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Objective—Inosine is a naturally occurring nucleoside degraded from adenosine. Recent studies have demonstrated that inosine has potent immunomodulatory and neuroprotective effects. In the present study, we further investigated the inhibitory effects of inosine on platelet activation in vitro and in vivo, as well as in attenuating middle cerebral artery occlusion (MCAO)-induced focal cerebral ischemia in rats.

Methods and Results—Inosine concentration-dependently (0.5 to 6.0 mmol/L) inhibited platelet aggregation stimulated by agonists. Inosine (1.5 and 3.0 mmol/L) inhibited phosphoinositide breakdown, $[Ca^{+2}]i$, and TxA_2 formation in human platelets stimulated by collagen (1 μg/mL). In addition, inosine (1.5 and 3.0 mmol/L) markedly increased levels of cyclic guanylate monophosphate (GMP) and cyclic GMP-induced vasodilator-stimulated phosphoprotein Ser^{157} phosphorylation. Rapid phosphorylation of a platelet protein of molecular weight 47 000 (P47), a marker of protein kinase C activation, was triggered by collagen (1 μg/mL). This phosphorylation was markedly inhibited by inosine (3.0 mmol/L). Inosine (1.5 and 3.0 mmol/L) markedly reduced hydroxyl radical in collagen (1 μg/mL)-activated platelets. In in vivo studies, inosine (400 mg/kg) significantly prolonged the latency period of inducing platelet plug formation in mesenteric venules of mice, and administration of 2 doses (100 mg/kg) or a single dose (150 mg/kg) of inosine significantly attenuated MCAO-induced focal cerebral ischemia in rats.

Conclusions—Platelet aggregation contributes significantly to MCAO-induced focal cerebral ischemia. The most important findings of this study suggest that inosine markedly inhibited platelet activation in vitro and in vivo, as well as cerebral ischemia. Thus, inosine treatment may represent a novel approach to lowering the risk of or improving function in thromboembolic-related disorders and ischemia-reperfusion brain injury. (*Arterioscler Thromb Vasc Biol*. 2005;25:1998-2004.)

Key Words: inosine ■ middle cerebral artery occlusion ■ platelet activation ■ protein kinase C ■ vasodilator-stimulated phosphoprotein

Ischemic hypoxic brain injury often causes irreversible brain damage. The cascade of events leading to neuronal injury and death in ischemia includes the release of cytokines, free radicals, and platelet activation.[1,2] The participation of activated platelets has been observed in brain microvessels of the ischemic microvascular bed after experimental middle cerebral artery occlusion (MCAO).[2] Microvascular thrombi continue to accumulate even after recanalization of the MCAO, contributing to postischemic hypoperfusion and ongoing neuronal damage.[3] Thus, platelet aggregation may play a crucial role in MCAO-induced cerebral damage. Furthermore, reactive oxygen species including the superoxide anion (O_2^-), hydroxyl radical, and peroxynitrite radical have been implicated in neuronal cell damage and death after cerebral ischemia.[4] Therefore, inhibition of production and enhanced degradation of reactive oxygen species with pharmacological agents have been found to limit the extent of brain damage after stroke-like events.[5]

Inosine is a naturally occurring nucleoside degraded from adenosine. Both adenosine and inosine participate in a wide variety of intracellular biochemical processes and serve as monomeric precursors of RNA and DNA. Adenosine is considered to be a potential immunomodulatory and neuroprotective agent.[6] Inosine was originally thought to have no biological effects. However, recent studies have demonstrated that inosine has potent immunomodulatory and neuroprotective effects by enhancing mast cell degranulation, attenuating cytokine production, and being protective in animal studies of sepsis.[6] Chen et al[7] demonstrated that inosine stimulates significant axonal reorganization after a stroke and leads to improved performance on several sensorimotor tasks. Moreover, inosine is released in large quantities from the ischemic heart and that from the heart has been found to constitute the major fraction of the accumulated inosine.[8] Inosine was also demonstrated to be a useful adjunctive agent in the treatment of acute myocardial ische-

mia.[8] Several investigators have found evidence of increased flow in coronary arteries during inosine administration[8] and a direct vasodilator effect on isolated coronary arteries.[9]

Although platelet activation is relevant in a variety of cardiovascular and cerebrovascular disorders, however, the biological function of inosine in platelets has not yet been studied, and no data are available concerning the detailed effects of inosine on platelet activation. We therefore systematically examined the influence of inosine in washed human platelets and on platelet plug formation in vivo. We also investigated the cerebroprotective effect of inosine after MCAO/reperfusion in rats. We used these findings to characterize the relationship between the antiplatelet activities of and cerebroprotection afforded by inosine.

Materials and Methods

Inosine, collagen (type I, bovine Achilles tendon), U46619, luciferin-luciferase, fluorescein sodium, Dowex-1, PGE$_1$, arachidonic acid (AA), phorbol-12,13-dibutyrate (PDBu), and bovine serum albumin were purchased from Sigma Chem. (St. Louis, Mo). Fura 2-AM was purchased from Molecular Probe (Eugene, Ore). Myo-2-[^{3}H] inositol was purchased from Amersham (Buckinghamshire, UK). TxB$_2$, cyclic AMP (cAMP), and cyclic guanylate monophosphate (cGMP) enzyme immunoassay kits were purchased from Cayman (Ann Arbor, Mich).

