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GT-repeat length polymorphism in heme oxygenase-1 promoter determines the effect of cilostazol on vascular smooth muscle cells

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ABSTRACT

Background: Cilostazol, a potent type 3 phosphodiesterase inhibitor, is found to reduce neointimal formation by inhibiting vascular smooth muscle cell (VSMC) proliferation. The aim of this study is to investigate whether the inhibitory effect of cilostazol on VSMC proliferation is operated via heme oxygenase-1 (HO-1).
Methods and results: In rat carotid arteries, cilostazol up-regulated HO-1 in the neointima of balloon-injured arteries. Treatment of human VSMCs with cilostazol enhanced the expression of HO-1, which was mainly regulated at the transcriptional level. Small interfering RNA knock-down of HO-1 attenuated the inhibitory effect of cilostazol on VSMC proliferation, suggesting the critical role of HO-1 in cilostazol effect. The transcriptional responsiveness of HO-1 to cilostazol was inversely correlated with the length of GT-repeat in human HO-1 promoter. Deletion and mutational analysis of HO-1 promoter along with chromatin immunoprecipitation showed that cyclic AMP response element (CRE)-binding protein (CREB) participated in cilostazol-induced HO-1 transcription. Furthermore, cilostazol triggered a linkage between the CRE and GT-repeat regions in the HO-1 promoter. The promoting effect of cilostazol on HO-1 expression, proliferation inhibition, and chromatin conformation in the HO-1 promoter was greater in VSMCs from subjects with shorter GT-repeat alleles than those with longer alleles.
Conclusions: Cilostazol inhibits VSMC proliferation involving an association between CREB and HO-1. The length polymorphism of GT-repeat in human HO-1 promoter determines the effect of cilostazol.

1. Introduction

Proliferation of vascular smooth muscle cells (VSMCs) plays a crucial role in the pathogenesis of neointimal formation and restenosis after angioplasty [1–3]. Normal mature VSMCs exhibit a quiescent, whereas activated VSMCs may switch to a proliferative phenotype [1–3]. Cilostazol, a selective type 3 phosphodiesterase inhibitor, possesses antiplatelet function by increasing intracellular cAMP concentrations [4,5]. Beyond its antiplatelet effect, we and others have demonstrated it to have another action on suppressing neointimal formation in balloon-injured rat carotid arteries [6,7] and restenosis after percutaneous transluminal coronary angioplasty [8,9] by inhibiting VSMC proliferation [7,10]. Furthermore, our previous study found that cilostazol suppresses VSMC proliferation mainly via cyclic AMP response element (CRE)-binding protein (CREB)-dependent pathway [10]. Nevertheless, whether other signaling pathways are responsible for this effect merits further clarification.

Heme oxygenase-1 (HO-1), an antioxidant defense enzyme induced by a variety of oxidative injuries, degrades heme into 3 products: Fe^{2+}, biliverdin, and CO [11]. It has been shown that the length polymorphism of (GT)n dinucleotide repeats in human HO-1 promoter may determine the responsiveness of HO-1 expression in individuals with different inherited settings [12,13]. Subjects with longer GT-repeat lengths exhibit weaker antioxidant capacities against various oxidative stresses due to the lower HO-1 response [12,14]. The association of HO-1 promoter polymorphism with cardiovascular diseases, such as coronary artery disease, restenosis after stenting, and atrial fibrillation, has been intensely investigated [13,15–18]. Nevertheless, mechanisms underlying the length of GT-repeat in determining the transcriptional activity

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of human HO-1 gene remain unresolved. Furthermore, because many studies have linked oxidative stress to VSMC dysfunction [19], we hypothesize that HO-1 may be involved in the beneficial effect of cilostazol on VSMC function.

The aim of this study is, therefore, to evaluate whether HO-1 and the GT-repeat length polymorphism in the HO-1 promoter are associated with the inhibitory effect of cilostazol on VSMC proliferation.

2. Materials and methods

2.1. Rat model of balloon injury

The animal study was approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital. Adult male Wistar rats weighting 350 to 400 g were anesthetized with an intraperitoneal injection of ketamine 100 mg/kg and xylazine 5 mg/kg. Angioplasty of the left external carotid artery was performed using an inflated 2F Forgarty embolectomy catheter. Cilostazol (100 mg/day/kg) was administered daily by gavage and balloon injury was performed on day 4. Cilostazol treatment was continued until rats were euthanized at 14 days after balloon injury. The uninjured and injured carotid arteries were removed, fixed with 4% paraformaldehyde, and paraffin-embedded for hematoxylin–eosin staining or immunohistochemical analysis.

