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Effect of carvedilol on neuronal survival and poly(ADP-ribose) polymerase activity in hippocampus after transient forebrain ischemia

Robert P. Strosznajder[1], Henryk Jesko[2], and **Jolanta Dziewulska[2]**

[1]Department of Respiratory Research, [2]Department of Cellular Signaling Medical Research Centre, Polish Academy of Sciences, 5 Pawińskiego St., 02-106 Warsaw, Poland

Abstract. Carvedilol a β-adrenoreceptor antagonist with potent antioxidant properties raises high expectations in therapy of ischemia. In this study the effect of carvedilol on neuronal survival after transient forebrain ischemia in gerbils was investigated. The role of poly(ADP-ribose) polymerase (PARP-1) in this process was evaluated. Our data indicated that carvedilol administered subcutaneously in a dose of 7 or 70 mg/kg b.w. directly after 5 min of transient forebrain ischemia protects significant population of neurons in hippocampal area CA1, but has no effect after induction of prolonged 10 min ischemia. Carvedilol significantly decreased PARP activity in hippocampus that was markedly increased after both 15 min and 4 days of reperfusion following 5 min of ischemia. Moreover, carvedilol prevented NAD^+ depletion after ischemic-reperfusion insult. These results indicated that carvedilol protects neurons against death and suggested that suppression of PARP activity during reperfusion could be involved in this process.

The correspondence should be addressed to: R.P. Strosznajder, Email: roberts@cmdik.pan.pl

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INTRODUCTION

Carvedilol 1-(9H-Carbazol-4-yloxy)-3-[[2-(2-methoxyphenoxy)ethyl]amino]-2-propanolol is a novel, beta adrenoreceptor antagonist with a multiple action potential. Carvedilol exerts a neuroprotective effect during global ischemia (Lysko et al. 1992), transient focal ischemia (Savitz et al. 2000), and ischemic-reperfusion renal injury (Singh et al. 2004). Moreover, carvedilol reduces oxidative stress in human failing myocardium. This compound exerts ameliorating effect on cardiac function, lowers the concentration of lipid peroxidation products determined by thiobarbituric acid-reactive substances (TBARS) and 4-hydroxy-2-nonenal (4-HNE) and protects proteins against oxidation (Book 2002, Chen et al. 1997, Gao 2000, Nakamura et al. 2002, Yue et al. 1994). The results of Habon and coauthors (2001) show that carvedilol can significantly modulate the reactive oxygen species mediated processes, activation of poly(ADP-ribosyl)ation and βNAD^+ catabolism in postischemic perfused hearts. Moreover, carvedilol possess also the additional neuroprotective activity as Na^+ channel modulator and glutamate release inhibitor (Lysko et al. 1994). The increased production of reactive oxygen and nitrogen species has been implicated in brain ischemia-reperfusion injury (Chalimoniuk and Strosznajder 1998, Chan 2004, Strosznajder and Chalimoniuk 1996, Strosznajder et al. 2003). The free radicals lead to DNA damage that triggers the activation of poly(ADP-ribose) polymerase (PARP, EC 2.4.2.30) the major player for the cell survival and death (Cookson et al. 1998, Lautier et al. 1994, Shah et al. 1996, Strosznajder et al. 2003, 2005, Zhang et al. 1995). The pathomechanism of brain ischemia and the role of PARP-1 in cell death are not fully elucidated. PARP-1 plays pivotal role in classical necrosis but the last data also indicate its active role in apoptosis (Chiarugi 2002, Strosznajder et al. 2005, Yu et al. 2002). This enzyme is activated by DNA single and double strand breaks and transfers ADP-ribose from its substrate nicotinamide adenine nucleotide (βNAD^+) to nuclear proteins mostly histones and PARP-1 itself. Subsequently, it synthesizes polymers of ADP-ribose [poly(ADP-ribose)]. The size of the branched polymer varies from a few to 200 ADP-ribose units. The increase of PARP activity is an early sensor of DNA damage. PARP over activation may lead to βNAD^+ and ATP depletion and to cell death (Skaper 2003, Szabo and Dawson 1998, Ying et al. 2005). Our previous study indicated that inhibitor of PARP-1, 3 aminobenzamide (3-AB) injected intravenously before or after transient forebrain ischemia protected over 50% of neurons in the hippocampal area CA1 exclusively after the short (3 min) ischemia but had no effect after prolonged (10 min) ischemic insult (Strosznajder et al. 2003). Other authors also reported the neuroprotective effect of PARP inhibitors in ischemia-reperfusion injury (Beneke et al. 2004, Endres et al. 1997, Thiemermann et al. 1997, Zingarelli et al. 1997). The aim of this study was to investigate the effect of carvedilol on neuronal survival in hippocampus after 5 and 10 min of ischemia and to evaluate the role of PARP in these processes.

