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Nitric Oxide- and Heme-Independent Activation of Soluble Guanylate Cyclase Attenuates Peroxynitrite-Induced Endothelial Dysfunction in Rat Aorta

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Abstract

Oxidative stress interferes with nitric oxide (NO)/soluble guanylate cyclase (sGC)/cyclic guanosine monophosphate (cGMP) signalling pathway through reduction of endogenous NO and formation of the strong intermediate oxidant peroxynitrite and leads to vascular dysfunction. We evaluated the effects of oral treatment with NO- and heme-independent sGC activator cinaciguat on peroxynitrite-induced vascular dysfunction in rat aorta. Sprague-Dawley rats were treated orally 2 times at an interval of 17 hours with vehicle or with cinaciguat (10 mg/kg). One hour after the last treatment, the animals were anesthetized, the thoracic aorta was removed, and the aortic segment preparations were incubated with and without the reactive oxidant peroxynitrite (200 μmol/L, 30 minutes). Endothelium-dependent (acetylcholine), -independent (sodium nitroprusside) vasorelaxations were investigated, and histopathological examination was performed. Incubation of aortic rings with peroxynitrite significantly attenuated the maximal endothelium-dependent relaxation (R_{max}) to acetylcholine (peroxynitrite, 44.5% $\pm$ 5.9% vs control, 93.2% $\pm$ 2.0%, $P < .05$) and decreased pD_2 values (-logEC_{50}, EC_{50} being the concentration of acetylcholine that elicited 50% of the maximal response) for the concentration–response curves as compared to control segments. Treatment of rats with cinaciguat significantly improved the decreased acetylcholine-induced vasorelaxation after exposure of aortic rings to peroxynitrite (cinaciguat + peroxynitrite, 67.1% $\pm$ 3.5% vs peroxynitrite, 44.5% $\pm$ 5.9%, $P < .05$). Incubation of aortic segments with peroxynitrite caused a significant shift of the sodium nitroprusside concentration–response curves to the right without any alterations in the R_{max}. Moreover, exposure of aortic rings to peroxynitrite resulted in increased nitro-oxidative stress and DNA breakage which were improved by cinaciguat. Treatment of rats with cinaciguat significantly increased intracellular cGMP levels in the aortic wall. Our results show under conditions of nitro-oxidative stress when signalling in the NO/sGC/cGMP pathway is impaired, acute activation of sGC by cinaciguat might be advantageous in the treatment of endothelial dysfunction in cardiovascular disease.

Keywords

peroxynitrite, endothelial dysfunction, soluble guanylate cyclase, DNA injury, cGMP

Introduction

Growing evidence indicates that in vivo formation of free radicals in the vascular wall plays important roles in different vascular diseases such as atherosclerosis, arterial hypertension, and restenosis. Peroxynitrite, a strong biological oxidant and nitrating species, is formed from the reaction of the free radicals nitric oxide (NO) and superoxide anion.[1,2] Under physiological conditions, several studies have shown that peroxynitrite exerts a prolonged vasorelaxant action in a variety of isolated vessels including dog coronary artery,[3] bovine pulmonary artery,[4] rabbit aorta,[5] as well as rat aorta.[6] However, in higher concentrations peroxynitrite is cytotoxic and has been

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demonstrated to cause nitro-oxidative damage to proteins,[7] lipids,[8] and DNA.[9] It is well established that in normal condition, binding of NO to the ferrous heme iron (Fe^{2+}) of the soluble guanylate cyclase (sGC) generates cyclic guanosine monophosphate (cGMP) which produces vasodilation through smooth muscle relaxation,[10] inhibits platelet aggregation,[11] and inhibits vascular smooth muscle growth and proliferation.[12] In endothelial dysfunction, signalling in the NO/sGC/cGMP pathway is altered because of the oxidation and subsequent loss of the sGC. Since impaired endothelial function is correlated with increased cardiovascular disease, therapeutic strategies aimed at limiting vascular oxidative stress and improving endothelial function may have clinical benefits. Organic nitrates act as a source of NO, but drug tolerance develops when used as sustained therapy.[13] Their efficacy is limited by the absence of clinically relevant antiplatelet activity[14] and the inability to activate NO-insensitive sGC. Moreover, the use of organic nitrates under oxidative conditions promotes peroxynitrite formation, which can prevent its beneficial effect.[15,16] As an alternative therapeutic approach, a novel class of drugs that modulate the sGC/cGMP signal transduction pathway has been developed. In preclinical studies, the sGC activator, cinaciguat (BAY 58-2667) has been shown to bypass the impaired NO/sGC/cGMP pathway by activation of the oxidized (Fe^{3+})/heme-free forms of sGC and to preferentially dilate the diseased versus nondiseased vasculature.[15,17-19] In a phase I clinical trial in healthy human participants, intravenously administered cinaciguat had a favourable safety profile and was well tolerated.[20] Moreover, a phase II clinical study is currently in progress in patients with acute decompensated heart failure.

