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## Irreversible Activation and Stabilization of Soluble Guanylate Cyclase by the Protoporphyrin IX Mimetic CinaciguatS

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### Abstract

Belonging to the class of so-called soluble guanylate cyclase (sGC) activators, cinaciguat and BAY 60-2770 are interesting therapeutic tools for the treatment of various cardiovascular pathologies. The drugs are supposed to preferentially stimulate oxidized or heme-depleted, but not native sGC. Since this concept has been challenged by studies demonstrating complete relaxation of nondiseased vessels, this study was designed to reinvestigate the mode of action in greater detail. To this purpose, the effect of cinaciguat was studied on vessel tone of porcine coronary arteries and rat thoracic aortas. Organ bath studies showed that the compound caused time- and concentration-dependent relaxation of precontracted vessels with a maximal effect observed at 90 minutes. The dilatory response was not affected by extensive washout of the drug. Cinaciguat-induced vasodilation was associated with a time- and concentration-dependent increase of cGMP levels. Experiments with purified sGC in the presence of Tween 20 showed that cinaciguat activates the heme-free enzyme in a concentration-dependent manner with an EC<sub>50</sub> value of ~0.2 μM and maximal cGMP formation at 10 μM. By contrast, the effect of cinaciguat on 1H-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one-oxidized (ferric) sGC was moderate, reaching ~10%–15% of maximal activity. Dilution experiments of cinaciguat/Tween 20–preincubated sGC revealed the irreversible character of the drug. Assuming a sensitive balance between heme-free, ferric, and nitric oxide-sensitive ferrous sGC in cells and tissues, we propose that cinaciguat by virtue of its irreversible mode of action is capable of shifting this equilibrium toward the heme-free apo-sGC species.

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## Introduction

Soluble guanylate cyclase (sGC) represents the major physiologic target of nitric oxide (NO) in vascular smooth muscle and many other tissues (Mayer and Hemmens, 1997). The enzyme is a  $\alpha/\beta$  heterodimer with a regulatory heme group bound to histidine-105 of the  $\beta$ -subunit (Wedel et al., 1994). High-affinity binding of NO to the ferrous heme moiety induces up to 400-fold activation of the enzyme (Humbert et al., 1990; Stone and Marletta, 1996), resulting in accumulation of cGMP (Koesling, 1999), which causes smooth muscle relaxation through stimulation of cGMP-dependent protein kinase (Feil et al., 2003). Focusing on sGC as a therapeutic target, synthetic drugs were developed a decade ago that are either able to increase the binding affinity of NO to ferrous sGC or activate the oxidized ferric and/or heme-free apoenzyme. Compounds such as YC-1 (3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole) (Friebe et al., 1998), riociguat, and BAY 41-2272 belong to the former group of so-called sGC stimulators, while drugs of the latter family including BAY 60-2770, ataciguat, and cinaciguat (BAY 58-2667) are commonly referred to as sGC activators (Evgenov et al., 2006).

Studies with sGC activators revealed beneficial effects of these drugs in various pathologies, including pulmonary hypertension and acute heart failure (Stasch et al., 2011). Based on these observations it has been concluded that oxidative stress causes oxidation of native ferrous sGC and subsequent loss of heme, resulting in increased levels of heme-free apo-sGC that is insensitive to NO but activated by cinaciguat and related compounds (Stasch et al., 2006). However, this view is challenged by studies showing that BAY 60-2770 promotes virtually complete relaxation of uninjured blood vessels (Tawa et al., 2014, 2015; Jabs et al., 2015). This observation could reflect dynamic regulation of the sGC subunit interaction in tissues with sGC activators causing a shift toward heme-free apo-sGC (Ghosh et al., 2014). Alternatively, binding of sGC activators to a small subset of apo-sGC that is physiologically present in intact blood vessels could be sufficient for full relaxation. This would be consistent with previous studies indicating that a small fraction (6%) of the total sGC pool is sufficient to mediate maximal vascular relaxation (Kollau et al., 2005; Mergia et al., 2006).

The present work was performed to explain the virtually complete relaxation of nondiseased blood vessels by the sGC activator cinaciguat. The drug induced complete and irreversible relaxation of intact micro- and macrovessels. The irreversible mode of action was also observed in experiments with cultured porcine endothelial cells and purified bovine lung sGC. These results might explain the severe hypotensive side effects of cinaciguat observed in a clinical trial on treatment of acute heart failure (Gheorghide et al., 2012).

## Materials and Methods

### Materials

Cinaciguat was purchased from Axon Medchem (Groningen, Netherlands). Stock solutions were prepared in dimethylsulfoxide and further diluted in buffer. Final concentration of the organic solvent did not exceed 0.1%. EDTA-free Complete Protease Inhibitor Cocktail Tablets were purchased from Roche Diagnostics GmbH (Vienna, Austria). 2,2-Diethyl-1-

nitroso-oxyhydrazine (DEA/NO) and 1*H*-[1,2,4]oxadiazolo-[4,3- $\alpha$ ]quinoxalin-1-one (ODQ) were obtained from Enzo Life Sciences (Lausen, Switzerland), purchased through Eubio (Vienna, Austria). DEA/NO was dissolved and diluted in 10 mM NaOH. All other chemicals were obtained from Sigma-Aldrich (Vienna, Austria).

