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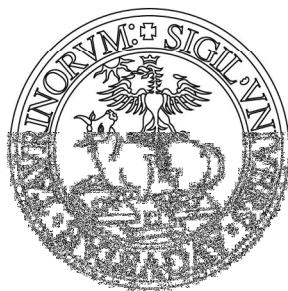
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Effects of NO-Donor Antioxidants Containing the Phenol Vitamine E Substructure and a Furoxan Moiety on Ischemia/Reperfusion Injury

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Abstract

Nitric oxide (NO)-donor antioxidants are a class of polyvalent drugs which is focus of great interest today; they are potentially useful for the treatment of many forms of cardiovascular diseases, including the myocardial ischemia/reperfusion (I/R) damage which seems to be due to both a burst of reactive oxygen species (ROS) and a reduced release of NO during reperfusion. In this paper the results of a study on the ability of new NO-donor antioxidants containing the phenol vitamin E substructure and furoxan moiety to attenuate I/R damage are reported. The compounds under study are obtained by combining the phenol moiety (6-hydroxy-2,2,5,7,8-pentamethylchroman) present in vitamin E with differently substituted furoxan substructures endowed with different capacity of NO-release. Their antioxidant and NO-dependent vasodilator activity are reported. The I/R experiments were performed on isolated rat heart preparations perfused at constant flow. After 20 min of stabilization, global ischemia was obtained by interrupting the perfusion for 30 min. After ischemia the hearts were reperfused for 2 hrs. The compounds were added to the perfusion buffer during the first 20 min of reperfusion. At the end of each experiment, infarct size was measured with nitro-blue tetrazolium. From the results it appears that the limitation of the infarct area is favoured by an appropriate balance between NO-donor and antioxidant properties and that these two actions are synergic.

1. Introduction

Some experimental evidence supports the notion that a burst of reactive oxygen species contributes significantly to myocardial ischemia/reperfusion (I/R) injury. This increase in ROS concentration occurs during the first few minutes after ischemic tissue is reoxygenated and it is principally due to the interaction of oxygen with the damaged mitochondrial respiratory chain, with the consequent overproduction of superoxide anion ($O_2^{\cdot-}$) [1-3]. The massive production of this radical can also be generated by other pathways involving xanthine oxidase, NADPH-oxidase and the arachidonic acid cascade [4]. Anion superoxide under the catalysis of superoxide dismutase (SOD) is transformed into hydrogen peroxide (H_2O_2), which, in the presence of metal ions, rapidly reacts with $O_2^{\cdot-}$ affording the very reactive and toxic hydroxyl radical (OH^{\cdot}) (Haber-Weiss reaction). The hydroxyl radical induces lipidic peroxidation, protein and DNA damage and mitochondrial alteration [5]. It has been shown that a number of antioxidants are able to display protection against cardiomyocyte disturbance during I/R, in cultured cell and animal models [6-8]. Endogenous nitric oxide (NO) produced by endothelial cells (EDRF) exerts a variety of physiological effects that control the vascular homeostasis, among these vasodilation, inhibition of platelet aggregation, attenuation of leukocyte adherence and aggregation, and inhibition of vascular smooth muscle cell proliferation. All of them can be dramatically affected by I/R [9]. It is known that I/R causes an impairment of endothelial function characterized by the reduced production of NO. Administration of NO or NO-donors immediately before ischemia or at the time of reperfusion can reduce the irreversible myocardial injury, as well as it is known that NO reduces lesion size in experimental cerebral infarct [9-11, 12]. The results of these studies are frequently in conflict, this depends on the species of animals and on the kind of NO-donor used [10]. Interesting attempts to limit myocardial reperfusion injury have been done using a combination of NCX-4016 (3-[nitrooxymethyl]phenyl 2-(acetyloxy)benzoate) and a number of antioxidants as well as resveratrol, a natural antioxidant polyphenol found in grapes and wine, which is able to inhibit low density lipoprotein (LDL) oxidation, block platelet aggregation and induce NO production [7, 13]. However, these compounds were infused before a prolonged ischemia. This pharmacological treatment in the clinical setting of acute myocardial infarction cannot be exploited because of the unpredictability of myocardial infarction.

Recently we described a new class of polyvalent drugs deriving from the combination of antioxidants, such as phenols [14], melatonin [15] and ascorbic acid [16] with appropriate NO-donor moieties. These products show a widely modulated balance between NO-donor and antioxidant properties and are potentially useful for the treatments of many forms of cardiovascular

diseases including myocardial I/R damage. In this paper we report the results of a study on the ability of compound **1** to attenuate the infarct size occurring in isolated rat hearts reperfused after induction of ischemia (Chart 1). We previously obtained this product [14] by combining the phenol moiety (6-hydroxy-2,2,5,7,8-pentamethylchroman (**4**) (Chart 1)) present in vitamin E, with the furoxancarboxamide substructure present in CAS 1609, an oral active NO-dependent vasodilator developed by Cassella Hoechst Company [17], and in its nitrogen containing analogue **1a** (Chart 1). CAS 1609 proved to be able of alleviating the irreversible myocardial I/R injury when tested in vivo on dogs [10]. Compounds **2**, **3** containing NO donor moieties related to the simple furoxans **2a**, **3a** (Chart 1), are also considered for a comparison.

