Effect of Aminoguanidine on Ischemia/Reperfusion Injury in Rat Small Intestine

Yusuke TAKIZAWA,* Takuya KITAZATO, Haruka ISHIZAKA, Naomi KAMIYA, Mikio TOMITA, and Masahiro HAYASHI

Department of Drug Absorption and Pharmacokinetics, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences; 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan.

Received June 27, 2011; accepted August 29, 2011; published online August 29, 2011

Ischemia/reperfusion (I/R) injury is induced by reactive oxygen species (ROS). During intestinal I/R, the amount of nitric oxide (NO), which is a ROS, is increased. In this study, we examined the protection against I/R injury by inhibition of NO generation. Wistar/ST rats were exposed to 1 h of ischemia, followed by reperfusion for 4 h. The rats were intravenously injected with 100 mg/kg aminoguanidine (AG), which is a selective inducible NO synthase (iNOS) inhibitor, for 5 min before ischemia. The increase in NO_2^- by intestinal I/R was significantly inhibited by AG 1 h after reperfusion. Moreover, the increase in area under curve of 0 to 1 h after reperfusion (AUC_{0-1}) of paracellular marker was inhibited. However, 3 h after reperfusion, the survival ratio of rats was significantly decreased in the intestinal I/R condition with AG. The amount of NO_2^- and AUC of 3 to 4 h after reperfusion (AUC_{3-4}) of paracellular marker in intestinal I/R groups were increased by AG compared with those in the I/R condition without AG 3 h after reperfusion. These data indicated that AG, which was given by single pre-administration, can clearly inhibit intestinal I/R injury 1 h after reperfusion. However, the injury occurs again 3 h after reperfusion and grows worse.

Key words intestinal ischemia reperfusion; nitric oxide; aminoguanidine; inducible nitric oxide; inflammatory cytokine

Ischemia/reperfusion (I/R) injury of the intestine is an important cause of organ dysfunction that is induced by the restoration of blood flow after diverse events, including external injury shock and intestinal transplantation. In grafting the small intestine, intestinal mucosal barrier dysfunction causes bacterial translocation,¹⁾ often leading to death; therefore, this dysfunction must be overcome by successful small intestinal transplantation. I/R injury occurs in the early phase of reperfusion, but most studies have focused on the relatively late phase.

In intestinal I/R injury, it has been reported that the influence of reactive oxygen species (ROS) is important.²⁾ During intestinal I/R, the expression level of nitric oxide (NO) is also changed, and vermiculation of the mucosal tract is induced by NO. It has been reported in models of I/R injury that NO causes tissue dysfunction, whereas in other reports, NO has been described as beneficial for I/R injury.^{3–5)} Thus, the role of NO in intestinal I/R injury is still controversial. NO is a transient product of inflammatory processes and is generated from L-arginine by NO synthase. This enzyme exists in three isoforms, neuronal (nNOS), endothelial (eNOS), and inducible (iNOS), and both nNOS and eNOS are Ca^{2+} - and calmodulin-dependent constructive isoforms.⁶⁾ iNOS, which is expressed in response to cytokines and growth factors, produces a large amount of NO, which contributes to the pathophysiology of I/R injury. Additionally, mice with iNOS knockout are more resistant to intestinal I/R-induced bacterial translocation and mucosal injury, further supporting the role of iNOS as an important mediator of I/R injury in the intestine.⁷⁾ NO has many beneficial effects in I/R injury: scavenging oxygen free radicals, reducing leukocyte adhesion to the mesenteric endothelium, and maintaining normal vascular permeability^{8–10)}; however, the presence of a large amount of NO as a free radical produced by iNOS has been implicated as a cytotoxic factor in a variety of pathophysiological processes, including various forms of inflammation

and circulatory shock.

We established an *in vivo* intestinal I/R model, and reported the opening of tight junctions (TJ) and dysfunction of P-glycoprotein (P-gp) in the rat ileum.^{11,12)} In our intestinal I/R model, it was clarified that NO, generated *via* iNOS, acts as an injury factor during reperfusion.¹³⁾

Aminoguanidine (AG) is a bifunctional molecule comprising the guanido group from L-arginine linked to hydrazine, and was first described by Corbett *et al.*¹⁴⁾ as a selective inhibitor of iNOS. Misko *et al.*¹⁵⁾ showed that AG selectively inhibits iNOS without increasing blood pressure; AG was 50-fold more effective on inhibiting the enzymatic activity of iNOS than eNOS or nNOS.¹⁶⁾

In the present study, we focused on NO as an injury factor and examined the inhibitory effect of AG, which is a selective iNOS inhibitor, on intestinal I/R injury for a longer term using our established *in vivo* intestinal I/R model.

