Investigations on the Pharmacology of the Cardioprotective Guanidine ME10092

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Abstract: The guanidine compound ME10092 (1-(3,4-dimethoxy-2-chlorobenzylideneamino)-guanidine), which possesses a strong cardioprotective effect to ischemia-reperfusion, was assessed for different pharmacological actions that may underlie its cardioprotective effect. In the living rat ME10092 decreased the blood pressure and heart rate in a dose-dependent manner. We found ME10092 to bind to α_1- and α_2-adrenoreceptors with moderate affinity (K_i values 1–4 μM), and to block adrenaline-elicited contractile responses in isolated guinea pig aortas. Our results indicate that ME10092 possesses a certain anti-oxidant profile. Thus, in a competitive manner and with low affinity it inhibited the bovine milk xanthine oxidase enzyme, as well as NAD(P)H oxidase driven oxyradical formation in membrane fractions isolated from the rat brain. By using electron paramagnetic resonance we here show that, after its systemic administration, ME10092 modulates the nitric oxide (NO) content in several tissues of the rat in a time-dependent manner. However, in vitro ME10092 inhibited the activities of nitric oxide synthases nNOS and eNOS, but not that of iNOS. Our data give evidence that the cardioprotective effect of ME10092 could be mediated through pharmacological mechanisms that include some modulation of NO production, as well as possible inhibition of radical formation during ischemia-reperfusion.

Key Words: guanidine, xanthine oxidase, adrenoreceptor, NAD(P)H oxidase, nitric oxide synthase

(*J Cardiovasc Pharmacol*™ 2004;44:178–186)

The complexity of ischemia and reperfusion-mediated biochemical events in the heart offers multiple possibilities for pharmacological intervention. Free radicals are generated by several enzyme systems (eg, xanthine oxidase, NAD(P)H oxidases, nitric oxide synthases (NOSs)) during reoxygenation following an ischemic period of the heart, and constitute a major factor causing abnormalities in cardiac functioning.[1,2] Inability of cellular membranes to maintain an ionic balance, as well as insufficient energy metabolism, also leads to the final pathways of tissue injury.

In our earlier studies, we found that an electron acceptor-inhibitor of xanthine oxidase, the N-hydroxy-compound PR5 (1-(3,4-dimethoxy-2-chlorobenzylideneamino)-3-hydroxy-guanidine)), possesses a remarkable cardioprotective effect, when administered to rats subjected to left coronary artery clamping followed by reperfusion.[3,4] Later we showed that ME10092, which is a guanidine metabolite to PR5, also exerts a similar cardioprotective action.[5] We found that the administration of ME10092 to rats subjected to 1 hour left coronary occlusion followed by 2 hours reperfusion reduced the heart infarction size by approximately 40%.[5] Moreover, ME10092 prevented the marked drop in mean arterial pressure seen during the reperfusion phase. In addition to these effects, ME10092 appeared to have a direct action on the heart as it caused a dose dependent but transient reduction in the heart rate and a slight and transient reduction in mean arterial blood pressure.[5]

We also showed that both PR5 and ME10092 are xanthine oxidase inhibitors.[6] Moreover, we found that PR5 becomes completely and rapidly metabolized to ME10092 in vivo as determined in rat serum after oral administration of the drug.[5] In view of the latter finding, and due to the practically indistinguishable pharmacological effects of PR5 and ME10092, we suggested that the cardioprotective effect of PR5 is mediated by a direct effect afforded by its metabolite, ME10092.[5]

The present study was undertaken to clarify the biochemical mechanisms underlying the cardioprotective effect of ME10092. We have here evaluated ME10092 for adrenergic activities, as several guanidine compounds structurally related to ME10092 are known to possess these properties.[7] Moreover, in addition to characterization of xanthine oxidase inhibitory activity, we also evaluated ME10092 for different antioxidant, ie, oxyradical and nitric oxide producing enzymes blocking properties in biochemical and in vivo test systems.

Received for publication January 22, 2004; accepted April 16, 2004.

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This study was supported by a grant from the Swedish Lung and Heart Foundation, a Latvian Council of Science Grant 01.0209, and the Swedish VT (04X-05957). Dr. Maija Dambrova was also supported by a stipend from Swedish Heart and Lung Foundation, and Syskonen Svenssons Foundation.

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METHODS

Animals

Male Wistar rats weighing 200 to 300 g were housed under standard conditions (21–23°C, 12-hour light:dark cycle) with unlimited access to food and water. All experimental procedures were performed in accordance with the regulations of the Animal Ethical Committee of BaltLASA, Riga, Latvia, and Local Ethics Committee for Animal Experiments, Uppsala, Sweden. This investigation also conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85-23, revised 1985).