We therefore decided to evaluate the effects of oral treatment with NO- and heme-independent sGC activator cinaciguat on vascular dysfunction induced by peroxynitrite in rat aorta functionally and to try to understand the possible pathways of its therapeutic efficacy histologically.

Methods and Materials

Animals

Male Sprague-Dawley rats (250-350 g; Charles River, Sulzfeld, Germany) were used in the experiments. The animals were housed in a constant room temperature (22 $\pm$ 2°C) and 12 hours light/dark cycles with free access to standard laboratory rat diet and water. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85-23, revised 1996). All procedures and handling of animals during the investigations were reviewed and approved by the appropriate institutional review committee.

Treatment

Rats were treated orally 2 times at an interval of 17 hours with vehicle (1% methylcellulose solution) or with cinaciguat (10 mg/kg). One hour after the last treatment, animals were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally), the descending thoracic aorta was removed and transferred to cold Krebs-Henseleit solution (118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, 1.77 mmol/L $CaCl_2$, 25 mmol/L $NaHCO_3$, 11.4 mmol/L glucose; pH = 7.4). After dissecting the adhering fat and connective tissue, segments (4-mm length) were placed in 30 mL Krebs-Henseleit solution supplemented with 10 mmol/L HEPES buffer, aerated with 95% O_2 and 5% CO_2, and incubated for 30 minutes with NaOH vehicle (4.7%) or peroxynitrite (200 μmol/L) to induce endothelial dysfunction. (In the present study, the concentration of peroxynitrite has been chosen on the basis of our previous work.[21])

Experimental Groups

The experimental groups were as follows: control group (rats pretreated orally with methylcellulose, then aortic rings incubated with NaOH), peroxynitrite group (pretreatment of rats with methylcellulose, exposure of aortic rings to peroxynitrite), cinaciguat + peroxynitrite group (pretreatment of rats with cinaciguat, exposure of aortic rings to peroxynitrite), and cinaciguat group (pretreatment of rats with cinaciguat, exposure of aortic rings to NaOH).

In Vitro Organ Bath Experiments

Isolated aortic rings (in each group, 12-15 independent experiments) were mounted on stainless steel hooks under 2 g resting tension in individual organ baths (Radnoti Glass Technology, Monrovia, California), containing 25 mL of Krebs-Henseleit solution gassed continuously with 95% O_2 and 5% CO_2 and warmed to 37°C. Special attention was paid during the preparation to avoid damaging the endothelium. Tissues were equilibrated for 60 minutes. During this period, tension was periodically adjusted to the desired level and the Krebs-Henseleit solution was changed every 30 minutes as a precaution against interfering metabolites. At the beginning of each experiment, maximal contraction forces of potassium chloride (KCl, 80 mmol/L) were determined and aortic rings were washed until the resting tension was again obtained. Aortic preparations were preconstricted with an α-adrenergic receptor agonist, phenylephrine (10^{-6} mol/L) until a stable plateau was reached, and relaxation responses were examined by adding cumulative concentrations of endothelium-dependent vasorelaxant acetylcholine (10^{-9}-10^{-4} mol/L). For testing relaxing responses of smooth muscle cells, a direct NO donor, sodium nitroprusside (10^{-10}-10^{-5} mol/L) was used. Tensions were recorded using isometric force transducers of a myograph (159901A, Radnoti Glass Technology), digitized, stored, and displayed with the IOX Software System (EMKA Technologies, Paris, France). Half-maximal response (EC_{50}) values were obtained from individual concentration–response by fitting experimental data to a sigmoidal equation using Origin 7.0 (Microcal Software, Northampton). Contractile responses to phenylephrine are expressed as percentage of the maximal

contraction induced by KCl. The sensitivity to vasorelaxants was assessed by $pD_2 = -\log EC_{50}$ (mol/L), vasorelaxation (and its maximum [R_{max}]) is expressed as percentage of the contraction induced by phenylephrine (10^{-6} mol/L).

