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YC-1-Mediated Vascular Protection through Inhibition of Smooth Muscle Cell Proliferation and Platelet Function

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YC-1, a synthetic benzyl indazole derivative, is capable of stimulating endogenous vessel wall cyclic guanosine monophosphate (cGMP) production and attenuating the remodeling response to experimental arterial angioplasty. In an effort to investigate the mechanisms of this YC-1-mediated vasoprotection, we examined the influence of soluble YC-1 or YC-1 incorporated in a polyethylene glycol (PEG) hydrogel on cultured rat vascular smooth muscle cell (SMC) cGMP synthesis, SMC proliferation, and platelet function. Results demonstrate that soluble YC-1 stimulated SMC cGMP production in a dose-dependent fashion, while both soluble and hydrogel-released YC-1 inhibited vascular SMC proliferation in a dose-dependent fashion without effects on cell viability. Platelet aggregation and adherence to collagen were both significantly inhibited in a dose-dependent fashion by soluble and hydrogel-released YC-1. Arterial neointima formation following experimental balloon injury was significantly attenuated by perivascular hydrogel-released YC-1. These results suggest that YC-1 is a potent, physiologically active agent with major anti-proliferative and anti-platelet properties that may provide protection against vascular injury through cGMP-dependent mechanisms.

Key Words: **YC-1; cGMP; SMC proliferation; platelets; balloon angioplasty; neointima.**

Arterial stenotic remodeling following balloon angioplasty and other forms of endovascular intervention presents a vexing and persistent iatrogenic complication in clinical medicine. Pathobiological neointima development is a complex process involving medial wall smooth muscle cell (SMC) proliferation and migration and deposition of extracellular matrix components, with major thrombogenic contributions from serum-derived factors such as thrombin and platelet-derived growth factor. Recent work from our laboratories and others has attempted to minimize the extent of neointima formation following experimental balloon injury through separation of luminal from medial factors at the site of injury (1), targeted delivery of pharmacologic agents to the arterial wall (2, 3), and localized vector-mediated gene therapy (4, 5). Results from these experiments suggest a potentially protective and clinically relevant strategy exists in the targeted application of therapeutic agents to the vessel wall subjected to luminal intervention.

The heme oxygenase (HO) system of heme catabolism has been hypothesized to provide vasoprotection against injury- or disease-induced remodeling (3, 6, 7, 8). Catalytic HO activity oxidatively forms the sequential antioxidants, biliverdin and bilirubin, and the diatomic gas carbon monoxide (CO). CO binds to the sixth position of the heme iron of soluble guanylyl cyclase (sGC), creating a six-coordinated heme moiety with the histidine-Fe bond remaining intact (9). A different sGC-activating mechanism exists for a similar diatomic gas, nitric oxide (NO), which breaks the iron-histidine bond and creates a conformational change leading to a five-coordinated complex (9, 10). This mechanistic difference in sGC activation between CO and NO has been suggested to contribute to the relatively poor sGC-stimulatory properties of CO *in vitro.* Under controlled conditions, CO stimulation of sGC leads to a 4- to 6-fold increase in enzymatic activity, while NO stimulation results in an up to a 400-fold increase in cyclase activity (11). However, despite this relatively poor ability of CO to stimulate sGC *in vitro,* CO remains a physiologically important, *in vivo* regulator of endogenous cyclic guanosine 3′,5′-

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monophosphate (cGMP) formation and subsequent cGMP-mediated effects (12).

The chemically synthesized 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole derivative YC-1 has been characterized as a potent sGC/cGMP activator (11, 13). YC-1 stabilizes the active configuration of sGC (14) while decreasing the dissociation rates of the gaseous ligands NO and CO from the activated enzyme (11). YC-1 has also been shown to inhibit cGMP-specific phosphodiesterase type 5 in platelets (15) and aortic extracts (13), thus inhibiting cGMP degradation. Recent work by our laboratory suggests a novel and physiologically significant strategy for YC-1 as a vasoprotective adjuvant to traditional balloon angioplasty (2). Results from this study demonstrate that YC-1 is capable of upregulating endogenous vessel wall cGMP and minimizing the extent of medial wall remodeling and neointima formation subsequent to experimental arterial balloon angioplasty.

We recently developed biocompatible polyethylene glycol (PEG) hydrogels that, upon photopolymerization, provide localized and sustained release of NO from a variety of NO donors that significantly reduces platelet adhesion to collagen and inhibits proliferation of cultured rat vascular SMCs (16). Therefore, we have now examined the influence of soluble YC-1 or YC-1 released from PEG hydrogels on cultured rat vascular SMC cGMP production, SMC proliferation, and platelet function, and the potential salutary properties of hydrogel-released YC-1 on balloon injury-induced arterial remodeling. Results suggest that YC-1 attenuates experimental injury-induced neointima development through suppression of SMC proliferation and inhibition of platelet function.

