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Study of the oxidative stress in a rat model of chronic brain hypoperfusion

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Abstract

A multiple analysis of the cerebral oxidative stress was performed on a physiological model of dementia accomplished by three-vessel occlusion in aged rats. The forward rate constant of creatine kinase, k_{for}, was studied by saturation transfer ^{31}P magnetic resonance spectroscopy in adult and aged rat brain during chronic hypoperfusion. In addition, free radicals in aging rat brain homogenates before and/or after occlusion were investigated by spin-trapping electron paramagnetic resonance spectroscopy (EPR). Finally, biochemical measurements of oxidative phosphorylation parameters in the above physiological model were performed. The significant reduction of k_{for} in rat brain compared to controls 2 and 10 weeks after occlusion indicates a disorder in brain energy metabolism. This result is consistent with the decrease of the coefficient of oxidative phosphorylation (ADP:O), and the oxidative phosphorylation rate measured in vitro on brain mitochondria. The EPR study showed a significant increase of the ascorbyl free radical concentration in this animal model. Application of α-phenyl-*N*-*tert*-butylnitrone (PBN) and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) spin traps revealed formation of highly reactive hydroxyl radical ($^{\bullet}$OH) trapped in DMSO as the $^{\bullet}CH_3$ adduct. It was concluded that the ascorbate as a major antioxidant in brain seems to be useful in monitoring chronic cerebral hypoperfusion.

Keywords: Creatine kinase; Saturation transfer ^{31}P NMR; Chronic cerebral ischemia; Animal model; EPR; Ascorbate free radical; Oxidative phosphorylation

1. Introduction

For more than 30 years, Alzheimer's disease (AD) has been classified and managed as a neurodegenerative disorder. However, it was recently proposed that sporadic (nongenetic) AD is a vascular disease (de la Torre, 2002a, b, review, Pratico and Delanty, 2000). This conclusion is based on common overlap of clinical AD and cognitive symptoms of vascular dementia. It is well known, that AD is heterogeneous and multifactorial nature, likely resulting from diverse presence or vascular risk factors or indicators of vascular disease.

In the present work a chronic pathophysiological animal model of dementia was used (Kašparová et al., 2000) for investigation of oxidative stress by means of two physical techniques—phosphorus-31 magnetic resonance spectroscopy (^{31}P MRS) and electron paramagnetic resonance (EPR) spectroscopy. We supposed that the characteristic pathology of AD involves microvascular degeneration and chronic cerebrovascular hypoperfusion as has recently been reported (de la Torre, 1999, 2002a, b). For an animal model of AD in rats it is proposed that two factors must be present before cognitive dysfunction and neurodegeneration is expressed in the AD brain: advanced aging and presence of a condition that lowers cerebral perfusion (de la Torre, 1999; de la Torre and Stefano, 2000). The model involves subjecting

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animals—aged rats to chronic cerebrovascular hypoperfusion for 2–10 or more weeks. With respect to reduced cerebral blood flow, it should be noted that prolonged brain ischemia could produce β-amyloid peptid precursor (APP) that was found increased in the hippocampus of AD brains, and which has been implicated in formation of senile plaques. A β-amyloid peptid-associated free radical model for neuronal death in AD brain has evolved from many observations. In this model, β-amyloid peptid-associated free radicals initiate lipid peroxidation and protein oxidation (Butterfield et al., 1999, review; Arivazhagan et al., 2002; Behl and Moosmann, 2002; Kaufmann et al., 2002). Thus, we supposed that EPR technique could be useful in investigation of free radical production in the above animal model of chronic cerebral hypoperfusion. Direct neurotoxicity of β-amyloid peptid can be related to its ability to associate with plasma membrane (Mattson et al., 1992), to induce Ca^{2+} influx and disrupt cell membrane functions (Mattson et al., 1992), to induce lipid peroxidation, to lead to protein oxidation (Bruce-Keller et al., 1998), to enhance glutamate toxicity (Mattson et al., 1992) and, in addition, to decrease the activity of several oxidative sensitive enzymes, including creatine kinase (Butterflied, 1997). Creatine kinase (CK) plays a central role in energy transfer in cells with highly energy flux or requirements and it is highly susceptible to oxidative inactivation (McCord and Russell, 1988). Dysfunction of the creatine kinase system under AD conditions has also recently been reported (Yatin et al., 1999; Askenov et al., 2000; David et al., 1998; Burbaeva et al., 1999, review). The causes of lower brain isoform creatine kinase (BB-CK) levels in the cell cytosol of the post-mortem brain in mental pathology are discussed (Burbaeva et al., 1999, review). BB-CK, a member of the CK gene family, is a predominantly cytosolic CK isoform in the brain and plays a key role in regulation of the ATP level in neural cells (Askenov et al., 2000). It can be expected that activity of the creatine kinase reaction in the brain in vivo is significantly changed under condition of the above animal model of dementia or during oxidative stress. Therefore, we studied reaction kinetics of a reversible exchange of the phosphate group in the creatine kinase reaction catalyzed by CK:

$$PCr^{2-} + MgADP^{-} + H^{+} \Leftrightarrow MgATP^{2-} + Cr \qquad (1)$$

in the adult and aged rat brains under conditions of severe hypoperfusion, using saturation transfer in vivo ^{31}P MRS technique (Mlynárik et al., 1998; Kašparová et al., 2000).

CK in brain exists in multiple forms, and the total enzyme activity in brain is high enough to ensure near equilibrium of the CK reaction, hence the CK flux is comparable to the ATP consumption (Erecinska and Silver, 1989). Thus, if the brain CK reaction is inhibited during modeled hypoperfusion and the ATP turnover correlates with the flux through the CK reaction, then we should be able to monitor cerebral effects of chronic hypoperfusion or oxidative stress in the rats by ^{31}P MRS saturation transfer experiments in vivo.

The purpose of our studies was to elucidate a relationship between the pseudo-first order rate constant, k_{for}, of the CK forward reaction (PCr ⇒ ATP) in aged rat brain under conditions of severe chronic cerebrovasular hypoperfusion, and the degree of the oxidative damage of brain cells via free radicals production monitored by means of the EPR spectroscopy. Therefore, after ^{31}P MRS measurements, the rat brains were subjected to biochemical analysis focused on mitochondrial oxidative phosphorylation parameters, and the data were also correlated with the free radicals EPR investigations. In AD, a possible mechanism, by which impaired electron transport chain function leads to cell death is a decrease in production of ATP, and an increase in production of free radicals or reactive oxygen species (Beal, 1995; Butterflied, 1997). Isoenzymes CK can be inactivated by hydrogen peroxide and by superoxide (Suzuki et al., 1992). No data relating CK system and free radicals production are presently available for animal model of dementia.

2. Materials

2.1. The spin trapping agents

The spin trapping agents, α-phenyl-*N*-*tert*-butylnitrone (PBN), α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN), and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), were purchased from Sigma Chemicals. Free 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy radical (TEMPOL) from Aldrich was applied for radical concentration determination. DMPO was freshly distilled before use and kept under argon in a freezer. Dimethylsulfoxide (DMSO) supplied by Fluka was used without further purification.

2.2. Animals

Male Wistar rats weighing 250–350 g were used. The animals were housed in air-conditioned room at 22 °C and fed standard Larsen's diet and water ad libitum up to the time experiments. Series of adult (3–6 months old) and aged (15–16 months old) Wistar rats were compared. Ketamine 50 mg/kg bw and xylazine 4 mg/kg bw intraperitoneal anesthesia was used for surgical procedure of cerebrovascular occlusion. In vivo ^{31}P MRS saturation transfer measurements on rats anesthetized in halothane were conducted on a SISCO 4.7 T imaging spectrometer. For in vitro EPR and biochemical experiments performed at the end of the experimental period (5 weeks, and/or 5 months) the rats were sacrificed by thiopenthal i.p. and subsequently the rat brain was removed.

