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The cyclic GMP modulators YC-1 and zaprinast reduce vessel remodeling through anti-proliferative and pro-apoptotic effects

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Abstract

Guanosine-specific cyclic nucleotide signaling is suggested to serve protective actions in the vasculature; however, the influence of selective pharmacologic modulation of cyclic guanosine monophosphate (GMP)-synthesizing soluble guanylate cyclase (sGC) or cyclic GMP-degrading phosphodiesterase (PDE) on vessel remodeling has not been thoroughly examined. In this study, rat carotid artery balloon injury was performed and the growth-modulating effects of the sGC stimulator YC-1 or the cGMP-dependent PDE-V inhibitor zaprinast were examined. YC-1 or zaprinast elevated vessel cyclic GMP content, reduced medial wall and neointimal cell proliferation, stimulated medial and neointimal cellular apoptosis, and markedly attenuated neointimal remodeling in comparable fashion. Interestingly, sGC inhibition by ODQ failed to noticeably alter neointimal growth, and concomitant zaprinast with YC-1 did not modify any parameter compared to individual treatments. These results provide novel in vivo evidence that YC-1 and zaprinast inhibit injury-induced vascular remodeling through anti-mitogenic and pro-apoptotic actions and may offer promising therapeutic approaches against vasoproliferative disorders.

Keywords

apoptosis; carotid artery balloon injury; proliferation; YC-1; zaprinast

Introduction

The soluble guanylate cyclase (sGC)/cyclic guanosine monophosphate (GMP) signal transduction system plays an important regulatory role in the cardiovascular system. The vasoactive influence of sGC/cyclic GMP-stimulatory nitric oxide (NO) has been extensively studied and well characterized. More recently, our laboratory has helped identify the heme oxygenase (HO)/carbon monoxide (CO) system as a robust cyclic GMP-stimulating pathway

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following vascular injury.[1-3] Synthesized cyclic GMP exerts physiologic and pathophysiologic effects through multiple signaling pathways including direct activation of cyclic GMP-dependent protein kinase (PK-G), direct and/or indirect cross-talk with the cyclic adenosine monophosphate (AMP)/cyclic AMP-dependent protein kinase (PK-A) system, or hydrolytic degradation by cyclic GMP-dependent phosphodiesterase (PDE-V).

Based principally on its protective effects in cardiac and vascular tissues, much emphasis has been placed on identifying upstream pharmacologic activators or stimulators of the sGC/cyclic GMP system. YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole), originally characterized as a potent sGC activator in platelets,[4,5] has been shown to protect against vascular smooth muscle injury through multiple growth-inhibitory properties.[6-10] In similar fashion BAY 41-2272, a potent YC-1-based sGC stimulator, has been recently suggested to serve beneficial actions against aberrant vascular smooth muscle growth.[11] An alternate route for inducing salutary cyclic GMP signaling is through selective inhibition of cyclic GMP-degrading PDE, an approach successfully used to combat a variety of vascular disorders with perhaps the most popular being those used to treat sexual dysfunction. Zaprinast [1,4-dihydro-5-(2-propoxyphenyl)-7H-1,2,3-triazolo[4,5-d]pyrimidine-7-one], a selective inhibitor of cyclic GMP-specific PDE-V, significantly elevates cyclic GMP and downstream signaling in the vasculature.[12,13] Several studies cite protective actions of zaprinast in hypoxic pulmonary vasculature,[14] during capillary leakage and edema,[15] or on platelet function following arterial injury,[16] while several others have shown beneficial effects of cyclic AMP-specific PDE-3 inhibition on vessel growth;[17-19] however, the influence of the PDE-V inhibitor zaprinast on the growth response to vascular injury is scientifically provocative yet has not been described in the literature.

Using YC-1 for comparison, the present study addressed the hypothesis that the selective cyclic GMP-dependent PDE-V inhibitor zaprinast attenuates remodeling in rat balloon-injured carotid arteries. Novel results demonstrate that both YC-1 and zaprinast elevate vessel cyclic GMP content, reduce vascular cell proliferation and stimulate apoptosis, and attenuate neointimal growth in similar manner. Furthermore, lack of significant ODQ and combined YC-1 and zaprinast effects suggest that YC-1 and zaprinast share common signaling pathways in the injury growth response and that these occur irrespective of upstream cyclase involvement.