2. Materials and methods

2.1 Chemicals

All the chemicals used for preparing the buffer solutions and compound **4** were purchased from Sigma (St. Louis- MO-USA). The reagents necessary to assess myocardial infarction were purchased from Merck (Darmstadt- Germany). Compounds **1**, **3**, **1a**, **2a**, **3a** were synthesised according to methods reported in literature [14, 15]. Compound **2** (2,5,7,8-tetramethyl-2-[(3-phenylfuroxan-4-yl-oxy)methyl]chroman-6-ol) was prepared according to the procedure reported below. Analysis (C, H, N) of the new compound was performed by REDOX (Monza, Italy) and the results are within $\pm 0.4\%$ of the theoretical.

To the solution of [6-(methoxyethoxymethoxy)-2,5,7,8-tetramethyl-3,4-dihydro-2*H*-chroman-2-yl]methanol [14] (0.49 g, 1.50 mmol) in THF dry (15 mL) placed under positive N₂ pressure NaH (70 mg, 1.75 mmol) was added at r.t. and the reaction was vigorously stirred for 30 min. Then 4-benzensulfonyl-3-phenylfuroxan (0.46 g, 1.50 mmol) was added and the reaction was stirred at r.t. for 3 h. After that time the reaction mixture was poured into H₂O (50 mL) and extracted with EtOAc (2×25 mL). The organic solvent was washed with brine, dried and evaporated. The resulting colourless oil was purified by flash chromatography (eluent 85/15 PE/EtOAc v/v) to give a protected compound as a colourless oil. The obtained oil was immediately dissolved in CH₂Cl₂ (20 mL) and CF₃COOH (2 mL) was added at r.t. The reaction mixture was stirred for 2 h, then the organic solvent was washed with H₂O (3×20 mL), brine, dried and evaporated. The obtained oil was purified by flash chromatography (eluent 9/1 PE/EtOAc v/v) to give a title compound as a white solid (yield: 60%). MS (EI): 396 (M)⁺; NMR ¹H (CDCl₃) δ : 1.45 (s, 3H, CH₃), 1.89 – 2.15 (m, 11H, 3ArCH₃ + 3-H₂), 2.69 – 2.73 (m, 2H, 4-H₂), 4.27 (s, 1H, OH), 4.46 – 4.54 (m, 2H, CH₂O), 7.47 – 7.51 (m, 3H), 8.10 – 8.18 (m, 3H) (C₆H₅); NMR ¹³C (CDCl₃) δ : 11.3, 11.8, 12.2, 20.2, 21.9, 28.9,

73.3, 74.8, 107.6, 116.9, 118.6, 121.4, 122.4, 122.8, 126.1, 128.9, 130.5, 144.6, 145.3, 162.4. Anal. (C,H,N).

All the reagents used in the *in vitro* experiments were of analytical grade and the purity of all the compounds tested was > 98% (HPLC detection).

2.2 Animals

All the animals used in this study (male Wistar rats, Harlan, S. Pietro al Natisone, Italy) were treated humanely in accordance with recognised guidelines on experimentation. They had ad libitum food and water until the start of the experimental procedure. As few rats as possible were used. The purposes and the protocols of our studies have been approved by the Ministero della Salute, Rome, Italy.

2.3 NO-release: vasodilator activity

Thoracic aortas were isolated from male Wistar rats weighing 200-250 g. The endothelium was removed and the vessels were helically cut: three strips were obtained from each aorta. The tissues were mounted under 1.0 g tension in organ baths containing 30 ml of Krebs-bicarbonate buffer with the following composition (mM): NaCl 111.2, KCl 5.0, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.0, NaHCO₃ 12.0, glucose 11.1, maintained at 37°C and gassed with 95% O₂ - 5% CO₂ (pH = 7.4). The aortic strips were allowed to equilibrate for 120 min and then contracted with 1 µM L-phenylephrine. When the response to the agonist reached a plateau, cumulative concentrations of the vasodilating agent were added. The effects of 1 µM ODQ (1*H*-[1,2,4]-oxadiazolo[4,3-*a*]quinoxalin-1-one) on relaxation were evaluated in separate series of experiments in which it was added to the organ bath 5 minutes before the contraction. Responses were recorded by an isometric transducer connected to the MacLab System PowerLab[®]. Addition of the drug vehicle (DMSO maximal amount 0.3%) had no appreciable effect on contraction level.