METHODS

Enzyme immunoassay for cGMP. Thoracic arota SMCs were isolated from male Sprague-Dawley rats via an enzymatic digestion technique (17). Vascular SMCs ere cultured (passage 3–7) in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 500 U penicillin, and 100 mg/L streptomycin at 37°C in a 5% CO_2 environment. SMCs were seeded in 96-well plates at 30,000 cells/cm^2 in 10% FBS. SMCs were incubated in sterile HEPES buffered saline solution (HBS) and pretreated with sterile 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). SMCs were incubated with varying concentrations of YC-1 (in DMSO) for 10 min. Cyclic GMP levels were measured using an enzyme immunoassay kit (Amersham Pharmacia Biotech, Piscataway, NJ). Briefly, SMCs were lysed with 0.5% dodecyltrimethylammonium bromide in 0.05 M sodium acetate buffer containing 0.02% BSA. Cyclic GMP was acetylated, and the cGMP-containing supernatant added to a microtitre plate coated with donkey anti-rabbit IgG. Rabbit anti-cGMP antibody was added, and the plate was incubated for 2 h at 4°C. Cyclic GMP was conjugated to horseradish peroxidase (HRP) and incubated at 4°C for 1 h. After rinsing, 3,3′,5,5′-tetramethylbenzidine was added for 30 min for chromogenic detection. The reaction was stopped with 1 M sulfuric acid, and the optical density (OD) was read at 450 nm on a microtitre plate reader (Bio-Tek Instruments, Inc.).

Release kinetics of YC-1 from PEG hydrogels. YC-1 was dissolved in DMSO and combined with a PEG hydrogel precursor (MW 6000 g/mol) containing 20% PEG-diacrylate, 4% triethanolamine, 0.15% *N*-vinylpyrrolidone as a solvent, and 1500 ppm 2,2-dimethoxy-2-phenyl acetophenone as a long wavelength UV initiator (16). The solution was gelled under a UV lamp (365 nm, 10 mW/cm^2, Blak-Ray B100AP, UVP, Upland, CA) for 30 s. Hydrogels were placed in closed containers in pH 7.4 HBS at 37°C. Based on the presence of multiple aromatic rings in its structure, scans were performed for YC-1 absorbance between 250 and 450 nm on a UV/V is spectrophotometer (Cecil Instruments CE 2041, Cambridge, England). YC-1 was determined to have an absorbance peak at 324.5 nm. YC-1 release was then measured at appropriate time points by removing the buffer solution and reading the OD at 324.5 nm.

Vascular SMC viability assay. SMCs were seeded in 24-well tissue culture dishes at 10,000 cells/cm^2 and allowed to adhere for 24 h in 10% FBS. YC-1 in sterile DMSO was added to the culture wells between 5 and 50 μM. Viability was assessed via Live/Dead calcein AM/ethidium bromide fluorescent staining (Molecular Probes, Eugene, OR), and cells were examined under fluorescence microscopy (Zeiss Axiovert 135, Thornwood, NY). As the Live/Dead staining assesses only the viability of adherent cells, cell counts on a Coulter Counter were performed on media that was removed from the wells prior to rinsing.

Vascular SMC proliferation assay with soluble YC-1. Rat vascular SMCs were seeded in 24-well tissue culture dishes at 5,000 cells/cm^2 and adhered for 24 h in 10% FBS. YC-1 in sterile DMSO was added to the wells at concentrations between 5 and 300 μM in order to determine an optimal concentration range for YC-1. Two days following the addition of YC-1 to the culture media, cells were trypsinized and counted on a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). After the initial dose-response determination, an appropriate YC-1 concentration range was selected (5–50 μM), and the experiment repeated in this range.

Vascular SMC proliferation assay with YC-1 released from PEG hydrogels. Rat vascular SMCs were seeded in 12-well tissue culture plates at a density of 5,000 cells/cm^2 in 10% FBS. Based on YC-1 release kinetics, YC-1-releasing PEG hydrogels were prepared containing varying amounts of YC-1 so that the final concentrations between 10 and 50 μM YC-1 would be achieved in the culture media. Twenty-four hours after seeding, hydrogels ± YC-1 were placed in the culture media, and cells were incubated with the hydrogel solutions for 48 h. Cells were then trypsinized and counted on a Coulter Counter.

Platelet aggregometry assay. Fresh whole blood was obtained from a human volunteer and anticoagulated with 1:6 acid citrate dextrose (ACD; 65 mM citric acid, 85 mM sodium citrate, 111 mM dextrose, pH 4.5):blood. Platelet-rich plasma (PRP) was obtained following centrifugation at 100× g. The PRP was centrifuged at 1000× g to obtain a platelet pellet and platelet-poor plasma. The pellet was resuspended in Tyrodes's solution (mM: NaCl 136.9, KCl 2.7; $MgCl_2$ 2.0, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, pH 7.4) to obtain approximately 10^8 platelets/ml. YC-1-releasing hydrogels were prepared in order to deliver between 25 and 100 μM YC-1 during the hydrogel-platelet co-incubation. Following co-incubation, thrombin was added (0.4 U/ml) and the extent of aggregation measured after 4 min using a dual sample light-transmission aggregometer (Sienco, Inc., Morrison, CO).