Drugs

Pentobarbital sodium was from Richter Pharma (Wien, Austria), N-(3,4-Dimethoxy-2-chlorobenzylideneamino)-guanidine (ME-10092, in some earlier studies also termed PR9) was synthesized by Nordic Synthesis, (Karlskoga, Sweden). [^{3}H]-prazosin (91 Ci/mmol), [^{3}H]-RX821002 (58 Ci/mmol) and L-[2,3,4,5-^{3}H]arginine HCl (53,4 Ci/mmol) were from Amersham Pharmacia Biotech. Dipheniliodonium (DPI), diethylenetriaminepentaacetic acid (DTPA), DMA (5-(N,N-dimethyl)amiloride), Tris-HCl, KH_2PO_4, phenylmethylsulfonyl fluoride (PMSF), propranolol, diethylthiocarbamate, L-nitroarginine methylester (L-NAME), $TBAHSO_4$ (Tetrabutyl Ammonium Hydrogen Sulfate) were from Sigma (St. Louis, MO). Ketamine was from Alfasan (Woeirden, Holland) and xylazine was from Bioveta AS (Ivanovice na Hane, Czech). Bovine milk xanthine oxidase, recombinant NOS bovine endothelial (eNOS), rat neuronal (nNOS), and mouse inducible (iNOS) (specific activity 0,3; 300 and 7 units/mg protein, respectively) were from Sigma (St. Louis, MO).

Blood Pressure and Heart Rate Measurements

Male Wistar rats (250–300 g) were anesthetized with ketamine 70 mg/kg and xylazine 10 mg/kg. Blood pressure was recorded by a transducer connected to the carotid artery. Heart rate was continuously computed from the blood pressure pulse wave using Quad Bridge Amp and PowerLab/400 (AD Instruments). A femoral vein was cannulated for peripheral drug administration.

Isolated Aorta Preparations

Male guinea pigs (350–450 g) were killed by decapitation. Their aortas were removed, and placed in 37°C warm modified Krebs-Henseleit solution (in mM: 2.5 $CaCl_2$, 11.1 glucose, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 118.0 NaCl, and 25.0 $NaHCO_3$, 1 μM ketoprofen) equilibrated with 95% O_2–5% CO_2. After removal of adjacent tissue from the aortas, rings of 2 to 3 mm of length were prepared using a pair of scissors. Each ring was then mounted into an organ bath and tied to an isometric MLT050 force transducer (AD Instruments), using a pair of cotton threads inserted through the lumen of the ring. The preparation was given an initial tension of 10 mN. When ME10092 was present it was added 15 minutes prior to the addition of (-)adrenaline. The contractile force was recorded using ADInstruments® Tech ware system equipped with a QUAD ML-118 bridge amplifier and PowerLab® 400, connected to a PowerMac computer. Data were calculated and plotted using GraphPad Prism 3.0 and Chart 4.0 for Windows software.

Electrically Stimulated Guinea Pig Ileum

Coaxial electrical stimulation of the ileum was performed essentially as described.[8] In brief, 2-cm ileum pieces were mounted in organ bath with an electrode inserted into the lumen and another in contact with the surrounding Krebs-Henseleit solution (for composition see above). Electrical stimulation was given with 1 millisecond pulses at 0.1 Hz using a voltage just above that which gave a maximal response. During these conditions twitch contractions are elicited, which are mediated via the stimulation of release of acetylcholine from intramural cholinergic neurons. Moreover, these twitch contractions can be inhibited by adrenergically active agonists such as (-)noradrenaline, by an action on prejunctional α_2-adrenoceptors situated in the cholinergic neurons.[8]

Expression of the Human α_{1A}-Adrenoceptor in COS1 Cells, and Human α_{2A}-, α_{2B}-, and α_{2C}-Adrenoceptors in Insect Sf9 cells, and Radioligand Binding Assays

The human α_{1A}-, α_{2A}-, α_{2B}-, and α_{2C}-encoding genes were cloned as described.[9,10] Radioligand binding was performed essentially as described.[9] Membranes expressing human α_{1A}-adrenoceptor (150 μL in final suspension) were incubated with 0.7 nM [^{3}H]-prazosin, and different concentrations of ME10092. Similarly, membranes expressing the human α_{2A}-, α_{2B}, or α_{2C}-adrenoceptors were incubated with 1 nM [^{3}H]-RX821002 and different concentrations of ME10092 (1 nM–1 mM). After filtering and washing on glass-fiber filters, the radioactivity retained on each filter was counted. The potencies of the tested drugs to displace the radioligands from the receptors were calculated from the data using law of mass action computer modeling with the BindAid software (Wan System).

Xanthine Oxidase Activity

The activity of xanthine oxidase was determined by assaying uric acid formation in a reaction mixture containing 0.005 units/ml of bovine milk xanthine oxidase, various concentrations of xanthine, and ME10092 in 50 mM Tris-HCl pH 7.4 at 25°C. The samples were preincubated with ME10092 for 5 minutes before starting the reaction by addition of xanthine. After 5 minutes the reaction was quenched by addition of ice-cold methanol (1:1 vol/vol). The concentration of uric acid in

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the samples was determined by HPLC on a 250 × 4.6 mm Alltech Select-B RP column. Mobile phase: linear gradient from 5% MeOH in 0.05M KH_2PO_4 (pH = 5.6, 0.05M TBAHSO$_4$) to 42% MeOH in 0.05M KH_2PO_4 (pH = 5.6, 0.05M TBAHSO$_4$), flow rate 1 ml/min, detection—UV at 291 nm, runtime—45 minutes. LOD of the procedure—3.3 mM/L of uric acid.