Histopathological Process

After incubation of aortic rings with NaOH or peroxynitrite, aortic segments were immediately fixed in buffered paraformaldehyde solution (4%) and embedded in paraffin. Then 5-μm thick sections were placed on adhesive slides.

Nitrotyrosine Immunohistochemical Staining

According to previously described methods,[22] we performed immunohistochemical staining on aortic rings for nitrotyrosine, a marker of peroxynitrite-mediated damage.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End-Labeling Assay

Terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) assay was performed for detection of DNA strand breaks (free 3′-OH DNA ends) according to the manufacturer's instructions (Chemicon International, Temecula, California). Rehydrated sections were digested with 20 μg/mL DNase-free Proteinase K (Sigma-Aldrich, Germany) to retrieve antigenic epitopes and endogenous peroxidases were blocked with 3% hydrogen peroxide. Free 3′-OH termini of the DNA ends were labeled with a reaction mixture of terminal deoxynucleotidyl transferase and digoxigenin–deoxyuridine triphosphate (dUTP) at 37°C for 1 hour (Chemicon International). Incorporated digoxigenin-conjugated nucleotides were detected using a horseradish peroxidase–conjugated anti-digoxigenin antibody and 3,3′-diaminobenzidine. Sections were counterstained with Gill hematoxylin. Dehydrated sections were cleared in xylene, mounted with Permount (Fischer Scientific, Germany), and coverslips were applied. Based on the intensity and distribution of labeling, semiquantitative histomorphological assessment was performed using conventional microscopy.

Cyclic GMP Immunohistochemical Staining

Cyclic GMP immunohistochemical staining was performed for identification of intracellular cGMP content. After rehydratation of the sections, nonspecific staining was blocked by incubation with a blocking serum (3% goat serum). A rabbit polyclonal anti-cGMP primary antibody (AbD Serotec, Düsseldorf, Germany) was used at a concentration of 1:1000 for 2 hours at room temperature followed by overnight incubation at 4°C.[23,24] Then, the sections were incubated with a secondary biotinylated anti-rabbit immunoglobulin E (BioGenex, California) which allowed reacting with alkaline phosphatase–conjugated streptavidin (BioGenex). A red reaction product at the site of the target antigen was then formed by the use of fast red substrate (DakoCytomation, Hamburg, Germany). Negative controls were performed by omitting the primary antibody. Sections were counterstained with Gill hematoxylin, mounted with Permount, and coverslips were placed on the section.

Quantification of Immunostainings and TUNEL-Positive Nuclei

For nitrotyrosine and cGMP stainings, semiquantitative histomorphological assessment was performed based on the intensity and distribution of labeling using conventional microscopy. After initially evaluating all corresponding tissue sections with ×200 magnification, the tissue section with the most intense labeling signals was used as a reference for maximum labeling intensity. Each specimen was characterized with the average of the 4 adjacent fields. Nitrotyrosine and cGMP levels were scored as follows: 0: *complete absence of immunoreactivity*, 1: *weak area of staining*, 2: *intermediate staining*, and 3: *extensive staining*. Using the Image J (version 1.42) software, we measured the area of the objects in each class in each field, assigned an area score ($1 \leq 10\%$ positive cells, $2 = 11\%$-50% positive cells, $3 = 51\%$-80% positive cells, and $4 \geq 80\%$ positive cells), and calculated an average score for the whole picture (intensity score multiplied by area score, 0-12).

For assessment of TUNEL-labeled cells, the number of positive cell nuclei/microscopic examination field with ×200 magnification was counted from 4 section fields for each sample, averaged, and the mean was calculated for each experimental group.

Histological evaluation was conducted by an investigator unaware of treatment status of the respective groups.

Preparation and Application of Chemical Reagents

Cinaciguat (BAY 58-2667), an amino dicarboxylic acid, was kindly provided by Bayer HealthCare (Wuppertal, Germany). It was suspended in 1% methylcellulose solution vehicle and administered orally at a dose of 10 mg/kg at a volume of 10 mL/kg. The application and dosage of cinaciguat have been determined according to the pharmacokinetic and dynamic properties[20] as well as to the results of previous rodent experiment.[25] Peroxynitrite (Calbiochem, San Diego, California) was diluted with 4.7% NaOH. Phenylephrine, acetylcholine, and sodium nitroprusside were dissolved in 0.9% saline (NaCl) and were bought from Sigma-Aldrich, Germany.

