学位論文

「Neuroprotective effect of nitrite-derived NO in brain injury mediated through the NOS-independent but the GC/COX/xanthine oxidase/PGIS-dependent pathway」

（亜硝酸由来の一酸化窒素の脳保護効果は NOS 非依存性で GC/COX/xanthine oxidase/PGIS 依存性である）

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著者の宣言

本学位論文は、著者の責任において実験を遂行し、得られた真実の結果に基づいて正確に作成したものに相違ないことをここに宣言する。

要旨

Purpose: Nitrite-derived NO has been shown to provide neuroprotection against brain ischemia-reperfusion injury. The present study was designed to examine the effect and mechanism of nitrite-derived NO on cerebral infarct volume in a chronic rat model.

Methods: Male Sprague-Dawley rats were divided into eight treatment groups：saline only, three groups with different doses of sodium nitrite ($NaNO_2$), L-NNA (NOS inhibitor) with saline and with $NaNO_2$, ODQ (soluble guanylate cyclase inhibitor) with saline and with $NaNO_2$, C-PTIO (NO scavenger) with saline and with $NaNO_2$, allopurinol (xanthine oxidase inhibitor) with saline and with $NaNO_2$, indomethacin (COX inhibitor) with saline and with $NaNO_2$, and U-51605 (prostacyclin synthase inhibitor) with saline and with $NaNO_2$. The rat was injected intraperitoneally with one of the above combinations, followed by one-hour occlusion of the middle cerebral artery and then by reperfusion. Five days later, the brain was stained for quantification of cerebral infarction area as percentage of the whole brain area.

Results: Nitrite significantly reduced the cerebral infarct area in a dose-dependent manner. The nitrite-induced reduction in cerebral infarct area was unaffected in rats injected with C-PTIO, ODQ, allopurinol, indomethacin and U-51605. However, injection of L-NNA augmented the reduction in nitrite-induced cerebral infarct area.

Conclusion: Nitrite-derived NO protects the brain against ischemia-reperfusion injury through NOS-independent but GC/COX/xanthine oxidase/PGIS-dependent pathways.

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本文

1. Introduction

Nitrite-derived nitric oxide (NO) has several physiological effects. Nitrite generates NO by the oxidation-reduction reaction as follows.

NO2-+H+⇆HNO2

3HNO2⇆HNO3+H2O+2NO

One peculiar property of nitrite-derived NO is its concentration-dependent multimodal effects, acting as a neuroprotective agent, e.g., in ischemia-reperfusion injury, or as a neurotoxic compound. [1, 2] However, the mechanisms and/or the pathways of these effects remain elusive. The present study was designed to examine the mechanisms and the optimum dose of nitrite-derived NO in reducing cerebral infarct volume following focal cerebral ischemia-reperfusion in rats.

2. Materials and Methods

2-1. Surgical and experimental setup

The experiments described in this study were conducted in 132 male Sprague-Dawley rats (weight, 300-400 g) after approval of the institutional animal care ethics review committee. To induce focal cerebral ischemia-reperfusion injury, the rat was anesthetized with pentobarbital sodium (50 mg/kg) intraperitoneally (i.p.). [3] An endotracheal tube was inserted for mechanical ventilation and end-tidal CO_2 was monitored continuously and maintained within the normal range (35-40 mmHg) throughout the experiment. A catheter was inserted into the femoral artery for monitoring blood pressure and heart rate, and another one into the femoral vein for administration of the muscle relaxant. Vecuronium bromide was infused continuously at a rate of 0.015-0.02 mg/kg/min for muscle paralysis. Pentobarbital sodium was administered as needed to maintain adequate level of anesthesia. Brain temperature was also measured with a 22-gauge stainless steel needle thermometer placed in the left temporal muscle, and normothermia was maintained by using an overhead heating lamp and a heating pad throughout the experiment. The middle cerebral artery was completely occluded using a nylon suture then released after one hour. Regional cerebral blood flow (rCBF) on the side ipsilateral to the occlusion was monitored using a laser-Doppler probe (Omegawave; Tokyo, Japan) placed 6 mm lateral and 2 mm posterior to the bregma, and during Middle Cerebral artery Occlusion (MCAO) the regional cerebral blood flow was maintained to be less than 50% of the baseline.

