Glutamate-Independent Calcium Toxicity

Introduction

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It is widely accepted that a critical factor in determining neuronal death during cerebral ischemia is the progressive accumulation of intracellular Na⁺ ([Na⁺]) and Ca²⁺ ([Ca²⁺]) ions, which can precipitate necrosis and apoptosis of vulnerable neurons. Whereas the detrimental action of [Na⁺], increase is attributable to both cell swelling and microtubular disorganization—2 phenomena that lead to cell necrosis—a change in [Ca²⁺], has been shown to be a key factor in ischemic brain damage, for it modulates several death pathways, including oxidative and nitrosative stress, mitochondrial dysfunction, and protease activation.

Reassessment of the Excitotoxicity Theory: Criticism to the Paradigm

Since Olney’s seminal work firstly suggested that excitatory aminoacids could elicit neurotoxicity, a large amount of work has been accumulated showing that glutamate extracellular concentrations briskly rise during acute brain injury, thus triggering an influx of Ca²⁺ and Na⁺ ions into neurons through ionotropic glutamate receptor subtypes. This evidence has led to the elaboration of the paradigm of glutamate excitotoxicity that explained ischemic neuronal cell death as a mere consequence of Na⁺ and Ca²⁺ influx through glutamate receptors. Although this theory has been guiding basic research in the field of neurodegeneration for almost 3 decades, more recently it has become the object of serious criticism and reassessment. What has aroused such skepticism among researchers has been the fact that although first, second, and third generation glutamate receptor antagonists have long yielded promising results in animal models of brain ischemia, they have failed to elicit a neuroprotective action in stroke and traumatic brain injury in humans. Therefore, the theory of excitotoxicity, though a fascinating paradigm, can only explain some of the events occurring in the acute phase of anoxic insult but cannot be seen as a major target for developing new therapeutic avenues for brain ischemia.

In the last 3 years, several seminal experimental works are markedly changing the scenario in this field. In fact, it has been shown that some integral plasma-membrane proteins, involved in the control of Ca²⁺ and Na⁺ ion influx or efflux and, therefore, responsible for maintaining the homeostasis of these 2 cations, might function as crucial players in the brain ischemic process. Indeed, these proteins, by regulating Na⁺ and Ca²⁺ homeostasis, may provide the molecular basis underlying glutamate-independent Ca²⁺ overload mechanisms in neuronal ischemic cell death and, most important, may represent more suitable molecular targets for therapeutic intervention.

TRPM7 and ASIC: 2 New Players in the Scenario of Ca²⁺ Overload Toxicity in the Ischemic Insult

In ischemia, TRPM7 and ASIC represent 2 new influx pathways. The former is a member of the melastatin subfamily of Transient Receptor Potential (TRP) named TRPM, which encompasses nonselective cationic channels highly expressed in the brain. TRPM7 is a Na⁺, Ca²⁺ and Mg²⁺ permeant ion channel that is regulated by changes in [Ca²⁺]o and [Mg²⁺]o. In particular, this Ca²⁺-sensing nonselective channel is regulated negatively by [Mg²⁺], and positively by [Ca²⁺], reactive oxygen (ROS) and nitric oxide (RNS) species, and, finally, phosphatidylinositol 4,5-diphosphate (PIP₂). Given that TRPM7 is activated by oxidative stress, it may play a relevant role in neuronal death. In particular, recent data suggest that TRPM7 carries an inward cationic current in response to combined oxygen-glucose deprivation (IOGD), a phenomenon that maintains ROS and RNS production. Indeed, when TRPM7 is silenced, OGD-induced neuronal degeneration is prevented by the suppression of [Ca²⁺]o rise and consequently by the reduction of NO and O₂⁻ production.

The other class of ion channels that received major attention from the perspective of the nonglutamatergic mechanisms of Ca²⁺ overload in ischemic cell death comprises the acid-sensitive ion channels (ASICs), which, like TRPM7, are widely expressed in the central nervous system. A peculiar aspect of these channels is that they carry an inward Na⁺ and Ca²⁺ current in response to extracellular acidification (Figure). Therefore, because extracellular pH drops early during the evolution of ischemic cerebral events, ASICs drew a major attention as putative Na⁺ and Ca²⁺ influx pathway in ischemic neurons. Importantly, cation influx through ASICs has been shown to have a crucial role in the progression of cell death. Indeed,
when these channels are selectively blocked with the tarantula toxin psalmotoxin-1, a pronounced neuroprotection is observed in models in vivo and in vitro of brain ischemia. Further arguments in favor of the detrimental role of ASIC activation in ischemia have been provided by the observation that ASIC1a knockout mice are protected against brain damage induced by experimental brain ischemia.