2.4 Antioxidant properties

Microsomal membranes from male Wistar rats (200-250 g) were prepared by differential centrifugation (8000g, 20 min; 120000g, 1 h) in a HEPES/Sucrose buffer (10 mM, 250 mM, pH = 7.4) and stored at -80°C. Incubation was performed at 37°C in a Tris-HCl/KCl buffer (100 mM, 150 mM, pH = 7.4) containing microsomal membranes (2 mg/mL), ascorbic acid (100 µM) and DMSO solutions of the tested compounds. An addition of DMSO alone (maximal amount 5%) did not significantly change the extent of peroxidation in the control experiments. Lipid peroxidation was initiated by adding FeSO₄ 2.5 µM. Aliquots were taken from the incubation mixture at 5, 15

and 30 min and treated with trichloroacetic acid (TCA) 10% p/v. Lipid peroxidation was assessed by spectrophotometric (543 nm) determination of the TBARS (2-thiobarbituric acid reactive substances) consisting mainly of malondialdehyde (MDA) and TBARS concentrations (expressed in nmol/mg protein) were obtained by interpolation with a MDA standard curve [18]. The antioxidant activity of tested compounds was evaluated as the % of inhibition of TBARS production with respect to control samples, using the plateau values obtained after 30 min of incubation.

2.5 Isolated heart preparation

Six month old Wistar rats were anaesthetised with urethane (1 g/kg intraperitoneally) ten minutes after heparin injection. The hearts were rapidly excised and placed in an ice-cold Krebs-Henseleit perfusion buffer. Then the aorta was attached to the cannula of the perfusion apparatus and the heart was perfused at constant flow with a Krebs solution at 37°C containing NaCl (127 mM), NaHCO₃ (17.7 mM), MgCl₂ (1.26 mM), KCl (5.1 mM), CaCl₂ (1.5 mM) and glucose (11 mM). Xylocaine (10 µg/ml) was added to Krebs solution to prevent the occurrence of arrhythmias (e.g. supraventricular tachycardias, ventricular flutter and fibrillation).

The perfusate buffer was saturated with a 95% O₂ and 5% CO₂ gas mixture as previously described [19, 20]. The constant flow was adjusted with a proper perfusion pump (Watson-Marlow 505DU, Falmouth, Cornwall, UK) to obtain a perfusion pressure of 80 mmHg during the stabilization. The mean flow to reach this value was (9 ± 1 mL/min/g). A small hole in the left ventricular wall allowed drainage of the thebesian flow.

After 20 minutes of stabilization, each heart underwent 30 minutes of global ischemia, obtained by arresting the perfusion pump. Ischemia was followed by 2 hours of reperfusion. At the moment of the reperfusion the hearts were infused for 20 min with vehicle (0.1% DMSO) or with studied compounds (fig. 1). The protocol was chosen since it has already been shown that a burst of ROS occurs during the early reperfusion even when a lack of NO is detected. The treatment started at the end of ischemia to mimic the ischemic postconditioning and lasted 20 minutes to include the period during which the most injuring effect takes place [18, 19].

2.6 Infarct size measurement

At the end of the experiments the hearts were rapidly removed from the perfusion apparatus. After isolation of the left ventricle, each heart was cut into 1 mm thick slices. After 20 minutes incubation in 0.1% solution of nitro-blue tetrazolium in phosphate buffer, stained viable tissue was separated from unstained necrotic tissue and then weighed. The necrotic tissue mass was expressed as a

percentage of the total left ventricle mass. The total left ventricle mass also corresponds to the risk area because a global ischemia was performed.

2.7 Statistical analysis

The values of vasodilator activity and infarct size are expressed as mean \pm standard error (SE). Antioxidant activities are expressed as mean \pm 95% CL. One-way analysis of variance and Student's "t" test for unpaired data were used to assess the statistical significance of the changes of the studied variables. No difference between the results of the two tests was found.

3. Results

3.1 Vasodilator activity

The products were able to relax the denuded rat aortic strips in a dose dependent manner. The behaviour of **2** is reported as an example (fig. 2a). Their vasodilator potencies, expressed as EC₅₀, namely the molar concentration able to induce 50% of relaxation of the precontracted tissue, are reported in Table 1. When the experiments were repeated in the presence of ODQ (1*H*-[1,2,4]-oxadiazolo[4,3-*a*]quinoxalin-1-one), a known inhibitor of the sGC, a strong decrease in the activity was observed.

3.2 Antioxidant properties

Products **1-3** were assessed as inhibitors of ferrous salt/ascorbate induced lipidic peroxidation of membrane lipids of rat hepatocytes using the TBA (2-thiobarbituric acid) assay. All the products were able to inhibit the generation of TBARS in a concentration dependent manner. The behaviour of **2** is reported as an example in fig. 2b. The antioxidant potencies expressed as IC₅₀, namely the minimal molar concentration able to inhibit 50% of the autoxidation, calculated by non linear regression analysis, are reported in Table 1 together with the corresponding values of the reference phenol **4** and of the reference NO-donor simple furoxans **1a-3a**.