Measurement of Lucigenin–Derived Chemiluminescence in Rat Brain Membranes

Rat brain membranes were isolated essentially as described previously.[11] In brief, rat forebrains were homogenized at 1500 rpm for 1 minute with a glass-Teflon homogenizer in ice-cold homogenization buffer (250 mM sucrose, 10 mM HEPES, 1 mM EDTA, 0.1 mM PMSF, 0.5% w/v bovine serum albumin, pH 7.4) at w/v ratio 1:20. The crude homogenate was spun at 500 × g for 10 minutes. The supernatant was then centrifuged at 17 500 × g for 20 minutes at 4°C and the pellet re-suspended in isotonic PBS, pH 7.4, containing 0.1 mM DTPA. The final protein concentration was adjusted to 4 mg/ml. The lucigenin reaction was carried out in 96-well Isoplates™ (Wallac Finland) at +37°C. ME10092 was incubated with the rat brain membranes (protein concentration 1 mg/ml) for 15 minutes. After that NAD(P)H (0.2 mM) and lucigenin (5 μM) were added and the luminescence immediately counted using a Wallac MicroBeta® TriLux liquid scintillation and luminescence counter. Luminescence in the blank samples (without cofactor) was less than 5% of control levels, which was subtracted from the luminescence readings of the test samples. Each data point represents average of 3 to 5 assays.

EPR Determination of Nitric Oxide in Rat Tissues after ME10092 Administration

The NO contents of rat brain, liver, heart, and testis were determined as described previously.[12,13] The experiment was started by an i.p. administration of saline or ME10092 in saline (5 and 10 mg/kg) to, respectively, control and ME10092 treated rats. Animals were killed and tissue samples frozen in liquid nitrogen 0.5 hours after spin trap agent administration.[12,13] EPR spectra were recorded by a Radiopan SE/X2544 EPR spectrometer. Measurement parameters were as follows: X-band operation, 25 mW, 9.24 GHz microwaves, 100 kHz modulation frequency, 5 G modulation amplitude, 2.5 mW microwave power, 5×10^{-5} receiver gain, time constant 1 second. NO concentration (ng/g of tissue) was calculated from an $NaNO_2$ calibration curve.

Nitric Oxide Synthase Activity Determination

Nitric oxide synthase activities were determined using a method based on the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. NOS isoforms (eNOS, nNOS, iNOS) were incubated in 25 mM Tris buffer (pH 7.4) for 30 minutes at 37°C in the presence of 1 mM NADPH, 100 μM H_4B, 10 μM FAD, 10 μM FMN, 1.5 mM $CaCl_2$, 2 μg/ml calmodulin, and 2.5 μM L-[^{3}H]arginine (approximately 100 000 dpm of L-[2,3,4,5-^{3}H]arginine HCl) and appropriate concentration of inhibitors (ie, ME10092 and L-NAME). The reaction was terminated by adding 0.6 mL Dowex-50W Tris form, equilibrated in 50 mM HEPES buffer (pH 5.5), containing 1 mM citrulline and 5 mM EDTA. After 1 hour incubation at room temperature, the solution was collected into OptiPhase SuperMix (Wallac) scintillation liquid and the radioactivity counted in a Rackbeta 1217 liquid scintillation counter. NOS activity was expressed as the amount of citrulline formed.

Statistical Analysis

The results are presented as the mean ± SEM. Statistical analysis was performed using unpaired two-tailed Student *t* test. Results were considered as significant when $P \leq 0.05$.

RESULTS

Effects on Blood Pressure and Heart Rate

As can be seen from Figure 1A and 1B, ME10092 induced clear hypotensive and bradycardic effects in the anesthetized adult rat. Thus, i.v. injections of ME10092 (1–10 mg/kg) dose dependently and significantly decreased diastolic blood pressure and heart rate. At the dose of 10 mg/kg the drop amounted to maximally 50% for both diastolic pressure (from initial 76 mm/Hg to 37 mm/Hg) (Fig. 1A) and heart rate (from initial 315 BPM to 189 BPM) (Fig. 1B). However, the systolic blood pressure was not influenced by ME10092, although there seemed to be a tendency toward lowered pressure. The effect on blood pressure lasted for approximately 30 seconds. Interestingly, the effect on heart rate followed a distinct pattern, with an initial fast drop (30 seconds) followed by some regain, which was then followed by a sustained gradual drop of heart rate until a plateau was reached and sustained for more than 30 minutes (Fig. 1C). (Statistical comparisons for the effect of ME10092 were in all above cases performed versus injection of saline, where the saline injections per se induced virtually no effects.)

α-Adrenergic Activities of ME10092 in Isolated Organs

The in vitro α-adrenergic activity of ME10092 was further examined in the guinea pig aorta preparation. As it can be seen in Figure 2, ME10092, in concentrations 3×10^{-6} to 10^{-4} M, caused a parallel rightward displacement of the dose response curve of (-)adrenaline ($3 \times 10^{-9} - 3 \times 10^{-5}$ M) without affecting the slope of the maximal response. The slope of the Schild plot computed from the data was 1.1 ± 0.1, suggesting that ME10092 is a competitive antagonist. The K_b calculated from the experiment was 4.6 μM, corresponding to a pA2 of 5.3.

