



Molecular mechanisms of naringenin modulation of mitochondrial permeability transition acting on F₁F₀-ATPase and counteracting saline load-induced injury in SHRSP cerebral endothelial cells

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ABSTRACT

Naringenin (NRG) was characterized for its ability to counteract mitochondrial dysfunction which is linked to cardiovascular diseases. The F₁F₀-ATPase can act as a molecular target of NRG. The interaction of NRG with this enzyme can avoid the energy transmission mechanism of ATP hydrolysis, especially in the presence of Ca²⁺ cation used as cofactor. Indeed, NRG was a selective inhibitor of the hydrophilic F₁ domain displaying a binding site overlapped with quercetin in the inside surface of an annulus made by the three α and the three β subunits arranged alternatively in a hexamer. The kinetic constant of inhibition suggested that NRG preferred the enzyme activated by Ca²⁺ rather than the F₁F₀-ATPase activated by the natural cofactor Mg²⁺. From the inhibition type mechanism of NRG stemmed the possibility to speculate that NRG can prevent the activation of F₁F₀-ATPase by Ca²⁺. The event correlated to the protective role in the mitochondrial permeability transition pore opening by NRG as well as to the reduction of ROS production probably linked to the NRG chemical structure with antioxidant action. Moreover, in primary cerebral endothelial cells (ECs) obtained from stroke prone spontaneously hypertensive rats NRG had a protective effect on salt-induced injury by restoring cell viability and endothelial cell tube formation while also rescuing complex I activity.

1. Introduction

Mitochondrial dysfunctions affecting the energy production of the cell are involved in a variety of human diseases, especially cardiovascular diseases (CVDs) which are a main cause of death worldwide.

Moreover, there is a consensus on the need to increase the prevention of CVDs based on the use of natural products that may delay their onset (Perna et al., 2019). In this regard, the search for new molecules that may counteract mitochondrial dysfunctions related to the risk of CVDs is encouraged by some promising hints coming from the study of

Abbreviations: NRG, naringenin; ECs, endothelial cells; QUE, quercetin; SHRSP, stroke prone spontaneously hypertensive rat; MPTP, mitochondrial permeability transition pore; SOX, anion superoxide; CVDs, cardiovascular diseases; PPPCs, polyphenolic phytochemicals; CRC, calcium retention capacity.

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phytochemical compounds. Phytochemical compounds, which include flavonoids (e.g. flavanones) and non-flavonoids (e.g. stilbenes) (Fig. 1), are actively studied for their potential to promote healthy life and disease prevention while counteracting chronic diseases (Pérez-Jiménez et al., 2010). In particular, flavonoids are a large family of natural polyphenolic compounds present in common fruits, medical herbs and vegetables with a great variety of therapeutic effects. They have a strong potential to function as free radical scavengers due to their ability to form stable radicals (Sandoval-Acuña et al., 2014). Naringenin (NRG), a flavanone present in the bergamot fruit (*Citrus bergamia*) has received increasing attention due to its broad range of biological activities (Carresi et al., 2020).

Cell protection against radical species by NRG results in a reduction of mitochondrial dysfunction. Moreover, NRG has been reported to reduce acute and chronic inflammatory responses and to exert cardiovascular beneficial properties in clinical trials (Salehi et al., 2019). In particular, the cardioprotective effects of NRG on coronary artery disease, hypertension, cardiac hypertrophy, myocardial infarction and ischemic stroke are most likely due to the hypocholesterolemic, antioxidant, anti-inflammatory and anti-ischemic activities of its flavone structure (Heidary Moghaddam et al., 2020). Nevertheless, studies regarding the precise mechanism of action of NRG in CVDs are lacking and additional efforts should be made to identify the molecular targets of NRG in different cellular contexts and its fine mechanism of action, with special attention to mitochondrial dysfunctions (Carresi et al., 2020).

The mitochondrial dysfunction associated with CVDs is highlighted by an altered cellular energy metabolism caused by an impaired oxidative phosphorylation leading to ATP depletion, increase of oxidative stress and activation of pro-death pathways, which in turn trigger the opening of the mitochondrial permeability transition pore (mPTP) (Bonora et al., 2019). Loss of inner mitochondrial membrane (IMM) permeability, which directly causes mitochondrial swelling, is

accompanied by disorders in mitochondrial dynamics (biogenesis, fission and fusion) that usually lead to mitochondrial impairment and degradation (mitophagy) (Forte et al., 2021). Interestingly, the polyphenolic phytochemicals (PPPCs) playing a scavenger action in mitochondria can counteract mitochondrial dysfunction (Algieri et al., 2022a). PPPCs might bind to a common region on the F₁ hydrophilic domain, facing the mitochondrial matrix. They act as F₁ inhibitors and block ATP hydrolysis without overlapping with the three catalytic sites of substrates ADP and Pi, and surprisingly they also confer resistance to mPTP opening (Algieri et al., 2019). NRG, which shares the polyphenolic structure of PPPCs, could itself inhibit ATP hydrolysis and mPTP formation, thus counteracting mitochondrial dysfunctions. However, small molecular differences can produce different biological effects as they may imply distinct chemical interactions with cellular proteins. Therefore, we aimed to test the effects of NRG in endothelial cells of stroke-prone spontaneously hypertensive rats (SHRSP), which offer an excellent model of target organ damage associated with mitochondrial dysfunction (Rubattu et al., 2016a). The NRG beneficial action on the impaired mitochondrial bioenergetics might sustain its use in therapy and address future cardiovascular drug design and development.

