Induction of tolerance in rat cortical neurons: hypoxic preconditioning

Ulrike Bruer[a], Markus K. Weih[a,*], Nikolaj K. Isaev[b], Andreas Meisel[a], Karsten Ruscher[a], Alexandra Bergk[a], George Trendelenburg[a], Frank Wiegand[a], Ilya V. Victorov[b], Ulrich Dirnagl[a]

[a]*Department of Neurology, Charité Hospital, Humboldt University, Schumannstr. 20–21, 10098 Berlin, Germany*
[b]*Brain Res. Inst., Russian Academy of Med. Science, Moscow, p. Obuha 5, 103064 Moscow, Russian Federation*

Received 10 July 1997

Abstract Sublethal ischemia leads to increased tolerance against subsequent prolonged cerebral ischemia in vivo. In the present study we modeled preconditioning mechanisms in a neuronal-enriched culture. Damage was significantly reduced (up to 72%) with 1.5 h of oxygen-glucose deprivation 48–72 h before 3 h oxygen-glucose deprivation. Tolerance was also elicited by Na^+-K^+-ATPase inhibition. No damage was observed when astroglial or endothelial cells were exposed to hypoxia for 3 and 6 h, respectively. We conclude that hypoxic preconditioning is a robust neuronal phenomenon in vitro with a similar temporal pattern and selective cellular vulnerability as the ischemic tolerance phenomenon shown in vivo.

Key words: Cell culture; Stroke; Ischemia; Ouabain

1. Introduction

Brief ischemia protects against subsequent prolonged cerebral ischemia in vivo, a phenomenon which, first described for the myocardium, has attracted considerable attention in brain research over the last years since its discovery in 1991 [1] (for review, see [2]). As possible underlying molecular mechanisms a great variety of mediators have been postulated: induction of heat-shock proteins [3], changing activity of respiratory enzymes [4], expression of anti-apoptotic factors [5], interleukin-1 [6], NMDA receptors [7] or adenosine receptors and ATP-dependent potassium channels [8].

In addition, tolerance to ischemia has been elicited by spreading depression [9] and inhibition of oxidative phosphorylation [10].

Exact analysis of tolerance mechanisms, however, has been hampered by the complexity of these systems in vivo. Eludication of this phenomenon in vitro might be useful to increase our understanding of drugs designed to prevent or ameliorate cerebral ischemia.

In the present work we developed and established an in vitro model of hypoxic and pharmacologic preconditioning in a neuronal-cell enriched culture as a basis for future studies of induced tolerance to ischemia in the brain.

2. Materials and methods

Neuronal cells were used for experiments after 10–12 days in vitro (DIV). For oxygen-glucose deprivation (OGD) cells were placed in a humidified, temperature controlled (36 ± 0.5°C) chamber at a pO_2 of 2–4 mmHg (5% CO_2/95% N_2) measured by a polarographic electrode (Licox pO_2, GMS, Kiel, Germany). Culture medium was washed out three times with PBS containing Ca^{2+}/$Mg^{2+}$and replaced by a deoxygenated solution containing (in mM): Na^+ 143.8; K^+ 5.5; Ca^{2+} 1.8; Mg^{2+} 0.8; Cl^- 125.3; HCO_3^- 26.2; $H_2SO_4^{2-}$ 1.0; SO_4^{2-} 0.8; glycine 0.01 at pH 7.4. The same solution with 25 mM D-glucose under normoxia served as control. OGD was then started by placing the cells in the hypoxia chamber and terminated by returning the stored culture medium and reexposure to normoxia. Cell injury was assessed using phase contrast microscopy and by measuring lactate dehydrogenase release (LDH) in the supernatant after 4 h, 24 h and 48 h. LDH has been shown to correspond to the number of damaged neurons [11]. The amount of LDH present in the medium supernatant of sham washed cultures was subtracted as background from values of treated cultures and OGD values were normalized to 100%.

Statistical difference between groups was calculated using an unpaired Student's *t*-test.

