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YC-1 Inhibits Neointima Formation in Balloon-Injured Rat Carotid Through Suppression of Expressions and Activities of MMP-2 and MMP-9

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Running title: YC-1 inhibits restenosis via a MMPs-dependent pathway

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Abbreviations: YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole; MMPs, matrix metalloproteinases; VSMCs, vascular smooth muscle cells; PTCA, percutaneous transluminal coronary angioplasty; sGC, soluble guanylyl cyclase; CMC, carboxymethyl cellulose; FBS, fetal bovine serum; PMSF, phenylmethyl sulfonyl fluoride; PBS, phosphate-buffered saline; DMSO, dimethylsulphoxide; ELISA, enzyme-linked immunosorbent assay.

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ABSTRACT

Matrix metalloproteinases (MMPs), particularly MMP-2 and MMP-9, and post-revascularization production of vascular smooth muscle cells (VSMCs) may play key roles in development of arterial restenosis. We investigated the inhibitory effect of YC-1, a benzyl indazole compound, on MMP-2 and MMP-9 activity in a balloon-injury rat carotid artery model. Injury was induced by inserting a balloon catheter through the common carotid artery; after 14 days, histopathological analysis using immunostaining and Western blotting revealed significant restenosis with neointimal formation that was associated with enhanced protein expression of MMP-2 and MMP-9. However, these effects were dose-dependently reduced by orally administered YC-1 (1-10 mg/kg). In addition, gelatin zymography demonstrated that increased MMP-2 and MMP-9 activity was diminished by YC-1 treatment. On the other hand, YC-1 inhibited hydrolysis of the fluorogenic quenching substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 by recombinant MMP-2 and MMP-9 with IC_{50} = 2.07 and 8.20 μM, respectively. RT-PCR analysis of MMP-2 and MMP-9 mRNA revealed that YC-1 significantly inhibited mRNA levels of MMPs. Finally, for the YC-1 treatment group, we did not observe elevation of cGMP levels using enzyme-linked immunosorbent assay, suggesting that YC-1 inhibition of neointimal formation is not through a cGMP-elevating pathway. These data show YC-1

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suppression of neointimal formation is dependent on its influence on MMP-2 and MMP-9 protein, mRNA expression, and activity, but not through a cGMP-elevating effect. YC-1 shows therapeutic potential for treatment of restenosis after angioplasty.

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Introduction

During the past 20 years, one focus of cardiovascular pharmaceutical research has been development of drugs that inhibit intimal hyperplasia. Despite many attempts, no clinical trial has proven there is an effective pharmacological solution to the problem (Garas et al., 2001; Bult, 2001). Intimal hyperplasia is a late response of the arterial wall to mechanical injury, and it is a major cause of restenosis after percutaneous transluminal coronary angioplasty (PTCA) (Lange et al., 1991). After vascular injury, inflammatory cells are recruited to the lesion, where they release several mitogens that activate vascular smooth muscle cells (VSMCs). Consequently, VSMCs are capable of proliferating and migrating from media to intima, releasing matrix and resulting in neointima formation.

Matrix metalloproteinases (MMPs) are a family of structurally related zinc-endopeptidases that degrade components of extracellular matrix associated with vascular remodeling during vascular injury-induced neointima formation (Pauly et al., 1994; Lijnen et al., 1999). MMP-2 and MMP-9, also called gelatinases A and B, respectively, are the major MMPs derived from VSMCs and inflammatory cells after vascular injury. Several lines of evidence indicate that MMP-2 and MMP-9 are upregulated and activated after PTCA and that they play a key role in regulating VSMC proliferation and migration (Yukihiro et al., 2001). In one study (Bendeck et

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al., 1994), MMP-9 was expressed within several hours of vascular injury, and MMP-2 activity was markedly increased after four days. Based on this hypothesis, MMP inhibitors have been demonstrated to inhibit intimal migration of rat carotid VSMCs *in vivo* and inhibit subsequent neointima formation (Bendeck et al., 1994; Cho et al., 2002).

Previous research reported that YC-1 is a nitric oxide (NO)-independent, NO-enhancing activator of (sGC); it has been used as a research tool for characterization of NO/sGC/cGMP signaling and function in various tissues (Di Fulvio et al., 2001; Gomez and Nasi, 2000; Suzuki et al., 2001). However, in cardiovascular systems, YC-1 causes an increase in cGMP level and relaxation in vascular smooth muscle (Galle et al., 1999; Wegener et al., 1997; Wegener and Nawrath, 1997). There is also specific HO-1 site overexpression in VSMCs (Imai et al., 2001). This action is a NO-independent, O_2^- sensitive activation of sGC in VSMCs. The additive or even synergistic responses to NO-donors of YC-1 in cultured VSMCs and isolated aortic rings apparently reflect the synergistic action of YC-1 and NO on sGC (Galle et al., 1999; Mulsch et al., 1997). On the other hand, YC-1 may inhibit VSMC proliferation to reduce neointima formation through endogenous CO- and/or NO-mediated, cGMP-dependent processes (Tulis et al., 2000; Wu et al., 1995). Despite experimental evidence that YC-1 suppresses intimal hyperplasia in animal

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models, the effects on MMP-2 and MMP-9 have not been well characterized. In the current work, we studied the *in vivo* effects of YC-1 on MMP-2 and MMP-9 expression and activity using a carotid artery injury model. Our data show that YC-1 significantly decreased levels of MMP-2 and MMP-9 protein and mRNA and decreased their activities on arterial tissue, almost completely preventing neointimal formation in the injured rat artery.

