

# Molecular Cardiology

## Pharmacological Activation of Soluble Guanylate Cyclase Protects the Heart Against Ischemic Injury

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**Background**—The role of the nitric oxide/cGMP/cGMP-dependent protein kinase G pathway in myocardial protection and preconditioning has been the object of intensive investigations. The novel soluble guanylate cyclase activator cinaciguat has been reported to elevate intracellular [cGMP] and activate the nitric oxide/cGMP/cGMP-dependent protein kinase G pathway in vivo. We investigated the effects of cinaciguat on myocardial infarction induced by isoproterenol in rats.

**Methods and Results**—Rats were treated orally twice a day for 4 days with vehicle or cinaciguat (10 mg/kg). Isoproterenol (85 mg/kg) was injected subcutaneously 2 days after the first treatment at an interval of 24 hours for 2 days to produce myocardial infarction. After 17 hours, histopathological observations and left ventricular pressure-volume analysis to assess cardiac function with a Millar microtip pressure-volume conductance catheter were performed, and levels of biochemicals of the heart tissues were measured. Gene expression analysis was performed by quantitative real-time polymerase chain reaction. Isolated canine coronary arterial rings exposed to peroxynitrite were investigated for vasomotor function, and immunohistochemistry was performed for cGMP and nitrotyrosine. The present results show that cinaciguat treatment improves histopathological lesions, improves cardiac performance, improves impaired cardiac relaxation, reduces oxidative stress, ameliorates intracellular enzyme release, and decreases cyclooxygenase 2, transforming growth factor- $\beta$ , and  $\beta$ -actin mRNA expression in experimentally induced myocardial infarction in rats. In vitro exposure of coronary arteries to peroxynitrite resulted in an impairment of endothelium-dependent vasorelaxation, increased nitro-oxidative stress, and reduced intracellular cGMP levels, which were all improved by cinaciguat. A cardioprotective effect of postischemic cinaciguat treatment was shown in a canine model of global ischemia/reperfusion.

**Conclusion**—Pharmacological soluble guanylate cyclase activation could be a novel approach for the prevention and treatment of ischemic heart disease. (*Circulation*. 2009;120:677-686.)

**Key Words:** contractility ■ genes ■ myocardial infarction ■ nitric oxide

Myocardial infarction (MI) is the rapid development of myocardial necrosis that occurs when a coronary artery is severely blocked so that there is a significant imbalance between the oxygen supply and the demand of the myocardium, causing damage or death of a portion of the myocardium. A better understanding of the processes involved in myocardial injury has stimulated the search for new drugs that could limit the myocardial damage. It has been proposed that the nitric oxide (NO)/soluble guanylate cyclase (sGC)/cGMP/cGMP-dependent protein kinase G pathway may play a pivotal role in myocardial protection and preconditioning. In the healthy endothelium, vascular NO binds to the ferrous

heme iron ( $\text{Fe}^{2+}$ ) and activates a key signal transduction enzyme, sGC, resulting in cGMP generation. This activation promotes various actions such as vasodilation, inhibition of platelet aggregation, and growth inhibition.<sup>1</sup> Cardiovascular disease is frequently associated with impaired NO/sGC/cGMP signaling. The concept has been advanced that under physiological conditions sGC exists as a pool of oxidized (or heme-free) NO-insensitive sGC and reduced NO-sensitive sGC and that this NO-insensitive sGC pool is increased under pathophysiological conditions.<sup>2</sup> Organic nitrates act as a source of NO, but their efficacy is limited by the development of tolerance after sustained administration,<sup>3</sup> the absence of

Received February 23, 2009; accepted June 19, 2009.

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The online-only Data Supplement is available with this article at <http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.109.870774/DC1>.

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*Circulation* is available at <http://circ.ahajournals.org>

DOI: 10.1161/CIRCULATIONAHA.109.870774

clinically relevant antiplatelet activity,<sup>4</sup> and the inability to activate NO-insensitive sGC. Therefore, compounds that activate NO-insensitive sGC with a profile of activity similar to that of organic nitrates but devoid of the problem of tolerance and the potential cytotoxic actions of NO may offer a new approach for treating cardiovascular disease. A novel sGC activator, cinaciguat (BAY 58–2667), which activates sGC in its NO-insensitive, oxidized (or heme-deficient) state, induces cGMP generation and vasodilation preferentially in diseased vessels.<sup>5</sup> Cinaciguat exhibits a potent vasorelaxant effect on isolated vessels, in vitro and in vivo antiplatelet activity, a strong blood pressure-lowering effect, and a hemodynamic profile comparable to that of organic nitrates.<sup>6</sup> Its pharmacokinetic and pharmacodynamic properties, tolerability, and safety have recently been assessed by phase I clinical trials.<sup>7</sup> A phase IIb clinical study for the indication of acute decompensated heart failure is currently underway.<sup>8</sup>

### Clinical Perspective on p 686

Isoproterenol, a synthetic catecholamine and  $\beta$ -adrenergic agonist, has been found to induce MI in rats,<sup>9</sup> and the acute phases of myocardial necrosis and repair mimic those that occur in patients: changes in serum enzymes,<sup>10</sup> ECG alterations,<sup>11</sup> and histological changes.<sup>12</sup> The rat model of isoproterenol-induced MI offers a standardized noninvasive technique for studying the effects of various cardioprotective therapeutic attempts.

In the present study, the effects of cinaciguat in isoproterenol-induced MI in rats were evaluated. We focus on the correlated histopathological, biochemical, and functional changes and on cardiac performance-related gene expression. In the pathogenesis of isoproterenol-induced MI, it has been proposed that reactive oxygen-derived free radicals play a crucial role.<sup>13</sup> The secondary aim of our experiment was to examine the effects of cinaciguat on vascular dysfunction induced by nitro-oxidative stress on isolated canine coronary arterial rings. Additionally, a clinically relevant canine model of global reversible cardiac ischemia/reperfusion injury was used to evaluate the effects of a postischemic treatment protocol with cinaciguat.

## Methods

### Animals

Male Sprague-Dawley rats (250 to 350 g; Charles River, Sulzfeld, Germany) and foxhound dogs of both sexes were used in these experiments. Rats were housed in a room at a constant temperature of  $22 \pm 2^\circ\text{C}$  with 12-hour light/dark cycles and were fed a standard laboratory rat diet and water ad libitum. The rats were acclimatized for at least 1 week before experiments and were randomly assigned to different groups. All animals received humane care in compliance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86–23, revised 1996). The experiments were approved by the ethics committee of the Land Baden-Württemberg for Animal Experimentation.

### Rat Model of Isoproterenol-Induced MI

#### Induction of Myocardial Injury

Isoproterenol was injected subcutaneously into rats (85 mg/kg) daily for 2 consecutive days to induce MI,<sup>14</sup> and the mortality rate of rats

(within 30 minutes and between 30 minutes and 24 hours) in different dosage groups was assessed.

### Experimental Groups

Rats were randomly divided into 5 groups. The control group received methylcellulose for 4 days and sterile distilled water on days 3 and 4. The isoproterenol-group received methylcellulose for 4 days and isoproterenol on days 3 and 4. The cinaciguat plus isoproterenol group received cinaciguat for 4 days and isoproterenol on days 3 and 4. The cinaciguat group rats received cinaciguat for 4 days and sterile distilled water on days 3 and 4. The isoproterenol plus cinaciguat group received cinaciguat 12 hours after the first isoproterenol injection and at the same time as the second injection of isoproterenol. The experiment was stopped 17 to 22 hours after the last administration of drugs.

### Hemodynamic Measurements

Left ventricular (LV) pressure-volume (PV) analysis to assess cardiac function with a Millar (Houston, Tex) microtip PV-conductance catheter was performed as described previously<sup>15</sup> (see the online-only Data Supplement).

### Canine Model of Global Reversible Myocardial Ischemia/Reperfusion

#### General Management and Surgical Procedure

Anesthetized dogs underwent global cardiac ischemia/reperfusion in the setting of cardiopulmonary bypass with hypothermic cardiac arrest. After initiation of cardiopulmonary bypass, the ascending aorta was cross-clamped, and the hearts were arrested with crystalloid cardioplegia (Custodiol). After 60 minutes, the aortic cross-clamp was released, and the reperfusion phase was initiated. Each animal underwent 60 minutes of cardiac ischemia and 60 minutes of reperfusion<sup>16</sup> (see the online-only Data Supplement).

