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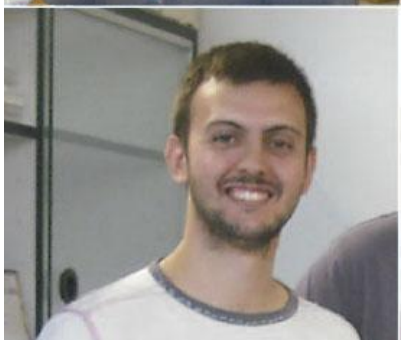
STUDY AND MANIPULATION OF Mfn2 GENE EXPRESSION IN EXCITOTOXICITY

Francesc Xavier Soriano Zaragoza
Faculty of Biology
University of Barcelona



1. Abstract

Ischemic stroke results from a transient or permanent reduction in cerebral blood flow that is restricted to the territory of a major brain artery. During an ischemic episode, extracellular glutamate builds up due to synaptic release and impaired/reversed uptake mechanisms. This glutamate induces excessive activation of NMDA receptor which results in excitotoxic cell death which can be necrotic or apoptotic-like depending on the duration and intensity of the activation. Various substances with antagonist properties against glutamate receptors have been tested for neuroprotective effects in clinical studies focused in stroke patients. Unfortunately, major side effects preclude their use in humans. We need new antiexcitotoxic strategies to expand the narrow repertoire of therapeutic opportunities.

Fragmentation of mitochondria has been shown to play a key role in cell death progression. Mfn2 is a fusion protein whose deletion causes fragmented mitochondria and bioenergetic defects. Deletion of Mfn2 gene in mice shows that Mfn2 is required for neuronal survival. Mfn2 expression protects neurons against ROS, DNA damage and excitotoxicity. In addition to its mitochondrial fusion property Mfn2 regulates mitochondrial metabolism, protects against apoptosis by interacting with Bcl-2 proteins and blocking Bax activation, controls ER-mitochondria interaction which is crucial for Ca^{2+} homeostasis, has a role in mitochondrial trafficking, and blocks cell cycle progression by inhibiting ERK/MAPK signaling. All these functions can provide protection against neuronal death.

We have found that that Mfn2 is the only member of the mitochondrial fusion/fission machinery whose expression is reduced irreversibly in in vitro and in vivo models of excitotoxicity in a delayed event of the excitotoxic process. Thus, Mfn2 reduction is a late event in excitotoxicity and its targeting may help to reduce excitotoxic damage and increase the currently short therapeutic window in stroke.

2. Results

1. Fusion/fission protein in excitotoxic conditions.

Mitochondrial dynamics plays a pivotal role in neuronal cell death. For a better understanding of the mechanism by which mitochondria are fragmented during excitotoxicity we exposed primary cortical cultures to moderate doses ($30\ \mu M$) of NMDA over a time course and analyzed the expression of the proteins of the mitochondrial fission/fusion machinery. We observed that only Mfn2 is downregulated in excitotoxicity (EXC) 4h after NMDA application (see Figure 1 in [1]).

To check the physiopathological relevance of these in vitro findings we created an ischemic insult in P12 rats by permanent middle cerebral artery occlusion followed by 90 minutes occlusion of the carotid artery. Western blot analysis of the protein samples revealed a similar pattern to in vitro (see Figure 1 in [1]). Both in vitro and in vivo samples were also normalized to porin as mitochondrial loading control with similar results. All these data show that Mfn2 is the only protein of the mitochondrial fusion/fission machinery altered in EXC and that our in vitro neuronal cultures recapitulate well the events of the in vivo excitotoxic model.

Although Mfn2 reductions occur four hours after initiating the insult, mitochondrial fragmentation kinetics in excitotoxicity is fast (30-60 minutes). We found that Drp1 recruitment to mitochondria plays a primordial role in mitochondrial fragmentation in an early phase, which depends on its nitrosylation and constriction of the actomyosin complex mediated by ROCK. Removal of the excitotoxic insult resulted in recovery of the Drp1 mediated mitochondrial fragmentation. We found a second phase in mitochondrial fragmentation which occurred hours after the application of the excitotoxic insult; it was dependent on Mfn2 downregulation and it was irreversible. It progressed even when the insult had ceased.

2. Mechanism by which Mfn2 levels are reduced in excitotoxicity.

We found that the reduction in Mfn2 levels was not due to a proteolytic process but to transcriptional regulation. We have characterized the mechanism by which this downregulation is produced. MEF2 is a transcription factor which is processed by caspases and calpain in excitotoxicity. Consistent with this, full length MEF2 levels were reduced in neuronal cultures exposed to excitotoxic conditions in a pattern that

correlated very well with Mfn2 expression. We obtained evidence that in neurons MEF2 regulates basal Mfn2 gene expression and MEF2 degradation in excitotoxicity causes Mfn2 downregulation. The evidence we found was the following:

1) Neurons transduced with AAV codifying for MEF2 dominant negative mutant (MEF2-DN) showed downregulated Mfn2 mRNA and protein expression.

2) In promoter reporter assays MEF2-DN repressed activity of human Mfn2 promoter specifically in neurons since it did not repress the activity of SESN2 promoter or the cell line 10T1/2 which expresses far less Mfn2 than neurons.