Materials and Methods

Materials. YC-1 was supplied by Yung-Shin Pharmaceutical Industry Co, Ltd, (Taichung, Taiwan); anti-rabbit IgG-HRP and anti-mouse IgG-HRP antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); TRIzol reagent was from Invitrogen (Carlsbad, California, USA); random primer and M-MLV RT were from Promega (Madison, WI, USA); pro Taq was from Protech (Taipei, Taiwan); anti-MMP-2 and anti-MMP-9 antibodies were from NeoMarkers (Fremont, California, USA). DAKO LSAB kit was from DAKO (DAKO, Glostrup, Denmark).

Balloon Injury Model. We utilized the established rat carotid artery (CA) model of balloon angioplasty to examine the *in vivo* arterial response to injury (Pan et al., 2003). Briefly, male Wistar rats weighing an average 300 g were anesthetized with pentobarbital (60 mg/kg) and the left CA exposed. A Fogarty 2F embolectomy balloon catheter was inserted into the left external carotid artery through an

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arteriotomy incision and advanced to the aortic arch. The balloon was inflated and withdrawn three times with rotation at the same pressure. The catheter was removed and the external carotid was ligated. The overlying tissue was sutured and the skin closed with rodent wound clips. Rats were euthanatized by pentobarbital, the left and right (collateral control group) were harvested for study.

YC-1 Dosing. YC-1 suspended in 0.5% carboxymethyl cellulose (CMC) was orally administered (1, 5, and 10 mg/kg) daily to rats beginning from 3 days prior to and lasting for 14 days after balloon injury. In contrast, control animals received only 0.5% CMC daily. And, the collateral right CA served as an un-manipulated control.

Tissue Processing. Tissues for enzyme examination were obtained from the sacrificed animals and immediately snap-frozen. For each indicated time studies examining morphologic remodeling of the vessel wall, carotid arteries were immersion-fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 hours. Then, tissues were transferred to 70% alcohol and processed by standard procedures, and paraffin-embedded. Five-µm sections were cut using a rotary microtome and placed on pretreated slides.

Histological Examination and Immunohistochemistry. Histological sections (5 µm in thickness) were stained with hematoxylin-eosin. Morphometric analysis of cross-sectional areas was performed on arterial sections by using computer-assisted

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image analysis (NIH image). Furthermore, sections were immunohistochemically stained for MMP-2, and MMP-9. In brief, 5-µm paraffin sections were deparaffinized and endogenous peroxydase was destroyed with 0.3% H_2O_2 in 100% methanol. Antigen was unmasked by 0.1% trypsin for 10 min at 37°C. Tissues were incubated with a monoclonal antibody that recognized the MMP-2 and MMP-9 for 1 hour at room temperature. A standard LSAB technique (DAKO, Glostrup, Denmark) was used to detect the reaction products.

Zymography. Proteins with gelatinolytic activity were identified by electrophoresis in the presence of sodium dodecyl sulfate (SDS) in 10% polyacrylamide gels containing 1 mg/mL gelatin (Mulsch et al., 1997). After sonication, tissue extracts were loaded into gels directly. After electrophoresis, the proteins in the gels were renatured by removing SDS with Triton X-100 (two 15-minute incubation in 2.5% Triton X-100). Subsequently, gels were incubated at 37°C in 50 µM Tris-HCl, pH 7.4, containing 10 µM $CaCl_2$ and 150 µM NaCl for 24 hours. At the end of incubation, gels were stained with 0.25% Commassie Blue for 1 hour. All results were quantified with densitometer.

Measurements of MMP-2 and MMP-9 activity by enzyme-linked immunosorbent assay. Substrate-linked enzyme-linked immunosorbent assay (ELISA) techniques were used to quantify enzymatic activity of individual MMPs.

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The samples were thawed on ice and all reagents needed for the assay were brought to room temperature. The MMP-2 and MMP-9 activity assay (Amersham Biotech, Freiburg, Germany) was performed according to the manufacturer's instructions.

Western Blot Analysis. Arterial tissues were homogenized in cold lysis buffer, sonicated, and SDS–PAGE was performed on 7.5% gels for MMP-2 and MMP-9. The blots were electrophoretically transferred to PVDF membranes and blocked with 5% non-fat milk for 1 hour. Membranes were incubated with either a monoclonal antibody specific for mouse MMP-2 or MMP-9 in PBS overnight at 4°C. After incubation with appropriate secondary antibodies, blots were incubated in enhanced chemiluminescence reagents (Amersham) and exposed to photographic film to detect protein expression.

RT-PCR Analysis. RNA was extracted from homogenized tissue with Trizol reagent by a standard protocol (Invitrogen, Carlsbad, CA). Reverse transcription was performed with 5 μg mRNA and random primer at 65°C for 5 min, then mixed with M-MLV RT to react at 37°C for 1 hour to obtain cDNA. Gene amplification was followed with reverse transcriptase-polymerase chain reaction. Primer sequence was as shown in Table 1. Reaction cycles for MMP-2, MMP-9 and GAPDH includes 94°C for 5 min, 30 cycles of 94°C for 1 min, 60°C for 1 min (55°C for GAPDH), 72°C for 1 min, and a final incubation at 72°C for 10 min. PCR products were analyzed on

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1.5% agarose gel in the presence of 1 μg/ml of ethidium bromide. The intensities of the cDNA bands were normalized to GAPDH band intensities.

