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Neuroprotective Effects of Carnosine and Homocarnosine on Pheochromocytoma PC12 Cells Exposed to Ischemia

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The development of neuroprotective drugs against ischemic insults is hampered by the lack of pharmacological in vitro models. We developed an ischemic model using PC12 cell cultures exposed to oxygen-glucose-deprivation (OGD) followed by reoxygenation (18 hr) under regular atmospheric oxygen level. The toxicity induced in this model, that is partially caused by generation of reactive oxygen species (ROS), was measured morphologically as well as by the release of lactate dehydrogenase (LDH) and the prostaglandin PGE_2 from the cells. Carnosine and homocarnosine, histidine dipeptides antioxidants, found in high concentration in the brain, have been suggested to provide neuroprotection. Using the OGD model we found that 5 mM carnosine and 1 mM homocarnosine provided maximal neuroprotection of about 50% against OGD insult. This neuroprotective effect was similar to that of a known antioxidant, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (tempol), and was not observed in a serum-deprivation toxicity model of PC12 cells, indicating that carnosine and homocarnosine may act as antioxidant-neuroprotective agents in the brain. Our ischemic model may provide a useful tool for investigating the mechanisms involved in the neuroprotection afforded by histidine dipeptides.

Key words: PC12; ischemia; cell death; histidine dipeptides; neuroprotection

Ischemic injury (oxygen and glucose deprivation, OGD) occurs when the blood supply to a tissue is cut off. Insults to the brain that interrupt its blood supply, as in ischemia, or its oxygen supply, as in hypoxia (>1% O_2) and anoxia (0% O_2), lead to rapid neuronal death. Restoration of the blood supply, renewing reoxygenation to the tissue, usually increases the damage because of the generation of reactive oxygen species (ROS) (Chalmers-Redman et al., 1997). The biochemical mechanism of ischemic brain damage has not been fully elucidated, but considerable evidence exists for the involvement of three major factors: an increase in the intracellular cytosolic calcium concentration, acidosis, and the production of reactive oxygen species (ROS) (Traystman et al., 1991; Chalmers-Redman et al., 1997; Kristian and Siesjo, 1998).

To understand the mechanism of ischemia-induced cell death at the cellular and molecular level, in vitro models of neuronal cultures are used (Goldberg et al., 1997). A well known neuronal model for in vitro studies of cell death is provided by pheochromocytoma PC12 cells (Fujita et al., 1989). The rat pheochromocytoma cell line PC12, which displays phenotypic characteristics of both adrenal chromaffin cells and sympathetic neurons (Greene and Tischler, 1976), is a useful system for exploring neuroprotective drugs (Abu-Raya et al., 1993, 1999). Undifferentiated PC12 cells provide a useful experimental system to study the mechanism of action of NGF and other growth factors because they survive and proliferate in culture (Fujita et al., 1989). These cells that differentiate into fully sympathetic neurons upon NGF treatment and synthesize and release catecholamines, constitute a paradigm for studying neurotransmitter release and neuronal differentiation (Fujita et al., 1989). Using a special device and undifferentiated PC12 cells, we established an in vitro model for ischemia by a combination of oxygen and glucose deprivation (Abu-Raya et al., 1993). In this model, PC12 cells exposed to OGD, released high amounts of the prostaglandin PGE_2, reduced by 50% the intracellular ATP level and entered into a process of cell death, measured by the release of lactate dehydrogenase (LDH) (Abu-Raya et al., 1993). The use of this cell death model to develop neuroprotective drugs was validated with rasagiline, a monoamine oxidase type B (MAO-B)

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inhibitor used to treat Parkinson disease (Abu-Raya et al., 1999, 2000).

