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# Ribonucleotide Reductase Inhibitors Reduce Atherosclerosis in a Double-Injury Rabbit Model

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**Atheroproliferative disorders such as atherosclerosis are an important health problem and one of the leading causes of morbidity and mortality in the United States. Minimally invasive therapeutic procedures, including angioplasty with stent deployment, are used frequently for obstructive coronary artery disease. However, restenosis, a proliferative vascular response, is a common sequela to this procedure. The current study investigated the effect of inhibiting ribonucleotide reductase (RR), an enzyme necessary for cellular proliferation, in an attempt to ameliorate the proliferative response. Two RR inhibitors, didox and hydroxyurea, were chosen for their potent antiproliferative properties. Studies were carried out by using a double-injury rabbit model, in which endothelial denudation was followed by the administration of a high-fat diet. At 4 wk after initial endothelial denudation, the developing atherosclerotic lesion was subjected to transluminal balloon dilation to simulate clinical intervention with percutaneous transluminal angioplasty. The degree of restenosis and atheroproliferation was assessed at 8 wk. Histologic evaluation of the lesion demonstrated that treatment with didox and hydroxyurea significantly decreased lesion area and lumen loss. These results suggest that RR inhibition may be an effective new tool for the treatment of atheroproliferative disorders.**

**Abbreviation:** RR, ribonucleotide reductase.

Coronary artery disease and atherosclerosis, in particular, are multifactorial processes that include numerous molecular and cellular cascades culminating in inflammatory and proliferative vascular responses involving the endothelium, vascular smooth muscle cells, and leukocytes. Among these responses, impaired endothelial function, manifested as impaired nitric oxide production, and increased signaling through reactive oxygen species have been recognized as critical components in the pathogenesis of atherosclerosis. The complex nature of the atherogenic process has hindered the development of animal models that mimic human atherosclerosis.[27]

Rabbits typically are used for studies of atherosclerosis because they are one of the few species that can develop atheromatous foci with many of the characteristics of human atherosclerotic lesions. Primary rabbit models of atherosclerosis include both genetically altered strains and models that are diet-induced. The lesions in atherosclerotic rabbits whose disease is induced through consumption of high-cholesterol diet exhibit several pathologic features associated with human lesions, including fatty streaks, accumulation of foam cells, and fibrous plaque formation. The atheromatous changes in this model manifest quickly, with marked lesion formation within 4 to 12 wk of initiation of a high-cholesterol diet.[19,28] Prolonged (8 mo or more) consumption of high-cholesterol diet causes lesions that are rich in smooth muscle cells and closely resemble human lesions. To induce more advanced lesions in these rabbits, consumption of a high-cholesterol diet can be combined with vascular endothelial denudation by using a single- or double-balloon injury.[16,26] Moreover, the double-balloon injury model closely approximates the clinical setting of balloon dilatation of a diseased atheromatous vessel.

Currently, although percutaneous transluminal coronary angioplasty with stent deployment is the mainstay of treatment of obstructive coronary artery disease therapy, this procedure is plagued by a high incidence of restenosis, or vessel renarrowing, which is responsible for 30% to 40% of long-term failures.[1,2] Drug-eluting stents recently have come to the forefront as a promising treatment modality for restenosis, but some evidence suggests that the clinical benefits may be overestimated, given that drug-eluting stents have been implicated in causing late-developing fatal thrombosis.[14,17,18] Therefore, prevention of restenosis after successful percutaneous transluminal coronary angioplasty remains one of the most challenging tasks in the treatment of obstructive coronary artery disease, and alternative pharmacologic approaches are currently being pursued.

Through a cascade of molecular events, the vascular trauma associated with percutaneous transluminal coronary angioplasty initiates vascular smooth muscle cells to undergo modulation from a contractile to a synthetic phenotype. Vascular smooth muscle cells proliferate in the tunica media and migrate to the tunica intima, resulting in intimal hyperplasia referred to as 'neointimal formation.'[7,8,25,33] The result of neointimal formation constitutes restenosis. Pharmacologic agents that impede the proliferation and migration of vascular smooth muscle cells are being investigated to ameliorate this response.

