Theme Issue Article

The indazole derivative YD-3 inhibits thrombin-induced vascular smooth muscle cell proliferation and attenuates intimal thickening after balloon injury

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Summary

Proliferation of vascular smooth muscle cells (VSMCs) is postulated to be one of the key events in the pathogenesis of atherosclerosis and restenosis. We investigated whether YD-3, a low-molecular weight, non-peptide compound, could modulate proliferation of VSMCs *in vitro* and restenosis after balloon angioplasty *in vivo*. We examined the effect of YD-3 on thrombin-induced VSMC proliferation by [^{3}H]thymidine incorporation assay. The data demonstrated that YD-3 inhibited VSMC proliferation in a concentration-dependent manner. To define the mechanisms of YD-3 action, we found that YD-3 showed a profound inhibition on thrombin-induced Ras and ERK1/2 activities by using Western blotting analysis. Furthermore, oral administration of YD-3 exhibited a marked reduction in neointimal thickness using the carotid injury model in rats. Using immunochemical detection, our experiments also revealed that YD-3 significantly suppressed expression of the PAR-1 receptor, and markedly inhibited PAR-1-activating peptide (SFLLRN)-induced VSMC proliferation in a concentration-dependent manner. These results suggest that YD-3 inhibits thrombin-induced VSMC growth via the Ras- and ERK1/2-mediated signaling pathway. Moreover, YD-3 also shows a developmental potential in the treatment of atherosclerosis and restenosis after vascular injury.

Keywords

YD-3, vascular smooth muscle cells, thrombin, restenosis, signal transduction

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Introduction

Thrombin, a serine protease derived from its precursor prothrombin, plays an important role in thrombosis and haemostasis as well as mediator of cellular effects that contribute to inflammation reactions and proliferation of vascular smooth muscle cells in the vessel wall (1, 2). Many of the functions of thrombin are mediated via activation of G protein-coupled protease-activated receptors, PAR-1, PAR-3, or PAR-4 (3-5). PARs are activated in an unusual, irreversible proteolytic mechanism in which the protease binds to and cleaves the amino-terminal exodomain of the receptor. This new amino terminus then binds intramolecularly to the body of the receptor to initiate transmembrane signaling (6).

Percutaneous transluminal coronary angioplasty (PTCA) is commonly used to repair occluded atherosclerotic blood ves-

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sels. Coronary angioplasty has resulted in a restenosis rate as high as 30-50% at 6 months (7-9). VSMC migration and proliferation are central to the pathology of injury-induced intimal hyperplasia and restenosis (10, 11). In fact, increased thrombin receptor expression was observed in neointimal cells throughout the vascular lesion formation (12, 13) and in the atherosclerotic plaque (14). However, the role of thrombin receptor activation in restenosis after balloon angioplasty and in progression of atherosclerosis remains unclear owing to the lack of suitable antagonists that can inhibit specifically the thrombin receptor signaling pathway.

In the previous study (15), we showed that YD-3, a newly synthetic indazole derivative, selectively inhibits rabbit platelet aggregation and phosphoinositide breakdown caused by thrombin. Alternatively, we also found that YD-3 selectively inhibits PAR-4-dependent platelet activation through blockade of PAR-4 and PAR-4-mediated thromboxane formation (16, 17). Recently, there are several reports finding that the thrombin receptor, especially PAR-1, is up-regulated in VSMC in response to vascular injury in animal models (13, 18). In the present study, we have identified that YD-3 significantly inhibits the thrombin-induced signal via Ras/extracellular-signal-regulated kinase (ERK), which critically influences cell proliferation in VSMC *in vitro* and attenuated the restenosis after balloon angioplasty *in vivo*.

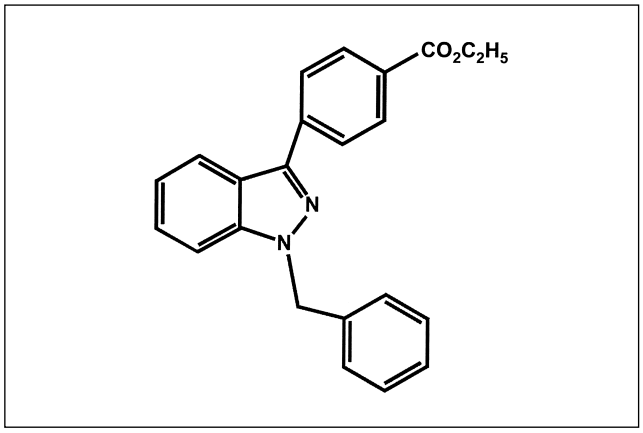

Figure 1: Structure of YD-3 [1-benzyl-3(ethoxycarbonyl-phenyl)-indazole].