Statistical Analysis

All data are expressed as means ± standard error of the mean. Intergroup comparisons were performed by using 1-way analysis of variance followed by a Student unpaired *t* test with Bonferroni correction for multiple comparisons. A value of $P < .05$ was considered statistically significant.

Table 1. Values of Maximal Relaxation (R_{max}, %) and pD_2 to the Vasorelaxant Action of Acetylcholine (ACh) and Sodium Nitroprusside (SNP), and Contraction Induced by Phenylephrine (% of Potassium Chloride, KCl) in Thoracic Aortic Rings

	Control	Peroxynitrite	Cinaciguat + Peroxynitrite	Cinaciguat
R_{max} to ACh (%)	93.2 ± 2.0	44.5 ± 5.9[a]	67.1 ± 3.5[a,b]	93.9 ± 1.1[b]
pD_2 to ACh	7.6 ± 0.1	6.6 ± 0.2[a]	7.0 ± 0.1	7.9 ± 0.1[b]
R_{max} to SNP (%)	100.1 ± 0.2	100.2 ± 0.3	100.2 ± 0.2	101.6 ± 0.2
pD_2 to SNP	8.8 ± 0.2	8.2 ± 0.1[a]	8.2 ± 0.1[a]	9.1 ± 0.3[b]
Phenylephrine (% of KCl)	73 ± 5	114 ± 3[a]	108 ± 5[a]	78 ± 5[b]

[a] $P < .05$ versus control.
[b] $P < .05$ versus peroxynitrite group.

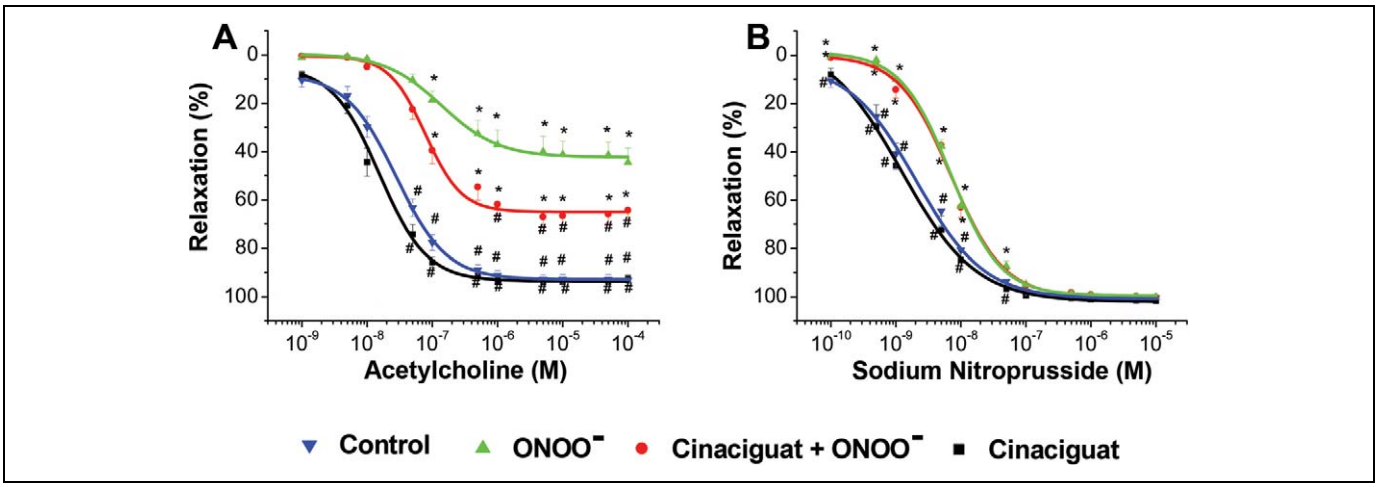

Figure 1. Cinaciguat enhances endothelium-dependent vasorelaxation in aortic rings exposed to peroxynitrite. A, Acetylcholine-induced endothelium-dependent vasorelaxation. B, Sodium nitroprusside–induced endothelium-independent vasorelaxation. *$P < .05$ versus control; #$P < .05$ versus peroxynitrite group.

