

## Induction of Metallothionein Synthesis by Cilostazol in Mice and in Human Cultured Neuronal Cell Lines

Shinya SUZUKI,<sup>\*a</sup> Yosuke MASUI,<sup>a</sup> Mieko OHNOKI,<sup>a</sup> Goro MIYAKODA,<sup>b</sup> Toyoki MORI,<sup>b</sup> Katsuyuki NAKAJIMA,<sup>b</sup> and Masao SATO<sup>a</sup>

<sup>a</sup> Faculty of Pharmaceutical Sciences, Tokushima Bunri University; 180 Yamashiro-cho, Tokushima 770–8514, Japan; and

<sup>b</sup> Research Institute of Pharmacological and Therapeutical Development, Otsuka Pharmaceutical Co., Ltd.; 463–10 Kagasuno, Kawauchi-cho, Tokushima 771–0192, Japan. Received December 16, 2006; accepted January 16, 2007

**In this study, we examined the effect of Cilostazol to induce metallothionein (MT) *in vivo* and *in vitro*. Intraperitoneal injection of Cilostazol increased the expression of both MT-1 and MT-2 mRNA and total MT protein in the mouse liver. Cilostazol also augmented MT-1 mRNA levels in the murine brain. *In vitro* exposure to Cilostazol significantly augmented intracellular MT protein levels in cultured human brain microvascular endothelial cells (HBMEC) and in the neuroblastoma cell line IMR32. Taken together, these findings suggest that Cilostazol is an inducer of MT in the murine liver and brain, and that it has the potential to directly induce MT in cells. The contribution of the anti-oxidative effect of MT to the anti-stroke effect of Cilostazol was discussed.**

**Key words** metallothionein; cilostazol; brain

Cilostazol, a quinolinone derivative, is approved by the Food and Drug Administration for the treatment of intermittent claudication.<sup>1)</sup> It has been shown that Cilostazol has an inhibitory effect on platelet aggregation and thrombosis in feline cerebral ischemia.<sup>2)</sup> Cilostazol has also been shown to significantly reduce the risk of recurrent strokes without affecting the occurrence of intracranial hemorrhage. Recently, the Cilostazol Stroke Prevention Study revealed that Cilostazol reduced the risk of secondary stroke by 41.7% as compared with a placebo<sup>3)</sup> suggesting that Cilostazol has a specific effect on small-vessel disease, although the mechanism for this is not well known. It has been shown that Cilostazol and its metabolites reduced the intracellular reactive oxygen species (ROS) induced by LPS in human umbilical vein endothelial cells by directly scavenging hydroxyl radicals.<sup>4)</sup> However, the anti-stroke effect of Cilostazol *in vivo* seems not to be due to its antioxidative effect, because its blood clearance is so rapid, and its concentration in the brain would be too low to directly scavenge ROS. Thus, Cilostazol may cause an anti-stroke effect *via* the induction of a putative anti-oxidative molecule in the brain.

Metallothionein (MT) is a small sulfhydryl-rich protein, the level of which is elevated by various inducers of metals, hormones and cytokines. The induction of MT synthesis has been reported in various tissues, but rarely in the brain. MT is thought to be a multifunctional protein detoxifying heavy metals such as cadmium and inorganic mercury, maintaining zinc and copper homeostasis, and regulating the biosynthesis and activity of zinc-binding proteins such as zinc-dependent transcription factors. MT also has the ability to scavenge ROS.<sup>5,6)</sup> Concerning the anti-oxidative activities of MT, a role of MT in neurodegenerative diseases has been proposed.<sup>7)</sup> Thus, in this experiment, we examined the induction of MT by Cilostazol in the mouse brain and liver, and also in human cultured neuronal cells in order to confirm the possibility that Cilostazol directly induces MT in the human brain as an anti-oxidative effector molecule.

## MATERIALS AND METHODS

**Materials** Cilostazol was obtained from Otsuka Pharm. Co., Ltd. (Tokushima, Japan). Anti-metallothionein antibody, E-9, was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). FITC-conjugated rabbit anti-mouse immunoglobulin antibody was from DAKO Co. (Denmark). All other chemicals were of reagent grade or better.

