ISSN 0006-2979, Biochemistry (Moscow), 2011, Vol. 76, No. 10, pp. 1079-1086. © Pleiades Publishing, Ltd., 2011.
Published in Russian in Biokhimiya, 2011, Vol. 76, No. 10, pp. 1325-1334.

REVIEW

Pleiotropic Functions of Brain Proteinases: Methodological Considerations and Search for Caspase Substrates

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Received March 16, 2011
Revision received April 17, 2011

Abstract—Analysis of the literature and our own data suggest that the so-called "apoptotic" proteinases play important roles in brain function. However, mechanisms of their involvement in normal neuronal plasticity remain obscure. One of the main reasons for this is broad substrate specificity of proteinases; the number of potential substrates of each can reach several thousands. Obviously, a real approach to study functions of "apoptotic" proteinases, caspase-3 in particular, is to identify their intracellular substrates. It is the nature of a substrate that defines the direction of signal transduction or metabolic changes; therefore, identification of molecular partners of particular proteases should be the key study, not just measuring its activity or respective protein or mRNA expression. This approach will allow studying regulatory mechanisms not only for proteinases, but also for other pleiotropic enzymes usually possessing broad substrate specificity.

DOI: 10.1134/S0006297911100014

Key words: proteinases, caspase, proteinase substrates, broad substrate specificity, plasticity, apoptosis, brain

During the last two decades, many studies have been focused on brain proteinases because of their apoptotic function. Indeed, a key role in neuronal cell death has been demonstrated for several proteinases, specifically for the enzymes of cysteine proteinase family [1]. From this perspective, the most unexpected result from the studies of "apoptotic" brain proteases turned out to be the fact that inhibition of these enzymes aimed to correct neurodegenerative processes induced disturbances in neuronal functioning [2]. This result motivated us to analyze the data from different groups and suggest that so-called "apoptotic" proteases, in fact, are intimately involved in normal functions of the nervous system, in other words, in normal neuronal plasticity [3-5]. Our studies in this field [3] have been further supported by several groups in Russia and abroad.

There is now no doubt that regulated proteolysis is critically involved in synaptic remodeling, learning, memory, and development of the nervous system [5, 6]. Thus, it should not be surprising that so-called apoptotic brain proteolytic enzymes, caspase-3 in particular, are essential not only for apoptosis [3-5]. Indeed, caspase-3 is among the most active executors of the apoptotic program in brain during prenatal and early postnatal periods of ontogenesis when a significant fraction of neurons are dying in the embryonic and newborn brain [7]. However, inhibition of caspase-3 in the adult brain results in impairment of plastic processes and decrease of brain adaptive capabilities [8]. Pleiotropicity of the enzyme function in the brain makes it difficult not only to understand mechanisms of its involvement in normal plasticity, but also to deliberately modulate enzyme activity in the brain in an effort to prevent or treat cerebral pathologies. So far, the mechanism of switching between apoptotic and non-apoptotic functions of caspase-3 remains obscure. Revealing mechanisms of caspase-3 regulation in normal and pathological situations will allow controlling them in pathological situations, preventing caspase-dependent neuronal cell death (e.g. in neurodegeneration) or stimulating it (e.g. during carcinogenesis).

Enzymes with broad substrate specificity as switches of signal transduction and metabolic pathways: key role of nature of substrate. Considering pleiotropicity of caspases from the perspective of the problem under discussion, it should be noted that this is just a special case within the framework of a general rule, this rule becoming obvious

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when analyzing the data of studies of the last two-three decades. Which key enzymes occupy places in junction points of signal transduction and metabolic pathways? These are protein kinases, protein phosphatases, and proteinases. The enzymes having up to tens of thousands of potential substrates [9] are functioning on the principal signaling-metabolic crossroads exactly because they can switch pathways by processing *different* substrates. This is particularly clear for protein kinases, enzymes catalyzing cascades of consecutive phosphorylations of substrates, the nature of these substrates just ensuring targeted signal transduction, while the plurality of substrates permits switching between different signal transduction pathways [10]. Obviously, this reasoning is also true for protein phosphatases, the enzymes from the regulatory and functional opposites of protein kinases.