Materials and methods

Cell culture

VSMC were isolated from the thoracic aorta of Wistar rats and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Passages before 7^{th} generation were used for the experiments. In order to characterize the isolated and cultured VSMCs and to exclude contamination by endothelial cells and fibroblasts, the cells were identified by immunofluorescent staining method using a monoclonal antibody of α-actin.

[^{3}H]Thymidine incorporation assay

Confluent VSMCs were trypsinized and seeded at 1.0×10^4 cells per well into 96-well plates. Cells were incubated with or without YD-3 (Fig. 1) (Yung-Shin Pharmaceutical Industry Co, Ltd, Taichung, Taiwan) treated with thrombin (2 U/ml) for 24 hours, and then harvested for the detection of DNA synthesis. Before the harvest, cells were incubated with [^{3}H]thymidine (1 μCi/ml, Amersham Pharmacia) for 16 hours and then processed and harvested with Filter-Mate (Packard), and the incorporated radioactivity was determined.

Western blot analysis

Cultured VSMCs were lysed in a standard lyses buffer. The cell lysate was centrifuged and the supernatant was used for the Western blotting analysis as described previously (19). ERK1/2 and phosphorylated ERK1/2 antibodies (Santa Cruz Biotechnology) diluted 1 : 1000 in phosphate-buffered saline (PBS), were used as primary antibodies and were applied for overnight incubation at 4°C. After several washing procedures and the conjugation with anti-rabbit immunoglobulin-HRP secondary antibodies, the membranes were subjected to enhanced chemiluminescence (ECL, Amersham) to obtain the protein expressions.

Determination of Ras activity

The active GTP-bound form of Ras was detected by using glutathione S-transferase (GST) fusion protein corresponding to the Ras-binding domain (RBD) of Raf-1 bound to glutathione-agarose according to the manufacturer's protocol (Upstate Biotechnology). The proteins were resolved by 12% SDS-PAGE and transferred onto PVDF membranes. Ras was detected by immunoblot analysis with anti-Ras antibody.

Restenosis model and histological examination

The Wistar rats were anaesthetized with an intraperitoneal administration of sodium chlorohydrate (37 mg/kg) and a 2F Fogarty arterial embolectomy balloon catheter was inserted into the left carotid artery. YD-3 (10 mg/kg/day) was orally administered into the rat 3 days before to 14 days after the vascular injury. However, fourteen days after injury, rats were euthanatized with intraperitoneal administration of pentobarbital (60 mg/kg), left and right (control group) carotid arteries were removed, fixed with 4% formaldehyde, stained with hematoxylin-eosin. Six sections in each slide were analyzed by means of computerized morphometric analysis (NIH image) by an investigator blinded to the type of the experimental group. The degree of neointimal thickening was expressed as the ratio between the neointima area to the area of the media (N/M).

Immunohistochemistry assay

PAR-1 was characterized by immunohistochemistry on paraffin sections. In brief, 5-μm paraffin sections were deparpaffinized and endogenous peroxidase was quenched with 0.3% H_2O_2 in 100% methanol. Antigen was unmasked by heating the sections for 15 min in 10 mM citrate buffer pH6.0 using a microwave, followed by 60 min cooling in the same buffer. Tissues were incubated with a monoclonal antibody that recognizes the PAR-1 (BD Transduction Lab.) for 45 min at room temperature. A standard Labelled Streptavidin-biotin (LSAB) Technique (Dako, Glostrup, Denmark) was used to detect the reaction products. Sections were counterstained with hematoxylin and mounted.

Statistical analysis

Data are presented as the mean ± 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.

