Dysregulation of Autophagy, Mitophagy, and Apoptotic Genes in the Medial Temporal Lobe Cortex in an Ischemic Model of Alzheimer's Disease

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Abstract. Ischemic brain damage is a pathological incident that is often linked with medial temporal lobe cortex injury and finally its atrophy. Post-ischemic brain injury associates with poor prognosis since neurons of selectively vulnerable ischemic brain areas are disappearing by apoptotic program of neuronal death. Autophagy has been considered, after brain ischemia, as a guardian against neurodegeneration. Consequently, we have examined changes in autophagy (BECN 1), mitophagy (BNIP 3), and apoptotic (caspase 3) genes in the medial temporal lobe cortex with the use of quantitative reverse-transcriptase PCR following transient 10-min global brain ischemia in rats with survival 2, 7, and 30 days. The intense significant overexpression of BECN 1 gene was noted on the 2nd day, while on days 7–30 the expression of this gene was still upregulated. BNIP 3 gene was downregulated on the 2nd day, but on days 7–30 post-ischemia, there was a significant reverse tendency. Caspase 3 gene, associated with apoptotic neuronal death, was induced in the same way as BNIP 3 gene after brain ischemia. Thus, the demonstrated changes indicate that the considerable dysregulation of expression of BECN 1, BNIP 3, and caspase 3 genes may be connected with a response of neuronal cells in medial temporal lobe cortex to transient complete brain ischemia.

Keywords: Alzheimer's disease, BECN 1, BNIP 3, brain ischemia, caspase 3, genes, rat, selective vulnerability, temporal cortex

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INTRODUCTION

Brain ischemia leads to chronic metabolic and structural changes within special brain regions: the hippocampus and temporal lobe [1–14]. Brain ischemia has been found as the most effective predictor for the later development of Alzheimer-type dementia [1–7]. Additionally, medial temporal lobe atrophy among ischemic brain survivors creates higher risks for memory dysfunction and the diagnosis of dementia [15]. Those patients with moderate to severe medial temporal lobe atrophy demonstrated poor performance on measures of learning, story recall, visual reproduction, visual spatial reasoning, and processing speed [15]. Post-ischemic dementia might be the result of direct influence of ischemia [12, 16–22] (also Pluta et al. unpublished results), ischemic white matter changes [23, 24], and Alzheimer-type neuropathology [12–14, 25–27], or combinations of these three [1].

Neuropathological changes associated with Alzheimer's disease (AD) begin in the hippocampus including medial temporal lobe structures [28, 29]. Hippocampus damage in AD features neuron loss in the CA1 area and in medial temporal lobe early in the process. It has not been finally determined whether the primary event of pathology in AD starts in the hippocampus and proceeds to medial temporal lobe and other parts of brain or may be started in the medial temporal lobe itself [30]. There some studies underpinning the fact that memory impairment is directly correlated to the atrophy of medial temporal lobe during development of AD [31, 32]. Some other reports suggest that patients with ischemic brain injury and post-ischemic medial temporal lobe atrophy might have preclinical AD, which is clinically revealed by ischemic brain lesions [1, 33]. Currently, AD still lacks an etiology, early prediction, diagnosis, and effective treatment. AD can been called the "disease of theories", e.g., amyloid hypothesis. While amyloid investigations have pivotal role in understanding some interactions and pathways, nothing is as powerful as an accurate animal model. Many transgenic models of AD have undoubtedly been of great benefit but the majority of mice genetically altered to overproduce amyloid-β peptide do not show significant tau protein pathology and have relatively little neurodegeneration [34, 35]. In summary, the results of the systematic review have revealed that mice bred to show increased levels of amyloid-β peptide do not perform significantly worse in cognitive tests than