MATERIALS AND METHODS

Materials Fluorescein isothiocyanate (FITC)-dextran 4000 (FD-4) was purchased from Sigma Aldrich Co., Ltd. (Tokyo, Japan). All other reagents were of analytical grade or better.

Animals and Experimental Design Male Wistar/ST rats (8 weeks old) were purchased from Japan SLC Ltd. (Shizuoka, Japan). All animal experiments were performed according to the guidelines of Tokyo University of Pharmacy and Life Sciences. The animals were fasted for 18 h before starting the experiment. Water was freely available during fasting. We used an *in vivo* intestinal I/R model established using a spring balance and surgical sutures as previously reported.^{11–13)} Briefly, the superior mesenteric artery and vein in rats were occluded by hanging using surgical sutures (Shirakawa, Tokyo, Japan) connected to a spring balance for 60 min (ischemia condition), followed by reperfusion by cut-

* To whom correspondence should be addressed. e-mail: takizawa@toyaku.ac.jp

ting the sutures (reperfusion condition). The hanging force on the blood vessel during ischemia was 50 g (50 g load group) or 100 g (100 g load group). All experiments were performed under anesthesia with Somnopentyl® (pentobarbital sodium, 50 mg/kg), and the body temperature was maintained at 37 °C with a heat lamp. In CTRL, same surgical operation was treated but the ischemia process was deleted (sham operation). Because we had a lot of data about the change in ileum after intestinal I/R, we chose ileum as an experimental region.^{11–13)}

Pretreatment with iNOS Inhibitor The rats were pretreated with a selective iNOS inhibitor, aminoguanidine (AG) (Sigma Aldrich, Tokyo, Japan). The rats were injected (intravenously) with 100 mg/kg^{13,17,18)} AG dissolved in 1 ml of physiological saline (0.9% NaCl) for 5 min before ischemia. Rats that were not administered AG were intravenously injected with 1 ml of physiological saline (0.9% NaCl) 5 min before ischemia.

Determination of Nitric Oxide Level NO production was quantified as the nitrite concentration in phosphate-buffered saline in an ileal loop (10 cm length) and plasma. The nitrite concentration in the samples was measured by the Griess reaction with 2,3-diaminonaphthalene according to the manufacturer's protocol (Dojindo, Kumamoto, Japan). The nitrite concentration was calculated by comparison with standard solutions of sodium nitrite prepared in saline solution.

Determination of Total Protein Level and Phospholipid An *in situ* ileum loop (60 cm length) was isolated in each rat at 60 min after reperfusion and mucosa scraped off was homogenized in buffer containing 0.05 mg/ml phenylmethylsulfonylfluoride, 300 mM mannitol, 12 mM Tris, and 5 mM ethylene glycol bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) (pH 7.1) with a tissue homogenizer. Protein was determined by a method using the Micro bicinchoninic acid assay (BCA) Protein Assay Reagent Kit (Thermo, Rockford, U.S.A.).

The phospholipid released from the ileal mucosa into phosphate-buffered saline in the ileal loop (10 cm length) during 1 h was determined. The phospholipid concentration in the samples was measured using a Phospholipid-C-Test Wako Kit (choline oxidase-*N*-ethyl-*N*-(2-hydroxy-3-sulfo-

propyl)-3,5-dimethoxyaniline (DAOS) method), according to the manufacturer's protocol (Wako, Osaka, Japan). The phospholipid concentration was calculated by comparison with standard solutions of choline chloride and phospholipid.