One of the major causes of neuronal cell death is the formation of ROS, which subsequently damage various biological molecules, including lipids, proteins and nucleic acids (Braughler and Hall, 1989; Werns and Lucchesi, 1990; Traystman et al., 1991; Coyle and Puttfarcken, 1993). Therefore, in recent years synthetic and natural antioxidants have gained tremendous interest because of their potential use as neuroprotective compounds in the clinic (Callaway et al., 2001; Herin et al., 2001; Mackensen et al., 2001). Antioxidants were found to decrease reactive oxygen species-induced brain damage produced in different experimental models, as well as after ischemic insults (Uyama et al., 1990; Clements et al., 1993; Watanabe et al., 1994). Carnosine (β-alanyl-L-histidine) and carnosine-related compounds (CRC) such as homocarnosine (γ-aminobutyryl-L-histidine) are present in skeletal muscles and in the central nervous system (Neidle and Kandera, 1974; Sobue et al., 1975; Nadi et al., 1980), at concentrations of 1–20 mM in skeletal muscles, and from 0.7–10 mM in the mammalian brain depending on the region in the brain (Nadi et al., 1980; Scriver et al., 1983). Carnosine is under metabolic control and is produced by the enzyme carnosine synthase (Kalyankar and Meister, 1959; Horinishi et al., 1978). Carnosine and CRCs may act as neuroprotective antioxidant compounds in the brain or peripheral tissues (Boldyrev et al., 1988; Kohen et al., 1988; Boldyrev, 1993). Clarification of the major biological roles of carnosine and CRCs could be facilitated by an in vitro neuronal model system. We present a modified OGD device adjusted to measure neuroprotection against ischemic (OGD) cell death in PC12 cells, in attempt to comprehend the potential neuroprotective role of CRC such as carnosine and homocarnosine.

MATERIALS AND METHODS

Materials

Prostaglandin PGE_2, dextran, L-carnosine, L-homocarnosine sulfate, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (tempol) were purchased from Sigma (St. Louis, MO). [^{3}H]PGE_2 (120–200 Ci/mmol) was purchased from New England Nuclear (Boston, MA), anti-PGE_2 antibody was purchased from Bio-Yeda (Rehovot, Israel). β-NGF purified by HPLC from mouse submaxillary glands, was the kind gift of Alomone Labs (Jerusalem, Israel).

PC12 Cultures

PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 7% FCS, 7% horse serum, 100 μg/ml streptomycin, and 100 U/ml penicillin (Beit Haemek, Afula, Israel). The cultures were maintained in an incubator at 37°C in a humidified atmosphere of 6% CO_2. The medium was changed twice weekly and the cultures were split at a 1:6 ratio once a week (Abu-Raya et al., 1993). In the ischemic experiments, an identical number of cells (1–1.5 × 10^6 cells) was plated on 35 mm diameter petri dishes (Nunclon Delta, NUN, Denmark) coated with rat tail type I collagen (0.1 mg/ml) (Beit Haemek).

Ischemic Device

To induce the ischemic insult (OGD), PC12 cells were cultured on petri dishes and introduced into an ischemic device (Fig. 1), described previously by us (Abu-Raya et al., 1993), now modified and upgraded. The ischemic device is composed of two connected ischemic chambers (Figs. 1B-5, Fig 1C) maintained at 37°C by warmed, circulating water (Fig. 1B) with the aid of a heating system (Fig. 1A-3). The air in the chambers is replaced by a stream of a mixture of 95% N_2 and 5% CO_2 (Fig. 1A-1) through a cylinder containing a water trap (Fig. 1A-2) to prevent PC12 cultures drying due to medium evaporation as a result of gas flow. The oxygen level within the device was kept below 1% by tightly closing the petri dishes (Fig. 1C-7) in the ischemic chamber with metal screws (Fig. 1C-8). The oxygen level in the gas leaving the ischemic chamber (Fig. 1B-5), was measured on line by an electronic oxygen sensor coupled to an oxygen monitor (HUDSON RCI, Hanover, Germany) (Fig. 1B-4). This level of oxygen represents a hypoxic insult. During the exposure to the hypoxic conditions, the PC12 cells were maintained in glucose-free DMEM, so that there was both oxygen and glucose deprivation, i.e., ischemic-like condition.