Cortical neurons were prepared from whole cerebral cortices of fetal Wistar rats (E16–17) as described previously [12]. After removing of the meninges the tissue was minced and digested with trypsin (0.005%; 0.002% EDTA; 15 min; 37°C) followed by mechanical dissociation. Cells were seeded in 4-well plates (250 000/cm^2) and grown in neurobasal medium with B-27 serum-free supplement (Gibco, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin and 2 mM L-glutamine. 80–90% of cells stained positive for neuronal specific enolase (NSE), 10–15% of cells for glial fibrillary acidic protein (GFAP) and less than 5% for OX 42, suggesting the presence of microglial cells. Antibodies were purchased from DAKO, Hamburg, Germany, media and supplements from Biochrom KG, Berlin, Germany, unless otherwise noted.

Primary cultures of cerebral endothelial cells were prepared from 3-week-old Wistar rats as described previously [13] and used for experiments at DIV 6–8.

Highly purified astroglial cell-cultures were prepared as described previously [14] and used for experiments at DIV 8–10.

For DNA laddering as an indicator of apoptosis, approximately 10^6 cells were harvested after an OGD interval of 180 min and following reoxygenation periods of 24 and 48 h, respectively. DNA was extracted using a modified commercial Easy-DNA Kit (Invitrogen, Netherlands). After resuspension in 80 μl TE-buffer (10 mM Tris, 1 mM EDTA, pH 7.8) DNA concentration was measured by the absorbance at 260 nm. For each lane 0.25 μg of DNA were [γ-^{32}P]ATP-labeled by T4 polynucleotide kinase (NEB) reaction at 37°C for 30 min. DNA was loaded on a 1% agarose gel and separated at 70 V for 180 min. After separation, gel was vacuum-dried and exposed afterwards to an X-ray film (Hyperfilm ECL, Amersham) film at −70°C for 24 h. For quantification of fragmented DNA, bands on the dried gel were analyzed by a Phosphoimager (Molecular Dynamics, CA).

3. Results

Ninety min oxygen-glucose deprivation (OGD) did not cause the LDH-release or morphological alterations of neurons. In contrast, 180 min hypoxia increased LDH release by 70 ± 8% at 48 h after hypoxia. Under phase contrast microscopy 70–90% of neurons appeared degenerated. Since many cells exhibited vacuolization and nuclear condensation we tested whether this might be due to apoptotic mechanisms.

*Corresponding author. Fax: (49) (30) 2802-8810 or -5047.
E-mail: mweih@neuro.charite.hu-berlin.de

PII S0014-5793(97)00954-X

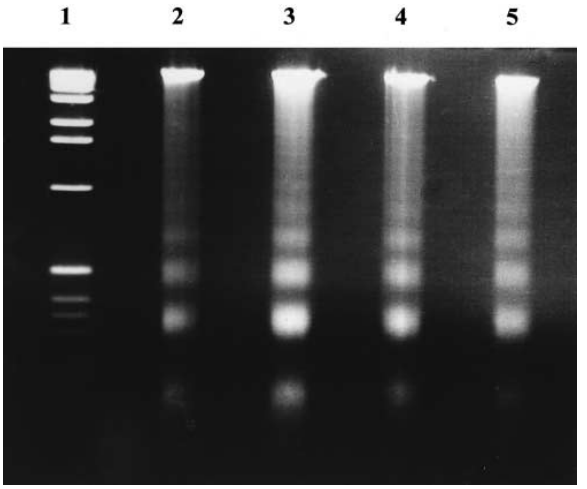

Fig. 1. DNA fragmentation from primary cortical neurons after OGD. Cortical neurons were stimulated by OGD. After 24 and 48 h total DNA was extracted and 0.25 μg of DNA were separated on a 1% agarose gel, and stained with 50 μl/l ethidium bromide. Lanes: 1, 1-kbp ladder; 2 and 4, control after 24 and 48 h, respectively; 3 and 5, DNA fragmentation 24 and 48 h after 180 min OGD.

As shown in Fig. 1 and Table 1, DNA fragmentation was already observed in the control and further augmented after 180 min of OGD followed by 24 h and 48 h reoxygenation, respectively. At 24 h after OGD a 3.4-fold increase in typical DNA ladder pattern with oligonucleosome-sized fragments of 180 base pairs was observed. An adjustment of DNA fragmentation of stimulated and control cells was detectable at 48 h after hypoxia.

