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Effects of naloxone on lactate, pyruvate metabolism and antioxidant enzyme activity in rat cerebral ischemia/reperfusion

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Abstract

Whether naloxone may modulate energy metabolism and endogenous antioxidant enzyme activities in ischemic cortex was studied. Cerebral ischemia/reperfusion (I/R) was produced by occluding two common carotid arteries and the right middle cerebral artery for 90 min followed by reperfusion in anesthetized Sprague–Dawley rats. Both pre-treatment (0.03 or 0.3 mg) and post-treatment (0.3 mg) of naloxone by intracerebroventricular infusion significantly reduced cortical infarct volumes. Pre-treatment with 0.03 mg reduced ischemia-induced suppression of extracellular pyruvate level and enhancement of lactate/pyruvate ratio as well as cerebral I/R-induced increases of endogenous catalase, glutathione peroxidase, and manganese superoxide dismutase activities. In conclusion, neuroprotective effects of naloxone in terms of reducing brain infarction involve attenuation of the disturbance of cellular functions following cerebral I/R via restoration of mitochondrial activities or energy metabolism.

Keywords: Energy metabolism; Free radical; Microdialysis; Opioid antagonist; Opioid peptide; Stroke

Cerebral ischemia results in a low oxygen and glucose supply, and a decrease in adenosine triphosphate (ATP) formation. Various ATP-dependent processes, such as the membrane-bound pumps which are important for maintaining the homeostasis of metabolites or ions, become impaired. The levels of energy metabolites, such as pyruvate and lactate are also severely influenced. Thus, changes in lactic acid, pyruvate, and their ratios have been used as important biochemical markers of cerebral ischemia in experimental animals and clinical studies [13,17].

Oxygen free radicals are likely participants in the pathogenesis of cerebral ischemia/reperfusion (I/R) injury. Recent studies provide direct and indirect experimental evidences that oxygen free radicals are elevated during I/R because of the failure of metabolic reactions [14,15]. Moreover, free radical scavengers, antioxidant enzymes, and spin trap agents attenuate I/R brain injury [6,16,18]. Because of the potential participation of oxygen free radicals in transient ischemic injury, it will be important to characterize antioxidant enzyme activities following cerebral I/R. The cellular endogenous antioxidant defense system against the excessive production of free radicals includes three concerted action enzymes, superoxide dismutase (SOD), glutathione peroxidase, and catalase [7,8,11].

Naloxone, an opiate antagonist, has neuroprotective effects in animal models of focal brain ischemia [4,5,9,10,12,20]. Naloxone also improves cerebral blood flow, reduces seizure activity, and enhances survival rate in gerbils following temporary occlusion of bilateral common carotid artery [2,4,10,20]. It, thus, interesting to know whether naloxone would improve or preserve energy metabolism as indicated by the changes in pyruvate and lactate. These metabolic improvement could be expected to result in reducing the free radical leakage from mitochondrial electron transport chain. Therefore, it is also important to examine the effects of naloxone on the I/R-induced changes in the endogenous antioxidant enzyme activities, a defence or compensatory response against overproduction of free radicals.

Transient focal cerebral ischemia followed by reperfusion was carried out on adult male Sprague–Dawley rats (300–350 g) as described previously [19]. Briefly, the rat's head was mounted in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) with the nose bar positioned 3.3 mm

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below the horizontal line. Following a midline incision, the skull was exposed and one burr hole was made on the skull for the placement of a microdialysis probe (4 mm in length, CMA/12, Carnegie Medicin, Stockholm, Sweden). The microdialysis probe was stereotaxically implanted into the cortex (AP 0 mm, ML 5.5 mm, DV −4.0 mm from Bregma). Dialysis probes were perfused with Ringer's solution (147 mM Na^+, 2.2 mM Ca^{2+} and 4 mM K^+; pH 7.0) at 2 ml/min using a CMA/100 microinfusion pump. Dialysates were collected every 15 min into a CMA/140 autoinjector. Dialysates (5 μl) were directly injected onto a liquid chromatographic (LC) system with a UV detector (BAS UV-116, Bioanalytical Systems, West Lafeyette, IN) for the measurement of pyruvate and lactic acid [13].