**Animals and Treatments** Male C57BL/6J mice were purchased from Clea Japan Inc. (Tokyo Japan). They were housed in plastic cages at 23 °C with a 12 h light/dark cycle and were given lab food and tap water *ad libitum*. Mice (7 weeks old) were randomly assigned to treatment groups and were injected i.p. with 0.5% Cilostazol suspended with 0.5% carboxymethyl cellulose solution or vehicle alone. After various time intervals, the liver and brain were removed from each mouse under anesthetization with sodium pentobarbital and were then stored at –80 °C for the determination of MT level. The mice received humane care throughout the experiments according to the guidelines of Tokushima Bunri University.

**Cell Cultures** IMR-32 cells, a human neuroblastoma cell line, were obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.), and maintained in Dulbecco's modified essential medium supplemented with 10% heat-inactivated fetal bovine serum. Human brain microvascular endothelial cells (HBMEC) were purchased from Dainihon Pharmaceutical Co. (Osaka, Japan), and cultured in HuMedia-EG2 medium (Kurabo Co., Osaka, Japan) with 5% fetal bovine serum at 37 °C in an incubator under 5% CO<sub>2</sub>.

**Measurement of hepatic MT** Liver tissue was homogenized in Tris buffer (50 mM Tris–HCl, pH 8.0), and the total level of MT protein in the cytosol fraction of the homogenate was determined by the Cd-hem method<sup>8,9)</sup> with partial modification. Since Cilostazol treatment did not alter the liver weight, MT level was expressed in terms of micrograms per gram of wet tissue.

**Measurement of Intracellular MT Levels** Intracellular MT level was estimated by flow cytometry using a monoclonal antibody E-9. Since E-9 recognizes a common epitope

\* To whom correspondence should be addressed. e-mail: suzukis@ph.bunri-u.ac.jp

of murine and human MT-1 and MT-2 proteins, this analysis revealed the total level of both MT-1 and MT-2 isotypes. HBMEC or IMR32 cells in confluent culture adhered to petridishes were mixed with Cilostazol and cultured for 12 h in a humidified CO<sub>2</sub> incubator, and then the cells were harvested by trypsinization followed by washing twice with ice-cold PBS. The cells were fixed with 4% paraformaldehyde PBS followed by permeabilization with 0.1% Triton X-100 PBS at r.t. for 30 min, and then treated with 50-fold-diluted E-9 for 1 h at 37 °C. After washing twice with ice-cold PBS, cells were mixed with FITC-conjugated rabbit anti-mouse immunoglobulin antibody (50-fold dilution) and incubated at 37 °C for 1 h. After washing as above, cell fluorescence was estimated using an ELITE flow cytometer (EPICS). Ten thousand counting events were recorded and the mean fluorescence intensity (MFI) was calculated for each sample as relative MT protein level.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis** A tissue section (0.1 g) was homogenized in 1.0 ml Trizol reagent (Gibco BRL, Gaithersburg, MD, U.S.A.), and then total RNA was extracted with phenol-chloroform. Cultured cells ( $2 \times 10^6$  cells) adhered to petridishes were washed with PBS twice and solubilized with 1 ml Trizol reagent to extract total RNA as above. RT-PCR was performed using an Access RT-PCR Kit (Promega, Madison, WI, U.S.A.) according to the manufacturer's procedure.

The sequences of the primers used for amplification are as follows: Mouse MT-1 sense, 5'-ATG GAC CCC AAC TGC TCC TGC TCC ACC-3'; antisense, 5'-GGG TGG AAC TGT ATA GGA AGA CGC TGG-3'; mouse MT-2: sense, 5'-ATG GAC CCC AAC TGC TCC TGT GCC TCC-3'; antisense, 5'-GGT CTA TTT ACA CAG ATG TGG GGA CCC-3'; mouse MT-3: sense, 5' GTC CCT GAG ACC GTC CCC TGT CCT-3'; antisense, 5'GGC CTC TGC CTT GGC CCC CTC TTC ACC-3'; human MT-1A: sense, 5'CTC GAA ATG GAC CCC AAC T-3'; antisense, 5'ATA TCT TCG AGC AGG GCT GTC-3'; human MT-1E: sense, 5'GC, T TGT TCG TCT CAC TGG TG-3'; antisense, 5'CAG GTT GTG CAG GTT GTT CTA-3'; human MT-1F: sense, 5'AGT CTC TCC TCG GCT TGC; antisense, 5'ACA TCT GGG AGA AAG GTT GTC-3'; human MT-1X: sense, 5'TCT CCT TGC CTC GAA ATG GAC-3'; antisense, 5'GGG CAC ACT TGG CAC AGC-3'; human MT-2A: sense, 5'CCG ACT CTA GCC GCC TCT T; antisense, 5'GTG GAA GTC GCG TTC TTT ACA-3'; GAPDH: sense, 5'GTG AGG GGC CGG TGC TGA GT; antisense, 5'TTG CTG GGG TGG GTG GTC-3'. The expected PCR product sizes were 259 bp for mouse MT-1, 225 bp for mouse MT-2; 219 bp for human MT-1A, 284 bp for human MT-1E; 232 bp for human MT-1F, 151 bp for human MT-1X, 259 bp for human MT-2A, and 773 bp for GAPDH. Each PCR product was electrophoresed on a 4.0% agarose gel containing 0.5 µg/ml ethidium bromide.

