SHOCK, Vol. 30, No. 3, pp. 274–279, 2008

YC-1 INDUCES HEAT SHOCK PROTEIN 70 EXPRESSION AND PREVENTS OXIDIZED LDL-MEDIATED APOPTOSIS IN VASCULAR SMOOTH MUSCLE CELLS

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Received 19 Jul 2007; first review completed 28 Aug 2007; accepted in final form 14 Nov 2007

ABSTRACT—Heat shock protein 70 (hsp70) functioning as molecular chaperon in physiological conditions is induced under stress environment, which affords a defensive mechanism for cells to escape cellular damage. Hence, it is a critical issue to develop a nontoxic hsp70-inducing compound against cellular death. The present study was conducted to evaluate whether 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl-indazol (YC-1) can effectively induce hsp70 expression and protect vascular smooth muscle cells (VSMCs) against oxidized low-density lipoprotein–induced cytotoxicity. We showed that YC-1 enhanced hsp70 expression in VSMCs through a concentration- and time-dependent manner with maximum expression at 18 and 24 h without involving the cyclic guanosine monophosphate and reactive oxygen species signal in the pathway. Furthermore, we did not observe significant cytotoxicity after YC-1 treatment through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, lactic dehydrogenase, and fluorescence activating cell sorting scan assays. We demonstrated that the nuclear level of heat shock transcription factor 1 increased at 2 h after YC-1 treatment, and hsp70 expression was directed by the up-regulation of *hsp70* mRNA, which peaked at 6 h and was followed by a decline. Hence, translocation of heat shock transcription factor 1 and increased level of *hsp70* mRNA would account for Hsp70 expression. Finally, we found that YC-1 protects VSMCs from oxidized low-density lipoprotein–inducing apoptosis. According to our observations, YC-1 would be an effectively pharmacological hsp70 inducer that can be used as a cytoprotective agent in vascular diseases.

KEYWORDS—YC-1, heat shock protein 70, heat shock transcription factor 1

INTRODUCTION

Atherosclerosis, a chronic inflammatory disease, results from endothelial cell dysfunction, lipid accumulation, and cytokine release, which together finally lead to vascular smooth muscle cell (VSMC) apoptosis, plaque rupture, and thrombosis (1, 2). Maintenance of atherosclerotic plaque stability lies in the number of VSMCs, but excess oxidized low-density lipoprotein (ox-LDL) would result in apoptosis of VSMCs owing to activation of caspase 3 and increased level of ceramide (3, 4). However, these cells spontaneously develop native defensive systems, including heat shock protein (hsp) families, to protect themselves. It has been reported that lower levels of cardiovascular disease are found in the presence of higher levels of human hsp70 (5). In transgenic mice, it has been well demonstrated that overexpression of hsp70 increases the resistance of myocytes to ischemia injury (6, 7).

According to their molecular weight, hsps are divided into several families: 110, 90, 70, 60, and 40 kd. Low molecular weight hsps possess a variety of functions, including cytoprotection and transportation of nascent proteins (8–10). Heat shock protein 70, consisting of constitutive hsp73 and inducible hsp72, is also involved in proper protein folding and inhibition of both intrinsic and extrinsic apoptotic signaling pathways (11). Reported recently, high expression of hsp70 in lipid-laden macrophages, endothelial cells, and smooth muscle cells plays an important role against cell damage and apoptosis in atherosclerosis (9, 12). The production of hsp70 is regulated by specific transcriptional factors known as heat shock transcription factors (HSFs), and four HSFs have been identified so far. Among them, HSF-1 is the primary HSF involved in regulating expression of hsps in mammals (13). Under normal conditions, HSF-1 remains as a monomer in cytosol but translocates into the nucleus, where it binds to the heat shock element present in the promoter of heat shock genes and initiates transcription and synthesis of hsps after activation (14).

3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl-indazol (YC-1), an NO-independent soluble guanylate cyclase (sGC) activator first discovered in our laboratory, inhibits rabbit platelet aggregation (15). Several lines of evidence have shown that YC-1 provided protection against balloon injury–induced vascular injury and neurological disorders (16–18). Therefore, in this study, we tried to determine whether YC-1 can enhance hsp70 expression and prevent VSMCs from cellular death.