Platelet adhesion assay. The platelet adhesion assay protocol has been previously described (16). Briefly, fresh whole blood was obtained from a human volunteer, anticoagulated with heparin, and fluorescently labeled with mepacrine (10 μM). A solution of 2.5 mg/ml collagen type I in 3% glacial acetic acid was applied to glass slides while soluble YC-1 or hydrogel ± YC-1 were incubated with the labeled blood. YC-1 treatments were removed, and the blood was

applied to the coated slides for 20 min at 37°C. Platelet counts were made under fluorescent microscopy.

Rat carotid artery balloon injury. The procedure for balloon injury in rat carotid arteries has been previously described (2, 3). Male Sprague Dawley rats (472 ± 3.8 grams; Harlan, Indianapolis) were anesthetized with a combination anesthetic (ketamine, zylazine, and acepromazine; 0.5–0.7 ml/kg, IM), and the left carotid artery (LCA) and associated vasculature exposed. A Fogarty 2F embolectomy catheter (Baxter Healthcare Corp.) was introduced through an external carotid arteriotomy incision, advanced to the aortic arch, and inflated and withdrawn with rotation 3 times. The catheter was removed, the external carotid branch ligated, and the exposed artery topically treated with empty PEG hydrogel or PEG hydrogel plus YC-1 (1 mg; Sigma-Aldrich) in a random and blind fashion. The solution was polymerized under a UV lamp (365 nm, 10 mW/cm^2, distance 6 in.) for 30 s, and solidification of the gel was verified. The incision was closed, and upon full recovery from anesthesia, rats were returned to the animal care facility and provided standard rat chow and water containing acetaminophen (300 mg/kg, Sigma) *ad libitum*. At specific times, rats were anaesthetized to overdose and sacrificed via pneumothorax and exsanguination. For histology, animals were perfusion-fixed with PBS and buffered formalin phosphate and the carotids removed. For Western blots, tissues were freshly removed and snap-frozen. All animal protocols complied strictly with the guidelines of the institutional animal care and use committee.

Sham-operated control experiments. Sham-operated control experiments were performed to analyze surgery-associated effects on arterial remodeling without the influence of balloon injury. The details for animal anesthesia and surgical preparation are the same as described above. However, in these experiments, the LCA and associated vasculature were exposed and the LCA was not balloon-injured. Empty gel was topically applied to the artery and polymerized. Animals were closed, and tissues obtained at various time points.

Tissue processing and staining. Perfusion-fixed tissues were processed in graded alcohols and xylene and paraffin-embedded as routine (2, 3). Standard staining procedures were employed for hematoxylin and eosin. For Verhoff's elastin staining, slides were deparaffinized, rehydrated, and stained with Verhoff's solution of alcoholic hematoxylin, ferric chloride, and potassium iodine. Slides were differentiated with ferric chloride and counterstained with acid fuchsin and picric acid. For Masson's Trichrome stain, slides were treated with Bouin's histologic reagent and hematoxylin-counterstained. Slides were treated with Biebrich-Scarlet acid fuchsin solution followed by phosphotungstic-phosphomolybdic acid treatment. Slides were stained with aniline blue, exposed to acetic acid, dehydrated, cleared and coverslipped. Standard techniques for microscopic analyses and quantitation of morphologic parameters were performed (3).

Western blot analysis. The protocol employed for Western blot analysis of HO-1 has been previously described (2, 3). Protein was homogenized from frozen arteries, and whole-cell lysates were boiled and sonicated. SDS-PAGE was performed using 20 μg protein per tissue. The separated blots were transferred to nitrocellulose membranes, blocked, and incubated with an anti-HO-1 polyclonal antibody (1:500, StressGen Biotechnologies Corp.) in PBS. Membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:7500). Blots were washed, incubated in enhanced chemiluminescence reagents (Amersham Corp.), and exposed to photographic film. Densitometric analyses of gels were performed using an Ultroscan XL Enhanced Laser Densitometer (Pharmacia LKB Biotechnology, Sweden).

Statistical analyses. Data were stored and analyzed on personal computers using Excel 2000 (Microsoft) and Sigma Plot 2001 v. 7.0 with Sigma Stat 2.03 (SPSS, Inc.). *In vitro* data sets were compared using a one-way Analysis of Variance with Tukey's post-hoc multiple comparison test. Data for injured distal LCA sections were groups according to treatment, and comparisons between treatments made using a two-tailed unpaired Student's t-test. A two-tailed paired Student's t-test was used to compare data from individual injured LCA proximal and distal sections. All data are represented as mean ± standard error (SE) of the mean. A P value of <0.05 is considered statistically significant for all comparisons.