### Animals and Tissue Preparation

Porcine hearts were obtained from a local abattoir and immediately transported to the laboratory. The right coronary artery was carefully explanted, cleaned from connective tissue, and immediately used for assessment of vessel function. In addition, thoracic aortas were harvested from unsexed Sprague-Dawley rats (Charles River, Sulzfeld, Germany) that were housed at the local animal facility in approved cages. Rats were fed standard chow (Altromin 3023; obtained from Königshofer Futtermittel, Ebergassing, Austria) and received water ad libitum. Animals were euthanized in a box that was gradually filled with CO<sub>2</sub> until no more vital signs (cessation of respiration and circulation) were noticed. Subsequently, the thorax was opened and the thoracic aorta was removed and placed in chilled buffer, and then immediately used for functional studies. All animal experiments were performed in compliance with the Austrian law on experimentation with laboratory animals (last amendment 2012; <https://www.ris.bka.gv.at/eli/bgbl/I/2012/114>).

### Isometric Tension Vasomotor Studies

For the isometric tension measurements, vessel rings were suspended in 5-ml organ baths containing oxygenated Krebs-Henseleit buffer (118.4 mM NaCl, 25 mM NaHCO<sub>3</sub>, 4.7 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 11 mM D-Glucose; pH 7.4), as described previously (Neubauer et al., 2013). After equilibration for 60 minutes at the optimal resting tension (i.e. 1 g for rat aortas or 2 g for porcine coronary arteries), maximal contractile activity was determined with a depolarizing solution containing 100 mM K<sup>+</sup>. Rings that did not elicit adequate and stable contraction to high K<sup>+</sup> were considered to be damaged and were omitted from the study. After washout, tissues were precontracted to ~60% of maximal contraction with a depolarizing solution containing 30 mM K<sup>+</sup>. After a stable tone had been reached (~20 minutes), cinaciguat was added at the indicated concentrations, and vasorelaxation was monitored over 90 minutes. Subsequently, rings were thoroughly washed with standard Krebs-Henseleit buffer for 60 minutes to test for the reversibility of cinaciguat-induced relaxation. During the washout period the bath solution was replaced with fresh buffer at least 10 times. Finally, contraction to 30 mM K<sup>+</sup> was measured again.

### Determination of cGMP Levels in Vascular Tissue

Vessel rings were freeze-clamped at the time points indicated in Fig. 2 and homogenized in 5% trichloroacetic acid. The acid was removed by diethyl ether extraction, and the aqueous extracts were appropriately diluted and analyzed for cGMP by radioimmunoassay as previously described (Kukovetz et al., 1979).

## Determination of sGC Activity

sGC was purified from bovine lung as previously described (Russwurm and Koesling, 2005). The enzyme (50 ng; ~0.3 pmol) was incubated at 37°C in a final volume of 0.1 ml of assay mixture containing 50 mM TEA/HCl (pH 7.4), 0.5 mM [ $\alpha$ -<sup>32</sup>P]GTP (~200,000 cpm), 3 mM MgCl<sub>2</sub>, and 1 mM cGMP. Other compounds were present as indicated. Reactions were started by addition of DEA/NO or cinaciguat (final concentrations as indicated). Basal enzyme activity was determined in the absence of activator. Reactions were terminated by the addition of 0.45 ml of 120 mM Zn(CH<sub>3</sub>COO)<sub>2</sub> and 0.45 ml of 120 mM Na<sub>2</sub>CO<sub>3</sub>. After centrifugation (20,000g at 4°C for 10 minutes) supernatants were applied onto Al<sub>2</sub>O<sub>3</sub> columns pre-equilibrated with 0.1 M HClO<sub>4</sub>. After washing the columns with distilled water, [<sup>32</sup>P]cGMP was eluted with 50 mM NaCH<sub>3</sub>COO and quantified by liquid scintillation counting (Schultz and Böhme, 1984). Blank values were measured in the absence of sGC. For time course experiments, sGC was stimulated with increasing concentrations of cinaciguat and incubated in the assay mixture in the absence or presence of ODQ (10  $\mu$ M). At selected time points, aliquots (0.1 ml) were removed from the incubation mixture, reactions were terminated, and [<sup>32</sup>P]cGMP was quantified as previously described. In another series of experiments, sGC (50 ng) was preincubated in the assay mixture in the absence or presence of 10  $\mu$ M ODQ for increasing periods of time (0–90 minutes; 37°C). At the indicated time points, incubations (5 minutes) were started by addition of DEA/NO or cinaciguat to yield final activator concentrations of 1  $\mu$ M. Incubations with cinaciguat were performed in the presence of 0.5% Tween 20 (v/v), which effectively removes the heme moiety from sGC (Foerster et al., 1996; Schmidt et al., 2003). To test for reversibility of cinaciguat-induced enzyme activation, sGC (50 ng) was preincubated in 50 mM TEA/HCl buffer (pH 7.4) in the presence of 1  $\mu$ M cinaciguat and 0.5% Tween 20 for 5 minutes at 37°C, diluted 50-fold in assay mixture, and then incubated in the absence or presence of 1  $\mu$ M cinaciguat for up to 60 minutes.

## Statistical Analysis

Data are presented as mean  $\pm$  S.D. values of *n* experiments. To establish relaxation curves, different ring segments from a single animal were averaged and counted as an individual experiment (*n* = 1). Analysis of variance with post hoc Bonferroni test was used for comparison between groups using IBM SPSS Statistics (version 22.0) (IBM Corp, Armonk, NY). Data of the concentration-response curve were fitted according to the Hill equation. The effect of ODQ on the activity of purified sGC (Fig. 3B) was analyzed by comparing the data of the combined time points (10, 45, and 90 minutes) for the three applied cinaciguat concentrations (1, 10, and 100  $\mu$ M) with a paired *t* test. Significance was assumed at *P* < 0.05.