MATERIALS AND METHODS

Materials

3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl-indazol was supplied by Yung-Shin Pharmaceutical Industry, Co., Ltd., (Taichung, Taiwan). Dulbecco's modified Eagle's medium, fetal bovine serum, antibiotics, and all other tissue culture reagents were obtained from GIBCO/BRL Life Technologies (Grand Island, NY). Leupeptin, dithiothreitol (DTT), dimethyl sulfoxide (DMSO), phenylmethylsulfonyl fluoride (PMSF), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, Mo). TRIzol reagent was

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This study was supported by the National Science Council of the Republic of China (grant no. NSC 95-2320-B-002-008).
DOI: 10.1097/SHK.0b013e318162c63a

from Invitrogen (Carlsbad, Calif); random primer and Moloney murine leukemia virus reverse transcriptase (RT) were from Promega (Madison, Wis); pro Taq was from Protech (Taipei, Taiwan). Antibodies against hsp70, HSF-1, actin, horseradish peroxidase–conjugated antimouse, antirat, and antirabbit immunoglobulin (Ig)Gs were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif).

Cell culture conditions

Vascular smooth muscle cells were isolated from the thoracic aorta of male Wistar rats as described in previous procedures (19). Vascular smooth muscle cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) at 37°C in a 5%/95% air atmosphere. Passages before eight generation were used in all the experiments.

Western blot analysis and preparation of nuclear fraction

For analysis of protein expression, cells were seeded in a 6-cm dish and treated with YC-1, 8–cyclic guanosine monophosphate (GMP), SIN-1, *S*-nitroso-*N*-acetylpenicillamine (SNAP), and sodium nitroprusside (SNP) for 24 h, or 50 μM YC-1 for different periods (4, 8, 12, 18, or 24 h). Several inhibitors (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one [ODQ], pyrrolidine dithiocarbamate [PDTC], *N*-acetylcysteine [NAC], and 6-hydroxy-2,5,7,8–tetramethyl chroman–2–carboxylic acid [Trolox]) were used to incubate with cells for 1 h and then challenged by YC-1 for 24 h to evaluate the signaling pathway. Finally, cells were harvested in ice-cold lysis buffer to evaluate protein expressions. Nuclear proteins were extracted as follows. Briefly, phosphate-buffered saline (PBS)–washed cells were resuspended in buffer A (10 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM PMSF, and 0.5 mM DTT). After incubation on ice for 15 min, cells were centrifuged at 3,000 rpm for 5 min. Pellets were resuspended in buffer C (20 mM Hepes [pH 7.9], 420 mM NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF), incubated on ice for 20 min, and cells were centrifuged at 13,000 rpm for 10 min. The blots were electrophoretically transferred to polyvinylidene difluoride membranes, and signal was detected with an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, U.K.).

Immunohistochemistry

3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl-indazol suspended in 0.5% carboxymethyl cellulose (CMC) was orally administered (10 mg/kg) daily to rats for 14 days. In contrast, vehicle-treated animals received only 0.5% CMC daily. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. The right carotid arteries were obtained from the killed animals and paraffin embedded. Histological sections were stained for IgG and hsp70 antibodies. In brief, 5-μm paraffin sections were deparaffinized, and endogenous peroxidase was destroyed with 0.3% H_2O_2 in 100% methanol. Antigen was unmasked by 0.1% trypsin for 10 min at 37°C, and tissues were incubated with the antibody that recognized the IgG and hsp70 overnight at 4°C. A standard labeled streptavidin biotin technique (DAKO, Glostrup, Denmark) was used to detect the reaction products.

Cytotoxicity assay

Cells were incubated in the absence or presence of YC-1 for 24 h, washed once, and incubated with 0.5 mg/mL MTT at 37°C for 1 h. Tetrazolium salts are reduced and form an insoluble blue formazan product. After incubation, formazan products were dissolved with DMSO and quantified with a spectrophotometer. The optical density was compared against the standard curve derived from serial dilution of cells, and the number of all groups was divided by vehicle-treated group to give the percentage of cellular viability.

Lactic dehydrogenase assay

A CytoTox 96 nonradioactive cytotoxicity kit from Promega was used to measure the amount of lactic dehydrogenase (LDH). Briefly, cells were incubated in the absence or presence of YC-1 for 24 h, and the supernatants were collected to measure the release of LDH that converted pyruvic acid to lactic acid.