Materials and Methods

Rat carotid artery balloon injury model

Experimental balloon injury was performed on the rat left carotid artery (LCA) as described.[20] Briefly, male Sprague Dawley rats (520 ± 10 g; Harlan, Indianapolis, IN) were anesthetized (ketamine, xylazine, and acepromazine; 0.5 - 0.7 ml/kg IM; VetMed Drugs) and a Fogarty 2F embolectomy catheter (Baxter Healthcare Corp.) was introduced through an external carotid arteriotomy site and advanced through the LCA, inflated, and withdrawn thrice. The exposed portion of the injured LCA was treated accordingly (see Dosing Protocol below), tissues were closed in layers, and animals were given buprenorphine (0.5 mg/kg, SC) for analgesia. At specific times, anesthetized rats were euthanized by pneumothorax and exsanguination and tissues were harvested for specific protocols. All procedures conformed to the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996), and were approved by the Institutional Animal Care and Use Committee (IACUC).

Dosing protocol

Topical administration of pharmacologic agents immediately followed balloon injury.[6,21] Two hundred μl of a 25% copolymer gel solution (Pluronic F-127; BASF) containing 1 mg zaprinast (Calbiochem-Novabiochem Corp.), YC-1 (Calbiochem, LaJolla, CA), or the specific sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Calbiochem-Novabiochem Corp.), or a combination thereof, in 50 μl DMSO was administered to the exposed LCA adventitia. Control animals received 200 μl gel containing 50 μl DMSO. Animals were closed and allowed to recover until euthanasia.

Tissue processing and staining

Perfusion-fixed, paraffin embedded tissues were used for standard staining techniques, and microscopic analysis and quantitation of morphologic parameters were performed.[21] Fresh tissues were snap-frozen and used for the cyclic GMP assay.[6]

Cyclic GMP radioimmunoassay

The protocol used for measuring cyclic GMP in vascular tissue followed that supplied with the ^{125}I-labeled cyclic GMP competitive radioimmunoassay (RIA) kit (Amersham Biosciences).[6] Forty-eight hours after injury animals were sacrificed and fresh tissues were obtained and snap-frozen. Similarly-treated tissues were pooled (n = 3-5 animals/group) and the RIA was performed. Tissues were homogenized in cold 6% trichloroacetic acid, centrifuged, and the cyclic GMP-containing supernatant removed and ether-washed. Cyclic GMP-containing extract was dried and resuspended in 200 μl assay buffer. Samples were acetylated and working standards were prepared. Sections of rat thoracic aorta were used as positive controls for cyclic GMP.[22]

Proliferating cell nuclear antigen (PCNA) and TUNEL assays

Immunostaining for PCNA for estimating cell proliferation was performed on balloon-injured rat LCAs.[1] Tissues were treated with an anti-PCNA monoclonal antibody (PC-10; 1:25; Sigma) complemented with a biotinylated anti-mouse secondary antibody (1:100), followed by an avidin-biotin block and DAB chromogen with nuclear fast red counterstain. Data are represented as a PCNA labeling index (LI), defined as the percentage of total cells positive for PCNA staining within a given area.[1] Rat balloon-injured LCAs were stained for apoptotic nuclei using an In Situ Cell Death Detection kit (Roche Molecular Biochemicals) as described.[1] Tissues were treated with proteinase K, endogenous peroxidase activity was blocked, and tissues were exposed to avidin-biotin block with antigen retrieval. Tissues were incubated with TUNEL reaction mixture and treated with converter-peroxidase solution. Tissues were serially treated with DAB and hematoxylin. Data are represented as a TUNEL LI. Conventional light microscopy was performed on TUNEL-positive cells to confirm morphologic characteristics of apoptosis.

Statistical analysis

Data were stored and analyzed on personal computers using Excel 2003 (Microsoft), Sigma Plot 8.0 with Sigma Stat for Windows (v. 3.0; SPSS, Inc.), Instat (v. 3.06; GraphPad Software Inc.), and Origin 7 SR1 (v. 7.0; OriginLab Corporation). Data were grouped according to treatment and analyzed using a one-way ANOVA with post-hoc Holm-Sidak test for pairwise comparisons. All data are represented as mean ±standard error of the mean (SEM). A p-value < 0.05 is considered statistically significant for all comparisons.