Thus, it becomes clear that it is not primarily the enzyme activity that defines the direction of further signaling/metabolic events (especially since it will have overlapping substrate specificity with enzymes of the same group), but the nature of a particular substrate. The fact that many intracellular proteins are arranged in dynamic multiprotein complexes confirms this idea suggesting a possibility for a "point" regulation of signaling/metabolic pathways by a specific spatial approaching with the enzyme of the target substrate. All aforesaid is fully applicable to proteinases, the enzymes using as substrates virtually *all proteins* of living organisms.

One of most important consequence of the broad substrate specificity of the above-mentioned enzyme families is their pleiotropicity and multimodality involving one and the same enzyme in a variety of different functions, often just opposite ones.

Pleiotropicity of caspase-3. Brain caspase-3 is a typical pleiotropic enzyme. In cerebral pathologies, it mediates both neuronal cell death and compensatory processes necessary for neuronal survival and normal functioning of the brain in general. It is obvious that opposite functions of caspase are based on cleaving different substrates of the enzyme (potentially, about 35,000 proteins with respective consensus amino acid sequence) [9]. That is why attempts to control processes of neuronal cell death and survival based on caspase-3-catalyzed reactions may only be successful after the identification of key substrates of the enzyme. Here is the main reason for the crushing failures of attempts of brain caspase-3 blockage for treatment of cerebral pathologies, though some positive effects of caspase inhibitors have been reported in cell cultures and animal models.

Caspase-3 is a major caspase of mammals responsible for the majority of proteolytic events during apoptosis [11]. In particular, in the brain caspase-3 is a main executor of the apoptotic program [1, 12]. Unlike thoroughly studied involvement of caspase-3 in apoptotic processes, no systematic investigation of non-apoptotic function of caspase-3 in the brain has been performed yet. In particular, no attention has been focused on the identification of substrates – molecular partners of caspase-3 in normal brain.

Non-apoptotic functions of caspase-3. The term "non-apoptotic functions of caspase-3" may be misleading since it may mean different concepts. For example, involvement of caspase-3 in non-apoptotic cell death has been demonstrated [13] (as numerous data on caspase-independent apoptosis are published). These problems are out of the scope of this review; we will focus only on caspase functions not related to cell death.

During the last decade, a number of reviews appeared summarizing a few evidences about caspase functions in the normal organism, in particular in the brain [14, 15]. Since that time a number of relevant experiments has been performed in this area (which we will discuss further), though no real breakthrough has occurred. Obviously this is a result of methodological difficulties of identifying definite substrates, the cleavage of which by caspase could conform to a definite functional mechanism or event.

A substantial number of studies related to non-apoptotic functions of caspase-3 deal with models of cell differentiation, unfortunately, mostly of non-brain origin. The involvement of caspases in differentiation can be dual. Depending on the type of cell, differentiation may involve processes resembling or reproducing some features of apoptosis or have no similarities with apoptosis. Respectively, differentiation of keratinocytes, lens cells, and red blood cells resembles apoptosis stopped at the stage of nuclear destruction. In these cell types, caspases are involved in differentiation [16-18]. However, even in these cells caspase function may not resemble that in apoptosis. For example, caspase inhibitor blocks erythrocyte differentiation at the stage preceding nuclear destruction; administration of caspase-3 siRNA has the same effect [16]. In differentiating lens cells a similar situation has been demonstrated, while *in vivo* activation of caspases occurs several days earlier than activation of proteolysis [17]. The authors of these studies emphasize that activation of caspases is strictly time-limited, and this period does not coincide with the period of nuclear destruction; this means that processes in differentiating cells are quite different from those in apoptosis. Several groups managed to identify substrates of caspases in differentiating cells. These are lamin B and acinus in erythroid cell differentiation [16] and protein kinase Cδ in keratinocytes [18]. Interestingly, PARP (poly(ADP-ribose) polymerase) is not cleaved by caspase-3 during erythroid cell and keratinocyte differentiation, though PARP is cleaved in differentiating lens cells. Most probably, the above-mentioned proteins as caspase-3 substrates represent only some of the substrates cleaved by caspase during differentiation. Since apoptotic activation of caspase results in cleavage of hundreds of proteins [19], we can suggest that the number of substrates involved in processes not related to cell death is rather large.