mice that do not have elevated amyloid-β peptide levels [36]. These results suggest that amyloid-β peptide may be considered as a biochemical "side effect" in the AD neuropathology [36]. Because of the large discrepancy in the behavioral findings observed across the AD mouse models, a question arises whether we are really any closer today to determining what these mechanisms actually are. In addition, another argument that complicates the use of transgenic models based exclusively on amyloid-β protein precursor and/or tau protein mutations is that other mechanisms may be also of importance. Is this simply because mice do not live long enough to trigger tau protein pathology and the associated neuronal death? Finally, the above models often fail to reproduce the selective vulnerability in the hippocampus CA1 area and it is impossible to follow how the neuropathology spreads into the other parts of brain. In using a non-genetic model to examine AD-like neuropathologies, we remove the variability that is associated with transgenic animals. For instance, transgene integration is apparently random. Also, experiments reveal that the genetic surrounding of the inserted transgenic construct is modulating the expression pattern of the transgene itself both quantitatively and qualitatively. To fully elucidate mechanisms of AD, it is essential that a good model is available [12, 37]. Ischemic brain injury with dementia and AD share apparently common features: protein aggregation [12, 16–19, 26, 27, 38, 39], specific vulnerability of certain classes of neurons [11–14], inflammation [13, 25, 40], long incubation period, and finally global brain atrophy [12–14] with dementia [4, 6, 7, 17]. It may suggest that these features reflect common neuropathological pathways in ischemic brain injury and AD and that the ischemic model is more faithfully recapitulating AD. In our experimental approach, we allow for normal aging and disease onset to occur instead of forcing the system to a disease state.

Based on known anatomical topography, we hypothesized that the local ischemic thickness of CA1 hippocampus field [12, 14] would be associated with medial temporal lobe damage and atrophy [8], reflecting an association between AD-related injury to hippocampal neurons and neurodegeneration in medial temporal lobe. To test this hypothesis, we decided to study disease progression from the hippocampus to the medial temporal lobe in the ischemic model of AD [12, 13, 37]. In this study, we present for the first time the time course of expression of autophagy, mitophagy, and apoptotic gene levels, all

of which are implicated in AD, in the medial temporal lobe cortex subjected to transient complete brain ischemia.

MATERIALS AND METHODS

Brain ischemia, cortex sampling, and extraction of total cellular RNA

Female Wistar rats ($n = 37$, 2 month old, 160–180 g) were submitted to 10-min global brain ischemia due to *cardiac arrest* [37]. The rats were maintained in pairs per cage in a room temperature of $24 \pm 2°C$, with $55 \pm 5\%$ humidity, and with a 12 h light-dark cycle. All animals had free access to commercial laboratory chow and tap water *ad libitum*. All experimental procedures were performed during the light phase, under identical conditions. The rats used for experiments were treated in strict accordance with the NIH Guide for Care and Use of Laboratory Animals and European Communities Council Directive 142, as well as with the approval of the local Ethical Committee. After brain ischemia, the animals were allowed to survive 2 ($n = 11$), 7 ($n = 10$), and 30 days ($n = 16$). Sham-operated rats ($n = 37$) were exposed to the same procedures as ischemic animals but without induced *cardiac arrest* and thus served as controls.

Prior to sampling, the brains were perfused with cold 0.9% NaCl *via* the left ventricle in order to flush blood vessels. After removing the brain from the skull, the brain was put on an ice chilled Petri dish. The ischemic and control pooled samples circa 1 mm^3 volume of medial temporal lobe cortex left and right (all cortical layers) were taken with a narrow scalpel and were immediately placed in RNALater solution (Life Technologies, USA) [20–22].

Total cellular RNA isolation was performed according to the method described by Chomczynski and Sacchi [41]. The RNA quality and quantity was assessed using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) [20–22]. Obtained RNA was stored in 80% ethanol at –20°C for further analysis [20–22].

