resting platelets [$(1.4 \pm 0.3$ vs. $3.7 \pm 0.2) \times 10^{-3}$ cpm, $n = 5$, $P < 0.001$] (data not shown). In the presence of midazolam (15 and 30 μM), the radioactivity of IP formation in thrombin-stimulated human platelets markedly decreased [15 μM, $(2.4 \pm 0.2) \times 10^{-3}$ cpm, $n = 5$, $P < 0.01$; 30 μM, $(1.7 \pm 0.2) \times 10^{-3}$ cpm, $n = 5$, $P < 0.001$] (data not shown).

3.3. Effects of midazolam on cyclic AMP, cyclic GMP, and nitrate levels in washed human platelets

The level of cyclic AMP in unstimulated platelets was low (29.4 ± 1.5 pmol/ml). Addition of PGE_1 (10 μM) increased the cyclic AMP level to 520.8 ± 58.6 pmol/ml (Table 1). When platelet suspensions were preincubated with various concentrations of midazolam (15 and 30 μM), the cyclic AMP levels were increased to 168.7 ± 14.4 and 192.7 ± 13.0 pmol/ml, respectively (Table 1). We also performed similar studies measuring the cyclic GMP response. The level of cyclic GMP in unstimulated platelets was also very low, but when nitroglycerin (10 μM) was added to the platelet suspensions, the cyclic GMP level increased from the resting level to 256.7 ± 3.3 pmol/ml (Table 1). However, addition of midazolam (15 and 30 μM) resulted in no significant increase in platelet cyclic GMP levels (Table 1), even at a higher concentration (60 μM) (data not shown).

On the other hand, NO was quantified using a sensitive and specific ozone redox-chemiluminescence detector. As shown in Table 1, thrombin (0.05 U/ml) caused about a 2.4-

Table 1
Effect of midazolam on cyclic AMP, cyclic GMP, and nitrate formation in washed human platelets

	Concentration	Cyclic AMP (pmol/ml)	Cyclic GMP (pmol/ml)	Nitrate (μM) (pmol/ml)
Resting		29.4 ± 1.5	13.3 ± 0.9	4.9 ± 0.8
Prostaglandin E_1	10 μM	520.8 ± 58.6*	–	–
Nitroglycerin	10 μM	–	256.7 ± 3.3*	–
Thrombin	0.05 U/ml	–	–	11.6 ± 0.5*
Midazolam	15 μM	168.7 ± 14.4*	13.2 ± 1.7	6.0 ± 0.8
	30 μM	192.7 ± 13.0*	16.3 ± 1.6	5.2 ± 0.5
Thrombin + Midazolam	0.05 U/ml 30 μM	–	–	12.3 ± 0.7*

Platelet suspensions were preincubated with midazolam (15 and 30 μM) or combined with thrombin at 37 °C. Addition of prostaglandin E_1, nitroglycerin, and thrombin into the platelet suspensions served as a positive control. Data are presented as means ± S.E.M. ($n = 5$).

* $P < 0.001$ as compared with the resting groups.

fold rise in nitrate formation, compared to that in resting platelets. In the presence of midazolam (15 and 30 μM), nitrate production did not significantly increase after incubation with platelets for 6 min (Table 1), even prolongation of the incubation time to 30 min (data not shown).

3.4. Effect of midazolam on thrombin-evoked pHi changes in platelets

Fig. 2 shows pHi changes triggered by thrombin (0.05 U/ml) in BCECF-AM loaded platelets. Resting platelet pHi values were about 7.10 ± 0.03 ($n = 5$) in washed human platelets. Addition of thrombin (0.05 U/ml) resulted in an increase in BCECF fluorescence equivalent to an increase in pHi values of about 0.03 ± 0.001 ($n = 5$) (Fig. 2). This thrombin-evoked increase in pHi was markedly inhibited in the presence of midazolam (15 μM, 65%; 30 μM, 98%; $n = 5$).