MMPs Activities Assay. The exzymatic activity of MMPs was assayed using the fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2. Briefly, samples were mixed with 50 mM HEPES (pH 7.2), 10 mM $CaCl_2 \bullet 2H_2O$, 10 μM $ZnCl_2$, 0.05% Brij 35, and DMSO (1% v/v), containing 2 μM substrate. Each assay was carried out at 37°C for 3 hr, in the dark. After incubation, substrate hydrolysis was measured using spectrofluorimeter, with excitation and emission wavelengths set at 325 and 387 nm, respectively.

Determination of Arterial cGMP Content. All procedures followed manufacturer's instructions (Amersham Pharmacia Biotech). Briefly, after sonication, 100 μg samples were loaded into 96-well cGMP assay plate. Sample competes with cGMP conjugate to the cGMP binding site on the plate, and then as substrate is injected into plate, substrate interacts with cGMP conjugate to produce blue color. Hence, the more dark blue is the color, the less cGMP is the sample.

Statistical Analysis. Data are presented as the means plus or minus SEM for the indicated number of separate experiments. Statistical analysis of data was performed with one-way analysis of variance (ANOVA) followed by a *t*-test and *P*-values less than 0.05 were considered significant.

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Results

Effect of YC-1 on neointimal thickness. The effect of YC-1 on neointimal hyperplasia was quantified by histomorphometric analysis of carotid arterial sections after balloon injury. As shown in Figure 1, uninjured collateral arteries did not exhibit intimal thickening (Fig. 1A); on the fourteenth day after balloon injury to the left carotid artery, its neointima was markedly increased (Fig. 1E). Orally administered YC-1 at 1 mg/kg/day had a low potency in inhibiting neointima formation; we observed uneven intimal hyperplasia of the vascular wall (Fig. 1F). In contrast, YC-1 at 5 or 10 mg/kg/day markedly decreased intimal thickening (Fig. 1G, 1H). YC-1 had no effect on vascular structure of collateral arteries from the same rats (Fig. 1B-1D).

Effect of YC-1 on MMP-2 and MMP-9 protein expression after injury. In order to examine the effect of YC-1 on expression of MMP-2 and MMP-9 after balloon injury, vascular sections were stained for specific MMP antibody. Morphometric analysis of common carotid arteries revealed a marked positive staining for both MMP-2 and MMP-9 protein in the intima of injured carotid arteries (Fig. 2C and 2E) compared with control arteries (anti-IgG group, Fig. 2A) on the fourteenth day after balloon injury. However, oral administration of 10 mg/kg YC-1 significant inhibited MMP-2 and MMP-9 protein expression in carotid artery sections after balloon injury (Fig. 2D

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and 2F). Next, we analyzed artery extracts for MMP-2 and MMP-9 protein expression using Western blot analysis. MMP-2 and MMP-9 protein expression markedly increased in injured artery, but were inhibited to baseline with YC-1 treatment of 5 or 10 mg/kg (Fig. 2G).

Effects of YC-1 on post-injury MMP activity by gelatin zymography and ELISA assay. We studied the suppressive effects of YC-1 on MMP-2 and MMP-9 activity in balloon-injured carotid vessels using zymographic analysis and ELISA assay. As shown in Figure 3, YC-1 (1-10 mg/kg) inhibited injury-evoked MMP-2 and MMP-9 activity in dose-dependent manner on the fourteenth day after balloon injury.

Effects of YC-1 on MMP activity by quenched fluorescence substrate hydrolysis. In order to directly test for inhibition of enzyme activity, we performed a concentration-effect study of YC-1 action on recombinant MMP-2 and MMP-9 activity with use of the fluorescence quenching substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2. Concentration-dependent inhibition of YC-1 on MMP-2 and MMP-9 activity is shown in Table 2. Significant inhibition was demonstrated at YC-1 concentrations as low as 1 μM; at 10 μM, YC-1 almost completely abolished enzyme activity. The IC_{50} values for YC-1 were 2.07 and 8.20 μM for MMP-2 and MMP-9, respectively.

Effect of YC-1 on MMP mRNA expression. In addition, we analyzed mRNA

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expression of MMPs by RT-PCR. Each experiment included a pool of 3~6 independent animals treated with the same conditions. We observed that levels of both MMP-2 and MMP-9 mRNA were increased on the fourteenth day after balloon injury and that YC-1 suppressed both, but was more effective at inhibiting MMP-9 than MMP-2 (Fig. 4). These data suggest that YC-1 inhibits MMP-2 and –9 protein productions, activities, and mRNA expressions after balloon injury.

Effect of YC-1 on cGMP content. To evaluate the effect of balloon injury and YC-1 on cGMP formation in rat carotid arteries, sections from injured and uninjured vessels in 0.5% CMC- or YC-1-treated groups were assayed using a cGMP enzyme-linked immunosorbent assay (ELISA) kit. After balloon-injured 5th hour and 14th day time point, we did not observe any elevation of cGMP levels in the presence or absence of YC-1-treated rats (Fig. 5). This result indicates that YC-1 suppressed MMP-2, MMP-9 activity and expression via a cGMP-independent pathway.