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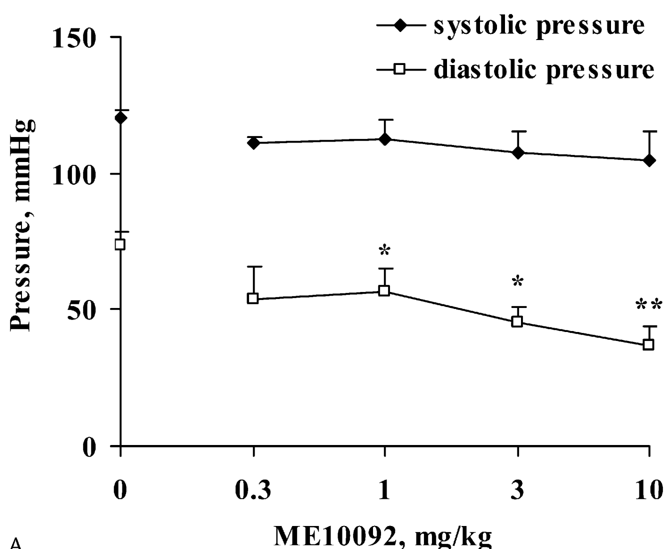

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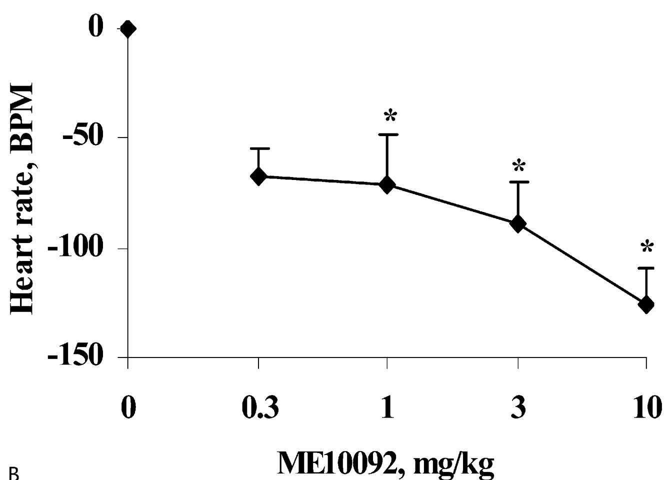

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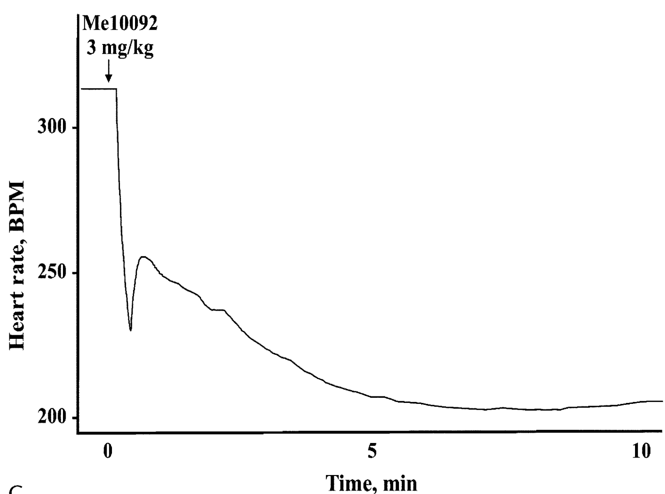

C

FIGURE 1. Influence of ME10092 on the arterial pressure and heart rate in the anesthetized rat. ME10092 in saline was administrated i.v. through the cannulated femoral vein and blood pressure recorded from the left carotid artery. Arterial pressure (A) values are expressed in mm Hg, heart rate (B) in percent of the basal rate (315 bpm). Shown is also the time course for change of heart rate after ME10092 administration in a single rat for 10 minutes after the compound administration (C). All values represent means ± SEM of at least 3 independent experiments. **P* < 0.05 compared with control, ***P* < 0.01 compared with control.

In the electrically stimulated isolated guinea pig ileum organ bath experiments, ME10092 inhibited the contractions of ileum with an IC_{50} of 25.3 μM (data not shown). Yohimbine (3 μM) did not alter the inhibitory effect of ME10092 (data not shown). In another experimental set-up we obtained inhibitory dose response for (-)noradrenaline. We then observed that the addition of 10 μM ME10092 caused a parallel shift of the (-)noradrenaline inhibitory dose response curve to the right (data not shown). Thus, these data indicate that ME10092 is a weak α_2-adrenoceptor antagonist on prejunctional α_2-adrenoceptors of the cholinergic neurones of the guinea pig ileum. The K_b of ME100092 calculated from these data amounted to 4.3 μM.