2.2. Cell culture

Human VSMCs were isolated from ascending aortas of patients receiving vascular surgery, cardiac transplant donors, or recipients using explant techniques. Some human VSMC clones were purchased from Gibco® (Rockville, MD) or LIFELINE (Frederick, MD). In all experiments, VSMCs between the fourth and seventh passage were used and cultured on conventional uncoated dishes. Most chemicals were purchased from Sigma (St. Louis, MO). Cilostazol (kindly provide by Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan) was dissolved in dimethysulphoxide (DMSO). The final concentration of DMSO in the culture medium was less than 0.1%, which was used as a vehicle. The protocols were approved by the Human Research Ethics Committee at Chang Gung Memorial Hospital (Chang Gung Medical Foundation Institutional Review Board 102-2897A3) and Tri-Service General Hospital (TSGHIRB # 2-102-05-104 and 2-102-05-105) and were conducted in concordance with the 1975 Declaration of Helsinki Principles. Written informed consent was obtained from each subject.

2.3. Western blot analysis

Western blot was performed using anti-tubulin (Santa Cruz, Delaware Avenue, CA), anti-HO-1 (Abcam, Cambridge, MA), anti-p-CREB, anti-CREB (Cell Signaling, Beverly, MA) antibodies as primary antibodies. Signals were developed using the ECL-detection method (Amersham, Netherlands) and quantified by densitometry. The amount of chosen protein was expressed relative to tubulin.

2.4. Immunohistochemical analysis

Immunohistochemical analyses were performed using anti-HO-1 (Abcam) antibody as primary antibody followed by Cy3 (red color, Chemicon, Temecula, CA)-conjugated secondary antibody and visualized by confocal immunofluorescent microscopy.

2.5. Measurement of HO activity

HO activities in VSMCs were measured as previously described [20]. Briefly, cells were homogenized and bilirubin was extracted with 0.5 ml chloroform and quantified by the absorbance difference between 464 and 530 nm, which was normalized by cellular protein concentrations.

2.6. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted using TRIzol reagent (Life Technologies, Rockville, MD) and real-time quantitative RT-PCR was performed as described previously [10]. GAPDH mRNA was used as the internal control.

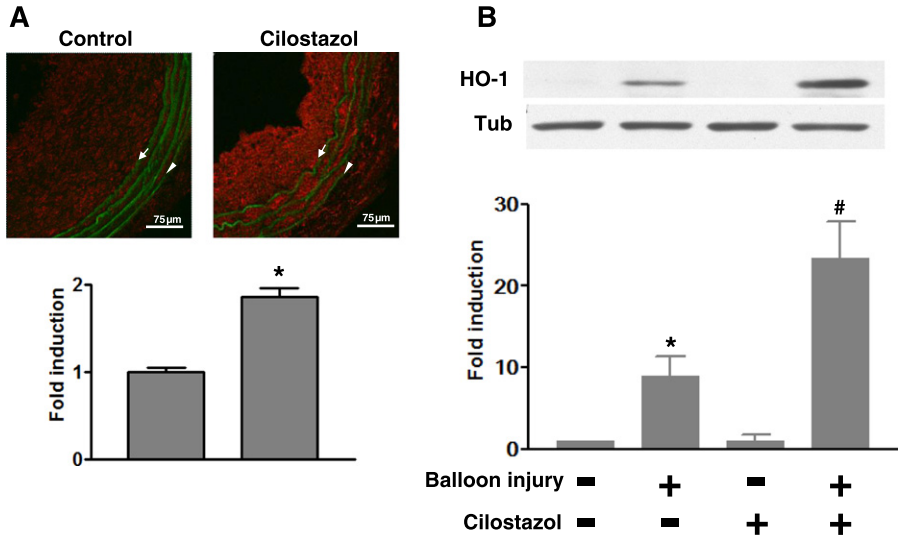

Fig. 1. A. Confocal immunohistochemical analysis shows that cilostazol up-regulates HO-1 in the neointima induced by balloon injury. Arrows denote the internal elastic lamina, and arrowheads denote the external elastic lamina. Relative intensity of HO-1 staining measured in VSMCs (co-localized by SM-α-actin) is quantified. Data are means ± SE (a total of >10 fields and 10 cells with scanning and averaging). The symbol (*) represents the significant difference. B. The levels of HO-1 and tubulin were detected by western blot (upper panels). The relative expression level of each protein are quantified by densitometry and expressed relative to the control condition (lower panels). Each value represents the mean ± SE of 5 rats. $P < 0.05$; *, #: the different symbols represent the significant difference among groups.