### Experimental Groups

Two groups of dogs were studied. The control group (n=6) received placebo; the cinaciguat group (n=6) received cinaciguat in a dose of 12.5  $\mu\text{g}$  in a short infusion for 10 minutes starting 5 minutes before the initiation of reperfusion.

### Hemodynamic Measurements

Heart rate (HR), mean arterial pressure (MAP), cardiac output, coronary blood flow, and PV loop–derived (conductance catheter) contractility indexes were assessed at baseline and at 60 minutes of reperfusion<sup>16</sup> (see the online-only Data Supplement).

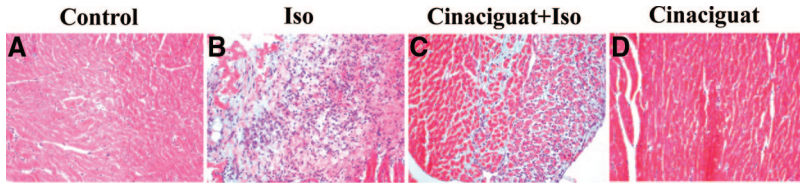
### In Vitro Model of Vascular Nitro-Oxidative Stress

#### Preparation of Isolated Canine Coronary Arterial Rings

Hearts were excised from anesthetized healthy control dogs, and the coronary arteries were isolated and placed in cold ( $4^\circ\text{C}$ ) Krebs-Henseleit solution (118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L  $\text{KH}_2\text{PO}_4$ , 1.2 mmol/L  $\text{MgSO}_4$ , 1.77 mmol/L  $\text{CaCl}_2$ , 25 mmol/L  $\text{NaHCO}_3$ , and 11.4 mmol/L glucose; pH=7.4). After dissection of surrounding tissue, segments (length, 4 mm) of coronary arteries were placed in 30 mL Krebs-Henseleit solution supplemented with 10 mmol/L HEPES buffer, incubated for 20 minutes with vehicle or cinaciguat ( $10^{-9}$  mol/L), and then exposed to vehicle or peroxynitrite (150  $\mu\text{mol/L}$ ) to induce endothelial injury

#### Experimental Groups, Measurement Protocol

The experimental groups were as follows: control group (vehicle treatment, exposure to vehicle), peroxynitrite group (vehicle treatment, exposure to peroxynitrite), cinaciguat plus peroxynitrite group (cinaciguat treatment, exposure to peroxynitrite), and cinaciguat group (cinaciguat treatment, exposure to vehicle). After the incubation time, a vessel ring from each group was used for functional isometric tension measurements as described previously.<sup>17</sup> Coronary preparations were precontracted with U46619 ( $5 \times 10^{-7}$  mol/L), and relaxation responses were examined by the addition of cumulative concentrations of acetylcholine ( $10^{-9}$  to  $5 \times 10^{-5}$  mol/L). For testing



**Figure 1.** Histological examinations of myocardium (hematoxylin and eosin staining; magnification  $\times 100$ ) in each group. Iso indicates isoproterenol.

relaxing responses of smooth muscle cells, sodium nitroprusside ( $10^{-10}$  to  $10^{-4}$  mol/L) was used. Relaxation is expressed as percent of contraction induced by U46619.

### Biochemical Estimations

Blood collected from the rats in EDTA tubes was immediately centrifuged, and plasma was separated. Plasma cGMP and cAMP concentrations were determined by competitive enzyme immunoassay with commercial kits (GE Healthcare, Buckinghamshire, UK). Lactate dehydrogenase activity was estimated with a commercial kit (Biotrend Chemikalien GmbH, Cologne, Germany), and the thiobarbituric acid reactive substances (TBARS) were measured by a commercial kit (ZeptoMetrix Corp, Buffalo, NY).

### Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from apex of the hearts (including both infarct and healthy areas) with the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany). RNA concentration and purity were determined photometrically (260, 280, and 230 nm). Reverse transcription was performed with the QuantiTect Reverse Transcription Kit (Qiagen) using 500  $\mu\text{g}$  RNA in a volume of 20  $\mu\text{L}$ . Quantitative real-time polymerase chain reactions were performed on the Light-Cycler480 system with the LightCycler480 Probes Master and Universal ProbeLibrary probes (Roche, Mannheim, Germany) (see the online-only Data Supplement).

### Histopathological Process

Hearts from rats and canine coronary arterial segments from each experimental group were fixed in buffered paraformaldehyde solution (4%) and embedded in paraffin. Then, 5- $\mu\text{m}$ -thick sections were placed on adhesive slides. Rat heart samples were stained with hematoxylin and eosin; for identification of intracellular cGMP,<sup>18</sup> cGMP immunohistochemistry was performed. According to previously described methods,<sup>19</sup> we performed immunohistochemical staining on coronary arterial rings for nitrotyrosine, and for cGMP. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed to detect DNA strand breaks (see the online-only Data Supplement).

### Chemical Reagents

Cinaciguat (BAY 58-2667; (4-[[[(4-carboxybutyl){2-[(4-phenethylbenzyl)oxy]phenethyl]amino methyl]benzoic]acid) was provided by Bayer HealthCare (Wuppertal, Germany). For a detailed description of preparation and application, see the online-only Data Supplement. Peroxynitrite (Calbiochem, San Diego, Calif) was diluted with 4.7% NaOH. Custodial was purchased from Dr Franz

Köhler Chemie GmbH (Alsbach-Hähnlein, Germany). Isoproterenol hydrochloride was dissolved in sterile distilled water; acetylcholine and sodium nitroprusside, in normal saline; and thromboxane A<sub>2</sub>-receptor agonist U46619 (9,11-dideoxy-11 $\alpha$ ,9 $\alpha$ -epoxy-methanoprostaglandin F2 $\alpha$ ), in ethanol. These reagents were bought from Sigma-Aldrich (Steinheim, Germany).

### Statistical Analysis

All values were expressed as mean  $\pm$  SEM. In the case of canine hemodynamic parameters, a paired *t* test was used to compare 2 means within a group (comparison of “before” and “after” values). Means between the groups were compared by an unpaired 2-sided Student *t* test (comparison of the control and cinaciguat groups). In all other cases, means between groups were compared by 1-way ANOVA followed by an unpaired *t* test with Bonferroni correction for multiple comparisons. Values of  $P < 0.05$  were considered significant.

## Results

### Histopathological Examination of Cardiac Tissues

Compared with the control group (Figure 1A), myocardial tissues from isoproterenol-treated rats revealed myofibrillar degeneration, which is related to a dense inflammatory infiltrate and interstitial edema (Figure 1B). Cinaciguat treatment exhibited a decreased degree of necrosis with less fragmentation of fibers and inflammation after isoproterenol administration (Figure 1C). Drug-treated groups showed the normal appearance of cardiac muscle fibers (Figure 1D). Table 1 shows the effect of cinaciguat on the degree of histological changes in myocardial tissues in control and isoproterenol-induced rats.

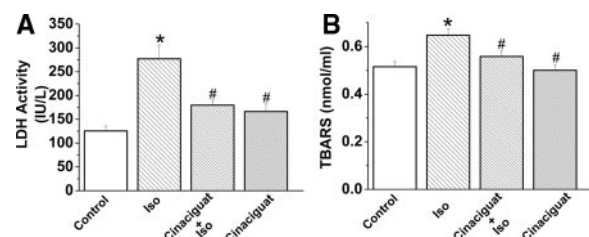
### Effect of Cinaciguat on Plasma Lactate Dehydrogenase Activity and TBARS Concentration

Increased plasma lactate dehydrogenase activity after isoproterenol treatment in rats was significantly reduced by cinaciguat administration, indicating amelioration of intracellular enzyme release (Figure 2A). Increased plasma TBARS concentration after isoproterenol treatment was significantly prevented by cinaciguat treatment, indicating reduced oxidative stress (Figure 2B).