Results

Rat carotid artery balloon injury model

Representative photomicrographs of rat balloon-injured LCAs 14 days post-injury are shown in Figure 1. An injured LCA treated with empty hydrogel (Fig. 1A) exhibits a concentric and significant neointima with a clearly defined medial wall and elastic laminae. Figure 1B shows an injured zaprinast-treated LCA with a significantly attenuated neointima, appearing sporadically as a thin non-concentric layer adjacent to the internal lamina. Figure 1C illustrates an injured YC-1-treated LCA and similarly shows reduced neointimal development. Figure 1D shows an injured LCA treated with combined zaprinast and YC-1, and a significantly diminished neointima is observed yet no additive effect is seen compared to individual zaprinast- or YC-1-treated sections. Figure 1E shows an ODQ-treated vessel with a significant and concentric neointima (similar to controls), while Figure 1F shows an ODQ and YC-1-treated vessel. Corresponding histomorphometric data obtained 14 days post-injury are shown in Figure 2. Figures 2A, 2C, and 2D clearly show significant and comparable attenuation of neointimal growth in injured LCAs treated with zaprinast, YC-1, or zaprinast plus YC-1 compared to vehicle controls. Separate cohorts of injured arteries treated with ODQ failed to show observable changes in neointima formation relative to vehicle controls, and arteries exposed to combined ODQ and YC-1 failed to fully reverse the YC-1-inhibitory effects on neointimal growth. Interestingly, LCAs exposed to zaprinast, either alone or in combination with YC-1, displayed significant medial wall enlargement compared to vehicle controls (Fig. 2B). Analyses of the circumferences of LCA internal and external elastic laminae between all treatment groups revealed no significant differences after 14 days (data not shown).

Cyclic GMP

Zaprinast stimulated a significant 2.7-fold increase in cyclic GMP content in injured LCAs compared to injured vehicle controls (61.61 ±5.62 vs. 23.04 ±2.30 fmol/mg protein, respectively) 48 hours post-injury. At this time YC-1 also markedly elevated vessel cyclic GMP levels compared to controls (35.45 ±2.2 vs. 22.12 ±4.3 fmol/mg protein, respectively), substantiating previously observed findings.[6] Interestingly, combined zaprinast and YC-1 failed to increase cyclic GMP over that observed in the zaprinast-only treated group (63.66 ±3.12 vs. 61.61 ±5.62 fmol/mg protein, respectively). Rat balloon-injured LCAs exposed to empty hydrogel (vehicle controls) did not demonstrate altered cyclic GMP compared to untreated injured sections, and uninjured LCAs treated with zaprinast and/or YC-1 did not show changes in cyclic GMP levels compared to uninjured untreated sections (data not shown).

PCNA and TUNEL

Temporal analyses of vessel wall DNA replication, performed by PCNA immunostaining, were performed 1, 2, 7 and 14 days following injury (Fig. 3). Data show markedly reduced PCNA staining in zaprinast- and YC-1-treated groups in both the medial wall (Fig. 3A) and neointima (Fig. 3B) at all time points. In complement, medial cell counts were significantly reduced in the zaprinast group compared to controls at 2 (304.5 ±7.5 vs. 339.3 ±7.3; $p < 0.05$) and 14 (371.3 ±24.7 vs. 603.5 ±71.9; $p < 0.05$) days post-injury, while neointimal cell counts were significantly reduced in the zaprinast group compared to controls after 14 days (226.8 ±26.2 vs. 1072.5 ±100.5; $p < 0.001$). Similarly, YC-1 potently reduced cellularity in the media after 1 (256.0 ±12.6 vs. 375.3 ±9.3; $p < 0.01$), 2 (260.7 ±4.7 vs. 339.3 ±7.3; $p < 0.001$), and 14 (401.3 ±32.8 vs. 603.5 ±71.9; NS, $p = 0.06$) days and in the neointima after14 days (172.8 ±37.5 vs. 1072.5 ±100.5; $p < 0.001$) compared to controls.

Figure 4 illustrates results from TUNEL immunostaining for LCA medial and neointimal cell apoptosis 14 days post-injury. Marked pro-apoptotic effects from zaprinast or YC-1 are clearly evident. Vessel wall TUNEL results at 1, 2, and 7 days post-injury revealed no specific trend

for apoptosis (data not shown). TUNEL-positive cells consistently exhibited marked cellular contraction, nuclear condensation, and intracellular cytoplasmic blebbing observed via light microscopy by an independent observer (data not shown).