## Results

### Vascular Relaxation

As shown in Fig. 1A, cinaciguat caused time- and concentration-dependent relaxation of isolated porcine coronary arteries. After 90 minutes of incubation, 1, 10, and 100 nM cinaciguat caused 32%  $\pm$  17%, 56%  $\pm$  16%, and 75%  $\pm$  12% relaxation of K<sup>+</sup>-precontracted vessels, respectively. A similar effect of the drug was observed with isolated rat aortas

inducing  $21\% \pm 15\%$ ,  $53\% \pm 29\%$ , and  $71\% \pm 14\%$  relaxation at concentrations of 1, 10, and 100 nM, respectively (Fig. 1C). The dilatory effect of cinaciguat was not reversed upon extensive washout of the drug for 60 minutes, as demonstrated in Fig. 1, B and D. Virtually identical results were obtained with the related sGC activator BAY 60-2770 (data not shown). Relaxation of coronary arteries to cinaciguat was not affected by removal of the endothelium, a procedure that led to ~80% loss of vasodilation to the endothelium-dependent receptor agonist bradykinin (Supplemental Fig. 1).

### Accumulation of cGMP in Porcine Coronary Arteries

To probe whether the effect of cinaciguat was indeed mediated by sGC activation in vascular tissues, cGMP levels of cinaciguat-treated porcine coronary arteries were measured before and after washout of the drug. As shown in Fig. 2A, slow relaxation of porcine coronary arteries to 100 nM cinaciguat was accompanied by time-dependent accumulation of cGMP in the tissue. Incubation of vessels for 90 minutes with 1, 10, and 100 nM cinaciguat increased cGMP levels from  $89 \pm 40$  (basal) to  $205 \pm 79$ ,  $245 \pm 83$ , and  $273 \pm 108$  fmol/mg wet weight, respectively (Fig. 2B). As observed in the functional studies, extensive washout of cinaciguat for 60 minutes had no effect, while cGMP levels of DEA/NO-treated rings were reversed to basal values after washout ( $91 \pm 48$  fmol/mg wet weight). Hydrolysis of [ $^{32}$ P]cGMP was not affected by cinaciguat, indicating that accumulation of vascular cGMP in response to the drug did not activate cGMP-dependent phosphodiesterases (Supplemental Fig. 2). Accumulation of cGMP in cultured porcine aortic endothelial cells triggered by 10 and 100  $\mu$ M cinaciguat was not significantly reversed upon washout of the drug for 60 minutes (Supplemental Fig. 3). Taken together, the data obtained with isolated blood vessels and cultured cells indicate that activation of vascular sGC by cinaciguat persisted for at least 60 minutes.

### Activation of Purified sGC

As illustrated in Fig. 3A, cinaciguat activated purified sGC with an  $EC_{50}$  value of  $194 \pm 15$  nM in the presence of 0.5% Tween 20. Maximal rates of cGMP formation in the presence of 10  $\mu$ M cinaciguat were comparable to sGC activity induced by 1  $\mu$ M DEA/NO in the absence of Tween 20 ( $24.4 \pm 1.0 \mu\text{Mol} \times \text{min}^{-1} \times \text{mg}^{-1}$ ). DEA/NO had no effect in the presence of the detergent, indicating loss of NO sensitivity through effective removal of sGC-bound heme.

To investigate whether cinaciguat activates the holoenzyme through time-dependent displacement of either reduced or oxidized heme, sGC was preincubated for 5 minutes with 10  $\mu$ M ODQ or vehicle, followed by incubation for 10, 45, and 90 minutes at 37°C with increasing concentrations of cinaciguat. As shown in Fig. 3B, the drug caused moderate activation of sGC corresponding to approximately 10% ( $2\text{--}4 \mu\text{Mol} \times \text{min}^{-1} \times \text{mg}^{-1}$ ) of maximal DEA/NO-stimulated activity. Preincubation with ODQ slightly enhanced the effects of 1 and 10  $\mu$ M cinaciguat ( $P = 0.0033$  and  $P < 0.0001$  for 1 and 10  $\mu$ M cinaciguat, respectively) but did not significantly affect sGC activation by 100  $\mu$ M of the drug. The degree of sGC activation was not increased upon prolonged periods of incubation, indicating that cinaciguat displaces neither ferrous nor ferric heme from the holo-enzyme within 90 minutes. Similar results were obtained with BAY 60-2770 (data not shown).

To test for stability of ferrous and ferric sGC, the enzyme was preincubated for increasing periods (up to 90 minutes) in the absence and presence of ODQ (10  $\mu$ M), and thereafter tested for DEA/NO-stimulated cGMP formation (Fig. 3C). Sensitivity of the enzyme to DEA/NO rapidly decreased, with a half-life of approximately 10 minutes, while sGC activity determined in the presence of ODQ was virtually stable for 90 minutes. Surprisingly, sGC that had been preincubated for 45 minutes was fully sensitive to activation by cinaciguat in the presence of Tween 20. These data indicate that preincubation of sGC causes loss of NO sensitivity through heme oxidation without affecting the functional integrity of the enzyme.

The results obtained from organ bath experiments and isolated cells indicate that sGC activation by cinaciguat was not reversed by washout of the drug. To study reversibility of sGC activation, the purified enzyme was incubated with 1  $\mu$ M cinaciguat for 5 minutes at 37°C in the presence of Tween 20. Samples were then diluted 50-fold (yielding a final cinaciguat concentration of 20 nM) and incubated for 5–60 minutes at 37°C with and without added cinaciguat (1  $\mu$ M). As shown in Fig. 3D, cGMP formation increased linearly with time but was not affected by 50-fold dilution of the preincubated samples. Formation of cGMP was not further increased by addition of cinaciguat to the diluted samples. These results demonstrate that no significant dissociation of the drug from the enzyme occurred within 60 minutes.