Another group of studies deals with cells that differentiate without nuclear loss, their differentiation not resembling apoptosis. Genetic deletion of caspase from myocytes results in their aberrant differentiation and changes in expression of a number of specific proteins; however, it does not affect the rate of apoptosis. Noteworthy, a caspase substrate, serine/threonine protein kinase 4 (MST1) involved in myocyte differentiation was identified [20]. Caspase-3 removes the inhibitory domain from this protein, and active MST1 is not only mediating differentiation, but it also restores the pattern of normal differentiation in cells with inhibited caspase-3. Thus, involvement in differentiation may be a major role of caspase-3 in myocytes. Serine/threonine protein kinase Raf exerts negative regulation of caspase-3-mediated differentiation of myoblasts, a similar cascade regulation revealed in erythroid cell differentiation [21]. Caspases are activated during differentiation of megakaryocytes and production of platelets [22]. Serine/threonine protein kinase MST1 is involved in megakaryocyte differentiation, though the role of caspase in this process has not been ascertained. Remarkably, during platelet production caspase-3 is locally activated in some cellular compartments, in contrast to apoptosis, when caspase-3 is activated over the whole cell [22]. Caspase inhibitor reduces platelet aggregation, a process similar to differentiation. Activation of caspases goes along with differentiation of monocytes to macrophages. This process is accompanied by release of cytochrome *c* from mitochondria and can be reduced by caspase inhibitor [23]. Moreover, deletion of the caspase-8 gene prevents the differentiation of hematopoietic cells. Interestingly, only some kinds of differentiation are affected, while others do not change. The protein acinus is cleaved by caspase during differentiation of monocyte, while PARP is not [23]. Another group made an attempt to identify all caspase substrates using this model [24]. Several dozens of proteins with their expression changed during differentiation have been demonstrated. Some of them undergo caspase-mediated cleavage. Caspase activation accompanies osteoblast differentiation as well [25]. Active caspase-3 is necessary for differentiation of cerebellar glial cells [26, 27]. Interestingly, immunohistochemical positive staining of neurons for caspase-3 is absent, while administration of caspase inhibitor results in a change of the portion of proliferating and differentiating glial cells. Caspase-3 activation accompanies differentiation of neuronal stem cells, while caspase inhibition induces decrease in activity of some pro-neurogenic kinases [28]. Maturation of dendritic cells is caspase-dependent, caspase inhibitor inducing changes similar to those characteristic for inflammatory process [29]. Tetrameric adapter protein AP-1 serves as a caspase substrate in this model.

As mentioned above, experiments demonstrating involvement of caspase-3 in differentiation of various cells type gave the first evidence about a non-apoptotic role of the enzyme [20, 25]. We made attempts to reveal the role of caspase-3 in differentiation of neuronal cells. According to our preliminary results, caspase-3 mediates some (but not all) scenarios of differentiation of neuroblastoma B103 cells as well as PC12 cells. Moreover, inhibition of caspase-3 changes morphological patterns of differentiation and duration of cell cycle phases. Importantly, increase in caspase-3 activity during differentiation is not accompanied by increases in activities of other apoptotic enzymes, while differentiated cells with higher caspase-3 levels are more resistant to cytotoxic stimuli.

The involvement of caspase-3 in proliferation is described in detail, though, mainly not for brain cells. A significant part of these studies deal with interaction of caspases with kinases, kinase inhibitors, and cyclins [30, 31]. Caspase inhibitor delays mitosis in HeLa cultures, the key protein cleaved by caspase-3 during induction of mitosis in these cells being BubR1, a protein kinase associated with serine/threonine protein kinase of mitotic check point Bub1 [30]. Analysis of caspase family protein expression and caspase activity during the cell cycle gave similar results [31].

Regulation of caspase activity in the cell: basic mechanisms remain unexplored. It is not clear so far how caspase activity is regulated and how caspases regulate various intracellular events. For example, it was shown that though all caspases are soluble proteins, caspase activity can be associated with membrane fraction [32, 33]. However, so far no mechanisms of caspase association with cellular membrane could be revealed. Activities of caspase family enzymes somehow depend on the N-terminal fragment cleaved from caspases during their activation [34, 35]. One of the mechanisms of caspase inactivation is poly-ubiquitinylation [36], caspases activating their inhibitor themselves creating a negative feedback.