Absorption Experiments Using the *in Situ* Loop Method Intestinal absorption was evaluated by the methods described previously with slight modification.¹⁹⁾ Anesthesia was induced using Somnopentyl® (pentobarbital sodium, 50 mg/kg) and rats were placed on a heating pad to maintain the body temperature at 37 °C. The abdomen was opened by a midline longitudinal incision and a 10 cm ileal segment was isolated and cannulated at both ends with plastic tubing. The segment was rinsed with phosphate-buffered saline, pH 7.4. Sixty minutes after starting ischemia using surgical sutures connected to a spring balance, 3 ml of Krebs Henselite Bicarbonate Buffer (KHBB, pH 7.4) solution containing FD-4 (15 mg/kg) was added to the loop. Blood was sampled from the portal vein until 1 h after administration of FD-4. The concentration of FD-4 in plasma was measured using a fluorescent spectrophotometer (FP-6500; HITACHI, Tokyo, Japan). The area under curve (AUC) of FD-4 was calculated by the trapezoidal method.

Preparation of RNA and cDNA Synthesis Total RNA was isolated from ileal and liver specimens using TRIzol reagents (Invitrogen Co. Ltd., Paisley, U.K.) according to the manufacturer's instructions. Complementary DNA (cDNA) was prepared from total RNA using ABI Prism 7000 for real-time polymerase chain reaction (PCR) according to the manufacturer's instructions. The two-step reaction mixture contained 2 µg of RNA, 100 ng of random hexamers, 0.5 mM deoxyribonucleotide triphosphate (dNTP) mix (deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), thymidine triphosphate (dTTP)), 10 mM Tris-HCl (pH 8.4), 25 mM KCl, 5 mM MgCl₂, 10 mM dithiothreitol (DTT), and 10 units of RNaseOUT recombinant ribonuclease inhibitor.

Analysis of Gene Expression Level by Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) To perform the real-time PCR, 96-well reaction plates with optical adhesive covers and ABI PRISM 7000 Sequence Detection System were used. Assay-on-Demand Gene Expression Products were purchased for interleukin

Table 1. Sequences of Primers Used for Real-Time RT-PCR

Gene		Sequence (5'—3')	Amplicon size	GenBank ID
IL-2	Forward	TGC TGG ACT TAC AGG TGC TCC	108 bp	NM-053836
	Reverse	TCA ATT CTG TGG CCT GCT TG		
IL-4	Forward	GAG AAC GAG CTC ATC TGC AGG	105 bp	NM-201270
	Reverse	CAG TTC ACC GAG AAC CCC AG		
IL-10	Forward	CTG CTC TTA CTG GCT GGA GTG A	107 bp	NM-012854
	Reverse	CTC AGC TCT CGG AGC ATG TG		
IFN-γ	Forward	AAG ACA ACC AGG CCA TCA GC	110 bp	NM-138880
	Reverse	CTT GGC GAT GCT CAT GAA TG		
TNF-α	Forward	GCA TGA TCC GAG ATG TGG AAC T	112 bp	NM-012675
	Reverse	GCC ACG AGC AGG AAT GAG AA		
iNOS	Forward	CTG GGT GAA AGC GGT GTT CT	101 bp	S-71597
	Reverse	CCG ACT TCC TTG TCT CAG TAG CA		
nNOS	Forward	GCC CAG TGC TCA ACT TCG AA	122 bp	AJ-305233
	Reverse	CCA TGT GAC CCC GAA AAG G		
GAPDH	Forward	TGA GGT GAC CGC ATC TTC TTG	102 bp	NM-017008
	Reverse	TGG TAA CCA GGC GTC CGA TA		

(IL)-1 β , IL-2, IL-4, IL-6, IL-10, interferon (IFN)- γ , tumor necrosis factor (TNF)- α , nuclear factor (NF)- κ B, iNOS, nNOS, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1). Reverse transcription was performed for 1 μ g of RNA using a cDNA High Capacity Archive kit (Applied Biosystems, Foster City, CA, U.S.A.) and random hexamers as primers. Quantitative PCR was performed on an SDS 7000 system from Applied Biosystems using a Universal MasterMix (Applied Biosystems, Foster City, CA, U.S.A.). The PCR conditions were 10 min at 90 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All assays were RNA-specific (spanning exon-exon junctions) pre-designed TaqMan Gene Expression Assays from Applied Biosystems (Table 1).

Statistical Analysis All results are expressed as the mean $\pm$ standard error (mean $\pm$ S.E.). Statistical significance between groups was analyzed using Tukey's test and $p < 0.05$ was considered significant. Statistical significance of survival ratio between groups was analyzed using Log rank test and $p < 0.01$ was considered significant.