The cDNA synthesis

The cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit, according to manufacturer's instructions (Applied Biosystems, USA). Each reactive mixture contained the following set of reagents: $1 \times$ RT buffer, 20 U RNase inhibitor, 50 U reverse transcriptase (Multiscribe Reverse Transcriptase), $1 \times$ RT Random Primers, 4 mM of each deoxynucleotide: dATP, dGTP, dTTP and dCTP plus examined 1 μg RNA in DNase-, RNase- and protease-free water (Sigma-Aldrich, USA) to complete the volume required for reaction [20]. Afterwards, the reactive components were thoroughly mixed and centrifuged to fuse them well. The cDNA was synthesized on Veriti Dx (Applied Biosystems, USA) under the following conditions: stage I: 25°C, 10 min, stage II: 37°C, 120 min, stage III: 85°C, 5 min, stage IV: 4°C.

The RT-qPCR protocol

The cDNA, which was obtained by reverse transcription (RT) procedure, was amplified by real-time gene expression analysis (qPCR) on 7900HT Real-Time Fast System (Applied Biosystems, USA), using the manufacturer's SDS software [20]. Reaction components included: 1.25 μl mixture of probe and oligonucleotide starters specific for genes examined, 12.5 μl buffer TaqMan Universal PCR Master Mix, 1 μl cDNA, DNase-, RNase- and protease-free water (Sigma Aldrich, USA) to complete the required reactive volume. The reaction was performed on an optic 25 μl-well reaction plate, using probe sets of TaqMan Gene Expression Assays (Applied Biosystems, USA) with FAM-NFQ markers and oligonucleotide starters for rat genes: autophagy (BECN 1), mitophagy (BNIP 3) and caspase 3 and the housekeeping gene: Rpl13a was used as an internal control gene [42]. Amplification protocol included the following cycles: initial denaturation: 95°C, 10 min and 40 cycles, each composed of two temperatures: 95°C, 15 s and 60°C, 1 min. The number of copies of DNA molecules was monitored and calculated on 7900HT Real-Time Fast System (Applied Biosystems, USA) in each amplification cycle. To calculate the number of examined DNA molecules present in the mixture at the onset of reaction, the number of PCR cycles after which the level of fluorescence exceeded the defined threshold cycle (C_T) RQ Study Software (Applied Biosystems, USA) was used. The C_T value for each sample of endogenous control gene (Rpl13a) was used to normalize the level of the examined gene expression. The relative level of gene expression was calculated according to the formula [43]: 1) Calculating C_T differences between the examined gene and the reference gene are presented below: for the examined gene after ischemia of medial temporal lobe cortex:

$\Delta C_{T\ \text{ischemic sample}} = C_{T\ \text{target gene from ischemic sample}} - C_{T\ \text{reference gene, ischemic sample}}$ and for the calibrator gene (control sample): $\Delta C_{T\ \text{calibrator}} = C_{T\ \text{target gene from control sample}} - C_{T\ \text{reference gene, control sample}}$. 2) Normalizing ΔC_T of the ischemic sample to the ΔC_T of the calibrator $\Delta\Delta C_T = \Delta C_{T\ \text{ischemic sample}} - \Delta C_{T\ \text{calibrator}}$. 3) Relative expression (RQ) of rat genes was calculated by the formula: $RQ = 2^{-\Delta\Delta C_T}$. The RQ defines the expression of the examined gene in the medial temporal lobe cortex taken from an ischemic rat with reference to the gene expression in the same structure of a control rat. Finally, the RQs were analyzed after their logarithmic conversion into logarithm of RQ (LogRQ) [20]. Thus, the obtained results were more legible. LogRQ = 0 means that gene expression in the calibrated sample and the ischemic one are the same. LogRQ <0 points to decreased gene expression in the ischemic sample, whereas LogRQ >0 points to increased gene expression in the ischemic sample compared to the calibrated one.

Statistical analyses

The results were statistically analyzed by SPSS v. 17 software by means of the non-parametric Kruskal-Wallis test and Spearman rho correlation analysis. Data were presented as mean $\pm$ SEM. The level of statistical significance was set at $p \leq 0.05$.