Caspase-3 can be activated as a result of a Ca^{2+}-dependent process [37], where the activation mechanism remains unknown and the activated enzyme is less active than it is after apoptotic activation. In addition, the following factors regulating caspase activity are discussed in the literature: availability of substrates, processing of substrates, activity of antiapoptotic factors, posttranslational modification of caspase-3 and its substrates, anchor proteins binding enzymes and/or their substrates in definite intracellular compartments. However, so far no experimental evidence confirms these suggestions.

Some additional mechanisms of caspase activity regulation can be suggested. Among them is alternative splicing – temporary, inducible, or persistent. In apoptotic cells several caspase-3 forms have been suggested [38], these forms differing in specific cell types, but the nature of caspase isoforms remained unclear. Unexpectedly, it has been shown that several caspase-8 isoforms can be expressed in the cell, some of them having antiapoptotic function [39]. Another potential factor of caspase-3 regu-

lation may be related to its possible function not associated with its catalytic activity. Such an unusual ability was demonstrated for cathepsin D: its catalytically inactive form is able to induce apoptosis [40].

It can be concluded that there is a considerable set of data on caspase roles beyond apoptosis. However, in general, this field remains descriptive and phenomenological. No doubt the situation will change dramatically if mechanisms of caspase intracellular regulation will become clear and caspase substrates will be identified in different physiological situations.

Pleiotropic functions of caspase-3 in the brain. In the brain, non-apoptotic functions of caspases, and caspase-3 in particular, have been studied much less that in other animal organs and tissues. First of all, the fact that caspase activities in the nervous system are significantly lower than in many other organs halts some investigators. Second, brain is an extremely difficult object for study — it is highly heterogeneous containing various regions and specific cell types with different functions.

Now it is clear that caspase-3 not only participates in several apoptotic scenarios in the brain (specifically, in neuronal cell death in early ontogenesis), but it is critically involved in important non-apoptotic functions unavoidable for survival and functioning of neurons, other brain cells, and the nervous system on the whole. An issue of primary importance becomes a question about regulation of the activity of the protein fulfilling several different functions; first of all it is a possibility to use different substrates, which should be cleaved to fulfill different tasks. In other words, the essence is in understanding mechanisms of "switch on" and "switch off" of different functions of one and the same protein by targeted choice of a specific substrate.

Our studies were among the first where non-apoptotic functions of brain caspase-3 were demonstrated using various models. Changes in caspase-3 activity over the hibernation cycle were demonstrated in brain regions of ground squirrels *Citellus undulatus* [41]. Both region-specific and hibernation stage-dependent alteration of caspase-3 activity were revealed, no TUNEL-positive apoptotic cells being noted in all brain regions studied. We suggested that caspase-3 is involved in seasonal changes in the brain of hibernators. One of the possible ways of caspase-3 involvement in this kind of neuronal plasticity is the ability of the enzyme to reorganize the actin cytoskeletal system. Neuronal shape is reported to change over the hibernation cycle [42, 43], this demanding rearrangement of cytoskeleton, and caspase-3 readily cleaves actin [44].

The results of another experiment suggested a new role of caspase-3 during early postnatal ontogenesis [45]. During this period cooperative changes in neuronal activity and caspase-3 activity were revealed in hippocampi of control and stressed rats. In hippocampi of stressed rats caspase-3 activity changed between postnatal days 19 and 23, during a period critical for synaptic reorganization. Since no increase of postnatal apoptosis could be demonstrated during this period in hippocampus, we suggested that caspase-3 activity is related with changes of synaptic connection strength. It is well known that rapid turnover of neuronal processes in hippocampus during early postnatal ontogenesis requires cytoskeletal reorganization [46], caspase-3 likely being involved in this process. This enzyme can also participate in postnatal modification of hippocampal receptors since it has been shown that caspase-3 controls activities of extra- and intracellular receptors relevant for neuronal plasticity [47, 48].