RESULTS

Inhibitive Effects of AG on Generation of NO $_2^-$, Leaked Phospholipid, and Total Protein Level in Ileal Mucosa 0–1 h after Reperfusion In both the 50 g and the 100 g load groups, the amounts of NO $_2^-$ (the active form of NO) that leaked in ileal loop were increased in a hanging-force-dependent manner during ischemia when compared with those in the control condition 0–1 h after reperfusion. In addition, the increase of NO $_2^-$ was inhibited by the addition of AG, and no significant increase of NO $_2^-$ generation in the 100 g load group compared with that in the control condition was observed (Fig. 1a).

Because it is general to use changes of the quantity of protein in mucosal membrane and leakage phospholipid for index of the gastrointestinal mucous lesion, the changes were examined. The amounts of phospholipid that leaked from the ileal mucosa to the ileal loop were increased in a hanging-force-dependent manner 0–1 h after reperfusion when compared with those in the control condition (Fig. 1b). In addition, total protein level in ileal homogenate was significantly decreased 0–1 h after reperfusion when compared with that in the control condition (Fig. 1c). By AG administration, the

Fig. 1. Effects of AG on Generation of NO $_2^-$ in Ileal Perfusate (a), Amounts of Mucosal Phospholipid Released from Ileal Lumen (b), and Amounts of Total Protein in Ileal Homogenates (c) 1 h after Reperfusion

Data represent means and S.E. ($n=6-10$ for each condition). Open column: the condition without AG; closed column: the condition with AG. * $p < 0.05$ vs. the control condition (CTRL). † $p < 0.05$ vs. the condition with AG. N.S.: not significantly different from CTRL.

amounts of phospholipid that leaked from the ileal mucosa to the loop tended to decrease, but the total protein level in the ileal homogenate was significantly increased (Figs. 1b,c).

Inhibitive Effect of AG on Changes in Paracellular Permeability 0–1 h after Reperfusion The opening and closing of TJ during intestinal I/R were examined by an ab-

sorption experiment using FD-4, a representative paracellular marker. The AUC of 0 to 1 h after reperfusion (AUC_{0-1}) of FD-4 was significantly increased in a hanging-force-dependent manner during ischemia when compared with that in the control condition. In both the 50 g and the 100 g intestinal I/R groups with AG administration, the AUC_{0-1} of FD-4

Fig. 2. Effect of AG on AUC_{0-1} of Plasma FD-4 Concentration during Intestinal I/R

Data represent means and S.E. ($n=5-12$ for each condition). Open column: the condition without AG; closed column: the condition with AG. * $p<0.05$ vs. the control condition (CTRL). # $p<0.05$ vs. CTRL with AG. N.S.: not significantly different from CTRL.

Fig. 3. Kaplan-Meier Analysis Showing Cumulative Survival during Reperfusion after Intestinal Ischemia with or without AG ($n=13-16$ for Each Condition)

Log rank test: ** $p<0.01$ vs. CTRL with AG, † $p<0.01$ vs. each condition without AG.

(a)

(b)

(c)

Fig. 4. Effects of AG on Generation of NO_2^- in Ileal Perfusate (a), Amounts of Mucosal Phospholipid Released from Ileal Lumen (b), and Amounts of Total Protein in Ileal Homogenates (c) 3 h after Reperfusion

Data represent means and S.E. ($n=5-10$ for each condition). Open column: the condition without AG; closed column: the condition with AG. * $p<0.05$ vs. the control condition (CTRL). † $p<0.05$ vs. the condition with AG. # $p<0.05$ vs. CTRL with AG. N.S.: not significantly different from CTRL.

tended to be smaller than that without AG (Fig. 2).

Effects of AG on Survival Rate by 3 h after Reperfusion In both the 50 g and the 100 g intestinal I/R groups without AG, changes of survival were not observed 3 h after reperfusion; their survival rates were 100%. However, in both the 50 g and the 100 g intestinal I/R groups with AG, the survival rates were significantly decreased by 4 h after reperfusion, with values of 55.9% and 46.7%, respectively (Fig. 3).