2. Material and methods

2.1. Preparation of the mitochondrial fractions

Swine hearts (*Sus scrofa domestica*) were collected at a local abattoir and transported to the lab within 2 h in ice buckets at 0–4°C. After removal of fat and blood clots as much as possible, approximately 30–40 g of heart tissue was rinsed in ice-cold washing Tris-HCl buffer (medium A) consisting of 0.25 M sucrose, 10 mM Tris(hydroxymethyl)aminomethane (Tris), pH 7.4 and finely chopped into fine pieces with scissors. Each preparation was made from one heart. Once rinsed, tissues

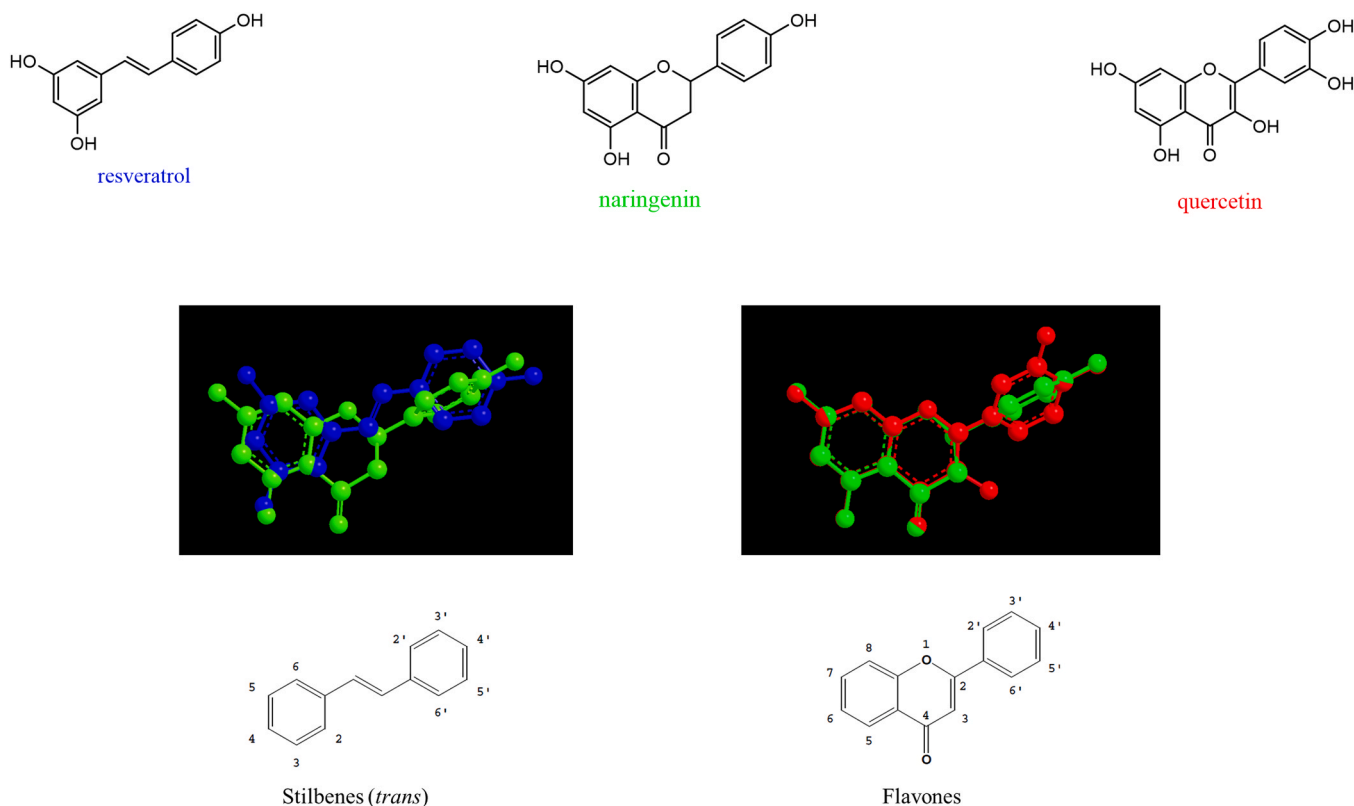


Fig. 1. Molecular structure of resveratrol, naringenin, and quercetin. Overlapping of resveratrol (blu) with naringenin (green) or quercetin (red) with naringenin (green) in the black box on the left or on the right, respectively. The typical structures of stilbene and flavone molecules are depicted.

were gently dried on blotting paper and weighed. Then tissues were homogenized in medium B consisting of 0.25 M sucrose, 10 mM Tris, 1 mM EDTA (free acid), 0.5 mg/mL BSA fatty acid free, pH 7.4 with HCl at a ratio of 10 mL medium B per 1 g of fresh tissue. After a preliminary gentle break-up by Ultraturax T25, the tissue was carefully homogenized by a motor-driven teflon pestle homogenizer (Braun Melsungen Type 853202) at 650 rpm with 3 up-and-down strokes. The mitochondrial fraction was then obtained by stepwise centrifugation (Sorvall RC2-B, rotor SS34). Briefly, the homogenate was centrifuged at 1,000xg for 5 min, thus yielding a supernatant and a pellet. The pellet was re-homogenized under the same conditions as the first homogenization and re-centrifuged at 1,000xg for 5 min. The gathered supernatants from these two centrifugations, filtered through four cotton gauze layers, were centrifuged at 10,500xg for 10 min to yield the raw mitochondrial pellet. The raw pellet was resuspended in medium A and further centrifuged at 10,500xg for 10 min to obtain the final mitochondrial pellet. The latter was resuspended by gentle stirring using a Teflon Potter Elvehjem homogenizer in a small volume of medium A, thus obtaining a protein concentration of 30 mg/mL (Nesci et al., 2014a). All steps were carried out at 0–4°C. The protein concentration was determined according to the colourimetric method of Bradford (Bradford, 1976a) by Bio-Rad Protein Assay kit II with BSA as standard. The mitochondrial preparations were then stored in liquid nitrogen until the evaluation of F₁F₀-ATPase activities.