3.3 Infarct size measurement

Infarct size is expressed as percentage of the left ventricular mass. The effects of compounds **1**, **1a** and **4** given at the concentration of 1 μ M and 10 μ M are reported in fig. 3. The concentrations were chosen inside the range in which the hybrid **1** showed both the NO-donating and antioxidant activity. In comparison with the control group (53 \pm 3%) at the concentration of 1 μ M infarct size was significantly reduced by **1** (22 \pm 5%, $p < 0.001$), but not by **1a** (37 \pm 7%) and **4** (48 \pm 8%). On

the other hand at 10 μM concentration infarct size was reduced by **4** ($24 \pm 3\%$) ($p < 0.001$), but not by **1** ($41 \pm 7\%$) and **1a** ($39 \pm 7\%$)

The activities of compounds **2**, **2a**, **3**, **3a** and **4** were evaluated at 1 μM concentration; in this group of compounds only **2** significantly reduced the extension of the infarcted myocardium ($33 \pm 5\%$, $0.01 < p < 0.05$) (Fig. 4).

4. Discussion

Analysis of the Table 1 shows that the products **1-3** behave as potent antioxidants. Their IC_{50} values are similar and close to those of the reference phenol **4** and occur at sub μM concentrations. By contrast the simple reference furoxans **1a-3a** are completely inactive in the concentration range in which the aforementioned compounds display radical scavenger actions. Therefore we can conclude that the antioxidant activity of the hybrid products are tightly related to the presence into their structures of the common phenol moiety.

In addition, analysis of Table 1 shows that compounds **1** and **2** are potent vasodilators, showing EC_{50} values in the μM concentration range, while **3** is a very potent vasodilator with an EC_{50} value = $4.4 \times 10^{-8}\text{M}$. It is generally accepted that the probable vasodilator mechanism of furoxan derivatives involves their interaction with endocellular thiols with consequent production of NO, which in turns activates soluble guanylate cyclase (sGC) of the vascular smooth muscle cells. This activation induces an increase of cyclic guanosine-3,5-monophosphate (cGMP), activation of cGMP dependent protein kinase (cGK-1) and finally vasorelaxation [21]. The decrease of the activity observed in the presence of ODQ is in keeping with the involvement of NO/cGMP mechanism in relaxation. Therefore, the vasodilator potencies of the three hybrids can be reasonably considered a measure of their capacity to release NO in a vessel.

Analysis of Figure 3 evidences that hybrid **1** displays a marked reduction of the infarcted area with respect to the control, while the simple furoxan **1a** and phenol **4** do not induce any protection when tested at 1 μM concentration. A different situation occurred when the experiments were carried out at 10 μM concentration. In this case neither **1** nor **1a** modified the infarcted size of the control, while the simple antioxidant **4** was protective. Since the ability of **1** and **1a** to release NO, evaluated on the basis of their vasodilator potencies, is similar, this picture suggests that the limitation of the infarct area is favoured by an appropriate concentration of NO, and in addition, by the simultaneous presence of an antioxidant effect and that these two actions are synergic. This hypothesis seems to be reinforced by the similar results we obtained carrying out the experiments at 1 μM concentration with products **2** and **2a**, which are endowed with NO-mediated vasodilator and antioxidant profiles close to those of **1** and **1a**. Moreover, working in the same conditions with the most potent NO-

donors **3** and **3a** any protective effects disappear. This overall picture is in keeping with the statement that high levels of NO promote apoptosis while basal levels of NO protect cardiomyocytes from apoptosis [22].

In summary, **1** appears to be a hybrid able to behave as an efficient pharmacological postconditioning agent as a consequence of appropriate balance between its NO-donor and antioxidant properties. *In vivo* studies should be performed to validate these results in a more complex model.