3. Experimental procedures

3.1. Surgery and measurement protocol

A minimally invasive surgical technique of 3-vascular occlusion by extrapleural transmanubrial approach and

spontaneous breathing (Horecky's modification of de la Torre technique, de la Torre et al., 1995) was used to eliminate blood flow through left carotid artery and through brachiocephalic trunk including right carotid and right vertebral arteries (severe hypoperfusion). This modification was made without thoracotomy, midline neck skin incision including manubrium sterni. Extrapleural part of the brachiocephalic trunk was exposed by spreading the sternohyoideus muscles from the midline with the retractor. After visualization, extrapleural part of the brachiocephalic trunk was occluded to eliminate blood flow through both the right common carotid and vertebral arteries. To minimize acute circulatory stress, the left common carotid artery was occluded 8 days later. Such preparation produced animals whose sensory-motor function was indistinguishable from intact controls (de la Torre, 1999). Mild hypoperfusion (two-vessel occlusion) was modeled in the rats by left and right carotid artery occlusion (Mlynárik et al., 1998). The groups of rats with severe cerebral hypoperfusion were measured 2 and 10 weeks after occlusion by saturation transfer in vivo ^{31}P MRS.

For EPR measurements the rat brain tissues were immediately transferred into the glass homogenizators containing 1 ml of DMSO with spin trapping agents (PBN, POBN, DMPO). DMSO was used as a stabilizer of ascorbyl free radicals (Pietri et al., 1994; Delmas-Beauvieux et al., 1998). After one minute of homogenization the cortex homogenizate was immediately frozen in liquid nitrogen (77 K). The prepared frozen rat brain tissues were stored before measurements of EPR spectra in liquid nitrogen.

3.2. Isolation of mitochondria and determination of mitochondrial function

After in vivo ^{31}P MRS studies the brain of animals were used for determination of oxygen consumption in brain mitochondria (before and/or after severe occlusion). In vitro oxidative phosphorylation of the rat brain mitochondria was measured polarographically using a Clark oxygen electrode and sodium glutamate as substrate. The mitochondria were isolated from the rat brain using the following procedure:

The brain was minced in the isolation medium (pH 7.4) containing in mol l^{-1} mannitol (2.25×10^{-1}), sucrose (7.5×10^{-2}) and EDTA (2×10^{-4}) (Palmer et al., 1977), and was homogenized (1:10 tissue to medium ratio). The homogenate was centrifuged at 4 °C (Hogeboom, 1955). Isolated mitochondria were resuspended. Suspension of 0.1 ml contained 2–4 mg of protein, as determined by the method of Lowry et al. (1951). Mitochondrial oxygen consumption was measured with a Gilson 5/6 oxygraph equipped with a Clark oxygen electrode at 30 °C. Incubation medium contained in mol l^{-1}: HEPES (1×10^{-2}), K_2HPO_4 (5×10^{-3}), KCl (1.2×10^{-1}), EDTA (5×10^{-4}), and dextran 2%, pH was adjusted to 7.2. Sodium glutamate (5×10^{-3} mol l^{-1}) was used as a NAD substrate.

The homogenate was centrifuged at 4 °C 10 min at $700 \times g$. This way we separated cell nuclei from the cell pellet. The supernatant was decanted and that from the first centrifugation was transferred to a Lusteroid tube and centrifuged for 12 min at $5600 \times g$. The sediment was resuspended and again centrifuged. To remove the microsomal fraction, this procedure was repeated three times. The final suspension of thrice sedimented mitochondria was reasonably pure on the basis of cytological criteria of homogenity and represents a yield of about 80% (Hogeboom, 1955).

To assess stimulated O_2 consumption, 500 nmol of ADP was added to the sample. The following parameters of oxidative phosphorylation were determined: the respiration control index (RCI), the coefficient of oxidative phosphorylation (ADP:O), ADP-stimulated rate of oxygen uptake (QO_2S_3), rate of basal oxygen uptake (QO_2S_4), and oxidative phosphorylation rate (OPR).

3.3. ^{31}P MRS technique

^{31}P MRS saturation transfer measurements were performed on a 4.7 T SISCO instrument. The static magnetic field was shimmed using the proton signal of water that showed a typical line width of 20–35 Hz.

We used a time dependent saturation transfer experiment, which allowed to measure simultaneously two parameters, T_1 and k_{for}. Saturation of the γ-ATP resonance for increasing time periods led to an exponential decay of the phosphocreatine resonance to a new steady state.

The evolution of longitudinal magnetization as a function of time is described by a McConnell equation (Eq. (2)) (Forsén and Hoffman, 1963):

$$\frac{dM_{PCr}}{dt} = -\left(k_{for} + \frac{1}{T_{1PCr}}\right)(M_{PCr} - M^0_{PCr}) + k_{rev}(M_{ATP} - M^0_{ATP}) \quad (2)$$

Starting with the system in equilibrium, the γ-ATP signal is saturated during the time interval t. The dependence of the longitudinal magnetisation of the PCr signal, M_{PCr}, on the irradiation time t is given by Eq. (3):

$$M_{PCr} = M^0_{PCr}\left\{1 - k_{for}T_{1sPCr}\left[1 - \exp\left(-\frac{t}{T_{1sPCr}}\right)\right]\right\} \quad (3)$$

where M^0_{PCr} is the equilibrium magnetisation of PCr in the absence of γ-ATP saturation, k_{for} is the forward creatine kinase reaction rate constant,

$$\frac{1}{T_{1sPCr}} = k_{for} + \frac{1}{T_{1PCr}}$$

is the apparent longitudinal relaxation rate in the presence of γ-ATP saturation and t is the irradiation time. The T_{1sPCr} value was calculated as a slope of the semilogarithmic plot of $M_{PCr} - M^\infty_{PCr}$ against t where

$$M^\infty_{PCr} = \frac{M^0_{PCr}(1/T_{1PCr})}{k_{for} + (1/T_{1PCr})}$$

is the steady-state magnetisation of PCr after a long-term irradiation of the γ-ATP signal. The pseudo first-rate constant k_{for} was calculated according to the equation

$$k_{for} = \frac{1 - (M^{\infty}_{PCr}/M^{0}_{PCr})}{T_{1sPCr}} \quad (4)$$

Instead of the magnetisation values, corresponding PCr signal intensities were used in these calculations. The value of M^{∞}_{PCr} was read from the spectrum with 10-s irradiation of the γ-ATP resonance, and M^{0}_{PCr} was obtained from the reference spectrum measured with the irradiation offset in the mirror position relative to the PCr resonance and with the irradiation time of 1 s. The saturation was accomplished by an on-resonance DANTE sequence consisting of a series of 10 μs radiofrequency pulses with interpulse delays 400 μs. The time of irradiation of the γ-ATP resonance was varied from 0.3 to 10 s and resulted in an exponential decay of the PCr signal (Clark et al., 1991). To verify the validity of the results, the T_{1PCr} values were calculated using the following equation (Eq. (5):

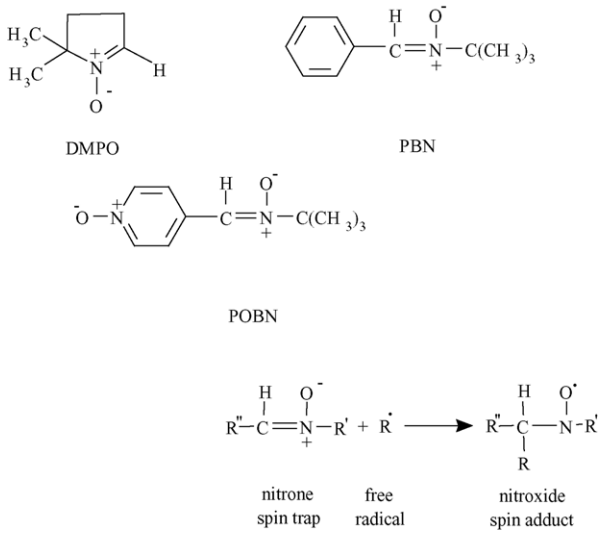
$$T_{1PCr} = T_{1sPCr}\frac{M^{0}_{PCr}}{M^{\infty}_{PCr}} \quad (5)$$

This parameter was supposed to be practically independent of the surgical intervention and was used as a validity check of the results.

3.4. EPR spectroscopy

The samples of homogenized brain tissue after thawing were transferred into a flat EPR cell, tightened with a stopper, and inserted into a standard TE_{102} cavity of a Bruker EPR 200D spectrometer working in X-band. Inside the cavity samples were equilibrated at 293 K using a Bruker temperature control unit ER 4111 VT. EPR spectra were recorded using an Aspect 2000 computer connected on line with the EPR spectrometer. The formation of short-lived radical species was evidenced by addition of DMPO, PBN or POBN spin trapping agents.