2.2. F₁ domain purification

After thawing, the mitochondrial suspensions of the swine heart were diluted with 50 mL of medium A to obtain a concentration of 20 mg/mL of protein, sonicated on ice with Sonicator MSE Soniprep 150 to an amplitude of 210 μm for 3 minutes three times with 30 second intervals and centrifuged at 10,000xg for 10 minutes. The supernatant from this first centrifugation was further centrifuged at 100,000xg for 2 hours. All these centrifugation steps were performed at 4°C. The pellet was resuspended in medium A plus 4 mM Na₂ATP, the pH was adjusted to 9.2 by adding small aliquots of 20% (w/w) NH₄OH solution and stored overnight at 4°C. The next day, the suspension, in which the pH was brought back to 8.0 by adding small aliquots of aqueous solution of 2 N HCl, was sonicated at an amplitude of 210 μm for 5 minutes and then centrifuged at 300,000xg for 1 hour (Penin et al., 1979). The resulting pellet was resuspended in 9 mL of medium A plus 2 mM EDTA, pH 7.6. After the addition of 4.5 mL of chloroform, the resulting mixture was vigorously vortexed for 15 seconds and centrifuged at 600xg for 10 minutes to allow for separation of the two phases. The upper aqueous phase was collected and further centrifuged at 100,000xg for 1 hour. The obtained pale yellow supernatant was supplemented with adequate aliquots of freshly prepared ATP solution to obtain a final 4 mM ATP concentration and with a 2 N NaOH solution to adjust pH to 8.0. After the dropwise addition of saturated solution (NH₄)₂SO₄ plus 5 mM EDTA under continuous stirring to obtain a saturation of 37% and a pH adjustment to 8.0 with a 1 N KOH solution (Penin et al., 1979), the suspension was centrifuged at 10,000xg for 15 minutes. Pellet was discarded and the collected supernatant was brought to 60% saturation with solid (NH₄)₂SO₄; the mixture was then adjusted to pH 8.0 with a 1 N solution of KOH and kept overnight at 4°C (Penin et al., 1979). Finally, pellet from the last centrifugation at 150,000xg for 90 minutes, was resuspended by gentle homogenization using a Potter Elvehjem Teflon homogenizer in a small volume of medium containing 100 mM Tris/H₂SO₄, 1 mM EDTA and 50% glycerol, pH 8.0, constituted the partially purified F₁ fraction (Algieri et al., 2019; Penin et al., 1979). Protein concentration was determined according to the Bradford colourimetric method by Bio-Rad Protein Assay kit II using BSA as standard (Bradford, 1976b). Once it was verified that in the partially purified F₁ fraction, ATPase activity, supported by Ca²⁺ or Mg²⁺, was completely insensitive to 1 μg/mL of oligomycin, thus demonstrating the detachment of the F₀ sector, the partially purified F₁ fraction was then

preserved in liquid nitrogen and no further purification was carried out.

2.3. Mitochondrial F₁F₀-ATPase activity assays

Thawed mitochondrial preparations were immediately used for F-ATPase activity assays. The capability of ATP hydrolysis was assayed in a reaction medium (1 mL). The optimal conditions to obtain the maximal activity of the F₁F₀-ATPase, which depend on substrates concentration and pH values, are at 0.15 mg mitochondrial protein and 75 mM ethanolamine-HCl buffer pH 9.0, 6.0 mM Na₂ATP and 2.0 mM MgCl₂ for the Mg²⁺-activated F₁F₀-ATPase assay, and 75 mM ethanolamine-HCl buffer pH 8.8, 3.0 mM Na₂ATP and 2.0 mM CaCl₂ for the Ca²⁺-activated F₁F₀-ATPase assay (Algieri et al., 2019; Nesci et al., 2017). These assay conditions were previously proven to elicit the maximal enzyme activities either stimulated by Mg²⁺ or by Ca²⁺ in swine heart mitochondria (Algieri et al., 2020). After 5 min pre-incubation at 37°C, the reaction carried out at the same temperature, was started by the addition of the substrate Na₂ATP and stopped after 5 min by the addition of 1 mL of ice-cold 15% (w/w) trichloroacetic acid (TCA) aqueous solution. Once the reaction was stopped, vials were centrifuged for 15 min at 3500 rpm (Eppendorf Centrifuge 5202). In the supernatant, the concentration of inorganic phosphate (Pi) hydrolyzed by known amounts of mitochondrial protein, which is an indirect measure of F-ATPase activity, was spectrophotometrically evaluated (Nesci et al., 2014b). According to the method employed, to detect the Pi release by the enzymatic reaction, the Pi released independently of the F₁F₀-ATPase activity should be quantified. To this aim, 1 μL from a stock solution of 3 mg/mL oligomycin in dimethylsulfoxide was directly added to the reaction mixture before starting the reaction. The total ATPase activity was calculated by detecting the Pi in control tubes run in parallel and containing 1 μL dimethylsulfoxide per mL reaction system. In each experimental set, control tubes were alternated to the condition to be tested. The employed dose of oligomycin, a specific inhibitor of F-ATPases which selectively blocks the F₀ subunit ensured maximal enzyme activity inhibition and was currently used in F-ATPase assays (Algieri et al., 2019).

NRG concentrations used for the analysis were solubilized in DMSO. The control sample contained the same aliquot of the solvent DMSO (≤10 μL) was employed to solubilize the NRG. In all experiments, the F₁F₀-ATPase activity was routinely measured by subtracting, from the Pi hydrolyzed by total ATPase activity, the Pi hydrolyzed in the presence of oligomycin (Nesci et al., 2014a). In all experiments, the F-ATPase activity, either activated by Ca²⁺ as cofactor or by Mg²⁺, was expressed as μmol Pi•mg protein⁻¹•min⁻¹.