Results

Endothelium-Dependent Vasorelaxation of Aortic Rings

In aortic rings precontracted with 10^{-6} mol/L phenylephrine, 10^{-9} to 10^{-4} mol/L acetylcholine induced a concentration-dependent relaxation. In contrast, exposure of aortic rings with the reactive oxidant peroxynitrite (200 µmol/L) for 30 minutes significantly attenuated the maximal relaxation to acetylcholine and decreased pD_2 values for the concentration–response curves as compared to control (NaOH only) segments (Table 1, Figure 1A). Treatment of rats with cinaciguat significantly improved the acetylcholine-induced, endothelium-dependent, NO-mediated vasorelaxation after exposure of aortic rings to peroxynitrite. In the absence of peroxynitrite, cinaciguat treatment did not alter maximal relaxation and the sensitivity to acetylcholine compared with the control group (Table 1, Figure 1A).

Endothelium-Independent Vasorelaxation of Aortic Rings

Figure 1B shows concentration-dependent relaxations induced by 10^{-10} to 10^{-5} mol/L sodium nitroprusside, an endothelium-independent vasodilator. In contrast to acetylcholine, maximal relaxation did not differ significantly between the different experimental groups (Table 1, Figure 1B). However, incubation of aortic ring with peroxynitrite caused a significant shift of the sodium nitroprusside concentration–response curves to the right. Cinaciguat has no effect on this level of damage (Table 1, Figure 1B). In the absence of peroxynitrite, treatment of rats with cinaciguat did not alter maximal relaxation and the sensitivity to acetylcholine compared with the control group (Table 1, Figure 1B).

Contractile Responses of Aortic Rings

The contractile responses of aortic segments to phenylephrine (10^{-6} mol/L), an α_1-adrenergic agonist, are shown in Table1. Incubation of aortic rings with peroxynitrite significantly increased the phenylephrine-induced maximum contraction compared with control rings. However, treatment of rats with cinaciguat did not significantly reduce increased contractile responses to phenylephrine. In the absence of peroxynitrite, cinaciguat treatment did not have any effect (Table 1).

Cinaciguat Decreases Nitro-oxidative Stress and DNA Strand Breaks in Aortic Rings Exposed to Peroxynitrite

To evaluate the levels of oxidative and nitrosative stress in the aortas after peroxynitrite exposure, we assessed nitrotyrosine