RESULTS

Changes in expression of BECN 1, BNIP 3, and caspase 3 genes in rats 2 days following brain ischemia

BECN 1 gene expression increased to a maximum of 1.029–fold change but both BNIP 3 and caspase 3 genes decreased to a minimum of –1,887 and –0.523 – fold change, respectively. The mean expression levels of genes were as follows: BECN 1 0.434 $\pm$ 0.115, BNIP 3 –0.877 $\pm$ 0.204, and caspase 3 –0.160 $\pm$ 0.110.

Changes in expression of BECN 1, BNIP 3, and caspase 3 genes in rats 7 days following brain ischemia

BECN 1 gene expression decreased to a minimum of 0.550 – fold change, but both BNIP 3 and caspase 3 genes increased to a maximum of 3.668 and 0.471 – fold change, respectively. The mean expression levels
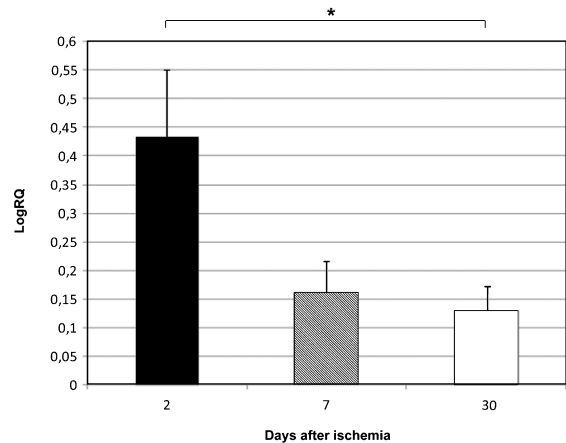

Fig. 1. The mean expression levels of BECN 1 gene in the medial temporal lobe cortex in rats 2, 7, and 30 days after 10-min of global brain ischemia. Marked SEM–standard error of the mean. Indicated statistically significant difference in levels of gene expression between 2 and 30 days after 10-min of global brain ischemia (Kruskal-Wallis test). $^*p \leq 0.05$.

of genes were as follows: BECN 1 0.161 $\pm$ 0.066, BNIP 3 1.487 $\pm$ 0.547, and caspase 3 0.196 $\pm$ 0.053.

Changes in expression of BECN 1, BNIP 3, and caspase 3 genes in rats 30 days following brain ischemia

Both BECN 1 and BNIP 3 genes expression decreased to a minimum of 0.490 and 0.437 – fold change, respectively. Caspase 3 gene increased to a maximum of 0.536 – fold change. The mean expression levels of genes were as follows: BECN 1 0.129 $\pm$ 0.048, BNIP 3 0.176 $\pm$ 0.037, and caspase 3 0.258 $\pm$ 0.040.

Summary of the mean levels of genes expression in medial temporal lobe cortex in rats 2, 7, and 30 days following brain ischemia