Table 1
Semiquantitative analysis of fragmented DNA

Fragmented-to-non-fragmented DNA ratio		
	Time after OGD	
	24 h	48 h
Control	140.9 (100%)	127.1 (100%)
OGD	491.7 (348%)	193.1 (152%)

0.25 μg of DNA were [γ-^{32}P]ATP-labeled by T4 polynucleotide kinase, loaded on a 1% agarose gel. Separated bands were analyzed by a Phosphoimager. Data presented as a ratio between fragmented and unfragmented DNA in control and hypoxic stimulated cells. Afterwards DNA fragmentation of stimulated cells is presented as a percentage of values found in control.

Substantial neuroprotection was observed by phase-contrast microscopy when cells were preconditioned by 90 min hypoxia 72 h prior to a 180 min hypoxia (Fig. 2 shows representative images). In some cases almost complete protection by a preconditioning stimulus was observed when only morphologic criteria were employed. The extent of neuronal injury after 180 min. OGD varied between different primary cultures but was comparable between sister cultures from a single plating. For all subsequent experiments the interval between preconditioning OGD (90 min) and prolonged OGD (180 min) was varied between 24 h, 48 h and 72 h.

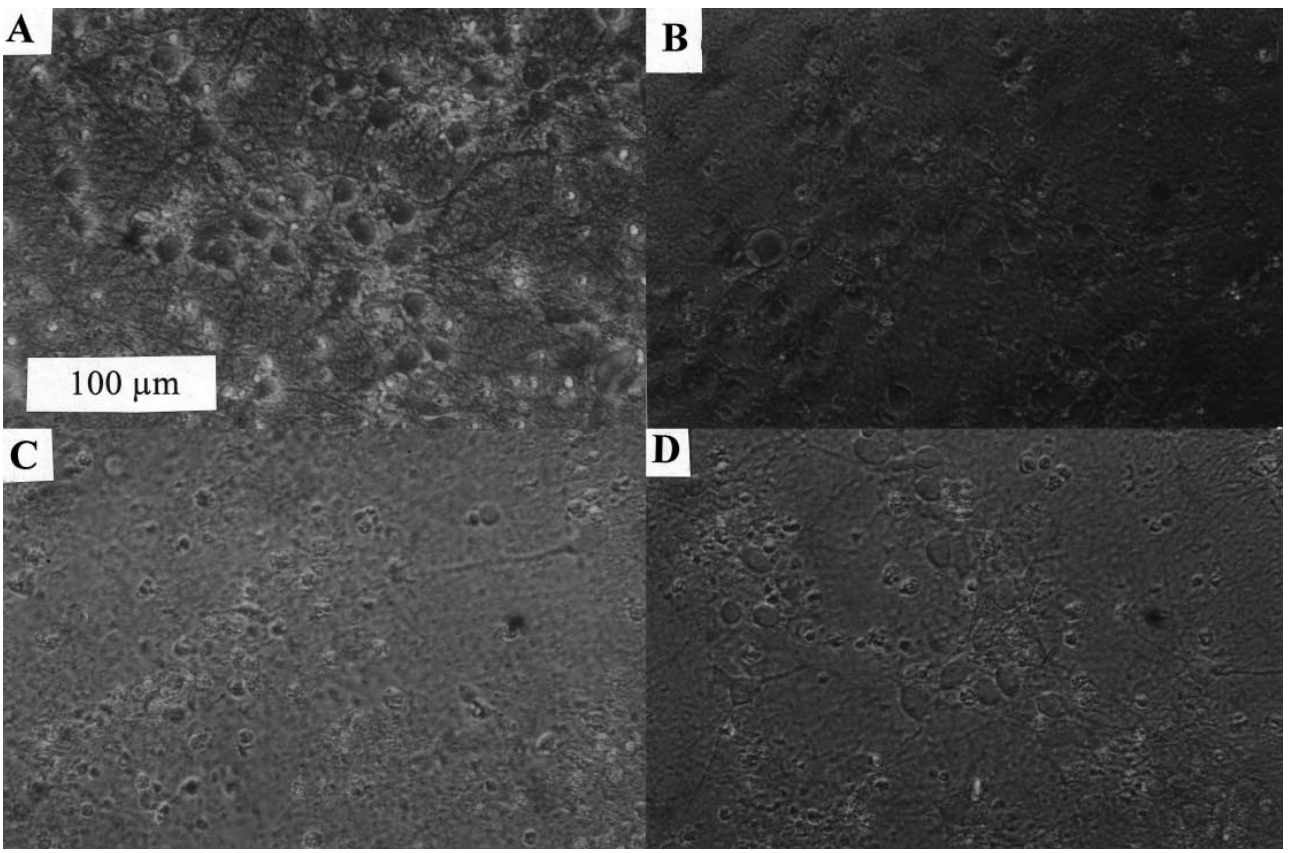

Fig. 2. Hypoxic preconditioning of cultured neuronal cells. Phase contrast photomicrographs: (A) neuronal cells at DIV 10 (control); (B) 24 h after 1.5 h OGD; (C) 24 h after 3 h OGD; (D) 1.5 h OGD 72 h before 3 h OGD; each of the images was recorded 24 h after OGD.