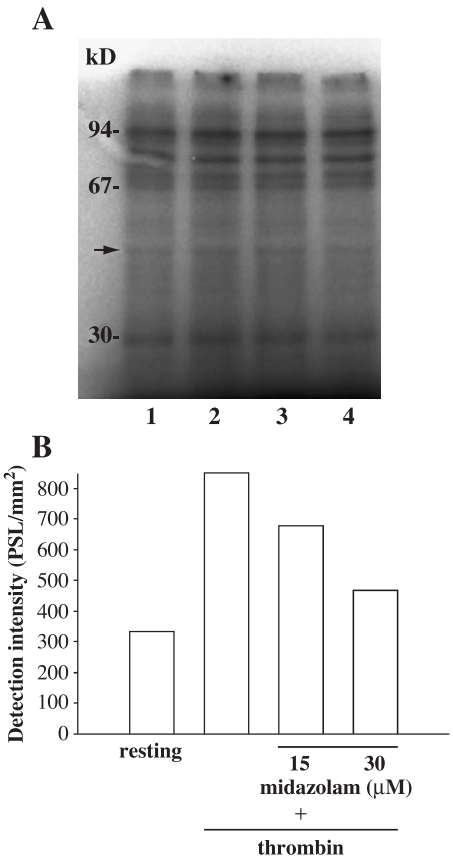

Fig. 3. (A) Effect of midazolam on phosphorylation of a protein of Mr. 47,000 (P47) in washed human platelets challenged with thrombin. Platelets were preincubated with midazolam (15 and 30 μM) for 3 min; thrombin (0.05 U/ml) was added for 1 min to trigger protein kinase C activation. Lane 1: platelets with Tyrode's solution; lane 2: platelets with thrombin (0.05 U/ml); lane 3: platelets with midazolam (15 μM); and lane 4: platelets with midazolam (30 μM) for 3 min followed by the addition of thrombin (0.05 U/ml). The arrow indicates a protein of Mr. 47,000 (P47). (B) Relative detection densities of radioactive bands expressed as PSL/mm² (PSL: photostimulated luminescence). Data are representative examples of five similar experiments.

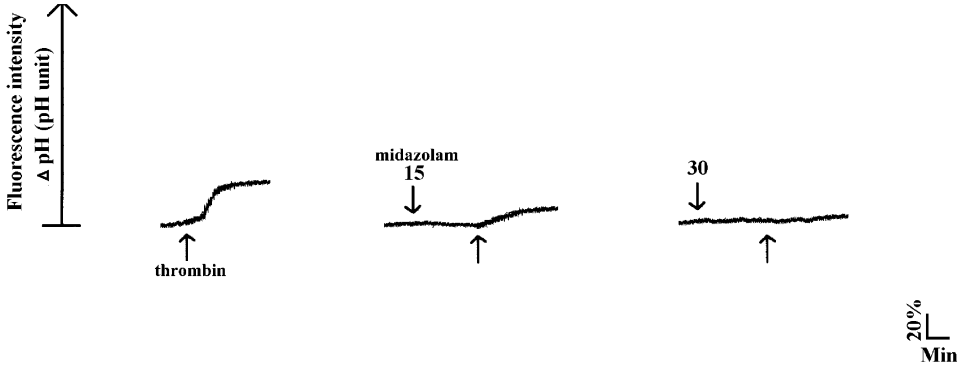

Fig. 2. Effect of thrombin-triggered intracellular pH increase on BCECF-AM-loaded platelets. BCECF-AM-loaded platelets preincubated with midazolam (15 and 30 μM) followed by the addition of thrombin (0.05 U/ml) to trigger intracellular alkalinization. The profiles are representative examples of five similar experiments.

3.5. Effect of midazolam on thrombin-stimulated phosphorylation of the 47-kDa 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 (Kirk et al., 1981). When thrombin (0.05 U/ml) was added to platelets prelabeled with $^{32}PO_4$, a protein with an apparent Mr. of 47,000 (P47) was predominately phosphorylated as compared with resting platelets (Fig. 3A and B). Midazolam (15 and 30 μM) significantly inhibited the phosphorylation of P47 in platelets stimulated by thrombin (0.05 U/ml) (15 μM, $P < 0.05$; 30 μM, $P < 0.01$, $n = 5$). 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.