Ribonucleotide reductase (RR) is an enzyme that, when activated by a free-radical intermediate, catalyzes the conversion of ribonucleotides to deoxyribonucleotides. This reductive reaction is a rate-limiting step in the biochemical pathway lead-

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ing to DNA synthesis and cell replication.[6,30,31] Hydroxyurea is a commercially available RR inhibitor that has been used for the treatment of various cancers. Didox is a more potent RR inhibitor than is hydroxyurea and has additional antiinflammatory and antioxidant properties.[4,10,21,29,32] Recently the use of the RR inhibitor didox in a rat model of balloon-mediated carotid artery injury led to reduction of restenosis and cell cycle arrest.[12] Because cellular proliferation and migration are involved in the formation of atherosclerotic plaques, the present study uses histologic analysis and the measurement of vascular reactivity to investigate the role of the RR inhibitors hydroxyurea and didox in preventing the development of atherosclerotic lesions.

## Materials and Methods

**Animals.** All procedures involving animals that were conducted in this study were approved by The Ohio State University Institutional Animal Care and Use Committee. All animals were housed in an AAALAC-accredited facility and in compliance with the *Guide for the Care and Use of Laboratory Animals*.[15] Male New Zealand White rabbits (*Oryctolagus cuniculus*; weight, 2 to 3 kg; Covance Research Products, Denver, PA) were specific-pathogen–free for myxoma virus, rotavirus, *Bordatella bronchiseptica*, cilia-associated repiratory bacillus, *Clostridium piliforme*, *Pasteurella multocida*, *Pastuerella pneumotropica*, *Listeria monocytogenes*, *Toxoplasma gondii, Eimeria* spp., *Salmonella* spp., *Encephalitozoon cuniculi*, and *Psoroptes cuniculi*. The rabbits were singly housed in standard rabbit caging (Lab Products, Seaford, DE) and maintained on a 12:12-h light:dark cycle. All rabbits had free access to food and water throughout the duration of the study.

**Surgical procedure.** All rabbits received ketamine (20 mg/kg IM) for induction of anesthesia. Intubation was performed once the swallowing reflex was minimized, and general anesthesia was maintained by using 2% to 3% isoflurane admixed with 100% oxygen. A 2.5-mm, wire-guided balloon catheter (Boston Scientific, Natick, MA) was inserted into the right common carotid artery through an arteriotomy in either the right or left femoral artery. Progress of the catheter and the location of the injury were monitored by using fluoroscopy (Figure 1). Endothelial injury of the carotid artery was initiated by inflating the balloon to 12 atm and moving the inflated balloon in a forward and retrograde direction. The balloon was deflated to enable removal of the catheter, the vessel was ligated, and the surgical site was closed by using a simple continuous intradermal pattern with 4-0 polydioxanone suture. Bupivicaine was infiltrated as a local analgesic into the tissues surrounding the incision site, and buprenorphine (0.05 mg/kg SC every 12 h) was used perioperatively for systemic analgesia as needed in response to evidence of lameness, guarding, or inflammation. After surgery, the rabbits were placed on a high-cholesterol diet containing 2% cholesterol and 1% peanut oil (Harlan Tekland, Oxford, MI) for the duration of the study.

At 4 wk after endothelial injury, balloon angioplasty of the developing atherosclerotic lesion was performed. A 2.5-mm, wire-guided balloon catheter (length, 25 mm) was inserted into the injured common carotid artery through an arteriotomy in the nonaccessed femoral artery. Lesion formation was verified by intravascular ultrasonography, and the balloon was inflated to 12 atm 3 times for 5 s each at the site of injury. After angioplasty, the catheter was removed, and the femoral artery was ligated. The rabbits recovered from surgery, and the high-cholesterol diet continued for an additional 4 wks. At the conclusion of the study, animals were placed under general anesthesia and euthanized by pentobarbital overdose (100 mg/kg IV) followed by removal of a vital organ (that is, carotid arteries, aorta). Blood was obtained at the time of euthanasia and evaluated for total cholesterol and white blood cell counts. The carotid arteries were removed for histologic evaluation and vascular reactivity studies.