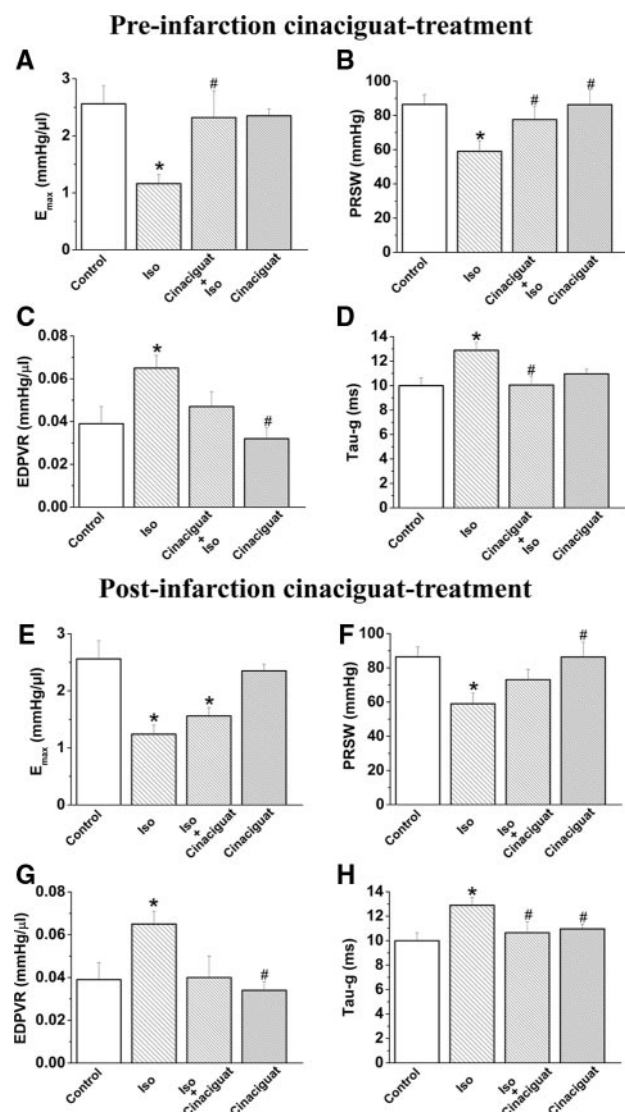
**Table 1. Effect of Cinaciguat on the Degree of Histopathological Changes in Myocardium in the Rat Model of Isoproterenol-Induced MI**

Groups	Necrosis	Edema	Inflammation
Control	A	A	A
Isoproterenol	++	++	+++
Isoproterenol + cinaciguat	+	+	++
Cinaciguat	A	A	A

A indicates no changes; +, mild changes; ++, moderate changes; and +++, marked changes.



**Figure 2.** Effect of cinaciguat on plasma lactate dehydrogenase activity (LDH; A) and TBARS (B) in isoproterenol (Iso)-induced MI in rats. All values are expressed as mean  $\pm$  SEM. \* $P < 0.05$  vs control; # $P < 0.05$  vs isoproterenol.



**Figure 3.** Effect of cinaciguat on LV contractility, end-diastolic stiffness, and cardiac relaxation in isoproterenol (Iso)-induced MI in rats. A and E, Slope ( $E_{max}$ ) of the LV end-systolic PV relationships; B and F, preload recruitable stroke work (PRSW); C and G, end-diastolic PV relationship (EDPVR); D and H, time constant of LV pressure decay ( $\tau$ ). A through D, Preinfarction cinaciguat treatment group; E through H, postinfarction cinaciguat treatment group. All values are expressed as mean  $\pm$  SEM. \* $P < 0.05$  vs control; # $P < 0.05$  vs isoproterenol.

### Effect of Cinaciguat on Cardiac Function in MI

Compared with the control group, isoproterenol treatment in rats was associated with significantly decreased systolic performance. The slope  $E_{max}$  of the end-systolic PV relationship and preload recruitable stroke work showed a marked reduction in isoproterenol-treated rats. Cinaciguat treatment resulted in a significant increase in these parameters, indicating improved LV contractility (Figure 3A and 3B). In contrast, the end-diastolic PV relationship was increased in isoproterenol-treated rats, indicating increased end-diastolic stiffness. Values of the end-diastolic PV relationship of cinaciguat plus isoproterenol-treated rats did not differ compared with the control group (Figure 3C). Isoproterenol treatment was associated with impaired cardiac relaxation, as

reflected by prolonged  $\tau$ , which was significantly improved by cinaciguat (Figure 3D). Isoproterenol treatment was associated with significantly increased HR and decreased ejection fraction and stroke volume, which were reversed to the level of controls after cinaciguat treatment. Isoproterenol treatment resulted in increased LV end-diastolic pressure, decreased stroke work and LV systolic pressure, and minimum pressure development ( $-dP/dt$ ). Cinaciguat had no effect on these parameters. Rats treated with cinaciguat plus isoproterenol had lower MAP and LV systolic pressure. Cinaciguat treatment alone had no effect on any of the hemodynamic parameters studied (Table 2). In the postinfarction cinaciguat treatment group, we detected significantly improved cardiac relaxation and a tendency toward improved systolic function (without reaching the level of statistical significance) compared with the isoproterenol group (Figure 3E through 3H; see Table I of the online-only Data Supplement).

### Effect of Cinaciguat on Mortality in MI

There were no deaths in the control and cinaciguat-treated groups. The rate of mortality (within 30 minutes) in the isoproterenol- and cinaciguat plus isoproterenol-treated rats was 6% and 0%, respectively. During the experimental period, the mortality rate of rats was 56% for the isoproterenol group and 33% for the cinaciguat plus isoproterenol group (see Table II of the online-only Data Supplement). Similarly, the mortality rate was markedly reduced after postinfarction cinaciguat treatment (56% versus 25%) (see Table III of the online-only Data Supplement).

### Effect of Cinaciguat on Plasma cGMP and cAMP Concentrations and on Myocardial cGMP Level

Significantly increased levels of plasma cGMP were observed in isoproterenol- and cinaciguat plus isoproterenol-treated rats compared with control and cinaciguat-treated rats (Figure 4A), whereas plasma cAMP level remained unchanged (Figure 4B). In the cinaciguat-treated group, the myocardial cGMP score was significantly higher compared with the control group (Figure 4C).

### Effect of Cinaciguat on Gene Expression

Quantitative real-time polymerase chain reaction from apical myocardium RNA extracts revealed that mRNA expression for atrial natriuretic factor (ANF), cyclooxygenase 2 (COX-2), transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ), and  $\beta$ -actin was significantly increased in the isoproterenol-treated group (Figure 5A through 5D), whereas  $\beta$ -myosin heavy chain and endothelial NO synthase mRNA expression remained unchanged (data not shown). Cinaciguat treatment was shown to prevent the increase in COX-2, TGF- $\beta_1$ , and  $\beta$ -actin mRNA expression in isoproterenol-treated rats, whereas the ANF mRNA level was not affected.

### Effect of Cinaciguat on Cardiac Function After Global Ischemia/Reperfusion

Table 3 shows the in vivo effects of posts ischemic cinaciguat treatment on global cardiac ischemia/reperfusion injury in our canine model. Baseline values did not differ between groups. MAP showed a decrease in both groups after ischemia/

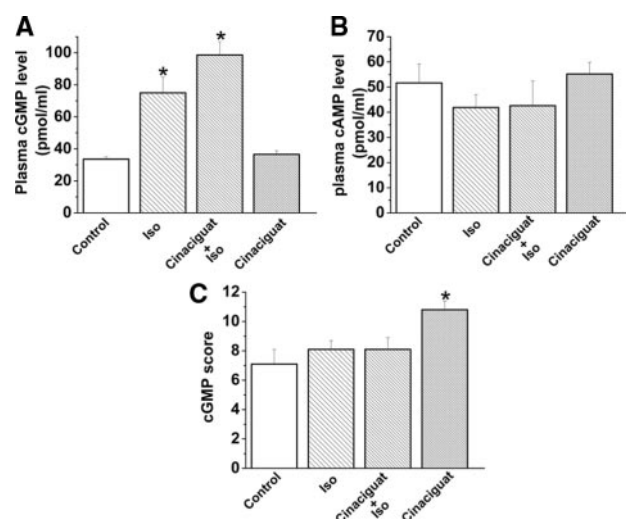
**Table 2. Effect of Cinaciguat on HR, MAP, Maximal LV Pressure, LV End-Diastolic Pressure, Ejection Fraction, Maximum Pressure Development, Minimum Pressure Development, End-Diastolic Volume, End-Systolic Volume, Stroke Volume, Efficiency, Systemic Vascular Resistance, Arterial Elastance, Stroke Work, and End-Diastolic PV Relationship in Isoproterenol-Induced MI in Rats**