A focal ischemic lesion was made by occluding two common carotid arteries (CCAs) and the right middle cerebral artery (MCA) for 90 min followed by reperfusion for 0, 4 and 24 h. In sham operated animals, all surgical procedures were same as above, but no arterial occlusion was performed. Body temperature was maintained at 37.0 ± 0.5°C by a heating pad. Either vehicle or naloxone (0.03, 0.3 mg) was infused through a cannula inserted perpendicularly into the right lateral ventricle at anteroposterially −0.9 mm, mediolaterally 1.5 mm from Bregma and dorsoventrally −3.5 mm from dural surface. The infusion was started 1 h prior to the occlusions and lasted for 4 h (pre-treatment). For post-treatment, the administration was started 30 min after occlusion of arteries and discontinued at 180 min after reperfusion.

For the determination of infarct volumes, animals were sacrificed at 24 h after reperfusion. Brains were sliced in 2 mm thickness and stained with 2, 3, 5-triphenyltetrazolium chloride (TTC stain) for measurement of infarct volume [1]. Another groups, rats were sacrificed at 0, 4 and 24 h after reperfusion. Cortical tissue samples were homogenized in potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide, then sonicated with three bursts of 10 s. The protein concentration in the supernatant was determined by the Bradford assay (Bio-Rad, Richmond, CA), thereafter, was used to determine antioxidant enzyme activity by colorimetric detection [16].

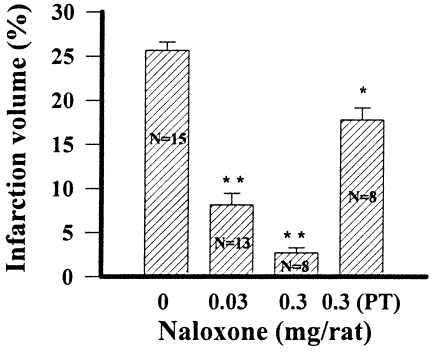

Fig. 1. Effects of naloxone on infarct volume. Infarct volume was determined by TTC stain in rats subjected to 1.5 h occlusion/24 h reperfusion. Pre- and post-treatments (PT) of naloxone (0, 0.03 and 0.3 mg) were infused and lasting for 4 h. Data were expressed as mean ± SEM, $*P < 0.05$ and $**P < 0.01$ vs. vehicle control as determined by Student's *t*-test.

Fig. 1 shows that pre-treatment of naloxone (0.03, 0.3 mg) dose-dependently reduced infarct volume by 70 and 90%, respectively. Post-treatment of naloxone (0.3 mg) also significantly reduced infarct volume by 30%.

Stable basal concentrations of pyruvate and lactic acid in dialysates were obtained 2 h after implantation of probes in the cortex. In the control (vehicle + I/R) group, pyruvate maximally decreased to 30% of basal level at 75 min after occlusions, and was maintained throughout the experiment (Fig. 2A). On the other hand, lactic acid markedly increased to 458% of basal level 75 min after occlusion, and then gradually decreased to 238% of baseline after reperfusion (Fig. 2B). Pre-treatment with naloxone significantly ($P < 0.05$) attenuated the ischemia-induced decrease in pyruvate level from 45 to 70% of the baseline during occlusions, with a gradual return to baseline at 120 min after reperfusion (Fig. 2A). However, pre-treatment with nalox-