2.4. F₁-ATPase activity assay

Immediately after thawing, partially purified F₁ domains were used for F₁-ATPase activity assays. The capability of ATP hydrolysis was assayed in a reaction medium (1 mL) containing 0.15 mg F₁ purified protein and 75 mM ethanolamine-HCl buffer pH 9.0, 6.0 mM Na₂ATP, and 2.0 mM MgCl₂ or 2.0 mM CaCl₂ for Mg²⁺-activated F₁F₀-ATPase and Ca²⁺-activated F₁F₀-ATPase assays, respectively. The methods and parameters of ATP hydrolysis and Pi detection were the same as those used for the mitochondrial F-ATPase activity assays. The sensitivity to 3 μg/mL oligomycin was tested to verify the detachment of F₀ domain (Algieri et al., 2019).

2.5. Kinetic analyses

The inhibition mechanism of NRG on the Ca²⁺- or Mg²⁺-activated F₁F₀-ATPases was explored by the graphical methods of Dixon and Cornish-Bowden plots, which complement one another (Cornish-Bowden, 1974). To this aim, the 1/v (reciprocal of the enzyme activity v) in Dixon plot or the S/v ratio in Cornish-Bowden plot were plotted as a function of NRG concentration. In all plots the enzyme-specific activity

was taken as the expression of v . To build these plots, different experimental sets were designed in which the F-ATPase activity was evaluated in the presence of increasing NRG concentrations at two ATP concentrations, keeping the divalent cofactor (Mg^{2+} or Ca^{2+}) concentration constant or *vice versa*. In these plots and in the definition of the binary or ternary complexes S indicates the ATP substrate or divalent cofactor. The values of K_i , which corresponds to the dissociation constant of the EI complex were calculated from the abscissa (changed to positive) of the intercept of the straight lines obtained in the Dixon plots. The values of K'_i , which represent the dissociation constant of the ternary ESI complex, were calculated as the abscissa (changed to positive) of the intercept of the straight lines obtained in the Cornish-Bowden plots (Algieri et al., 2021).

Kinetic studies have been conducted on the mutual exclusion of different inhibitors on the same F-ATPase activity. These analyses aimed to shed light on the possible interaction on the F_1 domain between compounds NRG and quercetin (QUE). To build Dixon-like graphs, in which reciprocal data on enzymatic activity ($1/v$) (y-axis) versus NRG (x-axis) concentration, F-ATPase activity were plotted was analyzed without and with a specific concentration of QUE and at constant concentration of ATP substrate. According to the graphical method (Yonetani, 1982) employed, when the straight lines show different slopes and intersection points, the enzyme inhibition mirrors the combined effect of two inhibitors. When F-ATPase is inhibited by two non-mutually exclusive compounds, for example, QUE (I_1) plus NRG (I_2), the enzyme can combine with both inhibitors to produce the ESI_1I_2 (Nesci et al., 2014b) quaternary complex. The value of $-\alpha K'_i$, which represents the dissociation constant of the quaternary complex ESI_1I_2 , was calculated from the abscissa (changed to positive) of the intersection point of the two lines obtained in the presence and absence of QUE.

2.6. Docking studies

The study was performed using the crystal structure of ATP synthase retrieved from the Protein Data Bank (<http://www.rcsb.org/>) with PDB code 2JJ2. Molecular docking was performed with AutoDock Vina (Trott and Olson, 2009), by keeping the docking parameters at default values. The geometries of QUE and naringenin (NRG) were previously optimized using quantum mechanics. Geometry optimizations and frequency calculations for stationary point characterization were carried out with Gaussian16 using the M06-2X hybrid functional (Zhao and Truhlar, 2008), the 6-31 G(d,p) basis set, and ultrafine integration grids. Bulk solvent effects in water were considered implicitly through the IEF-PCM polarizable continuum model (Scalmani and Frisch, 2010). The protein structure was processed with AutodockTools-1.5.6rc3 (Morris et al., 2009) to toggle problems of incomplete structures due to missing atoms or water molecules. Ligands and protein structure files were converted to PDBQT (Protein Data Bank Partial Charge and Atom Type) and the docking was executed using the Lamarckian algorithm. The grid box consisted of 25 grid points in all three dimensions (X, Y, and Z) separated by a distance of 1 Å between each one. As for the docking analysis of naringenin over the quercetin-inhibited enzyme a $45 \times 45 \times 45$ grid box was chosen in order to allow the algorithm to find the best docking position in the presence of QUE. For the docking analysis of naringenin over the quercetin-inhibited enzyme a $45 \times 45 \times 45$ grid box was chosen in order to allow the algorithm to find the best docking position in the presence of QUE. The docking protocol was validated by simulating the pose of QUE in the crystal structure, obtaining a low discrepancy between the pose of the docked ligand and the pose of the crystallographic ligand. Such a low discrepancy was assessed by considering a RMSD threshold value of 1.5 Å as done for our previous docking study towards F_1 -ATP synthase (C. Algieri et al., 2023). The crystallographic ligand was docked after adding hydrogen atoms with AutodockTools-1.5.6rc3 (Morris et al., 2009) and without optimizing its geometry. For all the simulations, the protein was kept rigid.