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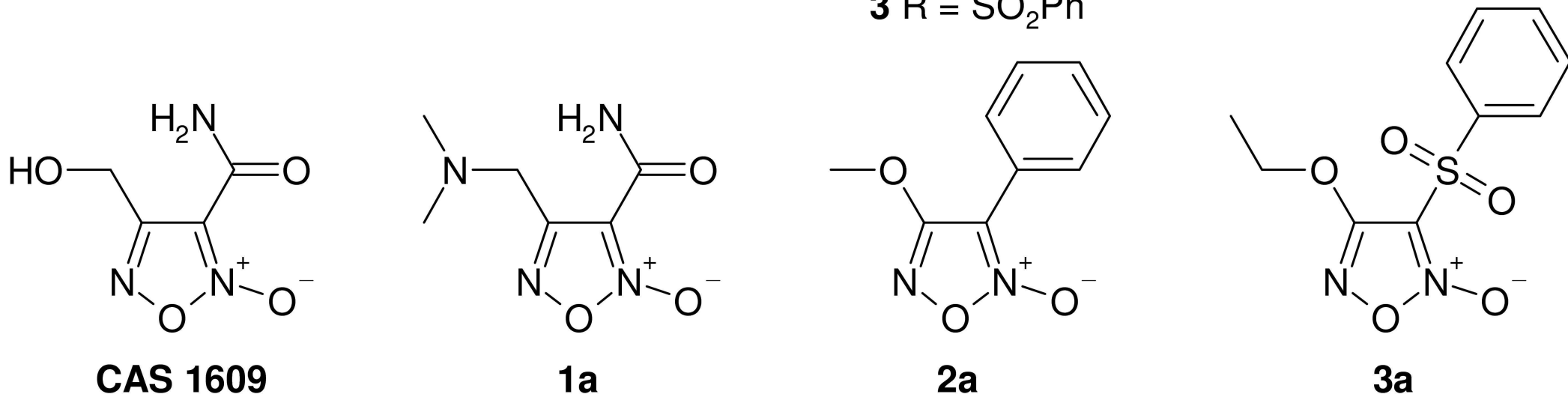
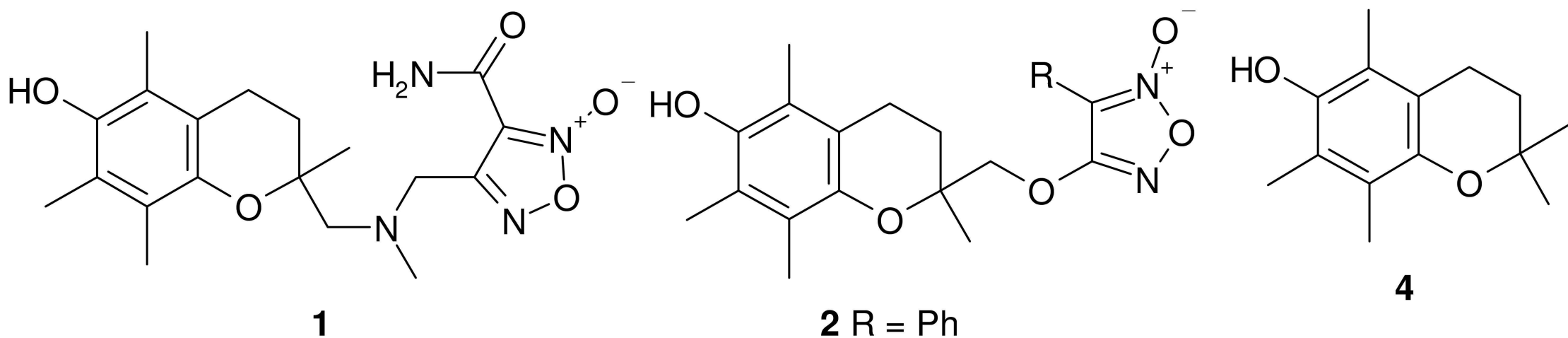


Chart 1.

Stabilization

20 min

Ischemia

30 min

Reperfusion

120 min



20 min

compound infusion

Hearts underwent infusion of vehicle (control) or the appropriate concentration of **1**, **1a**, **2**, **2a**, **3**, **3a** and **4** during the first twenty minutes of reperfusion

Fig.1: Time-line and protocol for ischemia/reperfusion.