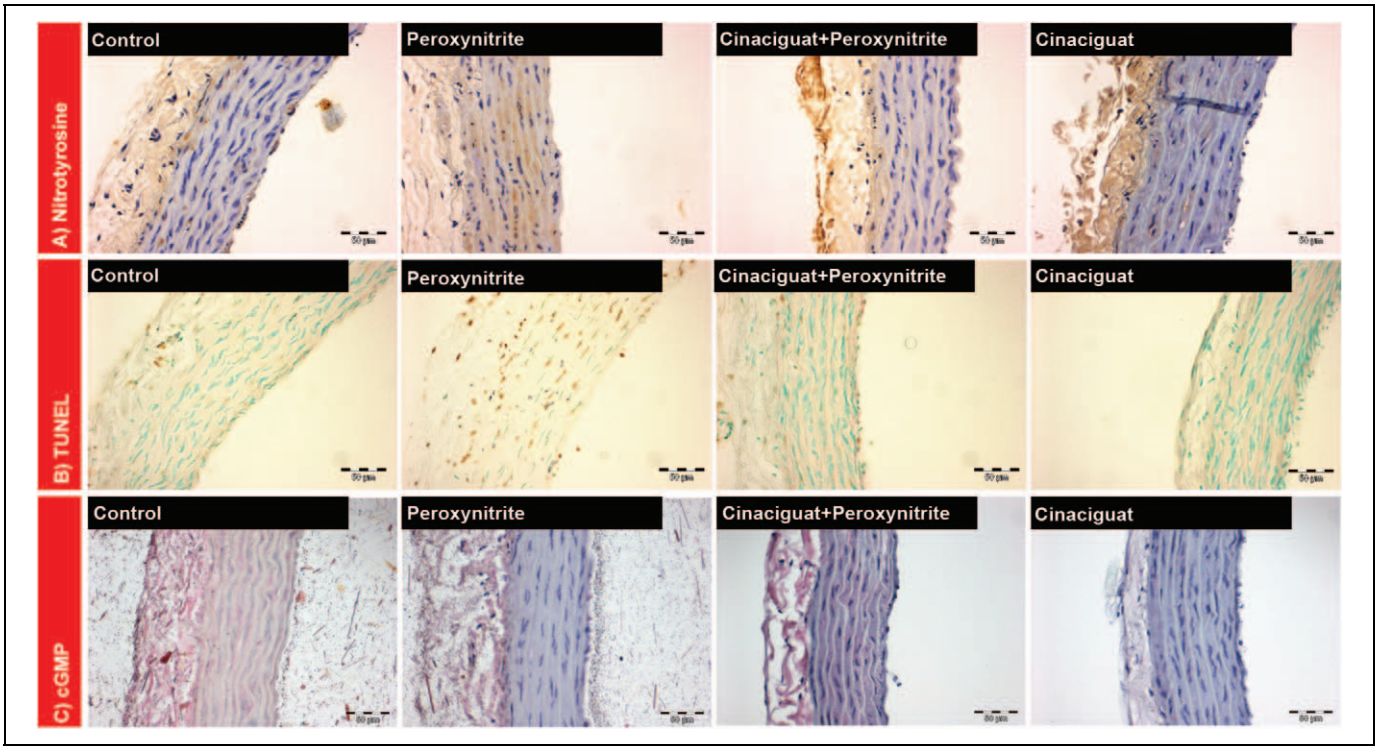

Figure 2. Effects of cinaciguat on nitro-oxidative stress, DNA strand breaks, and cyclic GMP levels in aortic rings exposed to peroxynitrite. Representative photomicrographs of (A) nitrotyrosine immunohistochemistry staining (brown staining), (B) TUNEL assay in the cell nuclei (brown staining), and (C) cyclic GMP immunohistochemistry staining (red staining) in the aortic vascular wall (magnification ×200, bar = 50 μm). GMP indicates guanosine monophosphate; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling.

immunoreactivity. There was a large increase in intensity of nitrotyrosine staining in the peroxynitrite-incubated rings compared to control segments which was reduced after cinaciguat pretreatment, as evidenced by decreased brown staining (Figures 2A and 3A).

Increased density of TUNEL-positive nuclei was observed in the wall of peroxynitrite-exposed aortic rings indicating DNA-fragmentation (Figures 2B and 3B). Pretreatment of rats with cinaciguat significantly decreased peroxynitrite-induced DNA strand breaks (Figures 2B and 3B).

Cinaciguat Increases cGMP Levels in Aortic Rings Exposed to Peroxynitrite

In peroxynitrite-exposed rings, we detected a tendency toward lower cGMP immunoreactivity compared with control (without reaching the level of statistical significance). However, after treatment of rats with cinaciguat, in the media of peroxynitrite-incubated rings a significantly higher score of cGMP staining was observed when compared with the peroxynitrite-incubated segments, as evidenced by increased red staining (Figures 2C and 3C).

Discussion

There is now good evidence for the contribution of elevated production of reactive oxygen and nitrogen species to cell dysfunction via induction of oxidative damage to cell macromolecules, such as lipids, DNA, and proteins. Peroxynitrite, the highly reactive coupling product of NO and superoxide, is an important mediator of tissue injury in various forms of inflammation, shock, and ischemia/reperfusion injury.[2]

In the present study, vascular rings exposed to peroxynitrite at a concentration of 200 μmol/L exhibited reduced endothelium-dependent vasorelaxations to acetylcholine. Stimulation of M3-muscarinic receptor by acetylcholine releases NO from the endothelium which then diffuses to smooth muscle cells where it binds to and activates sGC. This enzyme catalyzes the conversion of guanosine-5′-triphosphate (GTP) to cGMP. The elevation of cGMP ultimately initiates vascular smooth muscle relaxation.[26] Szabo et al also showed that exposure of peroxynitrite caused a marked impairment of the endothelium-dependent relaxation.[27] Peroxynitrite causes tyrosine nitration of the prostacyclin synthase, thereby inhibiting prostacyclin formation within the endothelium. The nitration of tyrosine residues to produce nitrotyrosine is a sensitive marker elicited by peroxynitrite. In the present study, in the aortic segments subjected to peroxynitrite, our immunohistochemical study has revealed a marked nitrotyrosine staining. The DNA damage induced by peroxynitrite is a well-known phenomenon and has been reviewed by Szabo and Ohshima.[28] In accordance with other biomolecular targets, DNA is damaged by peroxynitrite through nitration and oxidation. We therefore used TUNEL staining for detecting DNA fragmentation. In vascular segments incubated for only 30 minutes with peroxynitrite, we