Platelet Aggregation

Human platelet suspensions were prepared as previously described.[10] In this study, human volunteers gave informed consent. In brief, blood was collected from healthy human volunteers who had taken no medicine during the preceding 2 weeks and was mixed with acid/citrate/glucose. After centrifugation, the supernatant (platelet-rich plasma [PRP]) was supplemented with prostaglandin E$_1$ (PGE$_1$) (0.5 μmol/L) and heparin (6.4 IU/mL) and centrifuged for 10 minutes. The washed platelets were finally suspended in Tyrode's solution containing bovine serum albumin (3.5 mg/mL) and adjusted to $\approx$4.5$\times$10^8 platelets/mL. The final concentration of Ca^{2+} in Tyrode's solution was 1 mmol/L.

The turbidimetric method was applied to measure platelet aggregation[10] using a Lumi-Aggregometer (Payton, Canada). Platelet suspensions (0.4 mL) were prewarmed to 37°C for 2 minutes, and inosine was added 3 minutes before the addition of agonists. The reaction was allowed to proceed for at least 6 minutes, 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 minute before the addition of agonists, and ATP release was compared with that of the control.

Measurement of the Production of [^{3}H]-Inositol Phosphates

The method was performed as previously described.[10] 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 followed by centrifugation and were finally resuspended in Ca^{+2}-free Tyrode's solution (5$\times$10^8/mL). Inosine (1.5 and 3.0 mmol/L) was preincubated with 1 mL of loaded platelets for 3 minutes, and collagen (1 μg/mL) was then added to trigger aggregation. The reaction was stopped, and samples were centrifuged for 4 minutes. The inositol phosphates of the supernatants were separated in a Dowex-1 column. Only [^{3}H]-inositol monophosphate (IP) was measured as an index of total inositol phosphate formation.

Measurement of Platelet [Ca^{+2}]i by Fura 2-AM Fluorescence

Citrated whole blood was centrifuged at 120g for 10 minutes. The supernatant was incubated with Fura 2-AM (5 μmol/L) for 1 hour. Human platelets were then prepared as described. Finally, the external Ca^{+2} concentration of the platelet suspensions was adjusted to 1 mmol/L. The [Ca^{2+}]i increase 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.[11]

Measurement of TxB$_2$, cAMP, and cGMP Formation

Platelet suspensions (4.5$\times$10^8/mL) were preincubated for 3 minutes in the presence or absence of inosine (1.5 and 3.0 mmol/L) before the addition of collagen (1 μg/mL). Six minutes after the addition of agonists, 2 mmol/L EDTA and 50 μmol/L indomethacin were added to the reaction suspensions. The vials were then centrifuged, and TxB$_2$ levels of the supernatants were measured using an EIA kit. In addition, platelet suspensions were incubated with nitroglycerin (10 μmol/L), PGE$_1$ (10 μmol/L), and inosine (1.5 and 3.0 mmol/L) in the presence of IBMX (100 μmol/L) for 6 minutes. The incubation was stopped, and the solution was immediately boiled for 5 minutes. Fifty microliters of supernatant was used to determine the cAMP and cGMP contents with EIA kits.

Measurement of PKC Activity

Washed platelets (2$\times$10^9/mL) were incubated for 60 minutes with phosphorus-32 (0.5 mCi/mL). The [^{32}P]-labeled platelets were preincubated with inosine (1.5 and 3.0 mmol/L) for 3 minutes, and collagen (1 μg/mL) or PDBu (0.15 μmol/L) was then added to trigger PKC activation. Activation was terminated and analyzed by electrophoresis (12.5%; wt/vol) as described previously.[12] The relative intensities of the radioactive bands were analyzed using a Bio-imaging analyzer system (FAL2000; Fuji, Tokyo, Japan).

Western Blot Analysis of Vasodilator-Stimulated Phosphoprotein Phosphorylation

The method of Li et al[13] was followed. In brief, platelet lysates were analyzed by SDS-PAGE gel (10%) and electrotransfered to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat dry milk in Tris (tris(hydroxymethyl)-aminomethane)-buffered saline (pH 7.5), incubated with the monoclonal antibody 5C6 (CALBIOCHEM), specific for the phosphorylated Ser[157] site of vasodilator-stimulated phosphoprotein (VASP) (0.1 μg/mL). After 3 washes in Tris-buffered saline containing 0.05% Tween 20, the membrane was incubated with peroxidase-conjugated goat anti-mouse IgG (Amersham) for 2 hours. The band with peroxidase activity was detected by enhanced chemiluminescence detection reagents (ECL$^+$ system; Amersham). Densitometric analysis of specific bands was performed with a Photo-Print Digital Imaging System (IP-008-SD) with analytical software (Bio-1Dlight, V 2000).