	Control	Isoproterenol	Cinaciguat+Isoproterenol	Cinaciguat
HR, bpm	427±9	485±12*	448±12	413±10†
MAP, mm Hg	102±3	89±3	64±3*†	115±7†
LVSP, mm Hg	133±4	102±3*	88±4*†	138±4†
LVEDP, mm Hg	5.9±0.3	10.5±1.0*	9.9±0.8*	6.1±0.3†
EF, %	56±6	41±4*	46±4	54±6
LV dP/dt, mm Hg/s	8372±389	7607±370	8660±548	8426±520
LV -dP/dt, mm Hg/s	12 063±770	6474±457*	6608±430*	12 087±545†
EDV, $\mu$ L	280±42	329±39	348±29	246±16
ESV, $\mu$ L	213±26	270±33	230±21	171±10†
SV, $\mu$ L	192±12	137±16*	165±15	155±12
Efficiency, %	61.8±4.0	63.0±4.2	61.3±3.2	54.0±4.6
SVR, mm Hg·mL <sup>-1</sup> ·min	1.75±0.22	1.85±0.17	1.17±0.22†	1.79±0.13
Ea, mm Hg/ $\mu$ L	0.92±0.12	1.02±0.10	0.69±0.09†	1.07±0.09
SW, mm Hg· $\mu$ L	16 057±1172	9711±1211*	10 338±1141*	13 005±1345
+dP/dt-EDV, mm Hg·s <sup>-1</sup> · $\mu$ L <sup>-1</sup>	46.6±6.4	35.7±2.9	42.0±9.9	43.7±3.4

LVSP indicates maximal LV pressure; LVEDP, LV end-diastolic pressure; EF, ejection fraction; dP/dt, maximum pressure development; -dP/dt, minimum pressure development; EDV, end-diastolic volume; ESV, end-systolic volume; SV, stroke volume; SVR, systemic vascular resistance; SW, stroke work; and EDPVR, end-diastolic PV relationship. All values are expressed as mean±SEM.

\* $P$ <0.05 vs control; † $P$ <0.05 vs isoproterenol.

reperfusion. HR and cardiac output showed no differences between groups and over time. Coronary blood flow decreased significantly in the control group after ischemia/reperfusion, whereas it remained unchanged in the cinaciguat group. After ischemia/reperfusion injury, significantly decreased contractility (shown by decreased end-systolic PV relationship and preload recruitable stroke work) was observed in the control group, which was reversed by postischemic cinaciguat treatment.



**Figure 4.** Effect of cinaciguat on plasma cGMP (A), plasma cAMP (B), and myocardial cGMP (C) levels in isoproterenol (Iso)-induced MI in rats. All values are expressed as mean±SEM. \* $P$ <0.05 vs control; # $P$ <0.05 vs isoproterenol.

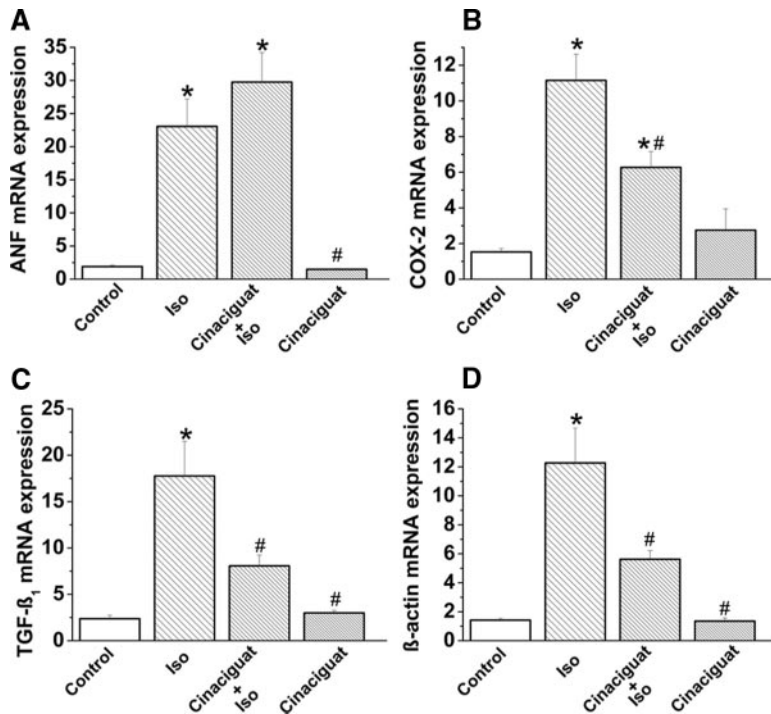
### In Vitro Effects of Cinaciguat on Vascular Function of Coronary Arterial Rings Exposed to Peroxynitrite

A marked impairment of endothelial function after exposure of isolated rings to the reactive oxidant peroxynitrite was demonstrated in our in vitro organ bath experiments. Acetylcholine-induced maximal relaxation was significantly attenuated after exposure of isolated segments to peroxynitrite, which indicates endothelial dysfunction. Cinaciguat significantly improved the acetylcholine-induced, endothelium-dependent, NO-mediated vasorelaxation after exposure of coronary rings to peroxynitrite (Figure 6A). Endothelium-independent vascular smooth muscle function, indicated by the vasorelaxation of coronary arteries to sodium nitroprusside, a direct NO donor, was not impaired by peroxynitrite. Incubation of rings with peroxynitrite only significantly enhanced the relaxation to 10<sup>-8</sup> mol/L sodium nitroprusside (Figure 6B). Contractile responses to U46619 did not show any significant differences in the groups studied (data not shown).

### In Vitro Effects of Cinaciguat on cGMP Levels, Nitro-Oxidative Stress, and DNA Strand Breaks in Coronary Arterial Rings Exposed to Peroxynitrite

A significantly lower score of cGMP staining (decreased red staining) was observed in the media of peroxynitrite-exposed rings compared with control. Cinaciguat treatment resulted in significantly higher immunoreactivity for cGMP but did not reach the level of statistical significance (Figures 7A and 8A).

Increased nitrotyrosine formation observed after exposure of coronary arteries to peroxynitrite was reduced after cinaciguat treatment, as evidenced by decreased brown staining



**Figure 5.** Effect of cinaciguat on gene expression in isoproterenol (Iso)-induced MI in rats. ANF (A), COX-2 (B), TGF- $\beta_1$  (C), and  $\beta$ -actin (D) mRNA expression. All values are expressed as mean  $\pm$  SEM. \* $P < 0.05$  vs control; # $P < 0.05$  vs isoproterenol.

(Figures 7B and 8B). We found pronounced DNA damage in the wall of peroxynitrite-exposed rings, as reflected by the quantitative assessment of TUNEL-positive cells. Cinaciguat treatment tended to decrease peroxynitrite-induced DNA strand breaks (Figures 7C and 8C).

### Discussion

The present study evaluated the effects of cinaciguat, an NO- and heme-independent sGC activator, on experimentally induced MI in rats. Our study showed that cinaciguat improves cardiac performance, prevents an increase in oxidative stress, and ameliorates histopathological changes and intracellular enzyme release in isoproterenol-induced MI. We also showed that cinaciguat has protective effects on coronary arteries and enhances endothelium-dependent vasorelaxation in an in vitro model of vascular nitro-oxidative stress.