## Discussion

The present study was designed to explain results from previous reports indicating that the sGC activators cinaciguat and BAY 60-2770 caused virtually complete cGMP-mediated relaxation of nondiseased isolated blood vessels (Tawa et al., 2014, 2015; Jabs et al., 2015). Probing the effect of cinaciguat on relaxation of porcine coronary arteries and rat thoracic aortas, our results confirm that sGC activators mediate virtually complete relaxation of uninjured blood vessels. In addition, our study demonstrates irreversibility of enzyme activation by sGC activators for the first time.

Previous reports showing a pronounced increase in cellular cGMP accumulation in response to cinaciguat upon preincubation with ODQ or 3-morpholinosydnonimine indicate that heme oxidation accelerates the shift from holo- to apo-sGC in cells and tissues (Stasch et al., 2006; Chester et al., 2009). This effect has led to the conclusion that the fraction of cGMP accumulation caused by sGC activators in the absence of heme oxidants gives an estimate of the amount of heme-free sGC in tissues. However, the present data showing that cGMP-mediated vascular relaxation increases with time questions this assumption.

According to Ghosh et al. (2014), cells and tissues contain a pool of heme-free sGC  $\beta$ -subunits that is shifted toward the  $\alpha,\beta$ -heterodimer by NO in the presence of sufficient cellular heme. Accordingly, sGC activators like cinaciguat or BAY 60-2770 may shift the equilibrium between NO-sensitive holoenzyme and heme-free apo-sGC toward latter species. Moreover, irreversible binding of the drug appears to promote a time-dependent transition toward permanently active cinaciguat-stimulated apo-sGC, leading to vascular relaxation even under physiologic conditions. Based on the concept of spare sGC NO

receptors (Mergia et al., 2006), even the conversion of a small fraction of the total sGC pool to this enzyme species could be sufficient for a maximal effect on blood vessel tone.

The effect of cinaciguat and BAY 60-2770 on vessel tone clearly depends on the intracellular ratio of ferrous, ferric, and apo-sGC. Diverse pathologies such as atherosclerosis, hypertension, and diabetes are associated with increased oxidative stress that affects the composition of the vascular sGC pool by shifting the equilibrium from NO-sensitive ferrous sGC toward ferric and apo-sGC. Irreversible activation of these sGC species by cinaciguat and analogs may be therapeutically beneficial but could have adverse effects by inducing severe hypotension. Indeed, pronounced hypotensive side effects of even low doses of intravenously applied cinaciguat led to early termination of the COMPOSE study on treatment of acute heart failure (Gheorghiadu et al., 2012).

The irreversible type of action that was observed in blood vessels and cultured cells was also evident in experiments with the purified enzyme. Interestingly, much higher concentrations of cinaciguat were necessary for maximal stimulation of purified sGC and in cultured cells ( $\sim 10 \mu\text{M}$ ) compared with isolated blood vessels ( $\sim 100 \text{ nM}$ ). Albeit we have no explanation for this observation it agrees well with previous reports (Stasch et al., 2002, 2006; Chester et al., 2009) and suggests a more complex mode of action of the drug in intact tissues.

Our results obtained with purified sGC suggest that cinaciguat stimulates the heme-free enzyme but does not displace ferric heme, since enzyme activation was virtually identical in the absence and presence of ODQ without a significant effect of incubation time. The modest enzyme activation by cinaciguat in ODQ-free samples (10%) is most likely due to stimulation of a small population of apo-sGC present in the enzyme preparation. The apparent lack of concentration dependence ( $1\text{--}100 \mu\text{M}$ ) may be due to irreversible saturation of a small fraction of apo-sGC. Assuming 10% of the enzyme preparation is heme deficient in the assay,  $\sim 0.03 \text{ pmol } \beta 1\text{-sGC}$  ( $50 \text{ ng } \alpha/\beta\text{-heterodimer total}$ ) was exposed to  $0.1\text{--}10 \text{ nmol cinaciguat}$ . Our observation is in good accordance with findings of Roy et al. (2008), which showed that the target of cinaciguat is heme-free sGC but not the ferric enzyme. However, for unknown reasons the data are in apparent conflict with a previous study showing that cinaciguat and ODQ synergistically activate sGC (Stasch et al., 2002).

Moreover, in a recent study Surmeli and Marletta (2012) reported that cinaciguat stimulates heme loss and (in contrast to our observations) cGMP formation with a linear correlation between heme loss and sGC activation. However, enzyme activation attained under those conditions was very modest ( $0.5\text{--}1.0 \mu\text{mol} \times \text{mg}^{-1} \times \text{min}^{-1}$ ), corresponding to only a few percent of maximal sGC activity (see Fig. 3, A and C) and is in fact quite similar to the activity we ascribe to a small fraction of apo-sGC that might already be present in the absence of cinaciguat (Fig. 3B). Moreover, the reported linear correlation between heme dissociation and sGC activation only held for the first 15 minutes, after which heme loss continued, while enzyme activity remained constant. Therefore, it appears that in both studies, i.e., the current study and that of Surmeli and Marletta (2012), activation of a small heme-free fraction of sGC by cinaciguat was observed, whereas the striking spectral changes of the oxidized heme of sGC induced by the drug do not result in enzyme activation.