OGD (Ischemic) Paradigm

On the day of the experiment, the regular high-glucose DMEM (4.5 mg/ml) was replaced with glucose-free DMEM supplemented with serum (Fig. 1D). The cultures were then introduced into the ischemic device to initiate the OGD insult. The insult entails two phases: Phase I, hypoxia and glucose deprivation (OGD) for 3–5 hr; Phase II, at the end of the OGD period, when a concentrated glucose solution is added to a final concentration of 4.5 mg/ml and the cultures (in 1 ml vol) are reintroduced in the tissue culture incubator for an additional 18 hr to regular atmospheric oxygen level (reoxygenation) (Fig. 1D). The reoxygenation of the PC12 cultures was performed in a tissue culture incubator and not in the ischemic device re-exposed to regular atmospheric oxygen level, because cell viability/death values were similar upon reoxygenation experiments in the ischemic device or the tissue culture incubator. Upon termination of the OGD insult-Phase I, the medium was not replaced to preserve all the mediators and enzymes (such as PGE_2 and LDH) released into the medium during the insult. These mediators may also contribute to cell death during the reoxygenation process. Control cultures were maintained in the incubator under atmospheric oxygen level and humidity conditions (normoxia) (Fig. 1D). Carnosine and homocarnosine were added to the cultures 18 hr before the OGD insult. Tempol was added 30 min before exposure to the OGD insult. All the tested compounds were present during the OGD insult and the reoxygenation period. The reason for choosing this relatively long period of incubation with carnosine and homocarnosine is based on previous reports in other models in which the neurons were incubated for 2–24 hr in vitro (Preston et al., 1998; Boldyrev et al., 1999a,b; Horning et al., 2000) or 7 days in vivo (Stvolinsky and Dobrota, 2000)

Cell Death

Cell death was measured at the end of the reoxygenation period by the release of lactate dehydrogenase (LDH) into the medium, using a Sigma Diagnostics LD-L reagent. LDH activity