2-2. Experimental protocol

Rats were divided into eight treatment groups: *1)* The saline control group (n=8), *2)* various doses of sodium nitrite [0.1 (n=8), 1.0 (n=7), and 10 mg/head (n=10)], *3)* 10 mg/kg of nitro-L-arginine (L-NNA) [a nitric oxide synthase (NOS) inhibitor] with saline (n=9) and with 1.0 mg/head sodium nitrite (n=8), *4)* 20 mg/kg of 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) [a soluble guanylate cyclase (GC) inhibitor] with saline (n=9) and with 1.0 mg/head sodium nitrite (n=9), *5)* 10 mg/head of 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO) [a NO scavenger] with saline (n=9) and with 1.0 mg/head sodium nitrite (n=7), *6)* 10 mg/head allopurinol [a xanthine oxidase (XO) inhibitor] with saline (n=8) and with 1.0 mg/head sodium nitrite (n=7), *7)* 5 mg/head indomethacin [a cyclooxygenase (COX) inhibitor] with saline (n=12) and with 1.0 mg/head sodium nitrite (n=5), and *8)* 400 μg/head U-51605 [a prostacyclin synthase (PGIS) inhibitor] with saline (n=11) and with 1.0 mg/head sodium nitrite (n=5). After surgical preparation, the above compounds were injected i.p. one hour before, and each sodium nitrite was injected i.p. 30 min before ischemia. The dose used in the present study for each drug was selected based on previous studies [1-3] and our preliminary observations (data not shown).

Five days after ischemia-reperfusion injury, the brain was dissected out carefully, sectioned immediately into seven 2-mm-thick coronal sections, and stained with 2, 3, 5-triphenyltetrazolium chloride (TTC). This method is commonly used for determining brain infarct volume and is highly reproducible. [4-7] Hematoxylin-and-eosin (H&E) staining was also performed to confirm the site of infarction (Fig. 1). The infarct area was measured in all brain slices and the infarct volume was expressed as a percentage of the total brain area (NIH ImageJ; Wayne Rasband, Bethesda, MD).

2-3. Statistical analysis

All data are expressed as mean±SEM. Differences in the infarct area between the control group and $NaNO_2$-treated group, and Baseline rCBF and mean arterial blood pressure (MABP) were compared by non-repeated analysis of variance (ANOVA), post-hoc with the Student-Newman-Keuls test for multiple comparisons while the effects of L-NNA, ODQ, C-PTIO, allopurinol, indomethacin and U-51605 on the $NaNO_2$ induced reduction of infarct area were conducted by unpaired t-test. Differences were considered statistically significant when $P<0.05$.

3. Results

Sodium nitrite significantly reduced cerebral infarct area compared with the saline group, and a weak dose-response effect was evident (Fig. 2). Further experiments designed to determine the mechanism of the neuroprotective effect of sodium nitrite showed that only L-NNA (a NOS inhibitor) significantly augmented the reduction in cerebral infarct area when injected before sodium nitrite, relative to the saline control. On the other hand, ODQ (a guanylate cyclase inhibitor), C-PTIO (a NO scavenger), allopurinol (a xanthine oxidase inhibitor), indomethacin (a nonselective inhibitor of COX), and U-51605 (a prostacyclin synthase inhibitor) had no effect on sodium nitrite-induced reduction of infarct area (Fig. 3). There was no significant difference in the changes of HR among the all experimental groups. Base line rCBF and MABP are shown in Table (Tables 1, and2).

4. Discussion

First, the results of the present study confirmed those of previous reports that NO derived from sodium nitrite exerts neuroprotective effects against cerebral ischemia-reperfusion injury, and this effect tended to be dose-dependent. We used the second highest dose of nitrite, because the highest dose lowered blood pressure, which could worsen the cerebral ischemia. Second, we examined the further mechanisms of this neuroprotective effect of nitrite-derived NO. Nitrite-derived NO-induced effect was observed in the presence of NOS-inhibitor, L-NNA; however, the effects disappeared in the presence of other inhibitor compounds, including ODQ (soluble guanylate cyclase inhibitor), C-PTIO (NO scavenger), allopurinol (xanthine oxidase inhibitor), indomethacin (nonselective inhibitor of COX) and U-51065 (prostacyclin synthase inhibitor). The neuroprotective effect of nitrite was inhibited in the presence C-PTIO, suggesting the NO-dependent action of nitrite. This was the same result as the previous study of Jung et al. [1] These findings suggest that the neuroprotective effects against brain ischemic-reperfusion injury is NOS-independent, and rather GC-, COX-, xanthine oxidase- and PGIS-dependent. L-NNA, ODQ, C-PTIO, allopurinol, indomethacin, and U-51605, all seem to have infarct-reducing effect themselves, like NaNO2 alone, compared with the saline group. However, we still found significant difference in experiments using L-NNA. Therefore, infarct-reducing effects of these inhibitors may not have critical impact on the present report, while having some confounding effects. The reduction of MABP was seen by the vasodilator effect of sodium nitrite; however this reduction did not exacerbate the cerebral infarction. Furthermore, although MABP was increased along with L-NNA administration, cerebral infarction area was not reduced. Therefore, it is suggested blood pressure fluctuation does not affect our results. This