Studies of the phenomenon of long-term potentiation (LTP) in hippocampal slices provided important evidence for the involvement of caspase-3 in neuronal plasticity [3, 49]. Slices of rat hippocampus were incubated for 30 min with caspase-3 inhibitor peptide Z-DEVD-FMK or another peptide Z-FA-FMK without inhibitory activity. Neither of the peptides affected basic characteristics of neuronal activity within 7 h after the incubation. Within the first hour after the incubation with either peptide, the slices demonstrated similar LTP initiation and retention curves. However, 1.5-3 h after the incubation with caspase-3 inhibitor LTP was significantly lower as compared with the control peptide, while after 3.5 h LTP was completely blocked. These results represent the first evidence that caspase-3 inhibition significantly decreases and even completely blocks LTP in the CA1 field of hippocampus, and that caspase-3 is essential for LTP. Other studies in hippocampal slices of rat suggest that caspase-3 is necessary for long-term depression phenomenon because caspase-3 regulates AMPA receptor transport [5]. Mechanisms of caspase-3 involvement in LTP phenomenon are not clear; however, effects on LTP are in concordance with data about impairment of long-term spatial memory in rats after intra-hippocampal administration of a caspase-3 inhibitor tested in a water maze [4]. We studied effects of caspase-3 inhibitor effects on other forms of learning and memory in rats [8]. Administration of caspase inhibitor Z-DEVD-FMK significantly affected behavior of rats in "dark-light chamber" test, while administration of control peptide Z-FA-FMK had no effect. Moreover, Z-DEVD-FMK impaired acquisition of some principal behavioral components in an active avoidance test. Caspase-3 activity was measured in a brain region involved in learning, and maximal decrease of the activity after administration of the specific inhibitor was evident in the fronto-parietal cortex. The degree of inhibition was rather low indicating the importance of local changes in specific brain cells. These data suggest that caspase-3 is important for some types of learning. These results indicate a relation between active caspase-3 and important processes in brain not associated with cell death. Caspase-3 is involved in neuroplasticity, including its highest forms (learning, memory), ensuring adaptation of neurons to changing environment.

Surprisingly enough, intracerebral administration of caspase-1 inhibitor improves memory [50] and, respectively, caspase-1 inhibitor amplifies LTP in hippocampal slices [51], demonstrating an effect opposite to those of caspase-3 inhibitor.

A decrease in brain caspase-3 activity was demonstrated under chronic stress, most probably reflecting processes leading to significant decrease of brain plasticity [52]. Another example of important biochemical rearrangements in the brain without significant neuronal death is a model of drug addiction in rats. We suggested that changes in rat brain induced by opiate administration affect brain caspase-3 activity [53]. Indeed, caspase-3 activity changed in brain regions specifically involved in drug addiction; neither the degree of caspase activation nor temporal pattern of its activation suggests the association of caspase-3 with apoptosis in this experimental situation. Like in general concerning non-apoptotic function of caspase-3, in this specific situation it is difficult to trace both pathways of the enzyme activation and its targets, though connection of caspase-3 with changes in glutamatergic synaptic transmission can be suggested. Different caspase-3 levels in brain regions of rats with different psychophysiological status may be also related to differences in glutamatergic neurotransmission [54]. For example, for emotionality brain regions of a genetically more anxious rat strain caspase-3 activity was several-fold lower [54]. It can be suggested that caspase-3 is involved in functioning of neuronal glutamate receptors taking into account that caspase-3 is able to cleave glutamate receptor subunits [55, 56].

Some studies demonstrate that "apoptotic" signaling controls synaptic plasticity. Plasticity and motility of growth cones is accompanied by local caspase-3 activation in the cone [47]. Local and rapid caspase-3 activation in growth cone of retinal cells is necessary for setting up correct neuronal communications [57]. Caspase-3 can control calcium current into the cell by cleaving GluR1 subunit of glutamate receptor [55, 56]. During development and aging, expression of caspase family proteins in rat brain is following a specific pattern; subcellular localization of these proteins in the adult brain is different [58, 59]. Localization of caspases in different cellular compartments is another confirmation of their potential non-apoptotic function since it is believed that only cytoplasmic fraction of caspases is important for apoptosis. Active caspase-3 was revealed immunohistochemically in proliferating regions of rat brain during the first two postnatal weeks [60]. This phenomenon is not related to apoptosis that at this time takes place in other brain regions, but, obviously, is associated with active plastic neuronal rearrangements. Using an *in vivo* model of ischemic tolerance, caspase-3 activation with no signs of apoptosis was demonstrated in preconditioned nervous tissue, while in a model of tolerance to excitotoxicity by oxidative phosphorylation blockage in neuronal culture caspase-3 inhibitor impaired ischemia-induced protection from NMDA [61]. The latter example indicates that a direct antiapoptotic function may be among the various functions of cappase-3.