Determination of α-Adrenoreceptor Binding Activities of ME10092

Radioligand binding competition assays of ME10092 were performed using cell membranes expressing human α_{1A}-, α_{2A}-, α_{2B}-, or α_{2C}-adrenoceptors and appropriate labeled com-

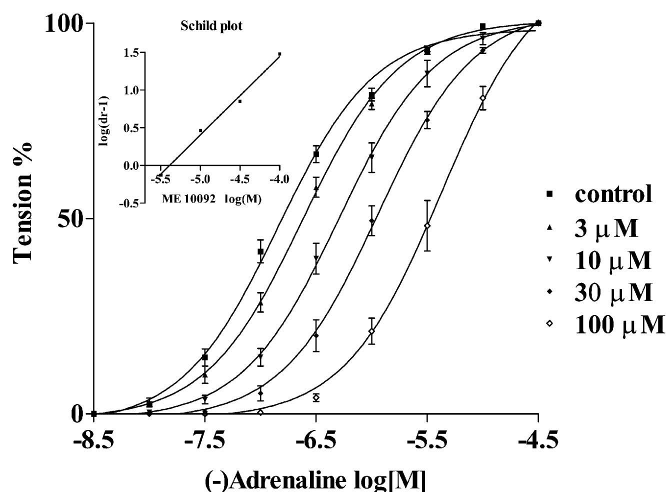

FIGURE 2. Influence of ME10092 on the contractile response of guinea pig aortic rings to (-)adrenaline. Values are expressed as the percent of the rings' maximally developed tension at the highest adrenaline concentrations used. All values represent the means ± SEM of at least 3 independent experiments. Insert shows the Schild plot calculated from the experimental data.

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TABLE 1. Binding Activities, K_i, of ME10092 on Recombinant Human α-Adrenoreceptor Subtypes Determined in Radiologand Binding Competition with [^{3}H] Prazosin (α_{1A}) and [^{3}H] RX821002 ($\alpha_{2A,B,C}$)

Receptor	K_i (μM) ± SEM
α_{1A}	4.1 ± 0.3
α_{2A}	1.2 ± 0.1
α_{2B}	4.1 ± 0.3
α_{2C}	3.1 ± 0.5

pounds. All obtained displacement curves were uniphasic, with Hill coefficients close to unity. The non-specific binding of [^{3}H]-RX821002 was negligible, and that of [^{3}H]-prazosin less than 5% of total binding (data not shown). As can be seen from Table 1, ME10092 showed 1 to 4 μM K_is to all α-adrenoceptor subtypes tested.

Inhibition of Xanthine Oxidase

Measuring the influence on formation of uric acid by using an HPLC-based assay was used to assess the effect of ME 10092 on xanthine oxidase mediated oxidation of xanthine. In control assays the speed of the uric acid formation amounted to about 10 μM per min and unit of xanthine oxidase (Fig. 3). Addition of 50 to 100 μM ME10092 inhibited the rate of uric acid formation. As seen in Figure 3, ME10092 substantially reduced the velocity at low concentrations of xanthine, but did not alter it appreciably at higher concentrations of the substrate. The competitive inhibition constant for ME10092 was estimated to be 24.4 μM, as it could be determined from the plot of the enzyme reaction K_m versus inhibitory concentrations of ME10092 (Fig. 3, inset).

Inhibitory Activity on NAD(P)H Oxidase Mediated Oxyradical Formation

Lucigenin-dependent luminescence was registered from samples containing rat brain membranes after addition of 200 μM of NADH or NADPH. The flavoenzyme inhibitor DPI[14] was used to control the specificity of the assay. We found that in the presence of 200 μM DPI the NADH and NADPH driven luminescence was inhibited by 80% and 60%, respectively (Fig. 4). As can be seen from the figure, ME10092 was a less effective inhibitor, the inhibition caused at the highest dose (500 μM) amounting to about 40% for both the NADH and NADPH driven luminescence (Fig. 4).

Inhibition of Nitric Oxide Formation in Rat Tissues In Vivo

The contents of NO were assayed in brain cortex, heart, liver, and lungs taken from rats 1 hour after i.p. administration of saline, or 5 and 10 mg/kg of ME10092, and spin trapping reagents, by use of EPR (see Methods for details). As controls served tissues from animals, treated identically, but without receiving ME10092. In the heart the NO contents were too low to be detectable both in control and ME10092-treated animals. In liver and brain cortex we found ME10092 to decrease the NO contents in a statistically significant manner (Fig. 5A). In

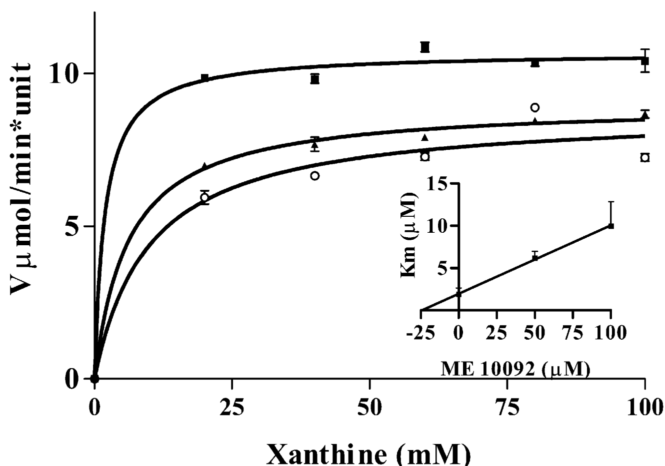

FIGURE 3. Influence of ME10092 on xanthine oxidase-mediated oxidation of xanthine. The formation of uric acid was measured by HPLC 5 minutes after initiating the reaction. Values are expressed as μM of uric acid formed in 1 minute per unit of xanthine oxidase. Squares, triangles, and rings indicate control, ME10092 50 μM, and ME10092 100 μM, respectively. All data points represent mean ± standard deviation of at least 3 independent experiments. Insert shows the plot of the enzyme reaction K_m calculated from the experimental data versus inhibitory concentrations of ME10092.