### Effects of Cinaciguat Pretreatment on Cardiac Dysfunction in MI

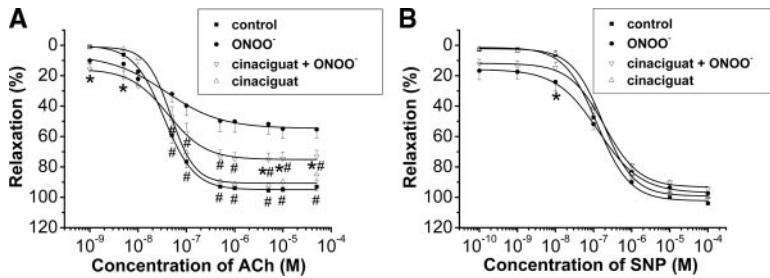
We show that isoproterenol treatment is characterized by decreased systolic performance accompanied by delayed relaxation and increased diastolic stiffness of the heart. Although we found that isoproterenol treatment was associated with decreased ejection fraction (known to be influenced by both preload and afterload), it cannot be used reliably to assess the contractile function in the models in which both preload and afterload are altered. End-systolic PV relationship ( $E_{max}$ ) and preload recruitable stroke work (2 well-established load-independent contractility indexes)<sup>20</sup> were decreased in isoproterenol-treated rats, suggesting decreased systolic performance. Another PV-derived index,  $dp/dt$ -end-diastolic volume, which is known to be sensitive to contractile changes and insensitive to preload, also has a tendency to be depressed in isoproterenol-treated rats. Impaired ventric-

**Table 3. Effect of Postischemic Cinaciguat Treatment on HR, MAP, Cardiac Output, Coronary Blood Flow, End-Systolic PV Relationship, and Preload Recrutable Stroke Work in the Canine Model of Global Cardiac Ischemia/Reperfusion**

	Before Ischemia/Reperfusion		After Ischemia/Reperfusion	
	Control	Cinaciguat	Control	Cinaciguat
HR, bpm	111 $\pm$ 6	131 $\pm$ 9	120 $\pm$ 12	139 $\pm$ 3
MAP, mm Hg	89.2 $\pm$ 3.5	90.1 $\pm$ 2.3	63.0 $\pm$ 4.9†	59.2 $\pm$ 2.8†
CO, L/min	2.99 $\pm$ 0.34	2.65 $\pm$ 0.30	2.39 $\pm$ 0.32	2.75 $\pm$ 0.58
CBF, mL/min	39.63 $\pm$ 5.35	33.83 $\pm$ 3.24	24.00 $\pm$ 2.59†	40.00 $\pm$ 4.13*
ESPVR, mm Hg/mL	5.04 $\pm$ 0.55	6.13 $\pm$ 0.78	2.62 $\pm$ 0.18†	4.27 $\pm$ 0.70*
PRSW, mm Hg	57.69 $\pm$ 3.28	75.11 $\pm$ 10.39	31.48 $\pm$ 1.88†	59.98 $\pm$ 12.01*

CO indicates cardiac output; CBF, coronary blood flow; ESPVR, end-systolic PV relation; and PRSW, preload recruitable stroke work. All values are expressed as mean  $\pm$  SEM.

\* $P < 0.05$  vs control; † $P < 0.05$  vs before ischemia/reperfusion.



**Figure 6.** Effect of cinaciguat on peroxynitrite (ONOO<sup>-</sup>)-induced vascular dysfunction in canine coronary arterial rings. A, Acetylcholine (ACh)-induced endothelium-dependent vasorelaxation. B, Sodium nitroprusside (SNP)-induced endothelium-independent vasorelaxation in each group. Values represent mean ± SEM. \**P* < 0.05 vs control; #*P* < 0.05 vs ONOO<sup>-</sup>.

ular relaxation (as reflected by the decreased  $-dp/dt$ , prolonged  $\tau$ , and increased LV end-diastolic pressure) and increased end-diastolic stiffness (as reflected by the increased end-diastolic PV relationship) were also detected in isoproterenol-treated groups. Relaxation, as an active process, depends mostly on Ca<sup>2+</sup> uptake by the sarcoplasmic reticulum during diastole, and end-diastolic stiffness is affected predominantly by alterations in myocardial structural components. Consistent with diastolic dysfunction, LV end-diastolic pressure was also increased in this group of rats, which may be influenced by changes in preload, and its increase may have resulted from increased venous return. Most of these parameters return to near-control levels after cinaciguat treatment, showing improvement in contractile pump function, LV relaxation, and diastolic stiffness.

Arterial elastance, *E<sub>a</sub>*, directly affects systolic function and is considered the most reliable index of LV afterload. In rats with MI, values of *E<sub>a</sub>* were not different from those in the control group, whereas cinaciguat significantly decreased afterload. When rats with MI are treated with cinaciguat, MAP is reduced (and presumably the LV afterload), and the ventricle can eject blood more rapidly, which increases stroke volume. After cinaciguat treatment, the ventricle will not fill to the same end-diastolic volume (preload) found before the afterload reduction because less blood remains in the ventricle after systole. During MI, stroke volume often falls because of a depression of LV contractility, resulting in a compensatory increase in afterload to maintain blood pressure within a physiological range. It has been shown that in experimental heart failure in dogs, intravenous administration of cinaciguat resulted in a reduction in cardiac preload and

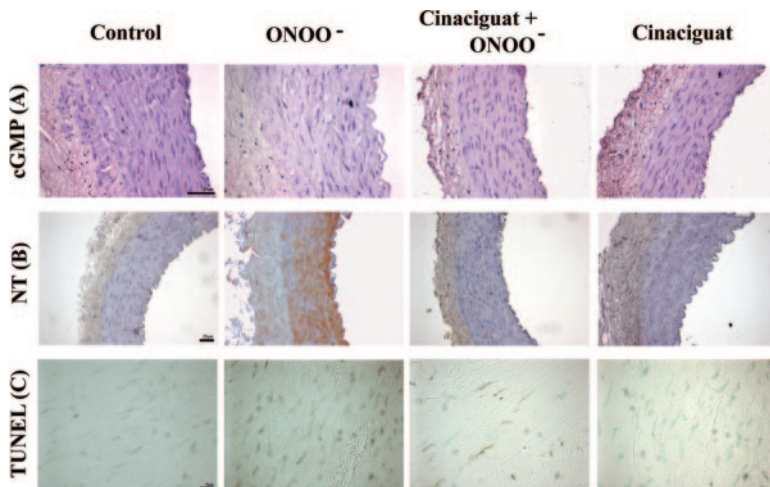
afterload.<sup>21</sup> Deteriorating myocardial status after isoproterenol administration is expected to affect HR. Increased HR observed in isoproterenol-treated rats, which reduces the time for diastole filling, can reduce the myocardial blood supply and cause ischemia. By decreasing afterload through its vasodilatory effect (shown by decreased MAP in cinaciguat plus isoproterenol-treated rats), cinaciguat may improve myocardial oxygen supply and decrease oxygen demand.

**Effect of Posts ischemic Cinaciguat Treatment on Cardiac Dysfunction in MI**

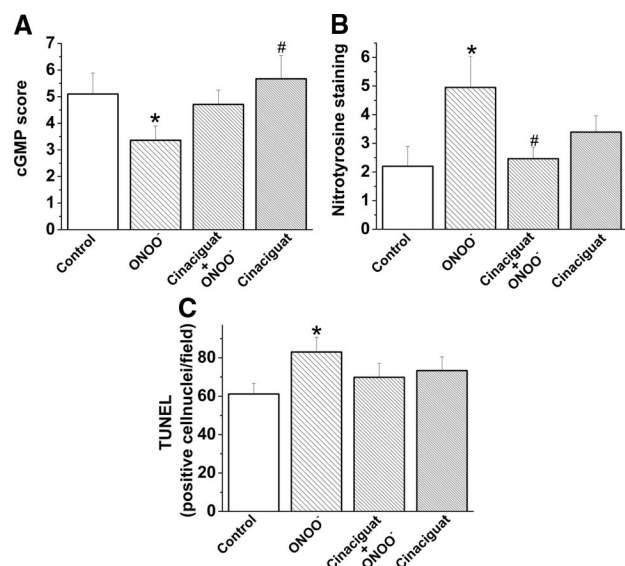
When applying a postinfarction treatment with cinaciguat, we observed an improvement only in diastolic function; LV contractility was not significantly ameliorated. In contrast, in the canine model of reversible global ischemia/reperfusion, systolic performance after posts ischemic cinaciguat treatment was significantly improved. The different results in these models might be explained by the type of ischemia (irreversible or reversible) and the timing of the treatment (because of the repetitive isoproterenol administration, the development of myocardial necrosis lingers in the rat MI model, so an exact timing was possible only in the canine model).