Discussion

In this study, we demonstrated that YC-1, an indazole derived compound, significantly inhibits balloon-injury-induced neointimal formation as well as production of MMP-2 and MMP-9, two proteins that have been implicated in intimal matrix breakdown. Proliferation and migration of VSMCs play a major role in intimal lesion formation (Liu et al., 1989; Pauletto et al., 1994). In previous studies, YC-1 has

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been shown to significantly inhibit VSMC proliferation through a cGMP-dependent pathway (Tulin et al., 2000; Wu et al., 2004) and to inhibit expression of TGF-β and FAX (Wu et al., 2004). However, all of these experiments have used topical administration of YC-1 to study inhibition of neointimal formation after balloon-induced carotid artery injury. In this study, we demonstrated that low-dose oral administration (1-10 mg/kg) of YC-1 markedly prevents neointimal formation. Base on pharmacokinetic analysis, this dose range do not induce sGC activity (unpublished data); for that to happen, the dose must be higher than 100 mg/kg. On the other hand, we evaluated the effect of balloon injury and YC-1 on cGMP formation in rat carotid arteries and did not find an increased level of cGMP; this observation is in contrast to previous reports (Tulis et al., 2000; Tulis et al., 2002). We also did not observe elevation of cGMP levels in YC-1-treated rats, suggesting that the therapeutic effect of YC-1 on restenosis may not involve a cGMP-elevating pathway.

It is well established that MMPs play important roles in mediating intimal thickening after vascular injury (Kuzuya et al., 2003; Galis et al., 2002). MMPs, important zinc-dependent proteinases responsible for matrix degradation, are involved in VSMC proliferation and migration, two key steps in intimal hyperplasia (Kuzuya et al., 2003; Galis et al., 2002). Southgate and colleagues (1996) also demonstrated that

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MMP-2 and MMP-9 mRNA expression increased markedly following vascular injury and that the proteinases played a role in migration and proliferation of VSMCs. Consistent with these latter observations, we observed marked thickening characteristic of neointimal formation on the fourteenth day after balloon injury. MMP-2 and MMP-9 clearly participated in neointima formation based on our demonstration that both activity and protein expression of MMP-2 and MMP-9 increased almost at the same time. In the present study, we demonstrated that YC-1 inhibited MMP activity as well as protein and mRNA expression. On the other hand, our data revealed that YC-1 could suppress MMP-2 and MMP-9 enzyme activity with an IC_{50} in the micromolar range. As far as we know, this is the first report to show that YC-1 is capable of inhibiting MMPs after balloon injury in rats.

Increased MMP activity is associated with a wide variety of pathological conditions such as restenosis and atherosclerosis (Kuzuya et al., 2003; Galis et al., 2002). The potential utility of MMP inhibitors as treatment for these diverse disease states is obvious, and several MMP inhibitors are currently being tested in clinical trials (Ramnath and Creaven, 2004). However, undesirable side effects have greatly limited clinical use to date (Overall and Lopez-Otin, 2002). Several recent studies have suggested that MMP-mediated vascular remodeling in response to inflammatory effects could be modulated by antioxidants (Galis et al., 1998). Our recent study has

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shown that YC-1 is capable of inhibiting LPS-induced iNOS, TNF-α, and cytokine release through down-regulation of NF-κB activity (Pan et al., 2005). It is possible that YC-1 down-regulates MMP-2 and MMP-9 via diminished transactivation because the 5'-flanking region of rat MMP genes contain binding sequences for the NF-κB transactivating molecule. Hence, YC-1 may inhibit MMP activation through a decreased inflammatory response, and this inspection needs more investigation. Moreover, we demonstrated that YC-1 inhibited MMP expression on the fourteenth day after balloon injury. This late phase was prone to a response of vascular VSMC proliferation and matrix secretion, but it was independent of the early post-injury inflammatory response. We demonstrated that MMP-2 and MMP-9 mRNA were upregulated on the fourteenth day after balloon injury and that YC-1 was capable of inhibiting MMP mRNA upregulation as well as other known MMP inhibitors. We conclude that YC-1 inhibits MMP activity through inhibition of late-phase VSMC proliferation, with the result of suppressing neointimal hyperplasia. In summary, we clearly demonstrated *in vivo* that oral administration of YC-1 inhibits neointima formation and MMP activation after balloon injury to the carotid artery. Based on these observations, YC-1 shows therapeutic potential for prevention of restenosis after angioplasty.

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Footnotes:

1. Yi-Nan Liu and Shiow-Lin Pan contributed equally to this work.

2. This work was supported by research grants from National Science Council of the Republic of China (NSC 94-2811-B-002-017).

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Legends for Figures

Fig. 1. Effects of YC-1 on the intimal hyperplasia of the carotid arteries 14 days after injury. Hematoxylin-eosin staining sections of carotid arteries from the control (A and E) and YC-1 (1-10 mg/kg)-treated (B-D and F-H) groups. Magnification for all photomicrographs is 40×. "N" represents neointima and "M" represents media. (I) Data are also quantified by the neointima/media ratio of common carotid arteries after balloon injury from each group of animal studies. Data are expressed as mean±SEM of six determinations. * $P < 0.05$ and ** $P < 0.01$ were compared with control group.