The *g*-values were determined with uncertainty of ±0.0001 using a manufacturer's supplied internal reference marker containing solid DPPH. The experimental EPR spectra were simulated using *WinEPR* and *SimFonia* programs (Bruker, Germany). Multi-component experimental EPR spectra were fitted as linear combinations of individual spectra simulations using a least-squares minimization procedure with the *Scientist Program* (MicroMath). Relative concentrations of radical adducts were calculated from contributions of individual spectra to the experimental spectrum after double integration procedure. Free TEMPOL from Aldrich was applied for determining ascorbyl radical concentration. The evaluated concentrations of ascorbyl free radical in DMSO rat brain homogenates were re-calculated per 1 mg of brain tissue.



Scheme 1. The structures of spin trapping agents DMPO, PBN and POBN and the key reaction of spin trapping technique.

3.5. EPR spin trapping technique

The EPR spin trapping method involves trapping of reactive short-lived free radicals by a diamagnetic EPR silent compound (spin trap) via addition to a spin trap double bond to produce a more stable free radical product (spin adduct). Spin adducts are paramagnetic, and have EPR spectra with hyperfine splitting constants and *g*-value characteristic of the type of free radical trapped (Li et al., 1988). Nitrone spin traps (DMPO, PBN, POBN) scavenge free radical species via addition to a carbon located in the alpha position relative to the nitrogen (Scheme 1) (Li et al., 1988).

3.6. Statistical analysis

The measured and calculated values were expressed as the mean ± S.D.

The χ-square test and Student's test were performed to compare the groups of animals. Statistical significance was established at $^{**}P < 0.01$ and $^{*}P < 0.05$.

4. Results

4.1. ^{31}P magnetic esonance spectroscopy

To investigate the metabolic consequences of the change of CK activity, ^{31}P MR spectroscopy was performed on aged rat brains in the conditions hypoperfusion. Dependence of the PCr signal intensity on the time of γ-ATP signal irradiation for the rat brain before and after induction of chronic hypoperfusion is shown in Fig. 1. From these spectra it can be seen that the decrease in signal intensity is faster,

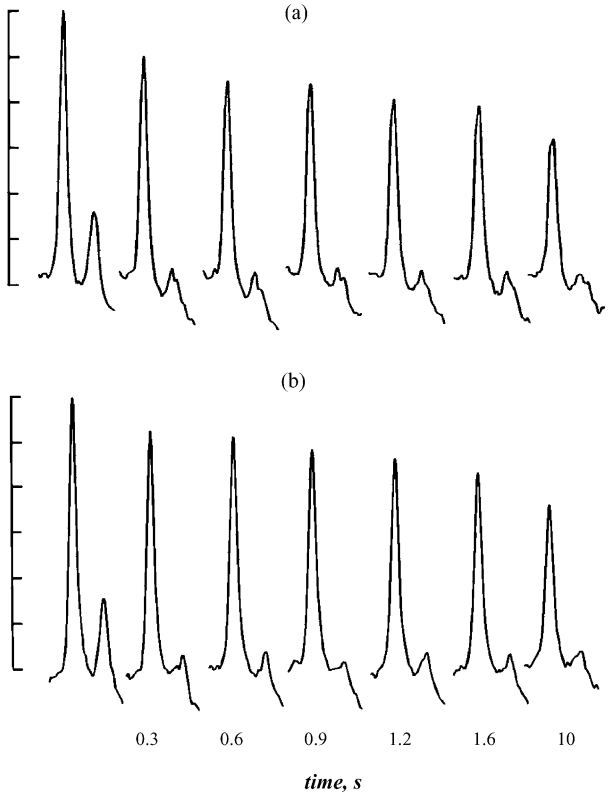


Fig. 1. Series of spectra taken during saturation transfer experiments on an aged rat before (a) and 10 weeks after induction of severe chronic ischemia (b). Only the spectral region containing signals of PCr and (-ATP is shown. From left to right: reference spectrum, spectra with 0.3, 0.6, 0.9, 1.2, 1.6 s irradiation of the (-ATP signal, steady-state spectrum with 10 s irradiation of (-ATP.

and the steady state intensity is lower in the healthy brain, which corresponds to a higher value of k_{for}. For simplicity, a two-site model for the exchange between γ-ATP and PCr was assumed in data analysis, supported by the previous finding in two-compartment model in the mouse and monkey brains (Holtzman et al., 1998; Mora et al., 1992). Thus, a kinetic parameter, k_{for} measured by ^{31}P MRS could be considered as the PCr ⇒ ATP turnover relating to CK reaction in the living animal brain corresponding to the equilibrium, or near-equilibrium state.

From our dynamic measurements of ^{31}P MRS saturation transfer it is clear that the hypoperfused aged rats showed statistically significant ($^{**}P < 0.01$) decrease in k_{for} both 2 and 10 weeks after occlusion compared to the control group of healthy aged rats (Table 1). For comparison, younger adult rat brains were also investigated. The group of adult rats (mean age of 6 months) showed only small, but statistically significant ($^{*}P < 0.05$) reduction of k_{for} after 3-vessel occlusions (Table 1).

4.2. Parameters of oxidative phosphorylation

The following parameters of oxidative phosphorylation were determined: the respiration control index (RCI), the coefficient of oxidative phosphorylation (ADP:O), ADP-stimulated rate of oxygen uptake (QO_2S_3), rate of basal oxygen uptake (QO_2S_4), and oxidative phosphorylation rate. Basal oxygen uptake (QO_2S_4) and ADP stimulated (QO_2S_3) oxygen uptake were unchanged compared to controls—aged rat brains (Table 2). However, the ATP production was lowered in the mitochondria isolated from hypoperfused brains compared to controls (Table 2). The coefficient of oxidative phosphorylation (ADP:O) was significantly decreased in aged rat brains after occlusion ($^{**}P < 0.01$). The oxidative phosphorylation rate (OPR) was also reduced in aged rat brains after occlusion ($^{*}P < 0.05$) as summarized in Table 2.

Table 1
Forward rate constants of CK, k_{for} (PCr ⇒ ATP) and spin lattice relaxation times of PCr (T_1) in various groups of the animals, before and/or 2 and 10 weeks after occlusion (mild and/or severe occlusion)

Subject—rats	k_{for} (s^{-1}) $M \pm$ SEM	T_{1PCr} (s) $M \pm$ SEM
Adult, 1 controls ($n = 10$)	0.34 ± 0.03	3.5 ± 0.2
Adult, severe CCI, 2 weeks ($n = 10$)	$0.28 \pm 0.04^{*}$	3.2 ± 0.2
Adult, severe CCI, 10 weeks ($n = 6$)	$0.26 \pm 0.04^{**}$	3.0 ± 0.4
Aged, 2. controls ($n = 8$)	0.30 ± 0.04	3.4 ± 0.3
Aged, mild CCI, 2 weeks ($n = 6$)	$0.25 \pm 0.02^{**}$	3.2 ± 0.3
Aged, severe CCI, 2 weeks ($n = 6$)	$0.21 \pm 0.04^{**}$	3.5 ± 0.4
Aged, severe CCI, 10 weeks ($n = 6$)	$0.20 \pm 0.01^{**}$	3.6 ± 0.1

n: number of experimetal rats; *M*: arithmetic mean; SEM: standard error of the mean, TTEST CCH: chronic cerebral hypoperfusion.
* $P < 0.05$.
** $P < 0.01$.