**Effect of Cinaciguat Pretreatment on Myocardial Ischemic Tissue Damage**

Myocardial tissue damage induced by isoproterenol was reduced by cinaciguat, as shown by decreased inflammatory infiltrate, degeneration, and interstitial edema. These data showed the cardioprotective action of cinaciguat in rats with MI. The increased activity of the cardiac enzyme lactate dehydrogenase in the plasma of isoproterenol-treated rats was



**Figure 7.** Representative photomicrographs of cGMP and nitrotyrosine (NT) immunohistochemistry staining and TUNEL assay in the vessel wall of coronary arterial rings in each group. A, cGMP (red staining; magnification ×400); B, nitrotyrosine (brown staining; magnification ×200); C, TUNEL (brown staining; magnification ×1000). Note the decreased red and increased brown staining, indicating decreased cGMP immunoreactivity, nitrotyrosine formation, and DNA damage in the peroxynitrite-exposed rings.



**Figure 8.** Scoring of cGMP and nitrotyrosine immunohistochemistry and TUNEL-assay. Immunohistochemical scores for cGMP (A) and nitrotyrosine (B) of the coronary artery wall of control, peroxynitrite-exposed (ONOO<sup>-</sup>; 150  $\mu$ mol/L), and cinaciguat-treated ONOO<sup>-</sup>-exposed coronary arterial rings. C, Average number of TUNEL-positive cell nuclei in a microscopic field. Values represent mean  $\pm$  SEM. \* $P$  < 0.05 vs control; # $P$  < 0.05 vs ONOO<sup>-</sup>.

decreased by cinaciguat. When myocardial cells are damaged or destroyed as a result of deficient oxygen supply or glucose, the cell membrane becomes permeable or may rupture, which results in the leakage of this enzyme from damaged tissues into the bloodstream.<sup>22</sup> In this study, mRNA expression of  $\beta$ -actin (a cytoskeletal protein important in the maintenance of cytoarchitecture) was markedly increased by isoproterenol and significantly downregulated by cinaciguat. These observations suggest that  $\beta$ -actin mRNA expression could be important in mediating the structural alterations that occur in the damaged myocardium.

### Mechanisms of Cardioprotective Effects of Cinaciguat Against Ischemic Injury

In animal models of MI, gene expression of ANF is reportedly upregulated<sup>23</sup> and correlates strongly with diastolic wall stress and stretch.<sup>24,25</sup> Our present results on myocardial ANF mRNA content showed a marked increase in this factor in isoproterenol- and cinaciguat plus isoproterenol-treated rats, suggesting end-diastolic wall stress. Recent studies also showed that production of cGMP by activation of natriuretic peptides receptor just before therapeutic reperfusion has a significant anti-infarct effect in both animals<sup>26</sup> and humans.<sup>27</sup> Our results also showed a correlation between increased myocardial ANF mRNA expression and plasma cGMP levels in these groups of rats, which could be a phenomenon that opposes MI. In heart failure, increased cGMP concentrations in extracellular fluids, including plasma<sup>28</sup> and urine,<sup>29</sup> are believed to result from its passage through the cellular membrane<sup>30</sup> as plasma cGMP is seen as an overspill of intracellular cGMP. However, plasma cGMP levels do not necessarily reflect intracellular cGMP levels or the activation of sGC by intracellular cinaciguat because the overspill from the intracellular space to the plasma might also be interpreted

as an intracellular overproduction. Any relevant biological activity of cGMP in plasma remains to be elucidated.

In this study, we observed increased myocardial cGMP levels in cinaciguat-treated rats. The absence of increased myocardial cGMP content in cinaciguat plus isoproterenol-treated rats could be explained by the short half-life of cinaciguat<sup>5</sup> and the relative long interval (17 to 22 hours) between the last treatment and the experiment. According to the unchanged cAMP levels, a role for cyclic nucleotide cross-regulation in the observed cardioprotective effects of cinaciguat seems rather unlikely.

The cardioprotective effect of the sGC activator could be mediated by several other mechanisms. Cinaciguat induces cGMP generation and evokes vasodilation preferentially in diseased vessels.<sup>5</sup> The markedly increased COX-2 mRNA content in isoproterenol-treated rats was significantly downregulated after cinaciguat, suggesting the decrease of proinflammatory prostanoid production and ischemic inflammation. COX-2 is induced only in cardiomyocytes in response to stress such as ischemia<sup>31</sup> and therefore is not expressed in healthy myocardium. It has been shown that TGF- $\beta$ , a locally generated cytokine, is upregulated after acute MI in rats and has a deleterious role in the response of the heart to injury.<sup>32</sup> In the present study, increased TGF- $\beta$ <sub>1</sub> mRNA content (which could be due to inflammatory changes) in isoproterenol-treated rats was significantly downregulated by cinaciguat. These findings support the concept that cGMP-activated pathways interfere with TGF- $\beta$  signaling, presumably by cGMP-mediated inhibition of TGF- $\beta$  expression, as previously proposed.<sup>33</sup>

Enormous amounts of reactive oxygen species are produced during MI and are involved in membrane damage, leading to increased TBARS levels. We also showed a significant elevation of plasma TBARS concentration after isoproterenol that was prevented by cinaciguat, indicating reduced oxidative stress. It is also known that COX-2 may generate reactive oxygen species.<sup>34</sup> In the present study, increased COX-2 mRNA content after isoproterenol was significantly downregulated after cinaciguat, suggesting decreased reactive oxygen species generation. In conditions associated with increased free radical release and oxidative stress, intracellular cGMP accumulation has been shown to reduce tissue injury.<sup>35</sup>

### Protective Effect of Cinaciguat on Endothelial Dysfunction Induced by Peroxynitrite

In coronary arteries exposed to peroxynitrite, we observed that relaxation to acetylcholine was diminished; in contrast, sodium nitroprusside-induced relaxation remained unchanged. Thus, responsiveness of the vascular smooth muscle cell to NO does not appear to be impaired. Our findings suggest that the release of NO after activation of muscarinic receptors in endothelial cells is blunted, whereas the cGMP-mediated signal transduction system and contractile apparatus in vascular smooth muscle cells are functionally preserved in arteries exposed to peroxynitrite. Szabo et al<sup>36</sup> also showed that exposure of isolated vessel rings to high concentrations of peroxynitrite caused a marked impairment of the endothelium-dependent relaxations. In accordance, our immunohis-



tochemical staining for cGMP supports reduced NO bioavailability in coronary arteries exposed to peroxynitrite. Improvement in endothelial function could be due mainly to the cytoprotection of endothelium by cinaciguat.

### Study Limitations

Supramaximal doses of isoproterenol, a  $\beta$ -adrenergic agonist and well-known inducer of MI,<sup>14</sup> are widely used as a model of evaluating cardioprotective drugs.<sup>37</sup> Although this rat model does not exactly reflect the clinical situation in terms of coronary occlusion and regional MI, animals develop “infarct-like” lesions when injected with isoproterenol. In a rabbit model of coronary artery ligation, Krieg et al<sup>38</sup> recently showed that cinaciguat elicited an infarct-reducing effect. Our canine model does not reflect acute MI but routine cardiac surgery with extracorporeal circulation and cold cardioplegic arrest leading to a myocardial and endothelial dysfunction.

### Conclusions

The present results indicate that the sGC activator cinaciguat has a significant effect in the protection of the heart against MI in rats. Our results show that cinaciguat improves cardiac function, improves histopathological lesions, reduces oxidative stress, ameliorates intracellular enzyme release, and decreases COX-2, TGF- $\beta_1$ , and  $\beta$ -actin mRNA expression in experimentally induced MI. We also show that cinaciguat enhances the endothelium-dependent vasorelaxation in canine coronary arterial rings exposed to peroxynitrite, decreases nitro-oxidative stress, and increases intracellular cGMP levels. Although results of animal experimental studies should not be directly extrapolated to human biology, pharmacological sGC activation could be a novel approach for the prevention and treatment of ischemic heart disease.