Measurement of Free Radicals by Electron Spin Resonance Spectrometry

The electron spin resonance (ESR) method used a Bruker EMX ESR spectrometer as described previously.[14] In brief, platelet suspensions (4.5$\times$10^8/mL) were prewarmed to 37°C for 2 minutes, and then catalase (300 U/mL), inosine (3.0 mmol/L), or an isovolumetric vehicle solution (Tyrode's solution) was added for 3 minutes before the addition of collagen (1 μg/mL). The reaction was allowed to proceed for 5 minutes, followed by the addition of 100 μmol/L DEPMPO for the ESR study.

Fluorescein Sodium-Induced Platelet Thrombi in Mesenteric Microvessels of Mice

As we previously described,[15] mice were anesthetized and an external jugular vein was cannulated with PE-10 for administration of the dye and drug (by an intravenous bolus). A segment of the small intestine was placed onto a transparent culture dish for microscopic observation. Venules (30 to 40 μm) were selected for irradiation to produce a microthrombus. Filtered light for which wavelengths <520 nm had been eliminated was used to irradiate a

microvessel. Various doses of inosine (100, 200, and 400 mg/kg) or the isovolumetric solvent control (Tyrode's solution) were administered 1 minute after fluorescein sodium (5 mg/kg) addition. The time lapse for inducing thrombus formation leading to cessation of blood flow was measured.

MCAO-Induced Focal Cerebral Ischemia

Male Wistar rats (250 to 300 grams; College of Medicine, National Taiwan University) were used in this study. All animal experiments and care were performed according to the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996). Before undergoing the experimental procedures, all animals were clinically normal and free of apparent infection or inflammation and showed no neurological deficits.

Animals were anesthetized with a mixture of 95% O_2 and 5% CO_2 gases containing 3% isoflurane. The rectal temperature was maintained at $37 \pm 0.5°C$. The right MCA was occluded as described by Longa et al[16] Briefly, the right common carotid artery was exposed, and a 4-0 monofilament nylon thread coated with silicon was then inserted from the external into the internal carotid artery until the tip occluded the origin of the MCA. After closure of the operative sites, the animals were allowed to awake from the anesthesia. During another brief period of anesthesia, the filament was gently removed after 1 hour of MCAO. An observer blinded to the identity of the groups assessed neurological deficits at 1 and 24 hours after reperfusion (before euthanization) by the forelimb akinesia (also called postural tail-hang) test, whereas the spontaneous rotational test was used as a criterion for evaluating the ischemic insult.[17] Animals not showing behavioral deficits at these time points after reperfusion were excluded from the study.

Rats were euthanized by decapitation after 24 hours of reperfusion. The brains were cut into 2-mm coronal slices. Each stained brain (2% 2,3,5-triphenyl tetrazolium chloride) slice was drawn using a computerized image analyzer (Image-Pro plus). The calculated infarction areas were then compiled to obtain the infarct volumes (mm^3) per brain. Infarct volumes were expressed as a percentage of the contralateral hemisphere volume by using the formula: (the area of the intact contralateral [left] hemisphere − the area of the intact region of the ipsilateral [right] hemisphere) to compensate for edema formation in the ipsilateral hemisphere.[18]

All animals were divided into 4 groups: (1) a sham-operated group; (2) a solvent control (Tyrode's solution); and (3) groups treated with (a) 2 doses (50 or 100 mg/kg, intraperitoneal) or (b) a single dose (100 or 150 mg/kg, intravenous) of inosine. In the group treated with 2 doses of inosine, rats were given inosine (50 or 100 mg/kg) 15 minutes before MCAO and 15 minutes before reperfusion. In the group treated with a single dose of inosine, rats were given inosine (100 or 150 mg/kg) 15 minutes before MCAO. Rats in the solvent control were administrated isovolumetric Tyrode's solution instead of inosine at the same time points (15 minutes before MCAO).

Data Analysis

The experimental results are expressed as the means $\pm$ SEM and are accompanied by the number of observations. Paired Student t test and Student unpaired t test were used to determine significant differences in the studies of platelet plug formation and MCAO-induced cerebral ischemia, respectively. The other experiments were assessed by the method of analysis of variance. If this analysis indicated significant differences among the group means, then each group was compared using the Newman-Keuls method. $P < 0.05$ was considered statistically significant.