A "caspase–cathepsin mimicry": to what degree does the activity measured in the brain belong to caspase-3? Theoretically, presence in one and the same cell of proteins with slightly different structure and with similar substrate specificity but differing in the structure of regulatory domain might explain various functions of different caspase-3 isoenzymes. Regulation of these slightly different forms of the same enzyme may also involve different pathways; for example, selective posttranslational modifications or selective translocation of isoforms to various intracellular compartments. We made attempts to verify this hypothesis and tried to characterize the enzyme of rat brain possessing caspase-3 activity. At neutral pH usually used for assessment of caspase-3 activity one protein, caspase-3 itself, displays this activity. Surprisingly, acidification to pH 6.5 involves other enzymes into cleavage of caspase-3 synthetic substrate. We managed to isolate and characterize one of them; it turned out to be lysosomal proteinase cathepsin B [62]. Now we have more reasons to suggest that not all substrates attributed to caspase-3 are cleaved by caspase-3. In living tissues acidification takes place quite often, and it is not necessarily a pathological process. For example, decrease in pH accompanies normal synaptic activity [63, 64].

Studies of caspase activity at low pH were further performed on neuronal cell cultures. It has been shown that in specific situations not related to cell death neurons can secrete an enzyme cleaving caspase-3 substrate at acidic pH [65]. Again, it was cathepsin B. Thus, other proteinases in the brain can possess caspase-3-like activity, this activity not necessarily being related to apoptosis. We can assume that local pH change may be one of the factors regulating caspase activity.

Nevertheless, we should not consider that non-apoptotic caspase activity is, in fact, the activity of other enzymes erroneously regarded as that of caspase. It has been shown in different experimental situations that it is one and the same enzyme involved in both apoptotic program and non-apoptotic processes. We have shown that caspase activity in differentiated neuroblastoma cells belongs to "apoptotic" enzyme caspase-3 (unpublished data), while in the impairment of LTP by caspase-3 inhibitor may also involve another enzyme with similar substrate specificity [49]. Thus, the fact that the same substrates may be cleaved by different proteinases on slight changes of pH illustrates the variety of ways for proteolytic enzyme regulation.

Indeed, the phenomenon of switching of proteinases, in particular, cathepsin B, from one substrate to another depending on pH [62, 65] deserves special attention and thorough studying as: i) a principally new mechanism of functional involvement of proteases in signaling

and metabolic processes with a possibility to switch between these pathways as a basis for pleiotropicity of these enzymes; and ii) as a mechanism that, when decoded, may become a basis for targeted modulation of proteinase-mediated pathologies.

Another, so far not studied in full, regulatory factor for caspase activity is the presence of caspase inhibitors in the organism, these inhibitors being differently expressed in organs and tissues in different functional states. We attempted to find such inhibitors in human cerebrospinal fluid [66, 67] and demonstrated the presence of previously unknown regulators of proteolytic enzyme activities in liquor, though their nature remains obscure. The mechanism for control of expression of such inhibitors (presumably, these are peptides) in the organism has not been studied yet.

Methodological approaches to study of non-apoptotic roles of caspases in the brain. Several reliable approaches are used for studying intermolecular interactions. Among traditional and successful methods number one is still affinity chromatography including immunoprecipitation using immobilized antibody to caspase-3. Other methods are related to the nature of the protein under study; in our case proteolytic enzymatic activity should be taken into account. For these enzymes, inhibitory analysis and identification of cleavage fragments by electrophoresis or chromatography are routinely applied. Additionally, in search for molecular partners of caspases, a new method using cross-linkers should be used. This method allows finding a cross linker with high specificity, which is impossible employing other approaches. In such experiments purified proteins, as well as recombinant proteins or cell homogenates, can be analyzed. Neuronal cell cultures or animals can be used for experiments with cross-linkers.