### Acknowledgments

The expert technical assistance of Heike Ziebart is gratefully acknowledged. The authors acknowledge Bayer HealthCare AG for the donation of cinaciguat.

### Source of Funding

This work was supported by the Land Baden-Württemberg.

### Disclosures

None.

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### CLINICAL PERSPECTIVE

Although clinical care is improved, public awareness is raised, and health innovations are widely used, myocardial infarction remains the leading cause of death worldwide. The nitric oxide/soluble guanylate cyclase/cGMP/cGMP-dependent protein kinase G signaling cascade has been shown to be an important sequence in both preconditioning and postconditioning. Alteration of this pathway has been thought to be involved in the pathogenesis of various cardiovascular diseases and is subject to the development of new therapeutic agents. Cinaciguat, the novel nitric oxide- and heme-independent activator of soluble guanylate cyclase, is currently being evaluated for the treatment of chronic heart failure in early clinical trials and appears to be well tolerated. Its nitric oxide independence may make it an ideal candidate for a pharmacological approach in acute myocardial infarction. In this study, we demonstrate that in a rat model of myocardial infarction induced by isoproterenol, cinaciguat treatment improves histopathological lesions, improves cardiac performance, improves impaired cardiac relaxation, reduces oxidative stress, and ameliorates intracellular enzyme release. Furthermore, we demonstrate cardioprotective effect of postischemic cinaciguat treatment in a clinically relevant canine model of global cardiac ischemia/reperfusion injury. Collectively, these findings make cinaciguat a candidate for the prevention and treatment of acute myocardial ischemia in humans.

## SUPPLEMENTAL MATERIAL

### 1. Supplemental Methods

#### **Hemodynamic measurements**

Rats were anesthetized with sodium pentobarbital (60mg/kg, i.p.), tracheotomized, intubated and artificially ventilated. Animals were placed on controlled heating pads, core temperature was measured via a rectal probe and was maintained at 37°C. Heart rate (HR), left ventricular (LV) systolic pressure (LVPS), LV end-diastolic pressure (LVEDP), mean arterial pressure (MAP), maximum pressure development ( $dP/dt_{max}$ ) and minimum pressure development ( $dP/dt_{min}$ ), ejection fraction (EF), stroke volume (SV), end-diastolic volume (EDV), end-systolic volume (ESV), stroke work (SW), end-diastolic PV relationships (EDPVR) were calculated. Ventricular relaxation was assessed by the time constant of LV pressure decay (Tau). It has been calculated by the Glantz method (Tau-g; regression of  $dP/dt$  versus pressure). LV pressure-volume relations were assessed by transiently compressing the inferior vena cava. The slope ( $E_{max}$ ) of the LV end-systolic PV relationships (ESPVR), preload recruitable stroke work (PRSW) and  $dP/dt_{max}$ -EDV as well as efficiency of LV work were calculated as load independent indexes of LV contractility.

At the end of each experiment, 0.1ml hypertonic saline was injected into the jugular vein, and from the shift of PV relations parallel conductance volume ( $V_p$ ) was calculated by the software and used for correction for cardiac mass volume. After completing the hemodynamic measurements, blood samples were collected from the inferior vena cava for preparation of blood plasma for further biochemical measurements. The volume calibration of the conductance system was performed as described previously. Briefly, nine cylindrical holes in a block 1cm deep and with known diameter ranging from 2 to 11mm were filled with fresh heparinized whole rat blood. In this calibration, the linear volume-conductance regression of

the absolute volume in each cylinder versus the raw signal acquired by the conductance catheter was used as the volume calibration formula.

### **Canine model of reversible global myocardial ischemia/reperfusion injury**

#### ***General management and surgical procedure***

Surgical preparation and general management: The dogs were premedicated with propionylpromazine and anesthetized with a bolus of pentobarbital (15mg/kg initial bolus and then 0.5mg/kg/h i.v.), paralyzed with pancuronium bromide (0.1mg/kg as a bolus and then 0.2mg/kg/h i.v.) and endotracheally intubated. The dogs were ventilated with a mixture of room air and O<sub>2</sub> (FiO<sub>2</sub>=60%) at a frequency of 12-15/min and a tidal volume starting at 15ml/kg per minute. The settings were adjusted by maintaining arterial partial carbondioxide pressure levels between 35-40mmHg. A urine catheter was inserted through the urethra to drain the urinary bladder. The femoral artery and vein were cannulated for recording aortic pressure and taking blood samples for the analysis of blood gases, electrolytes and pH, and parameters of blood coagulation and inflammation. Basic intravenous volume substitution was carried out with Ringer's solution as necessary. According to the values of potassium, bicarbonate and base excess, substitution included administration of potassium chloride and sodium bicarbonate (8.4%). Neither catecholamines nor other hormonal or pressor substances were administered. Rectal temperature and standard peripheral electrocardiogram were monitored continuously. After left anterolateral thoracotomy in the fourth intercostal space, pericardiotomy and isolation of the great vessels a perivascular ultrasonic flow probe was attached to the ascendent aorta. Aortic pressure was monitored with 5F Millar catheter tip manometer (Millar Instruments, Inc., Houston, TX, USA).

Cardiopulmonary bypass (CPB): After systemic anticoagulation with sodium heparin (300U/kg) the left subclavian artery was cannulated for arterial perfusion. The venous cannula was placed in the right atrium. The extracorporeal circuit consisted of a heat exchanger, a

venous reservoir, a roller pump and a membrane oxygenator primed with Ringer lactate solution (1000ml) supplemented with heparin (150U/kg) and 20ml sodium bicarbonate (8.4%). Hypothermic CPB was performed for 90min at a lowest temperature of 28°C. After initiation of CPB, the hearts were arrested with 1000ml crystalloid cardioplegia (Custodiol®). After 60min of cardiac arrest, the aorta was declamped and the heart was reperfused with normothermic blood in the bypass circuit. All animals were weaned from CPB without inotropic support 20min after the release of the aortic crossclamp. After weaning from CPB, heparin was antagonized by protamine iv. over 10min and the animals were monitored for one hour. Each animal underwent 60min of cardiac ischemia and 60min of reperfusion.

### ***Hemodynamic measurements***

Cardiac output (CO) as the equivalent of aortic flow was monitored continuously. The volume signal provided by the conductance catheter was registered continuously (Sigma F5, Leycom, Leiden, The Netherlands) and computed by the IOX software (EMKA Technologies, France). LV pressure-volume loops were constructed on-line. Vena cava occlusions were performed to obtain a series of loops for calculation of the slope of the ESPVR. In addition, PRSW was calculated as load-independent indices of myocardial contractility.

Coronary blood flow was measured by an ultrasonic flow meter placed on the left anterior descendent coronary artery.

**Quantitative real time polymerase chain reaction (PCR).** The conditions for PCR were as follows: 95°C for 10min (1-cycle), 95°C for 10s, 60°C for 30s (single; 45-cycle quantification), 40°C for 10s (1-cycle). Reaction volume was 20µl. Efficiency of the PCR reaction was confirmed with standard curve analysis. Sample quantifications were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression by using a pool of all cDNA's from the control group (positive calibrator). Primers were obtained from TIB Molbiol (Berlin, Germany) and their sequences were as follows: atrial natriuretic factor