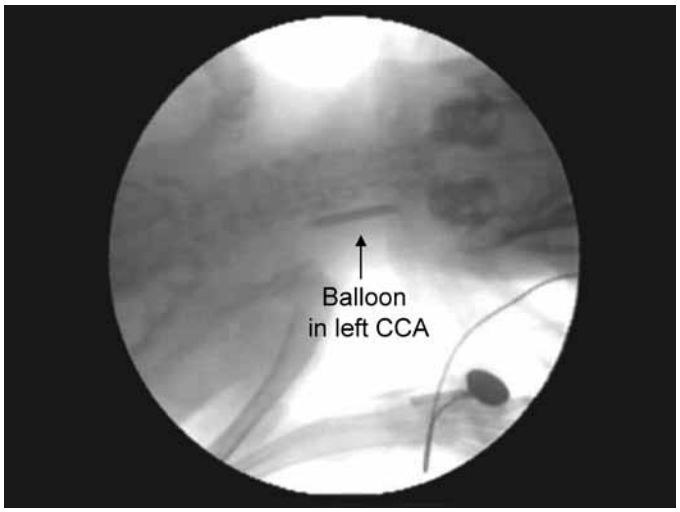


**Figure 1.** Fluoroscopy image depicting a 3-mm wire-guided balloon in the left common carotid artery (CCA) of a New Zealand White rabbit under general anesthesia. Balloons were inserted through the left or right femoral artery and inflated to 12 atm. The inflated balloons were moved in a forward and retrograde direction to produce endothelial injury.

**Pharmacologic treatments.** After the initial injury, rabbits were given either didox (200 mg/kg SC) or hydroxyurea (400 mg/kg SC). The dosages used for these studies were based on previously published reports and are sufficient to inhibit RR activity without causing significant toxicity.[13,22,23] Both didox and hydroxyurea were provided by Molecules for Health (Richmond, VA). Injections were given subcutaneously 3 d each week throughout the duration of the study. Blood levels were monitored by using high-performance liquid chromatography to ensure that the animals maintained the desired therapeutic levels. Treatment groups ($n$ = 4 to 6 rabbits per group) comprised untreated, uninjured (no balloon injury; control); untreated injured (balloon injury only); didox-treated, injured (balloon injury + didox); and hydroxyurea-treated, injured (balloon injury + hydroxyurea) rabbits.

**Vascular reactivity.** Constriction and relaxation of isolated carotid rings from untreated and treated rabbits were measured by using a wire myography system (Danish Myo, Colorado Springs, CO). Briefly, carotid arteries were harvested and maintained in ice-cold PBS ($Ca^{2+}$- and $Mg^{2+}$-free) during transportation before mounting on the wire myograph. The rings were allowed to equilibrate in Krebs–Henseleit solution aerated with 95% $CO_2$–5-%$O_2$ at 37 °C for 60 min. Contractile responses to phenylephrine (0.5 μM) were measured by using a force transducer interfaced with Chart software (ADI Instruments, Colorado Springs, CO) for data analysis. After a 60-min equilibrium period, the rings were stretched to generate a tension of 1.5 g. The optimum resting force of the carotid rings was determined by comparing the force developed by 40 mM KCl under different resting forces. After precontraction of the vascular rings with phenylephrine, the

relaxation response was determined by using increasing concentrations of acetylcholine (1.0 nM to 5 μM).

**Histologic assessment.** Injured and contralateral (uninjured) carotid arteries were fixed with neutral buffered formalin and paraffin-embedded. The tissues then were sectioned at 8 μm and stained with hematoxylin and eosin, trichrome, or elastic Van Gieson stain. Morphometric analysis of the cross sections was performed by using the image analysis software SPOT Advance (Spot Imaging Solutions, Sterling Heights, MI).

**Statistical analysis.** Data are presented as mean ± SE. Statistical analysis was performed by Sigma Stat (Systat, San Jose, CA). An ANOVA was used to detect significant differences in multiple comparisons. An unpaired Student *t* test was used to detect significant differences when 2 groups were compared. A *P* value of less than or equal to 0.05 was considered to be significant.

## Results

**Effects of RR inhibition on atherosclerosis.** The double-injury rabbit model with hypercholesterolemia generated an atherosclerotic lesion with pathologic characteristics similar to those seen in human atherosclerotic plaques (Figure 2 A). The atheroma area in the uninjured, untreated (control) rabbits was 1.13 $mm^3$. Treatment with didox (200 mg/kg) and hydroxyurea (400 mg/kg) significantly ($P < 0.05$) reduced the lesion area to 0.60 $mm^3$ and 0.57 $mm^3$, respectively (Figure 2 B). Loss of luminal diameter was estimated at 43% in the injured, untreated rabbits, whereas didox- or hydroxyurea- treated animals showed no significant lumen loss as compared with controls (Figure 2 C).