% relaxation

100

50

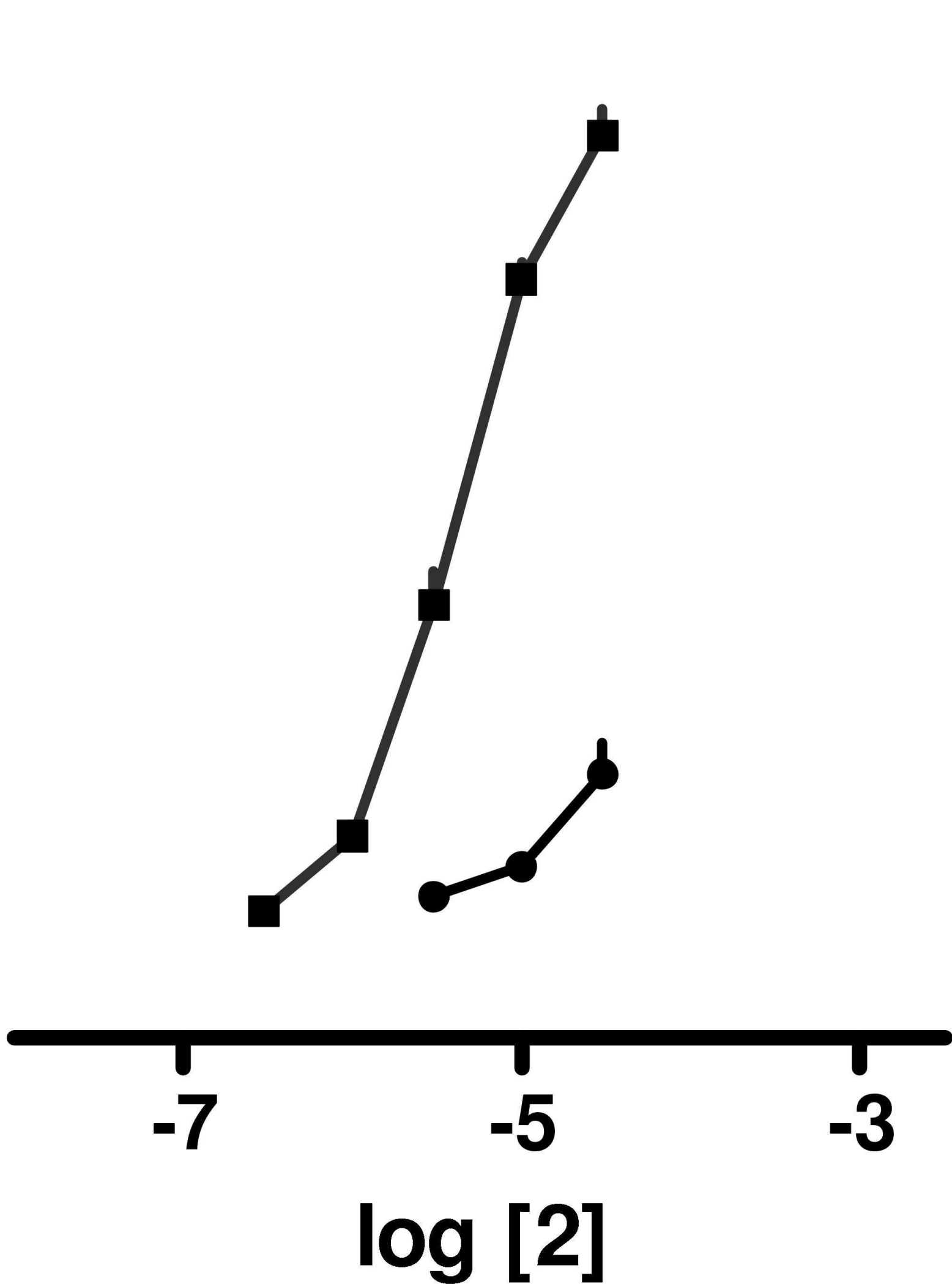
0

-7

-5

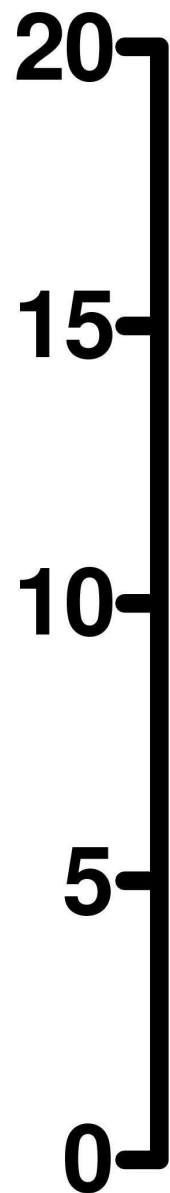
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log [2]



TBARS

(nmol/mg protein)



0

10

20

30

time (min)

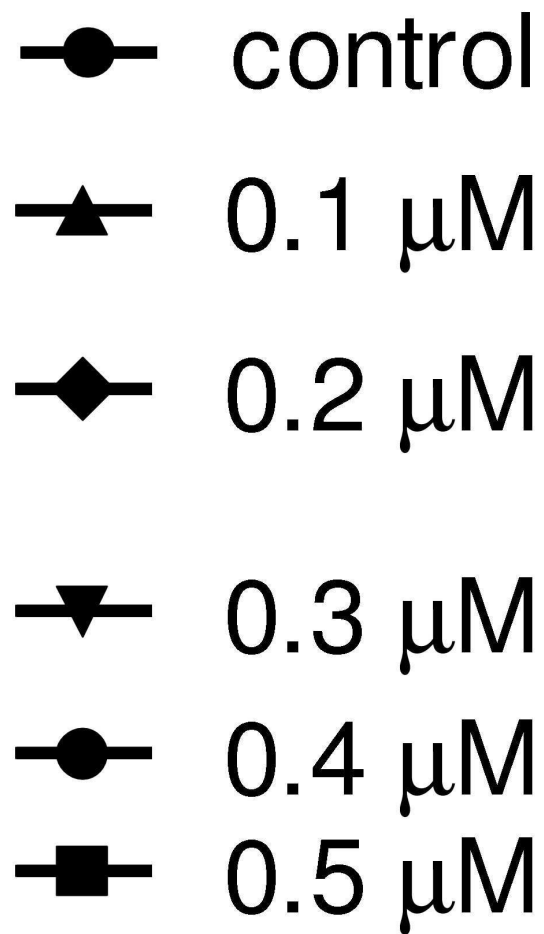


Fig. 2: a Concentration-response curves for vasodilator activity of compound **2** in the absence (square) and in the presence (circle) of ODQ. **b** Effect of compound **2** on time course of lipid peroxidation.