Results

Effects of Inosine on Platelet Aggregation

Inosine (0.5 to 6.0 mmol/L) concentration-dependently inhibited platelet aggregation and ATP release stimulated by collagen (1 μg/mL) (Figure 1A) and AA (60 μmol/L) but not by U46619 (1 μmol/L), a prostaglandin (PG) endoperoxide
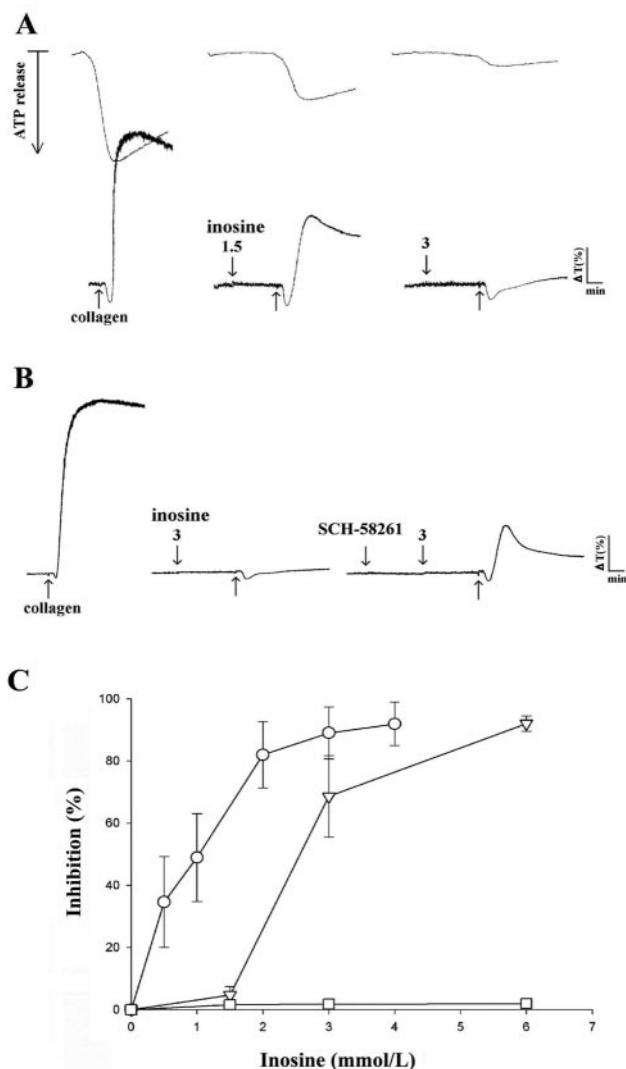

Figure 1. Tracing curves (A, B) and concentration-inhibition curves (C) of inosine on collagen-induced (1 μg/mL, ○), AA (60 μmol/L, ▽)-induced, and U46619 (1 μmol/L, □)-induced platelet aggregation in washed human platelets. Platelets were incubated with (A, C) inosine (0.5 to 6.0 mmol/L) or (B) preincubated with SCH-58261 (10 μmol/L) for 3 minutes, followed by the addition of inosine (3 mmol/L); agonists were then added to trigger aggregation (lower tracings) and ATP release (upper tracings) (A). Data are presented as a percentage of the control (means $\pm$ SEM; n=4).

analogue, in washed human platelets (Figure 1C) and PRP (data not shown). It similarly inhibited ADP (20 μmol/L)-induced platelet aggregation in the PRP. The IC_{50} values of inosine for platelet aggregation induced by collagen and AA were $\approx$1.7 and 3.0 mmol/L, respectively. Furthermore, SCH-58261, an adenosine A_{2A} receptor antagonist,[19] partially reversed the inhibitory effect of inosine (3 mmol/L) in collagen-induced platelet aggregation (Figure 1B). In subsequent experiments, we used collagen as an agonist to explore the inhibitory mechanisms of inosine in platelet aggregation.

Effects of Inosine on [Ca^{+2}]i, Phosphoinositide Breakdown, and PKC Activation

As shown in Figure 2A, collagen (1 μg/mL) evoked a marked increase in [Ca^{+2}]i, and this increase was markedly inhibited
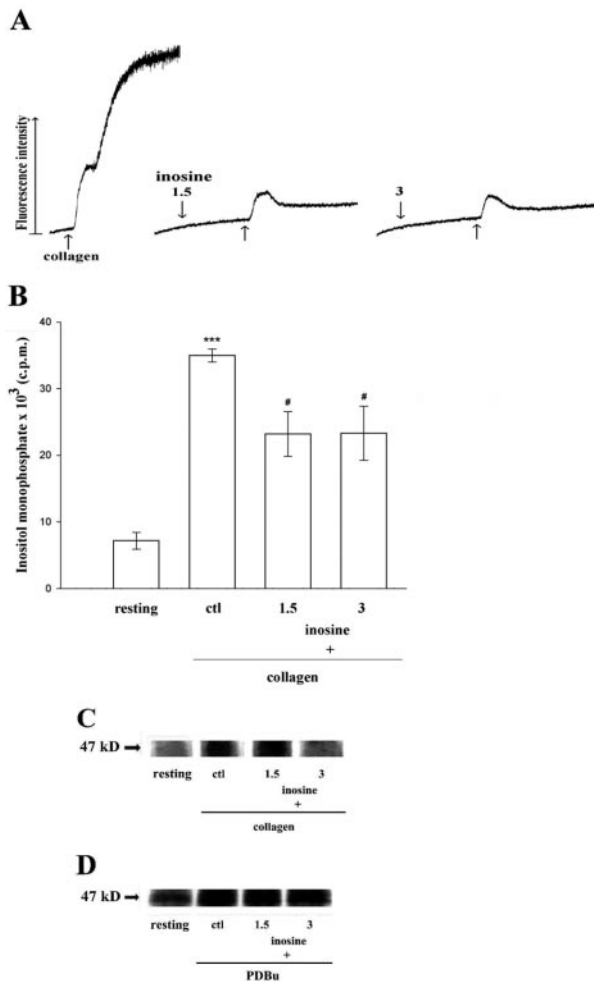
Figure 2. Effects of inosine on (A) collagen-induced $[Ca^{+2}]i$, (B) collagen-induced IP formation, and (C) collagen-induced and (D) PDBu-induced PKC activation in human platelets. Labeled platelets were preincubated with inosine (1.5 and 3.0 mmol/L) or isovolumetric solvent control (Tyrode's solution; ctl), followed by the addition of collagen (1 μg/mL) or PDBu (0.15 μmol/L) to trigger platelet activation as described in "Materials and Methods." Profiles (A, C, and D) are representative examples of four similar experiments; (B) data are presented as the means$\pm$SEM (n=4). ***$P<0.001$ as compared with the resting group; #$P<0.05$ as compared with the collagen group.