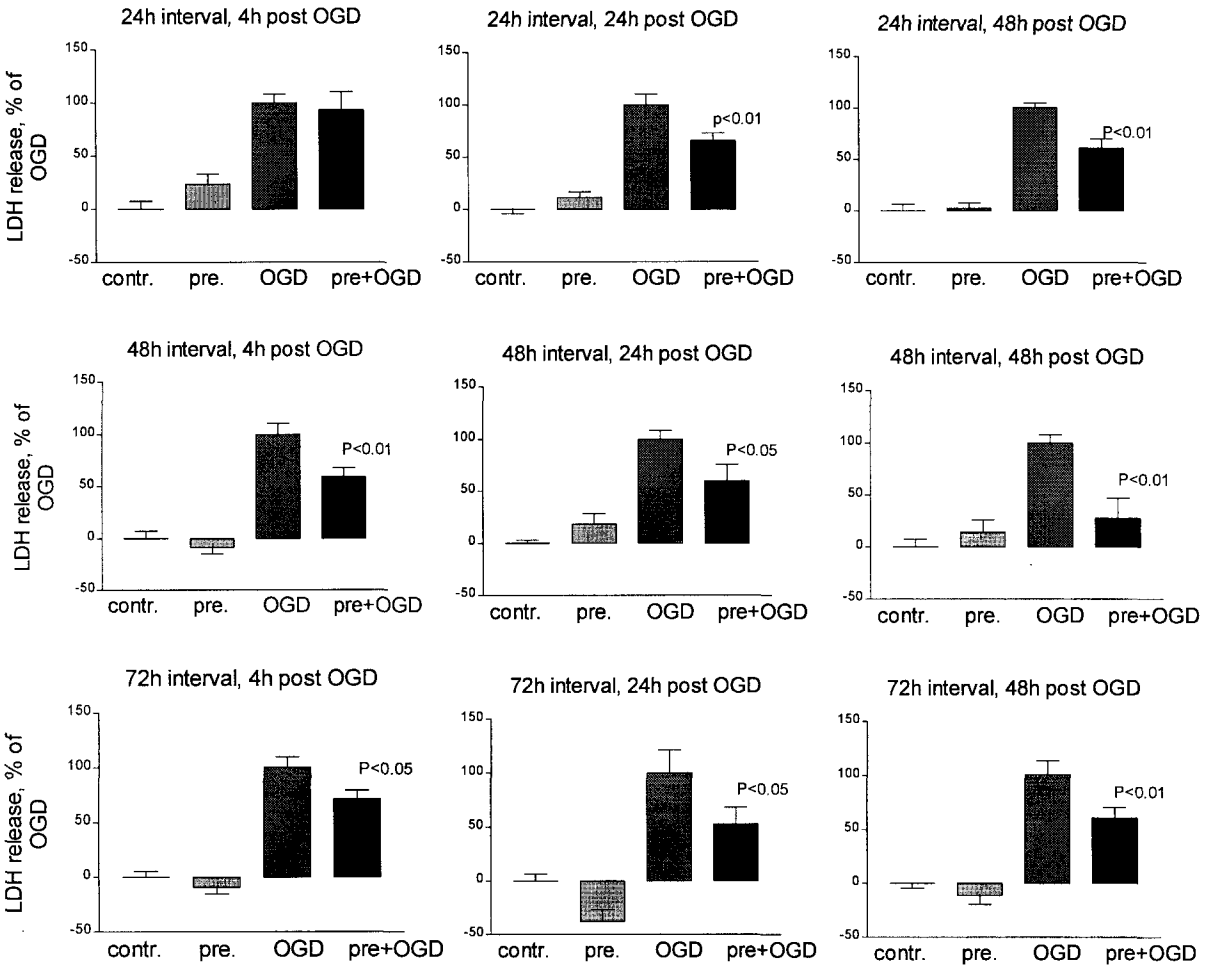

Fig. 3. Hypoxic preconditioning of cultured neuronal cells (LDH release): control = sham wash; pre. = 1.5 h OGD alone; hyp. = 3 h OGD alone; pre+hyp = 1.5 h OGD 24 h (first row); 48 h (second row) and 72 h (last row) before 3 h OGD. LDH levels after OGD varied between 103 and 268 U/ml and were normalized to 100%. Data are expressed as means ± standard error.

Shorter intervals than 24 h did not induce tolerance (data not shown).

Hypoxic LDH-release was attenuated by 6 ± 17%, 34 ± 7% and 39 ± 9% (24 h interval); 41 ± 9%, 40 ± 16% and 72 ± 19% (48 h interval) and 38 ± 9%, 62 ± 14% and 55 ± 11% (72 h) 4 h, 24 h or 48 h after prolonged OGD, respectively (Fig. 3). Note that for some time points (e.g. 72 h interval) there is a tendency towards neuroprotection by preconditioning alone as compared to control. Values are means from 8–16 values from at least three independent experiments ± standard error.

To further address the question whether induction of tolerance might be due to disturbance of ionic homeostasis, cells were pretreated for 2 h with 10 μM ouabain, a potent Na^+-K^+-ATPase inhibitor [15]. Hypoxic LDH-release was attenuated by 48 ± 13%, 25 ± 7% and 34 ± 14% (24 h interval) and 0 ± 11%, 45 ± 19% and 13 ± 15% (48 h interval) 4 h, 24 h or 48 h after prolonged hypoxia, respectively (Fig. 4). Statistical difference, however, was reached only for the 24 h interval. The ouabain concentrations used showed no toxicity (data not shown).

LDH release from cerebral endothelial cells decreased from 49.6 ± 1.7 to 24.6 ± 1.4 U/ml 24 h after 3 h OGD. Astrocytes released 58.2 ± 1 U/ml LDH in the control group and 57 ± 5 U/ml LDH 24 h after 6 h of OGD (data are means ± standard error).

4. Discussion

The fact that short hypoxic periods in the central nervous system induce tolerance to otherwise lethal hypoxia remains mechanistically and therapeutically interesting, but enigmatic. To our knowledge, in contrast to the growing in vivo results there are few in vitro data regarding hypoxic tolerance. Sakaki et al. [16] reported induction of tolerance by 4 h of mild hypoxia (calculated pO_2 of 40 mmHg) in undifferentiated neuronal cells at 24 h in vitro and a unknown proportion of non-neuronal cells present.

Lobner at al. [17] reported pharmacologic preconditioning with protein synthesis inhibitors in a mixed cortical culture at 14–16 days in vitro which was possibly due to reduced glutamate release.

Compatible with results shown in hypoxic brain slices [10] and in vivo models of global [1] and focal ischemia [18] and F. Wiegand and U. Dirnagl (personal communication) we found that brief, non-lethal oxygen-glucose deprivation can protect neurons from hypoxic damage.