Gene Products of the Na\(^{+}/\)Ca\(^{2+}\) Exchanger NCX1, NCX2 and NCX3 as Arbiters of Na\(^{+}\) and Ca\(^{2+}\) Intraneuronal Homeostasis in the Ischemic Insult

Besides TRPM7 and ASICs, another pivotal player in brain ischemia is the Na\(^{+}/\)Ca\(^{2+}\) Exchanger (NCX), an antipporter that can operate either as a Ca\(^{2+}\)-efflux/Na\(^{+}\)-influx pathway (forward mode) or as a Ca\(^{2+}\)-influx/Na\(^{+}\)-efflux pathway (reverse mode), depending on [Ca\(^{2+}\)]\(_{\text{e}}\) and [Na\(^{+}\)]\(_{\text{e}}\) (Figure). During anoxic conditions, owing to the compromise of the 2 plasma-membrane ATP-dependent pumps Na\(^{+}/\)K\(^{-}\) ATPase and Ca\(^{2+}\) ATPase, NCX assumes a relevant role in controlling the intracellular homeostasis of these 2 cations.

Interestingly, whereas the function of TRPM7 and ASIC activation in brain ischemia is unequivocally detrimental, the function played by NCX seems to be predominantly neurobeneficial. In fact, in the early phase of neuronal anoxic insult, because of the initial blockade of Na\(^{+}/\)K\(^{-}\) ATPase, (1) [Na\(^{+}\)] critically increases, (2) voltage-sensitive Na\(^{+}\) and Ca\(^{2+}\) channels open in response to the ensuing depolarization, and (3) ionotropic glutamate receptors are activated. As a consequence of Na\(^{+}\) overload, NCX is forced to operate in the reverse mode, thus extruding Na\(^{+}\) ions, while promoting Ca\(^{2+}\) influx. Although this reverse mode of operation in the early phase of anoxia does undoubtedly elicit an increase in [Ca\(^{2+}\)]\(_{\text{e}}\), its effect could be beneficial for neurons because it contributes to decrease Na\(^{+}\) overload, a phenomenon which would otherwise lead to cell swelling and, thus, sudden necrotic neuronal death. Conversely, in the later phase of neuronal anoxia, when Ca\(^{2+}\) overload takes place, NCX working in the forward mode, a condition that promotes Ca\(^{2+}\) efflux and Na\(^{+}\) influx, can contribute to the lowering of Ca\(^{2+}\) concentrations, and thus can protect neurons from Ca\(^{2+}\) overload neurotoxicity and their subsequent cell death.

A further element of complexity in the role played by this antipporter in the cascade of events leading to anoxic neurodegeneration is the existence of 3 different gene products NCX1, NCX2 and NCX3. These 3 isoforms display different sensitivity to intracellular ATP levels. In fact, whereas ATP is required for NCX1 and NCX2 activity, NCX3 is still able to operate in the absence of this nucleotide. Therefore, even though the intraneuronal ATP depletion, induced by ische-
nia, is expected to lead to a derangement of NCX1 and NCX2 activity, it should not interfere with NCX3 activity.

This differential sensitivity to intraneuronal ATP changes of the 3 NCX gene products may have relevant consequences if considered within the context of the different ischemic brain regions. In particular, during the early phase of brain injury, [Na+]i, relevantly increase in the ischemic core region owing to Na+/K+ ATPase failure, which is determined by the remarkable lowering of ATP levels. Hence, NCX, whose mode of operation is governed by the electrochemical gradient of Na+ and Ca2+ ions, is forced to operate in the reverse mode as a Na+ efflux-Ca2+ influx pathway, thus helping neurons to counteract Na+-overload–dependent acute necrotic cell death.6 Therefore, considering the dissimilar sensitivity of the NCX gene products to ATP levels, the fate of neurons in the ischemic core could be largely determined by NCX3, given that this NCX isoform, which is normally expressed in the brain regions12 of the ischemic core, is the only one able to preserve its activity despite the occurrence of intense ATP depletion. This hypothesis is fully supported by our laboratory results, showing that cells transfected with the NCX3 isoform have a reduced vulnerability to hypoxic insult compared with host cells stably expressing NCX1 or NCX2.13 By contrast, because in the penumbral region Na+/K+ ATPase activity is still operative thanks to the minor depletion of ATP levels, it is conceivable that in this specific area all the 3 NCX isoforms may be operative in the forward mode. As a result, in the later phases of the ischemic process, the exchanger, by extruding Ca2+ ions, may participate in the reduction of Ca2+ overload neurotoxicity occurring in neurons and axons located in the penumbra. Therefore, the cooperative intervention of the 3 NCX isoforms might contribute to neuronal rescue much more efficiently than in the ischemic core where only NCX3 should be active.