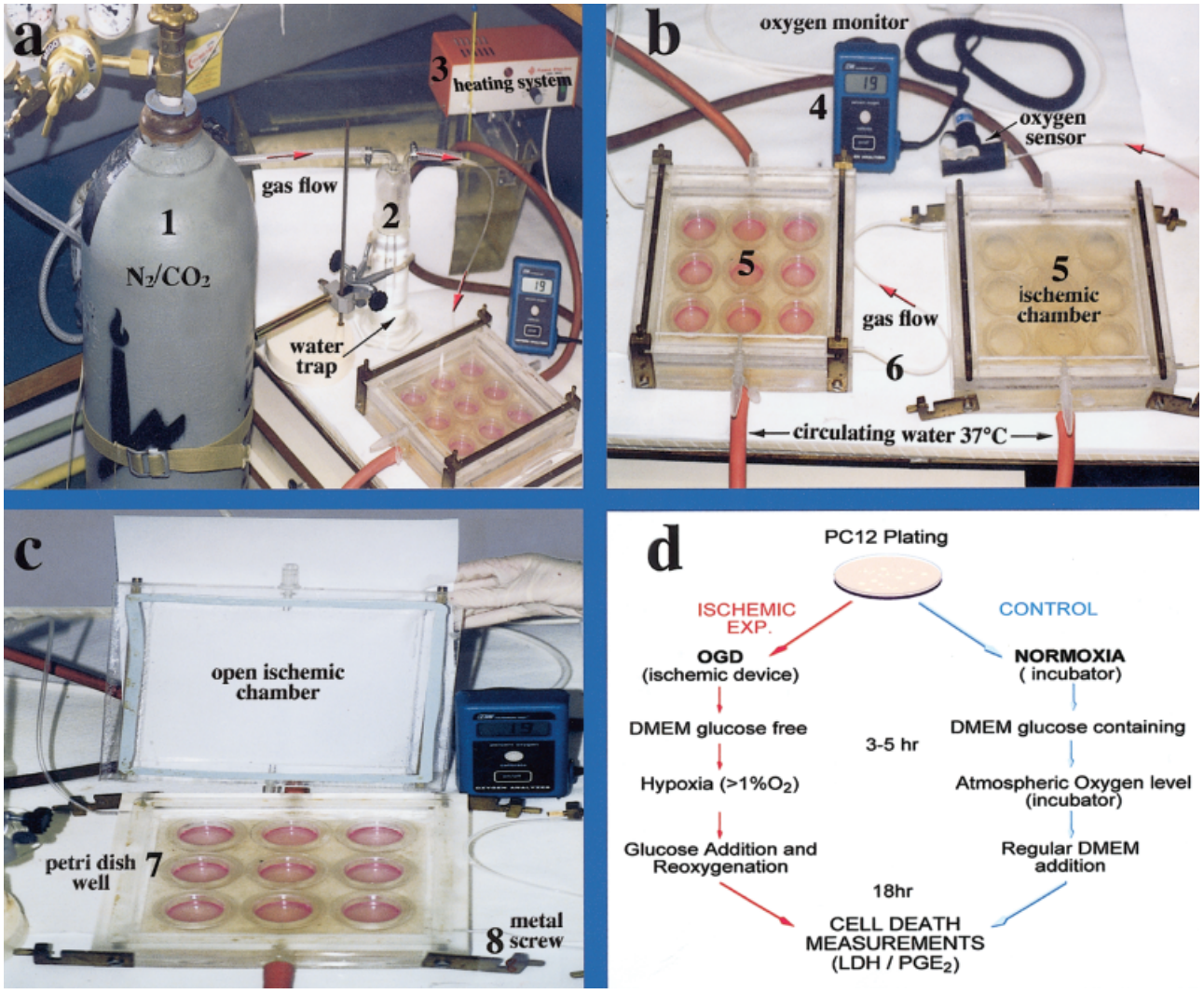

Fig. 1. Ischemia (oxygen-glucose-deprivation) device and protocol. **A:** ischemic system: (1) gas tank (N_2/CO_2 95:5%); (2) cylinder containing water; (3) water bath with heating system and pump. **B:** Ischemic chambers and oxygen detection: (4) oxygen monitor and oxygen sensor; (5) ischemic tissue culture chambers; arrow, flow of N_2/CO_2 gas through Teflon tube; (6) connecting consecutively the two chambers of the ischemic device (5). **C:** Photograph of an open tissue culture ischemic chamber: housing the petri dish containing the PC12 cells (7) and tightly closed by metal screws (8) to seal the chamber. **D:** Ischemia and normoxia protocols.

was determined spectrophotometrically at 340 nm by following the rate of conversion of oxidized nicotinamide adenine dinucleotide (NAD^+) to the reduced form of (NADH). Total LDH (extracellular + intracellular) was obtained by freezing and thawing the cultures. The basal LDH release measured in cultures maintained under normoxia was subtracted from all the experimental values. OGD-induced LDH release was expressed as the percentage of the total LDH value. The neuroprotective effect is defined as the percent decrease in LDH release in the presence of the tested compounds relative to that from untreated OGD cultures (100%). Each experiment was performed 3–4 times ($n = 3–6$).

PGE_2 Release

The amount of prostaglandin PGE_2 released into the medium was determined by radioimmunoassay (RIA) as described (Abu-Raya et al., 1993). In brief, the extracellular medium was collected after reoxygenation, centrifuged at 4°C for 10 min at 1,000 × *g*, and aliquots were removed for RIA. After 18–24 hr incubation of samples and standards with antiserum and radioligand, free and bound compounds were separated by dextran coated with activated charcoal, and the radioactivity in the supernatant was counted. The amount of PGE_2 in the medium was calculated according to a standard reference curve.

Serum Deprivation Protocol

Before the experiment, the cell cultures were gently washed three times with serum-free DMEM containing antibiotics. The cultures were then incubated with the tested compounds for an additional 48–72 hr. The duration of the incubation was determined by microscopical evaluation of PC12 cell death. The experiment was terminated when ~50% cell death

was confirmed by LDH release. Control cultures were maintained in regular DMEM with serum under identical conditions.

Microscopic Evaluation of PC12 Cultures

Live, triplicate PC12 cultures under normal conditions or after OGD were examined under an inverted Zeiss light microscope at ×200 or ×400 magnification. Representative fields from each culture were photographed.

Statistics

The results are presented as the mean ± SEM Statistically significant differences between experimental groups were determined by analysis of variance program (ANOVA), and they were considered significant when *P*-values <0.05 were obtained. Each experiment was performed four times in triplicate.

