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Effect of lipid peroxidation conditions on calcium-dependent activity of phosphodiesterase 3',5'-cAMP in the rat brain

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Abstract: 3',5'-cAMP plays an important role as a second messenger molecule controlling multiple cellular processes in the brain. Its levels are decreased by phosphodiesterases (PDEs), responsible for hydrolysis of intracellular cAMP. A part of the PDE activity is dependent on the effect of calcium, mediated by its binding to calmodulin. During oxidative stress, precisely these changes in calcium concentration are responsible for cell damage. We have examined the effects of oxidative stress conditions on the activity of PDE in rat brain homogenates. We found a different influence of activated lipid peroxidation conditions (Fe^{2+} with ascorbate and increased temperature) on the calcium-dependent and calcium-independent PDE activity. The inhibition of Ca^{2+}-dependent PDE was observed, while Ca^{2+}-independent PDE was not influenced. We assume that it might be the impact of lipid peroxidation products or any mechanism activated by the higher temperature on the interaction of the Ca^{2+}-dependent isoform of PDE with the complex calcium-calmodulin. Another explanation might be that the formation of the functioning calcium-calmodulin complex is impossible in these conditions.

Key words: phosphodiesterase; 3',5'-cAMP; lipid peroxidation; calcium-calmodulin.

Abbreviations: LPX, lipid peroxidation; PDE, phosphodiesterase.

Introduction

As in other tissues, oxidative stress can damage neurones and glia by several mechanisms including increased lipid peroxidation (LPX), oxidative damage of DNA, damage of proteins and induction of apoptosis and necrosis.

The brain and nervous tissues are sensitive to oxidative damage for several reasons: (1) The high input of oxygen can generate oxygen radicals. (2) Neuronal membrane lipids contain a high amount of polyunsaturated fatty acids, which are substrates for LPX. (3) The antioxidant capacity of the brain is low, ascorbate being the most important antioxidant in the central nervous system (Halliwell & Gutteridge, 1999). (4) Iron was found throughout the brain. Several brain areas (e.g. substantia nigra, globus pallidum, caudate nucleus) have high iron content (Halliwell & Gutteridge, 1999) that in ionized state is an important factor of oxidative stress in tissues. A linear relationship was found between endogenous iron content in brain regions and their ability to produce lipid peroxides *in vitro* (Zaleska & Floyd, 1985). (5) The increase of intracellular calcium concentration across neuronal membranes, caused by decreased ATP amount, is considered to be an important factor promoting cell damage by radical production (Ošťádal et al., 1999; Arundine & Tymianski, 2003).

The excessive Ca^{2+} influx into a cell and its suppressed outflow during oxidative stress state trigger metabolic cascades. This lead to (i) damage of proteins after activation of Ca^{2+}-dependent proteases (Ceňa et al., 2004), as well as of lipids via activation of Ca^{2+}-dependent phospholipases; and (ii) change of activities of kinases, phosphatases, endonucleases (Siesjő et al., 1995), NO-syntase (Kuneš et al., 2004*)* and other calcium dependent processes.

In addition to the direct effect of calcium on a cell, its influence can be realized after its binding to Ca^{2+}-binding proteins, e.g. calmodulin (James et al., 1995). The increase in cytosolic Ca^{2+} has effect on more processes either directly, or by second messengers, e.g. 3',5'-cAMP (Samigullin et al., 2005). In neurons, the cAMP level, which is increased in response to neurotransmitters and neuropeptides, is negatively regulated by the cyclic nucleotide phosphodiesterases (PDEs) that hydrolyse cAMP to 5'-AMP. Therefore, PDEs are important regulators of the cAMP signaling in cells. In mammalian cells, PDEs are classified into 11 families based on sequence similarity, inhibitor sensitivity and

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biochemical properties (SASAKI et al., 2004). Only some of them are calcium-dependent. PDE1s were among the first identified targets for calmodulin and their isoenzymes from the brain, heart and lungs showed differences in the affinity for calmodulin (CONTI, 2000).