Changes in Generation of NO_2^- , Leaked Phospholipid, and Total Protein Level in Ileal Mucosa 3–4 h after Reperfusion Although in both the 50 g and the 100 g load groups the amounts of NO_2^- that leaked in ileal loop and phospholipid that leaked from the ileal mucosa to the ileal loop tended to increase in a hanging-force-dependent manner during ischemia compared with those in the control condition 3–4 h after reperfusion, the rate of increase tended to be smaller than that 0–1 h after reperfusion. In both the 50 g and the 100 g load groups with AG, the amounts of leaked phospholipid tended to increase compared with those without AG (Figs. 4a,b). Moreover, the amounts in the 100 g load group with AG were significantly increased compared with

Fig. 5. Effect of AG on AUC_{3-4} of Plasma FD-4 Concentration during Intestinal I/R

Data represent means and S.E. ($n=5-12$ for each condition). Open column: the condition without AG; closed column: the condition with AG. * $p<0.05$ vs. the control condition (CTRL). † $p<0.05$ vs. the condition with AG. # $p<0.05$ vs. CTRL with AG. N.S.: not significantly different from CTRL.

those in the control condition.

At 3 to 4 h after reperfusion, the degree of the decrease of total protein level became smaller, and significant decrease was observed in the 100 g load group. In the condition of AG, the increase was not observed (Fig. 4c).

Changes in Paracellular Permeability 3–4 h after Reperfusion At 3 to 4 h after reperfusion, we carried out an absorption experiment of FD-4, and calculated AUC of 3 to 4 h after reperfusion (AUC_{3-4}). Although the AUC_{3-4} of FD-4 was significantly increased in a hanging-force-dependent manner during ischemia compared with that in the control condition, the rate of increase was smaller than that 0–1 h after reperfusion. However, in the group administered AG, the absorption of FD-4 in both the 50 g and the 100 g groups was significantly increased in a hanging-force-dependent manner during ischemia compared with that in the control condition. Moreover, the increased values 3–4 h after reperfusion were significantly higher than those 0–1 h after reperfusion (Fig. 5).

Effects of AG on Changes in mRNA Expression Level of Inflammatory and Anti-inflammatory Cytokines 1 and 3 h after Reperfusion At 1 and 3 h after reperfusion, although the mRNA expression level of nNOS did not change by intestinal I/R, the mRNA expression levels of other cytokines and iNOS were increased by intestinal I/R. At 3 h after reperfusion, although mRNA of inflammatory cytokines such as IL-2 and TNF- α tended to decrease compared with the levels 1 h after reperfusion, levels of mRNA of anti-inflammatory cytokines such as IL-4 and IL-10 were significantly increased compared with those 1 h after reperfusion.

Upon AG administration, it was shown that the increase of IFN- γ mRNA tended to be inhibited, and TNF- α mRNA was significantly inhibited 1 h after reperfusion. But 3 h after reperfusion, the mRNA expression level of inflammatory cytokines such as IL-2 and TNF- α were significantly increased compared with that without AG. On the other hand, the increase of mRNA of anti-inflammatory cytokines such as IL-4 and IL-10 were significantly inhibited by administration of AG.

Although the mRNA expression level of IL-4 was not changed between its value of 1 h and 3 h after reperfusion

Table 2. Effects of AG on the mRNA Expression Level of NOSs and Cytokines in Ileum 1 and 3 h after Reperfusion

		CTRL	I/R 50 g (% of CTRL)		I/R 100 g (% of CTRL)	
			No additive	+ Aminoguanidine	No additive	+ Aminoguanidine
IL-2	R=1	100±8.2	172.4±24.5	196.3±30.9	126.4±5.9	243.3±33.1*†
	R=3	100±8.4	118.9±14.1	249.7±38.8*†	115.7±9.0	247.0±29.8*†
IL-4	R=1	100±13.9	198.65±37.3	112.2±10.9	202.3±33.7	194.4±28.1
	R=3	100±18.4	235.1±21.8	153.0±3.2	601.8±81.2*#	281.5±37.0†
IL-10	R=1	100±10.2	188.4±35.3	136.2±5.6	153.9±18.6	216.7±33.3
	R=3	100±3.4	272.2±35.9*	139.7±11.0	534.2±93.8*#	371.3±105.1
IFN- γ	R=1	100±9.1	176.6±14.6	130.4±6.7	172.7±29.8	135.6±7.8
	R=3	100±11.0	162.4±25.9	148.1±9.9	362.1±72.9*	227.5±14.1
TNF- α	R=1	100±8.1	493.1±161.5*	184.8±27.3†	856.4±176.0*	181.8±4.0†
	R=3	100±14.4	306.0±73.1*	386.5±47.8*	795.7±102.1*	1074.9±134.5*#
iNOS	R=1	100±10.7	229.9±57.0	210.0±44.2	287.7±115.4	274.7±31.4
	R=3	100±19.2	294.5±100.5	134.2±23.4	682.7±202.7*	402.0±76.6*
nNOS	R=1	100±2.9	94.6±8.0	96.3±12.5	82.8±10.2	92.2±18.7
	R=3	100±6.4	95.3±5.8	122.9±11.8	140.8±28.1	160.1±3.3