4.3. EPR spectroscopy

EPR spectra of rat cortex DMSO homogenate without addition of the spin trapping agents gave a two-line EPR signal ($a_H = 0.185$ mT; $g = 2.0053$) corresponding to the formation of ascorbyl/ascorbate free radical. At physiological pH 7.4, 99.95% of vitamin C is present as $AscH^-$; and only 0.05% as $AscH_2$ (Fig. 2). Thus, the antioxidant chemistry of vitamin C is the chemistry of $AscH^-$. $AscH^-$

Table 2
Parameters of oxidative phosphorylation in brain mitochondria in aged rats (control) and in aged rats after 3-vessel occlusion

Mitochondrial oxidative phosphorylation parameters	Control ($n = 8$), $M \pm$ SEM	After occlusion ($n = 6$), $M \pm$ SEM
RCI [$S_3S_4^{-1}$]	3.22 ± 0.16	3.03 ± 0.15
ADP:O [nmol ADP $nAtO^{-1}$]	2.51 ± 0.08	$2.21 \pm 0.03^{**}$
QO_2S_3 [nAtO mg $prot^{-1}$ min^{-1}]	52.97 ± 2.40	51.35 ± 1.19
QO_2S_4 [nAtO mg $prot^{-1}$ min^{-1}]	16.72 ± 0.85	17.13 ± 0.91
OPR [nmolATP mg $prot^{-1}$ min^{-1}]	132.19 ± 6.48	$113.35 \pm 4.14^{*}$

n: number of experimental rats; *M*: arithmetic mean; SEM: standard error of the mean; RCI: respiration control index; ADP:O: coefficient of oxidative phosphorylation; $QO_2(S_3)$: the rate of mitochondrial oxygen uptake stimulated with ADP (State 3); $QO_2(S_4)$: the rate of basal mitochondrial oxygen uptake (State 4); OPR: oxidative phosphorylation rate.
* $P < 0.05$.
** $P < 0.01$

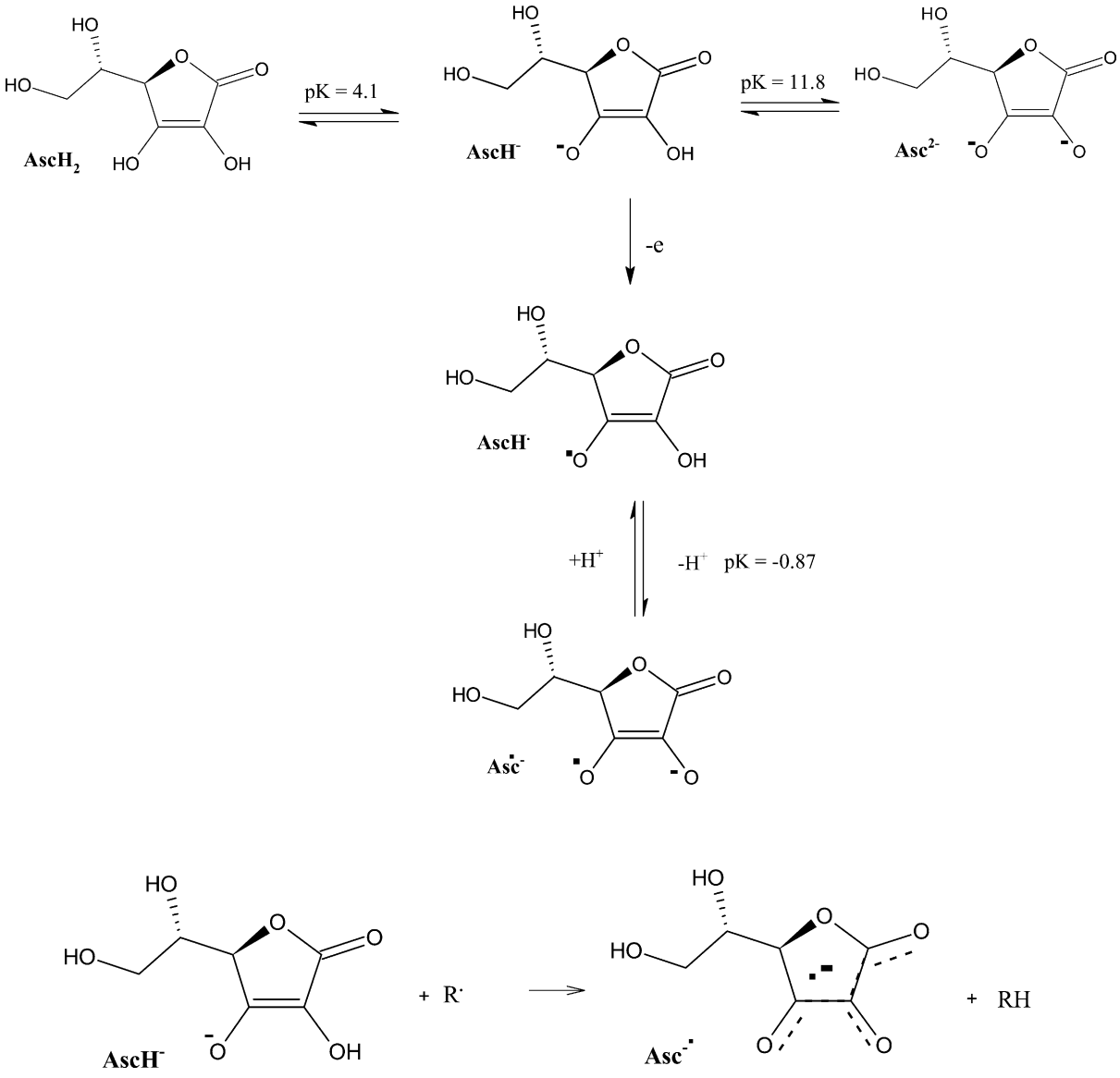


Fig. 2. Forms of Vitamin C at various pH and its reactions.

reacts with radicals and donates a hydrogen atom ($H^{\bullet}$ or $H^{+} + e^{-}$) to an oxidizing radical to produce the resonance-stabilized tricarbonyl ascorbate free radical, $AscH^{\bullet}$. This has a pK_a of -0.87; thus, under physiological conditions it is not protonated and will be present as $Asc^{\bullet -}$ (Fig. 2). The unpaired electron of $Asc^{\bullet -}$ (referred to as AFR below) is delocalized and resides in the π-system that includes the tricarbonyl moiety of ascorbate.

Fig. 3 represents the EPR spectra of AFR measured in DMSO rat brain homogenates obtained from the reference rat (trace *a*), as well as from a rat 5 months after 3-vessel occlusion (trace *b*), measured 20 min after sample thawing. The EPR spectra clearly demonstrated increased AFR formation in the aged ischemic brain tissue, since the concentration of AFR in this sample was of 1.95 ± 0.1 nM/1 mg brain tissue in comparison to 0.92 ± 0.1 nM/1 mg brain tissue measured in reference sample. An analogous raise of AFR radical concentration in brain homogenates was measured also in samples obtained after 5 weeks after occlusion.

After thawing the DMSO homogenate of rat brain, the significant decrease of EPR intensity of AFR was observed during measurements in accord with observation of Schneider et al., 2003. The experimental data were fitted using least-squares analysis by the exponential functions analogous to the formal first-order kinetics, and the formal half-life of 27 min was evaluated under given experimental conditions (Fig. 4).

Fig. 5 represents EPR spectra measured in rat cortex DMSO homogenates (from a rat 5 month after 3-vascular occlusion) in the presence of DMPO and PBN spin trapping agents 15 min (traces *a*) and 120 min after sample thawing (traces *b*), respectively. In these EPR spectra, paramagnetic species were identified, which correspond to the formation

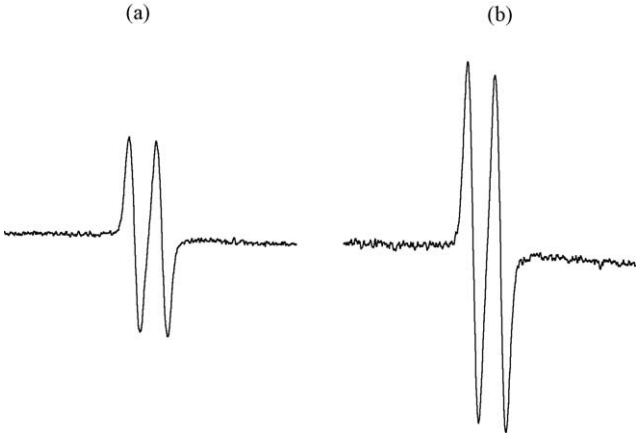


Fig. 3. EPR spectra of ascorbyl free radical (AFR) measured in DMSO homogenates of rat brains 20 min after samples thawing. (a) a control rat (AFR concentration 0.92 ± 0.1 nM/1 mg brain tissue); (b) a rat 5 months after occlusion (AFR concentration 1.95 ± 0.1 nM/1 mg brain tissue). Spectrometer settings: center field, 336.5 mT; sweep width, 2 mT; gain, 1.0×10^5; modulation, 0.1 mT; scan time, 50 s; microwave power, 20 mW; number of scans, 5; time constant, 100 ms; temperature, 293 K.

of ascorbyl free radical ($a_H = 0.185$ mT; $g = 2.0053$) and carbon-centered adducts, most probably $^{\bullet}$DMPO-CH_3 ($a_N = 1.511$ mT, $a_H = 2.22$ mT; $g = 2.0057$) or $^{\bullet}$PBN-CH_3 ($a_N = 1.543$ mT, $a_H = 0.33$ mT; $g = 2.0058$) radicals. Methyl radicals are produced from the DMSO solvent by its rapid reaction with generated hydroxyl radicals (the rate constant of the reaction of DMSO with $^{\bullet}$OH is 7.0×10^9 dm^3 mol^{-1} s^{-1} at 25 °C (Veltwisch et al., 1980), according to the equation

$$(CH_3)_2SO + {}^{\bullet}OH \rightarrow {}^{\bullet}CH_3 + CH_3SO(OH)$$

The time profile of EPR spectra indicates that ascorbate is a major antioxidant in brain, and consequently the concentration of spin adducts is relatively low until ascorbate is exhausted (Fig. 5).