in the presence of inosine (1.5 mmol/L, 69.9$\pm$3.4%; 3.0 mmol/L, 70.4$\pm$2.3%). Phosphoinositide breakdown occurs in platelets activated by many different agonists. In this study, collagen (1 μg/mL) induced the rapid formation of radioactive IP, IP_2, and inositol triphosphate (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 2B, the addition of collagen (1 μg/mL) resulted in an increase of IP formation of $\approx$ 4.8-fold compared with that in resting platelets ([7.2$\pm$1.3 versus 35.0$\pm$1.0] $\times10^3$ cpm). In the presence of inosine (1.5 and 3.0 mmol/L), the radioactivity of IP formation in collagen-stimulated platelets was markedly inhibited (Figure 2B).

Furthermore, stimulation of platelets with a number of different agonists, PDBu in particular, induces activation of PKC, which then phosphorylates proteins of molecular

weight 40 000$\approx$47 000. In this study, phosphorylation experiments were performed to examine the role of inosine in PKC activation in human platelets. When collagen (1 μg/mL) or PDBu (0.15 μmol/L) was added to human platelets prelabeled with $^{32}PO_4$, a protein with an apparent molecular weight of 47 000 (P47) was predominately phosphorylated as compared with resting platelets (Figure 2C and 2D). Inosine (3.0 mmol/L) markedly inhibited the phosphorylation of P47 stimulated by collagen (Figure 2C) but not by PDBu in human platelets (Figure 2D).

Effects of Inosine on cAMP, Cyclic GMP, and TxB_2 Formation, and VASP Phosphorylation

As shown in Figure 3A and 3B, levels of cAMP and cyclic GMP in resting platelets were relatively lower compared with those of PGE_1 (10 μmol/L)-treated and nitroglycerin (10 μmol/L)-treated platelets, respectively. Addition of inosine (1.5 and 3.0 mmol/L) significantly increased the level of cyclic GMP but not cAMP (Figure 3A and 3B). The inosine (3.0 mmol/L)-induced cyclic GMP formation was markedly inhibited in the presence of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-1one (ODQ) (20 μmol/L), a guanylate cyclase inhibitor (Figure 3B).[20] Moreover, resting platelets produced relatively little TxB_2 compared with collagen-activated platelets. Inosine (1.5 and 3.0 mmol/L) markedly inhibited TxB_2 formation in platelets stimulated by collagen (1 μg/mL) (Figure 3C).

Furthermore, it is presumed that cyclic GMP can induce VASP Ser[157] phosphorylation in human platelets.[13] In this study, nitroglycerin (10 μmol/L) markedly induced VASP Ser[157] phosphorylation, and this phosphorylation was inhibited by ODQ (20 μmol/L) (Figure 3C). Inosine (1.5 and 3.0 mmol/L) concentration-dependently triggered VASP Ser[157] phosphorylation, and this phosphorylation was also inhibited in the presence of ODQ (20 μmol/L) (Figure 3C).

Free Radical-Scavenging Activity of Inosine in Collagen-Activated Platelets

A typical ESR signal of the hydroxyl radical in activated platelets was observed, as shown in Figure 4B. Catalase (300 U/mL) and inosine (3.0 mmol/L) markedly suppressed hydroxyl radical formation in collagen-activated platelets, respectively (Figure 4C and 4D). This observation may provide in vitro evidence of the usefulness of inosine for its free radical-scavenging activity in activated platelets.

Effect of Inosine on Thrombus Formation in Microvessels of Fluorescein Sodium-Pretreated Mice

Inosine was administered at 100 and 200 mg/kg in mice pretreated with fluorescein sodium (5 mg/kg), and the occlusion time was not significantly prolonged. But with 400 mg/kg of inosine, the occlusion time was markedly prolonged as compared with the solvent control (Figure 5).