Data represent means and S.E. ($n=5$ for each condition). R=time after reperfusion (h). * $p<0.05$ vs. the control condition (CTRL). † $p<0.05$ vs. without AG (no additive) condition. # $p<0.05$ vs. each condition of R=1.

without AG administration, its value of 3 h after reperfusion with AG administration was significantly decreased compared with 1 h after reperfusion with AG administration (Table 2).

DISCUSSION

The blood supply to small intestine was interrupted by occlusion of superior mesenteric artery and vein, followed by reperfusion for 4 h. First, we examined the changes in generation of NO_2^- and mucosal lesion until 1 h after reperfusion. The amounts of NO_2^- that leaked in the ileal loop and phospholipid that leaked from the ileal mucosa to the ileal loop were significantly increased in a hanging-force-dependent manner during ischemia compared with those in the control condition. In contrast, the amount of total protein was significantly decreased by this intestinal I/R. For these changes due to the intestinal I/R, AG as a selective iNOS inhibitor showed inhibitive effects in both the 50 g and the 100 g groups, resulting in the return of the levels to the range not significantly different from those of the control (Fig. 1).

Furthermore, increase of AUC_{0-1} of FD-4 as a paracellular marker owing to the intestinal I/R was inhibited by administration of AG (Fig. 2). We have already reported that changes in the function and expression of ileal P-gp, which is an important ATP-binding cassette transporter, by intestinal I/R.^{11,12,20,21} Moreover, we reported AG significantly inhibited the decrease of ileal P-gp function to excrete P-gp substrate, rhodamine 123, into the ileal lumen and expression by intestinal I/R until 1 h after reperfusion.¹³ These results showed that AG can inhibit intestinal I/R injury until 1 h after reperfusion.

We continued to examine the effects of AG until 4 h after reperfusion. In our *in vivo* intestinal I/R model, 24 h after reperfusion, the survival ratios in the 50 g and 100 g intestinal I/R groups were 90% and 60% (data not shown), but the survival ratios in the 50 g and 100 g intestinal I/R groups by 3 h after reperfusion were both 100% (data not shown). However, the survival ratios in the 50 g and 100 g intestinal I/R groups administered AG 3 h after reperfusion were significantly decreased, at 55.9% and 46.7%, respectively (Fig. 3). Because the effects of AG were not observed in the control condition, the toxicity of AG was not observed. Therefore, it was thought that I/R injury was induced by the effects of AG except toxicity 3 h after reperfusion. No significant differences of NO_2^- generation and phospholipid leakage were observed between the control condition and the intestinal I/R group 3 h after reperfusion, but they were significantly increased by administration of AG (Fig. 4). It is thought that AG was effective to $R=1$, but the effect was not observed at $R=3$ for intestinal I/R injury. The reason is unknown and the detail should be further examined. The increase of the NO_2^- may be caused by increase of iNOS protein level and/or its activity 3 h after reperfusion. Moreover, the AUC_{3-4} of FD-4 in the 100 g intestinal I/R group became significantly greater by the administration of AG (Fig. 5). These results showed that AG recovered intestinal I/R injury 1 h after reperfusion, but thereafter the injury was induced again. Because increased membrane permeability may cause bacterial translocation in clinical contexts, the above occurrence is extremely dangerous.