Figures 1–3 show changes in the mean expression levels of genes with statistical significance: BECN 1 (Fig. 1), BNIP 3 (Fig. 2), and caspase 3 (Fig. 3) after 10-min complete brain ischemia in rats with survival 2, 7, and 30 days. Significant correlations between the expression of genes in the medial temporal lobe cortex 2, 7 and 30 days after global brain ischemia was evident for BNIP 3 and caspase 3 (Spearman rho correlation test $p \leq 0.01$).
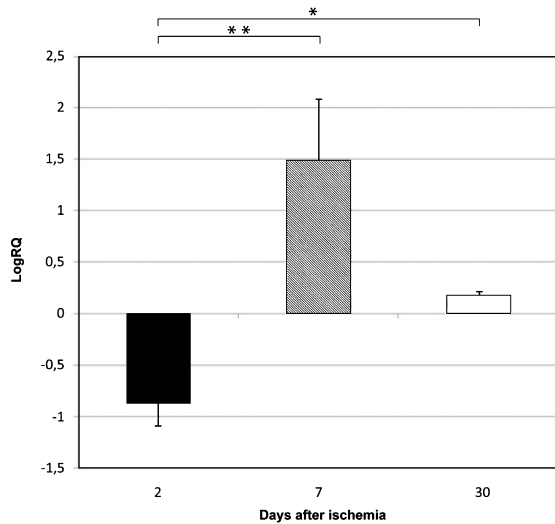
Fig. 2. The mean expression levels of BNIP 3 gene in the medial temporal lobe cortex in rats 2, 7, and 30 days after 10-min of global brain ischemia. Marked SEM–standard error of the mean. Indicated statistically significant differences in levels of gene expression between 2 and 7 and between 2 and 30 days after 10-min of global brain ischemia (Kruskal-Wallis test). *$p \leq 0.05$, **$p \leq 0.01$.
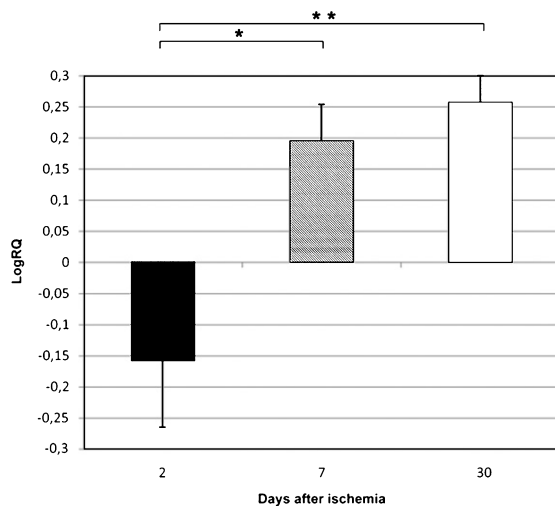

Fig. 3. The mean expression levels of caspase 3 gene in the medial temporal lobe cortex in rats 2, 7, and 30 days after 10-min of global brain ischemia. Marked SEM–standard error of the mean. Indicated statistically significant differences in levels of gene expression between 2 and 7 and between 2 and 30 days after 10-min of global brain ischemia (Kruskal-Wallis test). *$p \leq 0.05$, **$p \leq 0.01$.

DISCUSSION

In the present study, we provide evidence for the first time for the activation of an autophagic BECN 1 gene in the medial temporal lobe cortex during 30 days after brain ischemia. The overexpression of the BECN 1 gene was accompanied by an early (during 2 days) downregulation of both BNIP 3 and caspase 3 genes. Next, during 7–30 days expression of both genes was significantly upregulated. It has been suggested that ischemia induces classical apoptotic neuronal death in the cortex [44–46] but our data have indicated that the situation is more complex and that this form of neuronal death in temporal cortex is to some extent delayed for 7 days by protective overexpression of BECN 1 gene.

Overexpression of BECN 1 gene in the medial temporal lobe cortex was noted in all studied times after brain ischemia and suggest a possible efficient role of autophagy-mediated neuronal protection [47, 48]. Some studies indicate that the autophagy has an important role in amyloid protein precursor processing as well as in the autophagic clearance of aggregation-prone proteins in neurodegeneration [48–50]. We have postulated that an efficient autophagic response in the temporal cortex might protect cortical neurons from acute and/or fast cell death triggered by ischemia [44–46], which is not observed in the hippocampus (Pluta et al., unpublished results).

In this study, we have noted that BNIP 3 gene is significantly downregulated 2 days after ischemia, which is not in accordance with data from the focal brain ischemia [51] and the ischemic hippocampus (Pluta et al., unpublished results). Next during 7–30 days post-ischemia, this gene expression was significantly upregulated. In this context, it is important to stress that there is an association between the increased activity of BNIP 3 gene and different mitochondrial neurotoxic proteins such as AIF, Endo G, or PARP 1 [52–55], which are involved in neuronal death. Brain ischemia appears to cause redistribution of BNIP 3, AIF, Endo G, and PARP 1 from the mitochondria to the neuronal nucleus [52, 53, 55–59]. It has been shown that BNIP 3 induces cell death mostly *via* mitochondrial dysfunction, in that homodimeric BNIP 3 inserts into mitochondrial outer membrane to increase its permeability leading to a release of different cytotoxic proteins such as Endo G, AIF, and others [60]. In addition, it has been shown that BNIP 3 interacts with LC3 to target damaged mitochondria to autophagosomes, initiating the process of mitophagy in the ischemic neurons [61]. Moreover, double immunostaining for BNIP 3 and caspase 3 indicates that most ischemic neurons were stained for both proteins [51, 62]. However, some BNIP 3 positive neurons did not stain for caspase 3