The method consists in incubation of cells containing active caspase-3 with a chemical, cross-linker, able to covalently link the enzyme with macromolecules in its vicinity. Then the covalent complex formed can be isolated using immunoprecipitation and its composition determined using mass spectrometric analysis [68]. Cross-linkers of different chemical nature are available. Successful results of experiment would be identification of caspase-3 molecular partners, first of all substrates, in different functional state of the cell as well as information about conformation of caspase-substrate complex. The same approach is valid for search for intracellular caspase inhibitors. The current state of intermolecular interaction study method utilizing cross-linkers, as well as examples of some elegant experiments using this approach, are analyzed in the review [68]. Obviously, successful application of this approach for identification of molecular partners of a particular enzyme, e.g. caspase-3, will facilitate applications of this method for other experimental tasks as well.

We have performed the first experiments to find caspase-3 partners with cross-linkers [69]. Using animals and cell cultures we showed that caspase-3 is associated with several intracellular proteins that are not identified yet. The spectrum of caspase-3 molecular partners is different in normal and pathological conditions, as shown in primary cerebellar cultures. Covalent cross-linking of caspase-3 with its partners decreases enzyme activity suggesting that proteins—caspase-3 partners—can inhibit its activity, at least in some situations [69]. Thus, application of cross-linkers, indeed contributes to understanding of molecular interactions.

Good old methods and models can be used to study roles of caspase-3 in the brain. First of all, these are: i) assessment of purified caspase-3 in different *in vitro* conditions and of effects of different chemical and biological modulators; ii) proteolytic cleavage by caspase of proteins in tissue homogenates with subsequent evaluation of proteolysis products by electrophoretic separation and mass spectrometric analysis of low molecular weight fragments; iii) revealing intracellular and regional distribution of the enzyme using ultracentrifugation; iv) degradation by caspase of polypeptides in gels with subsequent electrophoresis to another direction (diagonal electrophoresis with proteolytic treatment). Recent methods of caspase-3 studies include co-immunoprecipitation and detection of protein-partners using caspase-3 protein itself, labeled in different ways, e.g. with biotin (far-western blotting) or immobilized on a carrier (affinity chromatography).

Addition of purified enzyme to cell homogenates results in selective cleavage of some peptide bonds and appearance of new N-terminal peptides. These newly formed peptides can be labeled with strongly hydrophobic substance, so that during chromatographic separation these peptides will behave differently [70] and, therefore, can be easily isolated and identified. This is the method of hydrophobic shift. So far it has not been applied to caspase-3 substrate identification, but the authors intend to proceed with this study shortly.

Methods suggested for search of caspases molecular partners in the brain are, in fact, major methods of modern protein research. Some of them have more than half a century long history of use in experimental biology, their validity being verified by hundreds of groups and confirmed with hundreds of publications. These are reliable, accurate, and reproducible methods, and their targeted use will hopefully result in a breakthrough in identification of caspase-3 molecular partners.

Caspases, including caspase-3, participate in non-apoptotic processes in the brain. Evidences that caspase-3 is not just an apoptotic enzyme have been demonstrated on different levels, including whole organism, organ, cell, and synapse. Nevertheless, biochemical mechanisms of most phenomena remain obscure. Studies of caspase-3 activity regulation and, most importantly, switching of enzyme functions in different situations is the area of interest of dozens of groups in the world, but methodolog-

ical difficulties of assessing particular substrate cleavage in different compartments of brain cells prevent rapid development of these studies. Overcoming this methodological barrier would inspire not only basic studies on the involvement of caspase in normal brain function, but also elaboration of approaches to pathogenically substantiated modulation of caspases for treatment of cerebral pathologies.

This study was supported by Russian Foundation for Basic Research grants No. 09-04-00174 (AY), 10-04-01071 (NG), and FCP contracts No. 14.740.11.0637 AY), No. 16.512.11.2091 (NG).

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