2.7. mPTP, membrane potential, and anion superoxide evaluation in mitochondria

Fresh swine heart mitochondria (1 mg/mL) were suspended and energized in assay buffer (130 mM KCl, 1 mM KH_2PO_4 , 20 mM HEPES, pH 7.2 with Tris) incubated at 37 °C with 1 µg/mL of rotenone and 5 mM succinate as a respiratory substrate. The opening of mPTP was induced by the addition of low concentrations of Ca^{2+} (20 µM) at fixed time intervals (1 min). Calcium retention capacity (CRC) was evaluated spectro-fluorophotometrically in the presence of 0.8 µM of Fura-FF. The probe has different spectral properties in the absence and presence of Ca^{2+} , i.e. it shows excitation/emission spectra of 365/514 nm in the absence of calcium (Fura-FF low Ca^{2+}) and moves to 339/507 nm in the presence of calcium concentrations (Fura-FF high Ca^{2+}). PTP opening, implying a decrease in CRC, was detected by increasing the fluorescence intensity ratio (Fura-FF high Ca^{2+})/Fura-FF low Ca^{2+} . Membrane potential ($\Delta\psi$) was evaluated in the presence of 0.5 µM JC-10. In polarized mitochondrial membranes, this probe selectively generates an orange JC-10 aggregate (excitation/emission spectra of 540/590 nm). The JC-10 monomers, generated when $\Delta\psi$ decreases, cause a green shift (excitation/emission spectra of 490/525 nm). Consequently, membrane depolarization (decrease in $\Delta\psi$) attributed to mPTP formation was detected by the increase in the fluorescence intensity ratio, which corresponds to an increase in the aggregate JC-10/monomers JC-10 ratio (C. Algieri et al., 2023). Anion superoxide (SOX) was detected by mitoSOX Red indicator. The production of superoxide by mitochondria after addition of 1 µM antimycin A was observed as MitoSOX fluorescence intensity (a.u.) increase at absorption/emission spectra of 396/610 nm. 1 mM MgADP was used as mPTP inhibitor (C. Algieri et al., 2023). All measurements were processed by LabSolutions RF software.

2.8. Rat primary cerebral endothelial cells isolation and culture

Primary cerebral endothelial cells (ECs) were isolated, as previously reported (Forte et al., 2020; Stanzione et al., 2023), from SHRSP newborn (1–3 day-old) rat brains by enzymatic and mechanic digestions and subsequent positive selection using microbeads magnetically labeled with CD31 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). ECs were cultured in DMEM/F12 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% FBS (Euroclone Srl, Pero, Italy) and ECGS (Sigma Aldrich–Merck, Darmstadt, Germany) on gelatin-precoated dishes at 37°C and 5% CO_2 in a humidified incubator. Cells were used between passages 1–4 for all experiments. *In vivo* experiments for EC isolation were performed in accordance with the European Commission guidelines (DlG 2010/63/EU) and the protocol was approved by the Italian Ministry of Health (protocol n.: 448/2022-PR).

2.9. Cell treatments

ECs were exposed to salt loading as previously reported (C. Algieri et al., 2023; Forte et al., 2020; Stanzione et al., 2023). Different NRG concentrations (10, 20, 30, 40 µM) were tested on cell viability, NAD/NADH ratio (as a measure of complex I activity) and angiogenesis to identify the most suitable dose able to counteract the harmful effects of high salt loading in our cellular model. Therefore, ECs were exposed for 72 hrs to the following treatments: NaCl 20 mM, 10 µM NRG, 20 µM NRG, 30 µM NRG, 40 µM NRG, and 20 mM NaCl + NRG (from 10 to 40 µM). Both NaCl and NRG were diluted in the cell medium. In these experimental conditions, we evaluated the cell proliferation, complex I activity, and angiogenesis as described below.

2.10. Cell proliferation assay

To test the effect of NRG on cell viability, ECs were plated in 96-well plates at a density of 1×10^4 cells/well and, the day after, they were

exposed for 72 hrs to the different concentrations of NRG either in the absence or in presence of NaCl 20 mM. The proliferation evaluation was performed using the quantitative colorimetric MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay (Sigma Aldrich–Merck). At the end of the treatment, ECs were incubated for 2–3 hrs with 10 μ L of MTT reagent (5 mg/mL) in a 37°C, 5% CO₂ incubator. Then, 100 μ L of DMSO were added to each well, and the absorbance of the solubilized substrate was measured with a microplate reader (Bio-rad, Hercules, CA, USA) at a wavelength of 570 nm.

2.11. NAD/NADH ratio assay

NAD and NADH levels were measured in ECs by using commercially available colorimetric kits (Abcam ab65348). For this purpose, 1×10^6 EC cells were plated in 100 mm cell culture dishes and then exposed to the chosen concentrations of NRG either in the absence or in presence of NaCl 20 mM for 72 hrs. At the end of the treatment, NAD and NADH values were read at 450 nm by a microplate reader and the NAD/NADH ratio was calculated as reported by the manufacturer's protocol.

2.12. Angiogenesis assay

The angiogenesis assay was performed by using a Matrigel matrix (Corning, Sigma Aldrich-Merck). In the specific, 50 μ L of Matrigel matrix were added to each well of a 96-multiwell plates and allowed to solidify for 1 hr at 37°C. To test the effects of NRG on angiogenesis, ECs were exposed for 72 hrs to the different concentrations of NRG either in the absence or in the presence of NaCl 20 mM. At the end of the treatment, 1×10^4 ECs were plated on top of the Matrigel layer and incubated for 3–4 hrs. Images were taken with EVOS Cell Imaging Systems (Thermo Fisher Scientific). The number of master junctions, segments and meshes was considered to evaluate the angiogenesis. These parameters were quantified using a specific plugin "Angiogenesis analyzer" of Image J software (National Institutes of Health, Bethesda, MD, USA) (Carpentier et al., 2020).

2.13. mPTP opening in cells

Experiments were conducted in intact SHRSP cerebral ECs, and mPTP opening has been assessed as mitochondrial membrane potential decay over time upon addition of 80 mM NaCl. Mitochondrial membrane potential was measured by loading cells with 40 nM tetramethyl rhodamine methyl ester (TMRM) for 45 min at 37 °C and then imaged over time using a 3D Cell Explorer-fluo microscope (Nanolive, Switzerland) as previously described (C. Algieri et al., 2023). Basal levels were normalized on fluorescence in the presence of 10 μ M FCCP [carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone], a protonophore that induces a rapid collapse of the membrane potential.