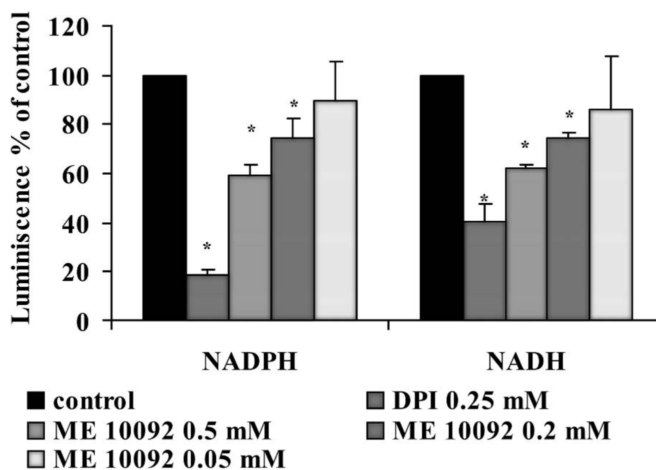

FIGURE 4. Influence of ME10092 on NAD(P)H oxidase-mediated oxyradical formation in rat brain membranes. Chemiluminescence measurements were performed immediately after addition of NADH or NADPH and lucigenin to membrane suspensions preincubated with ME10092 or DPI. Values are expressed as percents of controls, with blanks subtracted. All data points represent mean ± standard deviation of at least 3 independent experiments performed with tetraplicates. *$P < 0.05$ compared with control.

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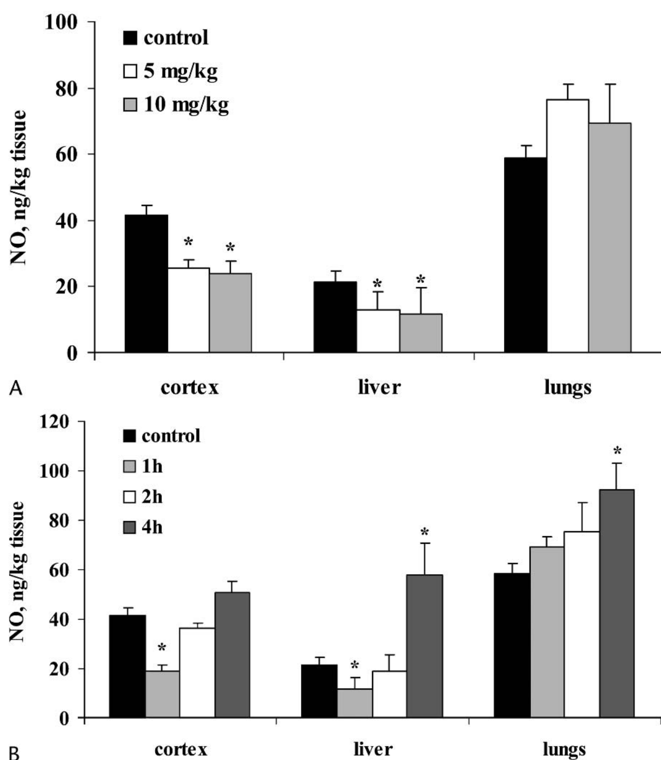

FIGURE 5. Dose-dependent (A), and time-dependent (B) influence of ME10092 on nitric oxide level in rat tissues. Values are expressed as ng NO/g tissue. Data of (A) are obtained 1 hour after ME10092 administration. Data of (B) are obtained for ME10092 10 mg/kg. All data points represent mean ± SEM of at least 6 independent experiments. *$P < 0.05$ compared with control.

the brain cortex ME10092 decreased the NO content to approximately half of the control level—that is from approximately 40 to approximately 20 ng/kg tissue, 1 hour after drug administration. In the liver the effect was even more pronounced, and the NO levels were decreased from 22 ng/kg in controls to 13.2 and 11.9 ng/kg for, respectively, 5 and 10 mg/kg of ME10092 administered. However, in lungs ME10092 did not statistically significantly decrease the NO content (Fig. 5A).

The effect of ME10092 on NO was time dependent. As seen from the series of experiments shown in Figure 5B, the NO content in brain cortex and liver was significantly inhibited 1 hour after administration of ME10092. However, after 2 hours the NO contents were only slightly decreased in all tissues, compared with the control levels. After 4 hours the NO contents in the brain cortex had reached control values, but in the liver it had become significantly elevated, from 20 to approximately 60 ng/kg tissue (Fig. 5B). In liver, the NO contents were slightly elevated 1 and 2 hours after administration of ME10092, but the effect was statistically significant only after 4 hours. In rat cerebellum, testis, and kidney ME10092 affected the NO contents in a similar manner as in brain cortex and liver (data not shown).