Results

YD-3 selectively inhibits thrombin-induced cell proliferation on rat VSMCs

In our previous study, YD-3 was found to be a potent PAR-4 antagonist to human platelets (15). To examine the effect of YD-3 on the thrombin (2 U/ml)-mediated rat VSMCs growth, [^{3}H]thymidine incorporation was used to determine the DNA synthesis. As demonstrated in Figure 2, YD-3 significantly inhibited the thrombin-induced increase of DNA synthesis in a concentration-dependent manner (IC_{50} = 7.3 × 10^{-6} M) (Fig. 2A). However, the thrombin-antagonizing action of YD-3 (1-100 μM) was verified by its failure to inhibit VSMC proliferation stimulated by either platelet-derived growth factor (PDGF) (Fig. 2B), a growth factor to binding protein tyrosine kinase receptor, or 5-hydroxytryptamine (5-HT) (Fig. 2C), a strong mitogen to binding G-protein coupled receptor. Furthermore, YD-3 shown little influence on the release reaction of lactate dehydrogenase (data not shown) revealing that the antiproliferative action of YD-3 was not due to its cytotoxic effect. These results suggested that YD-3 was a specific thrombin inhibitor in depressing rat VSMC proliferation.

Effect of YD-3 on ERK1/2 phosphorylation induced by thrombin

It has been established that MAPKs, one group of components in the signalling pathway, have shown to be activated during stimulation of cell proliferation (20). Therefore, we determined whether YD-3 inhibited thrombin-induced activated ERK1/2 on rat VSMCs. The data showed that thrombin induced a profound increase of ERK1/2 activation. Both YD-3 and PD98059, a