Fig. 2. Effect of YC-1 on expression and location of MMP-2 and MMP-9 after balloon injury. Immunohistochemical staining was performed by MMP-2 and MMP-9 antibody from the control (A, C and E) and YC-1 (10 mg/kg)-treated (B, D, and F) groups after balloon-injured carotid arteries, as detailed in the Methods section. Staining was performed in A and B, C and D, E and F using anti-IgG, anti-MMP-2 and anti-MMP-9 antibody, respectively. Magnification×200. Representative immunostaining sections are shown from six animals in each treatment group. (G) MMP-2 and MMP-9 proteins expression were identified by Western blot analysis using anti-MMP2 and anti-MMP-9 antibodies.

Fig. 3. Effects of YC-1 on MMP-2 and MMP-9 gelatinase activities at 14 days after balloon injury. Arteries were harvested from rats treated with 0.5% CMC、YC-1 (1, 5,

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and 10 mg/kg/day) for 3 days before injury. After injury, rats were treated with the same drug for the following 14 days. Proteins were harvested from injured and uninjured carotid arteries after balloon injury. (A) Equal amounts of protein (10 μg) were resolved on 10% gelatin zymography gels containing 0.1% gelatin. Zymograms were scanned, and MMP-2 (B) or MMP-9 (C) activity was quantified. "Basal group", arteries were harvested from uninjured carotid arteries. "Control group", arteries were harvested from injured carotid arteries. * $P < 0.05$ compared with uninjured carotid arteries of rats, and # $P < 0.05$ compared with injured carotid arteries of rats at 14 days after balloon injury. Data are means ± SEM for six independent experiments.

Fig. 4. Effects of YC-1 on MMP-2 and MMP-9 mRNA expression at 14 days after balloon injury. (A) MMP-2 and MMP-9 mRNA expression by RT-PCR and (B) quantified by densitometry. "Basal", arteries were harvested from uninjured carotid arteries. Data are presented as mean ± SEM. Each result is from 2-4 independent experiments, and each experiment includes mean of 3-6 independent animals in panel. # $P < 0.05$ compared with uninjured carotid arteries of rats, and * $P < 0.05$, *** $P < 0.001$ compared with injured carotid arteries of rats at 14 days after balloon injury.

Fig. 5. Effect of YC-1 on cGMP formation after balloon injury. Arteries were harvested at 5th hour or 14th day after balloon injury. Rats were treated with 0.5% CMC or YC-1 10 mg/kg/day, respectively. Data are presented as mean ± S.E.M.

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Different time points are 6 independent animals.

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Table 1

Synthetic oligonucleotide primers used for reverse transcription-polymerase chain reaction (PT-PCR)

Target gene	**Primer sequence**	**Length of PCR product (base pairs)**
MMP-2	Forward: 5'-CCCCTATCTACACCTACACCAAGAAC-3' Reverse: 5'-CATTCCAGGAGTCTGCGATGAGC-3'	576
MMP-9	Forward: 5'-AGTTTGGTGTCGCGGAGCAC-3' Reverse: 5'-CATTCCAGGAGTCTGCGATGAGC-3'	755
GAPDH	Forward: 5'-TGATGACATCAAGAAGGTGGTGAAG-3' Reverse: 5'-TCCTTGGAGGCCATGTGGGCCAT-3'	240

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Table 2

IC_{50} values and inhibition for YC-1 on the activity of MMP-2 and MMP-9

	MMP-2		MMP-9	
YC-1 concentration (μM)	% inhibition	IC_{50} (μM)	% inhibition	IC_{50} (μM)
10	102		59	
1	18	2.07 ± 0.11	3	8.20 ± 0.34
0.1	4		2	
0.01	2		0	

Value are given as means ± s.e.m. (n=3). The IC_{50} values for YC-1 (0.01-10 μM) was determined using the MMPs substrate assay. Details are described in the "Methods" section.

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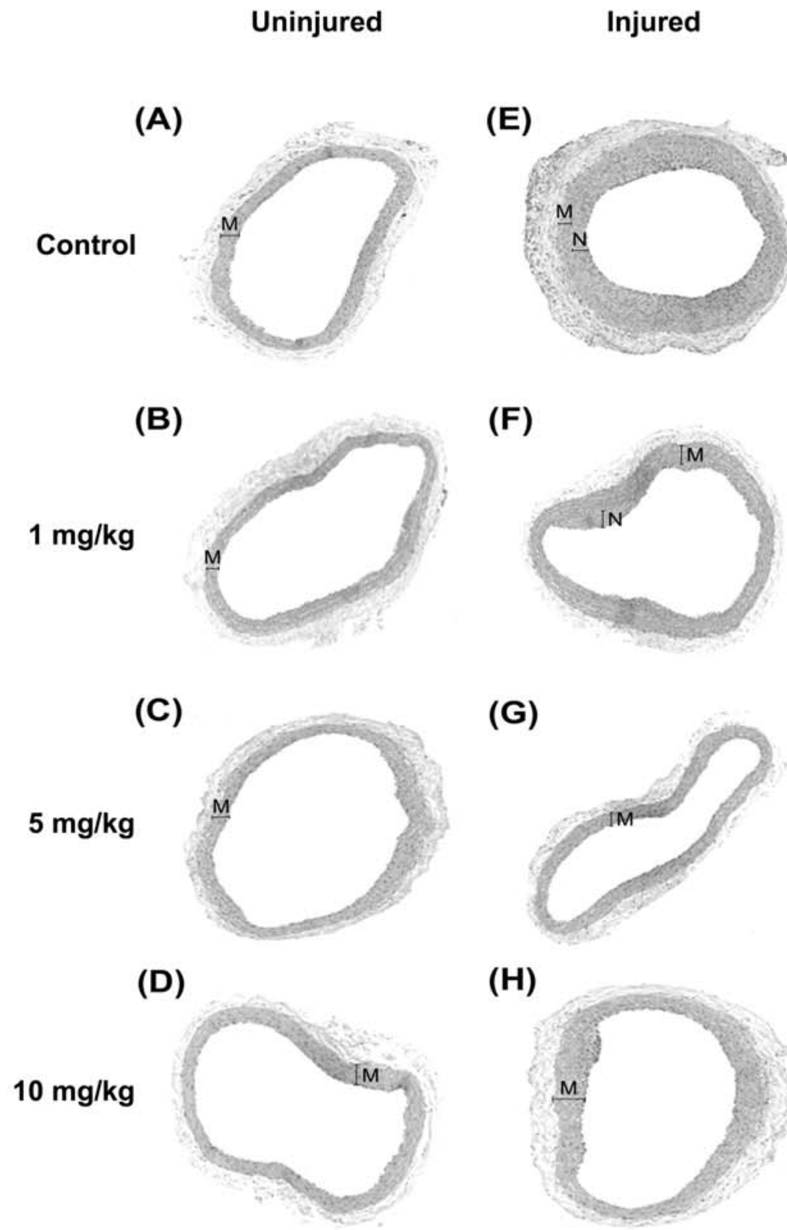
Fig. 1