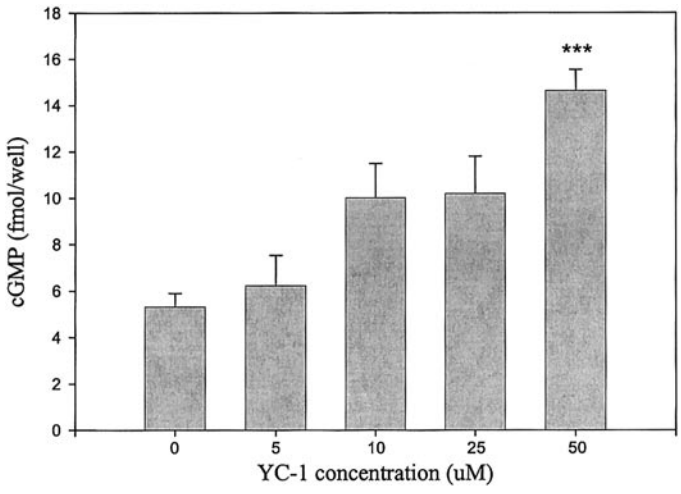

FIG. 1. Rat vascular SMC cGMP levels were significantly ($P <$ .001, ANOVA) increased in a dose-dependent fashion between 5 and 50 μM soluble YC-1. ***$P <$ .001 versus 0 μM control, Tukey test for pairwise multiple comparisons following one-way ANOVA; $n = 4$ per treatment group.

RESULTS

Figure 1 illustrates that soluble YC-1, between 5 and 50 μM, significantly ($P <$ .001, ANOVA) stimulated cultured rat vascular SMC cGMP production in a dose-dependent fashion. We next examined the release of YC-1 from a copolymer PEG hydrogel. Under *in vitro* physiological conditions (pH 7.4, 37°C) over a period of 6 h, approximately 50% of the initial YC-1 dose was released from PEG hydrogels, with approximately 60% released by 24 h (Fig. 2). No further increase in YC-1 release was observed after 72 h.

As shown in Fig. 3, soluble YC-1 or YC-1 released from PEG hydrogels consistently and significantly inhibited vascular SMC proliferation in a dose-dependent manner between 10 and 50 μM ($P <$ .001 for soluble YC-1, $P <$ .001 for hydrogel-released YC-1, ANOVA). At 50 μM, YC-1 caused approximately an 80% inhibition of SMC growth. A marked reduction in SMC proliferation following exposure to hydrogen-released YC-1 indicates that incorporation of YC-1 into the hydrogel material did not deleteriously affect drug bioactivity or availability. There was a small increase ($\leq$6%) in the number of detached SMCs in the culture media at higher concentrations of YC-1. However, at

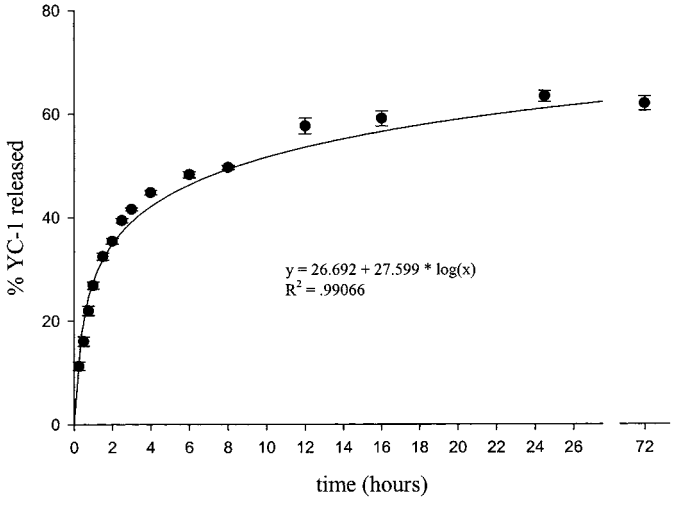

FIG. 2. Release kinetics of YC-1 from activated PEG hydrogels. Approximately 50% of initial YC-1 dose was released from PEG hydrogels under *in vitro* physiological conditions (pH 7.4, 37°C) over a period of 6 h, with approximately 60% released by 24 h. No further YC-1 release was found after 72 h. $n = 5$ per time point.

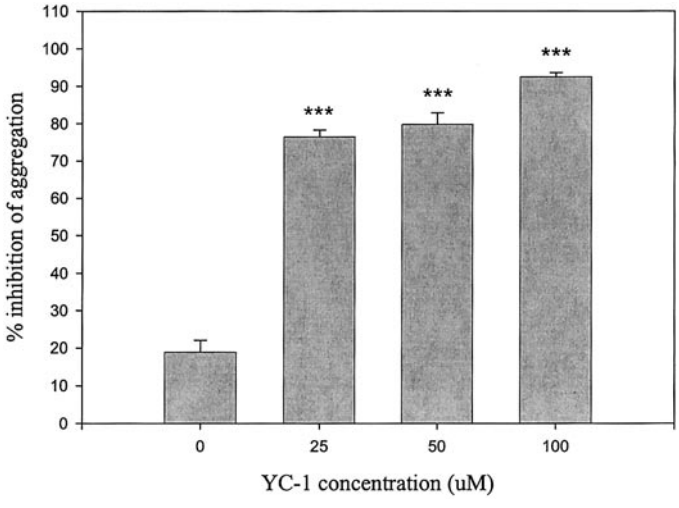

FIG. 4. Platelet aggregation was significantly ($P < .001$, ANOVA) inhibited by the release of YC-1 from PEG hydrogels in a dose-dependent manner. ***$P < .001$ versus 0 μM control, Tukey test for pairwise multiple comparisons following one-way ANOVA; $n = 3$ per treatment group.

all concentrations studied YC-1 did not significantly affect the percentage of viable adherent SMCs, or the percentage of total viable SMCs when the detached cells were included as non-viable.