Inhibition of Nitric Oxide Synthase Activity In Vitro

The influence of ME10092 on NOS was determined by assessing the inhibition of NOS mediated ^{3}H-arginine conversion using in vitro assays with recombinant NOS isoforms. As can bee seen in Figure 6, ME10092 exerted isoform-specific inhibitory actions. ME10092 caused a dose-dependent inhibition of the activities of both nNOS and eNOS with IC_{50} values 230 μM and 30 μM, respectively. Thus, ME10092 appeared to possess an 8-fold higher inhibitory activity for eNOS than for nNOS. However, the iNOS activity was not influenced at all by ME10092, even at the highest concentration of ME10092 tested, 1 mM (Fig. 6). The pre-incubation of NOS enzymes in the presence of ME10092 before substrate addition did not significantly influence the inhibitory profile of the compound (data not shown).

DISCUSSION

We have shown previously that ME10092 affords a remarkable cardioprotective action in an in vivo rat heart infarction model.[5] In view of the powerful cardioprotective effect of ME10092, while lacking any clear understanding on how this effect is brought about, the present study was broadly undertaken to clarify the pharmacology of ME10092. In some preceding studies we had found that the parent compound PR5 functions as an electron acceptor for xanthine oxidase during anaerobic conditions, while during aerobic conditions it inhibits xanthine oxidase.[3] PR5 shows strong protective effect in heart infarction models.[4] However, in vivo PR5 is very rapidly metabolized to ME10092.[5] This observation, and the virtu-

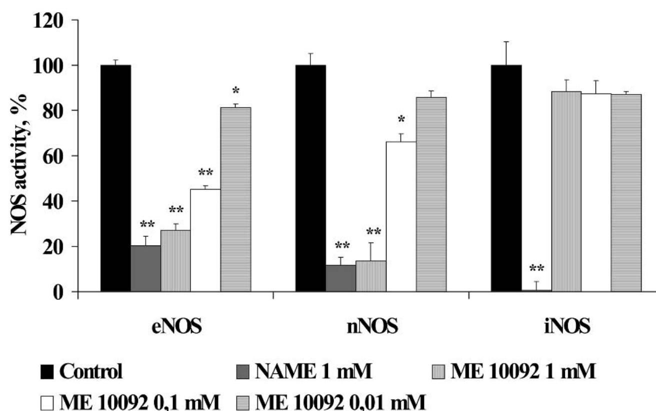

FIGURE 6. Influence of ME10092 on nitric oxide synthase isoform activity in vitro. Values are expressed as percents of amount of citrulline formed without ME10092. All data points represent mean ± standard deviation of at least 3 independent experiments performed in triplicates. *$P < 0.05$ compared with control, **$P < 0.01$ compared with control.

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ally indistinguishable in vivo pharmacology of PR5 and ME10092,[5] lend support to the idea that xanthine oxidase-mediated mechanisms might be responsible for the cardioprotective effects of ME10092.[5]

It is generally accepted that oxyradical formation contributes significantly to the size of the necrotic zone during ischemia-reperfusion.[15,16] If the formation of superoxide could be effectively prevented during reperfusion, the tissue damage would accordingly become reduced. Our previous studies supply some evidence that antioxidative mechanisms are significant for the pharmacological actions of ME10092. This is because we found PR5 treatment to lower the contents of the oxidative stress marker malondialdehyde in the ischemic heart tissue.[4] This finding thus links an antioxidant effect to ME10092, accepting the idea that the observed actions of PR5 are indeed mediated by its metabolite ME10092.

One major source for oxyradical formation may be xanthine oxidase.[16,17] During an ischemia and reperfusion period the xanthine dehydrogenase enzyme is converted to xanthine oxidase, and the latter uses molecular oxygen as electron acceptor, leading to formation of toxic superoxide.[15] Our present biochemical studies show that ME10092 is only a weak inhibitor on the xanthine oxidase enzyme, the K_i of ME10092 being 24 μM. This may be compared with the K_i of 0.7 μM reported for allopurinol.[18] An effect on xanthine oxidase of ME10092 seems therefore a less likely explanation for its cardioprotective properties. Moreover, even the role of xanthine oxidase inhibition as a mechanism for cardiac protection during ischemia reperfusion has been questioned, and quite contradictory data exist for a beneficial effect of allopurinol, and other effective xanthine oxidase inhibitors, in experimental heart infarction.[19,20] Real proof of pathophysiological role of xanthine oxidase generated superoxide during ischemia-reperfusion remains still elusive.

Another powerful enzymatic source of oxyradical formation is NAD(P)H oxidases.[21] Our results show that ME10092 inhibits free radical formation by this pathway to some extent. Although inhibition of the activity is seen in the high micromolar concentration range it matches the concentration of the cofactor NAD(P)H used in the assay. Still very high concentrations of ME10092 were needed to induce an effect, and the compound is considerably less potent than the flavoenzyme inhibitor DPI. It seems therefore quite unlikely that the cardioprotective effect of ME10092 can be explained solely due to its interaction with xanthine oxidase and NAD(P)H oxidase enzyme systems. However, the effect might still result in some antioxidant actions in vivo.