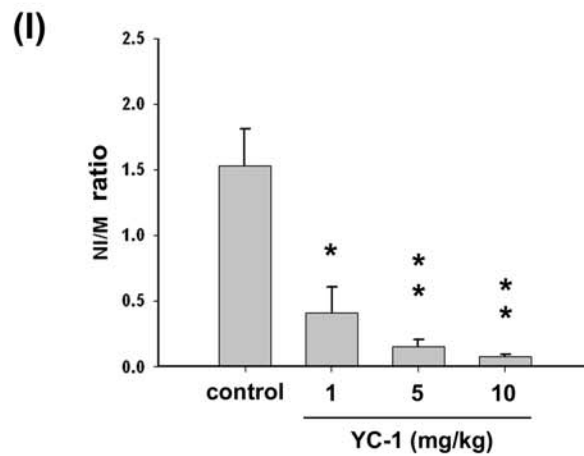


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Fig. 2

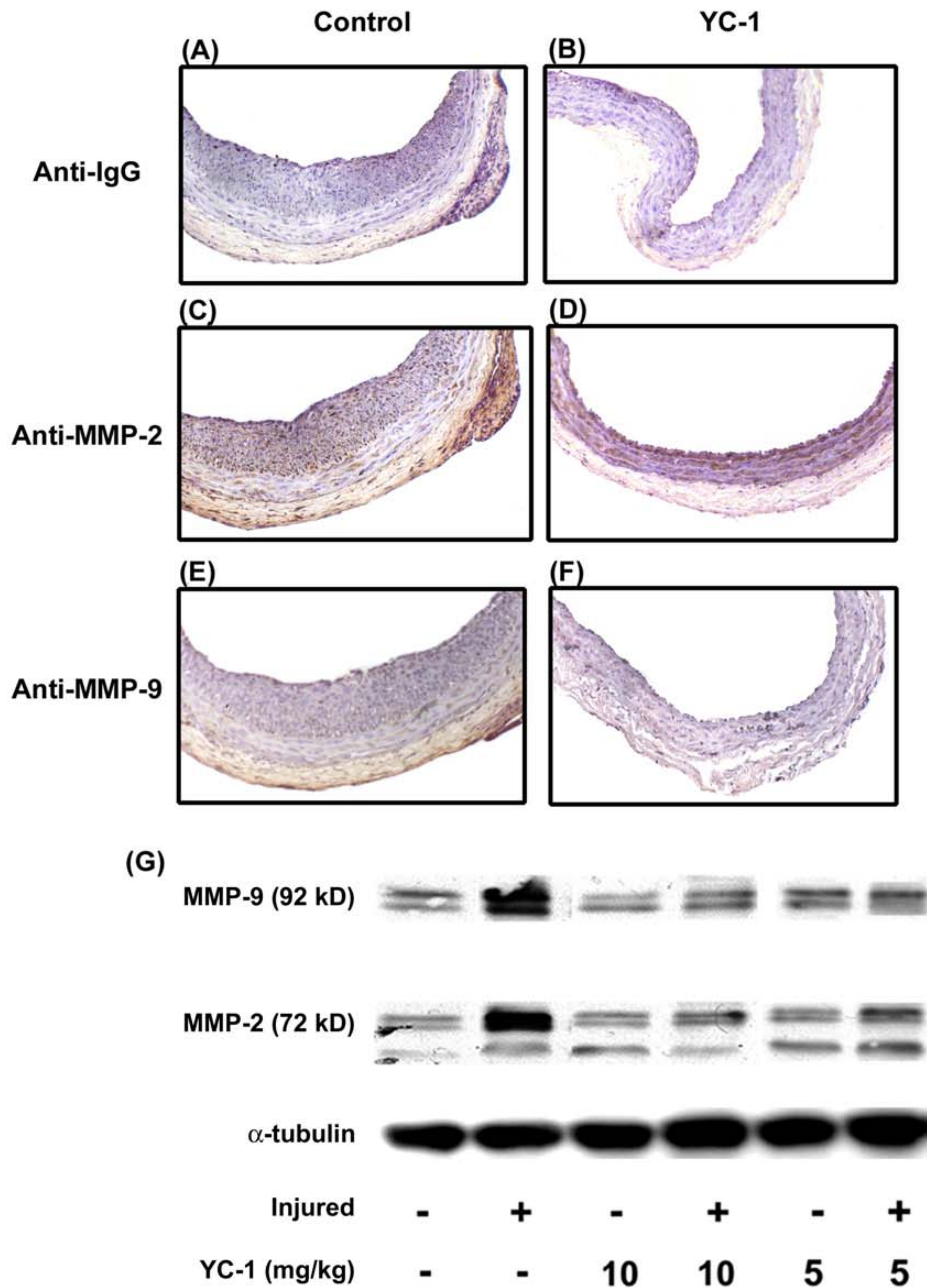