The lack of effect of ODQ observed with the purified enzyme indicates that heme oxidation is not sufficient to promote significant accumulation of apo-sGC within 90 minutes, pointing to an additional, yet undefined, driving mechanism that mediates loss of the heme moiety in vascular tissue. The process might be related to the dynamic interplay between sGC subunits and Hsp90 (Ghosh and Stuehr, 2017); however, further work is necessary to clarify the underlying mechanism.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

<b>DEA/NO</b>	2,2-diethyl-1-nitroso-oxyhydrazine
<b>NO</b>	nitric oxide
<b>ODQ</b>	1 <i>H</i> -[1,2,4]oxadiazolo-[4,3- <i>a</i> ]quinoxalin-1-one
<b>sGC</b>	soluble guanylate cyclase
<b>YC-1</b>	3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole

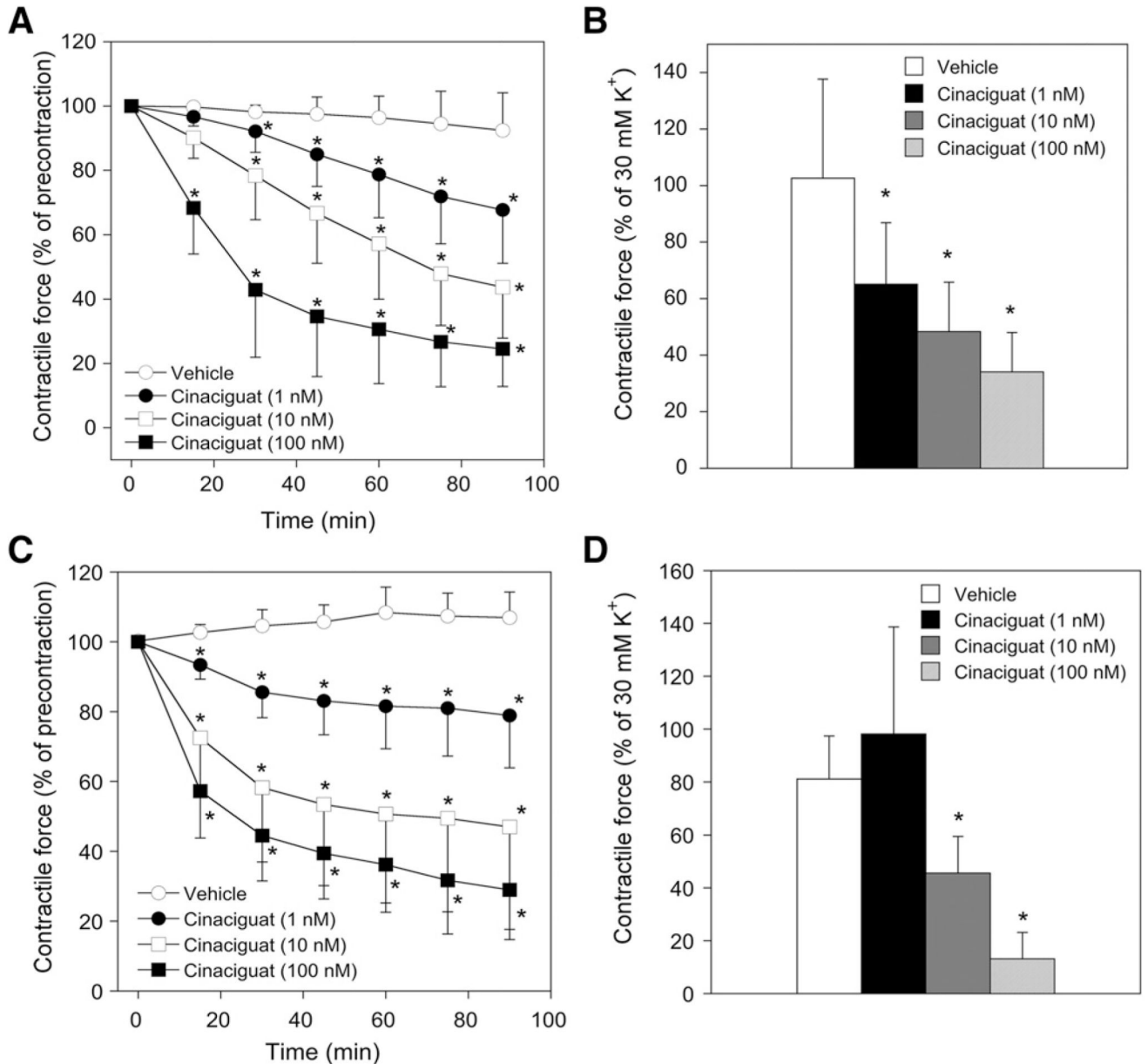
## References

- Chester M, Tourneux P, Seedorf G, Grover TR, Gien J, Abman SH. Cinaciguat, a soluble guanylate cyclase activator, causes potent and sustained pulmonary vasodilation in the ovine fetus. *Am J Physiol Lung Cell Mol Physiol.* 2009; 297:L318–L325. [PubMed: 19465519]
- Evgenov OV, Pacher P, Schmidt PM, Haskó G, Schmidt HHHW, Stasch JP. NO-independent stimulators and activators of soluble guanylate cyclase: discovery and therapeutic potential. *Nat Rev Drug Discov.* 2006; 5:755–768. [PubMed: 16955067]
- Feil R, Lohmann SM, de Jonge H, Walter U, Hofmann F. Cyclic GMP-dependent protein kinases and the cardiovascular system: insights from genetically modified mice. *Circ Res.* 2003; 93:907–916. [PubMed: 14615494]
- Foerster J, Harteneck C, Malkewitz J, Schultz G, Koesling D. A functional heme-binding site of soluble guanylyl cyclase requires intact N-termini of  $\alpha_1$  and beta  $\beta_1$  subunits. *Eur J Biochem.* 1996; 240:380–386. [PubMed: 8841402]
- Friebe A, Müllershausen F, Smolenski A, Walter U, Schultz G, Koesling D. YC-1 potentiates nitric oxide- and carbon monoxide-induced cyclic GMP effects in human platelets. *Mol Pharmacol.* 1998; 54:962–967. [PubMed: 9855623]
- Gheorghiane M, Greene SJ, Filippatos G, Erdmann E, Ferrari R, Levy PD, Maggioni A, Nowack C, Mebazaa A, COMPOSE Investigators and Coordinators. Cinaciguat, a soluble guanylate cyclase activator: results from the randomized, controlled, phase IIb COMPOSE programme in acute heart failure syndromes. *Eur J Heart Fail.* 2012; 14:1056–1066. [PubMed: 22713287]
- Ghosh A, Stasch JP, Papapetropoulos A, Stuehr DJ. Nitric oxide and heat shock protein 90 activate soluble guanylate cyclase by driving rapid change in its subunit interactions and heme content. *J Biol Chem.* 2014; 289:15259–15271. [PubMed: 24733395]