The utilization of PBN spin trap reflected analogous behavior of the experimental systems, EPR signal corresponds to AFR (relative concentration 75%) and $^{\bullet}$PBN-CH_3 (23%) 15 min after defrosting, compared to AFR (12%) and $^{\bullet}$PBN-CH_3 (88%) 120 min after defrosting (Fig. 5). It should be noted here that EPR spectra measured in reference rat brain homogenates were fully compatible with those obtained in traumatized brains after 3-vessel occlusion, and the experimental systems containing spin trapping agents are due to their complexity unsuitable for the quantitative evaluation of radical concentration.

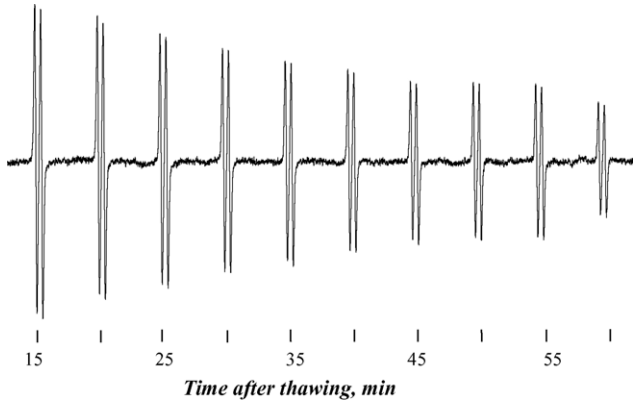


Fig. 4. Set of EPR spectra of AFR measured in a DMSO homogenate of rat brain 5 months after occlusion. The measurement was started 10 min after sample thawing. Spectrometer settings are described in Fig. 3.

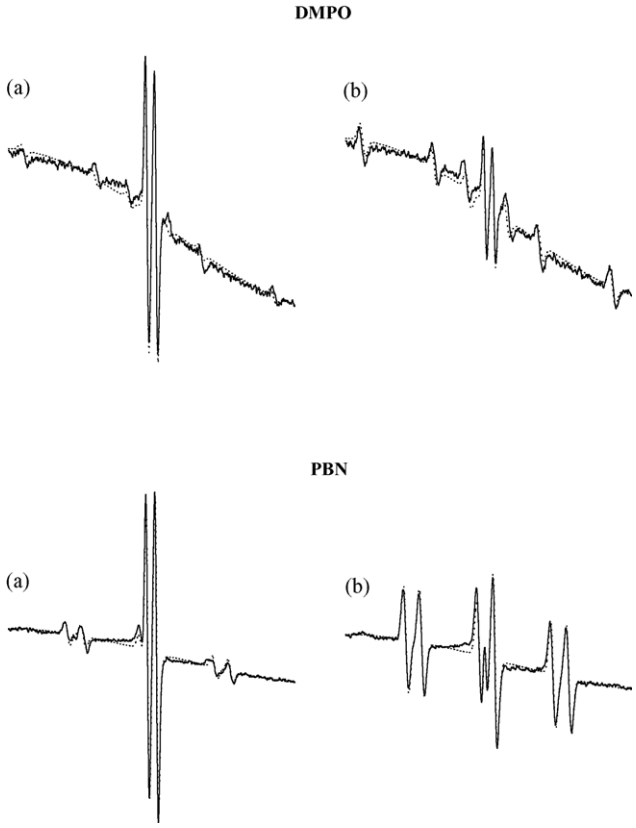


Fig. 5. Experimental (solid line) and simulated (dotted line) EPR spectra of DMPO and PBN adducts measured in the DMSO rat brain homogenates originated from an animal 5 months after 3-vessel occlusion in DMSO suspension ($c_{DMPO} = 0.02$ mol dm^{-3}; $c_{PBN} = 0.05$ mol dm^{-3}): (a) 15 min after sample thawing; (b) 120 min after sample thawing. Spectrometer settings: center field, 336.5 mT; sweep width, 6 mT; gain, 1.0×10^5; modulation, 0.1 mT; scan time, 50 s; microwave power, 20 mW; number of scans, 5; time constant, 100 ms; temperature, 293 K.

The application of POBN spin trap was under given experimental conditions unsuccessful, since no $^{\bullet}$POBN-adducts were measured, and consequently, only AFR was evident in EPR spectra.

5. Discussion

It is well known that regional cerebral hypoperfusion is one of the earlier clinical manifestations in a sporadic form of AD. Chronic cerebral hypoperfusion can affect metabolic, anatomic, and cognitive function adversely (de la Torre and

Stefano, 2000; Sarti et al., 2002). Our modification (see Section 2) of de la Torre and Fortin surgical technique involves subjecting aged rats to severe chronic cerebrovascular hypoperfusion for 1–10 weeks (de la Torre and Fortin, 1994). Such chronic cerebral hypoperfusion is reported to cause progressive spatial memory impairment, selective CA1 neuronal damage, reactive astrocytosis in the hippocampus, increase in membrane phospholipid synthesis in hipocampal region, reduction of cerebral pre-terminal noradrenalic varicosities, decreased immunoreactivity of microtubule associated protein 2 and significant reductions of hippocampal-parietal cortex blood flow (de la Torre and Stefano, 2000). All changes observed in the above rat model have been described in patients with confirmed dementia, particularly AD (Nitsch et al., 1991; Prohovnik et al., 1988; Frederickson, 1992; Hoyer, 1986). These findings have prompted many authors to propose the concept that advanced aging in the presence of a vascular risk factors can lead to a high level of cerebral hypoperfusion that triggers regional brain microcirculatory disorder and impairs optimum delivery of substrates needed for normal brain cell function (de la Torre and Stefano, 2000; Lythgoe et al., 2003).

The described monitoring of the kinetic MRS parameter (the rate constant of CK k_{for}) serves to compare normal and pathological physiology—chronic cerebral hypoperfusion in the rat brain. Turnover rates reflect functional activity of an organ. This is certainly true and generally accepted, e.g., for transmitters in the CNS, where the turnover of a transmitter rather than its overall concentration will reflect the functional activity of the neuron for which it is acting (Cooper et al., 1982).

It is well known that the steady-state phosphorus-31 magnetic resonance spectroscopy (^{31}P MRS) is used for studying metabolism in living tissues including brain. Using this technique it is possible to monitor changes of concentration of high-energy metabolites (ATP, PCr) and inorganic phosphate in the animal or human brain during various *acute* pathologic conditions such as an anoxia, hypoxia or acute ischemia (Sharkey et al., 1989; Nascimben et al., 1995; Lythgoe et al., 2001; Wardlaw et al., 1998; Saunders, 2000). However, the steady-state high-energy metabolite concentrations do not sufficiently reflect the tissue function, i.e., synthesis and/or consumption of ATP in *chronic* forms of these pathological states (Sauter and Rudin, 1993; Kašparová et al., 2000). This also seems to be the case of ATP, whose steady-state levels remain remarkably constant over a wide range of tissue functional activities. The levels of ATP do not accurately reflect brain activity because the stability of the ATP levels is not due to buffering, i.e., small changes within a large pool, but rather depends on an effective regulation of its turnover. The steady-state ATP levels are therefore a poor indicator of metabolic state of the brain (Rudin and Sauter, 1989). On the other hand, we can monitor rate constants of CK reaction by magnetization transfer in vivo ^{31}P MRS in the brain, which is sensitive on the oxidative stress. This technique is ideally suited for studying the CK reaction in vivo. ^{31}P magnetic resonance spectra of the brain are generally dominated by signals from ATP and phosphocreatine (PCr), a substrate and a product of the creatine kinase reaction (Eq. (1)).