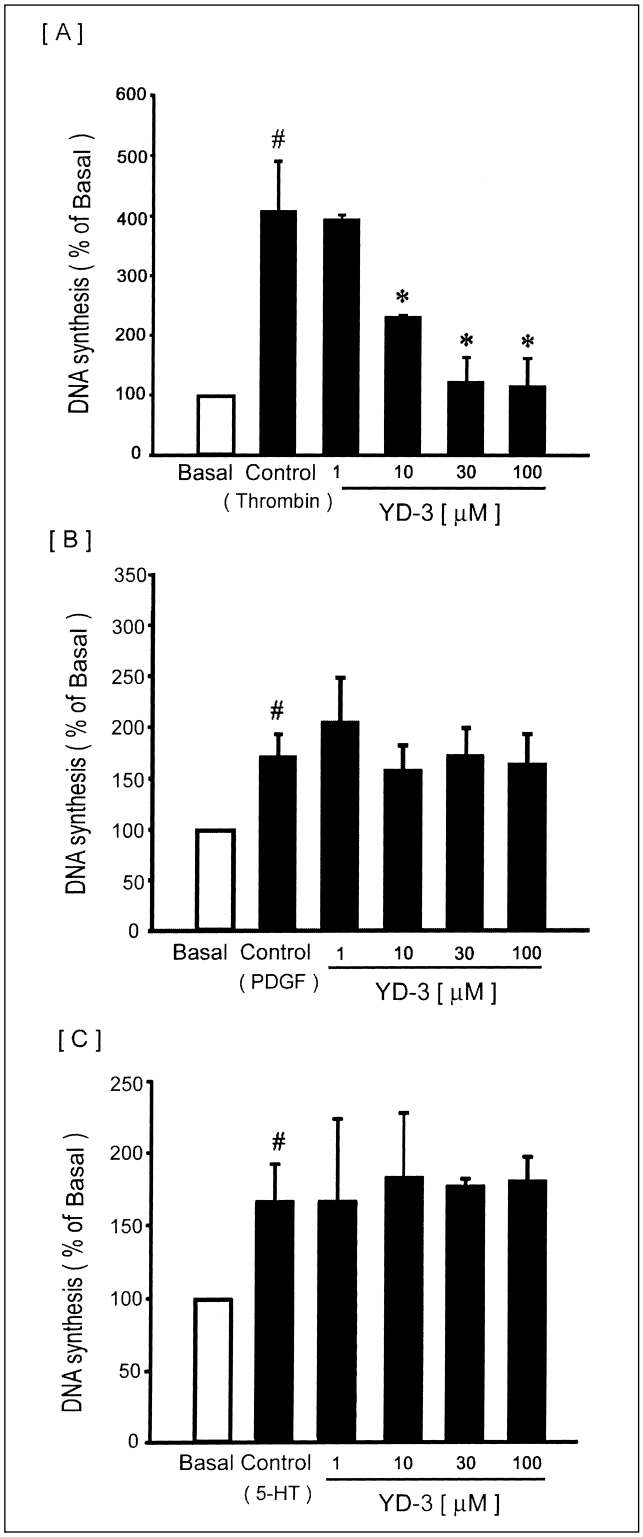

Figure 2: Effects of YD-3 on thrombin-, PDGF- and 5-HT-induced cell proliferation in rat VSMCs. Rat VSMCs were stimulated with thrombin (2 U/ml) (A), or PDGF (50 ng/ml) (B), or 5-HT (50 μM) (C) in the absence or presence of various concentrations of YD-3 for 24 hours, and incubated with [^{3}H]thymidine (1 μCi/ml) for 16 hours. Data were expressed as mean ± s.e.mean of three determinations. #P <0.05, *P <0.05 were compared with basal and control group, respectively.

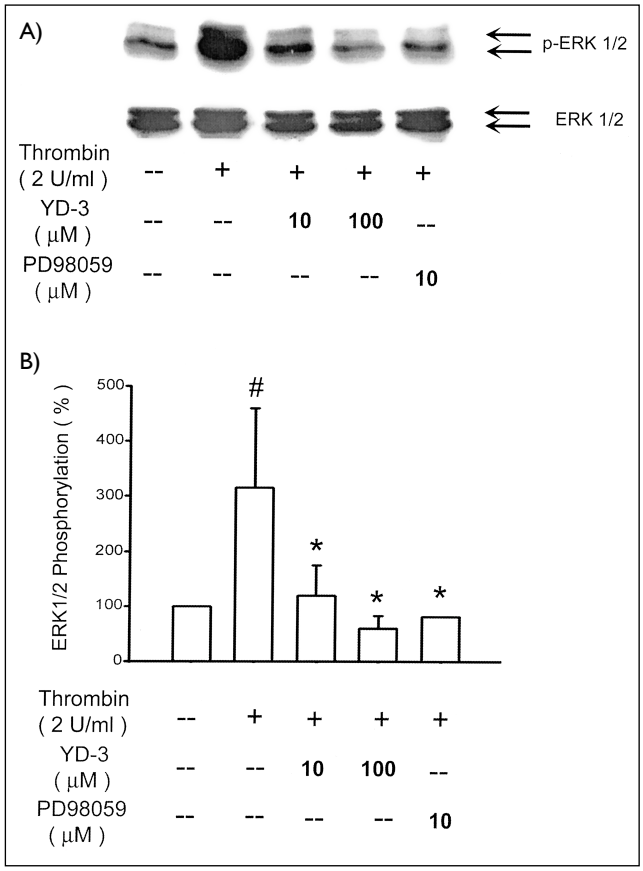

Figure 3: Effect of YD-3 on ERK1/2 phosphorylation induced by thrombin. Rat VSMCs were pretreated 1 hour with YD-3 or PD98059, and stimulated with 2 U/ml thrombin (A) for 10 min. Then cells were lysed, and equal protein quantities were electrophoresed and Western-blotted as described in the "Materials and methods" sections. (B) The density of specific band was scanned and quantified with an imaging analyzer. The ratio of phosphorylated ERK1/2 to total ERK1/2 is shown. The ratio of untreated cells was designated as 1.0. Data were expressed as mean ± s.e.mean of three determinations. #P <0.05, *P <0.05 were compared with non-treated and thrombin-treated group, respectively.