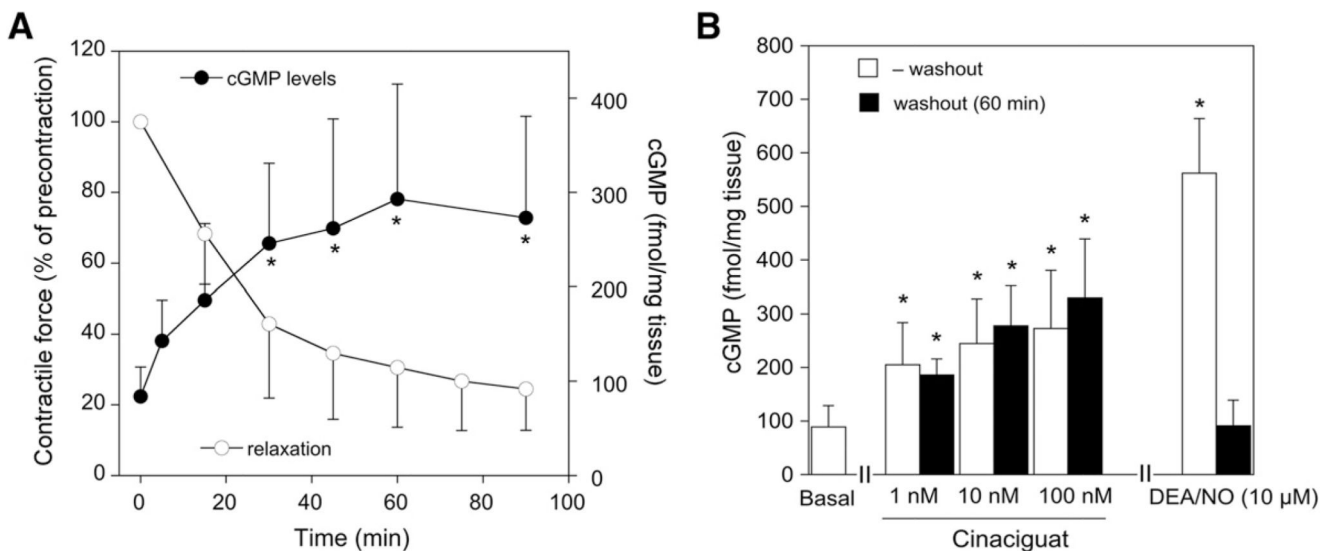
- Ghosh A, Stuehr DJ. Regulation of sGC via hsp90, cellular heme, sGC agonists, and NO: new pathways and clinical perspectives. *Antioxid Redox Signal*. 2017; 26:182–190. [PubMed: 26983679]
- Humbert P, Niroomand F, Fischer G, Mayer B, Koesling D, Hinsch KD, Gausepohl H, Frank R, Schultz G, Böhme E. Purification of soluble guanylyl cyclase from bovine lung by a new immunoaffinity chromatographic method. *Eur J Biochem*. 1990; 190:273–278. [PubMed: 1973095]
- Jabs A, Oelze M, Mikhed Y, Stamm P, Kröller-Schön S, Welschof P, Jansen T, Hausding M, Kopp M, Steven S, et al. Effect of soluble guanylyl cyclase activator and stimulator therapy on nitroglycerin-induced nitrate tolerance in rats. *Vascul Pharmacol*. 2015; 71:181–191. [PubMed: 25869522]
- Koesling D. Studying the structure and regulation of soluble guanylyl cyclase. *Methods*. 1999; 19:485–493. [PubMed: 10581148]
- Kollau A, Hofer A, Russwurm M, Koesling D, Keung WM, Schmidt K, Brunner F, Mayer B. Contribution of aldehyde dehydrogenase to mitochondrial bioactivation of nitroglycerin: evidence for the activation of purified soluble guanylate cyclase through direct formation of nitric oxide. *Biochem J*. 2005; 385:769–777. [PubMed: 15377279]
- Kukovetz WR, Holzmann S, Wurm A, Pösch G. Evidence for cyclic GMP-mediated relaxant effects of nitro-compounds in coronary smooth muscle. *Naunyn Schmiedebergs Arch Pharmacol*. 1979; 310:129–138. [PubMed: 231208]
- Mayer B, Hemmens B. Biosynthesis and action of nitric oxide in mammalian cells. *Trends Biochem Sci*. 1997; 22:477–481. [PubMed: 9433128]
- Mergia E, Friebe A, Dangel O, Russwurm M, Koesling D. Spare guanylyl cyclase NO receptors ensure high NO sensitivity in the vascular system. *J Clin Invest*. 2006; 116:1731–1737. [PubMed: 16614755]
- Neubauer R, Neubauer A, Wölkart G, Schwarzenegger C, Lang B, Schmidt K, Russwurm M, Koesling D, Gorren AC, Schrammel A, et al. Potent inhibition of aldehyde dehydrogenase-2 by diphenylethylidonium: focus on nitroglycerin bioactivation. *Mol Pharmacol*. 2013; 84:407–414. [PubMed: 23793290]
- Roy B, Mo E, Vernon J, Garthwaite J. Probing the presence of the ligand-binding haem in cellular nitric oxide receptors. *Br J Pharmacol*. 2008; 153:1495–1504. [PubMed: 18204474]
- Russwurm M, Koesling D. Purification and characterization of NO-sensitive guanylyl cyclase. *Methods Enzymol*. 2005; 396:492–501. [PubMed: 16291256]
- Schmidt P, Schramm M, Schröder H, Stasch JP. Preparation of heme-free soluble guanylate cyclase. *Protein Expr Purif*. 2003; 31:42–46. [PubMed: 12963339]
- Schultz G, Böhme E. Guanylate cyclase GTP pyrophosphate-lyase (cyclizing). *Method Enzymatic Analysis*. Bergmeyer, HU. Bergmeyer, J., Graßl, M., editors. Verlag Chemie; Weinheim, Germany: 1984. p. 379–389.
- Stasch JP, Pacher P, Evgenov OV. Soluble guanylate cyclase as an emerging therapeutic target in cardiopulmonary disease. *Circulation*. 2011; 123:2263–2273. [PubMed: 21606405]
- Stasch JP, Schmidt P, Alonso-Alija C, Apeler H, Dembowski K, Haerter M, Heil M, Minuth T, Perzborn E, Pleiss U, et al. NO- and haem-independent activation of soluble guanylyl cyclase: molecular basis and cardiovascular implications of a new pharmacological principle. *Br J Pharmacol*. 2002; 136:773–783. [PubMed: 12086987]
- Stasch JP, Schmidt PM, Nedvetsky PI, Nedvetskaya TY, HS AK, Meurer S, Deile M, Taye A, Knorr A, Lapp H, et al. Targeting the heme-oxidized nitric oxide receptor for selective vasodilatation of diseased blood vessels. *J Clin Invest*. 2006; 116:2552–2561. [PubMed: 16955146]
- Stone JR, Marletta MA. Spectral and kinetic studies on the activation of soluble guanylate cyclase by nitric oxide. *Biochemistry*. 1996; 35:1093–1099. [PubMed: 8573563]
- Surmeli NB, Marletta MA. Insight into the rescue of oxidized soluble guanylate cyclase by the activator cinaciguat. *ChemBioChem*. 2012; 13:977–981. [PubMed: 22474005]
- Tawa M, Geddawy A, Shimosato T, Iwasaki H, Imamura T, Okamura T. Soluble guanylate cyclase redox state under hypoxia or hypoxia/reoxygenation in isolated monkey coronary arteries. *J Pharmacol Sci*. 2014; 125:169–175. [PubMed: 24859780]