**Statistical Analysis** Significant difference was calculated using one-way ANOVA followed by Bonferroni's multiple comparisons test, or where applicable, Student's *t*-test.

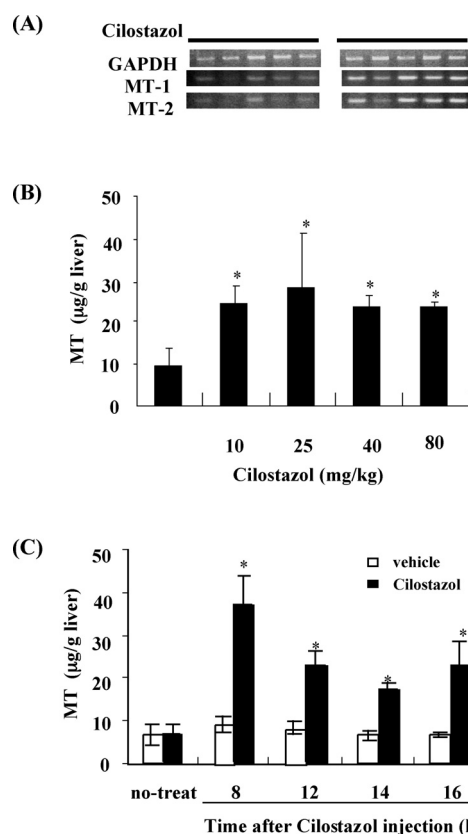


Fig. 1. Hepatic MT Levels after Cilostazol Injection

(A, B) Mice were injected i.p. with Cilostazol at the indicated dose, and the livers were excised, and then the levels of hepatic MT mRNA and protein were determined as described in Materials and Methods. Representative RT-PCR products of MT-1, MT-2 and GAPDH and hepatic MT protein levels on the factor of the Cilostazol dose 12 h after Cilostazol injection are respectively shown in A and B. (C) Kinetic changes in hepatic MT protein levels after Cilostazol injection (40 mg/kg) are shown in C. Hepatic MT levels were evaluated at the indicated times after administration of Cilostazol. In (B) and (C), columns and bars indicate, respectively, the mean MT level (µg/g liver) and S.D. of the mean ( $n=5$ ). \* Significantly different from the respective vehicle-treated control ( $p<0.05$ ).

## RESULTS

**Induction of Hepatic MT in Mice** We first examined the expressions of MT-1 and MT-2 mRNA in the liver of mice inoculated i.p. with Cilostazol using RT-PCR. As shown in Fig. 1A, the expressions of both MT-1 and MT-2 mRNA significantly increased compared with the control at 12 h after Cilostazol administration. We next investigated the hepatic total MT protein level in Cilostazol-inoculated mice (Fig. 1B). Twelve hours after inoculation, Cilostazol induced increases in hepatic MT levels of approximately 3-fold the control at dose ranges of 10 to 80 mg/kg. Maximal induction was observed at 25 mg/kg and further dose inoculations did not accelerate the induction. The kinetic changes of hepatic MT protein levels are also shown in Fig. 1C. Hepatic MT levels significantly increased at 8 h after the administration of Cilostazol by about 5-fold as compared with the non-treated control group, gradually decreased to 14 h, and then re-increased at 16 h.