Discussion

The current study shows for the first time that the selective cyclic GMP-dependent PDE-V inhibitor zaprinast confers protection against vascular growth following experimental injury. In the rat carotid artery balloon injury model, peri-operative zaprinast significantly elevated vessel cyclic GMP levels and markedly inhibited neointimal growth through anti-proliferative and pro-apoptotic avenues. Analogous effects were observed with the sGC stimulator YC-1, and combined YC-1 with zaprinast failed to show discernable effects over individual treatments, thus suggesting that these agents operate via similar signaling pathways. Moreover, cyclase inhibition by ODQ failed to exert demonstrable impact on vessel growth, implying that these phenomena occur in sGC-independent fashion. Indeed, these provocative results offer in vivo insights into the mechanisms of action of these upstream and downstream cyclic GMP modulators and suggest promising therapeutic approaches for the treatment of vascular proliferative disorders.

In this study zaprinast significantly elevated cyclic GMP in injured arteries in the same range as that reported in rat primary vascular smooth muscle following growth factor stimulation[23] and in rat cardiac tissue after natriuretic peptide stimulation.[24] We observed that YC-1 also increased vessel cyclic GMP after injury, substantiating earlier results in this same model[6] and in a separate study using rat primary vascular smooth muscle cells.[7] These results support bioactivity of cyclic GMP signaling by zaprinast and YC-1 in this experimental in vivo model. Interestingly, in this study concomitant zaprinast with YC-1 failed to induce cyclic GMP over that observed with zaprinast alone, suggesting common pathways exist for these agents in this experimental setting. This is highly plausible considering that YC-1 exerts potent (IC_{50} = 10 μM) PDE-V inhibition in addition to its well documented cyclase-stimulatory actions.[25,26] These remarkable results suggest that YC-1 may operate primarily through downstream PDE-V inhibition in vascular tissues following trauma. A recent article showing that YC-1 attenuates homotypic neutrophil aggregation primarily through inhibition of PDE activity[27] supports this theory. Conversely, these findings may also infer that sGC is fully activated in the zaprinast treated injured vessel and is therefore unable to synthesize additional cyclic GMP in the presence of a stimulatory agonist such as YC-1.

Vascular growth is largely dependent upon a balance between tissue gain and loss, critical parameters for therapeutic targeting. Zaprinast or YC-1 significantly and equally attenuated injury-induced neointimal growth in the rat carotid artery (Figs. 1 and 2), suggesting that these agents operate via similar mechanisms in their growth-reducing effects. Conversely, these findings may also suggest that growth inhibition from these compounds is maximal and has reached a plateau and that further reduction in growth via additive, synergistic or potentiative avenues is not possible under these experimental conditions. Further study is warranted with these promising agents, perhaps at lower doses than those used in the current investigation, to address this possibility. Interestingly, zaprinast, alone or in conjunction with YC-1, resulted in medial wall enlargement in addition to its attenuating effects on neointimal formation. This is an apparent paradox, considering that zaprinast significantly inhibited medial cell DNA and cellular replication and stimulated medial cell apoptosis, processes that eventuate in loss of medial wall architecture and integrity. Current investigations into potential mechanisms that could contribute to an enlarged medial area from zaprinast, which may include directional cell migration, altered production of extracellular matrix (ECM) components, or changes in ECM-degrading metalloproteinases (MMPs), should prove insightful and may offer potential underlying mechanisms.

The inhibitory effects of zaprinast on vascular DNA replication and cellularity observed in this study support recent reports of the cytostatic actions of zaprinast on cells of different origins.[28-30] We now provide first evidence that zaprinast can directly reduce proliferation of vascular cells following experimental injury. Zaprinast has been found to potentiate NO-mediated apoptosis in megakaryocytes[31] and natriuretic peptide-induced apoptosis in rat cardiac myocytes[32] and pulmonary endothelium,[30] although zaprinast itself has been suggested to lack apoptotic capacity.[33] Considering earlier results showing that balloon injury upregulated sGC-sensitizing NO synthase and HO enzymes,[6] herein we provide novel data showing potent pro-apoptotic effects of zaprinast in vascular tissues after injury. The pro-apoptotic effects of YC-1 observed in this report corroborate previous findings that YC-1 stimulates apoptosis in neutrophils[34] and in adrenomedullary endothelial and chromaffin cells,[35] possibly through caspase-3-like protease activity in fashion independent of sGC.[35]

A recent article provides strong evidence that cyclic GMP/PK-G signaling is not involved in the protective effects of NO during vascular remodeling.[36] Experiments using ODQ in the current report suggest that sGC is not involved in zaprinast- or YC-1-mediated vascular growth inhibition, yet cumulative findings also suggest that zaprinast and YC-1 share common signaling pathways, that is, through inhibition of cyclic GMP-degrading PDE-V with ensuing cyclic GMP-dependent actions. Differences in experimental models (arterial ligation or wire denudation versus balloon distension) and species (mouse versus rat) as well as distinct upstream activating ligands (NO versus CO) must be considered when comparing such studies.