RESULTS

OGD Model Using PC12 Cells

As can be seen in Figure 1, we established an in vitro model for ischemia, using PC12 cells and an ischemic device designed in our laboratory for toxicity/neuroprotection measurements. Two major parameters affect cell death induced by OGD insult under these conditions: the cell number and duration of exposure. As evident from Figure 2, OGD-induced cell death increased progressively as a function of cell number after 3- and 4-hr insult as previously reported for neuronal primary cultures (Yavin and Billia, 1997). Based on the data presented in Figure 2, and to evaluate the neuroprotective effect of the compounds used, experiments were performed at a culture density of 1.25×10^6 cells/dish/ml with 3–5 hr exposure to OGD. Under these conditions, cell death measured was 20–60%, a range in which neuroprotective effects may be distinguished (at a higher percentage of cell death the neuroprotective effects of the drug tested was minimal (Abu-Raya et al., 1999, 2000). PC12 cells exposed to OGD showed typical morphological features, such as shrinkage, necrosis, clustering and debris (Fig. 3B), in contrast to cells under normoxia conditions that preserved their morphological integrity (Fig. 3A). When the percentage of dying cells was high, the culture detached from the collagen, with many cells dispersing into the medium and disintegrating into particles (data not shown).

Histidine Dipeptides Protect Against OGD Insult

Figure 4 shows the effect of carnosine and homocarnosine on OGD-induced cell death after 3 (open bars) and 4 hr (black bars) of exposure. The untreated control cultures exhibited 27% and 62% cell death, respectively. Treatment of the cultures with 1 mM homocarnosine or 5 mM carnosine conferred 70% neuroprotection after 3 hr of OGD insult; exposure to 4 hr of OGD insult conferred 40–55% neuroprotection. OGD insults lasting longer than 5 hr caused almost 100% cell death. At these levels of toxicity, carnosine and homocarnosine did not protect the cells. The neuroprotective effect of the histidine dipeptides required a 12–24 hr exposure of the cultures before the OGD insult (data not shown). Carnosine and homocarnosine concentrations below 1 mM did not protect PC12 cells against OGD insults in accordance with their high concentrations exceeding 1 mM levels in the brain (Nadi et al., 1980; Sobue et al., 1975). The neuroprotective level achieved (Fig. 4) was maximal at these concentrations in our ischemic model.

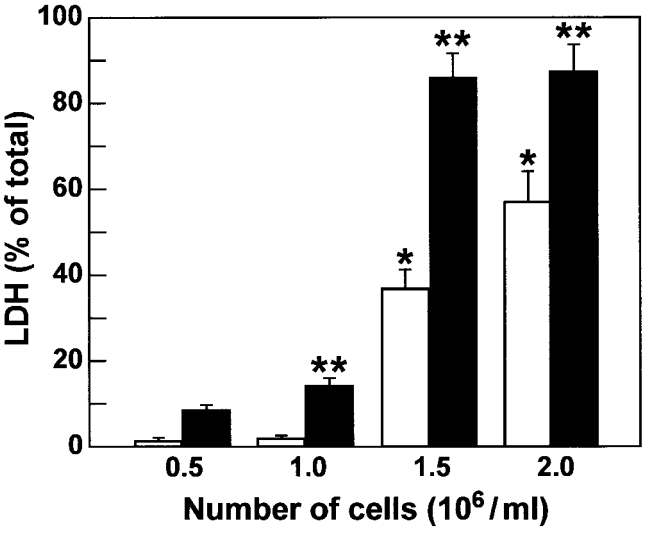

Fig. 2. The effect of cell density and time of exposure to OGD on PC12 cell death. PC12 cultures ($0.5–2 \times 10^6$ cells/dish/ml) were exposed to OGD for 3 hr (□) or 4 hr (■). The cultures were then reoxygenated for 18 hr. The values presented are the mean ± SEM of three independent experiments ($n = 3$). $\star P < 0.05$ compared to normoxia values.

The morphological neuroprotective effects of homocarnosine are presented in Figure 3C. The majority of the cells in the cultures exposed to the OGD insult in the presence of homocarnosine show a similar morphology to that of the control cells. A similar morphological neuroprotective effect was seen with carnosine (data not shown).

In another experiment (Fig. 5), we compared the neuroprotective effect of carnosine with that of tempol, a known antioxidant (Damiani et al., 2000; Rak et al., 2000). Under these OGD conditions, 5 mM carnosine and 1 mM homocarnosine (data not shown) protected against OGD-induced cell death by 50%, an effect similar to that of 0.5 mM tempol.