METHODS

Male Mongolian gerbils 60–70 g were used for the experiment. The animals were supplied from Medical Research Center Farm, Warsaw, Poland. The Local Medical Research Centre Ethics Committee that followed the European Communities Council Directive of 24 November 1986 accepted the use of these animals for described experiments.

[adenosine-^{14}C(U)]nicotinamide adenine dinucleotide NAD (587.80 Ci/mmol) was from Amersham, UK. Protease inhibitor cocktail Complete was obtained from Boehringer Mannheim GmbH, Germany. β-nicotinamide adenine dinucleotide (β-NAD), DL-dithiotreitol (DTT), dimethyl sulfoxide (DMSO) were obtained from Sigma. Carvedilol was obtained from Pharmaceutical Institute Warsaw, Poland.

Ischemia-reperfusion injury

Male Mongolian gerbils were anesthetized with halotane in 70% N_2O and 30% O_2 (2% halotane for induction, and 0.5% for maintenance of anesthesia). Brain ischemia was induced by ligation of both common carotid arteries using Heifetz clips for 5 or 10 min. The sham-operated animals served as a control. The groups of animals were allowed to survive for 15, 30 min, 1, 2 hours, 4 and 7 days after ischemia. The body temperature was kept at 37°C by using controlled heating pad and heating lamps during the time of ischemia and reperfusion. Then animals were quickly decapitated, the brains were removed, and hippocampus and cortex were isolated and homogenized in 50 mM Tris-HCl

pH 7.4 together with protease inhibitors Complete. Then homogenates were quickly frozen at -70°C and then used for biochemical determination.

Treatment with carvedilol

The animals were injected subcutaneously in a dose of 7 and 70 mg /kg b.w. directly after ischemia and allowed to survive 15 min, 4 and 7 days after ischemic episode. Carvedilol was dissolved in 100% DMSO then diluted in 0.9% saline to achieve the final concentration of 50 % DMSO in saline. The volume of administered solution did not exceed 200 µl.

Histological assessment

For the histological examination several groups of animals (6–7 animals per group) were used. Carvedilol in a dose of 7 or 70 mg/kg b.w. was injected subcutaneously directly after 5 or 10 min ischemia. Seven days after brain ischemia animals were transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline pH 7.4. The brains were rapidly removed and postfixed in the same perfusion solution for 7 days before paraffin embedding. Paraffin sections at the level of dorsal hippocampus 10 µm thick were cut on a microtome and stained with cresyl violet. Neurons in the hippocampal CA1 region were counted under the 200–400× magnification in the light microscope. In each group the average number of neurons per 1 mm length of section through the area CA1 was counted.

Preparation of brain homogenate and nuclear fraction

Brain homogenate (10%) from cerebral cortex and hippocampus was prepared in 0.32 M sucrose with Tris-HCL pH 7.4, 1 mM EDTA using Dounce glass homogenizer. To obtain crude nuclear fraction, homogenate was centrifuged at 900 g for 3 min and the obtained pellet was used for the PARP assay.