(ANF) forward 5'-CAACACAGATCTGATGGATTTC-3'; reverse 5'-CGCTTCATCGGTCTGCTC-3'; cyclooxygenase (COX-2) forward 5'-CTACACCAGGGCCCTTCC-3'; reverse 5'-TCCAGAACTTCTTTTGAATCAGG-3'; transforming growth factor-beta (TGF- $\beta_1$ ) forward 5'-TCAGACATTCGGGAAGCAGT-3'; reverse 5'-ACGCCAGGAATTGTTGCTAT-3';  $\beta$ -actin forward 5'-CTAAGGCCAACCGTGAAAAG-3'; reverse 5'-TACATGGCTGGGGTGTGA-3'; beta-myosine heavy chain ( $\beta$ -MHC) forward 5'-GCTGCAGAAGAAGCTCAAAGA-3'; reverse 5'-GCAGCTTCTCCACCTTGG-3'; endothelial NO synthase (eNOS) forward 5'-TGACCCTCACCGATACAACA-3'; reverse 5'-CGGGTGTCTAGATCCATGC-3' and GAPDH forward 5'-AGCTGGTCATCAATGGGAAA-3'; reverse 5'-ATTTGATGTTAGCGGGATCG-3'. Following UPL probes were used: #5 for COX-2 and eNOS, #56 for TGF- $\beta_1$ , #9 for GAPDH, #65 for ANF and  $\beta$ -MHC and #115 for  $\beta$ -actin. Evaluation was performed with the Light Cycler 480 SW 1.5 software (Roche, Mannheim, Germany). Mean values in the different groups are expressed with the formula:

$$[\text{efficiency}]_{\text{target}}^{-\text{(Cp}_{\text{sample}}-\text{Cp}_{\text{calibrator}})} / [\text{efficiency}]_{\text{reference}}^{-\text{(Cp}_{\text{sample}}-\text{Cp}_{\text{calibrator}})}$$

### **Histopathological process**

We performed immunohistochemical staining for cGMP for identification of intracellular cGMP content as well as for nitrotyrosine (NT is a product of the nitrating effect of peroxynitrite; a marker of nitrosative stress in general, and as “footprint of peroxynitrite” in particular<sup>1</sup>). TUNEL assay was performed for detection of DNA strand breaks. The detection was carried out using a commercial kit following the protocol provided by the manufacturer (Chemicon International, Temecula, CA, USA). Rehydrated sections were treated with 20 $\mu$ g/ml DNase-free Proteinase K (Sigma-Aldrich, Germany) to retrieve antigenic epitopes, followed by 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to quench endogenous peroxidase activity. Free 3'-OH termini were labeled with digoxigenin-dUTP for 1 hour at 37 °C utilizing a terminal

deoxynucleotidyl transferase reaction mixture (Chemicon International, Temecula, CA, USA). Incorporated digoxigenin-conjugated nucleotides were detected using a horseradish peroxidase conjugated anti-digoxigenin antibody and 3,3'-diaminobenzidine. Sections were counterstained with Gill's hematoxylin. Dehydrated sections were cleared in xylene, mounted with Permount (Fischer Scientific, Germany) and coverslips were applied. Based on the intensity and distribution of labelling, semi-quantitative histomorphological assessment was performed using conventional microscopy.

### **Quantification of cGMP-, nitrotyrosine-immunohistochemistry and TUNEL**

A digital camera was used to input microscopic pictures from a low power (100x) magnification of the whole section and a high power (400x) examination of four adjacent fields. For assessment of TUNEL-labelled cells, the number of positive cell nuclei/microscopic examination field (200x magnification) were counted (four fields characterizing each specimen), and an average value was calculated for each experimental group. For cGMP and nitrotyrosine staining, after initially evaluating all corresponding tissue sections (x400 and x200 magnification respectively), the tissue section with the most intense labelling signals was used as a reference for maximum labelling intensity. All other tissue sections were comparatively evaluated. The colours were measured using densitometry and put in four colour classes: one class for background staining and three classes for positively stained areas. On the basis of the measured intensity, the colour classes were coupled with score values as follows: 0: no positive staining, 1: weak staining, 2: intermediate staining, 3: extensive staining. Using the program, we measured the area of the objects in each class in each field, assigned an area score (1 = up to 10% positive cells, 2 = 11-50% positive cells, 3 = 51-80% positive cells, 4 = >80% positive cells), and calculated an average score for the whole picture (intensity score multiplied by area score, 0-12). Finally, each specimen was characterized with the average of the 4 adjacent fields.

### **Preparation and application of cinaciguat**

For *in-vivo* oral application, cinaciguat was prepared in a suspension in methylcellulose (1%) at a volume of 10 $\mu$ l/g and administrated orally at the dose of 10mg/kg and for *in-vivo* i.v. administration, it was dissolved in a 1:1 mixture of Transcutol<sup>®</sup> and Cremophor<sup>®</sup>. For *in-vitro* application, cinaciguat was prepared in dimethyl sulfoxide and in a solution of glycerol/distilled water/polyethyleneglycol400 (60/100/949=g/g/g). The application and dosage of cinaciguat have been determined according to the pharmacokinetic and –dynamic properties<sup>2</sup> as well as to the results of previous rodent experiments<sup>3</sup>.



## 2. Supplemental Tables

**Online Table 1:** Effect of post-infraction cinaciguat-treatment on heart rate (HR), mean arterial pressure (MAP), maximal left ventricular pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), ejection fraction (EF), maximum pressure development (+dP/dt), minimum pressure development (-dP/dt), end-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV), efficiency, systemic vascular resistance (SVR), arterial elastance (Ea), stroke work (SW), end-diastolic pressure-volume relationships (EDPVR) in isoproterenol-induced MI in rats. All values are expressed as mean±SEM. \*, P<0.05 versus control; #, P<0.05 versus isoproterenol.

	Control	Isoproterenol	Isoproterenol+Cinaciguat	Cinaciguat
HR (beats/min)	427±9	<b>485±12*</b>	494±13	<b>413±10<sup>#</sup></b>
MAP (mmHg)	102±3	89±3	<b>74±4*<sup>#</sup></b>	<b>115±7<sup>#</sup></b>
LVSP (mmHg)	133±4	<b>102±3*</b>	<b>92±4*<sup>#</sup></b>	<b>138±4<sup>#</sup></b>
LVEDP (mmHg)	5.9±0.3	<b>10.5±1.0*</b>	<b>8.0±1.0*<sup>#</sup></b>	<b>6.1±0.3<sup>#</sup></b>
EF (%)	56±6	<b>41±4*</b>	<b>48±6*<sup>#</sup></b>	54±6
LV+dP/dt (mmHg/s)	8372±389	7607±370	8507±403	8426±520
LV-dP/dt (mmHg/s)	12063±770	<b>6474±457*</b>	<b>6664±308*</b>	<b>12087±545<sup>#</sup></b>
EDV (μl)	280±42	329±39	331±32	246±16
ESV (μl)	213±26	270±33	298±41	<b>171±10<sup>#</sup></b>
SV (μl)	192±12	<b>137±16*</b>	171±18	155±12
Efficiency (%)	61.8±4.0	63.0±4.2	53.2±2.3	54.0±4.6
SVR (mmHg.l <sup>-1</sup> .min)	1.75±0.22	1.85±0.17	<b>1.09±0.12<sup>#</sup></b>	1.79±0.13
Ea (mmHg/μl)	0.92±0.12	1.02±0.10	<b>0.60±0.04<sup>#</sup></b>	1.07±0.09
SW (mmHg.μl)	16057±1172	<b>9711±1211*</b>	13017±1464	13005±1345
+dP/dt-EDV (mmHg/s/μl)	46.6± 6.4	35.7±2.9	42.0±9.9	43.7± 3.4

**Online Table 2:** Effect of pre-infarction cinaciguat-treatment on mortality rate in isoproterenol-induced MI in rats.

	Control	Isoproterenol	Cinaciguat+isoproterenol	Cinaciguat
0-30min after isoproterenol	0/7 (0%)	1/18 (6%)	0/15 (0%)	0/9 (0%)
30min-24h after isoproterenol	0/7 (0%)	9/18 (50%)	5/15 (33%)	0/9 (0%)
Cumulative	0/7 (0%)	10/18 (56%)	5/15 (33%)	0/9 (0%)

**Online Table 3:** Effect of post-infraction cinaciguat-treatment on mortality rate in isoproterenol-induced MI in rats.

	Control	Isoproterenol	Cinaciguat+Isoproterenol	Cinaciguat
0-30min after isoproterenol	0 (0%)	1/18 (6%)	0/36 (0%)	0 (0%)
30min-24h after isoproterenol	0 (0%)	9/18 (50%)	9/36 (25%)	0 (0%)
Cumulative	0 <b>(0%)</b>	10/18 <b>(56%)</b>	9/36 <b>(25%)</b>	0 <b>(0%)</b>

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*Circulation*. 2009;120:677-686; originally published online August 10, 2009;  
doi: 10.1161/CIRCULATIONAHA.109.870774

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231  
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:

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