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Original Article

The Effect of Nrf1 and Nrf2 siRNA-injection on NRF-1 Protein Level in three Regions of Male Wistar Rats' Brain

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Abstract

Introduction: Nuclear respiratory factor-1 (NRF-1) is an important factor involved in cellular growth and mitochondrial replication. The nuclear factor, erythroid-derived 2, -like2 (Nrf2) and Nuclear erythroid 2-related factor 1 (Nrf1) are two regulatory factors important for anti-oxidants defense system.

Materials and Methods: To understand the effect of Nrf2 and Nrf1 downregulation on NRF-1 protein level, the effects of Nrf1 and Nrf2 silencing through small interfering RNA (siRNA) on NRF-1 protein level were examined by western blotting in hippocampus, prefrontal cortex, and amygdala.

Results: According to the current data, downregulation of Nrf1 and Nrf2 significantly reduced the level of NRF-1 protein level in the amygdala (%33), 4 and 8h after injection. In prefrontal cortex, NRF-1 protein level decreased (%27) 8h after siRNA injection but it did not have any statistically significant effect on NRF-1 protein level in the hippocampus.

Conclusion: Overall, it is argued that antioxidant defense system is important in mitochondrial respiration as using siRNA against Nrf1 and Nrf2 could lower NRF-1 protein level.

Key words: NRF-1, Nrf1, Nrf2, siRNA

1. Introduction

Brain has different parts with different characteristics [1]. It is important to find the molecular adversity in different parts of the brain. New methods including genetic engineering have been used by scientists to discover molecular mechanism involved in brain function [2]. Small interfering RNA (**siRNA**) or short interfering RNA or silencing RNA with 20-27 base pairs, which is non-coding double-stranded RNA, interferes with mRNA to silence or downregulate translation of proteins [3]. Nuclear factor, erythroid-derived 2,-like 1(Nrf1) and nuclear factor, erythroid-derived 2,-like 2 (Nrf2) are in the NF-E2 basic leucine zipper family of factors which interact with antioxidant response element (ARE) sequences [4]. Nrf1 and Nrf2 are involved in inflammation responses; they contribute to transcription of proteasome subunits and emerging autophagosome. Their major roles are regulating the oxidants and electrophiles in cell by upregulating antioxidant signaling [5].

Changes in activities of cell organelles including antioxidant defense system accrue in response to Nrf1&2 Si-RNA injections [4]. Important organelle involved in cellular metabolisms is mitochondrion [6]. Some transcription elements like nuclear respiratory factor-1 (NRF-1) are involved in mitochondrial biogenesis [7,8]. When the redox balance of cell is disrupted, NRF-1 induces reactive oxygen species **(**ROS)-detoxifying proteins production [9].

Using siRNA to knockdown genes *in vivo* has been shown before [10]. Here, we aimed to silence Nrf1 and Nrf2 by intra-D3V siRNA injection in order to investigate the effect of this silencing on NRF-1 protein level.

2. Materials and Methods

2.1. Animals and Experimental Design

Male albino Wistar rats were provided from Pasteur Institute (Tehran, Iran), their weight was between 200-250 g. Three groups were considered for this study:

Control group one: five µl scrambled siRNA with no homology to any gene was injected in their D3V.

Control group two: five µl RNase-free water was injected in their D3V.

Nrf1&Nrf2-siRNA group: five µl Nrf1-siRNA and 5 µl Nrf2-siRNA (5 nmol siRNA/200 µl RNase-free water) made by ABCAM (Cambridge, UK) was injected in their D3V.

Each group explained above was split into two sub-groups; one decapitated 4 h after siRNA injection and the other 8 h after siRNA injection. D3V was selected for siRNA injection because, based on pervious research, it has been shown that siRNA injection in this region can transfer easily to other parts of the brain [23].

2.2. Stereotaxic Surgery and siRNA Administration in Rat Brain

Rats were insensible by an intraperitoneal injection of Ketamine Hydrochloride (50 mg/kg) and xylezine (4 mg/kg) then put in stereotaxic instrument. With surgery, guide cannula was fixed in their D3V (anteroposterior: –0.5 mm relevant to bregma, mediolateral: 0 mm, and dorsoventral: –3 mm from the skull surface) [11]. The silencer selects pre-designed siRNA to downregulation of Nrf1 in the rats' brain specific to Nrf1: 5´ -CAACCUGCCUGUAGAAGAAtt-3´ (ID: s165931), and for down regulation of Nrf2 in rats' brain the silencer selects predesigned siRNA specific to Nrf2: 5´ -GCUGAACUCCUUAGACUCAtt-3´ (ID: 136127) were provided from Ambion (Austin, TX, USA) and scrambled siRNA was purchased from QIAGEN (Germany): 5´ UUCUCCGAACGUGUCACGUdTdT-3´ .