Figure 4 shows that platelet aggregation in response to thrombin was significantly ($P < .001$, ANOVA) inhibited in a dose-dependent fashion by YC-1 released from PEG hydrogels. No differences in platelet aggregation were observed between control platelet suspensions without hydrogel and platelets that had been exposed to empty hydrogel, indicating that the PEG hydrogel alone did not have significant effects on platelet function. Soluble YC-1 or YC-1 released from PEG hydrogels also significantly ($P < .001$, $P < .05$, respectively, ANOVA) inhibited the attachment of platelets to collagen (Fig. 5). Interestingly, platelets exposed to higher YC-1 concentrations released from the hydrogels show subtle increases in numbers of attached platelets.

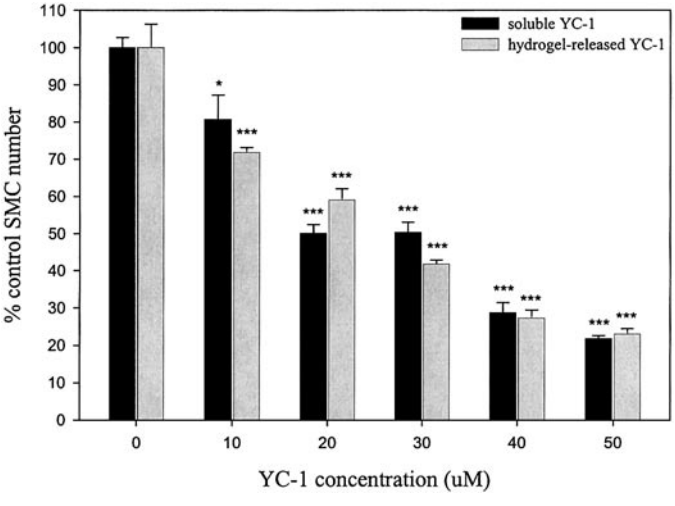

FIG. 3. Soluble YC-1 or YC-1 released from PEG hydrogels significantly ($P < .001$, $P < .001$, respectively; ANOVA) decreased rat vascular SMC proliferation in a dose-dependent manner between 10 and 50 μM. *$P < .05$, ***$P < .001$ versus 0 μM control, Tukey test for pairwise multiple comparisons following one-way ANOVA; $n = 3$ per treatment group.

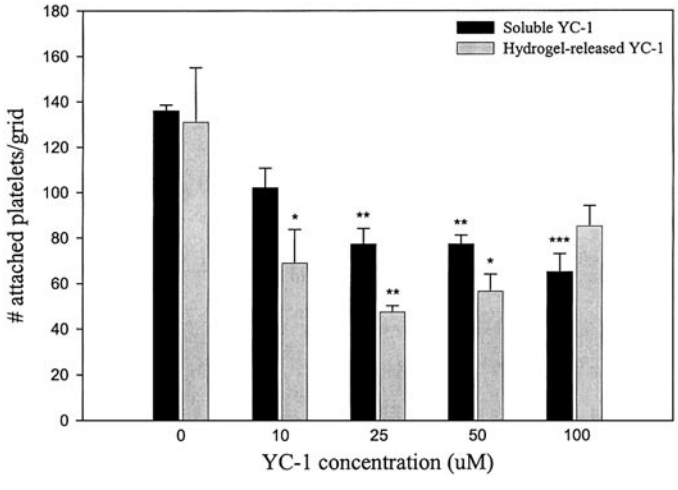

FIG. 5. Soluble YC-1 or YC-1 released from PEG hydrogels significantly ($P < .001$, $P < .05$, respectively, ANOVA) inhibited platelet adherence to collagen between 10 and 100 μM. *$P < .05$, **$P < .01$, ***$P < .001$ versus 0 μM control, Tukey test for pairwise multiple comparisons following one-way ANOVA; $n = 2$–3 per treatment group.

Representative photomicrographs of perfusion-fixed, balloon-injured LCA cross-sections 2 weeks post-injury are shown in Fig. 6, including an untreated proximal LCA section (A) and distal LCA sections exposed to either empty PEG hydrogel (B) or 1 mg YC-1 in hydrogel (C). Figure 7 illustrates morphologic data using unpaired statistical comparisons between groups. Injured but untreated proximal LCA sections between and within treatment groups did not show statistically significant differences in any morphologic parameter analyzed. Therefore, these data were combined (n = 12) and termed "Proximal Control" (Fig. 7). Results revealed no significant differences between Proximal Control and Empty Gel groups for any morphometric parameter analyzed. However, YC-1 treatment significantly and consistently reduced neointima development compared to the Empty Gel vehicle group (Figs. 7A, 7C, and 7D). Medial wall cross-sectional area was not affected by YC-1 (Fig. 7B).