Determination of poly(ADP-ribose) polymerase activity

PARP activity was determined using [adenine-^{14}C]NAD as a substrate. The incubation mixture in a final volume of 100 ml contained 200 µM βNAD and 2×10^5 dpm [adenine-^{14}C]NAD; 100 mM Tris–HCl buffer (pH 8.0); 10 mM $MgCl_2$; 5 mM DTT; 50 µM P-APMSF and 100 µg of protein. The mixture was incubated for 1 min at 37°C and the reaction was stopped by adding 0.8 ml of ice-cold 25% trichloroacetic acid (TCA). Precipitate were collected on Whatman GF/B filters, washed three times with 5% TCA and left overnight for drying. The radioactivity was measured using scintillator counter LKB, Wallach 1409.

Determination of NAD^+ concentration

NAD^+ concentration was determined according to the method of Nisselbaum and Green (1969) modified by Stern (Stern et al. 2002). Animals were quickly decapitated and the heads were put in liquid nitrogen. Then the nitrogen powder were prepared from isolated brain and dispersed in 0.1 M HCL and then incubated at 100°C for 2 min then chilled to 0°C and centrifuged Supernatant was collected and was neutralized and added to the reaction mixture containing 50 mM of Tris-HCL pH 8.0 1mM of phenazinmethosulfate, 0.25 mM of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, 0.2 mg of alcohol dehydrogenase, 300 mM of ethanol in a total volume of 2 ml. Reaction mixture was incubated at 37°C without ethanol and reaction was started by adding ethanol. The rate of increased absorbance was determined for 2 min at 560 nm. Blanks without NAD were used to correct background rate. NAD^+ concentration was calculated from standard curve.

The statistical analysis was performed by one-way ANOVA, using Newman-Keules *post-hoc* test.

RESULTS

Carvedilol protects CA1 hippocampal neurons against death after 5 min forebrain ischemia

Histological examination of hippocampal sections of the brains collected 7 days after ischemia showed that carvedilol injected subcutaneously in a dose of either 7 or 70 mg/kg b.w. directly after 5 min of transient forebrain ischemia protected a significant population of neurons (Fig.1). In the brains of seven control animals, the average number of neurons per 1 mm length of section through the area CA1 was 310 ± 10 and this value was taken as 100%. Without the carvedilol protection about 90–100% neurons of the hippocampal area CA1 died 7 days after 5 min

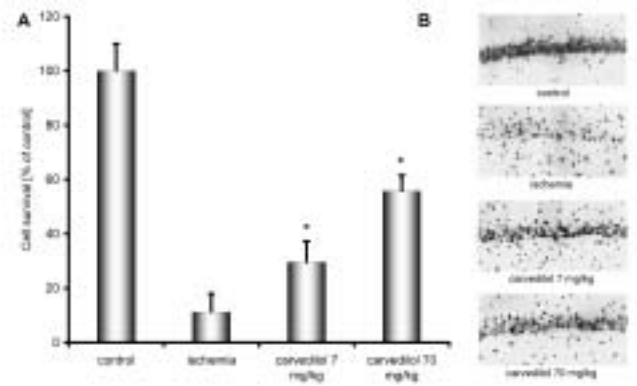

Fig. 1. (A) Statistical evaluation of the effect of carvedilol on CA1 pyramidal cells survival on the seventh day of reperfusion after 5 min of ischemic episode. Values are means ± SEM from 6–7 animals in each experimental group. (*) statistically significant $P<0.05$. (B) Histological assessment of carvedilol on CA1 pyramidal cells survival on the seventh day of reperfusion after 5 min of ischemic episode. Carvedilol was injected subcutaneously in a dose of 7 and 70 mg/kg b.w. directly after ischemia.

ischemia. Carvedilol applied in the dose of 7 mg/kg increased the number of surviving neurons by 48%, and in the higher dose by 58% (Fig. 1). However, carvedilol had no protective effect on cells death after 10 min of transient forebrain ischemia (data not shown).

Carvedilol protects brain against NAD^+ depletion after 5 min ischemia

Brain ischemia induced significant decrease of NAD^+ concentration in brain. After 15 min of reperfusion following 5 min of ischemia, the NAD^+ concentration was reduced to 30% of the control value. Carvedilol injected directly after ischemia protected NAD^+, so its concentration was equal to the control (Fig. 2).