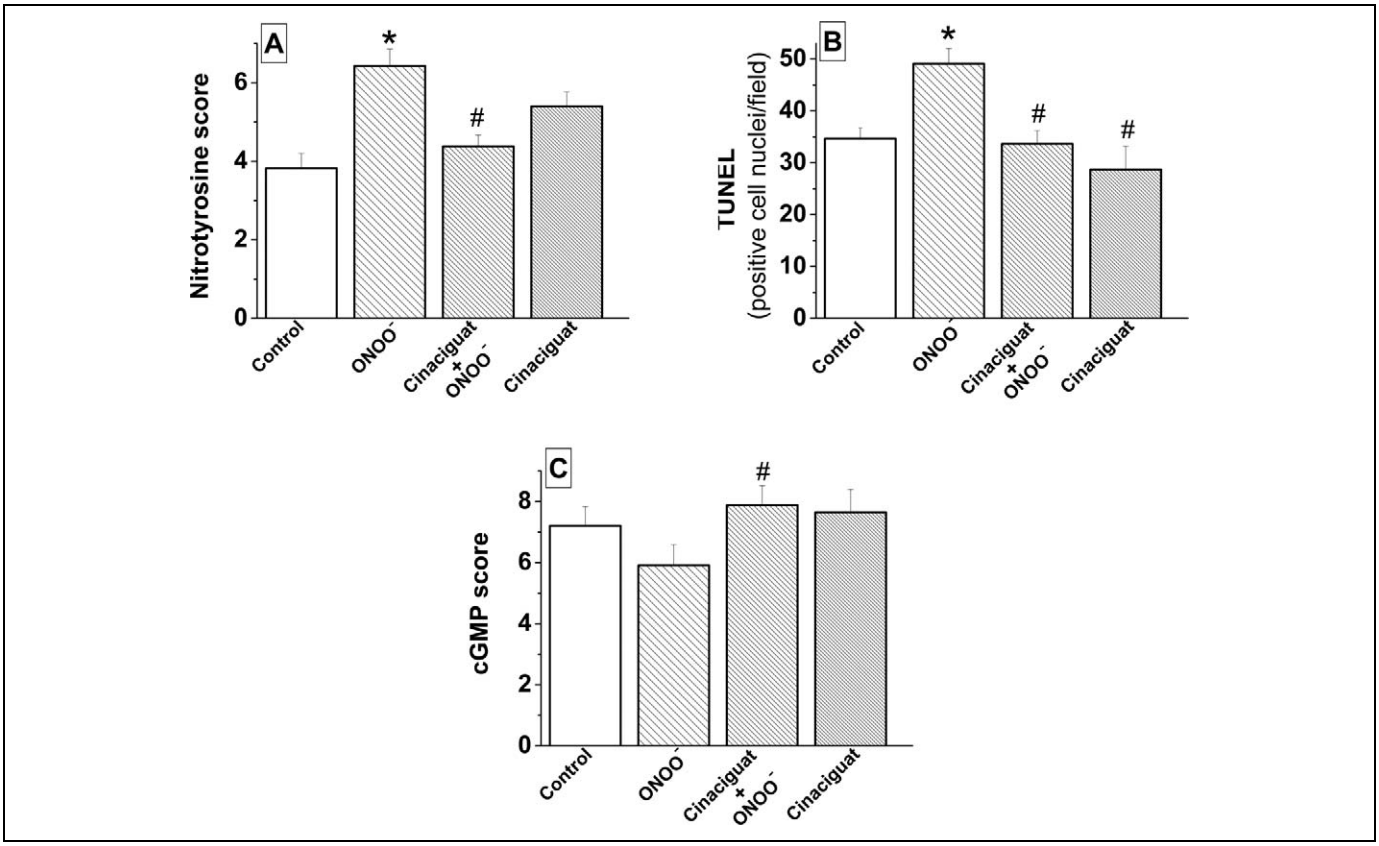

Figure 3. Scoring of nitrotyrosine and cGMP immunohistochemistry and TUNEL assay. Immunohistochemical scores for (A) nitrotyrosine, (B) average number of TUNEL-positive cell nuclei in a microscopic field, and (C) cGMP in the vessel wall of aortic rings. $^{*}P < .05$ versus control; $^{\#}P < .05$ versus peroxynitrite group. cGMP indicates cyclic guanosine monophosphate; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling.