conclusion is, in part, in agreement with the results of a recent study that demonstrated the generation of NO in a manner dependent on nitrite but independent on NOS inhibitors in the re-perfused myocardium. [8] Brain hypoxemia during occlusion-reperfusion injury could result in impairment of brain NOS since oxygen is essential for the reaction involved in the production of NO from L-arginine and catalyzed by NOS. Similar to myocardial ischemia, nitrite produced NO in our study, which could potentially serve to alleviate ischemic brain injury.

Our results showed that ODQ inhibit the effect of NO. This finding suggests that the neuroprotective effect of nitrite-derived NO in brain injury is mediated via GC, in agreement with the results of two previous reports. [2, 9] Another recent study showed that xanthine oxidase can generate NO by reducing nitrite in the presence of NADH. [10] The reaction occurred even under low tissue oxygen levels. Our results also showed that xanthine oxidase inhibitor suppressed the neuroprotective effects of nitrite-derived NO, in agreement with the results of previous studies. [10-12]

Both indomethacin and U-51605 inhibited the neuroprotective effects of nitrite-derived NO. Considered together with other findings of the present study, the results suggest that the neuroprotective effects of NO are mediated through COX and prostacyclin. In this regard, previous studies reported that the NO/COX/cAMP pathway is independent of GC and cGMP, and is involved in the opening of potassium channels and various physiological processes. [2, 13, 14] Our results also demonstrated the involvement of this pathway in the brain vasculature, consistent with other studies that showed the involvement of the same pathway in ocular vascular beds. [13-15] Our study may provide the therapeutic insight of nitrite for patient suffering from stroke and/or brain injury.

5. Conclusion

We have demonstrated in the present study that nitrite-derived NO protects the brain against ischemia-reperfusion injury probably through NOS-independent but GC-, COX-, xanthine oxidase-, PGIS-dependent pathways.

6. Competing interests

The authors declare that they have no competing interests.

7. Authors' Contributions

H.A. and H.O. conceived and design of the experiments. H.A. performed the experiments,

and collection and assembly of data together with Y.N. and Y.K, and M.A. performed the statistical analysis. H.A. and H.O. co-wrote the paper. All authors read and approved the final manuscript.

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9. Tables

Table 1. Baseline rCBF (perfusion unit).

(a)	Saline	L-NNA+Saline	ODQ+Saline	C-PTIO+Saline	Allopurinol+Saline	Indomethacin+Saline	PGISi+Saline
	21.39±7.56	26.58±8.86	26.02±8.67	24.31±8.10	21.51±7.60	23.02±7.67	20.99±6.32
(b)	$NaNO_2$ 1.0mg	L-NNA+$NaNO_2$	ODQ +$NaNO_2$	C-PTIO+$NaNO_2$	Allopurinol+$NaNO_2$	Indomethacin+$NaNO_2$	PGISi+$NaNO_2$
	23.02±8.70	21.27±7.52	22.73±8.59	20.81±7.86	19.44±7.35	19.78±7.47	22.98±10.27

We compared rCBF of the saline- and saline+inhibitor-treated groups (a), and the nitrite- and nitrite+inhibitor-treated groups (b). Baseline rCBF was not significantly different among the groups.

Data are mean±SEM of the indicated number of animals. *p<0.05.

Table 2. Baseline MABP (mmHg).

(a)	Saline	L-NNA+Saline	ODQ+Saline	C-PTIO+Saline	Allopurinol+Saline	Indomethacin+Saline	PGISi+Saline
	135.20±47.80	118.92±39.64	110.66±36.88*	124.45±41.48	116.78±41.29	120.59±40.19	122.74±37.00
(b)	NaNO2 1.0mg	L-NNA+NaNO2	ODQ +NaNO2	C-PTIO+NaNO2	Allopurinol+NaNO2	Indomethacin+NaNO2	PGISi+NaNO2
	113.38±42.85	126.21±44.62	124.54±47.07	126.44±47.79	118.16±44.66	114.47±43.26	114.45±51.18

We compared MABP of the saline- and saline+inhibitor-treated groups (a), and the nitrite- and nitrite+inhibitor-treated groups (b). The MABP of rats of the saline group treated also with ODQ was significantly lower than the saline alone group.

Data are mean±SEM of the indicated number of animals. *p<0.05.