Cell cycle analysis

Distribution of cell cycle was assessed by fluorescence activating cell sorting (FACS) scan. Briefly, cells were fixed in 70% ethanol and stained with propidium iodide staining solution (50 μg/mL propidium iodide, 100 U/mL of RNase A, 0.1% sodium citrate, and 0.1% Triton X-100). The cell cycle distribution is shown as the percentage of cells containing G_0/G_1, S, G_2, and M DNA as judged by propidium iodide staining and analyzed using CellQuest software.

RT–polymerase chain reaction

RNA was extracted with Trizol reagent by a standard protocol (Invitrogen). Reverse transcription was performed with 5 μg mRNA and random primer at 65°C for 5 min then mixed with Moloney murine leukemia virus RT to react at 37°C for 1 h to obtain cDNA. Gene amplification was followed with RT–polymerase chain reaction (PCR). Primer sequence was as described: *hsp70* antisense, 5′-AACTG-TACACAGGGTGGCAGTG-3′; *hsp70* sense, 5′-CAAGATCAGCGAGGCTGA-CAAG-3′; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) antisense, 5′-TCCTTGGAGGCCATGTGGGCCAT-3′; and *GAPDH* sense, 5′-TGATGA-CATCAAGA AGGTGGTGAAG-3′. Reaction cycles for *hsp70* and *GAPDH* includes 94°C for 5 min, 35 cycles of 94°C for 30 s, 58°C for 30 s (55°C for *GAPDH*), 72°C for 1 min, and a final incubation at 72°C for 10 min. Polymerase chain reaction products were analyzed on 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.

Preparation of ox-LDL

Low-density lipoprotein was isolated from health volunteers by ultracentrifugation and oxidized by incubation with 5 μM $CuSO_4$ for 8 h (20). The degree of oxidation measured by thiobarbituric acid reactive substances is equal to 20 nmol/mg LDL while compared with MDA (malondialdehyde), a by-product of lipid peroxidation. The freshly oxidized LDL is dialyzed against PBS, filtered through a 0.22-μm filter, and stored at 4°C.

Sulforhodamine B assay

For cellular viability assay of VSMCs, the cells were fixed *in situ* with 10% trichloroacetic acid after cell inoculation to represent a measurement of the cell population at the time of drug addition. The plates were incubated for the indicated time after drug addition, and the assay was terminated by 10% trichloroacetic acid. Sulforhodamine B) dye at 0.4% (wt/vol) in 1% acetic acid was added to stain cellular proteins. Unbound dye was removed by repeated washing with 1% acetic acid, and the plates were air-dried. Bound stain was subsequently soluble with 10 mM Trizma base, and the absorbance is read on a microplate reader at a wavelength of 515 nm.

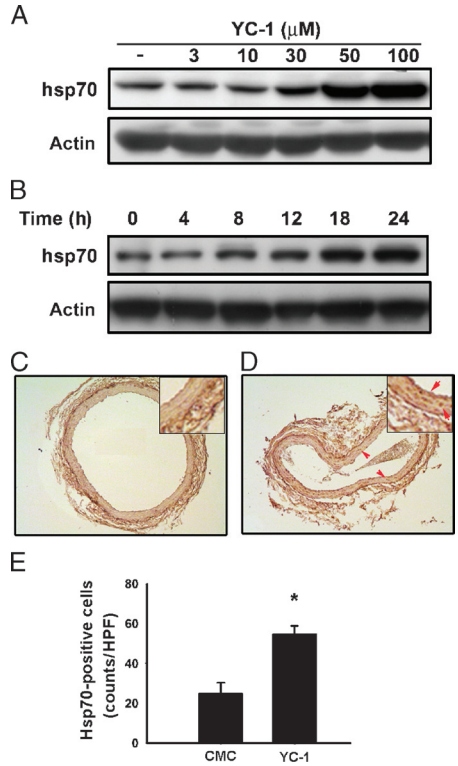


FIG. 1. **Expression of hsp70 protein in VSMCs treated with YC-1**. A, Vascular smooth muscle cells were incubated with vehicle (0.1% DMSO) or a different concentration of YC-1 for 24 h to detect hsp70 protein expression by Western blotting. B, Cells were incubated with vehicle or 50 μM YC-1 for the indicated time period and then harvested for the detection of hsp70 protein expression by Western blotting. Actin expression was used as a loading control. For immunohistochemistry, slices from carotid arteries of CMC-treated rats (C) or YC-1–treated rats (D) were hybridized with anti-hsp70 antibody to evaluate hsp70 expression, and arrowhead indicates the hsp70-positive stained cells. Magnification 200×. E, The Hsp70-positive stained cells were counted under HPF. *$P < 0.01$ compared with CMC-treated groups. The representative result of three separate experiments was shown. HPF indicates high-power field.