Complimenting the attenuated neointima observed in these 2-week YC-1 tissues, reduced neointimal SMC numbers (−60%, $P < .05$; compared to empty hydrogel) were also observed (data not shown). No differences were observed in medial SMC counts between vessels exposed to hydrogel ± YC-1. Trichrome staining of the injured distal LCA sections exposed to the hydrogel ± YC-1 revealed an abundant collagen-rich fibrous capsule in the perivascular space 2 weeks post-injury. This fibrous capsule was also evident in 2-week sham surgeries where balloon injury was not performed, suggesting that it was produced as an artifact from the surgery itself or from application and/or activation of the PEG hydrogel. The 2-week sham surgeries revealed no detectable neointima after exposure to empty gel (n = 3; data not shown). Western blot analysis on uninjured LCAs 48 h post-surgery indicated upregulated HO-1 protein in distal arterial sections exposed to UV light (4-fold), UV light plus empty hydrogel (6.7-fold), and UV light plus hydrogel containing 1 mg YC-1 (2.3-fold), compared to untreated, uninjured proximal LCA sections from the same animals (data not shown). These data suggest that some non-specific, surgery-associated stress response does occur in the vessel wall following intervention in the absence of balloon injury.

DISCUSSION

We report here that the benzyl indazole derivative YC-1, in either soluble form or following release from a copolymer PEG hydrogel, is capable of significantly inhibiting rat vascular SMC proliferation in a dose-dependent fashion and reducing platelet aggregation and adherence to collagen under cell culture conditions. Soluble YC-1 stimulated the production of cGMP by cultured vascular SMCs in a dose-dependent manner, and perivascular delivery of YC-1 from activated

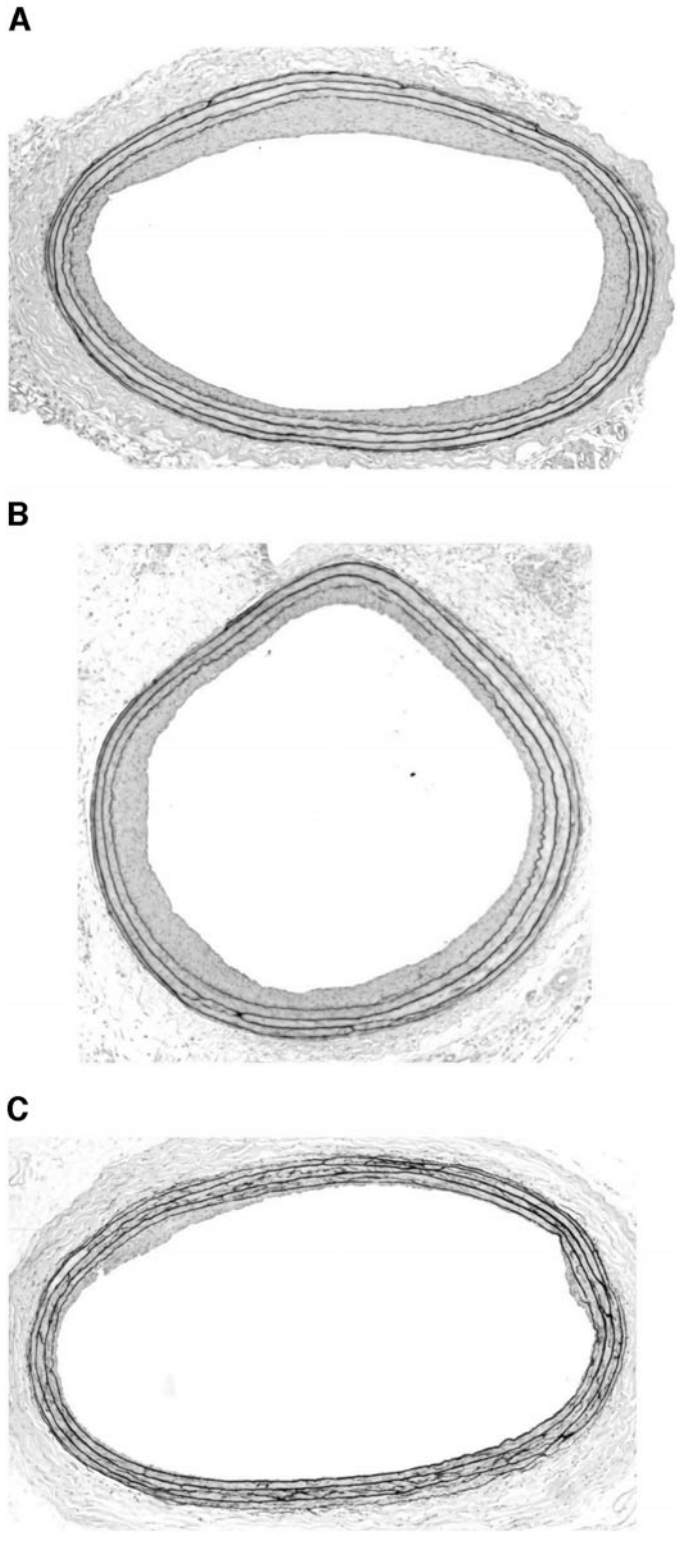

FIG. 6. Representative photomicrographs of perfusion-fixed, balloon-injured carotid artery cross-sections 2 weeks post-injury, with an untreated proximal section (A) and distal sections exposed to either empty PEG hydrogel (B) or 1 mg YC-1 in PEG hydrogel (C) immediately after injury.