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Fig. 3

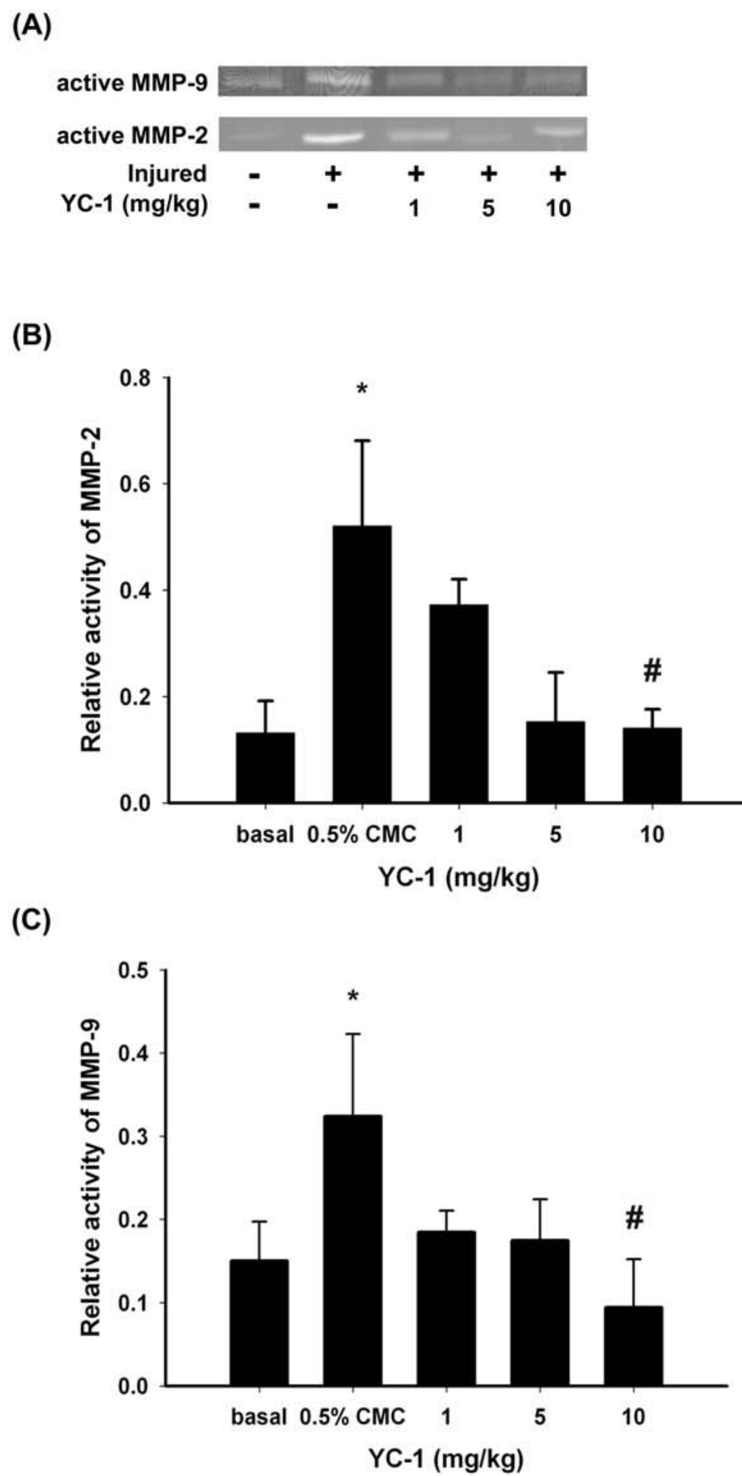

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Fig. 4

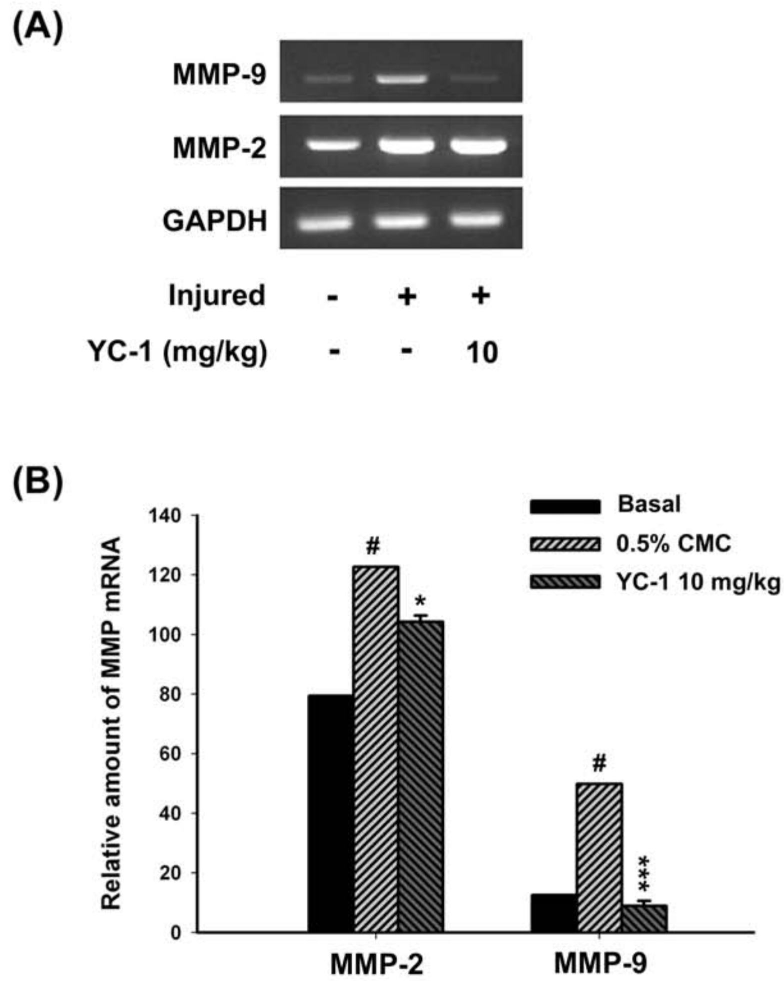