2.3. Western Blotting

Proteins were extracted from tissues (hippocampus, prefrontal cortex (PFC), and amygdala) by specific buffer with protease inhibitor cocktail. Then, they were centrifuged at 4000 × g at 4 ˚C [17].

Protein concentration was evaluated by Bradford's method [12]. Next, 60 µg of total protein of each sample was inserted in sodium dodecyl sulfate-polyacrylamide (SDS) PAGE separation gel. Transferring to Polyvinylidene fluoride (PVDF) membrane was from Millipore (Billerica, MA, USA). After that, blots were blocked with skim milk, probed with NRF-1 antibody purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) for 3 hours in room temperature, then incubated with secondary antibody. After 3 times washing, enhanced chemiluminescence method was used, blots were visualized by immunoreactivity; ECL was provided from Amersham Bioscience (Piscataway, USA). Densitometry analysis was also used by ImageJ software.

2.4 Data Analysis

Each experiment was performed 3 to 5 times. Mean ± SEM (standard error of mean) was used in order to express the data, which were analyzed by Graph Pad Prism® 5.0. One-way analysis of variance (ANOVA) and post hoc analysis Turkey's were used. P value less than 0.05 ($P < 0.05$) is statistically significant.

3. Results

Nrf1 and Nrf2 downregulation decreased protein Level of Nrf1 and Nrf2.

With western blotting, we evaluated the level of Nrf1 and Nrf2 protein level, 4 and 8 hours after siRNA injections in D3V. According to Table1, protein level of Nrf1

and Nrf2 significantly decreased after siRNA injection in three brain regions (hippocampus, PFC, and amygdala).

Table 1. siRNA injection induced Nrf1 and Nrf2 decrease in 3 areas of rat's brain. Evaluation of protein level (Western blotting) after direct in *vivo* injection of scrambled siRNA (control) and Nrf1&2 siRNA in D3V. *P < 0.05; **P < 0.01; ***P <0.001 compared to the control group.

Nrf1 and Nrf2 siRNA	Amygdala (4h)	Amygdala (8h)	PFC (4h)	PFC (8h)	Hippocampus (4h)	Hippocampus (8h)
Nrf1/Beta Actin Protein level(A.B.)	45% ***	19% **	36% **	29% *	33% **	30% **
Nrf2/ Beta Actin Protein level(A.B.)	40% ***	10% **	33% ***	25% *	22% ***	20% ***

Nrf1&2-siRNA application did not change NRF-1 protein level in Hippocampus but reduced it in PFC and Amygdala

The blots of western blotting are shown in Fig. 1A, The protein level of NRF-1 of hippocampus is shown in Fig. 1B there is a about 11% reduction in the NRF-1 protein level 4h and 8h after siRNA injection compared to the control group. Fig.1C shows the NRF-1 protein level in PFC which decreased about 27% 8h after siRNA use in comparisons with the control group(***P <0.001). Fig1.D shows the NRF-1 protein level in Amygdala which decreased about 33% 8h and 4h after siRNA application compared to the control group (***P <0.001).