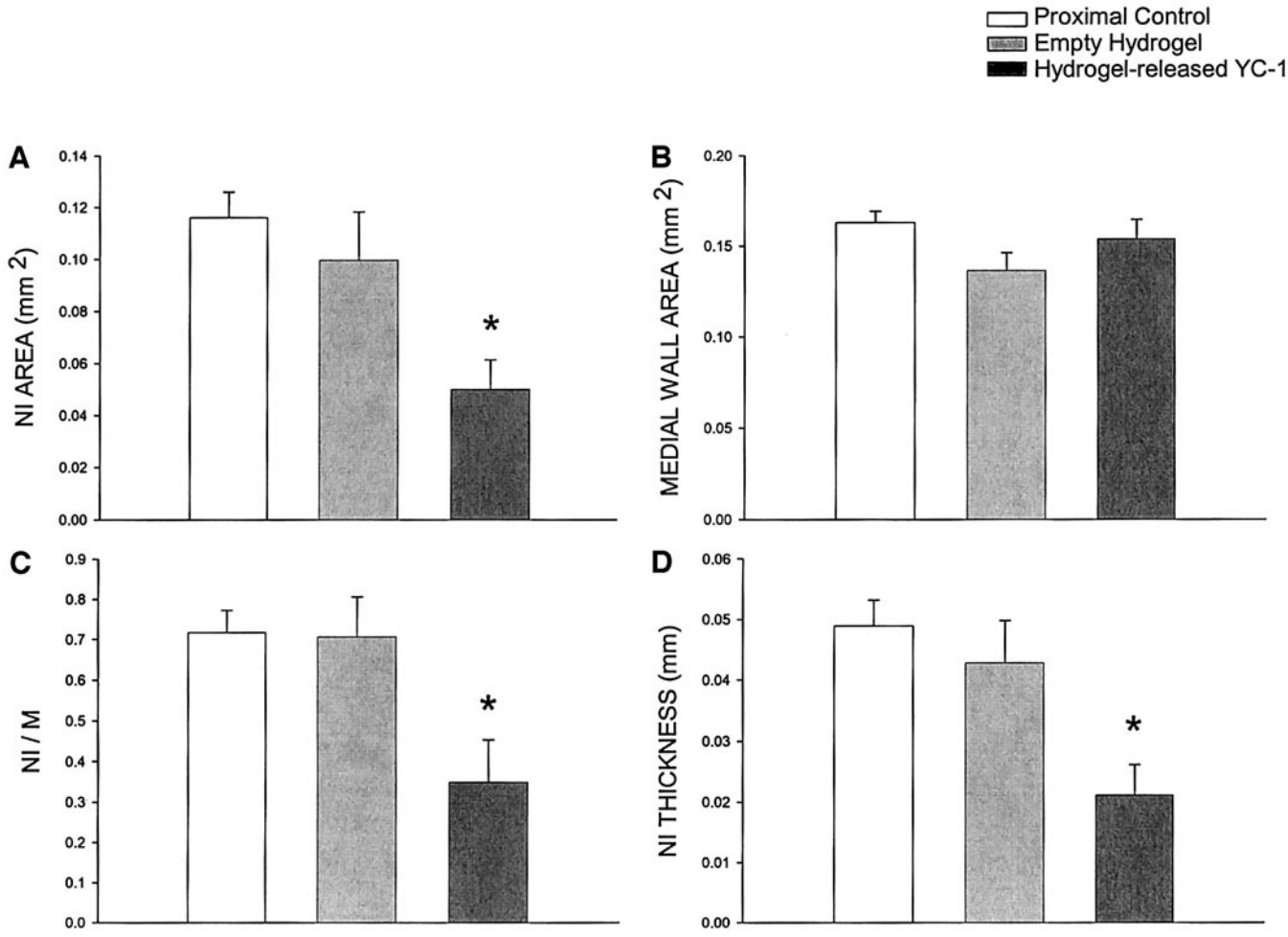

FIG. 7. Morphologic parameters 2 weeks following carotid artery balloon injury using unpaired Student's t-test comparisons between treatment groups. Effects from the empty hydrogel were not statistically different from untreated proximal controls. Hydrogel-released YC-1 (1 mg) significantly inhibited neointima development (A, C, D) compared to the empty hydrogel. *$P < .05$ versus empty hydrogel group; $n = 12$ for proximal controls; $n = 6$ for empty hydrogel and hydrogel-released YC-1 groups.