Many reported studies have examined the inhibitive effects of AG on ischemia/reperfusion injury in heart,²² brain,²³ small intestine,²⁴ and others. On the other hand, it has been reported that AG did not prevent intestinal mucosal injury after intestinal I/R²⁵ and hepatic I/R.²⁶ Therefore, the effects of AG on intestinal I/R injury remain controversial. However, there have been no reports of AG inducing intestinal I/R injury, as shown in this paper.

To clarify the change in the inflammation and the stress of the intestinal mucosa by AG administration, we examined the mRNA expression level of NOSs and cytokines as inflammation indexes in ileum 1 and 3 h after reperfusion. According to general classification of the inflammatory and anti-inflammatory cytokine, we selected IL-2, TNF- α and IFN- γ as inflammatory cytokines, and IL-4 and IL-10 as anti-inflammatory cytokines.

Most mRNA expression levels tended to increase by intestinal I/R 1 h after reperfusion. In particular, TNF- α mRNA was significantly increased in a hanging-force-dependent manner during ischemia compared with that in the control condition, but the increase was inhibited by AG. On the other hand, 3 h after reperfusion, the increases of IL-4 and IL-10 mRNA were significantly inhibited by AG. The decrease of IL-4 mRNA and the increase of TNF- α mRNA were observed with AG administration 3 h after reperfusion. From the changes of these inflammatory parameters, it was shown that AG induced inflammation of intestinal mucosa by intestinal I/R 3 h after reperfusion (Table 2).

NO has many beneficial effects in I/R injury, scavenging oxygen free radicals such as O_2^- , reducing leukocyte adhesion to the mesenteric endothelium, and maintaining normal vascular permeability.⁸⁻¹⁰ Peroxynitrite (NO_3^-) is a potent oxidant which is formed by a rapid reaction between NO and O_2^- , and plays various pathophysiological roles in the development of inflammation.²⁷⁻³⁰ It is thought that because AG inhibited generation of NO, the other reactive oxygen species (ROS) such as O_2^- and $\cdot\text{OH}$ are not scavenged. Accordingly, it was thought that the amount of O_2^- was increased by AG administration. Consequently, it is considered that intestinal I/R injury is induced by oxidation stress.

These results suggested that because the inhibition of iNOS during intestinal I/R induced generation of other ROS such as O_2^- and $\cdot\text{OH}$, intestinal I/R injury can be induced more than that in conditions without iNOS inhibitor. Therefore, it is expected that the removal ROS by scavengers is more useful than inhibition of ROS generation for the inhibition of intestinal I/R injury. Even if NO is an injury factor, the inhibition of the pathway of generation of endogenous substance cannot inhibit intestinal I/R injury. Moreover, it was shown that the inhibition of iNOS during intestinal I/R may have induced more intestinal I/R injury.

In conclusion, although AG as an iNOS inhibitor can temporarily inhibit intestinal I/R injury, the injury was again induced and survival rate was significantly decreased 3 h after reperfusion. Thus compensation reaction, that is, an injury induced by other ROS such as O_2^- , by the iNOS inhibition was observed during intestinal I/R, it was shown that it is difficult to inhibit the intestinal I/R in the long term after reperfusion injury by a single administration of AG.

Acknowledgments The authors thank Mr. Yasuhiko Ito,

Miss Yukako Ohno, Mr. Yoshifusa Tobe, Miss Nasa Sakamoto, and Miss Minami Nakagawa for technical assistance.