We examined the kinetics of CK reaction in living aged rat brain under conditions of severe chronic cerebral hypoperfusion, using a time dependent saturation transfer ^{31}P MRS experiment. As it is seen from our dynamic measurements of ^{31}P MRS saturation transfer, the hypoperfused rats showed statistically significant decrease in k_{for} both 2 and 10 weeks after occlusion compared to the control group of healthy aged rats (Table 1). It should be noted here that adult and aged rats kept for 10 weeks under conditions of severe and/or mild hypoperfusion, both showed no statistically important changes in conventional in vivo ^{31}P MRS spectrum (Mlynárik et al., 1998; Kašparová et al., 2000). Thus, under the above conditions a significantly reduced metabolic phosphorus flux is observed. Our data suggest that the creatine kinase reaction could play an important role in the energetic systems of the aged brain under conditions of chronic cerebral hypoperfusion and/or during oxidative stress. The exact mechanism responsible for the reduction of the pseudo-first-order rate constant for the CK reaction k_{for} is presently not clear. We suppose that CK is sensitive to oxidation, and that the changes observed in this study result from free radical damage. Impairment of creatine-creatine phosphate system was observed in human vascular dementia and the level of cytosolic BB-CK showed a significant decrease in postmortem AD brain (Burbaeva et al., 1999, review). These facts were confirmed ‘‘in vivo’’ by measuring a kinetic parameter of ^{31}P MRS in our animal model of vascular dementia or severe chronic cerebral hypoperfusion. The reduction in BB-CK levels is not a result of postmortem delay, but could be a result of toxicity by the beta-amyloid peptid (APP) precursor during severe hypoperfusion. APP was identified in the animal model of dementia (de la Torre and Stefano, 2000). The decline in CK activity in AD brain may be related to altered endogenous antioxidant levels (Maret et al., 1999). CK activity is also inhibited by β-amyloid peptid (Yatin et al., 1999), and this loss of activity is blocked by Vitamin E, further supporting the concept of β-amyloid peptid associated free radical oxidative stress (Yatin et al., 1999).

As it was recently demonstrated, BB-CK energy transfer in the brain is important for habituation and special learning behaviour (Jost et al., 2002). ^{31}P MR spectroscopy revealed a strongly reduction of PCr $\Rightarrow$ ATP, phosphorus flux in mice with ablation of cytosolic BB-CK (Jost et al., 2002), while ATP and PCr levels were unaffected in these mice. Thus, the rate constant of CK k_{for} in the brain depends mainly on the changes of BB-CK activity, which are induced by oxidative stress. This fact is in agreement with the decrease of CK activity in the AD brain due to posttranslational modification of BB-CK (Askenov et al., 2000). These authors demonstrated that the increase of protein carbonyl content in BB-

CK provides evidence that oxidative posttranslational modification of BB-CK causes the loss of BB-CK activity in AD (Askenov et al., 2000). Examining the level of cytosolic BB creatine kinase in postmortem AD and schizophrenic's brain structures showed a significant decrease in BB creatine kinase as compared with the similar control brain structures. There was the maximum decline in AD cases (Burbaeva et al., 1999). We suppose that a reduction of k_{for} could be result of severe reduction of cerebral blood flow leading to microvascular degeneration and a global cerebral hypometabolism, or common oxidative stress in brain cells.

It has been hypothesized that free radicals play a central role in the brain cell damage during various chronic neurodegenerative diseases including AD (de la Torre et al., 1998; Pratico and Delanty, 2000). However, the chemical nature of free radicals makes them extremely reactive and short-lived, which renders their direct detection and quantification extremely difficult (Cheng et al., 2002).

The significant role and numerous actions of ascorbate in organisms were described in literature (Rice, 2000, review; Lee et al., 2000). The production of reactive radical species in brain tissues under conditions of oxidative stress induced by hypoxia (Bågenholm et al., 1997; Sharma, 1997), ischemia (Matsuo et al., 1995) or traumatic injury (Awasti et al., 1997; Tyurin et al., 2000) was demonstrated previously and it was concluded that ascorbic acid plays the dominant role in the prevention of the cell damage initiated by radical species (Langemann et al., 2001), because of its ability to terminate reactive intermediates by rapid electron-transfer forming AFR (Buettner and Jurkiewicz, 1993, 1996). Consequently, the concetration of AFR produced, evaluated by EPR can be used as a measure of oxidative stress in various biochemical or biological systems, as was demonstrated previously for traumatic brain injury (Gilgun-Sherki et al., 2002), hypoxic ischemic brain damage (Wang et al., 2000), open-heart surgery (Zweier, 1998), myocardial ischemic and post-ischemic injury (Arroyo et al., 1987; Nagy et al., 1996; Hotta et al., 1999), and ischemic lungs (Sanders et al., 2000). Our EPR study in the rat brain under conditions of severe hypoperfusion (3-vessel occlusion) shows significant increase of the ascorbyl free radical concentration (Fig. 3). These results, along with detailed model experiments in vitro indicate that ascorbate is a major antioxidant in the brain, and that the EPR assay of ascorbate radicals may be used to monitor production of free radicals in the brain tissue during chronic cerebrovascular insufficiency and/or under condition oxidative stress in the aging brain.

The EPR detected increase of AFR concentration upon 2 and 10 weeks of occlusion seems to correlate with significant reduction of forward rate constant of creatine kinase, k_{for}, in rat brain compare to control experiments. Such pronounced disorder in brain energy metabolism is also in accordance with the consistent decrease of a coefficient of the oxidative phosphorylation (ADP:O), and the oxidative phosphorylation rate measured in vitro on brain mitochondria. These changes in the oxidative phosphorylation parameters indicate a decrease of energy production in the mitochondria isolated from hypoperfused rat brains and suggest that the metabolic capacity in this organ was deteriorated. The findings obtained can be also related to the decrease of the forward rate constant of CK, k_{for}.

However, one should bear in mind that in addition to β-amyloid associated formation of free radicals, there are indisputably other routes of free radical generation. It is well established that one of the biochemical hallmarks of ischemia of CNS is increased release of free fatty acids in the tissue. The release of free fatty acids may therefore contribute to the generation of oxygen radicals and lipid peroxidation, most probably accelerated by iron (Kondo et al., 1995; Campbell et al., 2001; Zhuang et al., 2002). It is impossible to distinguish between various routes of free radical generation experimentally (Floyd and Hensley, 2000; Kucukkaya et al., 1996). Regardless of the origin of radicals, they all contribute to the experimentally observed (EPR) chain-terminal AFR.

In summary, our concerted experiments revealed that the increase of ascorbyl radical concentration in aged rats exposed to hypoperfusion correlate with significant reduction of in vivo measured forward rate constant of creatine kinase, k_{for} which is in turn in accordance with the consistent decrease of a coefficient of the oxidative phosphorylation (ADP:O), and the oxidative phosphorylation rate measured in vitro on brain mitochondria.

It is believed that the described ^{31}P MRS technique can be used as a relatively noninvasive in vivo biomarker for age-related neurodegenerative diseases and that the CK rate constant k_{for} can be used for predicting energy metabolism disorder in the brain which is not yet detectable by conventional MRS methods.

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References

Arivazhagan, P., Thilakavathy, T., Ramanathan, K., Kumaran, S., Panneerselvam, C., 2002. Effect of DL-alpha-lipoic acid on the status of lipid peroxidation and protein oxidation in various brain regions of aged rats. J. Nutr. Biochem. 13, 619–624.

Arroyo, C.M., Kramer, J.H., Dickens, B.J., Weglicki, W.B., 1987. Identification of free radicals in myocardial ischemia/reperfusion by spin trapping with nitrone DMPO. FEBS Lett. 221, 101–104.

Askenov, M., Askenova, M., Butterfield, D.A., Markesbery, W.R., 2000. Oxidative modification of creatine kinase BB in Alzheimer's disease brain. J. Neurochem. 74 (6), 2520–2527.

Awasti, D., Church, D.F., Torbadi, D., Carey, M.E., Pryor, W.A., 1997. Oxidative stress following traumatic brain injury in rats. Surg. Neurolog. 47, 575–581.

Bågenholm, R., Nilsson, U.A., Kjellmer, I., 1997. Formation of free radicals in hypoxic ischemic brain damage in the neonatal rat, assessed by an endogenous spin trap and lipid peroxidation. Brain Res. 773, 132–138.

Beal, M.F., 1995. Aging, energy, and oxidative stress in neurodegenerative diseases. Ann. Neurol. 38, 357–366.

Behl, C., Moosmann, B., 2002. Oxidative nerve cell death in Alzheimer's disease and stroke: Antioxidants as neuroprotective compounds. Biol. Chem. 383, 521–536.

Bruce-Keller, A.J., Begley, J.G., Fu, W., Butterfield, D.A., Bredesen, D.E., Hutchins, J.B., Hensley, K., Mattson, M.P., 1998. J. Neurochem. 70, 31–39.

Buettner, G.R., Jurkiewicz, B.A., 1993. Ascorbate free radical as a marker of oxidative stress: An EPR study, 1993. Free Rad. Biol. Med. 14, 49–55.

Buettner, G.R., Jurkiewicz, B.A., 1996. Catalytic metals, ascorbate and free radicals: Combinations to avoid. Radiat. Res. 145, 532–541.

Burbaeva, G.Sh., Savushkina, O.K., Dmitriev, A.D., 1999. Brain izoforms of creatine kinase in health and mental diseases Alzheimer's disease and schizophrenia. Vestn. Ross. Akad. Med. Nauk (1), 20–24.

Butterfield, D.A., Howard, B., Yatin, S., Koppal, T., Drake, J., Hensley, K., Askenov, M., Askenova, M., Subramaniam, R., 1999. Elevated oxidative stress in models of normal brain ageing and Alzheimer's disease (Review). Life Sci. 65 (18/19), 1883–1892.

Butterflied, D.A., 1997. β-Amyloid-associated free radical oxidative stress and neurotoxicity: Implications for Alzheimer's disease. Chem. Res. Toxicol. 10, 495–506.

Campbell, A., Smith, M.A., Sayre, L.M., Bondy, S.C., Perry, G., 2001. Mechanism by which metals promote events connected to neurodegenerative diseases. Brain Res. Bull. 55, 125–132.

Cheng, F.-C., Jen, J.-F., Tsai, T.-H., 2002. Hydroxyl radical in living systems and its separation methods. J. Chromatogr. B 781, 481–496.

Clark, J.F., Harris, G.I., Dillon, P.F., 1991. Multisite saturation transfer using DANTE and continuous wave. Magnet. Reson. Med. 17, 274–278.

Cooper, J.R., Bloom, F.H., Roth, R.H., 1982. The Biochemical Basis of Neurofarmacology, 4th ed. Oxford University Press, London, pp. 138–149.

David, S., Shoemaker, M., Haley, B.E., 1998. Abnormal properties of creative kinase in Alzheimer's disease brain: correlation of reduced enzyme activity and active site photolabeling with aberrant cytosol-membrane partitioning. Brain Res. Mol. Brain Res. 54, 276–287.

de la Torre, J.C., Fortin, T., 1994. A chronic physiological model of dementia. Behav. Brain Res. 63, 35–40.

de la Torre, J.C., Butler, K., Kozlowski, P., Fortin, T., Saunders, J.K., 1995. Correlates between nuclear magnetic resonance spectroscopy, diffusion weighted imaging, and CA1 morphometry following chronic brain ischemia. J. Neurol. Res. 41, 238–245.

de la Torre, J.C., Nelson, N., Sutherland, R.J., Pappas, B.A., 1998. Reversal of ischemic-induced chronic memory disfunction in aging rats with a free radical scavenger glycolytic intermediate combination. Brain Res. 779, 285–288.

de la Torre, J.C., 1999. Critical threshold cerebral hypoperfusion causes Alzheimer's disease? Acta Neuropathol. 98, 1–8.

de la Torre, J.C., Stefano, G.B., 2000. Evidence that Alzheimer's diseae is a microvascular disorder: the role of constitutive nitric oxide. Brain Res. Rev. 34, 119–136.

de la Torre, J.C., 2002a. Alzheimer's disease: How does it start? J. Alzheimer's Dis. 4, 497–512.

de la Torre, J.C., 2002b. Alzheimer disease as a vascular disorder, Nosological evidence. Stroke 33, 1152–1162.

Delmas-Beauvieux, M.-C., Peuchant, E., Thomas, M.-J., Dubourg, L., Pinto, A.P., Clerc, M., Gin, H., 1998. The place of electron spin resonance methods in the detection of oxidative stress in Type 2 diabetes with poor glycemic control. Clin. Biochem. 31, 221–228.

Erecinska, M., Silver, I.A., 1989. ATP and brain function. J. Cereb. Blood Flow Metabol. 9, 2–19.

Floyd, R.A., Hensley, K., 2000. Reactive oxygen species: From radiation to molecular biology. Ann. N Y Acad. Sci. 899, 222–237.

Forsén, F., Hoffman, R., 1963. A. Study of moderately rapid chemical exchange reactions by means of nuclear magnetic double resonance. J. Chem. Physiol. 39, 2892–2901.

Frederickson, R.C., 1992. Astroglia in Alzheimer's disease. Neurobiol. Ageing 13, 239–253.

Gilgun-Sherki, Y., Rosenbaum, Z., Melamed, E., Offen, D., 2002. Antioxidant therapy in acute central nervous system injury: current state. Pharmacol. Rev. 54, 271–284.

Hogeboom, G.H., 1955. Fractionation of cell components of animal tissues. In: Colowik, S.P., Kaplan, N.O. (Eds.), Methods in Enzymology. Academic Press Inc., Publishers, New York, pp. 17–19.

Holtzman, D., Mulkern, R., Mayers, R., Cook, C., Allred, E., Khait, I., Jensen, F., Tsuji, M., Laussen, P., 1998. In vivo phosphocreatine and ATP in piglet cerebral gray and white mater during seizures. Brain Res. 783, 19–27.

Hotta, Y., Otsuka-Murakami, H., Fujita, M., Nakagawa, J., Yajima, M., Liu, W., Ishikawa, N., Kawai, N., Masumizu, T., Kohno, M., 1999. Protective role of nitric oxide synthase against ischemia-reperfusion injury in guinea pig myocardial mitochondria. Eur. J. Pharmacol. 380, 37–48.

Hoyer, S., 1986. Senile dementia and Alzheimer's disease, brain blood flow and metabolism. Progr. Neuropsychopharmacol. Biol. Psychiatry 10, 447–478.

Jost C.R., Van der Zee, C.E.E.M., in't Zandt, H.J.A., Oerlemans F., Verheij, M., Streijger, F., Fransen J., Heerschap A., Cools A.R., Wieringa B., 2002. Creatine kinase B-driven energy transfer in the brain is important for habituation, and spatial learning behaviour, mossy fibre field size and determination of seizure susceptibility. Eur. J. Neurosci. 15, 1692–1706.

Kašparová, S., Dobrota, D., Mlynárik, V., Pham, T.N., Liptaj, T., Horecký, J., Braunová, Z., Gvozdjáková, A., 2000. A Study of Creatine Kinase Reaction in Rat Brain under Chronic Pathological Conditions – Chronic Ischemia and Ethanol Intoxication. Brain Res. Bull. 53, 431–435.

Kaufmann, J.A., Bickford, P.C., Taglialatela, G., 2002. Free radical-dependent changes in constitutive nuclear factor kappa B in the aged hippocampus. Neuroreport 13, 1917–1920.

Kondo, Y., Ogawa, N., Asanuma, M., Ota, Z., Mori, A., 1995. Regional differences in late-onset iron deposition, ferritin, transferritin, astrocyte proliferation, and microglial activation after transient forebrain ischemia in rat-brain. J. Cerebral Blood Flow Metabol.: Official J. Int. Soc. Cerebral Blood Flow Metabol. 15, 216–226.

Kucukkaya, B., Haklar, G., Yalcin, A.S., 1996. NMDA excitotoxicity and free radical generation in rat brain homogenates: application of a chemiluminiscence assay. Neurochem. Res. 21, 1535–1538.

Langemann, H., Feuerstein, T., Mendelowitsch, A., Gratzl, O., 2001. Microdialytical monitoring of uric and ascorbic acids in the brains of patients after severe brain injury and during neurovascular surgery. J. Neurol. Neurosurg. Psychiatry 71, 169–174.

Lee, J.W., Bobst, E.V., Wang, Y.G., Ashraf, M.M., Bobst, A.M., 2000. Increased endogenous ascorbyl free radical formation with singlet oxygen scavengers in reperfusion injury: an EPR and functional recovery study in rat hearts. Cell. Mol. Biol. (Noisy-le-grand) 46 (8), 1383–1395.

Li, A.S.W., Cummings, K.B., Roethling, H.P., Buettner, G.R., Chignell, C.F., 1988. A spin-trapping database implemented on the IBM PC/AT. J. Magnet. Reson. 79, 140–142.

Lowry, H.O., Rosenbrough, N.J., Farr, A.L., Randal, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265.

Lythgoe, D., Simmons, A., Pereira, A., Cullinane, M., Williams, S., Markus, H.S., 2001. Magnetic resonance markers of ischaemia: their correlation with vasodilatory reserve in patients with carotid artery stenosis and occlusion. J. Neurol. Neurosurg. Psychiatry 71 (1), 58–62.

Lythgoe, M.F., Sibson, N.R., Harris, N.G., 2003. Neuroimaging of animal models of brain disease. Br. Med. Bull. 65, 235–257.

Maret, W., Jacob, C., Vallee, B.L., Fischer, E.H., 1999. Inhibitory sites in enzymes: zinc removal and reactivation by thionein. Proc. Natl. Acad. Sci. U.S.A. 96 (5), 1936–1940.

Matsuo, Y., Kihara, T., Ikeda, M., Ninomiya, M., Onodera, H., Kogure, K., 1995. Role of neutrophils in radical production during ischemia and reperfusion of the rat brain: effect of neutrophil depletion on extracellular ascorbyl radical formation. J. Cerebral Blood Flow Metabol. 15, 941–947.

Mattson, M.P., Barger, S.W., Cheng, B., Lieberburg, I., Smith-Swintosky, V.L., Rydel, L.E., 1992. β-amiloid protein metabolites and loss of neuronal Ca^{2+} homeostasis Alzheimer's disease. Trends Neurosci. 16, 409–414.

McCord, J.M., Russell, W.J., 1988. Inaktivation of creatine phosphokinase by superoxide during reperfusion injury. In: Simic, K.A., Word, J.F., Von Sonntag, C. (Eds.), Oxygen Radicals in Biology and Medicine. Plenum Press, New York, pp. 869–873.

Mlynárik, V., Kašparová, S., Liptaj, T., Dobrota, D., Horecký, J., Belan, V., 1998. Creatine kinase reaction rates in rat brain during chronic ischemia. Magnetic resonance materials in physics, biology, and medicine. MAGMA 7, 62–165.

Mora, B.N., Narasimhan, P.T., Ross, B.R., 1992. ^{31}P Magnetization Transfer Studies in the Monkey Brain. Magnet. Reson. Med. 26, 100–115.

Nagy, K., Takacs, I.E., Pankucsi, C., 1996. Age-dependence of free radical-induced oxidative damage in ischemic-reperfused heart. Arch. Gerontol. Geriatr. 22, 297–309.

Nascimben, L., Friedrich, J., Liao, R.L., Paulleto, P., Pessina, A.C., Ingwall, Enalapril, J.S., 1995. Treatment increased cardiac performance and energy reserve via creatine kinase reaction in myocardioum of Syrian myopathic hamsters with advanced heart failure. Circulation 91, 1824–1833.

Nitsch, R., Blustajn, J., Wurtman, R., Growdon, J., 1991. Membrane phospholipid metabolites are abnormal in Alzheimer's disease. Neurology 41 (Suppl. 1), 269.

Palmer, J.W., Tandler, B., Hoper, L.C., 1977. Biochemical properties subsarcolemmal and interfibrillar mitochondria isolated from cardiac muscle. J. Biol. Chem. 252, 8731.

Pietri, S., Séguin, J.R., D'Arbigny, P., Culcasi, M., 1994. Ascorbyl free radical: A noninvasive marker of oxidative stress on human open-heart surgery. Free Rad. Biol. Med. 16, 523–528.

Pratico, D., Delanty, N., 2000. Oxidative injury in diseases of the central nervous system: focus on Alzheimer's disease. Am. J. Med. 109 (7), 577–585.

Prohovnik, I., Mayeux, R., Sackheim, H., Smith, G., Stern, Y., Alderson, P.Y., 1988. Cerebral perfusion as a diagnostic marker of early Alzheimer's disease. Neurology 36, 931–937.

Rice, M.E., 2000. Ascorbate regulation and its neuroprotective role in the brain. TINS 23, 209–216.

Rudin, M., Sauter, A., 1989. Dihydropyridine calcium antagonists reduce the consumption of high-energy phosphates in the rat brain. A Study using combined $^{31}P/^{1}H$ magnetic resonance spectroscopy and ^{31}P saturation transfer. J. Pharmacol. Exp. Therapeut. 251, 700–706.

Sanders, S.P., Bassett, D.J., Harrison, S.J., Pearse, D., Zweier, J.L., Becker, P.M., 2000. Measurements of free radicals in isolated, ischemic lungs and lung mitochondria. Lung 178, 105–118.

Sarti, C., Pantoni, L., Bartoloni, L., Inzitari, D., 2002. Cognitive impairment and chronic cerebral hypoperfusion: What can be learned from experimental model. J. Neurol. Sci. 203-204, 263–266.

Saunders, D.E., 2000. MR spectroscopy in stroke. Br. Med. Bull. 56 (2), 334–345.

Sauter, A., Rudin, M., 1993. Determination of creatine kinase kinetic parameters in rat brain by NMR magnetization transfer. J. Biol. Chem. 682, 13166–13171.

Schneider, M., Niess, A.M., Rozario, F., Angres, C., Tschositsch, K., Battenfeld, N., Schaffer, M., Northoff, H., Dickhuth, H.H., Fehrenbach, E., Trommer, W.E., Biesalski, H.K., 2003. Vitamin E supplementation does not increase the Vitamin C radical concentration at rest and after exhaustive exercise in healthy male subjects. Eur. J. Nutr. 42, 195–200.

Sharkey, S.W., Elspberger, K.J., Murakami, M., Apple, F.S., 1989. Canine myocardial ceatine kinase isoenzyme response to coronary occlusion. Am. J. Physiol. 256, H501–H514.

Sharma, P., 1997. Consequences of hypoxia on the cell size of neuropeptide-Y neurons and the role of ascorbate in cultured neurons from chick embryo. Neurochem. Int. 30, 337–344.

Suzuki, Y.J., Edmondson, J.D., Ford, G.D., 1992. Inactivation rabbit muscle creatin kinase by hydrogen peroxide. Free Rad. Res. Commun. 16, 131–136.

Tyurin, V.A., Tyurina, Y.Y., Borisenko, G.G., Sokolova, T.V., Ritov, V.B., Quinn, P.J., Rose, M., Kochanek, P., Graham, S.H., Kagan, V.E., 2000. Oxidative stress following traumatic brain injury in rats: Quantitation of biomarkers and detection of free radical intermediates. J. Neurochem. 75, 2178–2189.

Veltwisch, D., Janata, E., Asmus, K.-D., 1980. Primary processes in the reaction of OH-radicals with sulfoxides. J. Chem. Soc., Perkin Trans. 2, 146–153.

Wang, L.M., Han, Y.F., Tang, X.C., 2000. Huperzine A improves cognitive deficits caused by chronic cerebral hypoperfusion in rats. Eur. J. Pharmacol. 398, 65–72.

Wardlaw, J.M., Marshall, I., Wild, J., Dennis, M.S., Cannon, J., Lewis, S.C., 1998. Studies of acute ischemic stroke with proton magnetic resonance spectroscopy: relation between time from onset, neurological deficit, metabolite abnormalities in the infarct, blood flow, and clinical outcome. Stroke 29, 1618–1624.

Yatin, S.M., Askenov, M., Butterfield, D.A., 1999. The antioxidant vitamin E modulates amyloid β-peptid-induced creatine kinase activity inhibition and increased protein oxidation: Implications for the free radical hypothesis of Alzheimer's disease. Neurochem. Res. 24, 427–435.

Zhuang, H., Pin, S., Christen, Y., Dore, S., 2002. Induction of heme oxygenase 1 by Ginkgo biloba in neuronal cultures and potential implications in ischemia. Cell. Mol. Biol. 48, 647–653.

Zweier, J.L., 1998. Free radical generation in human endothelial cells exposed to anoxia and reoxygenation. Transplant. Proc. 30, 4228–4232.