3.6. Effect of midazolam on free radical formations in platelets

In this study, a typical ESR signal of the hydroxyl radical was markedly increased in activated platelets compared with the resting platelets (Fig. 4A and B). Midazolam (30 μM) did not significantly attenuate the hydroxyl radical formation in human platelets.

3.7. Effect of midazolam on thrombus formation in the microvessels of fluorescein sodium-pretreated mice

When fluorescein sodium was given at 15 μg/kg, the occlusion time required was approximately 181 ± 17 s (Fig. 5). When midazolam was administered at 5 μg/g, the occlusion time was not significantly changed until it was

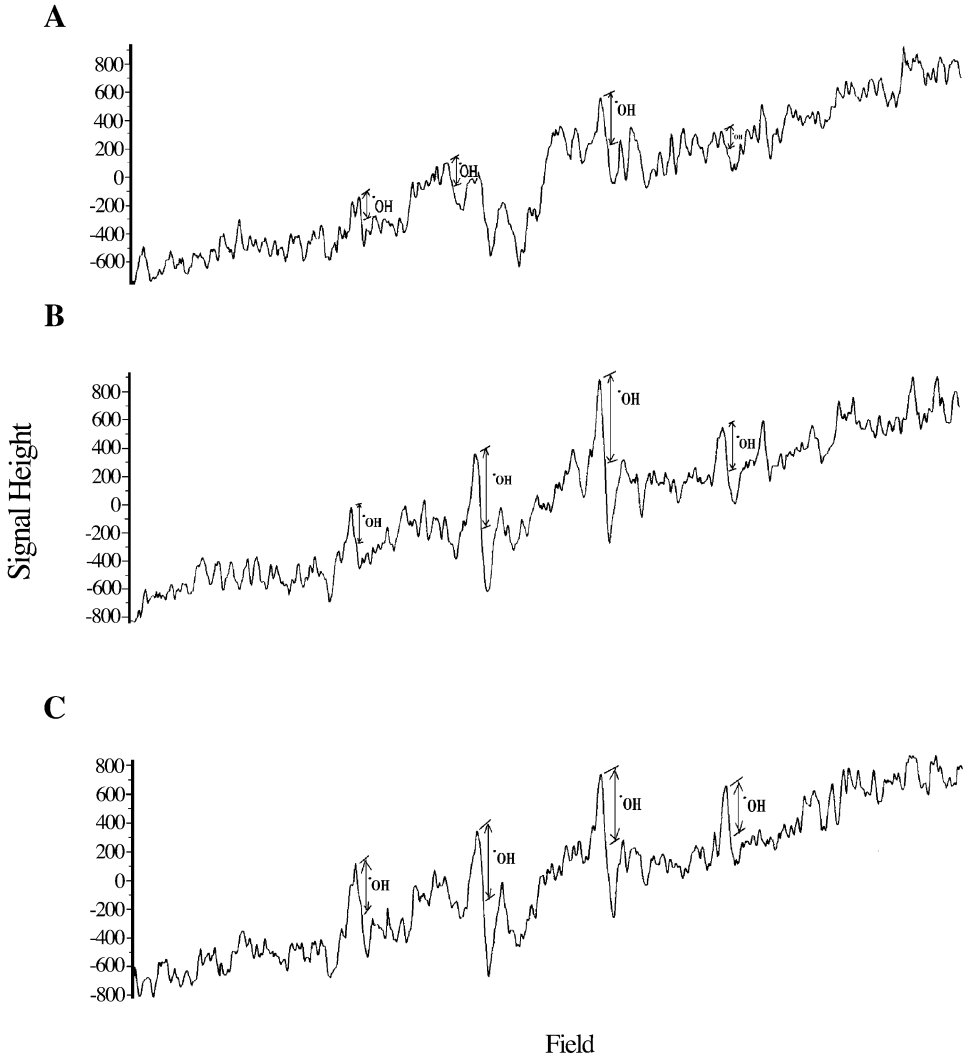

Fig. 4. Effect of midazolam in hydroxyl radical formation in thrombin-activated platelets. Platelet suspensions (0.4 ml) were preincubated with Tyrode's solution or midazolam (30 μM) for 3 min, and then thrombin (0.05 U/ml) was added to trigger platelet aggregation. The reaction was allowed to proceed for 5 min, followed by the addition of DMPO (100 mM) for electron spin resonance (ESR) experiments. (A) Resting platelets (control); (B) platelets with thrombin (0.05 U/ml); (C) platelets with midazolam (30 μM) followed by the addition of thrombin (0.05 U/ml). The spectrum is a representative example of five similar experiments.

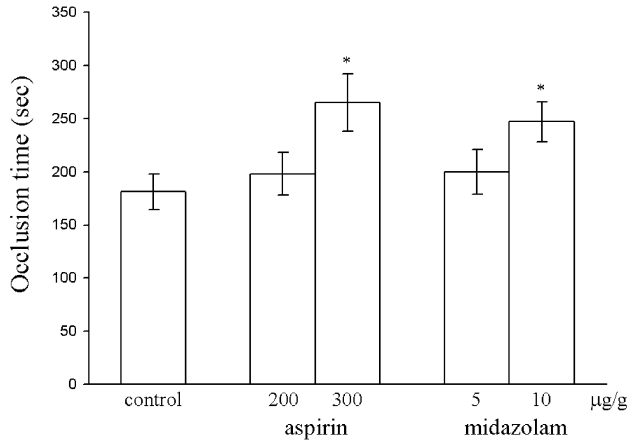

Fig. 5. Effect of midazolam on occlusion time for induction of thrombus formation upon light irradiation of mesenteric venules of mice pretreated with fluorescein sodium (15 μg/kg). Data are presented as the occlusion time (s) of platelet plug formation (means ± S.E.M.; $n=10$). *$P<0.05$ as compared with the control group (normal saline).

administered at 10 μg/g in mice (Fig. 5). Aspirin also exhibited a similar activity in this experiment. When aspirin was administered at 200 μg/g, the occlusion time was not significantly changed until 300 μg/g was administered in fluorescein sodium (15 μg/kg)-inducing platelet plug formation (Fig. 5).

4. Discussion