2.14. Statistical analysis

All values are expressed as mean \pm standard error (SEM) or standard deviation (SD). Comparisons between the experimental groups were performed by one-way ANOVA followed by Bonferroni post hoc test or Student–Newman–Keuls test. A *P* value of <0.05 was considered significant. GraphPad Prism (Ver 5.01 GraphPad Software, Inc., La Jolla, CA, USA) statistical software was used for the statistical analysis.

3. Results

3.1. Effect of the NRG on F₁F₀-ATPase

Dose-response effect of NRG was evaluated on Mg²⁺- and Ca²⁺-activated F₁F₀-ATPase. The ATP hydrolysis is supported by a divalent cofactor-dependent way and, in the range of 0.1 μ M – 4 mM NRG, different power of inhibition was exerted on the enzyme. NRG displayed

a higher inhibition efficiency on Ca²⁺-activated F₁F₀-ATPase than on Mg²⁺-activated F₁F₀-ATPase. The maximal concentration of NRG tested (4 mM) inhibits Mg²⁺- and Ca²⁺-activated F₁F₀-ATPase by 49% and by 76%, respectively (Fig. 2A and B).

Some polyphenols, such as resveratrol, quercetin, and piceatannol, cause the F₁F₀-ATPase inhibition by non-covalent bond (Gledhill et al., 2007) (Fig. 3A), preventing the mPTP opening (Algieri et al., 2019) and subsequent cell death. To give a clue on the possible molecular interactions of NRG and QUE on F₁F₀-ATPase, we performed several docking analyses by exploiting the crystallographic structure already available in the Protein Data Bank for the quercetin-inhibited enzyme (PDB, link: <https://www.rcsb.org/>) (see Figure S1 for the details). Such analyses must be considered only valid for the Mg²⁺-activated condition since the Ca²⁺-activated F₁F₀-ATPase structure is not available yet. In doing so, we performed a preliminary docking analysis wherein we attempted to reproduce the crystallographic pose of QUE to validate our docking protocol (Figure S1A,B). Then, we performed the docking analysis of NRG within the empty QUE active site, assessing that NRG shows a very similar affinity toward the same site in comparison with QUE (Figure S1C,D).

Lastly, an additional docking analysis was performed over the active site occupied by QUE. Comparable affinity values were obtained for NRG even in this case (Fig. 3B). Therefore, it might be reasonable to propose that NRG could interact near the QUE-active site whilst this latter is present. Of course, it might be reasonable to speculate the other way round (NRG within the QUE-active site), but since a crystallographic structure of F₁-ATPase in complex with NRG is not available, such a docking analysis would be excessively speculative. However, the docking score indicated that the F₁ domain of the enzyme could be a strong NRG target, confirming the interesting features of NRG.

3.2. The inhibition mechanism of NRG revealed by inhibition kinetics analyses

The enzyme-NRG interaction related to the presence or absence of the ATP substrate or cation cofactors (Mg²⁺ or Ca²⁺) was evaluated by kinetic analysis based on the building of Dixon and Cornish-Bowden plots. The inhibition exerted by NRG on the enzyme showed an uncompetitive mechanism with respect to the ATP substrate for Mg²⁺- (Fig. 4A,B) and Ca²⁺-activated F₁F₀-ATPase (Fig. 4C,D). According to the mechanism of uncompetitive inhibition, usually observed when the enzyme has two or more binding sites, NRG binds to a different site than that of the ATP substrate, but only when the enzyme–substrate (*ES*) complex is already formed, in order to yield the enzyme–substrate-inhibitor (*ESI*) complex. Moreover, the dissociation constants of the *ESI* complex known as the *K_i'* values of the F₁F₀-ATPase were the same for Mg²⁺- and Ca²⁺-activated F₁F₀-ATPase (3.11 \pm 0.30 mM vs 2.90 \pm 0.21 mM). These *K_i'* values indicated that NRG formed the ternary complex (enzyme-ATP-NRG) irrespective of the divalent cation which acted as a cofactor.

Studying the inhibition mechanism exerted by NRG on both Mg²⁺- and Ca²⁺-activated F₁F₀-ATPase with respect to the divalent cation cofactor (*C*), a mixed-type inhibitory effect was depicted. The mixed-type mechanism indicated that the inhibitor can bind to the free enzyme and form the binary complex *EI* (enzyme-NRG) or the ternary complex *ECI* (enzyme-cofactor-NRG). However, considering the dissociation constants of the *EI* complex (*K_i*) and the *ECI* complex (*K_i'*), since Mg²⁺-activated F₁F₀-ATPase (Fig. 4E,F) showed *K_i* value (6.30 \pm 0.48 mM) higher than *K_i'* value (2.70 \pm 0.29 mM), the formation of the ternary complex (enzyme-Mg²⁺-NRG) was preferred. Conversely, the mixed-type inhibition exerted by NRG on Ca²⁺-activated F₁F₀-ATPase indicated that the formation of the binary complex (enzyme-NRG) was favoured with respect to that of the ternary complex (enzyme-Ca²⁺-NRG) displaying a *K_i* value (1.13 \pm 0.05 mM) lower than *K_i'* value (3.23 \pm 0.13 mM). On balance, the dissociation constants values were lower in the presence of Ca²⁺ than in the presence of Mg²⁺, thus pointing out that