We previously demonstrated that there is a direct correlation between cell death (LDH release) and stimulation of the arachidonic acid cascade (prostaglandin PGE_2 release) in PC12 cell cultures exposed to OGD (Abu-Raya et al., 1999). Therefore, we evaluated the effect of carnosine and homocarnosine on OGD-induced PGE_2 release from PC12 cells (Table I). These cultures release ~50 pg/ml PGE_2 under normoxic conditions. Upon exposure to OGD, the cells synthesize and release a higher level of ~1,000 pg/ml of PGE_2. Exposure of the cells to the OGD insult in the presence of 1 mM homocarnosine and 5 mM carnosine reduced the level of PGE_2 released into the medium by 60–70%, respectively (Table I). These

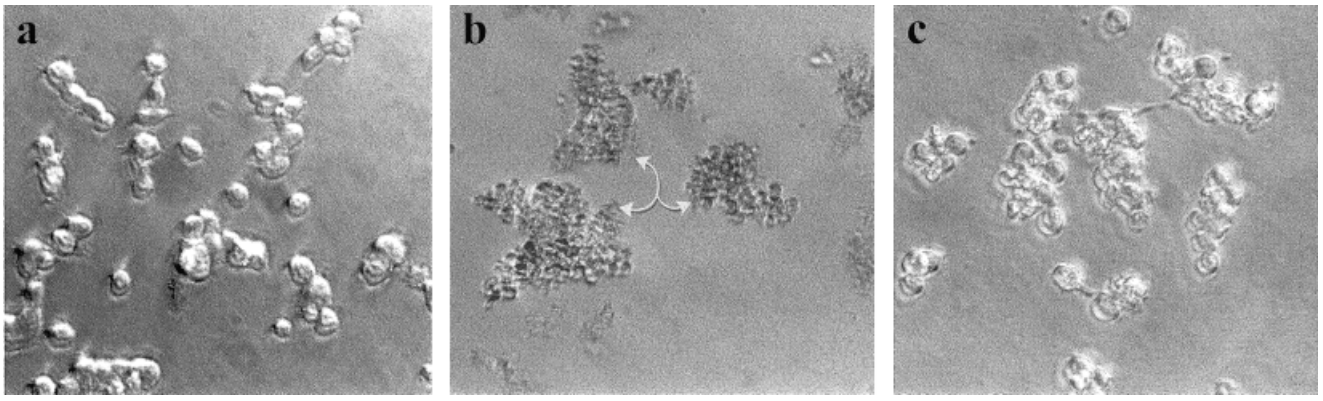

Fig. 3. Light micrographs of PC12 cells exposed to OGD. PC12 cells were maintained at normal conditions (**A**, ×400), or exposed to OGD for 4 hr in the absence (**B**, ×200) or presence of 1 mM homocarnosine (**C**, ×400), followed by reoxygenation for 18 hr.

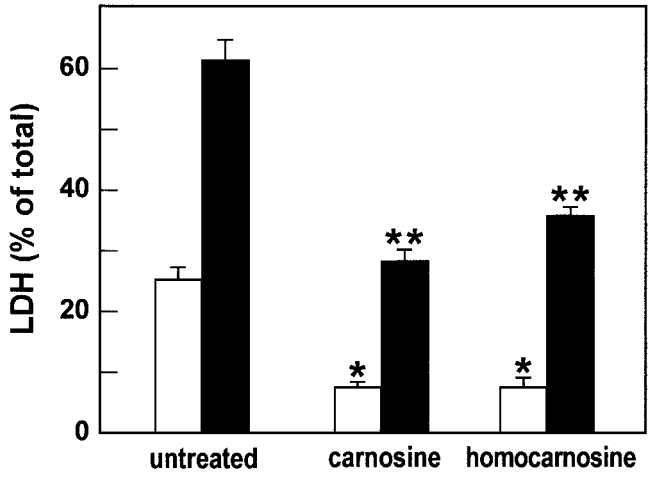

Fig. 4. The effect of carnosine and homocarnosine on OGD-induced cell death. PC12 cultures treated with carnosine (5 mM) or homocarnosine (1 mM) were exposed to OGD for 3 hr (□) or 4 hr (■) followed by reoxygenation for 18 hr. The values presented are the mean ± SEM of a representative experiment ($n = 4$). ⋆,⋆⋆$P < 0.05$ compared to OGD alone.