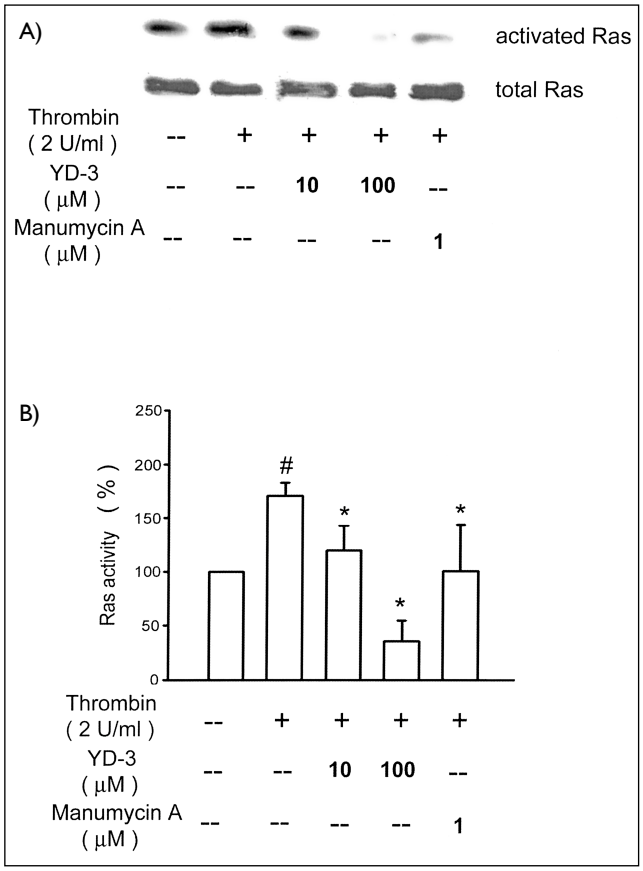

Figure 4: Effect of YD-3 on Ras acitivity induced by thrombin. (A) Cells were made quiescent for 24 hours, then incubated in the absence or presence of YD-3 or manumycin A for 1 hour, and vehicle or thrombin was added to the cells for another 5 minutes. Cells were harvested and the pan-Ras activity was measured as the amount of Ras precipitated with Raf-1(RBD)-GST as described in the "Materials and methods" section. (B) The density of specific band was scanned and quantified with an imaging analyzer. The ratio of activated Ras to total Ras is shown. The ratio of untreated cells was designated as 1.0. Data were expressed as mean ± s.e. mean of three determinations. #P <0.05, *P <0.05 were compared with non-treated and thrombin-treated group, respectively.

selective MAPK kinase (MEK) inhibitor, markedly inhibited the effects of thrombin (Fig. 3).

Effect of YD-3 on Ras activity induced by thrombin

Several lines of evidence have suggested that activated Ras stimulates the signaling cascades of cell proliferation (21). However, thrombin appears to exert its mitogenic effects through the activation of Ras/Raf/MEK/MAPK pathway (22). In addition, YD-3 inhibited thrombin-induced phosphorylation of ERK1/2, a downstream signalling pathway of activated Ras. We also examined the activity and total protein expression of Ras in the present work. As showed in Figure 4, thrombin caused rapid activation of Ras; however, YD-3 and manumycin A, a Ras farnesyltransferase inhibitor, profoundly inhibited the thrombin-induced effect (Fig. 4). These results suggest that YD-3 inhibits thrombin-induced mitogenic function through a Ras/MAPK signaling pathway.

Effect of YD-3 in a rat restenosis model

Since thrombin-mediated VSMC responses associated with vascular injury (e.g. cell proliferation) was inhibited by YD-3, this agent would be a good candidate to assess the role of thrombin in a rat balloon angioplasty model of vascular injury. The Wistar rats were assigned to receive oral administration of a vehicle control (0.5% carboxymethyl cellulose) or YD-3 (10 mg/kg/day) beginning three days before balloon angioplasty. At day 14 after angioplasty, animals were euthanized for the detection of