4,6-Diamidino-2-phenylindole stain

Cells were incubated for the indicated drug and fixed with ice-cold methanol. After being washed twice with PBS, cells were stained with 1 μg/mL 4,6-diamidino-2-phenylindole (DAPI) and imaged with a fluorescence microscope.

Statistical analysis

All data are represented as mean ± SE of the mean. An unpaired Student *t* test was used to compare the same data. *P* values less than 0.05 were considered statistically significant for all comparisons.

RESULTS

YC-1 induced hsp70 expression in VSMCs

First, we evaluated whether YC-1 increased hsp70 expression in VSMCs. We showed that hsp70 constitutively expressed in vehicle-treated subjects, and the level increased in a concentration-dependent manner after YC-1 treatment (Fig. 1A). Furthermore, we evaluated the variation of hsp70 expression after YC-1 treatment. Results showed that hsp70 expression increased at the eight hour and reached peak at the 18th and 24th hour after YC-1 treatment (Fig. 1B). To translate these results *in vivo*, we determined the effect of YC-01 on hsp70 expression in rat carotid arteries. In control subjects, morphological analysis revealed the moderate constitutive hsp70 stain on vessels and a marked positive stain for inducible and constitutive hsp70 in the smooth muscle cells of the media layer of carotid arteries in the YC-1–treated group (Fig. 1, C and D). Hence, these results show that YC-1 can significantly induce hsp70 protein expression *in vitro* and *in vivo*.

YC-1 was not toxic to VSMCs

Heat shock protein 70 is known as a kind of stress protein that increases as cells encounter toxic substances, environmental stress, and apoptotic stimuli (11). To exclude doubts about the possibility that YC-1 is harmful to VSMCs, we investigated the survival rate after YC-1 by MTT assay. Data showed that YC-1 did not elevate cytotoxicity at concentration ranges between 10 and 100 μM (Fig. 2A). On the other hand, we also simultaneously used the LDH assay to test whether YC-1 resulted in acute cellular toxicity, with the result that YC-1 still did not induce necrosis of VSMCs even at the high concentration of 100 μM (Fig. 2B). Furthermore, we also confirmed that YC-1 would not disturb cell-cycle distribution with FACScan analysis (Fig. 2C). Hence, we considered that induction of hsp70 by YC-1 did not result from cytotoxicity as previously reported (16).

YC-1 induced hsp70 expression through A cGMP- and reactive oxygen species–independent manner

As several studies have indicated, YC-1 is capable of activating sGC and elevating cellular cGMP level with 100 μM SNP or 300 μM SNAP to provide a protective function on VSMCs against cytotoxic agents (21). Thus, we investigated whether YC-1 increased hsp70 expression through the