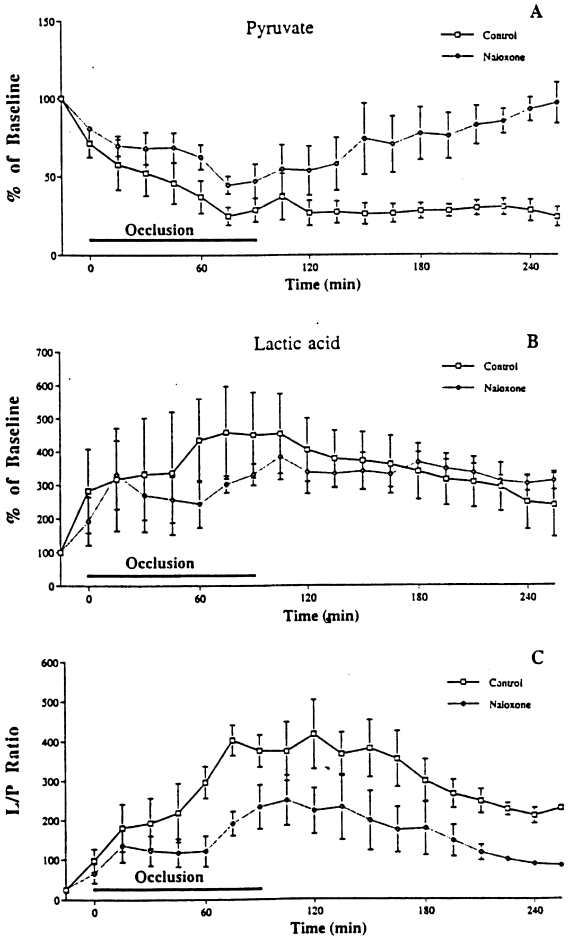

Fig. 2. Time profiles of naloxone on the changes in extracellular levels of (A) pyruvate, (B) lactic acid, and (C) the lactic acid/pyruvate ratio in rat cortex during 1.5 h occlusion and 3 h reperfusion. Naloxone (0.03 mg) infusion was started 1 h prior to occlusions and lasting for 4 h. Data were presented as mean ± SEM ($n = 4$).

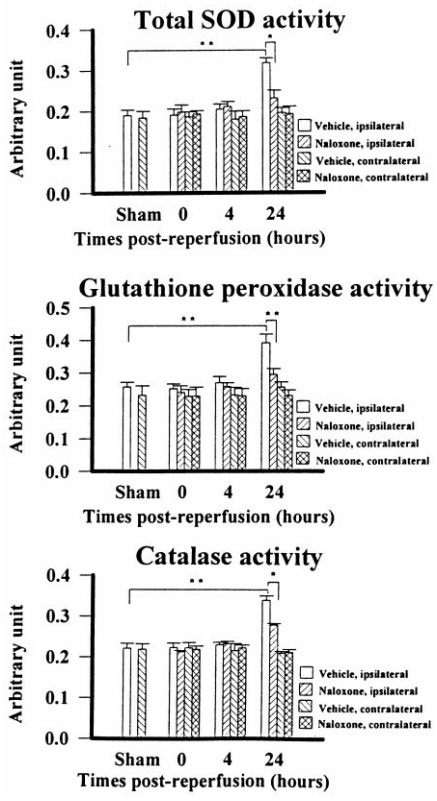

Fig. 3. Effects of naloxone on antioxidant enzyme activities after cerebral I/R. Enzymatic assay was carried out in sham operated (sham), vehicle, and naloxone (0.03 mg) infusion groups. Enzymatic activities were expressed in arbitrary unit which was defined as the ratio of optical absorbance to the protein amount. Data were presented as mean ± SEM, ($n = 4$–6), $^{*}P < 0.05$ and $^{**}P < 0.01$ as determined by Student's *t*-test.

one seemed but slightly attenuate the increase in lactic acid during the late period of the occlusion, whereas exerted no significant effect during reperfusion period (Fig. 2B).

The L/P ratio increased drastically to a peak of 403 at 75 min after occlusions then, gradually decreased to 210 at the end of 3 h reperfusion in the control group (Fig. 2C). These data are in agreement with those of other investigators [3,13]. Pre-treatment of naloxone drastically suppressed ($P < 0.05$) the L/P ratio from 403 to 250 at the end of occlusion and from 250 to 84 at the end of 3 h reperfusion (Fig. 2C).

The time courses of endogenous antioxidant enzyme activities after cerebral I/R are shown in Fig. 3. The total SOD, catalase, and glutathione peroxidase activities were significantly enhanced in the vehicle + I/R cortex at 24 h but not at 0 and 4 h after reperfusion. There were no significant changes in these enzyme activities in the contralateral, non-ischemic side as compared with the sham operated group. The enhancement of enzyme activities were substantially decreased in the naloxone pre-treated group.

