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The effects of MK-801 and U-83836E on post-ischemic reperfusion injury in rat brain

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Abstract. Rats were subjected to incomplete cerebral ischemia induced by occlusion of common carotid arteries for 30 min, and subsequent reperfusion for 15 min. The concentrations of reduced glutathione (GSH), malondialdehyde (MDA) and superoxide dismutase (SOD) activity were determined in the dorsal hippocampus in order to evaluate their changes during ischemia and reperfusion following ischemia. The depletion of GSH was observed during ischemia with a further depletion during post-ischemic reperfusion ($P<0.001$), while a significant increase in SOD activity and MDA levels was found only after reperfusion following ischemia ($P<0.001$). Animals in which ischemia was followed by reperfusion were treated with a non-competitive NMDA receptor antagonist, MK-801 (1 mg/kg, i.v.), and a radical scavenger, U-83836E (5mg/kg, i.v.), prior to ischemia. Although a full recovery of GSH levels was not observed following MK-801 and U-83836E pretreatment as compared to control ($P<0.05$), MK-801 was more potent than U-83836E in the partial protection of the GSH pool ($P<0.05$ *and* $P<0.01$, respectively). The rise in SOD activity and MDA level were brought close to those of control due to the effects of both MK-801 and U-83836E ($P>0.05$). In conclusion, the tissue changes in GSH concentrations evoked by ischemia and reperfusion were partially prevented by the effects of both drugs, MK-801 having the grater effect. This suggests that the NMDA receptor activation may play a role in the generation of reactive oxygen species. On the other hand, the inhibition of lipid peroxidation brought about by both MK-801 or U-83836E suggests the therapeutic efficiency of these agents in ischemia/reperfusion injury.

Key words: rat brain, ischemia and reperfusion, glutathione, superoxide dismutase, lipid peroxidation

INTRODUCTION

It has been postulated that the stimulation of the N-Methyl-D-Aspartate (NMDA) receptor by excitatory amino acid neurotransmitters, mainly glutamate or aspartate, results in a lethal influx of calcium into the cells (Chio 1988, Greenamyre and Porter 1994). It has been shown that these amino acids are released into brain extracellular space in a variety of ischemia and trauma models (Faden et al. 1989, Butcher et al. 1990). A non-competitive NMDA antagonist, dizocilpine maleate (MK-801), has been reported to reduce calcium accumulation in cells and histologic damage following focal cerebral ischemia (Greenberg et al. 1990)

Another proposed mechanism leading to cell damage in traumatic, ischemic and especially in the reperfused brain is the formation of reactive oxygen species (ROS), which attack the polyunsaturated fatty acids (PUFAs) of plasma membranes, resulting in lipid peroxidation (Braughler and Hall 1989, Cheeseman and Slater 1993). Malondialdehyde (MDA) is a known end product of lipid peroxidation. Various endogenous enzymatic and nonenzymatic defence mechanisms are activated against oxidative stress (Cheeseman and Slater 1993). Reduced glutathione (GSH), a sulfhydryl tripeptide, plays an important role as a major intracellular scavenger in all cells. It also protects sulfhydryl-containing proteins from oxidation. The conversion of GSH into oxidised glutathione (GSSH) can be catalysed by the glutathione peroxidase reaction, resulting in the reduction of hydrogen peroxide (H_2O_2) or fatty acid hydroperoxides. Thereafter, glutathione reductase recycles GSSG back into GSH (Meister and Anderson 1983). The enzyme, superoxide dismutase (SOD) can scavenge the superoxide radical by a reaction known as dismutation with concomitant production of H_2O_2 (McCord and Fridovich 1969).

The 21-aminosteroids (Lazaroids) are potent inhibitors of iron-catalysed lipid peroxidation in neural tissue and have been developed for the acute treatment of central nervous system injury and ischemia (Braughler et al. 1989) One of them, (-) 2-((4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl)methyl)-3,4dihydro-2,5,7,8-tetr amethyl-2H-1-benzopyran-6-ol dihydrochloride (U-83836E) is a novel antioxidant and the minus enantiomer of the racemic compound U-78517F, which shows vitamin E-like activity with the amine portion of its molecule (Hall et al. 1991).

The aim of this study was to evaluate the therapeutic value of either a non-competitive NMDA receptor antagonist, MK-801 (dizocilpine), or a radical scavenger, U-83836E, on oxidative tissue damage in the rat brain subjected to incomplete ischemia and subsequent reperfusion.