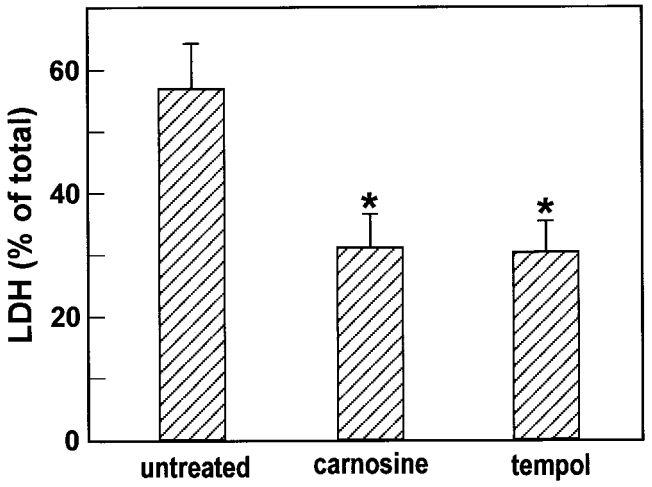

Fig. 5. The effect of carnosine and tempol on OGD-induced cell death. PC12 cultures treated with carnosine (5 mM) or tempol (0.5 mM) were exposed to OGD for 3.5 hr followed by reoxygenation for 18 hr. The values presented are the mean ± SEM of a representative experiment ($n = 6$). ⋆$P < 0.05$ compared to OGD alone.

findings suggest that the lower release of PGE_2 by PC12 cells treated with carnosine or homocarnosine may reflect a direct effect on PGE_2 production or a neuroprotective effect resulting from decreased cell death.

Lack of Neuroprotection of Histidine Dipeptides After Serum-Deprivation Insult

Histidine dipeptides were tested for neuroprotection in another neuronal cell death model - serum deprivation, using a modified procedure (Rukenstein et al., 1991). As a positive control we measured the neuroprotective effect of NGF against serum-deprivation cell death (Rukenstein et al., 1991, Satoh et al., 1995). PC12 cell cultures exposed for 48 hr to serum deprivation showed 40% cell death (Table II). Treatment of PC12 cells with 50 ng/ml NGF during the serum-deprivation insult protected the cells by 50% (Table II). Both carnosine (5 mM) and homocarnosine (1 mM) were not effective, however, indicating lack of neuroprotection afforded by histidine dipeptides in the serum-deprivation PC12 cell death model.

DISCUSSION

The present study focused on two major issues: 1) the establishment of a model system for studying OGD toxicity/neuroprotection effects on PC12 cells in culture and; 2) elucidation of neuroprotective role of the histidine dipeptide-related compounds carnosine and homocarnosine in the model system. We show, for the first time, the neuroprotective effect of carnosine and homocarnosine in the OGD cell death model, using PC12 cells and a novel ischemic device designed in our laboratory.

TABLE I. The Effect of Carnosine on PGE_2 Release From PC12 Cells†

Treatment	Compound	PGE_2[a] (pg/ml)
Normoxia	—	42 ± 5
	Untreated	1039 ± 42
OGD	Homocarnosine	421 ± 76⋆
	Carnosine	313 ± 24⋆

†PC12 cultures treated with carnosine (5 mM) or homcarnosine (1 mM) or left untreated (—) were exposed to OGD 3 hr. The cultures were then reoxygenated for 18 hr. The experiment was performed in triplicate and the data is presented as the mean ± SEM.
[a]The medium was assayed by RIA for PGE_2 content.
⋆$P < 0.05$ compared to OGD alone.