Effect of Inosine in MCAO-Induced Focal Cerebral Ischemia

All animals in this study showed similar physiological values for rectal temperature, mean arterial blood pressure, plasma
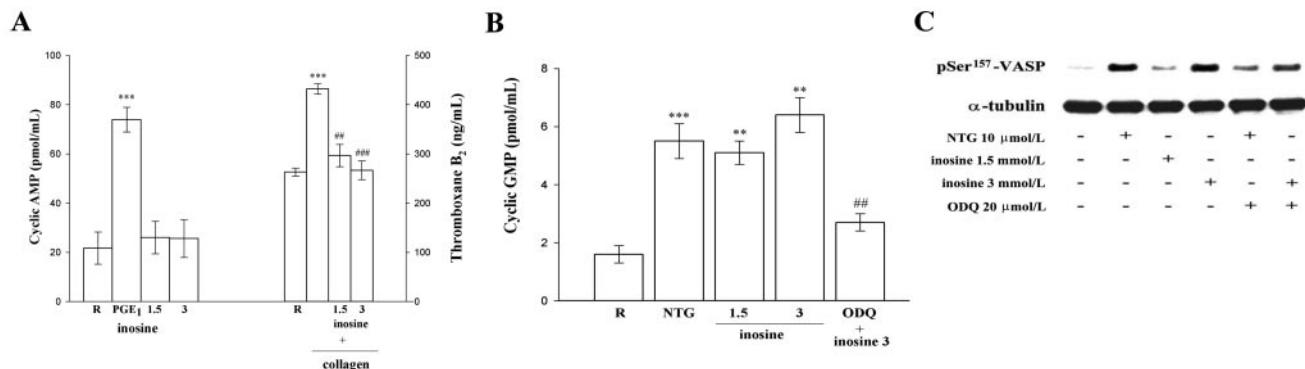
Figure 3. Effects of inosine on (A) cAMP and TxB$_2$ formation, (B) cyclic GMP formation, and (C) agonist-induced phosphorylation of VASP at Ser[157] in human platelets. A and B, Platelets were incubated with prostaglandin E$_1$ (PGE$_1$) (10 μmol/L), nitroglycerin (NTG) (10 μmol/L), and inosine (1.5 and 3.0 mmol/L) in the absence or presence of ODQ (20 μmol/L) for measurement of cAMP, cyclic GMP, and TxB$_2$ formations as described in "Materials and Methods." The addition of PGE$_1$ and nitroglycerin to platelets served as a positive control of cAMP and cyclic GMP, respectively. C, In addition, platelets were incubated with nitroglycerin (10 μmol/L) and inosine (1.5 and 3.0 mmol/L) in the absence or presence of ODQ (20 μmol/L) and solubilized directly in SDS-PAGE sample buffer. Phosphorylation of VASP at Ser[157] was detected by immunoblotting with a monoclonal antibody (mAb) specifically recognizing Ser[157]-phosphorylated VASP. A, Data are presented as the means±SEM (n=4); **$P<0.01$ and ***$P<0.001$ as compared with the resting groups (R); ##$P<0.01$ and ###$P<0.001$ as compared with the collagen group. B, Data are presented as the means±SEM (n=4 to 7); **$P<0.01$ and ***$P<0.001$ as compared with the resting groups (R); ##$P<0.01$ as compared with the inosine (3.0 mmol/L) group. C, Profiles are representative examples of four similar experiments.

glucose, and hematocrit (%) before, during, and after MACO among groups (data not shown). Figure 6A shows coronal sections of the solvent control from an ischemic brain (lane 1, white area) and coronal sections pretreated with a single dose (lane 2, 100 mg/kg; lane 3, 150 mg/kg) of inosine before the ischemic insult. Administration of 2 doses of inosine, at a dosage of 100 mg/kg (infarct volume, 18.9%, n=8), or a single dose of 150 mg/kg (infarct volume, 20%, n=9), showed significant reductions in infarct size compared with the solvent control (33.8%, n=18) ($P<0.01$) (Figure 6B).
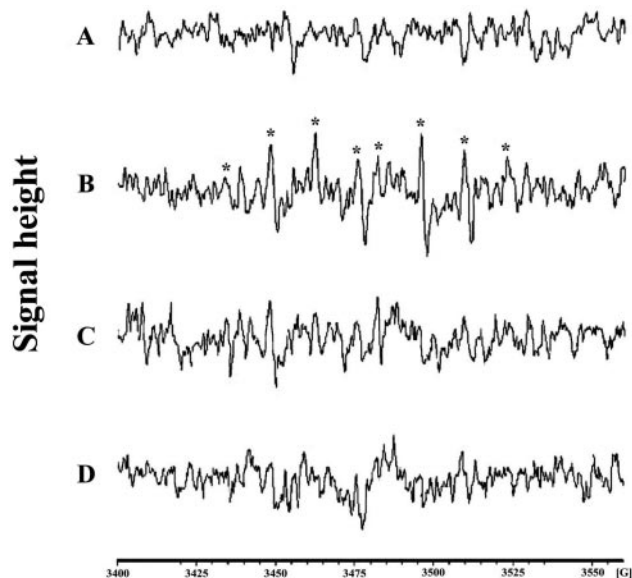

Figure 4. ESR spectra of inosine in the inhibition of hydroxyl radical formation in collagen-activated platelets. Platelet suspensions were preincubated with (A) Tyrode's solution alone or (B) Tyrode's solution, (C) catalase (300 U/mL), and (D) inosine (3.0 mmol/L), followed by the addition of collagen (1 μg/mL) for ESR experiments. Spectra are representative examples of 5 similar experiments.