METHODS

Experimental protocol

A total of 40 adult male Sprague-Dawley rats weighing 300-380 g were used. Anaesthesia was induced with intraperitoneal injection of a combination of *ketamine* (60 mg/kg) and xylazine (12 mg/kg). Rats were cannulated *via* right femoral artery to measure arterial blood pressure using a Nihon-Kohden recorder system with a transducer (TP-200T). The experiment was performed under normotensive conditions and rectal temperature was maintained at close to 37°C by an automated heat lamp. Ischemia was achieved by clamping the bilateral common carotid arteries with microaneurysmal clips for 30 min, as previously described (Shivakumar et al. 1992), and the subsequent 15 min reperfusion was obtained by removing the clips.

The experiment was performed in two sets of animals. The first one was divided into three groups. The first group (n = 6), undergoing a sham operation to expose only the common carotid arteries at the neck under anaesthesia for 30 min, was used as a control. The second group (n = 7) was subjected to ischemia and the third group (n = 7) to ischemia followed by reperfusion. The second set of animals subjected to ischemia and reperfusion were treated with MK-801 (n = 7), U-83836E (n = 7) and saline (n = 6) through the left femoral vein, 5 min prior to ischemia for a period of 2 min. MK-801 (from ICN) was given at a dose of 1 mg/ kg (1 mg/ml in 0.9 % saline solution). U-83836E (from Biomol GmbH) at 5 mg/kg was given in sterile 0.9 % saline solution (5 mg/ ml) in the same protocol. The vehicle group was treated with the same volume of 0.9 % saline (1 ml/kg). After the animals were decapitated, the brains were quickly removed, frozen in liquid nitrogen, and stored in a deep freezer until the study.

Biochemical analyses

The dorsal hippocampal samples were dissected after thawing of the whole brains at + 4°C and homogenised in buffers appropriate to each analysis using an Ultra Turrax (T25, Janke and Kunkel, IKA Labortechnik).

The homogenates were then sonicated with a Bandelin Sonopuls HD 70.

GSH levels were determined as the amounts of non--protein sulfhydryl groups, according to the method of Ellman (Ellman 1959), and defined as μmol per g wet weight of tissue. In this method, samples were deproteinized with acidification. The (DTNB) 5-5-dithiobis (2-nitrobenzoic acid), a disulfide chromogen, is readily reduced by sulfhydryl compounds. The absorbance of the reduced chromogen is recalculated to GSH concentration using the molar extinction coefficient.

SOD activity was determined by the method of Winternbourn et al. (Winternbourn et al. 1975). This method depends on the superoxide-dependent inhibition of nitroblue tetrazolium (NBT) reduction assay with riboflavin, and oxygen as SOD substrate. One unit of SOD is defined as the amount of enzyme causing half the maximum inhibition of NBT reduction, and the results were expressed in terms of unit/mg protein.

The measurement of MDA levels by colorimetric reaction with thiobarbituric acid (TBA) at 532 nm was carried out according to method of Ohkawa et al. (1979) and expressed as n mol/g wet weight. Protein was measured using a commercial protein detection kit based on Lowry method (Lowry et al. 1951).

Statistical analysis: The significance of differences between the mean values of groups was ascertained by automated one-way analysis of variance, followed by Tukey's multiple comparison test (Motulsky et al. 1994-1995). All results were expressed as the mean ± standard error of the mean (SEM).

RESULTS

Bilateral carotid occlusion for 30 min caused a significant depletion (down to 48 % of the control) of GSH levels in the hippocampus ($P<0.001$). A further decrease (to 25 % of the control) in GSH concentrations was observed after reperfusion for 15 min following ischemia ($P<0.001$, Fig. 1A). On the other hand, the depletion of GSH levels during reperfusion was not significant when compared to that of the ischemia group ($P>0.05$). Pretreatment with either MK-801 or U-83836E did not fully recover the tissue changes in GSH evoked by reperfusion following ischemia ($P<0.05$), but MK-801 was more potent than U-83836E in the partial protection of dorsal hippocampal GSH content as compared to the control ($P<0.05$ *versus* $P<0.01$, Fig. 1B).