PEG hydrogels significantly attenuated experimental balloon angioplasty-induced neointima development in the rat carotid artery. These injured, YC-1-treated arteries demonstrated significantly reduced neointimal SMC numbers with unaltered medial wall SMC counts. These results provide physiologically significant mechanisms for the inhibitory actions of YC-1 on angioplasty-induced neointima formation and support and extend our earlier report citing vasoprotective properties of pluronic gel-incorporated YC-1 (2). The original findings presented here suggest that YC-1 confers vasoprotection against injury-induced pathological remodeling by directly inhibiting SMC proliferation and platelet function, possibly through cGMP-dependent processes.

Following arterial injury and experimental and clinical atherogenesis, vascular levels of HO-1 are stimulated at both the transcript and protein levels (2, 18, 19), resulting in elevated local concentrations of the heme metabolites, CO and biliverdin. Concomitant with HO-1 induction, impairment of the endothelial-dependent NO signaling pathway occurs under these inimical conditions. Local stimulation of HO-1 may therefore act as a compensatory mechanism to mediate vascular protection and repair where NO activity is lost. Enhanced HO-1 activity has been shown to inhibit acute vascular cell proliferation (3, 4) and stimulate medial wall cellular apoptosis (5) following experimental injury, subsequently inhibiting vessel wall remodeling. Additionally, HO-1 has been shown to possess anti-atherogenic properties under conditions of experimental atherosclerosis (7, 8). These HO-1-mediated protective phenomena have been suggested to occur, at least in part, through the vasodilatory, anti-inflammatory, and antimitogenic properties of heme-derived CO (6, 20, 21). The physiological role of CO has

been challenged, however, as it possesses relatively low potency in stimulating the sGC/cGMP pathway compared to NO (11). Nevertheless, as shown by this and other studies, YC-1 is capable of sensitizing CO-activated sGC and stimulating cGMP production to a high degree through several redundant and physiologically active pathways.

Cyclic GMP is a vital intracellular second messenger capable of stimulating diverse cellular responses through cGMP-dependent serine/threonine kinase (PKG)-mediated phosphorylation events. Elements of the sGC/cGMP/PKG system have been identified as pivotal mediators of the arterial remodeling response to balloon injury (22, 23). The actions of YC-1 on this signaling pathway include allosteric stabilization of the active configuration of sGC (14), inhibition of dissociation rates of agonists from the activated cyclase (11), and inhibition of the cGMP-dependent phosphodiesterase type 5 (13, 15). We now report that, under *in vitro* conditions, vascular SMC cGMP is increased in a dose-dependent fashion following exposure to YC-1. These findings support earlier reports of YC-1-induced sGC activation and cGMP production in bovine lung (9, 11), human platelets (24), isolated rabbit aortic rings (13), and cultured rat vascular SMCs (14). The increases in SMC cGMP content found in this study parallel the marked inhibition of SMC proliferation and reduced platelet function observed in the same YC-1 concentration range.

Proliferation of vessel wall SMCs is a pivotal event in the pathogenesis and progression of occlusive vascular diseases as well as in the adaptive remodeling response to vascular injury. Various attempts have been made to inhibit or reduce the proliferative capacity of vascular SMCs exposed to inimical stimuli in an effort to minimize pathological and invasive cellular hyperplasia. In this study, we have found that YC-1, in either soluble form or following release from PEG hydrogels, significantly and reproducibly inhibited cultured vascular SMC proliferation in a dose-dependent fashion. In addition, carotid arteries exposed to hydrogel-released YC-1 after injury demonstrated reduced neointimal SMC numbers after 2 weeks. These are the first data reporting anti-proliferative properties of YC-1, under both controlled cell culture conditions and following experimental arterial injury. We propose that the reduced acute proliferative capacity of medial wall SMCs exposed to YC-1 inhibits consequent neointima formation.

Platelet-derived factors compliment vessel wall contributions to injury-induced vascular remodeling. Platelets provide the source of vasoactive agents, including thrombin (25) and platelet-derived growth factor (26), that regulate neointima development and stimulate migration of proliferating SMCs into the intima (27). Activated platelets also stimulate thrombogenesis that contributes to stenotic occlusion. We report here than YC-1 significantly inhibits several indices of platelet function under cell culture conditions. Platelet aggregation following thrombin stimulation was decreased in a dose-dependent fashion by hydrogel-released YC-1. These results corroborate recent studies using soluble YC-1 that showed reduced platelet aggregation under culture conditions (15, 24) and inhibition of the formation of platelet-rich thrombosis in mice (28). In this current study, platelet adherence to collagen was also inhibited by soluble YC-1 or by hydrogel-released YC-1, consistent with studies using soluble YC-1 (29). Thus, hydrogel-mediated delivery of YC-1 is potentially a physiologically and therapeutically relevant inhibitor of platelet function.

In conclusion, we provide here new evidence that YC-1 is capable of attenuating the neointimal response to experimental arterial injury through concomitant inhibition of vascular SMC proliferation and platelet aggregation and adhesion, probably through sGC/cGMP-dependent pathways. These results provide novel insights into mechanisms of YC-1-conferred vasoprotection and offer therapeutic potential for YC-1 treatment of occlusive vascular disorders and post-interventional complications.

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