The enzyme 3',5'-cAMP PDE participates in decreasing of cAMP, the important signaling molecule in the central nervous system. In addition, the activity of PDE1 family is regulated by the calcium-calmodulin complex; the level of calcium being changed during the oxidative stress. The aim of the present study was to investigate the possible effect of the oxidative stress conditions on 3',5'-cAMP PDE activity in the rat brain tissue.

Material and methods

Tissue preparation

Adult, male Wistar rats (7 animals in each group), weighing 300–400 g, fed on commercial rat food and water *ad libitum*, were used in our experiments. After decapitation, the brain was rapidly removed and 1% homogenates were prepared in 33 mM Tris-HCl (pH 7.5) at 0 °C.

Determination of 3',5'-cAMP phosphodiesterase activity

Phosphodiesterase (EC 3.1.4.17) activity was measured according to the method of CHEUNG (1967). The total PDE activity was measured in the reaction mixture containing 0.5 mM 3',5'-cAMP, 5 mM $MgCl_2$, 33 mM Tris-HCl (pH 7.5), 0.1 mM EDTA and 0.3 mM $CaCl_2$.

The Ca^{2+}-independent PDE activity was assayed in the reaction mixture containing 3',5'-cAMP, $MgCl_2$, Tris-HCl (pH 7.5), in the above-mentioned concentrations, with addition of 0.1 mM EDTA – for chelating of endogenous calcium. The Ca^{2+}-dependent PDE activity was calculated as a difference between the total activity and the Ca^{2+}-independent activity.

PDE activities were determined immediately after preparation of brain homogenate (0 minute) and after pre-incubation of brain homogenates for 30 min at 37 °C or 41 °C, without or with the LPX effectors (3:1, homogenate to effector). The used effectors were: group I – without effector, group II – with 125 μM ascorbate, and group III – with activator of LPX (125 μM ascorbate + 5 μM $FeSO_4$). These were, in special series of experiments, combined with 10 μM deferoxamine mesylate, the chelator of Fe^{3+} ions.

The reaction of PDE was started with 0.05 mL of 1% brain homogenate that was immediately or after the pre-incubation added to 0.35 mL of a particular reaction mixture with or without exogenous Ca^{2+} . The samples were incubated at 37 °C for 10 min, during which the brain PDE cleaved 3',5'-cAMP. Then, 0.05 mL of snake venom (Crotalus Atrox), containing enzyme 5'-nucleotidase, was added to each test tube. The mixtures were incubated for additional 5 min at 37 °C in order to release inorganic phosphate from 5'-AMP. The reaction was stopped with 0.05 mL of 60% trichloracetic acid. After centrifugation at 1000 × *g* for 10 min, inorganic phosphate was determined spectrophotometrically at 760 nm in supernatants according to AMES (1966).

Calibration graph was drawn using KH_2PO_4 as the standard solution in Tris-HCl (pH 7.5) in the calibration range 8–32 nmols, treated in the same way as samples during determination of inorganic phosphate.

Activities of PDE are expressed in nkat per mg of proteins. Proteins were measured according to LOWRY et al. (1951). The results are given as means ± SEM. Statistical analysis was performed using the Student's t-test.

Results

In conditions without any pre-incubation of samples (0 minute), ascorbate, added alone or together with Fe^{2+} ions, did not significantly influence any form of PDE activity in comparison to samples with no effector (Table 1).

After pre-incubation of homogenates at 37 °C, the Ca^{2+}-dependent PDE activity was significantly decreased in the condition with the activator of LPX (by 33%). Total PDE and Ca^{2+}-indepedent PDE were not significantly altered at this temperature (Table 1).

After the pre-incubations of brain homogenates at 41 °C (Table 1): (i) without effector of LPX, changes of the total, Ca^{2+}-independent and Ca^{2+}-dependent PDEs were measured in comparison to samples with no pre-incubation (0 minute). In fact, the decrease of the total PDE (by 30%) and Ca^{2+}-dependent PDE (by 70%) were statistically significant; (ii) with ascorbate alone, all forms of determined PDE were decreased in comparison to samples with no pre-incubation (0

Table 1. Effect of LPX and temperature on activities of PDE 3',5'-cAMP in rat brain homogenates.[a]