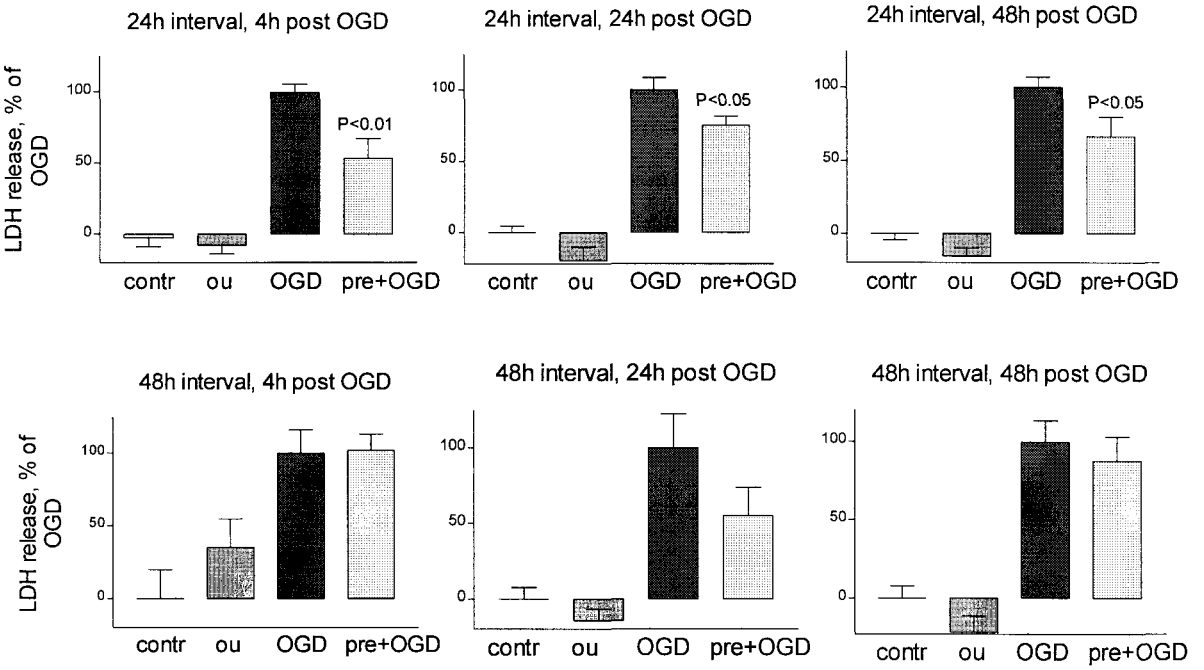

Fig. 4. Ouabain preconditioning of cultured neuronal cells (LDH release): control = sham wash; ou = 2 h ouabain 10 μM alone; hyp. = 3 h OGD alone; ou+hyp = 2 h ouabain 24 h (first row) or 48 h (second row) before 3 h OGD. Data are expressed as means ± standard error.

In addition we suggest that preconditioning is rather a neuronal phenomenon since astrocytes represent only a minor population and are not damaged by hypoxia in our system. However, we cannot rule out that astrocytes 'sense' hypoxia and subsequently produce neuroprotective or neurotrophic factors. In accordance to in vivo systems, neurons in vitro seem to maintain their 'selective vulnerability' as compared to other CNS cell types like astrocytes [19] or endothelial cells which have been shown to tolerate more prolonged hypoxic phases [20].

We observed that prolonged hypoxia seems to activate DNA fragmentation in our system. However, apoptosis is an inherent phenomenon in neuronal cell cultures [21]. We believe that our system, which can be maintained viable maximally 20 days (death occurs possibly due to a lack of trophic factors normally provided by astroglial cells), might be useful to study preconditioning but may not be less suited to distinguish necrosis from apoptosis after hypoxia.

In addition we demonstrated that preconditioning can also be elicited pharmacologically by ouabain, albeit to a lesser extent. We can only speculate why ouabain leads to tolerance induction. Inhibition of Na^+-K^+-ATPase by ouabain leads to imbalance in ionic homeostasis and might therefore mimic ischemic or hypoxic conditions [15]. Ouabain is a well established cardiac glycoside [22] with binding sites found also in brain [23]. Future studies will be needed to test whether this substance might be an interesting candidate for pharmacologic preconditioning.

In summary we have shown that induction of ischemic tolerance can be modeled in vitro. Our neuronal cell enriched system showed preconditioning within a time frame and with a 'selective' cellular vulnerability compatible to in vivo conditions. Future studies are needed to delineate the 'point of no return', where lack of oxygen turns from an signal in to an lethal condition. In future our model might prove useful to examine selective cellular expression of genes involved in regulation of apoptosis and or neurotrophic factors.