REFERENCES

- 1) Kubes P., Hunter J., Grander D. N., *Gastroenterology*, **103**, 807—812 (1992).
- 2) Nagira M., Tomita M., Mizuno S., Kumata M., Ayabe T., Hayashi M., *Drug Metab. Pharmacokinet.*, **21**, 230—237 (2006).
- 3) Wasserberg N., Pileggi A., Salgar S. K., Ruiz P., Ricordi C., Inverardi L., Tzakis A. G., *Int. J. Surg.*, **5**, 216—224 (2007).
- 4) Fotiadis C., Adamis S., Misiakos E. P., Genetzakis M., Antonakis P. T., Tsekouras D. K., Gorgoulis V. G., Zografos G. C., Papalois A., Fotinou M., Perrea D., *Acta Chir. (Belgium)*, **107**, 192—200 (2007).
- 5) Breithaupt-Faloppa A. C., Vitoretti L. B., Coelho F. R., dos Santos Franco A. L., Domingos H. V., Sudo-Hayashi L. S., Oliveira-Filho R. M., Tavares de Lima W., *Shock*, **32**, 55—61 (2009).
- 6) Xie Q., Nathan C., *Am. J. Physiol. Renal Physiol.*, **282**, F777—F784 (2002).
- 7) Suzuki Y., Deitch E. A., Mishima S., Lu Q., Xu D., *Crit. Care Med.*, **28**, 3692—3696 (2000).
- 8) Wink D. A., Hanbauer I., Krishna M. C., DeGraff W., Gamson J., Mitchell J. B., *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 9813—9817 (1993).
- 9) Lefer A. M., Lefer D. J., *Cardiovasc. Res.*, **32**, 743—751 (1996).
- 10) Kosonen O., Kankaanranta H., Malo-Ranta U., Moilanen E., *Eur. J. Pharmacol.*, **382**, 111—117 (1999).
- 11) Tomita M., Takizawa Y., Kishimoto H., Hayashi M., *Drug Metab. Pharmacokinet.*, **23**, 356—363 (2008).
- 12) Tomita M., Takizawa Y., Kishimoto H., Hayashi M., *J. Pharm. Pharmacol.*, **61**, 1319—1324 (2009).
- 13) Takizawa Y., Kishimoto H., Kitazato T., Tomita M., Hayashi M., *Eur. J. Pharm. Sci.*, **42**, 246—252 (2011).
- 14) Corbett J. A., Tilton R. G., Chang K., Hasan K. S., Ido Y., Wang J. L., Sweetland M. A., Lancaster J. R. Jr., Williamson J. R., McDaniel M. L., *Diabetes*, **41**, 552—556 (1992).
- 15) Misko T. P., Moore W. M., Kasten T. P., Nickols G. A., Corbett J. A., Tilton R. G., McDaniel M. L., Williamson J. R., Currie M. G., *Eur. J. Pharmacol.*, **233**, 119—125 (1993).
- 16) Corbett J. A., McDaniel M. L., *Methods*, **10**, 21—30 (1996).
- 17) Stabile A. M., Moreto V., Antunes-Rodrigues J., Carnio E. C., *Peptides*, **31**, 706—711 (2010).
- 18) Babai L., Szigeti Z., Parratt J. R., Végh A., *Clin. Sci.*, **102**, 435—445 (2002).
- 19) Iida A., Tomita M., Idota Y., Takizawa Y., Hayashi M., *Drug Metab. Pharmacokinet.*, **21**, 424—428 (2006).
- 20) Takizawa Y., Kitazato T., Kishimoto H., Tomita M., Hayashi M., *Eur. J. Drug Metab. Pharmacokinet.*, **35**, 89—95 (2011).
- 21) Takizawa Y., Kishimoto H., Kitazato T., Tomita M., Hayashi M., *Biol. Pharm. Bull.*, **34**, 408—414 (2011).
- 22) Parlakpınar H., Ozer M. K., Acet A., *Mol. Cell. Biochem.*, **277**, 137—142 (2005).
- 23) Vakili A., Zahedi-Khorasani M., *Pak. J. Pharm. Sci.*, **21**, 24—28 (2008).
- 24) Wu B., Iwakiri R., Tsunada S., Utsumi H., Kojima M., Fujise T., Ootani A., Fujimoto K., *Free Radic. Biol. Med.*, **33**, 649—658 (2002).
- 25) Uchida K., Mishima S., Ohta S., Yukioka T., *J. Trauma*, **63**, 603—607 (2007).
- 26) Hu M., Wang Z., Rao J., Cao Y., Jiang W., Zhang F., Li X., Wang X., *Transpl. Immunol.*, **23**, 6—11 (2010).
- 27) Huie R. E., Padmaja S., *Free Radic. Res. Commun.*, **18**, 195—199 (1993).
- 28) Beckman J. S., Koppenol W. H., *Am. J. Physiol.*, **271**, 1424—1437 (1996).
- 29) Okamoto T., Akaike T., Nagano T., Miyajima S., Suga M., Ando M., Ichimori K., Maeda H., *Arch. Biochem. Biophys.*, **342**, 261—274 (1997).
- 30) Yamakura F., Taka H., Fujimura T., Murayama K., *J. Biol. Chem.*, **273**, 14085—14089 (1998).