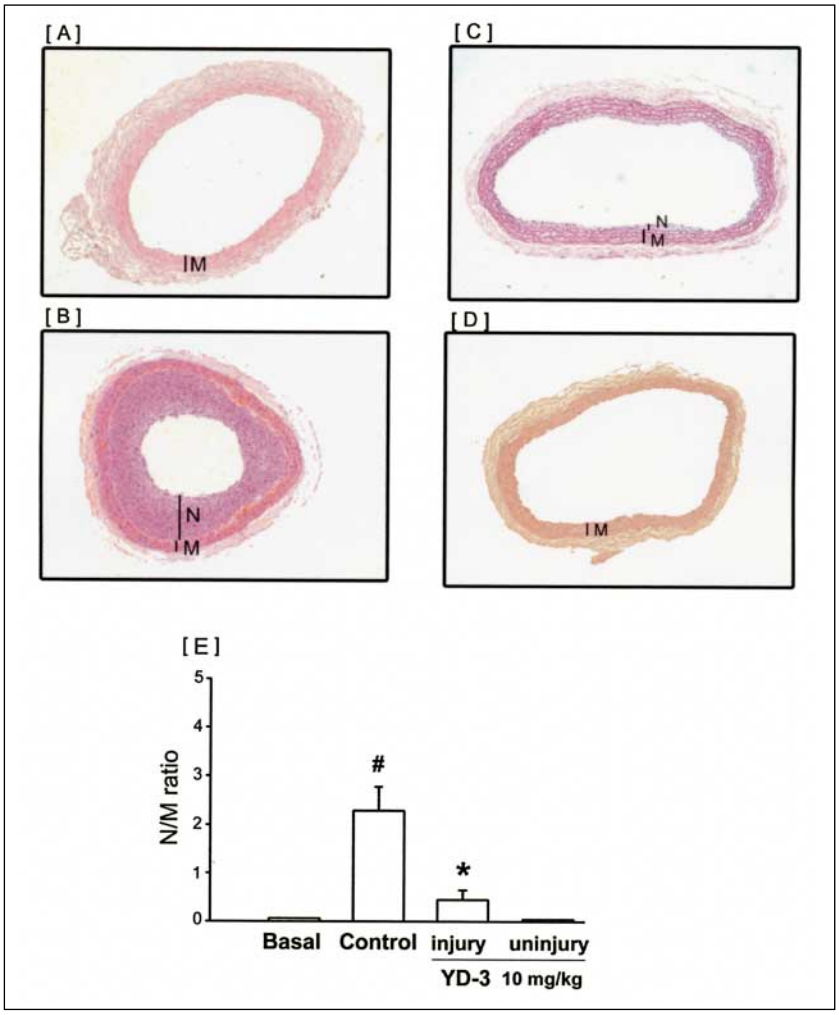

Figure 5: YD-3 inhibited neointima formation *in vivo*. Histological sections of rat carotid arteries 14 days after balloon-injury. (A) Artery from uninjured rats. (B) Artery from injured rats treated with vehicle (0.5% carboxymethoxyl cellulose) alone. Artery from injury (C) and un-injury (D) rats treated with orally administration of YD-3 (10 mg/kg). N: neointima; M: media. Magnification × 40. (E) 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 ± s.e. mean of five determinations. #P < 0.05, *P < 0.05 were compared with basal and control group, respectively.

vascular injury and intimal thickening. As demonstrated in Figure 5, the carotid artery without balloon angioplasty exhibited little changes in each area and thickness (Fig. 5A) and the injured vessel showed a profound neointimal thickening (Fig. 5B). However, there was a profound reduction in neointimal thickness in the YD-3-treated animals (Fig. 5C).

Effect of YD-3 on balloon injured-induced PAR-1 expression in carotid artery and PAR-1-induced inhibition of cell proliferation on rat VSMCs

To determine whether expressed PAR-1 is present in the biopsies of pigmented lesions after balloon angioplasty, immunohistochemical staining of paraffin-embedded vessel specimens were performed using a PAR-1 monoclonal antibody. As shown in Figure 6, the PAR-1 was displayed by balloon angioplasty (Fig. 6B). Oral administration of YD-3 significantly reduced the expression of PAR-1 in the balloon angioplasty rat (Fig. 6C). Based on this result, we examined whether YD-3 inhibited PAR-1-induced cell proliferation on rat VSMCs *in vitro*. Cells were incubated with YD-3 and a PAR-1-activating peptide (SFLLRN) using the [^{3}H]thymidine incorporation assay. As shown in Figure 6D, YD-3 significantly inhibited SFLLRN-induced cell proliferation in a concentration-dependent manner ($IC_{50} = 4.1 \times 10^{-6}$ M). These findings suggested that YD-3 might be a thrombin inhibitor, especially PAR-1, to regulate restenosis after balloon angioplasty in rat.