TABLE II. The Effect of Carnosine and Homocarnosine on Serum Deprivation Induced Cell Death in PC12 Cells†

Treatment	LDH (% of total)
Control	41 ± 2
NGF	19 ± 0.34⋆
Carnosine	42 ± 0.52
Homocarnosine	44 ± 2.5

†PC12 cultures treated with carnosine (5 mM), homocarnosine (1 mM) or NGF (50 ng/ml) were exposed to 48 hr serum deprivation. The medium was assayed for LDH. The experiment was performed in triplicate and the data are presented as the mean ± SEM.
⋆$P < 0.05$ compared to the control.

PC12 cells, both undifferentiated and differentiated with NGF have become a popular cellular model for neuroscience research on neuronal diseases such as Parkinson, Alzheimer, hypoglycemia, stroke, and ischemia. Although hypoglycemia can be easily achieved by glucose withdrawal from the culture medium (Tong and Perez-Polo, 1995; Chung and Hong, 1998), ischemic conditions are difficult to be obtained due to the lack of ischemic instruments. Most investigators use special tissue culture incubators connected to argon or N_2/CO_2 gas tanks to mimic in vivo ischemia (Boniece and Wagner, 1993), thereby limiting the use of in vitro ischemic models to highly specialized laboratories. In the present study we describe a simple device causing hypoxic injury. One of the advantages conferred by the OGD system described is the separation of hypoxia and hypoglycemia insults. Exposure of PC12 cells to an hypoxic insult alone (in the presence of glucose 4.5 mg/ml in the medium) revealed seven-fold less cell toxicity than the toxicity achieved upon exposure of PC12 cells to a combined hypoxic and hypoglycemic (absence of glucose) insult. Exposure of PC12 cells to an hypoglycemic insult alone caused about 30% cell toxicity after a long exposure for 24–48 hr. Therefore each individual insult alone causes mild and slow developing toxicity compared to the combined insult in the OGD model. Our OGD "ischemic" model is simple, reproducible, dependent on cell density and the duration of exposure to the insult and is well suited for acute and chronic studies of the mechanisms of hypoxic neuronal cell death and the development of neuroprotective drugs.

The histidine dipeptide related compounds have been known for about a century. A variety of roles and functions were suggested for these agents, which are present in high concentrations in brain tissue and skeletal muscles (Nadi et al., 1980; Scriver et al., 1983). The physiological role of these compounds, however, is not fully understood. The specific localization of these compounds in neurons and glia cells, their high mM concentration as well as their ability to scavenge many ROS (Kohen et al., 1988) suggests that they represent a family of neuroprotective agents in the brain. This notion is supported by our present study in which the neuroprotective effect of histidine dipeptides against OGD-induced cell death was observed both at the morphological and at the biochemical levels (reflected by the decrease in LDH and PGE_2 release from the cells). The neuroprotective effect was evident after the OGD insult but not after serum deprivation. The differential neuroprotective effect of carnosine and homocarnosine against the OGD compared to that against serum-deprivation insult requires further investigation. In view of their antioxidant properties (Boldyrev et al., 1988, 1997; Boldyrev, 1993), it is conceivable that they lower or inhibit the production of ROS. Alternatively, carnosine and homocarnosine might prevent the drop in intracellular pH induced by OGD. The importance of carnosine as a potent pH buffer has been noted previously (Abe, 2000). It was also found that carnosine forms complexes with copper, zinc, cobalt and ferrous ions (Baran, 2000), thereby possibly regulating the concentration of these ions in neurons and protecting them from damage (Horning et al., 2000). The contribution of these ions to OGD induced cell death and the potential neuroprotective effect of carnosine via this mechanism remain to be investigated in the PC12 model.

Carnosine is hydrolyzed at the peptide bond by carnosinase (located mainly in the kidney, liver and blood) (Quinn et al., 1992), acetylated at a free β-amino group (in the brain and heart of some mammals) (O'Dowd et al., 1990), or decarboxylated giving rise to carcinine, (in rat heart muscle) (Fitzpatrick et al., 1991) It will be interesting to verify the formation of these metabolites in PC12 cultures treated with CRCs. In view of the potential neuroprotective effect of CRC, it would be worthwhile to synthesize CRC derivatives resistant to the above degradation processes or with greater antioxidant properties for use in the clinical setting.

Our ischemic PC12 cell model may prove to be a convenient and reliable model for screening, developing and characterization of CRC prototype drugs and investigating their neuroprotective mechanism of action.

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