Discussion

This study demonstrates for the first time that inosine possesses antiplatelet activity in in vitro and in vivo studies. This inhibitory effect of inosine was demonstrable with the use of various agonists: collagen, AA, and ADP. The inhibition was directly proportional to the pharmacological (exogenous) concentrations of inosine used. It was found that in the canine heart within 10 minutes of ischemia, the myocardial content of inosine increased from less than 1 nmol/g to 700≈900 nmol/g of wet tissue (≈0.875 to 1.125 mmol/L).[21,22] During prolonged cardiac arrest, myocardial concentrations of inosine were reported to be even higher at 1 mmol/L after 5 minutes and 2.5 mmol/L after 35 minutes.[23] At present, it is unclear whether this endogenously produced inosine is sufficient to exert physiological effects.
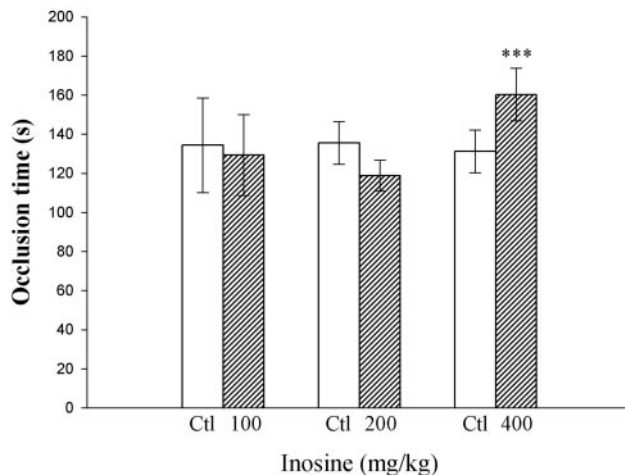

Figure 5. Effect of inosine on occlusion times for inducing thrombus formation on light irradiation of mesenteric venules of mice pretreated with fluorescein sodium (5 mg/kg). Data are presented as the means±SEM of occlusion time(s) of platelet plug formation (n=7). ***$P<0.001$ as compared with the individual control group (Tyrode's solution).
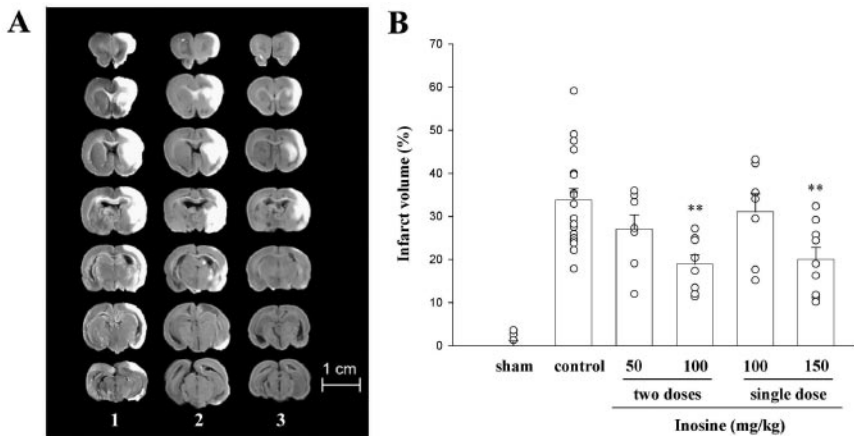
Figure 6. Effect of inosine in MCAO-induced focal cerebral ischemia in rats. A, Coronal sections of 2,3,5-triphenyl tetrazolium chloride-stained brains after MACO are from representative animals that received intra-peritoneal solvent control (lane 1, Tyrode's solution) and inosine (lane 2, single dose, 100 mg/kg; lane 3, single dose, 150 mg/kg). B, Dose-response curves of inosine in MCAO-induced focal cerebral ischemia. Rats received a solvent control (Tyrode's solution, n=18), 2 doses (50 mg/kg, n=7; 100 mg/kg, n=8) or single dose of inosine (100 mg/kg, n=7; 150 mg/kg, n=9) as described in "Materials and Methods." Infarct volumes are expressed as a percentage of the contralateral hemisphere, and data are presented as a superimposed scatterplot showing the infarct volume for each animal in the group as well as the means±SEM. **$P<0.01$ as compared with the control group (Tyrode's solution).

Exogenous administration of larger doses of inosine are reported to prevent ischemia-reperfusion injury in several tissues (including the heart and brain).[6] However, inosine is safe and appears to be nontoxic to humans, even when ingested at doses as high as 10 g/kg per day (>100 mmol/L per day).[6] In fact, inosine is widely available as a nutritional supplement in health food stores.[24]

In this study, platelet aggregation induced by agonists (ie, collagen) appeared to be affected in the presence of inosine. This partly infers that inosine may also affect Ca^{+2} release from intracellular Ca^{+2}-storage sites (Figure 2A). Although the action mechanisms of various platelet agonists, such as collagen, ADP, and AA, differ, inosine significantly inhibited platelet aggregation stimulated by all of them. This implies that inosine may block a common step shared by these inducers. These results also indicate that the site of action of inosine is not at the receptor level of individual agonists. However, adenosine receptor antagonist, SCH-58261, partially reversed the inhibitory effect of inosine (Figure 1B). The most likely explanation of this result is that (1) inosine may bind partially to adenosine receptor on platelet membrane; and (2) adenosine is a contaminant in Sigma's inosine, which is only guaranteed to be 99% pure.