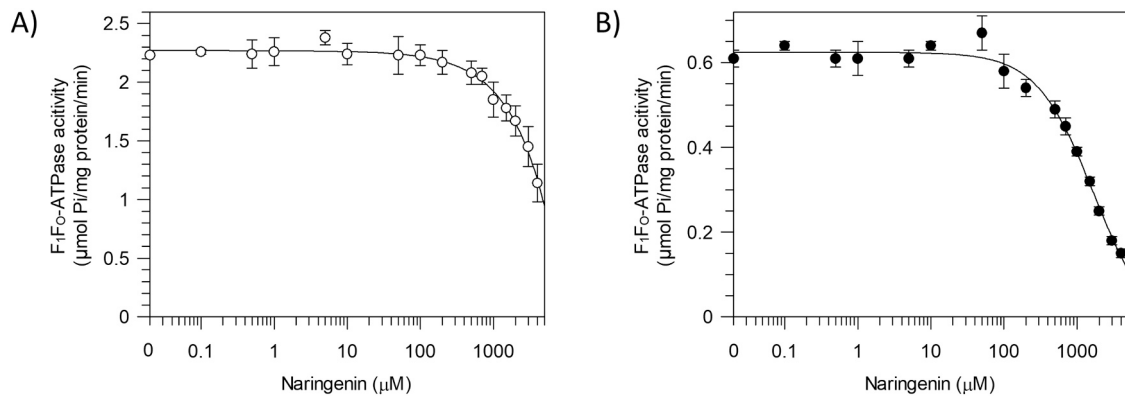


Fig. 2. Response of the mitochondrial Mg²⁺- and Ca²⁺-activated F₁F₀-ATPase activities to increasing naringenin concentrations. Data, expressed as ATPase activity of the Mg²⁺-activated F₁F₀-ATPase (A) and Ca²⁺-activated F₁F₀-ATPase (B), represent the mean ± SD from four independent experiments carried out on different mitochondrial preparations.

NRG preferably binds to Ca²⁺-activated F₁F₀-ATPase.

3.3. Multiple inhibition analysis on F₁ domain with NRG

To demonstrate the possible interaction of NRG with the hydrophilic F₁ domain of the enzyme, a mutual exclusion analysis approach has been adopted carrying out experiments with binary mixtures of NRG and QUE. The latter is known as an inhibitor of the F₁ domain (Algieri et al., 2019). The tests aimed at clarifying if NRG can combine with the enzyme-QUE complex to form the enzyme-QUE-NRG complex or if the binding of the QUE prevents the binding of the NRG. Thus, we tested if QUE and NRG are mutually exclusive (Fig. 5A,B). The reciprocal of Mg²⁺- or Ca²⁺-activated F₁F₀-ATPase activity in the presence and in the absence of fixed QUE concentration was plotted as a function of increasing NRG concentrations. Two straight lines intersecting above the x-axis in the presence of Mg²⁺ (Fig. 5A) were obtained. This result depicts a simultaneous interaction of NRG and QUE with the enzyme forming the enzyme-QUE-NRG complex (dissociation constant 1.1 ± 0.1 mM). On the contrary, NRG and QUE were mutually exclusive inhibitors in the interaction with Ca²⁺-activated F₁F₀-ATPase as illustrated by parallel straight lines in which the slopes were independent of the presence or absence of QUE (Fig. 5B). NRG and QUE could not bind simultaneously to the enzyme activated by Ca²⁺.

To verify that the binding site of NRG is placed on the F₁ domain of the enzyme, the flavone was tested on the purified catalytic portion that performs ATP hydrolysis. The F₁-ATPase activity was insensitive to oligomycin or inhibited by NBD-Cl, which are F₀ or F₁ domain-specific inhibitors respectively, in a manner independent from the activation with Mg²⁺ (Fig. 5C) or Ca²⁺ (Fig. 5D) of the purified portion of the enzyme. The tested flavones, QUE and NRG, had a dose-dependent inhibitory effect that was more enhanced on Ca²⁺-dependent F₁-ATPase rather than on Mg²⁺-dependent F₁-ATPase.

3.4. Mitochondrial bioenergetics modulated by NRG

By interacting with the F₁ domain, the inhibitory effect of NRG on Ca²⁺-activated F₁F₀-ATPase was related to a beneficial effect on mitochondrial bioenergetics. The mPTP opening was highlighted by a decreased CRC (Fig. 6A) and the dissipation of mitochondrial Δφ (Fig. 6B) when a threshold of Ca²⁺ pulse at 1 min intervals triggered the pore formation. As a consequence, the Fura-FF ratio and the JC-10 ratio increased, respectively. NRG blocked the mPTP formation preventing the increase in the Fura-FF ratio or JC-10 ratio. However, 40 μM or 200 μM NRG profiles vs Ruthenium Red (RR), an inhibitor of mitochondrial calcium uniporter, profile of CRC were different. The addition of 0.5 μM FCCP decreased the CRC value of control and 40 μM NRG. Conversely, the desensitization of CRC by 200 μM NRG was unaffected

by FCCP stimulation (Fig. 6A). The mPTP opening depolarized the mitochondria bypassing the FCCP effect except in the presence of MgADP which inhibited the pore formation. On the contrary, the FCCP addition to mitochondria in the presence of MgADP increased the JC-10 ratio emulating the effect of mPTP opening (Fig. 6B). The antioxidant effect of flavonoid structure of NRG was evaluated on the SOX production in mitochondria. Energized mitochondria with substrate for the first site of phosphorylation (complex I-linked substrates: pyruvate plus malate) (Fig. 6C,D) or with substrate for the second site of phosphorylation (complex II-linked substrate: succinate) were tested with two NRG concentrations (40 μM and 200 μM). NRG did not affect the detection of SOX revealed by mitoSOX™ probe in the presence of pyruvate plus malate (Fig. 6C) or succinate (Fig. 6E). Antimycin A added to energized mitochondria stimulated the SOX generation. The increase in mitoSOX intensity antimycin A-dependent by substrates for complex I was mostly decreased at low dose of NRG (Fig. 6D). Conversely, under the condition of succinate respiration blocked with antimycin A, NRG at both tested concentrations had a preventive effect on SOX production (Fig. 6F).