Conclusions

Herein we demonstrate that the cyclic GMP-dependent PDE-V inhibitor zaprinast provides protection against vessel growth after injury through inhibition of vascular cell proliferation and induction of apoptosis. Analogous results with the sGC stimulator YC-1 suggest that YC-1 and zaprinast operate through parallel pathways in the vascular growth response following trauma. Also, lack of noticeable effects from concomitant YC-1 and zaprinast suggest that these agents operate in sGC-independent fashion under these experimental conditions. This new evidence provides insights into the mechanisms and potential therapeutic applicability of the cyclic GMP modulators YC-1 and zaprinast for the treatment of vasculoproliferative disorders.

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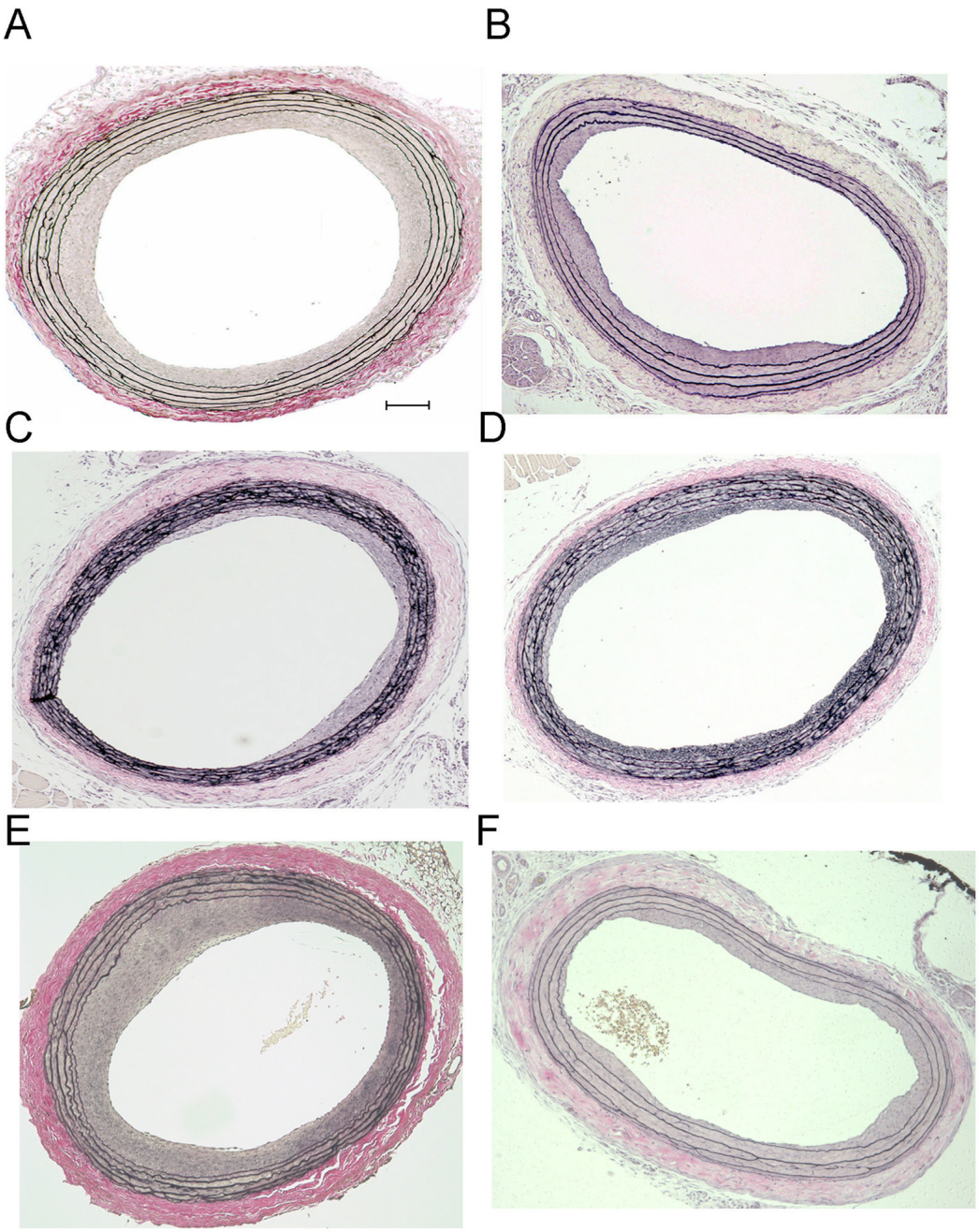