Besides inducing cardioprotective actions, ME10092 also caused lowering of blood pressure and reductions of heart rate in the normal artificial respirated anesthetized rat,[5] possibly indicating autonomic actions of the compound. ME10092 is a guanidine derivative, and guanidine derivatives, such as guanabenz, have been synthesized and applied clinically for use as centrally acting antihypertensive agents.[22] Guanabenz is known to stimulate α_2-adrenergic receptors in the brainstem and consequently to reduce the sympathetic outflow from the CNS, which results in a hypotensive effect. The effect is similar to that elicited by clonidine and guanfacine.[23–25] It is believed that α_2-adrenergic agonists, due to their autonomic effects, reduce the energy demand in the heart, and that they thereby could decrease the degenerative effects associated with cardiac ischemia. Guanabenz has been shown to restore the noradrenaline level and tyrosine hydroxylase activity in hypertrophic rat hearts.[7]

We studied the α-adrenoceptor activity of ME10092 using radioligand binding experiments on cells expressing recombinant α-adrenoceptors. Our results show that ME10092 binds to all the human α_1 and α_2-adrenoceptor subtypes with low micromolar affinity (Table 1). This may be compared with the activity of guanabenz, which shows nanomolar affinities for α_{2A}- and α_{2B}-adrenoceptors in rat kidney membranes.[26] A known selective α_1 and unselective β-adrenoceptor antagonist and potent antioxidant carvedilol shows high affinity (0,9–35 nM) for human recombinant adrenergic receptors and it also significantly reduces the necrotic area in rabbit heart ischemia and reperfusion model.[27] The affinity constants obtained in the binding experiments and functional studies of the present study show that ME10092 is a much weaker antagonist on α_1- and α_2-adrenoceptors than guanabenz and carvedilol. An α-agonistic mechanism of ME10092 can therefore be ruled out. Possibly an α_1-blocking activity could have a role for the blood pressure-lowering effect of the compound, but α-adrenoceptor blocking effects can not explain the effect on heart rate.

It has been reported that several bicyclic aroylguanidines act as very potent Na^+/H^+ exchange inhibitors and thereby possess antiarrhythmic activity.[28–30] The inhibition of the Na^+/H^+ exchanger is considered to be a promising approach for treating arrhythmia and cardiac dysfunction.[31] We have also tested the activity if ME10092 using a thrombocyte swelling assay, wherein swelling can be induced by blockade of the Na^+/H^+ exchanger. We found that ME10092 was a more than 10-fold weaker inhibitor, compared with the Na^+/H^+ exchange inhibitor DMA (Dambrova et al, unpublished observations).

We here also tested the hypothesis that ME10092 influences NO-related events. Several recent studies describe interactions of guanidine derivatives with subtypes of nitric oxide synthase (NOS). Thus, it is known that aminoguanidine itself is a selective inhibitor of inducible NOS.[32] Guanabenz was found to reduce the activity of rat penile NOS in vivo and in vitro.[33] It has also been shown that guanabenz may reduce neuronal NOS in cells in culture by enhancing the proteolytic degradation of NOS.[34] Our results show that administration of ME10092, in a dose-dependent manner, decreases the basal

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NO contents in the cerebral cortex and liver of the rat 1 to 2 hours after ME10092 administration. However, the reduction of NO in rat lungs was not observed. By contrast, 4 hours after the administration the NO levels had regained in all tissues evaluated, and it had even become significantly elevated above the control level in the liver and lungs (Fig. 5A). The later finding possibly could be explained by a cytochrome-dependent metabolism of guanidine derivatives with the oxidation of corresponding N-hydroxyguanidine compound in the liver microsomes and NO production.[35,36]

The role of NO in ischemic conditions remains controversial. However, there is some evidence that NOS subtype selective inhibition of NO synthesis may be useful for the treatment of clinical conditions, including neurodegeneration, ischemic tissue injury, and inflammatory diseases.[21,37,38] The cardioprotective effect of ME10092 in our rat myocardial infarction studies was observed after 3 mg/kg i.v. administration 5 minutes before 60 minutes occlusion and 1.5 mg/kg administration 5 minutes before 120 minutes reperfusion.[5] The inhibitory action on eNOS and nNOS 1 hour after ME10092 administration thus constitutes an interesting observation that has possible bearings on the observed in vivo pharmacological actions of ME10092. However, even though ME10092 possesses a similar inhibitory effect on NO content in rat tissues in comparison to aminoguanidine and guanabenz,[12] we show here that in vitro it inhibits eNOS activity but not that of iNOS. Thus, our data on NO inhibitory profile ME10092 does not allow us to draw a final conclusion about the degree of the involvement of NOS regulatory activity in the cardioprotective action of ME10092.

In conclusion, our data give evidence that the cardioprotective effect of ME10092 could be mediated through pharmacological mechanisms that include some modulation of NO production, as well as possible inhibition of radical formation during ischemia-reperfusion.

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