Pre-incubation			Total PDE	Ca^{2+}-independent PDE	Ca^{2+}-dependent PDE
no effector (I)	0 minute		1.28 ± 0.09	0.90 ± 0.06	0.38 ± 0.04
	30 minute at	37 °C	1.38 ± 0.13	1.04 ± 0.09	0.34 ± 0.04
		41 °C	0.92 ± 0.07**	0.79 ± 0.06	0.13 ± 0.04***
with ascorbate (II)	0 minute		1.29 ± 0.12	0.87 ± 0.07	0.42 ± 0.05
	30 minute at	37 °C	1.37 ± 0.34	1.03 ± 0.13	0.34 ± 0.06
		41 °C	1.00 ± 0.13**	0.82 ± 0.13	0.18 ± 0.05***
with LPX activator (III)	0 minute		1.23 ± 0.09	0.80 ± 0.05	0.43 ± 0.04
	30 minute at	37 °C	1.48 ± 0.10	1.18 ± 0.08	0.30 ± 0.06***
		41 °C	0.99 ± 0.06**	0.99 ± 0.08	0.00 ± 0.05***

[a]Results are presented as means ± SEM of 7 experiments; $^{**}p < 0.005$, $^{***}p < 0.001$: decrease of enzyme activity after pre-incubation versus non-pre-incubated samples (0 min) in a particular group (I, II or III).

minute); however, the significant decrease was measured in total PDE (by 23%) and Ca^{2+}-dependent PDE (by 58%); and (iii) with the activator of LPX, total PDE and Ca^{2+}-dependent PDE were significantly decreased comparing to non-pre-incubated samples (0 minute); Ca^{2+}-indepedent PDE was not significantly influenced, whereas the Ca^{2+}-dependent PDE activity was completely decreased in these conditions.

The effect of deferoxamine mesylate on the PDE activity was determined at 41 ℃, because the higher protective effect of deferoxamine mesylate on LPX was found (Ďurfinová et al., 2003) at this temperature (in comparison with 37 ℃). However, no significant influence of deferoxamine mesylate (10 μM) on any form of the brain PDE activity was found in the conditions of non-activated or activated LPX (ascorbate or complete activator of LPX) at 41 ℃.

Discussion

The determination of PDE activities in rat brain homogenates in conditions of activated LPX was used as a model approach for monitoring the impact of the LPX state on enzyme activities regulated by the calcium-calmodulin complex. We focused on the PDE activity because there were identified Ca^{2+}-independent as well as Ca^{2+}-dependent families of PDEs. The Ca^{2+}-dependent PDE connects two different signaling pathways, i.e. cAMP/cGMP and calcium (Conti, 2000).

We found a decrease of the total and Ca^{2+}-dependent PDE in the conditions of LPX activated by temperature and LPX effectors. This may be explained either by processes of LPX or some other mechanisms (e.g. proteolysis), both of which are activated during the incubation of tissue homogenates. One of the factors that activate LPX *in vitro* (Kozinková et al., 1998) and *in vivo* (Yang & Lin, 2002) is an elevated temperature. The amount of LPX products was increased 3.5-times at 41 ℃ at the presence of ascorbate in rat brain homogenates (Ďurfinová et al., 2003). We assume that the rise in peroxidation at the higher temperature might be caused by the increased ionized iron concentration. Iron, in the form of Fe^{2+} , enables the production of radicals, simultaneously being oxidated to Fe^{3+} (Štípek et al., 2000). Its subsequent reduction to Fe^{2+} (e.g. by ascorbate) stimulates further generation of radicals.

In this paper, we present a decrease of Ca^{2+}-dependent PDE and no influence of LPX conditions activated by temperature on Ca^{2+}-independent PDE. This finding might indicate some influence of LPX, its products or processes activated by temperature (41 ℃) on the interaction between calmodulin and Ca^{2+}-dependent PDE.

We consider it likely that this intervention of LPX or any influence of temperature in the mutual interaction between the Ca^{2+}-dependent isoform of the enzyme and calcium-calmodulin complex leads to a change or loss of the Ca^{2+}-dependent enzyme activity.