Figure 1. Photomicrographs of rat balloon-injured carotid arteries

Representative photomicrographs of rat balloon-injured, perfusion-fixed Verhoeff-van Gieson-stained left carotid arteries (LCAs) 14 days post-injury. Immediately following injury LCAs were treated with vehicle (A) or vehicle containing zaprinast (B), YC-1 (C), zaprinast + YC-1 (D), ODQ (E), or ODQ + YC-1 (F). Scale bar in (A) represents a length of 100 μm.

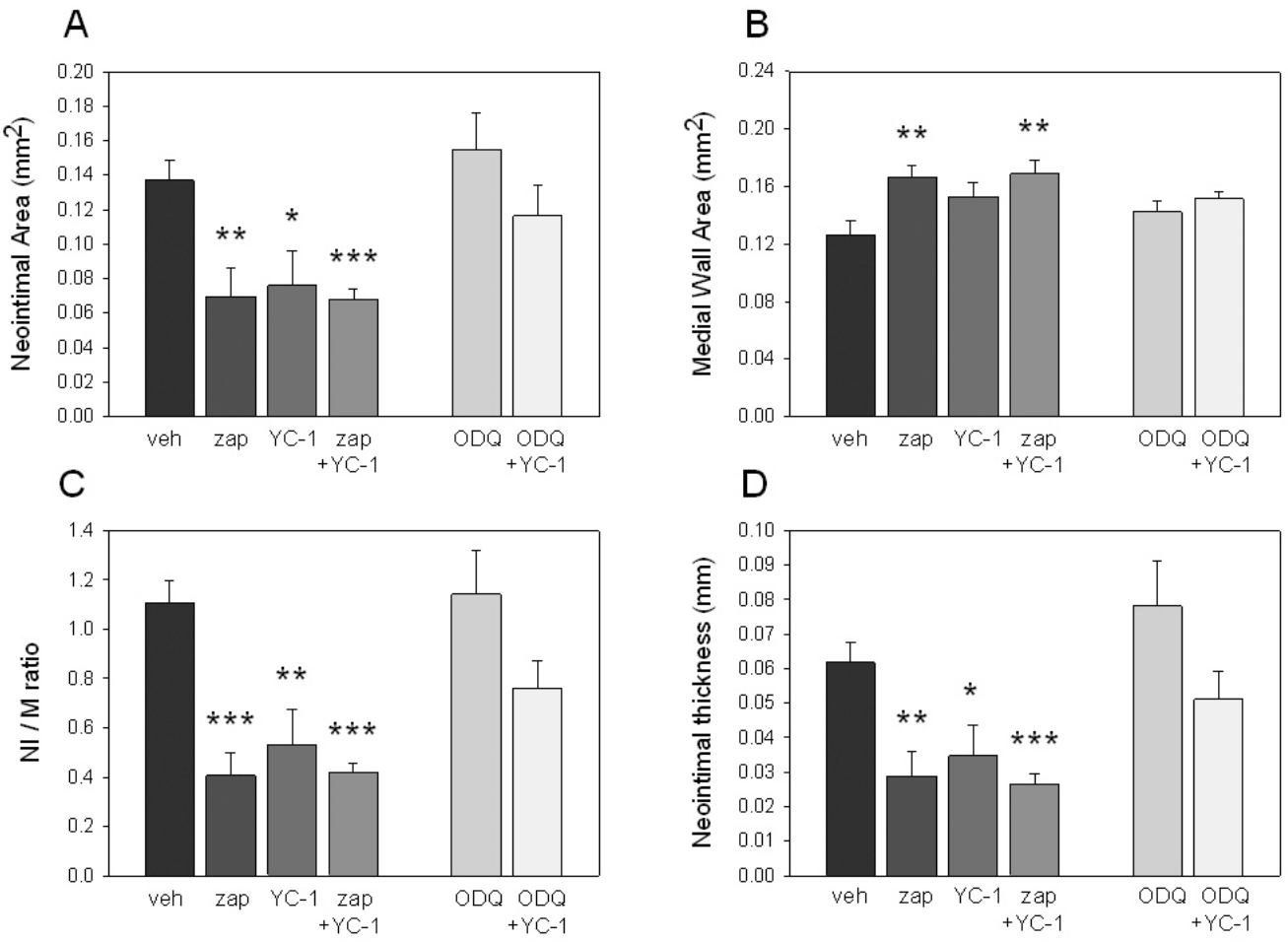

Figure 2. Histomorphometric data for rat balloon-injured carotid arteries

Morphologic parameters 14 days following balloon injury for rat left carotid arteries (LCAs) treated with vehicle, zaprinast, YC-1, zaprinast + YC-1, ODQ, or ODQ + YC-1 immediately after injury. Neointimal growth (A, C, D) is significantly attenuated in the zaprinast, YC-1, and zaprinast + YC-1 groups. Significant medial wall enlargement is detected in both zaprinast-treated groups (B). ODQ failed to significantly alter neointimal growth or medial wall morphology relative to controls. Animal numbers: n = 7 for vehicle, n = 9 for YC-1, n = 8 for zaprinast, zaprinast + YC-1, ODQ, and ODQ + YC-1 groups. Values represent mean ±SEM. * $p < .05$, ** $p < .01$, *** $p < .001$ versus vehicle.