**Induction of MT mRNA in Murine Brain** Figure 2 shows mRNA levels of MTs in the brains of mice inoculated with Cilostazol. Cilostazol injection (40 mg/kg) resulted in significant increases in brain MT-1 mRNA levels 12 h after injection. Representative chromatograms of PCR products

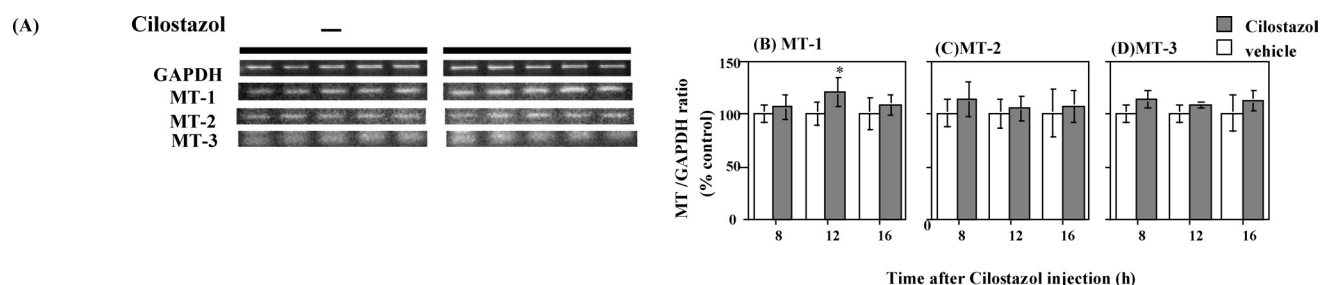


Fig. 2. RT-PCR Analysis of Brain MT-1, MT-2 and MT-3 mRNA Levels in Cilostazol-Injected Mice

Mice were treated with a vehicle or Cilostazol (40 mg/kg, i.p.) and at the indicated times after treatment, the whole brain were excised. Total RNAs were extracted from the brains and RT-PCR analyses of MT-1, MT-2, MT-3 and GAPDH were performed as described in Materials and Methods. GAPDH mRNA levels were measured as a standard for RNA recovery. Representative RT-PCR analyses of brain MTs mRNA in mice 12 h after Cilostazol inoculation are shown in (A). Kinetic changes of relative mRNA levels of MT-1 (B), MT-2 (C), MT-3 (D) are also shown. Relative densitometric intensities of each band normalized by that of the respective GAPDH band were calculated as the MT/GAPDH ratio. Columns and bars indicate, respectively, the percent of the mean MT/GAPDH ratio as compared with the respective non-treated control and S.D. of the mean (n=5). \* Significantly different from the vehicle-treated group (p<0.05).

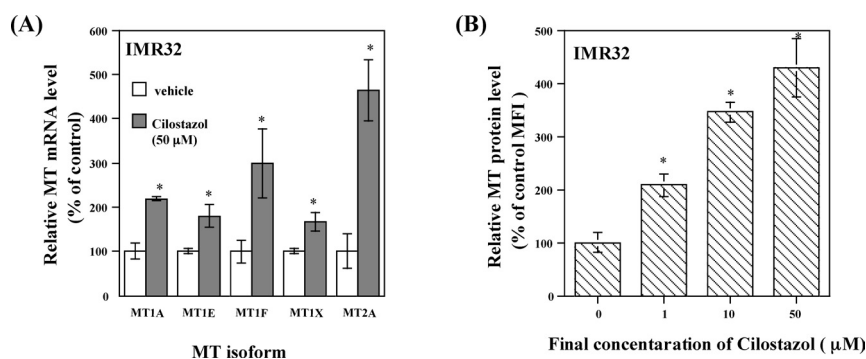


Fig. 3. Induction of MT mRNA and Protein by Cilostazol in IMR32 Cells

IMR32 cells were treated with Cilostazol (50 μM) for 12 h, and then, MT-1A, -1E, -1F, -1X, 2A, and GAPDH mRNA expression (A) and MT protein levels (B) in the cells were determined as described in Materials and Methods. Columns and bars indicate, respectively, the percent of mean value and S.D. of the mean (n=3). \* Significantly different from the vehicle-treated group (p<0.05).

are also shown in the upper field of Fig. 2. MT-2- and MT-3 mRNA levels tended to increase, although not significantly.

### Induction of MT in Cultured Human Neuronal Cells

In order to estimate whether Cilostazol induced MT in the murine brain, directly affecting neuronal cells, we examined MT synthesis in cultured human neuronal cell lines IMR32 and HBMEC in the presence of Cilostazol. Twelve hours after the addition of Cilostazol (50 μg/ml) to culture media, the expression of human MT-1A-, MT-1E-, MT-1F-, MT-1X- and MT-2A mRNA in IMR32 cells increased as compared with the non-treated control (Fig. 3A). Cilostazol also augmented the protein levels of MT in both IMR32 and HBMEC cells, as shown in Fig. 3B and Fig. 4, respectively. When IMR32 cells were cultured in the presence of 50 μg/ml Cilostazol, the MT protein level increased by about 300% as compared with the control level, while in HBMEC, about 100% augmentation was observed at a optimal dose of 1 μg/ml and a higher dose did not cause higher augmentation.