Reactive oxygen species, generated in excess, can directly damage neurons by different mechanisms. For example, by direct oxidation of amino acid residues in proteins, by some LPX products (malondialdehyde, 4-hydroxynonenal), which covalently bind to the -NH_2 groups of amino acids (Štípek et al., 2000), or by inhibition of intracellular/membrane bound enzymes with a consequent loss of ionic homeostasis (Vlkolinský & Štolc, 1999). We assume that the impact of LPX on PDE could be caused by the binding of LPX products to -NH_2 groups of regulatory domains either of the enzyme or calmodulin. The -NH_2 groups of regulatory domains might by "uncovered" by the conformation changes of the enzyme at the hyperthermia (41 ℃). Moreover, another possibility might include other conformation changes of the enzyme (caused by the elevated temperature), which could prevent the binding of the calcium-calmodulin complex to PDE. We suppose that the temperature did not influence the calmodulin, because it is considered to be a thermostable protein (Iurkov, 1981).

The calcium-calmodulin complex participates in the regulation of a number of enzymes (e.g. phosphorylase kinase, NO synthase, adenylate cyclase, myosin light chain kinase, calcium/calmodulin kinase I, II) involved in metabolic and functional processes. Therefore, the impact of higher temperature and/or LPX conditions on Ca^{2+}-dependent PDE activity may also be applied to other calmodulin-regulated enzyme systems.

In hypoxia and reoxygenation-mediated neuronal injury, an important role is ascribed to the ionized iron, which may participate in Fenton-type reactions and thus catalyze the generation of reactive oxygen species (Oubidar et al., 1994). Deferoxamine mesylate chelates ionized iron in Fe^{3+} state (Halliwell & Gutteridge, 1999), thus making it unavailable for catalytic action in a Fenton type reaction. In our previous experiments, we found that 10 μM deferoxamine mesylate inhibited the processes of LPX activated by iron + ascorbate by 82% at 41 ℃ in brain homogenates (Ďurfinová et al., 2003). In the present study, we did not detect any protective effect of 10 μM deferoxamine mesylate on any form of PDE activity after preincubation of the brain homogenate with the LPX activator at 41 ℃. Deferoxamine mesylate (10 μM) caused the inhibition of activated LPX, but it did not protect Ca^{2+}-dependent PDE in these conditions. Thus we suppose that the "loss" of the Ca^{2+}-dependent PDE activity was caused by the hyperthermia-activated processes.

In summary, the present study suggests that in conditions of the oxidative stress, caused by its activators and increased temperature, the total and the Ca^{2+}-dependent PDE activities in the rat brain were decreased. In these conditions, Ca^{2+}-independent PDE was not influenced. No protective effect of deferoxamine mesylate, the chelator of iron ions, was detected on the Ca^{2+}-dependent PDE in rat brain homogenates.

The brain, compared with other organs, seems to be very vulnerable to oxidative damage during a fever (MIURA et al., 1998). The inhibition of Ca^{2+}-dependent PDE activity in rat brain homogenates at higher temperature might thus point to the impact of hyperthermia on other calcium-calmodulin mediated processes.

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References

AMES, B.N. 1966. Assay of inorganic phosphate, total phosphate and phosphates. Methods Enzymol. **8:** 115–118.

ARUNDINE, M. & TYMIANSKI, M. 2003. Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. Cell Calcium **34:** 325–337.

CEÑA, V., FERNÁNDEZ, M., GONZÁLEZ-GARCIA, C. & JORDAN, J. 2004. Stroke and ischemic insults; chapter 34. In: HERDEGEN, T. & DELGADO-GARCIA, J.M. (eds) Brain Damage and Repair, Kluwer Academic Publishers, Dordrecht.

CHEUNG, W.Y. 1967. Properties of cyclic 3',5'-nucleotide phosphodiesterase from rat brain. Biochemistry **6:** 1079–1087.

CONTI, M. 2000. Phosphodiesterases and cyclic nucleotide signaling in endocrine cells. Mol. Endocrinol. **14:** 1317–1327.

ĎURFINOVÁ, M., BRECHTLOVÁ, M., KURAČKA, Ľ. & LÍŠKA, B. 2003. Consequences of iron and some factors that affect its action in activation of lipid peroxidation. Psychiatrie **7:** 28–29.