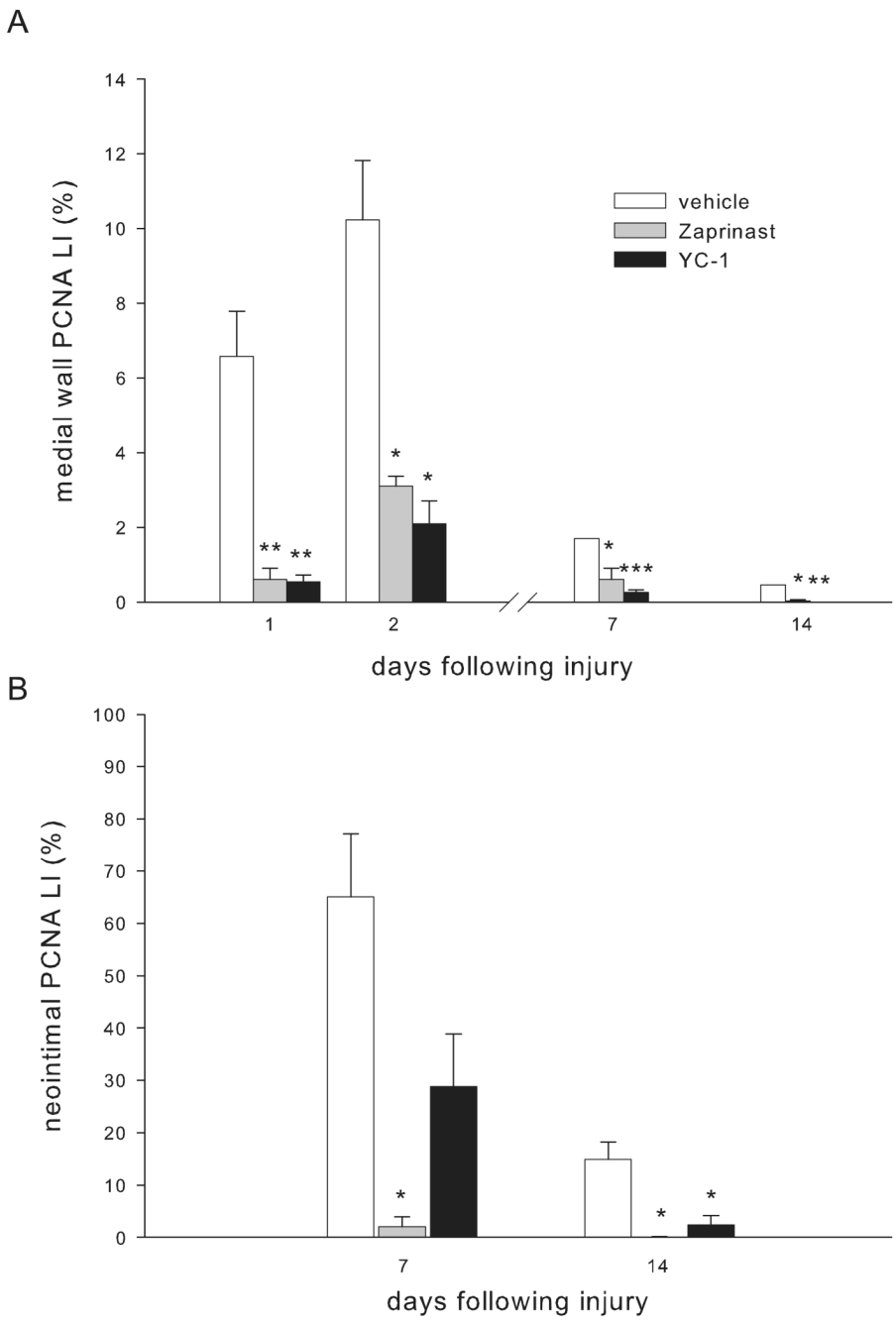

Figure 3. Vessel wall PCNA immunostaining

Medial wall (A) and neointimal (B) PCNA labeling indices (LI%) for rat left carotid arteries (LCAs) 1, 2, 7, or 14 days following balloon injury. Immediately following injury LCAs were treated with vehicle or with vehicle containing zaprinast or YC-1. Zaprinast or YC-1 robustly and equally reduced medial and neointimal cell proliferation at all time points compared to controls. Animal numbers: n = 