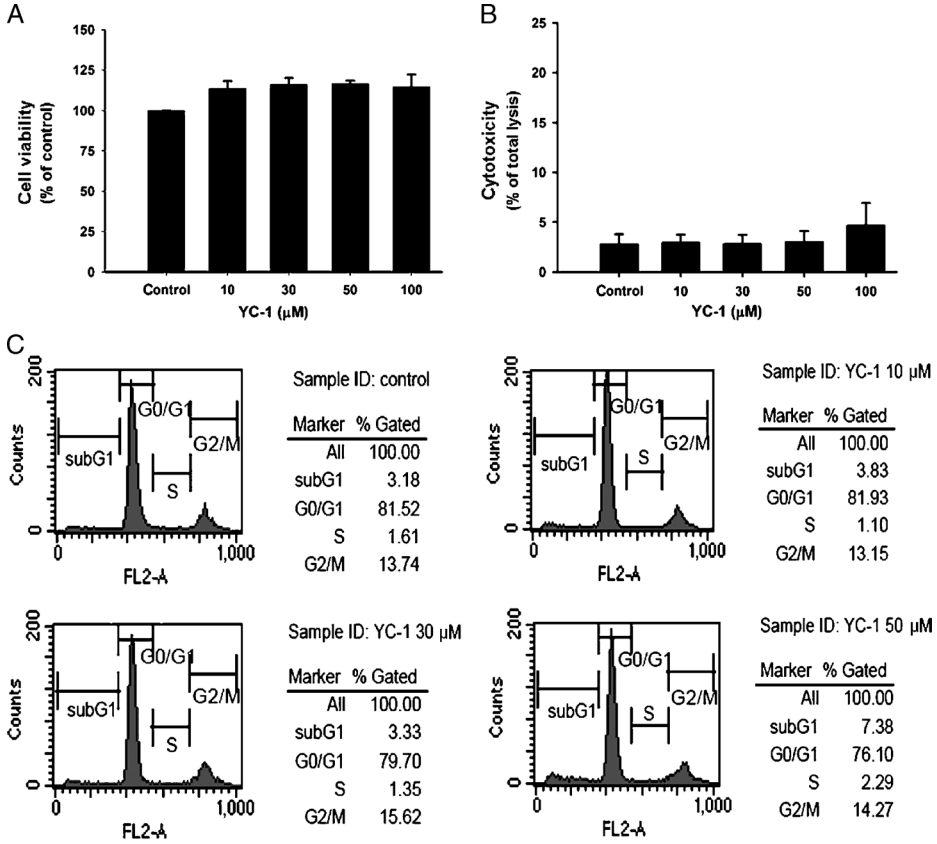


FIG. 2. **Effect of YC-1 on cellular viability**. Vascular smooth muscle cells were treated with 10, 30, 50, and 100 μM YC-1 or vehicle (0.1% DMSO) for 24 h, and the cytotoxicity was determined with MTT (A) and LDH assays (B) as described in "Materials and methods." The amount of LDH was expressed as the percentage of one of the completely lysed cells. C, Vascular smooth muscle cells were treated with the indicated concentration of YC-1 or vehicle for 24 h, and then the cells were harvested for the detection of cell cycle progression by FACScan flow cytometric analysis. These procedures were described in "Materials and methods." Results were expressed as the mean ± SE of three separate experiments.

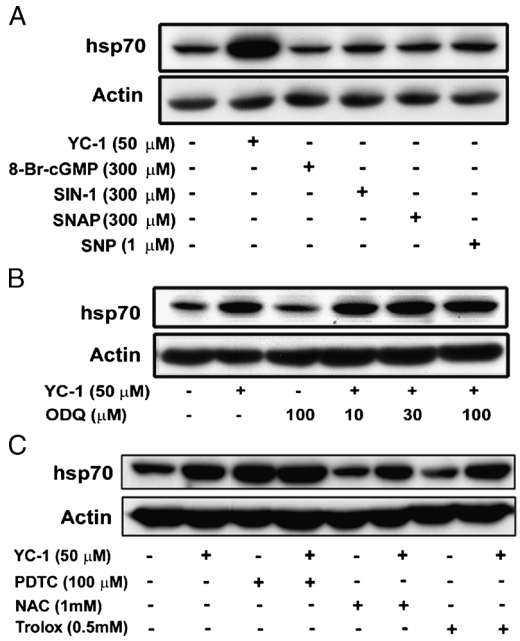


FIG. 3. **Effects of NO donors and ODQ on YC-1–induced hsp70 protein expression**. A, The cells were treated with vehicle (0.1% DMSO), YC-1 (50 μM), 8-Br-cGMP (300 μM), SIN-1 (300 μM), SNAP (300 μM), and SNP (1 μM) for 24 h to detect the expression of hsp70 protein by Western blotting. The cells were pretreated with ODQ (10, 30, or 100 μM) (B) or NAC (1 mM), PDTC (100 μM), and Trolox (0.5 mM) (C) for 1 h and then challenged with vehicle or 50 μM YC-1 for 24 h to detect the expression of hsp70 protein by Western blotting. Actin expression was used as a loading control. The representative result of three separate experiments was shown.