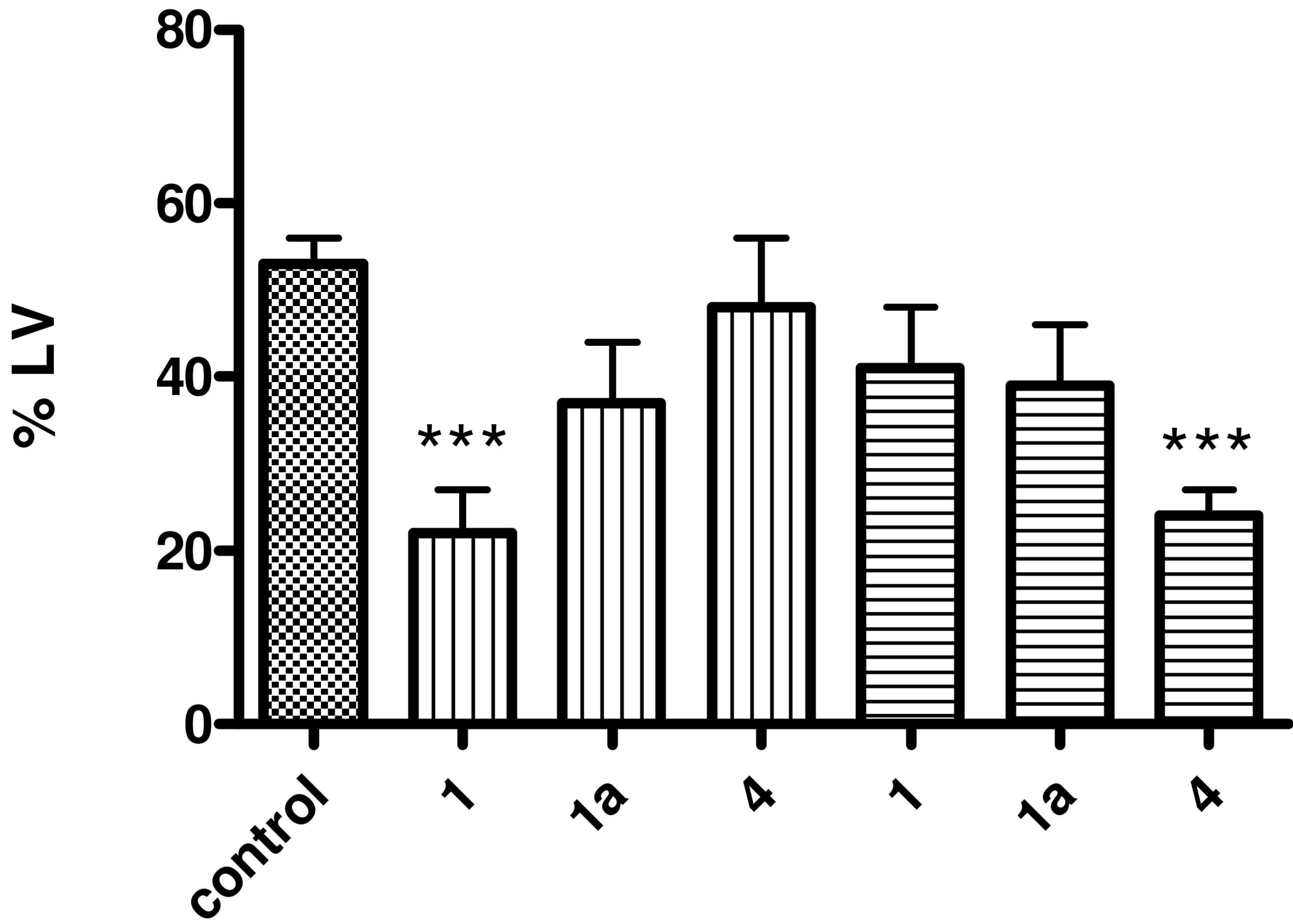


Fig. 3: Extension of the infarct size as a percent of the weight of the left ventricle (% of LV) when the compounds **1**, **1a** and **4** were administered at 1 μ M concentration (vertical line filling) and 10 μ M concentration (horizontal line filling). Results are expressed as mean \pm SE of 6-9 experiments.