PARP activity in hippocampus and cerebral cortex during reperfusion after 5 min of transient forebrain ischemia

PARP activity was determined in hippocampus and cerebral cortex during different reperfusion time 15, 30, 60, 120 min and 4 days after 5 min of forebrain ischemia in gerbil. The basal PARP activity in cerebral cortex and hippocampus was 17 and 28 pmol/mg protein/min respectively. In hippocampus PARP activity significantly increased by 60% 15 min after 5 min. of ischemic insult comparing to sham-operated animals (control). After 30 min, 1 and 2 h of reperfusion PARP activity slightly decreased and 4 days after ischemia enzyme activity significantly increased comparing to control (Fig. 3A). In the cerebral cortex there was no stimulation of PARP activity after 15 min and 4 days of reperfusion but a non-significant decrease of its activity at 30, 60 and 120 min after ischemic episode was observed (Fig. 3B). Carvedilol administrated in a dose of 7 mg/kg b.w. significantly decreased the PARP activity in hippocampus that was increased by ischemia and 15 min or 4 days of reperfusion (Fig. 4).

DISCUSSION

The idea to perform these experiments was connected with the expectation that carvedilol, being a multifunctional drug may be much more efficient protectant for brain ischemia pathology than specific compounds acting on one selected enzyme or pathway. It is obvious that ischemic pathology is very complex and many different molecular events are involved in the cell death (Lipton 1999). Our previous study demonstrated that inhibitor of poly(ADP-

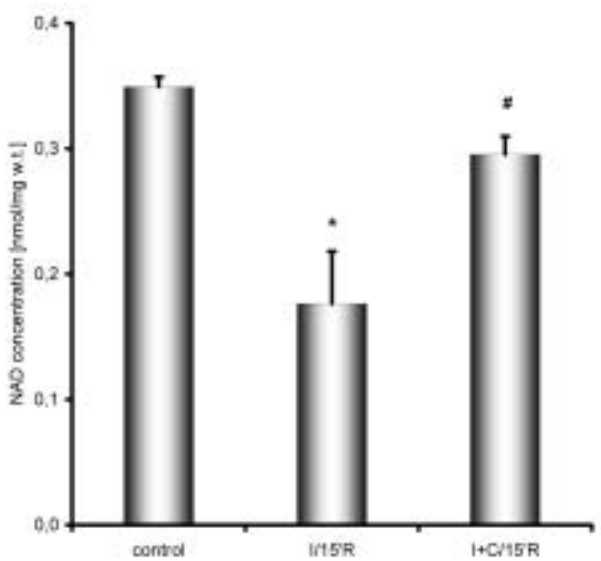

Fig. 2. Effect of carvedilol on NAD^+ concentration in the brain. (I) 3 min forebrain ischemia; (15'R) 15 min reperfusion; (C) carvedilol. Values are means ± SEM from 3 animals carried out in triplicate. (*) $P<0.05$ comparing to control; (#) $P<0.05$ comparing to ischemia reperfusion.