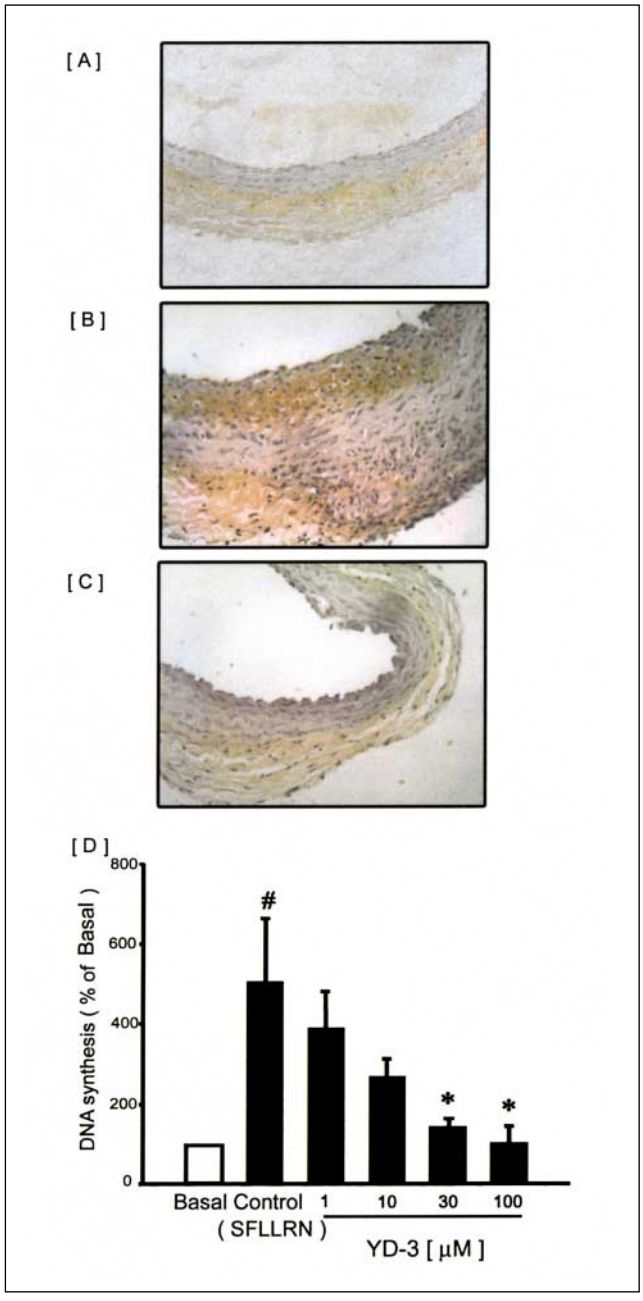

Figure 6: Effects of YD-3 on PAR-1 protein expression after vascular injury and PAR-1 agonist (SFLLRN)-induced cell proliferation in rat VSMCs. Immunohistochemical sections of rat carotid arteries 14 days after balloon-injury were performed in A. B. C. using the PAR-1 antibody 1 : 50 dilution by using the DAKO LASB$^+$ kit to give a brown final reaction product, and tissues were counterstained with hematoxylin. (A) Arteries from uninjured rats. (B) Arteries from injured rats treated with vehicle alone. (C) Arteries from injured rats treated with orally administration of YD-3 (10 mg/kg). Magnification × 200. (D) Rat VSMCs were stimulated with PAR-1 agonist (SFLLRN, 100 μM) in the absence or presence of various concentrations of YD-3 for 24 hours, and pulsed with [^{3}H]thymidine (1 μCi/ml) during the last 4 hours of stimulation. Data were expressed as mean ± s.e. mean of three determinations. #P < 0.05, *P < 0.05 were compared with basal and control group, respectively.

Discussion

There is considerable evidence that VSMC proliferation plays an important role in the development of atherosclerosis and restenosis following balloon angioplasty (23, 24). Vascular injury or coronary angioplasty results in activation of the coagulation cascade, which can induce more thrombin formation (25, 26). Thrombin plays an important role in coagulation and stimulates cell proliferation via the activation of G protein-coupled receptors in several cell types including VSMCs (27, 28).

In the previous studies, YD-3, a low-molecular weight, non-peptide compound, competitively inhibited thrombin-induced rabbit platelet aggregation and had no or little inhibitory effect on platelet aggregation elicited by PAF, collagen, arachidonic acid and U46619 (15). However, Wu determined that YD-3 selectively inhibited PAR-4-dependent platelet activation through the blockade of PAR-4. It is the first non-peptide, PAR-4 antagonist (16). In the present study, we demonstrated that YD-3 significantly inhibited thrombin-induced [^{3}H]thymidine incorporation in rat VSMCs. In contrast, YD-3 had no or little inhibitory effect on VSMC proliferation elicited by serotonin and PDGF.