An elevation in SOD activity up to 156 % of the control was observed directly after ischemia and a further increase to 302 % occurred following reperfusion (Fig. 2A). This latter result was significantly different from both the control and the ischemia group ($P<0.001$ and $P<0.01$, respectively). SOD activity was

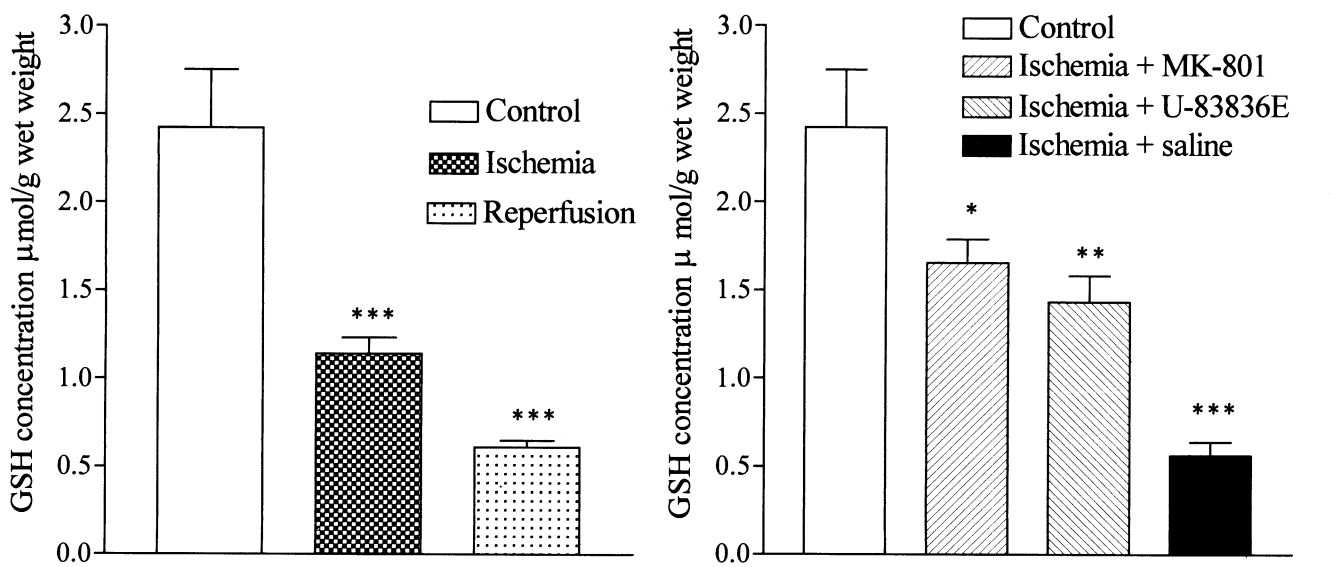

Fig. 1. Each bar shows the mean hippocampal GSH concentrations ± SEM Asterix denotes the significant difference from control. A, shows the GSH concentrations in control, ischemia and post-ischemic reperfusion groups. B, shows the comparison of GSH concentrations in control and post-ischemic MK-801, U-83836E and saline-treated groups. Both MK-801 and U-83836E--treated groups also differed significantly from saline-treated group ($P<0.001$ and $P<0.01$, respectively). * Significant at $P<0.05$, ** significant at $P<0.01$, *** significant at $P<0.001$.