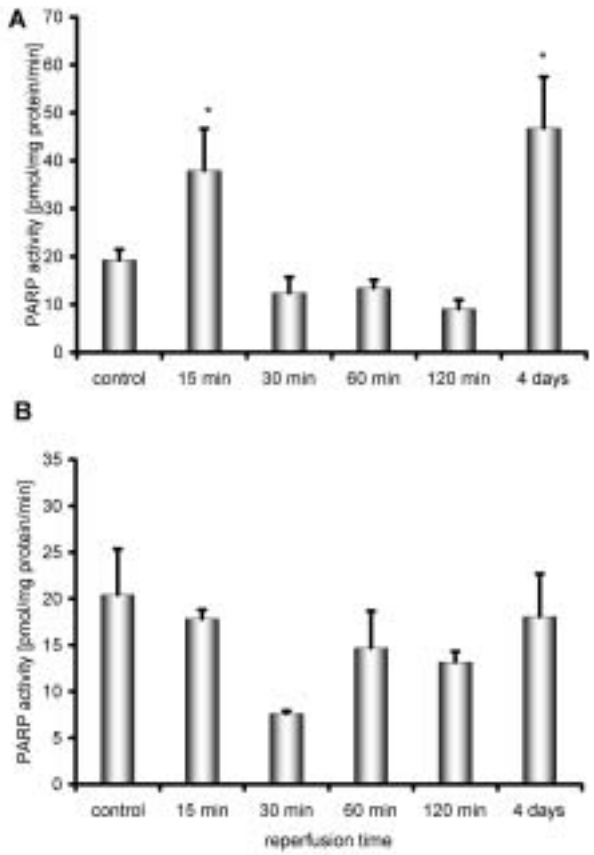

Fig. 3. (A) PARP activity in the hippocampus during reperfusion after 5 min of transient forebrain ischemia. Values are means ± SEM from 3–6 animals in each experimental group. The results are statistically significant at $P<0.05$. (B) PARP activity in cerebral cortex during reperfusion after 5 min of forebrain ischemia in gerbil. Values are means ± SEM from 3–6 animals.

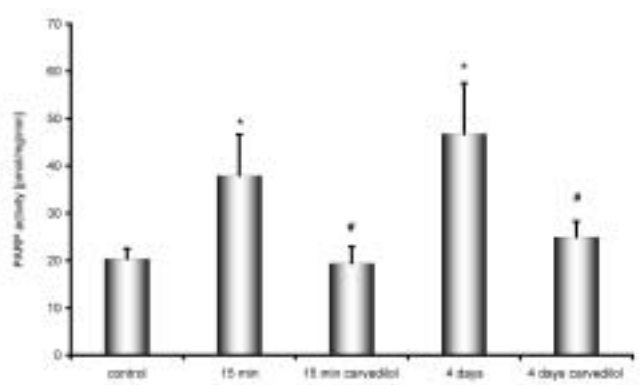

Fig. 4. Effect of carvedilol on PARP activity in the hippocampus of gerbils reperfused for 15 min and 4 days after 5 min ischemia. Values are means ± SEM from 4–5 animals. (*) $P<0.05$ comparing to control, (#) $P<0.05$ comparing to ischemia reperfusion.

ribose) polymerase 3-AB is able to protect more then 50% of neurons in CA1 layer of hippocampus after 3 min but not after 10 min ischemia. Lysko and coauthors (1992) and Habon and coauthors (2001) demonstrated the neuroprotective effect of carvedilol in *in vitro* and *in vivo* experiments. In *in vitro* experiments performed on cultured rat cerebral neurons carvedilol protects the cells in a dose dependent manner against glutamate-mediated excitotoxicity and also against free radicals generation after addition of ferrum ions. In *in vivo* experiments Lysko and coauthors (1992) used the gerbil global brain ischemia model. Carvedilol administered subcutaneously before or/and after ischemic episode had protective effect on CA1 hippocampal neurons. Habon and coauthors (2001) in the experiments performed on ischemic-reperfused hearts presented that carvedilol decreased ROS level and the single-strand DNA breaks and suppressed the self ADP-ribosylation of PARP. Moreover, it was shown that carvedilol in a concentration of 1 μM potently inhibited PARP activity isolated from rat liver. Till now there was no experimental evidences indicated possible interrelation between PARP activity and carvedilol action in postischemic brain. Our data demonstrate that the enhancement of PARP activity during first 15 min of reperfusion might be due to the elevation of oxidative stress and suggest the involvement of PARP in DNA repair mechanism. However, the second rise in PARP activity observed 4 days of reperfusion is probably associated with the massive DNA damage. The PARP over activation may lead to energy depletion and in consequence to cell death. Our results presented in this paper clearly show that carvedilol significantly decreased the PARP activity after short ischemic insults, 15 min and 4 days of reperfusion. Moreover, carvedilol protected ischemic brain against NAD^+ depletion.

CONCLUSIONS

We suggest that neuroprotection of CA1 hippocampal neurons after carvedilol treatment my correlate with diminished PARP activity during reperfusion after transient forebrain ischemia. We propose that carvedilol through its action prevents brain cells against PARP-1 over activation and subsequently against depletion of its substrate βNAD^+ and ATP exhaustion.

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