[51, 62]. These observations open the possibility that the ischemia-regulated pro-apoptotic protein BNIP 3 enters the nucleus and could interact with other proteins involved in DNA structure, transcription or mRNA splicing after brain ischemia. The precise mechanism by which BNIP 3, AIF, Endo G, and PARP 1 execute cortical neurons death is not fully understood, however, it likely involves both nuclear-independent and nuclear-dependent processes. Thus, our data suggest a novel insight into BNIP 3 gene in regulating neuronal damage and death in the ischemic medial temporal lobe cortex.

We have recently noted that caspase 3 gene is significantly downregulated 2 days after medial temporal lobe ischemia. These data are contradictory to caspase 3 overexpression in the hippocampus during 2 days after ischemia (Pluta et al., unpublished results). In the hippocampus, at that time caspase 3 appears to be a key trigger for apoptotic neuronal death. Seven and 30 days after ischemia, caspase 3 was significantly upregulated in the temporal cortex. Then, it is possible that the above mediated process in the medial temporal lobe cortex is delayed by 7 days as compared to the same process in the ischemic hippocampus (Pluta et al., unpublished results). On the other hand, it can suggest the mechanism of slow spreading neuronal pathology from the hippocampus into the medial temporal lobe cortex in ischemic brain with Alzheimer-phenotype and dementia [63]. Caspase can use some substrates such as PARP 1, an inhibitor of caspase-activated DNAse, amyloid-β protein precursor, presenilins [62], and tau protein [64–67]. Activated caspase 3 cleaves nuclear DNA repair enzyme, such as PARP 1, which leads to nuclear DNA damage and finally apoptosis [44–46]. Moreover, caspase 3 could cleave the GGA3 protein and thus increase BACE 1 activity [68, 69]. Importantly, as amyloid-β protein precursor has been elevated long-term after brain ischemia [10, 12, 14, 23, 24, 70, 71] and has been shown a substrate for caspase 3 [62] and caspase 3 can enhance BACE 1 activity [68, 69], this pathway may also have influence on the development of neuronal changes in the medial temporal lobe cortex. Recent studies have indicated that increased level of activated caspase significantly correlates to elevated levels of truncated tau protein and formation of neurofibrillary tangles [72, 73]. In addition, cognitive decline was significantly negatively associated with increased levels of caspase activity and tau protein truncated by caspase 3 [67].

Presented data suggest a possible efficient role of autophagy gene in slowing cortical neuronal damage and death mediated by ischemic injury [47]. Our data may suggest that brain ischemia induced neuronal death in the medial temporal lobe cortex with delay by 7 days as compared to hippocampus (Pluta et al., unpublished results), by caspase 3 and BNIP 3 gene activation. This study suggests that BNIP 3 gene regulates ischemia-induced caspase-independent cortical neuronal death probably through influence on Endo G, AIF, and PARP 1 in the neuronal cell nucleus [52, 74]. Mitochondrial BNIP 3 gene is probably an upstream signal of these proteins. The levels of anti- and pro-apoptotic proteins were studied before now in our model what recently is in accordance with gene induction [75]. For already examined proteins such as beclin 1 and BNIP 3 in the brain ischemia by other scientists [52, 53, 61, 76, 77], we have added now new data related to their genes. Caspase-dependent and caspase-independent neuronal death can occur coincidently within ischemic neurons of the medial temporal lobe cortex, leading to cell death of mixed neurochemical features [59, 62].

ACKNOWLEDGMENTS

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