The mitogenic effect induced by growth factors and G protein-coupled receptor agonists are mediated through the activation of MAPK, which regulates gene expression and cell cycle reentry to cell proliferation (29, 30). Several lines of evidence demonstrated that thrombin activates MAPK in a variety of cell types. In the present study, we demonstrated that thrombin-induced cell proliferation was mediated via activation of ERK1/2. However, thrombin-induced mitogenic signaling pathway was abolished by YD-3.

Extracellular signals transduced via receptor tyrosine kinases or G protein-coupled receptors activate Ras, a key switch in cellular signaling (31). Ras is a small guanosine triphosphate-binding protein that plays an important role in signal transduction pathways that influence cellular proliferation, apoptosis, cytoskeletal organization, and other important biological processes (32,33). In this study we observed that thrombin induced a rapid induction of Ras activity, which suggested that signaling via Ras was a major pathway responsible for the transduction of thrombin-induced mitogenesis in rat VSMCs. However, YD-3 significantly abrogated thrombin-induced Ras activity at the cellular level. In contrast, YD-3 did not inhibit PDGF-induced cell proliferation. Therefore, we suggest that YD-3 is not a specific Ras inhibitor to regulate thrombin-induced mitogenic function, and it may be to modulate the upper stream factors of the signaling pathway, such as activation of receptor and Src. Moreover, the signaling transduction of 5-HT is a G-protein coupled receptor/Src/ERK pathway (28). In the present study, YD-3 did not either suppress 5-HT-induced mitogenic function. Additional elucidation of detailed molecular mechanisms and the precise molecular target, such as receptor level, associated

with anti-thrombin function of YD-3 is the subject of further investigation.

In the previous report, thrombin is generated at the site of vascular injury. In normal arteries, the thrombin receptor is mainly expressed in platelets and arterial endothelial cells (14). In human atheroma, PAR-1 was found to be widely expressed within atheroma lesions, including macrophages and VSMCs (14). PAR-1 expression was induced at 6 hours after balloon injury of the carotid artery (13), and the upregulation of PAR-1 expression continued throughout vascular lesion formation for up to two weeks. These results suggest that the mechanisms of restenosis after balloon injury, thrombin and its receptor may play a central role in mediating the disordered proliferative actions for VSMCs (2, 14, 34). Since YD-3 effectively inhibited thrombin-mediated rat VSMCs proliferation, the *in vivo* effect would be carried out to examine its therapeutic potential. The balloon injury model has been the most frequently used *in vivo* model in restenosis study. Rats are regularly used because of the reproducible major formation of intimal hyperplasia within two weeks after balloon injury (35). Our data showed that balloon-catheter inflation caused injury in the rat common carotid artery and the intimal hyperplasia. YD-3 showed an *in vivo* potential to block the intimal thickening in the rat model. Therefore, based on this study, we were interested in determining whether there was a different distribution among neointima layers in the expression of thrombin receptors. In this study, we used immunohistochemical methods to describe PAR protein distribution in the neointima layer, showing PAR-1 over-expression in intimal hyperplasia; and then, the reduced expression of PAR-1 in the neointima layer seen in the YD-3-treated group, which accompanied significant inhibition of the neointima formation. In addition, it is well established that thrombin activates PAR receptors coupling to GTP-binding proteins, including PAR-1, PAR-3 and PAR-4 (36). However, on the basis of studies with human platelets, it appears that YD-3 specific block action of PAR-4. To date, neither functional data nor evidence of PAR-4 protein expression has been reported for rat VSMCs, and thus PAR-1 was considered to be the major thrombin receptor on these cells. In this study, we also demonstrated directly that the addition of YD-3 significantly inhibited the proliferative effect of PAR-1-activating peptide (SFLLRN) on rat VSMCs. These results revealed that YD-3 is major through PAR-1, however, do not rule out PAR-4 to limit thrombin-induced proliferation events after vessel injury.

In conclusion, we suggest that YD-3 exhibits the antiproliferative effect to thrombin action via the inhibition of Ras activity and the downstream effect on ERK1/2 phosphorylation. Oral administration of YD-3 may be a potential treatment for proliferative disorders of VSMCs such as atherosclerosis, or restenosis formation after percutaneous transluminal coronary angioplasty or carotid endarterectomy.

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