cGMP-dependent pathway. In the present study, we used a low concentration of 1 μM SNP to elevate cGMP, rather than a high concentration of 100 μM SNP because of cellular death under a high concentration of SNP (21, 22). However, we found that 8-Br-cGMP, a cGMP analog, and NO donors SIN-1 (morpholinosydnonimine), SNAP and SNP failed to increase hsp70 expression (Fig. 3A). Next, we used ODQ, a sGC inhibitor, to block the function of sGC and inhibit the accumulation of intracellular cGMP. Although VSMCs were pretreated with ODQ (10 – 100 μM), we still did not observe the reversal of hsp70 expression after YC-1 treatment, and ODQ itself was unable to affect hsp70 expression (Fig. 3B). Therefore, we excluded the possibility that cGMP was involved in the cascade of YC-1–induced hsp70 expression. In the study of Xu and Boysen, YC-1 was shown to induce the generation of reactive oxygen species (ROS) (23), and hsp expression was up-regulated after being challenged by ox-LDL and ROS (24). Hence, we treated VSMCs with several kinds of antioxidants to evaluate whether ROS was involved in the pathway. However, several antioxidants, 1 mM NAC, 100 μM PDTC, and Trolox, which were reported to inhibit production of ROS, were all useless in counteracting the function of YC-1 (Fig. 3C) (25, 26). Hence, these ROS-related signaling pathways did not contribute to the production of hsp70 induced by YC-1. What surprised us is that PDTC evoked significant expression of hsp70 alone. However, it has been demonstrated in previous research that PDTC can induce the heat shock response and increase HSF-1 activity in human endothelial cells as observed in our study (27).

YC-1 induced nuclear translocation of HSF-1 and up-regulated hsp70 mRNA

Heat shock protein genes are known to be regulated by HSF-1 (14). Heat shock transcription factor 1 is present mainly in cytosol, and its transcriptional activity is mediated via phosphorylation and translocation to nuclei. Therefore, we investigated whether YC-1 can affect HSF-1 level in nuclei. Compared with the vehicle-treated group, we found no band shift of HSF-1 in the YC-1–treated group, which indicates the hyperphosphorylation status of HSF-1. However, treatment with YC-1 caused an increased level of HSF-1 at the second hour in the nuclear fraction (Fig. 4A). Hence, induction of HSF-1 translocation would account for the effect of YC-1 on hsp70 induction. To investigate whether transcriptional activity resulted in hsp70 protein synthesis after translocation of HSF-1, the changes in *hsp70* mRNA expression after YC-1 treatment were studied by RT-PCR analysis. As shown in Figure 4B, YC-1 obviously increased *hsp70* mRNA levels at the fourth hour and peaked at the sixth hour, and the induction of *hsp70* mRNA corresponds to hsp70 protein expression observed at the eight hour after YC-1 treatment.

YC-1 protected VSMCs from ox-LDL–induced cellular death

It has been presented previously that hsps can keep cells from lethal injuries, including injuries from excess NO and ox-LDL, etc. (12, 21, 28). To determine whether YC-1 still possesses protective potential to VSMCs, we studied the effect of YC-1 (50 μM) on prevention of 200 μg/mL ox-LDL–induced VSMC cell death. A significant decline in cellular viability was observed after a 24-h period of ox-LDL incubation, dropping to approximately 65% compared with the control group (Fig. 5A). However, we observed that reduced cellular viability after exposure to high ox-LDL concentration (200 μg/mL) was reversed after pretreatment of YC-1 for 24 h. Furthermore, we adopted the DAPI assay *in situ* to observe nuclear condensation after incubation with YC-1 or ox-LDL, and we showed that YC-1 was able to reverse the apoptotic effect induced by ox-LDL (Fig. 5B).

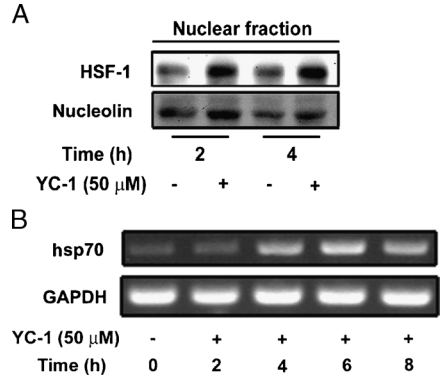


FIG. 4. **Effects of YC-1 on HSF-1 translocation and hsp70 mRNA expression**. A, Nuclear extracts were collected at the indicated period after vehicle or 50-μM YC-1 treatment and analyzed by Western blotting. Nucleolin was used as the loading control. B, Vascular smooth muscle cells were treated with 50 μM YC-1 for the indicated times, and 5 μg mRNA was subjected to the RT-PCR process to determine the level of hsp70 mRNA. Glyceraldehyde-3-phosphate dehydrogenase was used as the loading control. The representative result of three separate experiments was shown.