The principal objective of this study was to further ascertain the inhibitory activity of midazolam on platelet aggregation in vitro and antithrombotic activity in vivo. Midazolam is a familiar agent commonly used to produce sedation, and it is also effective in the treatment of generalized seizures, status epilepticus, and behavioral emergencies (Claassen et al., 2002). This inhibitory effect of midazolam was demonstrable with the use of various agonists: collagen, thrombin, and arachidonic acid (Sheu et al., 2002). Ramoska et al. (1991) demonstrated adequate sedation with effective intravenous doses of 1–3 mg (per adult) midazolam. Harper et al. (1985) reported that intravenous doses of 0.3 mg/kg may be required when midazolam is employed for sedation in surgical patients. In this study, midazolam was employed at concentrations which inhibited platelet aggregation induced by agonists at concentrations of about 15–30 μM. These results indicate that the concentrations of midazolam employed to inhibit platelet aggregation in vitro are possible and reasonable to those of blood concentrations obtained during midazolam-induced sedation in vivo.

Stimulation of platelets by agonists (i.e., thrombin) 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 (Kirk et al., 1981). There is strong evidence that inositol 1,4,5-trisphosphate induces the release of Ca^{2+} from intracellular stores (Kirk et al., 1981). Diacylglycerol activates protein kinase C, inducing protein phosphorylation and a release reaction (Kirk et al., 1981). In this study, phosphoinositide breakdown and phosphorylation of the 47-kD protein in thrombin-activated platelets was inhibited by midazolam, suggesting that inhibition of platelet aggregation by midazolam is related to inhibition of phospholipase C activation. It has been reported that increased NO/cyclic GMP formation negatively affects agonist-induced protein kinase C activation (Murohara et al., 1995). However, midazolam did not significantly increase the level of NO or cyclic GMP in this study (Table 1).