**Effects of RR inhibition on carotid vascular reactivity.** Carotid artery rings from injured (ipsilateral) and noninjured (contralateral) vessels were excised and evaluated in vascular reactivity studies using wire myography techniques. Results demonstrated significant ($P < 0.05$) impairment of vascular reactivity in the vehicle-treated balloon-injured carotid, with maximal endothelium-dependent relaxation of 27.5% ± 10.9% (Figure 3 A). Treatment with the RR inhibitor hydroxyurea did not significantly increase endothelial dependent relaxation (34.3% ± 17.6%). In contrast, didox-treated rabbits had significantly improved relaxation responses with a maximal acetylcholine-induced relaxation response of 53% ± 17.0% (Figure 3 A). Endothelium-dependent relaxation measured in the contralateral (uninjured) vessels demonstrated similar acetylcholine responses among all groups (Figure 3 B).

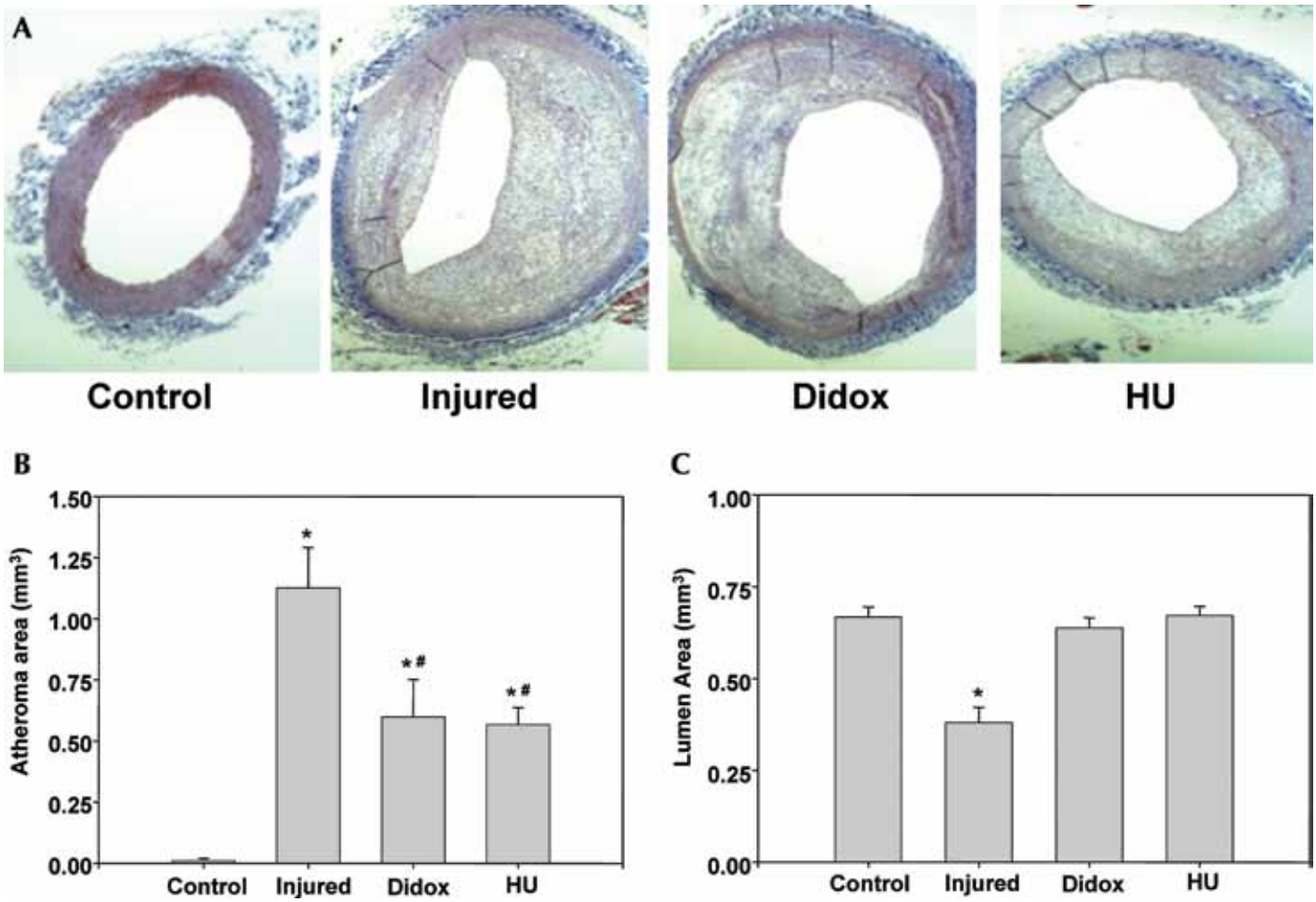


**Figure 2.** Effect of RR inhibitors on atherosclerosis. The groups comprised noninjured (control), untreated injured (injured), injured and didox-treated (200 mg/kg; Didox), and injured and hydroxyurea-treated (400 mg/kg; HU) animals. (A) Trichrome-stained sections from control, injured, and treated rabbits. (B) Atheroma area. (C) Lumen area. Data are presented as mean ± SE ($n = 5$ or) 6; *, value significantly ($P < 0.05$) from that of the control group; #, value significantly ($P < 0.05$) different from that for injured group.