- Tawa M, Shimosato T, Iwasaki H, Imamura T, Okamura T. Vasorelaxing effects of the soluble guanylyl cyclase activator BAY 60-2770 in nitrate-tolerant monkey and canine coronary arteries. *Naunyn Schmiedebergs Arch Pharmacol.* 2015; 388:381–385. [PubMed: 25582420]
- Wedel B, Humbert P, Harteneck C, Foerster J, Malkewitz J, Böhme E, Schultz G, Koesling D. Mutation of His-105 in the beta 1 subunit yields a nitric oxide-insensitive form of soluble guanylyl cyclase. *Proc Natl Acad Sci USA.* 1994; 91:2592–2596. [PubMed: 7908439]

**Fig. 1.**

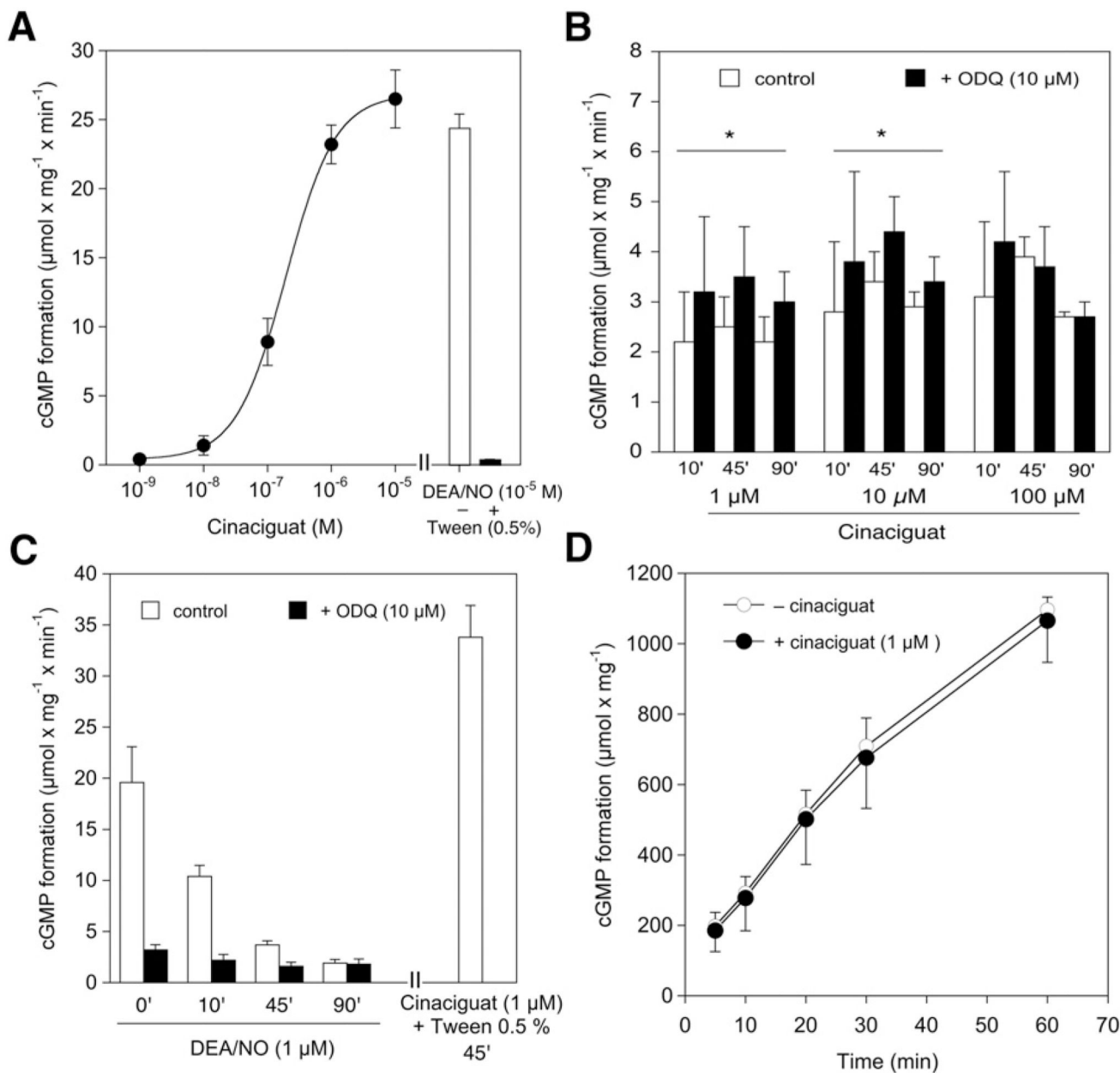
Effects of cinaciguat on vessel tone of isolated porcine coronary arteries and rat thoracic aortas. K<sup>+</sup>-precontracted rings from porcine coronary arteries (A) and rat aortas (C) were incubated in the absence or presence of cinaciguat (1, 10, and 100 nM) and the dilatory response was recorded up to 90 minutes. Effect of extensive washout (60 minutes) on relaxation of porcine (B) and rat (D) vessels that were pretreated with cinaciguat (1, 10, and 100 nM) for 90 minutes. Results are expressed as contraction percentage (effect of K<sup>+</sup> = 100%). Data obtained from different ring segments from the same vessel were averaged and counted as an individual experiment ( $n = 1$ ). Results represent mean  $\pm$  S.D. values of seven and eight individual animals for porcine and rat vessels, respectively. Results of Fig. 1, A and C were analyzed by two-way analysis of variance (ANOVA), while results of Fig. 1, B

and D were analyzed by one-way ANOVA with a Bonferroni post hoc test.  $*P < 0.05$  vs. vehicle.



**Fig. 2.**

Effect of cinaciguat on vascular cGMP formation. (A) Porcine coronary arteries were incubated with 100 nM cinaciguat for up to 90 minutes and relaxation was recorded. At the indicated time points, vascular rings were freeze-clamped and cGMP levels were measured by radioimmunoassay. Results represent mean  $\pm$  S.D. values of eight rings. (B) Porcine coronary arteries were incubated in the absence and presence of cinaciguat (1–100 nM) for 90 minutes. cGMP concentrations were measured before ( $t = 90$  