The importance of cyclic AMP in modulating platelet reactivity is well established (Karniguian et al., 1982). Elevated levels of cyclic AMP decreased $[Ca^{+2}]_i$ with uptake of Ca^{+2} into the dense tubular system, resulting in inhibition of most platelet responses (Zavoico and Feinstein, 1984). In this study, midazolam significantly increased the cyclic AMP level in washed platelets (Table 1). Therefore, cyclic AMP plays an important role in the mediation of the inhibitory effect of midazolam in platelet aggregation. On the other hand, activation of platelets by a variety of agonists (i.e., thrombin and ADP) is associated with stimulation of the Na^+/H^+ exchanger (Kimura et al., 1992). This mode of activation of the Na^+/H^+ exchanger usually induces a rise in cytosolic Ca^{+2}, granule secretion, stimulation of shape change, and aggregation (Kimura et al., 1992). Basal pHi is normally maintained within a narrow range, and even small changes in pHi may have significant effects on platelet activity. In many cell types, including fibroblasts, hepatocytes, and smooth muscle cells, Na^+/H^+ exchange activity is regulated by $[Ca^{+2}]_i$ (Nieuwland et al., 1994). Furthermore, Kimura et al. (1992) reported that cyclic AMP modulates Na^+/H^+ exchange in human platelets. Inhibition of Na^+/H^+ exchange by cyclic AMP has also been demonstrated in other cells (Felder et al., 1990). Thus, an agent (i.e., dopamine) known to stimulate adenylate cyclase in these cells can also inhibit the Na^+/H^+ exchanger (Felder et al., 1990). Relationships among phosphoinositide breakdown, $[Ca^{+2}]_i$, cyclic nucleotides, and the Na^+/H^+ exchanger may play an important role in mediating the antiplatelet activity of midazolam.

Reactive oxygen species (i.e., hydrogen peroxide and hydroxyl radicals) derived from platelets might affect cells with which they come into intimate contact, such as endothelium, and this could result in an amplification of platelet reactivity during thrombus formation. Therefore, the presence of free radical scavengers at the site of a developing thrombus can alter the pattern of the thrombotic lesion in response to endothelial cell injury. In this study, we found that midazolam (30 μM) did not effectively attenuate hydroxyl radical formation in activated platelets (Fig. 4). It is known that hydrogen peroxide exerts several biological effects via the Fenton reaction with metal catalysis to produce the very reactive hydroxyl

radical (Salvemini and Botting, 1993). Thus, the mechanisms of antithrombotic activity of midazolam may not be involved in the inhibition of free radical formation in activated platelets. In addition, electron microscopy has shown that the thrombi formed after irradiation injury are mainly composed of activated platelets, as are those induced by laser irradiation (Sheu et al., 1994). We have demonstrated that endothelial cell injury induced platelet aggregation and adhesion to the vessel wall in vivo (Sheu et al., 1999). Midazolam (10 μg/g) caused significant prolongation of the occlusion time in mice, probably mainly through its antiplatelet effects.

In conclusion, the inhibitory effect of midazolam in platelet aggregation may involve in the following mechanisms: (1) midazolam may inhibit the activation of phospholipase C, followed by inhibition of intracellular Ca^{+2} mobilization and phosphorylation of P47; (2) midazolam triggers the formation of cyclic AMP which subsequently inhibits the Na^+/H^+ exchanger. This leads to reduce intracellular alkalinization and intracellular Ca^{+2} mobilization and ultimately inhibition of platelet aggregation. Midazolam significantly attenuates the thrombus formation in vivo; however, the physiological relevance of a direct anti-aggregatory effect of midazolam still remains to be further studied.

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