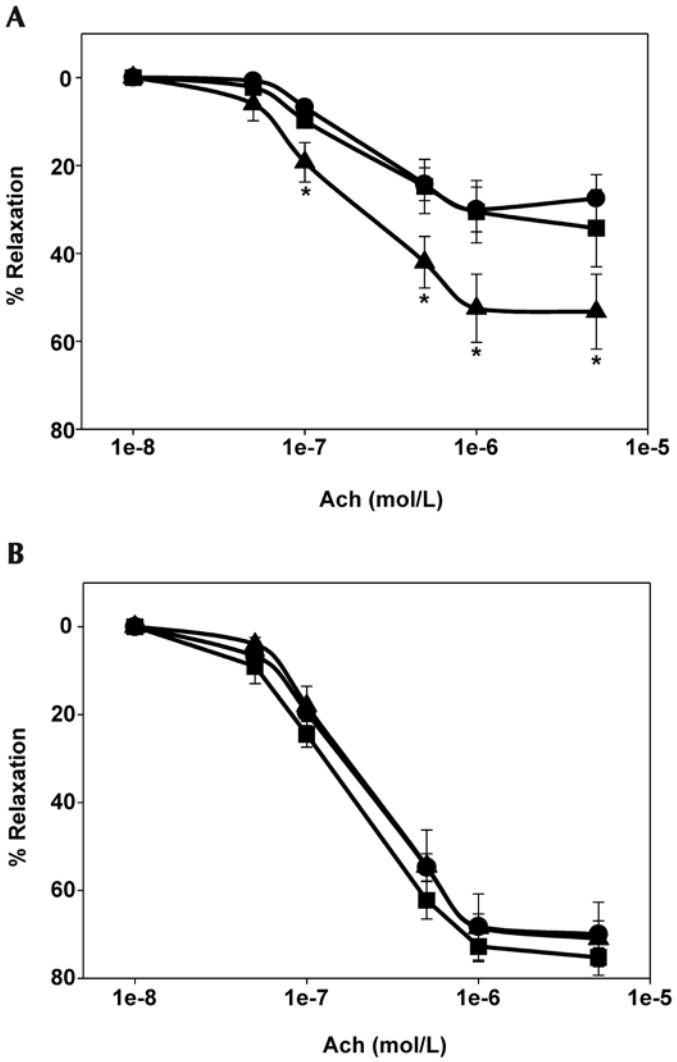


**Figure 3.** Effects of RR inhibitors on vascular reactivity. Endothelium-dependent relaxation was measured in untreated (circles), didox-treated (200 mg/kg; triangles), and hydroxyurea-treated (400 mg/kg; squares) animals. Rings were constricted with phenylephrine (0.5 µM), and the relaxation response to acetylcholine (Ach; 10 nM to 5 µM) was measured on a wire myograph. (A) Vascular relaxation response to acetylcholine from ipsilateral injured vessels. (B) Vascular relaxation response to acetylcholine from contralateral noninjured vessels. Data are presented as percentage relaxation compared to control (mean ± SE; $n = 6$). *, Value significantly ($P < 0.05$) different from that for control animals.

**Hematologic effects of RR inhibition.** Total plasma cholesterol was measured at euthanasia. All groups had an average plasma cholesterol level that exceeded 1200 mg/dL, with no significant differences in total cholesterol among groups. The RR-inhibitor-treated groups had lower numbers of circulating leukocytes, compared with nontreated controls. Didox treatment resulted in a 74% decrease in leukocyte counts, whereas hydroxyurea treatment resulted in a 59% decrease compared with controls (Table 1).