### DISCUSSION

Cilostazol has an inhibitory effect on phosphodiesterase 3 and the subsequent elevation of intracellular cAMP levels<sup>10)</sup> *in vitro*, and showed vasodilatory and antiplatelet actions *in vivo* probably *via* the cAMP-dependent pathway. In this study, we examined the effect of Cilostazol to induce MT *in vivo* and *in vitro*, involving the contribution of the anti-oxida-

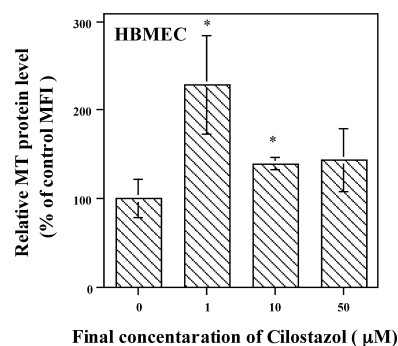


Fig. 4. Induction of MT Protein by Cilostazol in HBMEC

HBMEC were treated with Cilostazol (50 μM) for 12 h, and then, MT protein levels in the cells were determined as in Fig. 3. Columns and bars indicate, respectively, the percent of mean value and S.D. of the mean (n=3). \* Significantly different from the vehicle-treated group (p<0.05).

tive effect of MT to the anti-stroke effects of Cilostazol.

As shown in Figs. 1 and 2, i.p. Cilostazol injection resulted in a significant increase of MT mRNA levels in both the liver and brain, but the degree of increase of MT mRNA in the brain was very weak (Fig. 2B) and only in the MT-1 isotype. Cilostazol also augmented the MT protein level by about 3-fold in the liver, showing the potential to induce MT *in vivo* for systemically-inoculated Cilostazol, but did not induce a significant increase in the brain. This may show that MT induction occurred only in a small number of brain cells and the sensitivity of the assay system is higher in RT-PCR

than in the Cd-hem method.

In some cases, the injection of chemical compounds induces local inflammation at the injection site, resulting in increased blood levels of inflammatory cytokines. We previously observed that cytokines such as interleukin 6, tumor necrosis factor  $\alpha$ <sup>11)</sup> and interferon  $\alpha$ <sup>12)</sup> induced MT synthesis *in vivo*. In this study, did Cilostazol also induce MT *via* cytokine production? In order to clarify this in the human brain cell system, we next examined the *in vitro* inducibility of MT by Cilostazol. As in Fig. 3 and Fig. 4, the addition of Cilostazol into culture medium resulted in an increase of MT protein levels in IMR32 and HBMEC. This suggests the potential of Cilostazol directly induce MT synthesis in neuron cells and supportive brain vascular endothelial cells. In spite of the *in vitro* inducibility of MT in the neuron cell line, Cilostazol did not significantly augment total MT levels in the murine brain *in vivo*. This suggests that Cilostazol in the blood probably did not efficiently perturb the blood brain barrier in maternal neuronal tissue.

Brain microendothelial cells are one of the constituents of the blood brain barrier, which is thought to be sensitive to exogenous compounds. Rapid induction of MT-1 and MT-2 in response to cerebral ischemia and reperfusion has been reported, and MT plays a role in regulating the formation of brain edema and invasion of leukocytes.<sup>13)</sup> MT may also play a role in the regulation of angiogenesis, the blood aggregation system, and formation of atherosclerosis.<sup>14)</sup> In addition, Cilostazol inhibits endothelial-neutrophil adhesion by decreasing adhesion molecule expression *via* NO production, and leukocyte integrin mac, leading to a potential reduction in restenosis after coronary stent implantation.<sup>15)</sup> Further, it has been reported that MT can prevent kainic acid-induced hippocampus damage<sup>16)</sup>; therefore, it is noteworthy that

Cilostazol induced MT protein in cultured HBMEC.

Taken together, we hypothesized that the preventive effect of Cilostazol against secondary stroke would be mediated through the induction of MT in blood vessels in the brain, which has a function to scavenge reactive oxygen.

**Acknowledgements** The authors thank Dr. Kaji and Dr. Yamamoto (Hokuriku University, Japan) for their kind suggestions. This work was supported in part by a Grant-in-Aid for General Scientific Research from the Ministry of Education, Culture, Sports, Sciences and Technology of Japan.

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