minutes) and after a washout period of 60 minutes ( $t = 150$  minutes). For comparison, vascular cGMP levels in response to DEA/NO (10  $\mu$ M) were measured before (i.e. 2 minutes after addition of DEA/NO) and after washout. Data represent mean  $\pm$  S.D. values of 6–19 rings; Results of Fig. 2 were analyzed by one-way analysis of variance with a Bonferroni post hoc test. \* $P < 0.05$  vs. basal cGMP values.

**Fig. 3.**

Activation of purified bovine lung sGC by cinaciguat. (A) Purified sGC (50 ng) was incubated in assay mixture with increasing concentrations of cinaciguat in the presence of 0.5% Tween (v/v) for 10 minutes at 37°C and assayed for activity. Enzyme activation by DEA/NO (10  $\mu\text{M}$ ) in the absence and presence of 0.5% Tween (v/v) was measured as control. (B) sGC (50 ng) was preincubated for 5 minutes at 37°C in the absence or presence of ODQ (10  $\mu\text{M}$ ). Incubations were started by addition of cinaciguat (final concentrations of 1, 10, and 100  $\mu\text{M}$ ) and terminated after 10, 45, and 90 minutes. (C) sGC (50 ng) was preincubated at 37°C in the assay mixture in the absence or presence of ODQ (10  $\mu\text{M}$ ) for increasing periods of time (0–90 minutes). At the indicated time points, incubations (5

minutes) were started by addition of DEA/NO or cinaciguat to yield final activator concentrations of 1  $\mu\text{M}$ . Incubations with cinaciguat were performed in the presence of 0.5% Tween 20 (v/v). (D) To test for reversibility, sGC (50 ng) was preincubated in TEA/HCl buffer (50 mM, pH 7.4) containing cinaciguat (1  $\mu\text{M}$ ) in the presence of 0.5% Tween 20 (v/v) for 5 minutes at 37°C, diluted 50-fold with assay mixture, and incubated in the absence or presence of cinaciguat (1  $\mu\text{M}$ ) for the indicated periods of time (37°C). Data represent mean  $\pm$  S.D. values of three independent experiments. Results of Fig. 3B were analyzed by comparing the data of the combined time points (10, 45, and 90 minutes) for the three applied cinaciguat concentrations (1, 10, and 100  $\mu\text{M}$ ) with a paired *t* test. Significance was assumed at  $*P < 0.05$ .