Interaction of Naringenin with Ryanodine receptor in Human and Caenorhabditis elegans

Sandhya Pal[1] and Meenakshi Sharma[2]
[1]PhD Scholar, Molecular Genetics of Aging Laboratory, Dr. B.R. Ambedkar Center for Biomedical Research, University of Delhi, Delhi 110007, India
[2]Corresponding Author, Assistant Professor, Molecular Genetics of Aging Laboratory, Dr. B.R. Ambedkar Center for Biomedical Research, University of Delhi, Delhi 110007, India

Abstract: ***Calcium channels present on membrane and sarcoplasmic reticulum modulate the cytoplasmic calcium level which act as a secondary messenger thus regulate various activity of cell. Naringenin (Nar), a natural flavonoid present in citrus and grape fruits known for its antioxidant, cardiovascular effects. In this study, we are trying to elucidate the impact of binding of Nar on Ryanodine receptor (RyR) which is a principal channel for calcium release from sarcoplasmic reticulum in human (RyR2) as well as in Caenorhabditis elegans (ryr-1) along with its effect on its muscle health. To study the docking of Nar with RyR receptor in C. elegans we prepared homolog model via alpha fold2 server of ryr-1 receptor for C. elegans because of the absence of their X ray crystal structure. In this study, we observed that the Nar show the interaction with N-terminal domain (NTD) of RyR2 (4JKQ) and handle domain of ryr-1. Naringenin binding with these calcium channels might be helpful in controlling the dysregulation of calcium release from ryanodine receptor.***

Index Terms – Calcium channel, Naringenin, Ryanodine receptor, Sarcoplasmic reticulum

INTRODUCTION

Plants contain various secondary metabolites which are of health benefit for humans and received attention of researchers. Naringenin (Nar) is one of the phytochemicals, a most abundant flavonoid found in citrus and grape fruit also in tomatoes, coffee and fenugreek. It is well known for its antioxidant, anti-inflammatory, anti-diabetic, anti-cancer, cardiovascular, etc [1,2]. Calcium channel modulators are compounds that bind to these channels and regulate the activity of these channels. From previous studies it has been seen that Nar show its effect on calcium signaling. Various components involved in calcium signaling have been targeted directly or indirectly by Nar to improve muscle health. Nar is found to target AMPK and Protein kinase enzymes PKA and PKC activation of these enzymes are also regulated by intracellular calcium ions level in cells [3]. Endoplasmic reticulum (ER), storage house of calcium ions in cells regulates the intracellular calcium level via channels like SERCA and RyRs. Ryanodine receptors (RyRs) are the principal and the largest ion channel responsible for release of calcium ion from sarcoplasmic reticulum (SR) [4]. There are three isoforms of RyR identified in mammals: RyR1 generally present in skeletal muscles, RyR2 predominantly found in cardiac muscle also expressed in smooth muscles as well as neuronal tissues. RyR3 expressed at low level in various tissues of nervous system and involuntary smooth muscles [5].

RyR receptors are divided in 3 domains: N-terminal domain (NTD), SPRY domain and the C-terminal domain. The N terminal domain which forms the mushroom like structure towards the cytoplasmic side of cell and is further divided into three subdomains: A (1-223 residues), B (224-408) and C (409-643) subdomain. The ryanodine receptor consists of more than 5000 amino acids in which 1-606 are present in NTD in RyR2 of homo sapiens (4JKQ). The C sub domain forms the alpha solenoid which extend to the surface of the handle like region where it bends under the of A and B subdomains of NTD [6].

These ABC subunits together form a gating ring like structure near the pore of channel and thus play an important role regulating the opening and closing of the channel. There is an inter subunit pocket formation at the interface of A and B subunit [7]. Thus, the N-terminal binding site is mainly located in domain A and small proportion is located in domain B, this inter subunit interface pocket can be more effective target as it directly affects the conformational change that happens upon gating. The molecules that lock the gate during opening and closing will have some detrimental effect on normal channel function [7,8].

NTDs, the handle and helical domains forms the interaction scaffold with a multitude of peripheral

domains and channel modulators [7]. In this study, we try to find the site of action of Naringenin using in-silico approach. Binding of Nar with RyR might be helpful in managing the uncontrolled calcium release from ryanodine receptor. In this study, we have reported that the Nar show the interaction with NTD of RyR2 (4JKQ) at the potential binding site in the inter subunit pocket between both A and B sub domain interface.