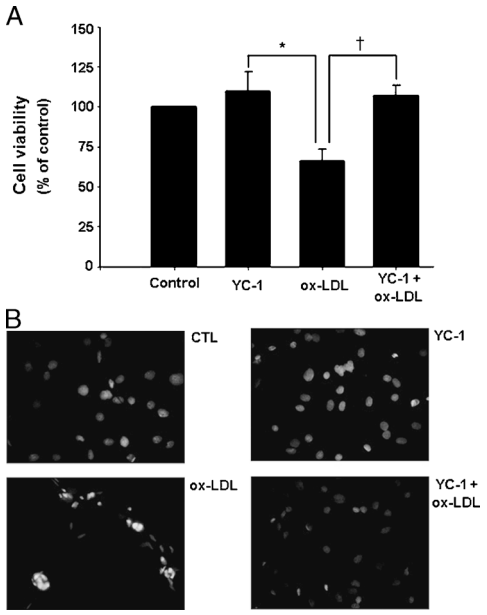


FIG. 5. **Effects of YC-1 on ox-LDL–stimulated apoptosis in VSMCs**. Vascular smooth muscle cells were treated with 50 μM YC-1 for 48 h in the presence or absence of 200 μg/mL ox-LDL for the later 24 h for the sulforhodamine assay (A) and DAPI stain (B) as described under " Materials and methods." The survival rate of all indications is expressed as the mean ± SE of four separate experiments. $^{*}P < 0.05$ and $^{\dagger}P < 0.01$ compared with vehicle-treated or ox-LDL–treated groups.

Hence, we concluded that up-regulation of hsp70 with YC-1 can strengthen VSMCs to confront extraordinary cellular stress such as high concentration of ox-LDL.

DISCUSSION

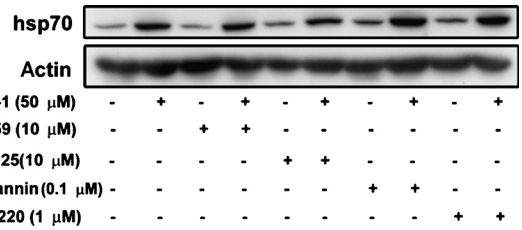
In the field of vascular disease, accumulating evidence suggests that hsp70 is highly expressed in atherosclerotic lesions of humans, rabbits, and apoenzyme E–deficient mice (12), and it is unevenly distributed in the center of atheroma where lipid-laden macrophages and apoptotic smooth muscle cells reside (28). Several lines of evidence have proven that pharmacological inducers of hsp70 would protect cells from damage or death (29). In the present study, we examined the effect of YC-1 on the modulation of hsp70 protein, and we found that YC-1 evoked expression of hsp70 in a concentration- and time-dependent manner. With treatment of ODQ, the induction of hsp70 by YC-1 was not reversed, and NO donors or cGMP analogs could not significantly increase the induction of hsp70. Treatment with antioxidants was also unable to reverse the effect, so it seemed that other mechanisms were involved in the modulation of Hsp70 without the effect of NO/sGC/cGMP and redox-mediated pathways. Finally, we found that YC-1 promoted the translocation of YC-1 with transcriptional activation, and it prevented ox-LDL–induced apoptosis in VSMCs.

Both stressors and apoptotic stimuli are the candidates as inducers of hsp, so it is critical to distinguish nontoxic inducers from stressors or apoptotic inducers. Several studies indicated that YC-1 was a cytotoxic agent against cancer cell lines, including Hep3B, HA22T, and PC-3 (30, 31). After treatment of YC-1, the percentage of G0/G1 phase increased in Hep3B, and the levels of the cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$ or $p27^{KIP1}$ also increased at a concentration of 30 μM. Using the cytotoxic assays (Fig. 2), YC-1 did not result in apoptosis and growth arrest in VSMCs as in Hep3B. We also studied the effect of YC-1 in Hep3B and PC-3 besides VSMCs; however, YC-1 only induced hsp70 expression in VSMCs but not in other cell lines (data not shown). According to our observations, we consider that the induction of hsp70 by YC-1 may be cell-type specific and is independent on its cytotoxicity.