10. Figure legends

Figure 1. Images of 2, 3, 5-triphenyltrazolium chloride-stained sections of a representative rat (a) of the control group and (b) 10 mg NaNO2-treated group. Note the lack of staining of the infarct area (white arrows). The infarct area is 15.5% in the control and 2.8% in the NaNO2 rat.

Figure 2. Comparison of the infarct area in the control group and NaNO2-treated groups. The infarct volume in the control group was 10.9% of the total brain volume. Intraperitoneal injection of NaNO2 significantly decreased the infarct area to 4.1% in the 0.1 mg group, 3.0% in the 1.0 mg group and 2.5% in the 10 mg group. Data are mean±SEM. *$p<0.05$ vs. the control group.

Figure 3. Effects of L-NNA, ODQ, C-PTIO, allopurinol, indomethacin and U-51605 on NaNO2-induced reduction of infarct volume. L-NNA＋NaNO2 group decreased cerebral infarction area significantly as compared with the L-NNA＋Saline group. In other groups, there were no significant difference in infarct volume after the injection of each compound followed by injection of NaNO2, compared with the compound alone. Data are mean±SEM. *$p<0.05$.

(11. Figures)

Figure 1. Images of 2, 3, 5-triphenyltrazolium chloride-stained sections of a representative rat (a) of the control group and (b) 10 mg $NaNO_2$-treated group.

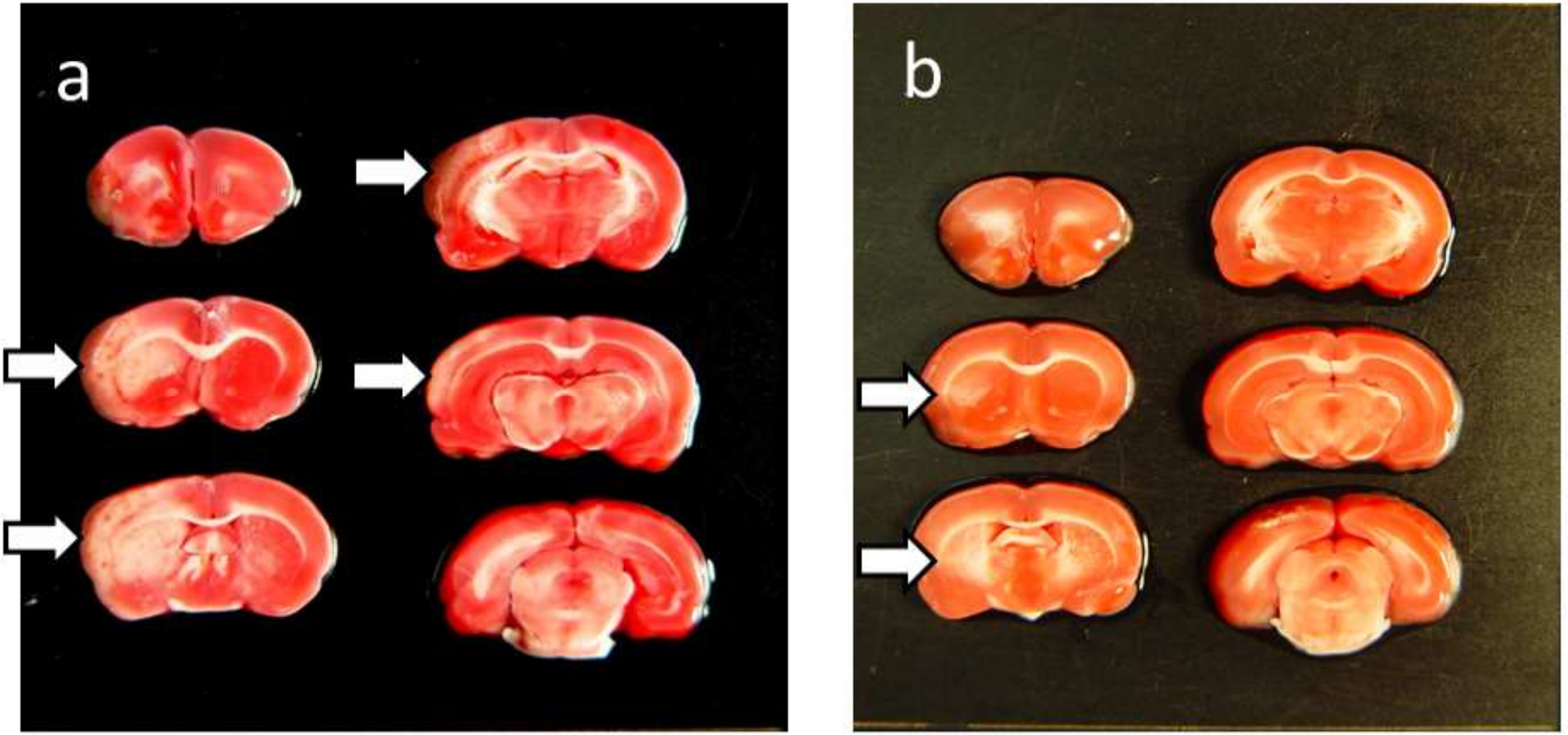

Figure 2. Comparison of the infarct area in the control group and $NaNO_2$-treated groups.