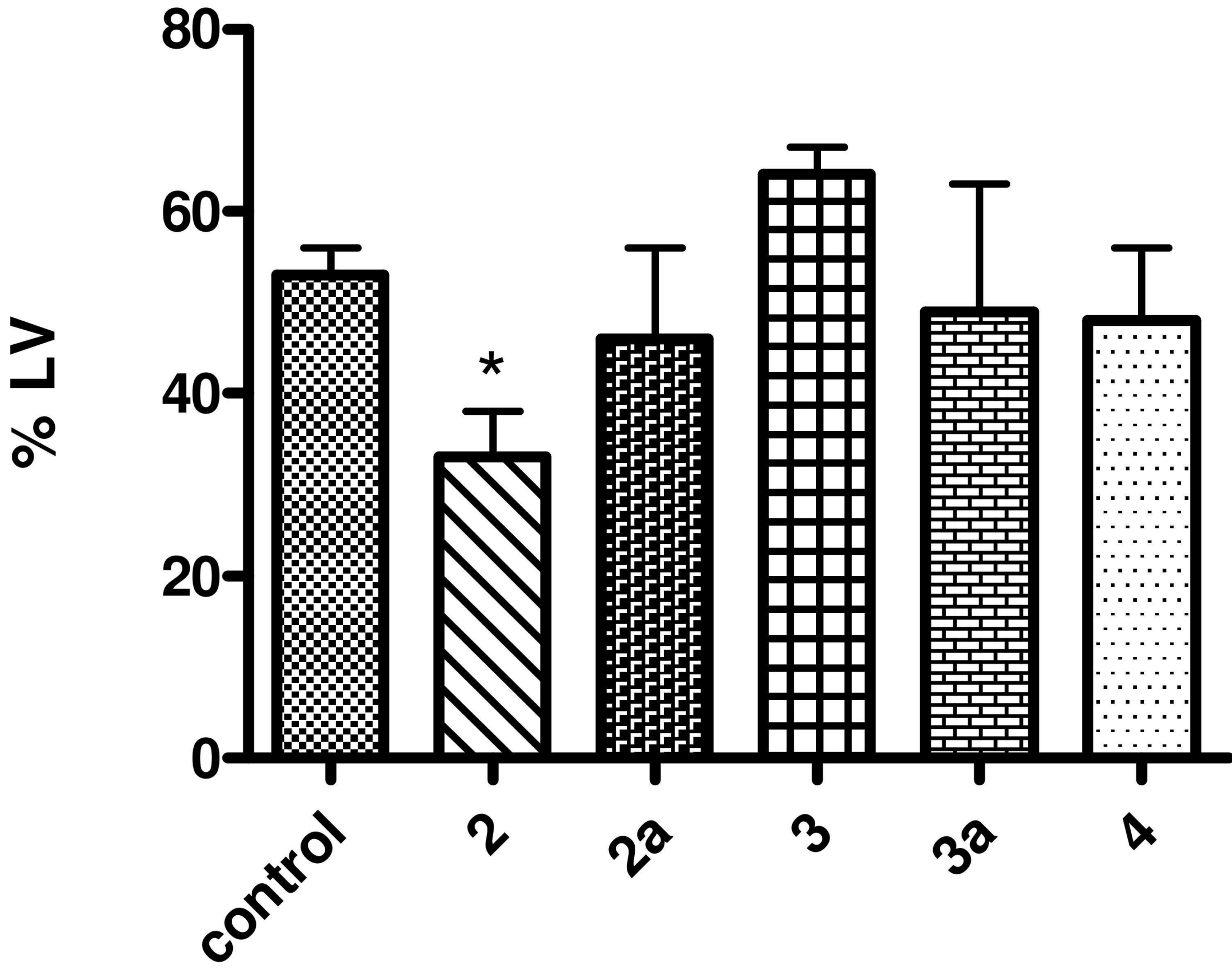


Fig. 4: Extension of the infarct size as a percent of the weight of the left ventricle (% of LV) when the compounds **2**, **2a**, **3**, **3a** and **4** were administered at 1 μ M concentration. Results are expressed as mean \pm SE of 6-10 experiments.

Table I. Antioxidant activity and vasodilator activity of the NO-donor Phenols **1-3**, the NO-donor parents **1a-3a** and the parent phenol **4**. The values are the means of at least 4 experiments.

Compound	TBARS-IC₅₀ (95% CL) (μM)	EC₅₀ ± SE (μM)	+ ODQ 1 μM EC₅₀ ± SE μM
1	0.14 (0.14-0.14) [14]	1.5 ± 0.1 [14]	19 ± 1 [14]
2	0.26 (0.23-0.28)	4.5 ± 0.4	> 30 ^a
3	0.49 (0.48-0.50) [14]	0.044 ± 0.004 [14]	0.67 ± 0.09 [14]
1a	- ^b [14]	3.1 ± 0.3 [14]	17 ± 3 [14]
2a	- ^b [15]	5.6 ± 0.5 [15]	90 ± 11 [15]
3a	- ^b [14]	0.012 ± 0.002 [14]	1.2 ± 0.2 [14]
4	0.17 (0.16-0.17) [15]	-	-

^a The maximal concentration tested (30 μM due to insolubility limits) cannot reach the 50% of the effect.

^b The product was inactive in the 2.5 μM – 0.05 μM concentration range.