It had been reported that NO-generating compounds such as SNP, SNAP, and spermine/NO complex caused a synthesis of the hsp70 protein and led to transcriptional activation of HSF in primary VSMCs (21). On the contrary, we observed no elevated expression of hsp70 after treatment of NO donors. We suggested that the concentration of SNP used in the research of Xu and Boysen was much higher than that in the present study (300 and 1 μM, respectively). The high concentration of NO led to cellular death in VSMCs and evoked spontaneous defensive mechanisms in most mammals, including hsps (13, 21, 32). However, the low concentration of 1 μM SNP was usually used as a sGC activator, which was not harmful to cells; hence, it was expected that cGMP was not involved in the induction of hsp70. Recently, the research of Kim et al. (33) demonstrated that carbon monoxide (CO) can also up-regulate expression of hsp70 protein in primary murine lung endothelial cells and fibroflasts. Their study showed that ODQ was unable to reverse the effect of CO, and they also ruled out the possibility that cGMP was essential in the cascade of CO-induced hsp70 expression. In addition, it was demonstrated that several pharmacological actions of YC-1 were independent on sGC/cGMP pathway (31, 34). Hence, we excluded the possibility that cGMP played a crucial role in the cascade of YC-1–induced production of hsp70 in our study as well as CO did.

It has been reported that regulation of hsps is modulated by HSF-1. The transcription of *hsp70* will be initiated when HSF-1 binds to heat shock element in the nucleus; however, it is still unclear which kinase is essential for phosphorylation of HSF-1. It has been indicated that mitogen-activated protein kinase (extracellular receptor kinase 1/2 and c-Jun N-terminal kinase), PI3K, and protein kinase C pathways are involved in the hyperphosphorylation of HSF-1 after being challenged with different stimulators (12, 24). In the present study, the reversal of hsp70 expression was not observed after treatment of PD98059 (mitogen-activated extracellular receptor kinase inhibitor), SP600125 (c-Jun N-terminal kinase inhibitor), Wortmannin (PI3K inhibitor), and RO318220 (protein kinase C inhibitor) (Supplement 1). Compared with the vehicle-treated group, we also did not observe any band shift after YC-1 treatment. It seemed that YC-1 was incapable of promoting hyperphosphorylation of HSF-1 to initiate hsp70 expression. However, we observed that YC-1 promoted the accumulation of HSF-1 in nucleus as geranylgeranylacetone did (29). It has been demonstrated that HSF-1 is a client protein of hsp90, and that hsp90-binding drugs are able to compete in the binding with HSF-1. After treatment of hsp90-binding drugs, HSF-1 will be released from hsp90 and translocate to the nucleus, followed by the initiation of

downstream signals. So far, we need more evidence to prove whether YC-1 belongs to the hsp90-binding drug family in a future experiment. In the present study, we have tried to transfect *hsp70* siRNA to eliminate the function of hsp70, but it is hard to accurately evaluate whether increased hsp70 expression would account for the cytoprotective effect of YC-1 because of low efficiency of transfection (20% – 30%) in primary cells. However, it has been well recognized that hsp70 is overexpressed in atherosclerosis as the protector against cellular injury (1, 2, 29). It is demonstrated that hsps can inhibit the activity of proapoptotic protein, caspase 3 and caspase 8, and modulate extrinsic apoptotic activation (11). Hence, we suggest that induction of hsp70 with YC-1 in VSMCs leads to cytoprotection against ox-LDL–induced caspase 3 activation and apoptosis (11, 35). Finally, the present study suggests that YC-1 seems to be a pharmacological inducer of hsp70, and it would be a good candidate as a protector against vascular diseases.



SUPPLEMENT 1. **Effects of several inhibitors on YC-1–induced hsp70 protein expression**. The cells were pretreated with PD98059 (10 μM), SP600125 (10 μM), Wortmannin (0.1 μM), and RO318220 (1 μM) for 1 h and then challenged with vehicle or 50 μM YC-1 for 24 h to detect the expression of hsp70 protein by Western blotting. The representative result of three separate experiments was shown.

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