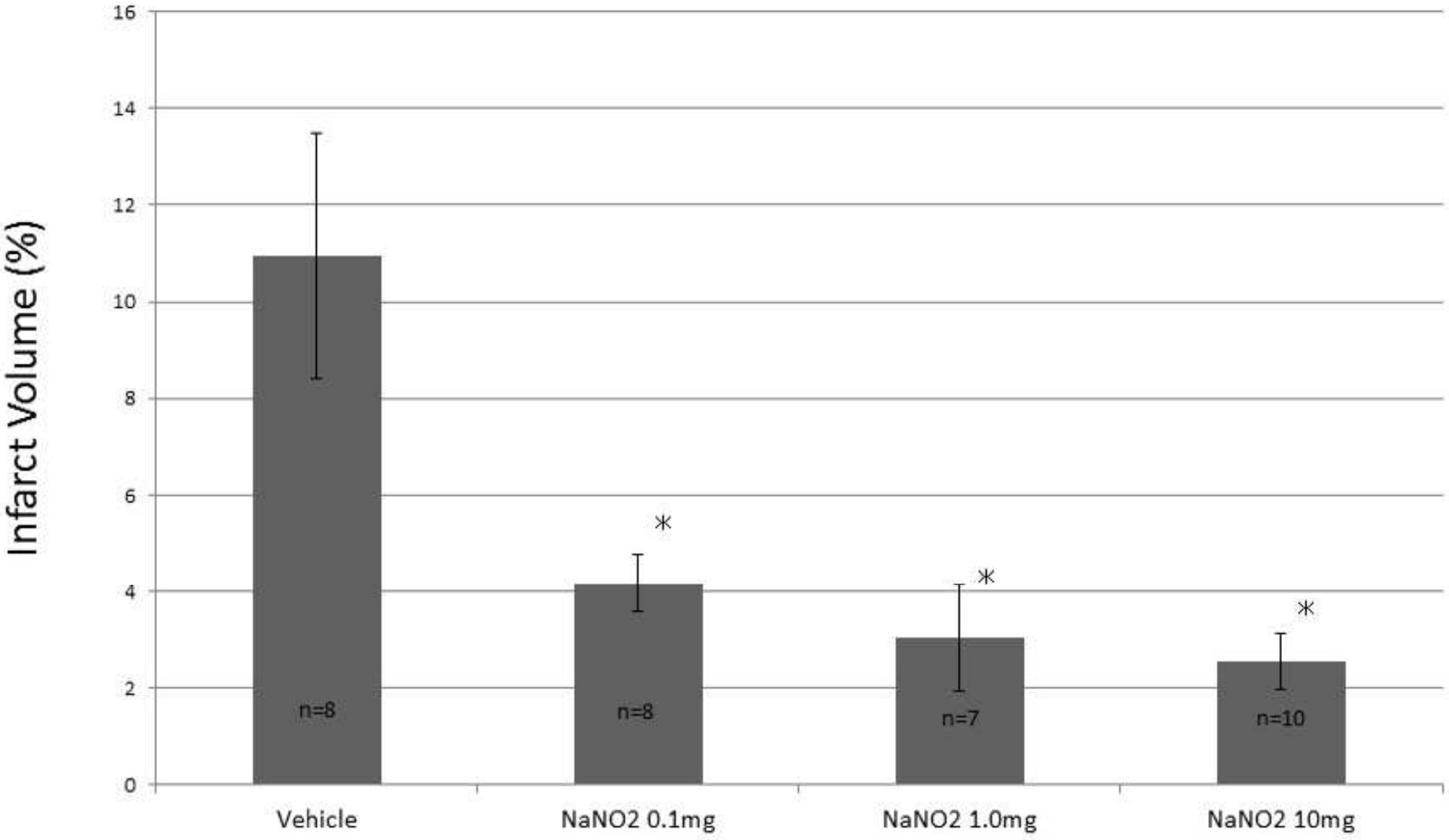

Figure 3. Effects of L-NNA, ODQ, C-PTIO, allopurinol, indomethacin and U-51605 on $NaNO_2$-induced reduction of infarct volume.