3-5/group/time point. Values represent mean ±SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle.

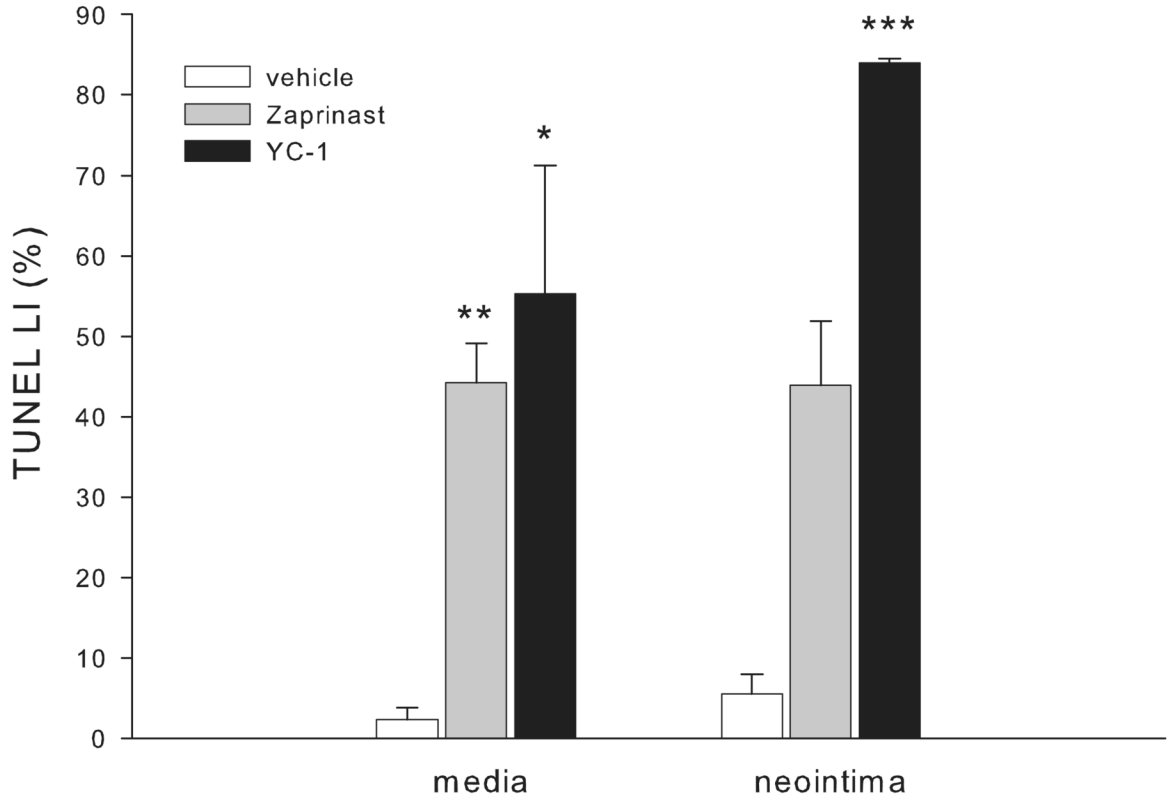

Figure 4. Vessel wall TUNEL staining

Medial and neointimal TUNEL immunostaining on rat balloon-injured left carotid arteries (LCAs) after 14 days reveals robust apoptosis in the zaprinast- or YC-1-treated LCAs compared to controls. Animal numbers: n = 3-5/group/time point. Values represent mean ±SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle.