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REVIEW

Role of Acidosis, NMDA Receptors, and Acid-Sensitive Ion Channel 1a (ASIC1a) in Neuronal Death Induced by Ischemia

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Abstract—This review collects data on the influence of intracellular and extracellular acidosis on neuronal viability and the effect of acidosis on neuronal damage progressing under brain ischemia/hypoxia. Particular attention is devoted to the involvement of ionotropic glutamic receptors and acid-sensitive ion channel 1a in these processes.

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It is well known that the index of intracellular and extracellular acidity or pH, the negative decimal logarithm of hydrogen ion concentration ($pH = -\log[H^+]$), plays a significant role in regulation of vital and functional activity of cells in living organisms. Different changes in cell functional state in most cases are accompanied by variation of intracellular pH (pH_i) by 0.10-0.16 and extremely rarely by more than 1.0 [1, 2]. The pH value is very different in different cell compartments, including its differences among cell organelles. For example, in lysosomes the proton concentration is about 10^{-5} M, and in mitochondrial matrix it is around 10^{-8} M. According to most reports, the neuronal cell cytoplasm of mammals is essentially neutral (pH_i is about 7.05-6.98) [3-7]. Taking into consideration the extremely high metabolic activity of neurons, this undoubtedly indicates the presence of strict mechanisms of intraneuronal pH stabilization. The pH_i value varies significantly in other types of cells. For example, in the state of rest the pH_i of an axolotl ovum is 7.2, but after fertilization this value reaches 8.3 [8].

The pH distribution inside the cell and its value that can vary in time is an important factor determining the functional state of a cell. This space–time pH_i heterogeneity might work as an effective way of administering the consistency of processes taking place in different cell compartments [2]. It should be noted that neuronal pH_i depends largely on extracellular pH (pH_e), and changes of pH_e lead to parallel changes in pH_i [5, 9]. In turn, the decrease in pH_i during brain ischemia/hypoxia lowers pH_e.

Detailed description of mechanisms of intracellular and extracellular pH regulation in normal state and in pathology can be found in many works [3, 10-13], so we will not discuss this issue in detail but will restrict ourselves to a short exposition. Metabolic systems leading to proton release and uptake in cytosol, systems of proton release and uptake from subcellular organelles, and intracellular and intra-organelle acid–base buffering systems are involved in pH homeostasis. However, the leading role in mechanisms of pH regulation in cell belongs to different systems transporting protons through the plasma membrane. Na^+/H^+-exchanger, Na^+-dependent and -independent HCO_3^-/Cl^--exchangers, Na^+-HCO_3^- symport, vacuolar proton ATPases, Ca^{2+}/H^+-exchanger, and

Abbreviations: ASIC1a) acid-sensitive ion channel 1a; CaMKII) Ca^{2+}/calmodulin-dependent protein kinase II; CGNs) cultured granular neurons; EIPA) 5-(N-ethyl-N-isopropyl)-amiloride; NMDA) N-methyl-D-aspartate; NMDARs) NMDA-subtype of glutamate receptors incorporating NR2B-subunit; ROS) reactive oxygen species.

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H^+-lactate and Na^+-lactate symports are involved in those systems. It is not only that each enzyme has its pH optimum, but also functioning of different transport systems directly depends on pH_i and pH_e. Cell metabolism, proliferation and differentiation, cytoskeleton structure, ion transport through membranes, and ATP generation by mitochondria—all these processes are pH-dependent.

INTRA- AND EXTRACELLULAR pH DECREASE DURING BRAIN ISCHEMIA/HYPOXIA

Limitation of oxygen entry into cells leads to a shift of energy exchange towards glycolysis, and during severe hypoxia complete transition to anaerobic metabolism—glycolysis—occurs. The efficiency of glycolytic ATP synthesis is much lower than that of aerobic (mitochondrial) ATP synthesis, and therefore ATP hydrolysis begins to dominate against its synthesis, this promoting proton accumulation in the cell. As a result of intensified glycolysis, its final product, pyruvic acid, is concentrated in cells and converted to lactate by lactate dehydrogenase. Intense withdrawal of protons and lactate out of cells lowers pH_e [14]. Normally pH_e in brain tissue is about 7.31-7.24 [3-5], and pH_i of neurons is about 7.05-6.98 [3-7], but during ischemia pH_e decreases to 7.0-6.43 [4-7] and pH_i to 6.86-6.15 [4-6, 15, 16]. Certainly, pH_e and pH_i in an ischemic center are heterogeneous. Choi in his review [17] cites data indicating that directly in an ischemic center $pH_i = 6.4$, whereas in the penumbral zone it is 6.7. According to this data, acidification of the medium during ischemia takes place outside the cell, as well as inside it, but these two parameters differ in absolute value, that is there is a distinct compartmentalization of the pH value. The $[H^+]$ compartmentalization is concerned not only with division of brain tissue as to pH_e and pH_i, but also with heterogeneity of cell composition of neural tissue including various types of glial cells, neurons, endothelium cells, blood cells, all inheriting different functions and H^+ concentration in cytoplasm. For example, in astrocytes pH_i value is 0.1 higher than in neurons [11]. This $[H^+]$ compartmentalization can exert a large influence on local pH changes in brain at different phases in the development of ischemia.

It should be noted that activation of ionotropic glutamic receptors might be another reason for pH_i lowering during ischemia. Stimulation of these receptors brings changes in Ca^{2+}, Na^+, and K^+ balance in neuronal cytosol and initiates drastic decrease in cytoplasmic pH level [18-20]. Acidification of neuronal cytoplasm under the action of Glu or N-methyl-D-aspartate (NMDA) can exceed 0.5 pH unit [18, 19]. The pH_i in neurons during activation of NMDA receptors decreases for many reasons, and one of them is calcium overload of neurons and competition between calcium ions and protons for protein binding sites. Wang and colleagues [21] have shown that neuronal cytoplasm acidification induced by Glu is directly connected with Ca^{2+} transport into mitochondria, since acidosis was prevented by a specific blocker of Ca^{2+} transport in mitochondria—ruthenium red. Later it was found that NMDA-induced acidification can be prevented by glycolysis inhibitors and inhibition of Ca^{2+}-ATPase of cytoplasmic membrane. This data shows that Ca^{2+}-ATPase of cytoplasmic membrane and glycolysis are involved in Glu-induced acidification of neuronal cytoplasm [22].

ACIDOSIS CAN INDUCE NEURONAL DEATH

It is quite difficult to investigate the influence of acidosis on neuronal survival in animals. It is much easier to model and control the intensity and duration of extracellular acidosis in *in vitro* systems. In light of this, the influence of acidosis on neuronal survival has been investigated mainly with the use of various systems of neuron cultivation. During these studies, it has been shown that the decrease in extracellular pH to 6.5-6.0 under normoxic conditions induces neuronal death in cell cultures and hippocampal slices [23-27], cultured brain cortical neurons [28], and also cultured granular neurons (CGNs) of rat cerebellum [29, 30]. It was shown on the same type of neuronal cultures that intracellular acidification of CGNs cytoplasm induced by inhibiting Na^+/H^+-exchange causes neuronal death [31].

Mechanisms of acidosis-induced neuronal death are now intensively studied. It has been shown that during acidification of the cultivation medium, hippocampal neurons perish not only by necrotic but also by apoptotic mechanisms, as the development of destruction can be restrained by protein or RNA synthesis inhibitors, and also by caspase inhibitors, which is not typical for necrotic death [27]. At the same time, in the case of intracellular cytoplasm acidification in granular neurons caused by inhibition of Na^+/H^+-exchanger, apoptotic death of these neurons is not sensitive to caspase inhibitors and is inhibited only by cycloheximide—a protein synthesis inhibitor [31].

It should be noted that neurochemical maturity can have high significance for neuronal sensitivity to the damaging action of acidosis. We have shown that neurochemically immature cultured cerebellar neurons are much more sensitive to acidosis than mature ones [30]. A product of anaerobic glucose metabolism, lactate, has even higher influence on the acidosis-induced neuronal damage; its presence in the cultivation medium increases toxic acidosis in those cells [27, 32].

In recent years, the question of acid-sensitive ion channels, specifically acid-sensitive ion channel 1a (ASIC1a), playing a role in acidosis-induced neuronal damage has been actively discussed. Earlier it was found that ASIC1a is expressed in many brain structures: amygdale, caudate nucleus, substantia nigra, hippocampus,

subthalamic nuclei, and cerebellum [33]. It was shown that channels of this type open at acidic pH, half-activation of ASIC1a occurring at pH about 6.2 [34, 35]. ASIC1a is permeable for sodium and calcium ions [36]. Calcineurin and kinase 150-binding protein are involved in the regulation of the channel activity [37]. Normally in neural tissue this channel is apparently connected to synapse functioning, as mainly it is localized in brain structures with high synaptic density [33, 38, 39]. The absence of ASIC was shown to disturb long-term potentiation in hippocampus. Knocked-out, homozygous $ASIC^{-/-}$ mice exhibit decreased exciting post-synaptic potentiation and activation of NMDA receptors during high-frequency stimulation. These knocked-out mice demonstrate obvious behavioral defects. It has been suggested that ASIC is involved in synaptic plasticity, learning, and memory [40].

It was shown using cultivated hippocampal and cortex neurons that external acidosis initiates entrance of calcium ions into neurons through ASIC1a, which does not depend on glutamate receptors and potential-dependent calcium channels. This calcium increase destroys neurons, and this destruction can be prevented by ASIC1a blockers or by lowering external calcium concentration [24, 28].

Another reason for destruction of neurons during acidosis might be the increase in production of free radicals in cells at low pH, with iron ions being involved in this process. This point of view found confirmation in experiments where it was shown that decrease in pH from 7.0 to 6.0 significantly increases free radicals production in brain homogenate [41]. Indirect evidence for the participation of reactive oxygen species (ROS) in acidosis-induced neuronal destruction is that a superoxide dismutase mimetic, catalytic superoxide anion trap EUK-8, decreases lipid peroxidation induced by acidosis in hippocampal slices [42]. Still, it is clear that the increase in ROS level in cells could be connected not only with the increase in their production, but also with decrease in antioxidant activity in cells. This state is probably true for acidosis as during this physiological event a decrease in activity of a few antioxidant enzymes takes place, in particular glutathione peroxidase and glutathione transferase, and also the amount of one of the main intracellular antioxidants (glutathione) decreases, as was shown on brain cortex cell cultures [43].

INFLUENCE OF DECREASE IN pH_e AND pH_i ON NEURONAL DAMAGE DURING ISCHEMIA/HYPOXIA AND GLUTAMATE TOXICITY

The literature data on the influence of decreasing pH_i and pH_e on neuronal damage during ischemia and glutamate toxicity is contradictory. In most studies using cultured neurons, a protective influence of extracellular acidosis on neuronal and other cell cultures subjected to oxygen-glucose insufficiency and glutamate toxicity has been shown [28, 44, 45]. Many authors connect a protective influence of a moderate external acidosis (pH value about 6.7) with the acidosis-induced inhibition of channels associated with NMDA receptors [28, 44, 45], which occurs due to the decrease in calcium flow through these channels [28, 44-46] or the ability of acidosis to block glutamate release from vesicles [47].

One way to decrease pH_i is to inhibit the Na^+/H^+-antiporter with 5-(N-ethyl-N-isopropyl)-amiloride (EIPA). It was found that lowering pH_i by this method prevents damage to hippocampal neurons initiated by ischemia/reperfusion of the Mongolian gerbil brain [48]. However, protective effect of this substance may be connected not with intracellular pH lowering, but with the ability of EIPA to block NMDA receptors [44]. At the same time, genetic disruption of the NHE1 isoform of Na^+/H^+-antiporter substantially decreases the oxygen–glucose deprivation-induced destruction of cultured mouse brain cortical neurons carrying this defect [49]. These data support the assumption that increasing proton concentration in cytoplasm by inhibiting Na^+/H^+-antiporter can extend neuron survival after ischemia. Furthermore, under condition of intracellular acidification during ischemia the release of endogenous excitatory amino acids (glutamate and aspartate) is inhibited. Since inhibiting of the Na^+/H^+-exchanger intensifies the work of the Na^+/Ca^{2+}-exchanger lowering the amount of Ca^{2+} in cells, that in turn decreases stimulation of Ca^{2+}-dependent phospholipase by calcium ions and decreases release of excitatory amino acids [48].

Other authors connect the protective effect of acidosis during a hypoxic period with its ability to prevent a decrease in activity of complex IV of the mitochondria respiratory chain caused by hypoxia [50]. However, these authors note that if acidosis protects neurons during a hypoxic period, then in the period of re-oxygenation the decrease in extracellular pH promotes increased neuronal damage. This idea that acidosis protects neurons during hypoxia but decreases their viability during the period of re-oxygenation is supported by other authors [51]. The decrease in activity level of a few antioxidant enzymes during acidosis [43] can also increase risk of neuronal damage by ROS during re-oxygenation, when free radical production in neural tissue is maximal. However, other authors using an *in vivo* ischemia model did not find any influence of acidosis on neuronal tissue damage by ROS [52]. Some authors believe conflicting data on the effect of extracellular acidosis on the development of neuronal damage is because toxic effect is observed during long-term acidosis and requires oxygen [25], whereas short-term pH lowering without oxygen exerts a protective effect [53]. As we already said, another factor of neuronal damage by acidosis during ischemia is acid-sensitive ion

channels, and especially ASIC1a subtype. It was shown on cultured cortical neurons that oxygen–glucose deprivation done under blockade of potential-dependent and glutamate-activated calcium channels causes much more damage to neurons at acid than at neutral pH values. This destruction clearly decreases when the cultivated medium contains ASIC1a-inhibiting substances. Neuronal death does not take place at all with cultures from brain of ASIC1a$^{-/-}$ mice. Knockout of the *ASIC1a* gene or intravenous injection of ASIC1a blockers protected animal brain from focal ischemia [28], even when blockers were introduced during the post-ischemic period [53].

As the data above indicate, the protective effect of acidosis is manifested mainly when modeling ischemia on cell models, whereas modeling brain ischemia on animals showed no protective influence of acidosis. Ou-Yang and coauthors note that although acidosis increase during ischemia slows the entrance of extracellular calcium into neurons through NMDA channels, in the end acidosis more likely increases the extent of brain damage rather than decreasing it [46]. In addition to the mechanisms of increasing neurodestructive influence of acidosis during ischemia described above, Yao and Haddad [54] note a number of mechanisms that can potentiate the effect of acidosis during this pathology. They included in this list the inhibition of glycolysis by acidosis, suppressing mitochondrial metabolism that can accelerate ATP exhaustion.

HYPERACTIVATION OF GLUTAMATE RECEPTORS AND ACIDOSIS — IS IT A UNIFIED MECHANISM OF NEURONAL DEATH DURING ISCHEMIA/HYPOXIA?

It is now an axiom that glutamate toxicity is a main pathogenetic factor during development of brain ischemia/hypoxia. However, there is accumulating data that point to the participation of direct acidosis in development of neuronal damage during this pathology. But at the same time, as we noted above, there is a whole set of studies showing that at acidic pH values not only the inactivation of NMDA receptors takes place, which protects neurons from destruction, but that it also lowers calcium flow into cells through potential-dependent calcium channels [46, 55]. Studies that settle this contradiction have appeared in recent years. It was shown on cultured hippocampal slices that at pH 6.4 in the presence of lactate, antagonists of NMDA or other subtypes of glutamate receptors lowered neuronal death, while in the absence of lactate at the same pH_e value Glu-receptor antagonists did not have significant influence on neuronal death. Acidic pH_e lowered [^{3}H]Glu capture significantly more in the presence of lactate. It was suggested that during ischemia acidosis caused by lactate accumulation is responsible for excitotoxic neuronal destruction initiated by an excess level of excitatory neurotransmitter [26]. This suggestion was confirmed by work done by Gao et al. [56], in which it is shown that ischemia increases ion entrance via ASIC1a in which Ser478 and Ser479 are phosphorylated, exacerbating ischemic cell death. This phosphorylation is catalyzed by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), whose activity is connected with activation of N-methyl-D-aspartate subtype of glutamate receptors incorporating NR2B subunit (NMDARs). Moreover, intensification of ion flow through ASIC caused by ischemia, increase in cytoplasmic calcium, and neuronal death are prevented if an NR2B-specific antagonist, CaMKII inhibitor is applied, or the expression of mutant forms of ASIC1a in which Ser478 or Ser479 is replaced by alanine (ASIC1a-S478A, ASIC1a-S479A) is increased. It was concluded that an NMDAR–CaMKII cascade is functionally connected with ASIC and endows acidotoxicity during ischemia [56].

Data cited above combines external acidosis and hyperactivation of glutamate receptors in a cascade of mechanisms of neuronal destruction during ischemia. However, works proving this theory are few at present, and further intensive studies devoted to its proof are needed. This is particularly relevant for investigating ASIC1a as a route of calcium ion entry into neurons and their role in mechanisms of regulation of $[Ca^{2+}]_i$ during the development of neuronal death caused by acidosis.

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