Substantial evidence in support of the differential role played by the different members of the NCX family in brain ischemia has been obtained in our laboratory. In particular, in 2004 we found that, after permanent middle cerebral artery occlusion, NCX1 and NCX3 protein levels are both significantly and persistently decreased in the ischemic core, whereas, in the penumbra region, only NCX3 protein levels are reduced, suggesting that these 2 proteins are degraded during the ischemic process.6 These data indicate that, owing to NCX proteinolysis in the neurons of the ischemic core, the functional activation of NCX1 and NCX3, needed to compensate ionic derangement, is profoundly depressed. Consequently, ischemic neurons should be extremely vulnerable to experimental maneuvers that further decrease NCX expression. Confirming this hypothesis, we found that the specific antisense knocking-down of NCX1 and NCX3 transcripts produces a significant worsening of ischemic brain injury induced by permanent middle cerebral artery occlusion, whereas the antisense knocking-down of NCX2 has no effect on the evolution of the ischemic brain damage.6

Interestingly, when we looked at the levels of mRNAs encoding for NCX1 and NCX3 proteins in the penumbra, we found that these transcripts markedly increased in the face of the decrease of protein levels, suggesting that a compensatory increase in NCX gene transcription does occur in response to increased NCX protein degradation.14 Conversely, NCX2 mRNA was downregulated in the same area, possibly as a consequence of neuron depolarization triggered by ischemia-induced repetitive spreading depression-like depolarization waves.15

One year later, in 2005, the idea that NCX proteins could undergo extensive protein degradation as a consequence of brain ischemia found an indirect confirmation in the article by Bano et al.16 They indeed reported that when cultured cerebellar granule cells are exposed to excitotoxic glutamate concentrations, NCX1 and NCX3 are cleaved by caspase-3 and calpain, respectively.

Closing Remarks

Overall, the experimental work briefly reviewed in this article leads to the conclusion that the classic excitotoxicity paradigm is an oversimplified interpretation of the chain of events determining ionic derangement in the different phases of brain ischemia. Furthermore, it emphasizes the need to revisit some of the current theory on the molecular pathophysiology and pharmacology of neuronal ischemic cell death.

On energetic deprivation of oxygen and glucose the reduction of ATP production by mitochondria, a marked increase of [Na+]i occurs, attributable to the failure of Na+/K+ ATPase, thus leading to a depolarization-induced opening of voltage-gated Ca2+ and Na+ channels which is followed by (1) a rapid decrease in [Na+]i and [Ca2+]i, and (2) by a release of glutamate in the extracellular space. Glutamate, once released, further amplifies the process opening Na+ and Ca2+ permeable glutamate receptors and therefore further lowering [Ca2+]i. This [Ca2+]i decrease triggers the flux of more Na+ and Ca2+ through TRPM7 channels (Figure). In parallel, the drift of intracellular metabolism toward anaerobic glycolysis leads to lactate accumulation and extracellular acidification. This change in extracellular pH is sensed by ASICs, whose activation allows much more Na+ and Ca2+ ions entering into the cell (Figure). All these events leading to intracellular Ca2+ overload trigger an increase in ROS and RNS generation which, in a positive feedback loop manner, further amplifies [Na+]i and [Ca2+]i accumulation by potentiating TRPM7 channel activity (Figure).

Finally, a third player of the nonglutamatergic neurotoxicity theory is NCX. Indeed, it has the potential to thwart TRPM7-, ASICs- and NMDA-dependent [Na+]i, and [Ca2+]i overload. Actually, this antiporter has the ability to quickly increase its activity in response to [Ca2+]i, increase to oxidative and nitrosative stress. Interestingly, the plasma-membrane phospholipid PIP2, which is the precursor of the IP3 and DAG, simultaneously regulates in a positive manner NCX and TRPM7 activity (Figure). Therefore, because PIP2 concentration may change during brain ischemia in some selective regions,17 a role for this phospholipid in the regulation of the ionic events taking place in the ischemic neurons because it simultaneously affects TRPM7 and NCX can be envisaged.

In conclusion, the crucial role played by TRPM7, ASIC, and NCX in the scenario of ischemic neuronal cell death paves the way for further molecular and electrophysiological studies aimed at developing new strategies for the treatment
of stroke, in the hope of overcoming the limitations of antiglutamatergic drugs.

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Disclosures
None.

References
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