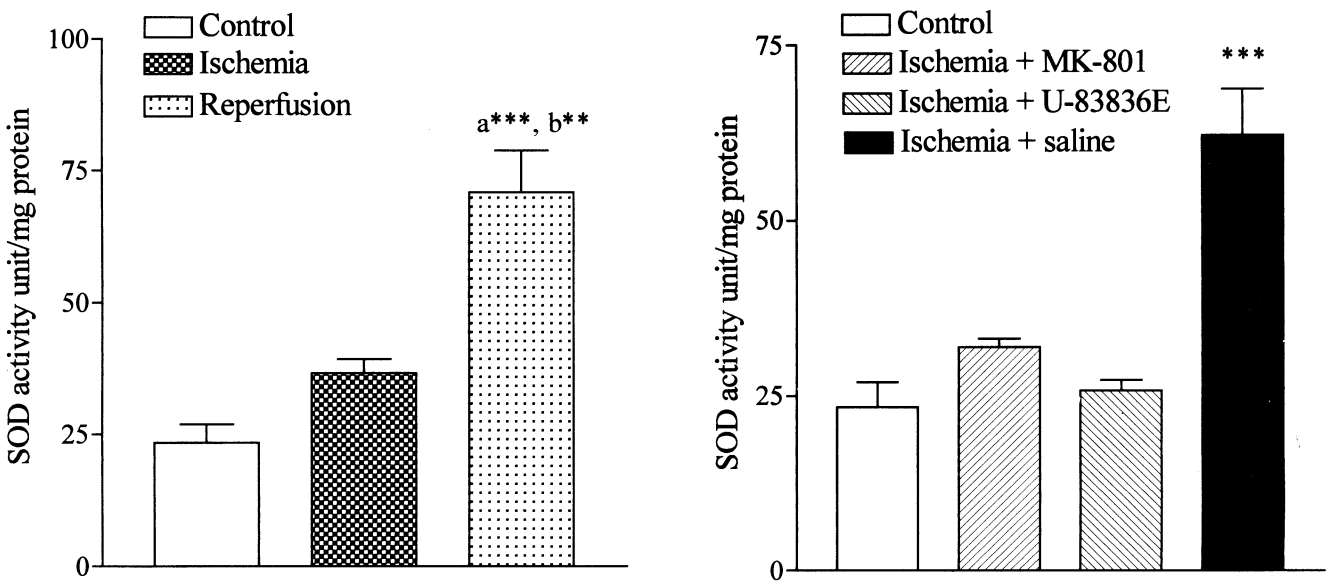

Fig. 2. Each bar shows the mean hippocampal SOD activity ± SEM Asterix denotes the significant difference. A, shows the SOD activity in control, ischemia and post-ischemic reperfusion groups. (a) denotes values significantly different from control group, (aa) also from ischemia-group. B, both MK-801 and U-83836E were effective in normalisation of SOD activity evoked by ischemia and subsequent reperfusion when compared to control ($P>0.05$). ** Significant at $P<0.01$, *** significant at $P<0.001$.

brought close to that of control due to the effects of MK-801 and U-83836E ($P>0.05$, Fig. 2B).

Ischemia alone caused no significant MDA elevation (up to 186 % of control) in the dorsal hippocampus ($P>0.05$). A significant elevation of MDA level (238 % of control) was observed only after reperfusion following ischemia ($P<0.001$, Fig. 3A). MK-801 was effective and U-83836E even more, in decreasing the changes in

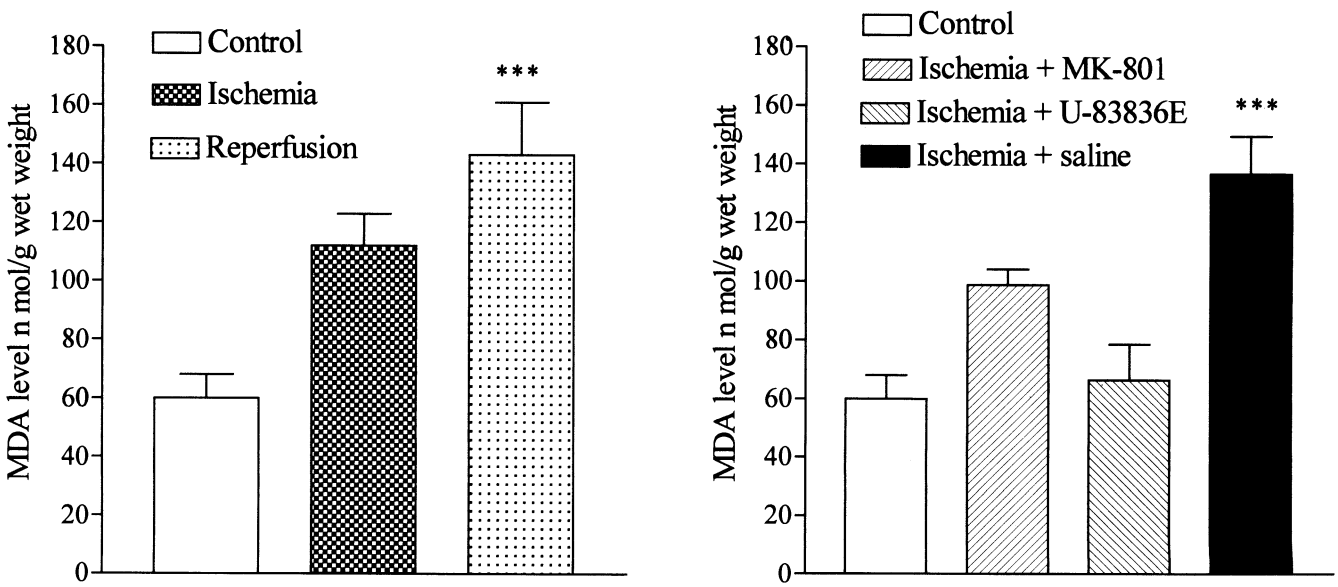

Fig. 3. Each bar shows the mean hippocampal MDA concentrations ± SEM Asterix denotes the significant difference from control. A, shows the MDA levels in control, ischemia and post-ischemic reperfusion groups. B, shows the comparison of MDA levels in control and post-ischemic MK-801, U-83836E and saline-treated groups. There was also a significant difference between U-83836E and saline-treated groups ($P<0.001$). *** Significant at $P<0.001$.