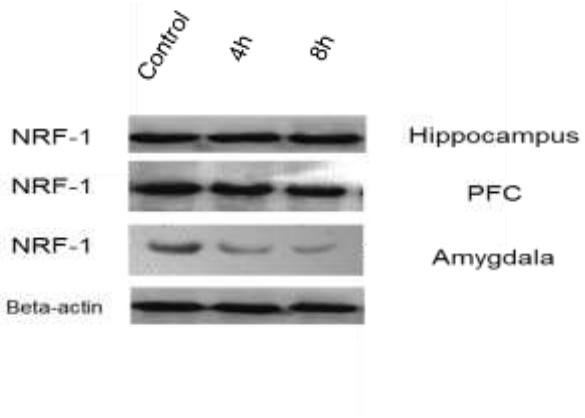

Fig 1A

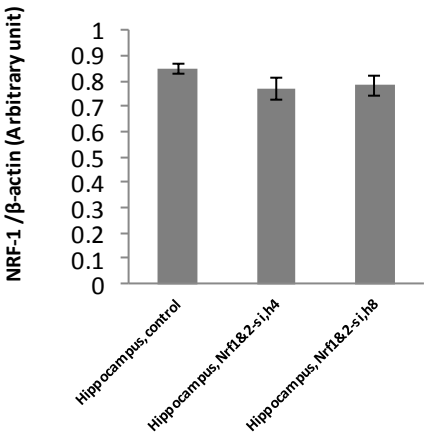

Fig 1B

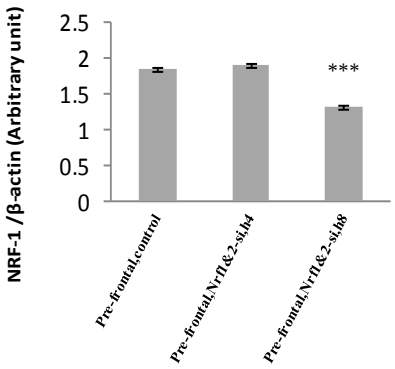

Fig 1C

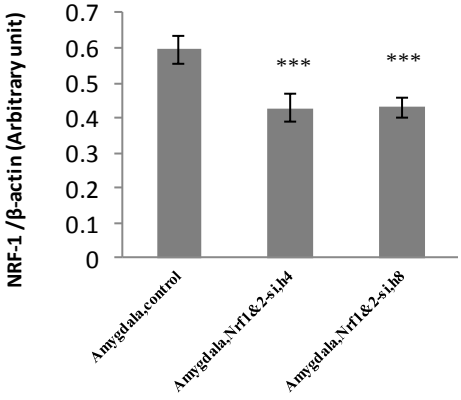

Fig 1D

Figure 1. Western blotting technique used to evaluate Nrf1&2-siRNA effect on NRF-1 protein level in three brain region. ***P < 0.001 compared to the control group.

4. Discussion

Hippocampus, amygdala, and PFC are the most important regions involved in behavior. Hippocampus is involved in learning and memory, PFC is related to making decisions and other aspects of personality, and amygdala regulates a number of behaviors such as aggressiveness [1, 2, 23].

All eukaryotic organisms have mitochondrion as an energy producer [13]. Mitochondria have their own DNA but they are not completely independent of genomics DNA. They are important in energy production and homeostasis and neuronal cell functions.

NRF-1 is a transcription factor which increases the transcription of some important genes regulating mitochondrial DNA transcription and replication [14]. Nrf2 is an important factor in cell signaling related to antioxidant proteins expression, increasing the power of cell to defend against oxidative damage and inflammation [16]. Nrf1 is an important regulator of cellular activities including oxidative stress regulation, differentiation, inflammatory response and hemostasis [17,18]. Application of siRNA has been useful in genetic engineering to downregulate gene expression in translation step [23]. To understand the effect of Nrf1&2 silencing on NRF-1 protein level, we have used Nrf1&2 siRNA. The results of the present study have indicated NRF-1 decrease as a result of Nrf1&2 silencing.

Different factors help cells against oxidative damages, like NRF-1 [19]. Our data show significant decrease in NRF-1 protein, after Nrf1&2 downregulation. Previously, it has been argued that NRF-1 protects cells against oxidative stresses [20].

Our results have demonstrated that NRF-1 protein level reduced in amygdala and prefrontal cortex following Nrf1&2 knockdowns. Wright et al. and Hatazawa et al. have also suggested the effect of Nrf2 on mitochondrial activity [21].

Khalifeh et al. have found that Nrf2 has an important role in biology related to mitochondrial activities [22]. And NRF-1 has been discussed as a transcriptional downstream of Nrf2 [23].

5. Conclusion

As proteins are the final effectors in cell functions, in this study the effect of Nrf1 and Nrf2 silencing on NRF-1 protein level was examined instead of evaluation of NRF-1 mRNA. According to the current data, it is argued that Nrf1 and Nrf2 are required for NRF-1 expression and that following Nrf1&2 silencing, the NRF-1 protein level goes down. Our study underscores the specific role of Nrf1 and Nrf2 in modulation of mitochondrial functions.

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Conflict of interest

The authors declare no conflict of interest.

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