The involvement of specific enzyme form of SOD in I/R stress was further determined by immunoblot analysis against cytosolic Cu/Zn-SOD and mitochondrial Mn-SOD. An increased protein content was found in Mn-SOD, but not Cu/Zn-SOD (Fig. 4). The pre-treatment of naloxone significantly attenuated the increase of Mn-SOD protein content.

The present investigation clearly demonstrated three important findings. First, both pre- and post-treatment of naloxone markedly reduced the infarct volume. Second, pre-treatment of naloxone significantly reduced the suppression of extracellular pyruvate and enhancement of L/P ratio in the early course of I/R. Third, pre-treatment of naloxone prevented I/R-induced enhancement of catalase, glutathione peroxidase, and Mn-SOD activities.

Morphological evidence of cellular death caused by an ischemic insult is commonly detected by TTC stain [1]. TTC is a sensitive histochemical indicator of mitochondrial respiratory enzyme function. Therefore, brain lesion identified by TTC stain indicates that tissues were irreversibly impaired in mitochondrial function and oxidative respiratory enzyme systems. Our results revealed that naloxone can restore mitochondrial activity following cerebral I/R stress, and thus reduced infarct volume. In concert with this result, naloxone obviously favored restoration of energy metabolism as indicated by reductions in cerebral I/R-induced extracellular accumulation of lactate, decrease of pyruvate and increase of L/P ratio (Fig. 2).

As far as the restoration of mitochondrial activity or energy metabolism by naloxone is concerned, some steps

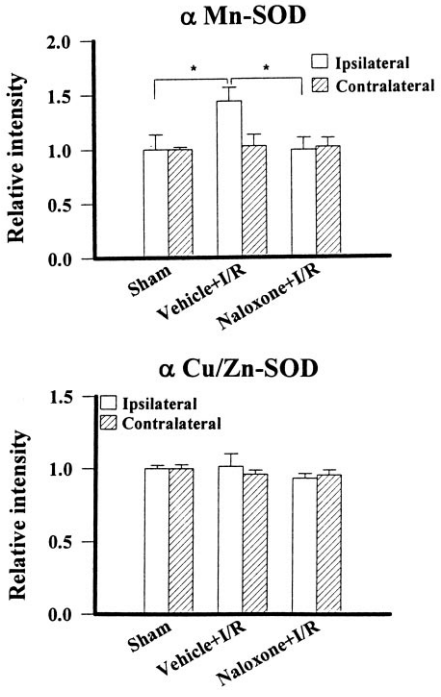

Fig. 4. Effects of naloxone on cerebral I/R-induced SOD protein level. Immunoblot analysis was performed in sham operated (sham), vehicle (vehicle + I/R), and naloxone (0.03 mg, naloxone + I/R) infusion groups with antibodies against Mn- and Cu/Zn-SOD after 24 h reperfusion. The relative protein intensity was expressed as folds of the content to ipsilateral side of the sham group. Data were presented as mean ± SEM, ($n = 4$–6), $^{*}P < 0.05$ as determined by Student's *t*-test.

in a complex cascade of detrimental metabolic events caused by cerebral I/R could be interrupted by naloxone. We assume that naloxone most likely may reduce the generation of free radicals. In support of this notion, the present study demonstrated that cerebral I/R induced significant increases in activities of Mn-SOD, catalase and glutathione peroxidase that could be markedly attenuated by naloxone (Fig. 3). Since substantial and significant increases in antioxidant enzyme activities in ischemic cortex could represent a compensatory mechanism of surviving cells attempting to suppress further oxygen-radical mediated injury, the reduction of these enzyme activities may indicate a decreased production of free radicals generated from impaired metabolic reactions. Whether a decreased production of free radicals is associated with restoration of the impaired metabolic reactions by naloxone should be further investigated.

Although intravenous or intraperitoneal administration of naloxone caused increase in cerebral blood flow [3,4], intracerebroventricular infusion did not alter the cerebral blood flow (data not shown). Therefore, these naloxone effects could not be attributed to the improvement of cerebral blood flow.

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