METHODOLOGY

Chemical and reagents

Naringenin was purchased from Sigma Aldrich (67604-48-2).

Strain

N2 Bristol wild type strain was selected for study and *Escherichia coli* (OP50) were provided by the Caenorhabditis Genetics Center (Minnesota, USA). The worms were cultured on Nematode Growth Medium (NGM) containing plate at 20°C. *E. coli* (OP50) food lawn was prepared on the surface of media as food source for worms.

Network Pharmacology

The proteins involved in calcium signaling in *C. elegans* were selected and the target protein UNC-68 was filled in single protein by name identifier and the species was selected as "*C. elegans*". The protein-protein interaction (PPI) network plot was constructed using STRING database (the score value was set as 0.700).

Molecular docking

For molecular docking the structure of Naringenin was retrieved from the Pub Chem Database (http://pubchem.ncbi.nlm.nih.gov) and the three-dimensional structures of hRyR2 (4JKQ) were retrieved from the RCSB Protein Data Bank (http://www.rcsb.org). As the crystal structure for *C. elegans* was not available using the homology modelling we prepared the crystal structure using AlphaFold2 server. The ligand and protein receptors were prepared and molecular docking was performed using AutoDock 4.0 and visualized using Discovery Studio.

Treatment

The stock solution of Nar was prepared in dimethyl sulfoxide (DMSO) and added to agar plates directly after NGM was cooled to obtain final concentration of 0.1% DMSO. The plate supplemented with different doses of Nar or the control solvent (0.1% DMSO) were prepared.

Pharyngeal pumping

The pharyngeal pumping assay was performed referring to the previously described method with some modifications. N2 (wild type) young adult worms were treated with Nar at 50µM and 100 µM concentration dissolved in 0.1% DMSO after 24 hrs of treatment. After treatment for 2 generations pharyngeal pumping of worms was recorded for a one-minute interval using a stereoscopic microscope. 3 worms were randomly selected for each group and the assay was carried out thrice independently.

Reproduction assay

Individual worms from synchronized L4 larvae were transferred to treatment NGM plates. Then, the worms from each previous plate transferred to fresh treatment NGM plate after every 24 hours every day. The number of progenies were counted each day. The experiments were repeated independently thrice.

RESULTS AND DISCUSSION

Interaction of ryr-1 protein in *C. elegans*

Ryanodine receptor channel functioning is regulated by other factors such as modulators, regulatory proteins, Ca^{2+} ions. Proteins such as FKB-2 homolog of human calstabin-2 directly binds to UNC-68 (ryr-1) and acts as a channel stabilizing subunit [9,10]. CSQ-1 show direct interaction with luminal loops region of UNC-68 [11]. Thus, molecules binding in or near by these regulatory subunits binding sites may regulate the disruption of calcium regulation.

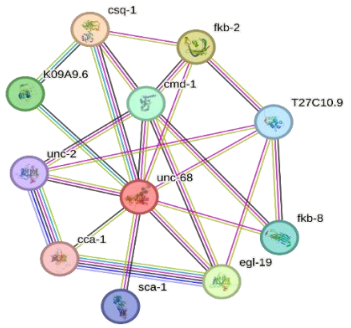

Fig.I: The possible interaction of UNC-68 (ryr-1) protein with other proteins involved in calcium signalling in *C. elegans.* In figure UNC-68 protein show the interaction with other regulatory units such as calsequestrin CSQ-1, calmodulin CMD-1, calstabin FKB proteins and SCA-1 calcium transporting ATPase as well as relation with other calcium receptor such as VGCC EGL-19 and CCA-1.

Nar binding with human RyR2

Nar binds with NTD of RyR2 with the amino acid residues Lys34, Gln23, Arg281 and Ala283 with H-bond and a pi donor hydrogen bonding is present at Val282. Covalent bonds interactions are also present such as pi sigma bond with Val282 and pi alkyl bond with Arg281. Nar is binding at the potential binding site present in the inter subunit pocket between both A and B sub domain interface.

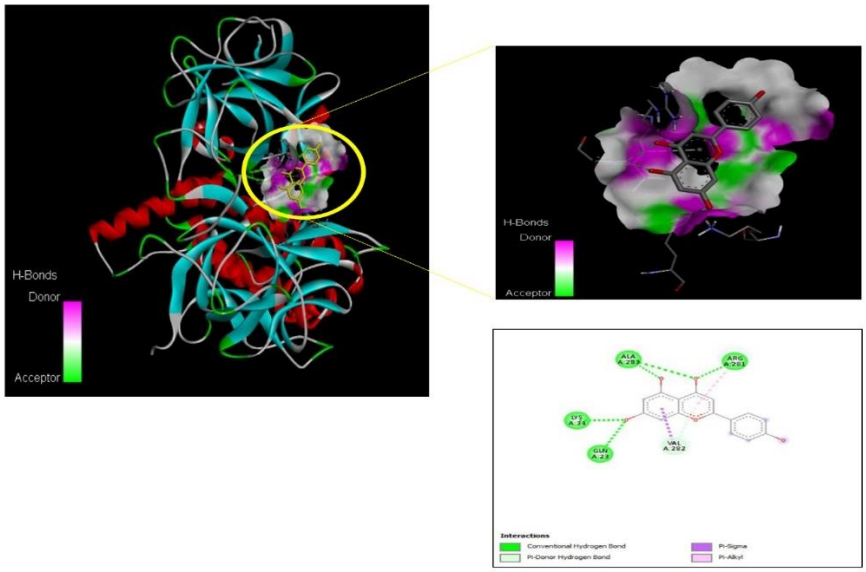

Fig. II: 3D diagram of interaction of Nar with N-terminal domain of Ryr2 on the upper region and the 2D interaction between Nar and RyR2 NTD are also depicted.

Nar binding with ryr-1 in *C. elegans*

We performed docking of Nar with *C. elegans* ryr-1 prepared with the help of alpha fold2 server. In *C. elegans* the Nar binding site is different from that with hRyR protein as in ryr-1 Nar binds in the alpha solenoid region and handle domain present after SPRY domain. Although together with NTD and helical domains it regulates the gating activity and also provide binding site for channel modulators. The Nar show 5 h bond similar to hRyR and Nar interaction although the binding site is different. The H bond interaction with His1804, Lys1805, Asp1908, Gku2247 and Gln2244 are present, covalent interactions such as Pi alkyl interaction with Lue1912, Ile2260 and Pi sigma bond with Leu2248. In addition, amide pi bonding at Lys1911 is also present in this interaction as shown in Fig.III.

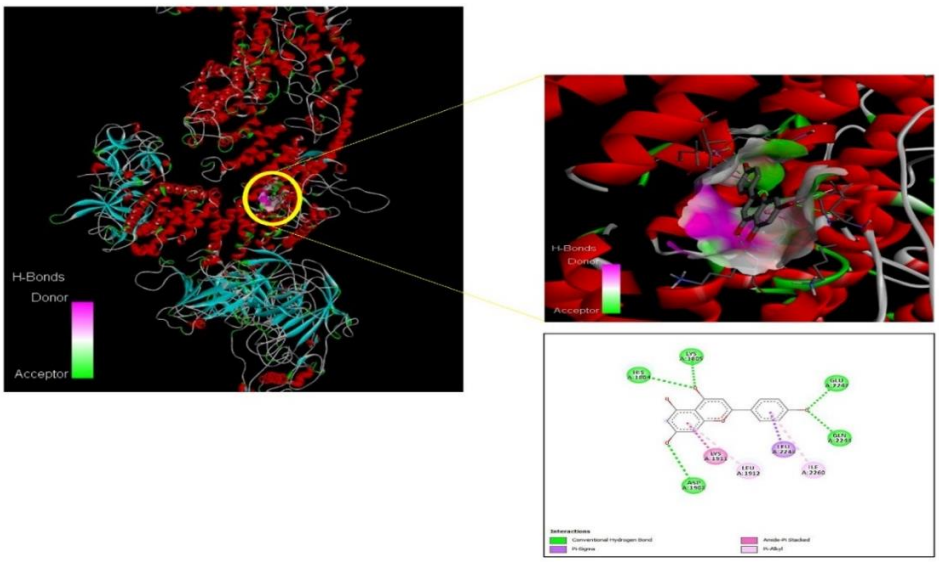

Fig.III: 3-D interaction between Nar with ryanodine receptor ryr-1 in *C. elegans* along with the 2-D interaction.

Table. I: Docking parameters of interaction of Nar with RyR2 and ryr-1.

Docking parameters	RyR2	ryr-1
Organism	*Homo sapiens*	*Caenorhabditis elegans*
Binding energy (kcal/mol)	-6.64	-6.07
Types of interaction	H-bond Pi donor hydrogen bond pi sigma pi alkyl bond	H-bond amide pi stacking pi sigma pi alkyl bond

Pharyngeal pumping assay

Average pharyngeal pumping while feeding on OP50 bacteria was evaluated. As shown in figure there is significant decrease (p-value <0.01) in pharyngeal pumping at 50µM and 100 µM concentration compared to control worms. But there is no significant change with change in the concentration of Nar as shown in Fig IV.

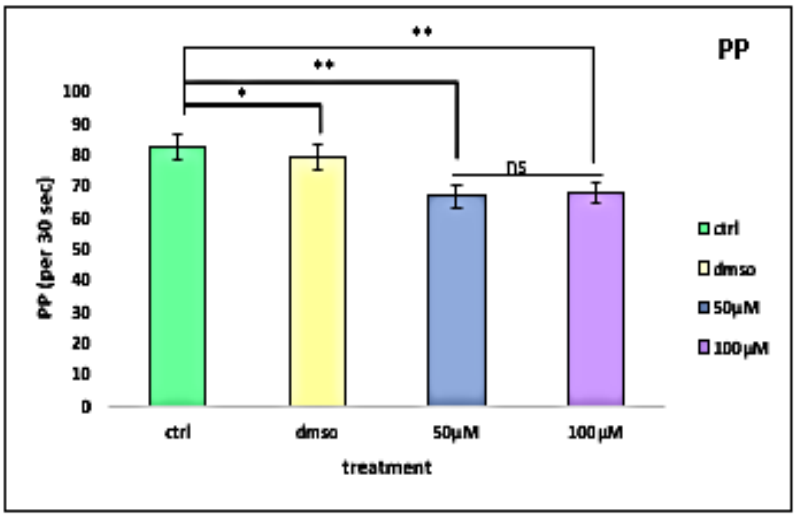

Fig. IV: Effect of Nar at two different concentrations 50 µM and 100 µM on pharyngeal pumping. Nar show decrease in the Pharyngeal pumping rate in *C. elegans* compared to control treatment.

Egg Laying Assay

Although there is no deviation in egg laying behaviour of treated and untreated N2 worms but compared to untreated control worms there is a significant decrease in eggs count compared to Nar and DMSO treated worms 50µM (p-value <0.05) and 100µM (p-value <0.01).

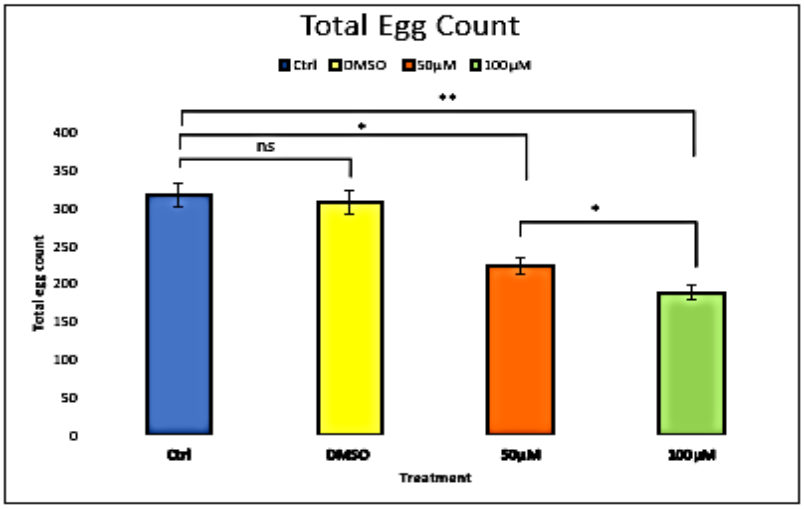

Fig. V: Effect of Nar at two different concentrations 50 µM and 100 µM on pharyngeal pumping. Nar show decrease in the pharyngeal pumping rate in *C. elegans* compared to control treatment.

CONCLUSION

In this study, first using in-silico approach we tried to find the site of action of Nar on RyR receptor in human as well as *C. elegans*. Nar is found to bind at the NTD in human and in *C. elegans* the molecule gets docked at the handle domain. We also observed the effect of Nar on muscle health related physiological activity such as pharyngeal pumping and reproduction assay and is found to decrease the pharyngeal pumping as well as egg laying in *C. elegans*.

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Conflicts of Interest

There are no conflicts of interest to declare.

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