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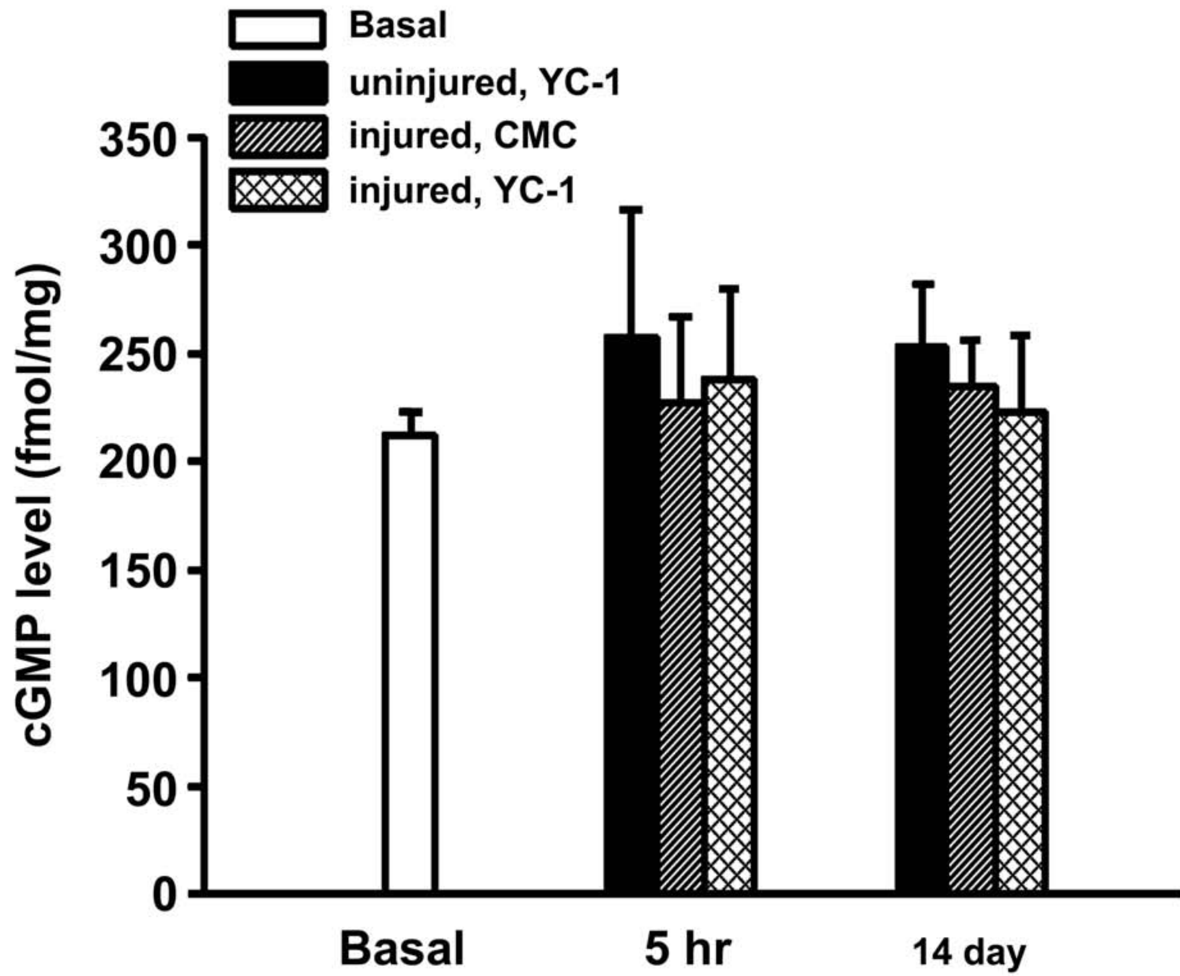
Fig. 5



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