Acknowledgements: This work was supported by DFG ME 1562/1-1 and DFG Di 454/8-1.

References

[1] Kitagawa, K., Matsumoto, M., Kuwabara, K., Tagaya, M., Ohtsuki, T., Hata, R., Ueda, H., Handa, N., Kimura, K. and Kamada, T. (1991) Brain Res. 561, 203–211.
[2] Chen, J.C. and Simon, R. (1997) Neurology 48, 306–311.
[3] Liu, Y., Kato, H., Nakata, N. and Kogure, K. (1993) Neuroscience 56, 921–927.
[4] Dagani, F., Marzatico, F., Curti, D., Zanada, F. and Benzi, G. (1984) J. Cereb. Blood Flow Metab. 4, 615–624.
[5] Shimazaki, K., Ishida, A. and Kawai, N. (1994) Neurosci. Res. 20, 95–99.
[6] Ohtsuki, T., Ruetzler, C., Tasaki, K. and Hallenbeck, J. (1996) J. Cereb. Blood Flow Metab. 16, 1137–1142.
[7] Kato, H., Liu, Y., Araki, T. and Kogure, K. (1992) Neurosci. Lett. 139, 118–121.
[8] Heurteaux, C., Lauritzen, I., Widmann, C. and Lazdunski, M. (1995) Proc. Natl. Acad. Sci. USA 92, 4666–4670.
[9] Matsushima, K., Hogan, M.J. and Hakim, A.M. (1996) J. Cereb. Blood Flow Metab. 16, 221–226.
[10] Riepe, M.W., Esclaire, F., Kasischke, K., Schreiber, S., Nakase, H., Kempski, O., Ludolph, A.C., Dirnagl, U. and Hugon, J. (1997) J. Cereb. Blood Flow Metab. 17, (3) 257–264.
[11] Koh, J.Y. and Choi, D.W. (1987) J. Neurosci. Methods 20, 83–90.
[12] Weikert, S., Freyer, D., Weih, M., Isaev, N., Busch, C., Schultze, J., Megow, D. and Dirnagl, U. (1997) Brain Res. 748, (1-2) 1–11.
[13] Abbott, N.J., Hughes, C.C., Revest, P.A. and Greenwood, J. (1992) J. Cell Sci. 103, 23–37.
[14] Bowman, P.D., Ennis, S.R., Rarey, K.E., Betz, D.L. and Goldstein, G.W. (1983) Ann. Neurol. 14, 396–402.
[15] Santos, M.S., Moreno, A.J. and Carvalho, A.P. (1996) Stroke 27, 941–950.
[16] Sakaki, T., Yamada, K., Otsuki, H., Yuguchi, T., Kohmura, E. and Hayakawa, T. (1995) Neurosci. Res. 23, 289–296.

[17] Lobner, D. and Choi, D.W. (1996) Neuroscience 72, 335–341.
[18] Kitagawa, K., Matsumoto, M., Tagaya, M., Hata, R., Ueda, H., Niinobe, M., Handa, N., Fukunaga, R., Kimura, K. and Mikoshiba, K. et al. (1990) Brain Res. 528, 21–24.
[19] Yu, A.C., Gregory, G.A. and Chan, P.H. (1989) J. Cereb. Blood Flow Metab. 9, (1) 20–28.
[20] Hess, D.C., Zhao, W., Carroll, J., McEachin, M. and Buchanan, K. (1994) Stroke 25, 1463–1467.
[21] Behrens, M.I., Koh, J., Canzoniero, L.M., Sensi, S.L., Csernansky, C.A. and Choi, D.W. (1995) Neuroreport 6, 545–548.
[22] Hamlyn, J.M., Hamilton, B.P., Manunta, P., Lathers, C.M., Lipka, L.J. and Klions, H. (1992) J. Hypertens. 10, S99–111.
[23] Harik, S.I., Mitchell, M.J., Kalaria, R.N., Leenen, F.H., Huang, B.S., Yu, H., Yuan, B., Koyama, Y., Ishibashi, T., Iwata, H., Baba, A., Peng, J.H., Zeng, Y.C., Parker Jr., J.C., Maki, A.A., Baskin, D.G. and Stahl, W.L. (1992) Arch. Neurol. 40, 771–779.