## Discussion

Endothelial dysfunction is thought to be the initiating step in the cascade of events leading to atherosclerotic plaque formation.[27] The double-injury rabbit model of atherosclerosis involves an initial injury of the endothelium. This approach creates a complex lesion that histologically demonstrates neointimal formation, smooth muscle cell proliferation and migration, and increasing numbers of lipid-laden macrophages, resulting in luminal stenosis.[9,24] Thus, this model provides a pathologic correlate of the human disease and enables the investigation of potential underlying cellular mechanisms involved in disease initiation and progression. Atherosclerotic plaques in humans tend to have additional attributes, such as areas of fibrocalcification and necrosis, much of the research done today is aimed primarily at the arterial response to injury. However, the vascular response of the double-injury rabbit model, as revealed through ultrasonography and histology, exhibits similar pathology to that in humans.[35]

The present study examined the role of RR in neointimal hyperplasia, atheroma production, and vascular remodeling. Because the vascular response to injury triggers a migratory and proliferative response from the smooth muscle cells, emphasis has been placed on developing pharmacologic therapy aimed at reducing the proliferative response.[5,14,25] Although treatment with RR inhibitors significantly decreased intimal hyperplasia in a rat model of balloon injury,[12] the experiments were performed in an otherwise healthy vessel and did not follow the clinical presentation, where dilation-associated injury is occurring in a diseased vessel. This inconsistency is important because the molecular mechanisms involved in proliferative responses differ between healthy and diseased vessels. The current study was performed by using a double-injury model with superimposed hypercholesterolemia. Results from this study demonstrated an almost 50% reduction in atheroma area after treatment with either of the RR inhibitors didox and hydroxyurea. Moreover, treatment with an RR inhibitor prevented the 43% lumen loss in the injured, untreated animals. These in vivo studies demonstrate that inhibiting RR limits the extent of atheroma formation and intimal hyperplasia after balloon injury.

In addition, didox possesses a variety of chemical attributes which may contribute to its protective effects. Didox inhibits NFκB and tissue factor and is a potent free-radical scavenger.[4,10,21,29,32] Evidence collected over the past 20 y suggests that endothelial dysfunction secondary to inflammation and oxidative injury is the molecular trigger for atheroma initiation. Our results demonstrated that didox-treated animals had significantly improved endothelium-dependent relaxation compared with controls, indicating conferment of an endothelium-protective effect. These results indicate that the endothelial protective effects elicited by didox are likely independent of RR inhibition, given that hydroxyurea was unable to confer similar augmentation of acetylcholine-induced relaxation.

Analysis of serum lipids demonstrated that neither didox nor hydroxyurea possessed cholesterol-lowering effects, because cholesterol levels did not differ among control and treated animals. However, hematology demonstrated greatly decreased white blood cell values in the RR-inhibitor-treated groups. Because atherosclerosis is ultimately an inflammatory disease characterized by leukocyte infiltration and foam cell formation, decreasing circulating leukocytes may contribute to the vascular protective effects of these compounds. In support, recent advances suggest a potential role for myeloid leukocytes, specifically monocyte subsets and mast cells, in the propagation of atheroproliferative disorders. These cell types are not just rapidly recruited but already reside in the vascular wall and initiate and perpetuate core mechanisms in plaque formation and destabilization.[34]

**Table 1.** Effects of RR inhibitors on serum cholesterol and blood cell counts (mean ± SE, $n = 4$)

| Group | Cholesterol (mg/dL) | RBC ($x^6/\mu L$) | WBC ($x^3/\mu L$) | Platelets ($x^3/\mu L$) |
|---|---|---|---|---|
| Control | 1412 ± 567 | 5.1 ± 0.8 | 6.1 ± 1.7 | 323 ± 122 |
| Didox | 1255 ± 481 | 3.93 ± 0.6 | 1.6 ± 0.9 | 224 ± 60 |
| Hydroxyurea | 1540 ± 617 | 4.55 ± 0.8 | 2.5 ± 1.3 | 262 ± 78 |

No significant difference between groups was present.

Although the incidence of restenosis has decreased markedly with the advent of drug-eluting stents, restenosis still occurs in as many as 20% of patients within the first year after stent placement. Long-term data on late lumen loss are still being gathered.[3,11,20] Because the use of coated stents may increase the risk of thrombosis, agents that can be administered systemically in patients at high risk for thrombotic events are needed urgently. Inhibition of RR is a pathway that can be targeted therapeutically through either local or systemic delivery. The observations detailed in the present study highlights the important therapeutic potential of RR inhibitors and implicate RR as a promising therapeutic target in the treatment of vascular proliferative disorders.

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