MDA levels caused by reperfusion following ischemia, as compared to the control ($P>0.05$, Fig. 3B).

DISCUSSION

Bilateral carotid artery occlusion in rats causes a 50 % decrease in cerebral blood flow in the brain without a significant energy failure (Eklof and Siesjö 1972). Oxidative stress has been shown to be generated in the brain during reperfusion following ischemia in this model (Shivakumar et al. 1992). The GSH concentration in the ischemic brain was found to be lower than those of the normal brain (Noguchi et al. 1989, Shivakumar et al. 1992). In the present study, the decrease in GSH concentrations was observed largely during ischemia, with a further decrease after reperfusion. In a previous study, a decrease was observed in GSH levels without a concomitant increase in GSSG, in the rat brain subjected to ischemia/reperfusion (Rehncrona et al. 1980). It was therefore concluded that the decrease in GSH might result from an imbalance between the breakdown and synthesis secondary to tissue energy failure (Rehncrona et al. 1980). The decrease in GSH values during ischemia may not be attributed to tissue energy failure because the energy-compensated ischemia model was used in our study. Therefore, it is hypothesised that this may be related to the consumption of GSH due to oxidative stress, possibly generated by partial tissue oxygenation. The further decrease in GSH levels during reperfusion may be due to enhancement of ROS formation by reoxygenation.

In our model of energy-compensated ischemia a significant elevation of SOD was not observed in dorsal hippocampus. SOD activity increased significantly only in the groups of animals where ischemia was followed by reperfusion, as has been previously reported (Horáková et al. 1991). Significant superoxide production was reported to have been detected in the early reperfusion period following the cerebral ischemia, but not in ischemic period nor in the control animals (Nelson et al. 1992). SOD is an important superoxide anion scavenger, so the increase which was observed in its activity after reperfusion in our study may reflect the activation of antioxidant defence system against oxidative stress.

Since the membrane lipids of the central nervous system (CNS) are enriched in PUFAs, the CNS may be especially vulnerable to oxygen free radical injury (Demopoulos et al. 1982). The oxidative destruction of PUFAs by oxidising radicals occurs during reperfusion in a self-perpetuating chain-reaction (Cheeseman and Slater 1993). It is generally accepted that production of ROS is preceded by reperfusion, is burst-like, and is dependent on the duration of the ischemic interval (Dirnagl et al. 1995). In the present work, MDA, the end product and acceptable indicator of lipid peroxidation, significantly increased in dorsal hippocampus only when ischemia was followed by reperfusion. This observation is also supported by the study of Sakamato et al. (Sakamato et al. 1991) showing that free radical formation and lipid peroxidation were enhanced more during reperfusion and there was a close relation between them.

In the present study, the immediate pretreatment by MK-801 did not fully recover the GSH depletion, but it was found to be more effective than U-83836E in partial protection of the GSH pool. On the other hand, it was effective in the prevention of lipid peroxidation and normalisation of SOD activity during ischemia/reperfusion. An interesting observation relating to MK-801 is that when given after, or at late point during ischemia, its neuroprotective effect was lost (Diemer et al. 1996, Margaill et al. 1996). This may suggest that the NMDA receptor activation plays only a transient role in the initiation of ischemic brain injury. It should be recalled that ischemia was induced under ketamine anaesthesia, which is also a weak non-competitive NMDA receptor antagonist. The 21-aminosteroids (lazaroids) have been shown to be neuroprotective in several experimental models of central nervous system ischemia and trauma (Hall et al. 1988, Hall et al. 1990). In our study, U-83836E completely inhibited the lipid peroxidation and prevented the tissue changes in SOD activity caused by reperfusion following ischemia. The lazaroids were suggested to inhibit lipid peroxidation by scavenging peroxyl and phenoxy radicals via vitamin E-like activity (Braughler and Pregenzer 1989, Audus et al. 1991).

Since both MK-801 and U-83836E were able to almost totally prevent the postischemic changes in SOD and MDA, and partially prevent changes in GSH level, it could be concluded that the activation of NMDA receptors and formation of ROS are equally important in postischemic lipid peroxidation. Thus, combined pretreatment with either NMDA antagonists or broad spectrum free radical scavengers like lazaroids would be beneficial.

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