observed increase in TUNEL-positive staining compared with the control. Consistently, Mihm et al have demonstrated that the preincubation of rat thoracic aorta segments with clinically relevant concentrations of 3-nitrotyrosine, a biomarker of peroxynitrite formation, observed in various pathophysiological states resulted in concentration-dependent impairment of endothelium-dependent vascular relaxation and induced DNA damage in vascular endothelial cells.[29] Endothelial dysfunction observed after peroxynitrite incubation can be associated with the accumulation of oxidized and heme-free sGC that cannot be activated by NO. It has been shown that oxidation of sGC from the Fe^{2+} to the Fe^{3+} state by peroxynitrite rende to endogenous NO and NO-releasing drugs, thereby inhibiting NO signaling.[13] Moreover, in higher concentration, peroxynitrite-mediated oxidation of tetrahydrobiopterin, an essential NO synthase (NOS) cofactor, leads to the dysfunction of NOS, thereby causing endothelial NOS uncoupling.[30] Pharmacological activation of sGC by cinaciguat can potently bind and activate the oxidized and/or heme-free sGC, producing selective sGC activation and vasodilation of diseased blood vessels.[15] In this work, oral pretreatment of rats with cinaciguat significantly ameliorated the peroxynitrite-induced endothelial dysfunction. The sGC stabilizing features of cinaciguat might help to overcome this imbalance by preventing sGC from degradation.[31]

In the present study, aortic rings incubated with peroxynitrite exhibited decreased sensitivity and normal maximal responses to the endothelium-independent vasodilator sodium nitroprusside. These results show a slight damage of the relaxation apparatus in the smooth muscle cells. Under normal conditions, sodium nitroprusside breaks down spontaneously to yield NO, thereby causing endothelium-independent vasodilation by the same effector mechanism as NO released from endothelium, that is, activation of sGC.[26] Li et al showed that direct exposure of primary rat aortic smooth muscle cells to peroxynitrite induces apoptosis in a concentration-dependent manner, as confirmed by means of quantitative fluorescence staining and TUNEL assay.[32] We found an increase in contractile responses of the smooth muscle cells to the α_1-adrenergic agonist phenylephrine in the peroxynitrite-exposed groups. This might be due to peroxynitrite-induced impairment of cGMP levels. It has been shown that the increase in intracellular cGMP production stimulates cGMP-dependent protein kinases, leading to the inhibition of calcium entry into the cell, thereby decreasing cytoplasmic Ca^{2+} concentrations and decreased vasoconstriction.[26] But in the present study, cinaciguat had no effect on the phenylephrine-induced increased maximal contraction. Enhanced contractile response to phenylephrine after peroxynitrite exposure, which occurs despite the

elevated levels of cGMP by cinaciguat may suggest the involvement of vascular structural and functional components that are not cGMP-mediated.

In this experimental setup, after oral treatment of rats, aortic segments are mounted in organ bath. According to the protocols, the tissues are subjected to repeated washings (aortic rings for histological purposes were also washed during the incubation time). Even though cinaciguat is washed out in this in vitro model, the intracellular cGMP levels remain elevated. These observations suggest that cinaciguat continues to exert its pharmacological effect in the aortic preparation previously washed.

Conclusion

In this study, we observed significant aortic endothelial dysfunction, nitro-oxidative stress, and DNA injury after brief incubation of vascular segments with peroxynitrite. By activating sGC and thereby increasing cGMP, oral treatment of rats with cinaciguat significantly improved endothelial function, reduced nitro-oxidative stress, and reduced DNA fragmentation. The sGC activator cinaciguat may have therapeutic potential to lower nitro-oxidative stress with the aim of improving clinical outcome in patients with vascular diseases. However, a limitation of our study was that the aortic rings were harvested (ex vivo) to study vascular reactivity with the lack of involvement of nonaortic tissue, the lack of blood flow, and absence of leucocytes activation. Therefore, confirmation of these observations in vivo is essential. Moreover, additional studies would have been interesting to investigate a deep mechanistic understanding on molecular level of cinaciguat in the vascular protection under nitro-oxidative stress.

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Declaration of Conflicting Interests

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