Stimulation of platelets by agonists (ie, collagen) results in phospholipase C-catalyzed hydrolysis of the minor plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, with concomitant formation of IP_3 and diacylglycerol.[25] There is strong evidence that IP_3 induces the release of Ca^{+2} from intracellular stores.[26] Diacylglycerol activates PKC, inducing P47 phosphorylation. In this study, collagen-induced phospho-inositide breakdown and P47 phosphorylation were inhibited by inosine, whereas inosine did not significantly inhibit PDBu-induced P47 phosphorylation (Figure 2D), suggesting that inosine preferred to inhibit phospholipase C activation rather than PKC. Collagen-induced TxB_2 formation, a stable metabolite of TxA_2, was markedly inhibited by inosine. TxA_2 is an important mediator of platelet aggregation. Phosphoinositide breakdown can induce TxA_2 formation via free AA release by diglyceride lipase or by endogenous phospholipase A_2 from membrane phospholipids.[27] Thus, it seems likely that inhibition of TxB_2

formation plays a role in mediating the inhibitory effect of inosine in human platelets.

Increased cyclic GMP can negatively affect agonist-induced phosphoinositide breakdown and $[Ca^{+2}]i$.[28] Inosine increased both cyclic GMP and cyclic GMP-induced VASP Ser^{157} phosphorylation in human platelets. It has been reported that the antiplatelet activity of adenosine is mediated by activation of adenylate cyclase.[29] Thus, different antiplatelet mechanisms of inosine and adenosine may be observed. In fact, different biological functions of both nucleosides have also been addressed.[30]

Platelet aggregation plays a pathophysiological role in cerebrovascular disorders. Inhibition of platelet aggregation by drugs may represent an increased therapeutic possibility for such diseases. We previously demonstrated that endothelial cell injury induces platelet aggregation and adhesion to vessel walls.[31] However, the role of reactive oxygen species (ie, the hydroxyl radical) in platelet physiology is relevant because tissue damage mediated in this manner can contribute to pathological situations in which platelets are involved. These include endothelial damage, reperfusion injury of ischemic myocardium, and thrombus formation.[32] Thus, inosine protects against platelet plug formation, and cerebral infarction may be involved, at least partly, in the inhibition of free radical formation in activated platelets. In the thrombotic study, the mesenteric venules were continuously irradiated by fluorescein sodium throughout the entire experimental period, thus leading to strong damage to endothelial cells as described previously.[15] Therefore, the dosage (400 mg/kg) of inosine used in this model was relatively higher than that (150 mg/kg) in MCAO-induced cerebral ischemia. Results from animal models of MCAO-induced cerebral ischemia have also been observed in many human ischemic stroke patients.[33] Cerebral ischemia restricted to the distribution of the MCAO gives rise to focal metabolic disturbances that result in infarction, neuronal necrosis, and brain edema.[33] Inosine has been demonstrated to be a small-molecule factor that stimulates axonal regrowth in vivo after corticospinal tract injury and stroke.[6] In this study, inosine significantly reduced the cerebral infarct volume after an MCAO. This effect may be mediated, at least partly, by inhibition of platelet activation and stimulation of

axonal regrowth in the central nervous system.[6] However, infiltration of neutrophils into the infarct areas after cerebral ischemia/reperfusion injury plays a crucial role in the development of cerebral infarction.[4,5] The inhibitory effect of inosine on neutrophil activation was also evaluated by formyl-Met-Leu-Phe (fMLP)-induced lucigenin-enhanced chemiluminescence, which is an index of respiratory bursts, by determining the production of superoxide anions.[34] When human neutrophils (2×10^6 cells/mL) were treated with fMLP (800 nmol/L), rapid generation of superoxide anions was observed with the lucigenin-enhanced chemiluminescence signal increasing as high as 2166 ± 295 relative light units per second (RLU/s; n=4; data not shown). Inosine (1.5 and 3.0 μmol/L) markedly inhibited this increase in chemiluminescence stimulated by fMLP to $\approx$35.5% and 46.2% as compared with the solvent control (Tyrode's solution) (n=4; data not shown).

In conclusion, the most important findings of this study suggest that inosine can markedly inhibit agonist-induced platelet aggregation. This inhibitory effect possibly involves the following mechanisms. Inosine may inhibit activation of phospholipase C, followed by inhibition of phosphoinositide breakdown and TxA$_2$ formation, thereby leading to inhibition of PKC activation and [Ca^{+2}]i. In addition, inosine increases cyclic GMP formation, resulting in inhibition of [Ca^{+2}]i, and finally inhibits platelet aggregation. The data presented herein also demonstrate for the first time to our knowledge the protective effect of inosine in in vivo studies of both platelet plug formation and ischemic brain injury. Inosine is a naturally occurring metabolite with few adverse effects. Thus, treatment using it may represent a novel approach for improving function after ischemia-reperfusion brain injury or thromboembolic diseases.

Acknowledgments

References

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