HALLIWELL, B. & GUTTERIDGE, J.M.C. 1999. Free Radicals in Biology and Medicine, Third Ed., Oxford University Press, Oxford, 936 pp.

IURKOV, V.A. 1981. Characteristics of cyclic nucleotide phosphodiesterase and calmodulin in the small intestinal mucosa of rabbits. Biull. Eksp. Biol. Med. **92:** 91–94.

JAMES, P., VORHERR, T. & CARAFOLI, E. 1995. Calmodulin-binding domains: just two faced or multi-faceted? Trends Biochem. Sci. **20:** 38–42.

KOZINKOVÁ, M., REGENDOVÁ, M., BRECHTLOVÁ, M. & LÍŠKA, B. 1998. Effect of some factors on lipid peroxidation in rat brain homogenates. Chem. Papers **52:** 531–532.

KUNEŠ, J., HOJNÁ, S., KADLECOVÁ, M., DOBEŠOVÁ, Z., RAUCHOVÁ, H., VOKOURKOVÁ, M., LOUKOTOVÁ, J., PECHÁŇOVÁ, O. & ZICHA, J. 2004. Altered balance of vasoactive systems in experimental hypertension: the role of relative NO deficiency. Physiol. Res. **53:** S23–S34.

LOWRY, O.H., ROSEBROUGH, J.N., FARR, A.L. & RANDAL, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem.**193:** 265–275.

MIURA, T., MURAOKA, S. & FUJIMOTO, Y. 1998. Temperature-dependent lipid peroxidation of rat brain homogenate. Res. Commun. Mol. Pathol. Pharmacol. **100:** 117–128.

OŠŤÁDAL, B., OŠŤÁDALOVÁ, I. & DHALLA, N.S. 1999. Development of cardiac sensitivity to oxygen deficiency: comparative and ontogenetic aspects. Physiol. Rev. **79:** 635–659.

OUBIDAR, M., BOQUILLON, C. MARIE, SCHREIBER, L. & BRALET, J. 1994. Ischemia-induced brain iron delocalization: effect of iron chelators. Free Radic. Biol. Med. **16:** 861–867.

SAMIGULLIN, D., BUKHARAEVA, E.A., VYSKOČIL, F. & NIKOLSKY, E.E. 2005. Calcium dependence of uni-quantal release latencies and quantal content at mouse neuromuscular junction. Physiol. Res. **54:** 129–132.

SASAKI, T., KOTERA, J. & OMORI, K. 2004. Transcriptional activation of phosphodiesterase 7B1 by dopamine D1 receptor stimulation through the cyclic AMP/cyclic AMP-dependent protein kinase/cyclic AMP-response element binding protein pathway in primary striatal neurons. J. Neurochem. **89:** 474–483.

SIESJŐ, B.K., ZHAO, Q., PAHMARK, K., SIESJŐ, P., KATSURA, K. & FOLBERGROVÁ, J. 1995. Glutamate, calcium, and free radicals as mediators of ischemic brain damage. Ann. Thorac. Surg. **59:** 1316–1320.

ŠTÍPEK, S., BOROVANSKÝ, J., ČEJKOVÁ, J., HOMOLKA, J., KLENER, P., LUKÁŠ, M., ŠPIČÁK, J., TESAŘ, V., ZEMAN, M., ZIMA, T. & ŽÁK, A. 2000. Antioxidanty a volné radikály ve zdraví a nemoci, Grada Publishing, s.r.o., Praha, 314 pp.

VLKOLINSKÝ, R. & ŠTOLC, S. 1999. Effects of stobadine, melatonin, and other antioxidants on hypoxia/reoxygenation-induced synaptic transmission failure in rat hippocampal slices. Brain Res. **850:** 118–126.

YANG, C.Y. & LIN, M.T. 2002. Oxidative stress in rats with heatstroke-induced cerebral ischemia. Stroke **33:** 790–794.

ZALESKA, M.M. & FLOYD, R.A. 1985. Regional lipid peroxidation in rat brain *in vitro*: possible role of endogenous iron. Neurochem. Res. **10:** 397–410.

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