3) Luciferase assays of truncated forms of Mfn2 promoter revealed a MEF2 binding site which responds and binds MEF2.

4) EMSA assay showed that MEF2 binds in vitro to the identified binding box.

5) By ChIP assay we demonstrated basal binding of MEF2 on Mfn2 promoter in neurons but not when the neurons were stimulated with NMDA.

3. Neuroprotective role of Mfn2 against excitotoxicity.

We have shown that Mfn2 downregulation is a late event of excitotoxicity. This is important because currently the only approved treatment for acute ischemic stroke is thrombolysis with tissue plasminogen activator administered within 4 hours of symptom onset. It is of great importance to find novel targets to extend the therapeutic window.

Several mechanisms are responsible for the NMDA-mediated cell death. It is generally accepted that necrosis occurs during early phases of excitotoxicity and apoptosis during the delayed one. We observed that Mfn2 reduction contributed to delayed apoptosis in excitotoxicity but had no effect on early necrosis. Overexpression of Mfn2 protected against excitotoxicity and Mfn2 knock down (KD) using shRNA sensitized neurons to subtoxic doses of NMDA. In line with the role of MEF2 degradation on Mfn2 transcriptional regulation, overexpression of MEF2-DN sensitizes neurons to subtoxic doses of NMDA in a similar way to Mfn2 downregulation and this sensitization is reversed by Mfn2 expression.

4. Mechanism by which Mfn2 protects against excitotoxicity.

Mitochondrial dysfunction and altered Ca^{2+} homeostasis are hallmarks of excitotoxicity. Respirometric assays showed that Mfn2 KD neurons had impaired mitochondrial function. Using another experimental approach, the quantification of mitochondrial

membrane potential, we observed similar results, and moreover the mitochondrial dysfunction was exacerbated when neurons were challenged with doses of NMDA in the threshold of toxicity.
Because mitochondria play a key role in buffering the increase in cytosolic Ca^{2+} produced during excitotoxicity, which depends on proper mitochondrial membrane potential, we reasoned that Ca^{2+} homeostasis may be impaired in Mfn2 KD neurons. The application of NMDA produced an increase in cytosolic Ca^{2+} that was much greater in Mfn2 KD cells. Concomitantly we observed reduced mitochondrial Ca^{2+} uptake in Mfn2 KD neurons.

In agreement with increased cytoplasmic Ca^{2+} in Mfn2 KD neurons we observed increased calpain activation, one of the mediators of neuronal death in excitotoxicity, which was greatly enhanced by subtoxic doses of NMDA.
We also found that Mfn2 downregulation facilitated Bax recruitment to mitochondria and cytochrome c release, in agreement with its role in delayed apoptotic death in excitotoxicity.

3. Relevance and possible clinical implications

This was a basic science project but with interesting clinical implications. Currently, the only approved treatment for acute ischemic stroke is thrombolysis with tissue plasminogen activator (tPA) administered within 4.5 hours of symptom onset, however the evolution of ischemic injury is progressive, lasting for minutes, hours and even days. Hence, it is of great importance to find novel targets to extend the therapeutic window. In this study we have shown that Mfn2 downregulation is a late event of excitotoxicity (four hours after the excitotoxic insult). Downregulation of Mfn2 causes mitochondrial dysfunction and altered Ca^{2+} homeostasis, and facilitates Bax recruitment to mitochondria during excitotoxicity. All that suggests that Mfn2 downregulation could determine the fate of neurons in the penumbra area, the most clinically relevant therapeutic target against ischemic stroke. Therefore, Mfn2 is a potential therapeutic target against excitotoxicity in stroke.

The finding that Mfn2 is downregulated mainly at transcriptional level and that Mfn2 reduction facilitates Bax translocation to mitochondria suggests therapeutic strategies.

For instance, to use a chemical library for high-throughput screening assay for Mfn2 promoter activation could provide us with a molecule that prevents Mfn2 mRNA downregulation in excitotoxicity. In the same line, synthetic modified RNA (modRNA) has emerged as a new therapeutic strategy to control the spatial and temporal delivery of gene products. A third possibility would depend on a better understanding of how Mfn2 deficiency facilitates mitochondrial Bax translocation. If it depends on changes on Mfn2 interaction with Bax or other Bcl-2 family protein members, a fine mapping of the domain implicated in these interactions would allow us to generate TAT fused peptides to interfere with or keep these interactions.

4. Publications

Martorell-Riera A, Segarra-Mondejar M, Reina M, Martínez-Estrada OM, Soriano FX. *Mitochondrial fragmentation in excitotoxicity requires ROCK activation*. **Cell Cycle.** 2015. 14(9): 1365-1369.

Martorell-Riera A, Segarra-Mondejar M, Muñoz JP, Ginet V, Olloquequi J, Pérez-Clausell J, Palacín M, Reina M, Puyal J, Zorzano A, Soriano FX. *Mfn2 downregulation in excitotoxicity causes mitochondrial dysfunction and delayed neuronal death.* **EMBO J.** 2014. Oct 16;33(20):2388-407