3.5. NRG counteracts the harmful effects induced by saline load in SHRSP primary cerebral ECs

NRG, at doses of 30 and 40 μM, exerted a significant protective effect against the harmful effect of high salt exposure on cell viability (Fig. 7A). On the contrary, NRG alone did not induce any significant effect on cell viability. We investigated mitochondrial function by assessing the activity of mitochondrial complex I. ECs exposed to high-salt loading showed a decreased NAD/NADH ratio, as an index of mitochondrial complex I dysfunction, similarly to what was previously reported in the SHRSP (Forte et al., 2020; Rubattu et al., 2016a), and in *in-vitro* studies (C. Algieri et al., 2023; Stanzione et al., 2023). NRG, at doses from 20 to 40 μM, was able to rescue the complex I activity in ECs exposed to high salt load (Fig. 7B). NRG alone did not induce any significant effect on complex I activity.

NRG at all doses was able to rescue the ECs ability to form vessel-like tubes on a matrigel substrate in the presence of high salt loading (Fig. 7C,D). Interestingly, NRG alone at the dose 10 μM significantly reduced the number of master junctions, of segments and meshes compared to untreated ECs, whereas NRG at the dose of 20 μM reduced only the number of segments and meshes compared to untreated ECs. Notably, acute exposure of SHRSP cells subjected to high salt conditions displays a rapid collapse of mitochondrial membrane potential, as a result of mPTP opening, which is completely prevented by NRG pre-treatment (Fig. 7E). Our results suggest that NRG represents a suitable approach to rescue endothelial function, through an improvement of mitochondrial function and by inhibiting mPTP functions, in conditions

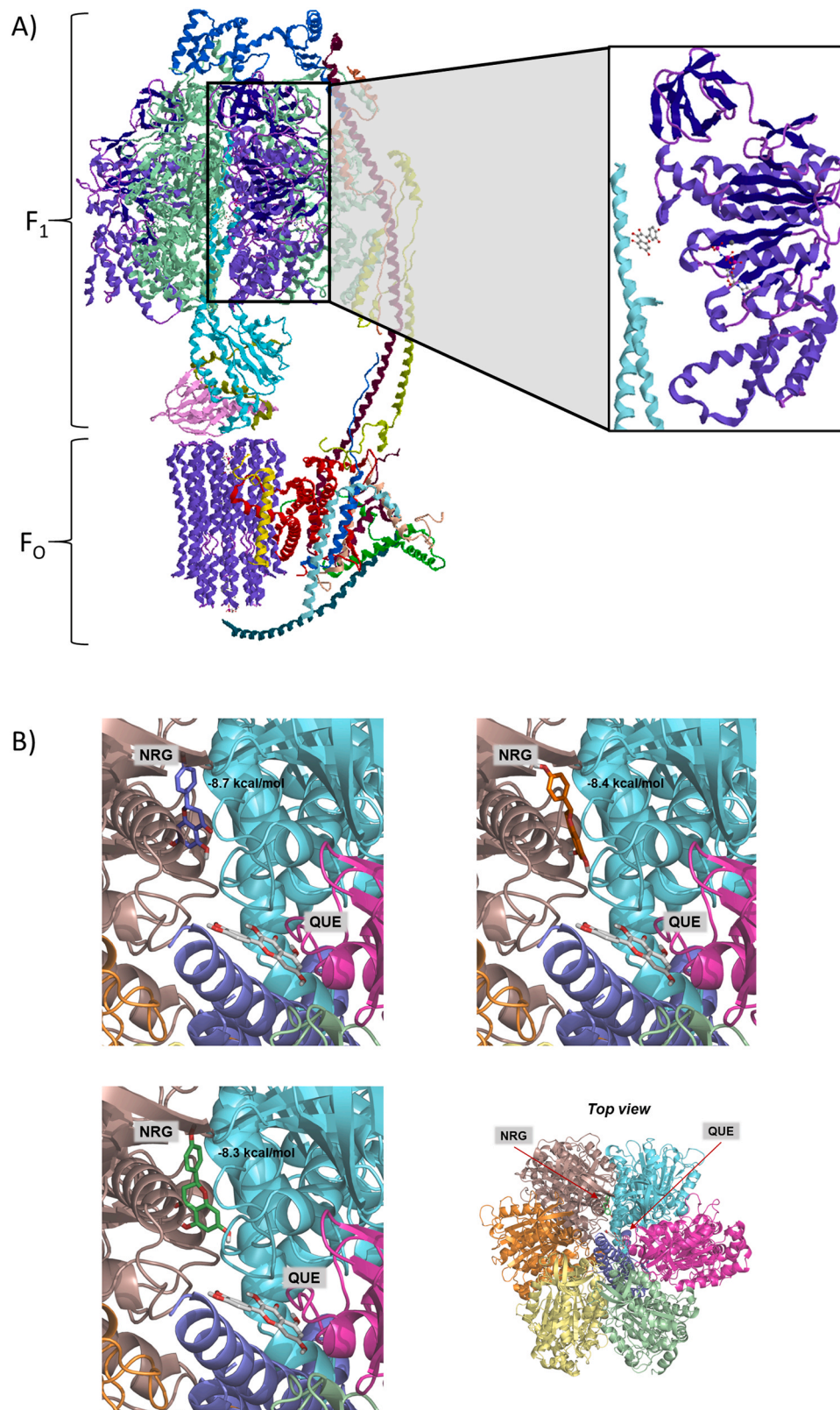


Fig. 3. Docking model of naringenin fitted in the phytochemical compounds binding region of the F_1 domain. A) Structure of F_1F_0 -ATPase drawn as ribbon representations obtained from modified PDB ID code: 6TT7. In the box the possible site of naringenin (in ball and stick model) between β (in purple) and γ (in light blue) subunits is highlighted. B) Best three docking poses of naringenin. The docking calculations were performed over the F_1 -ATP synthase active site already occupied by quercetin (modified PDB ID code: 2JJ2).

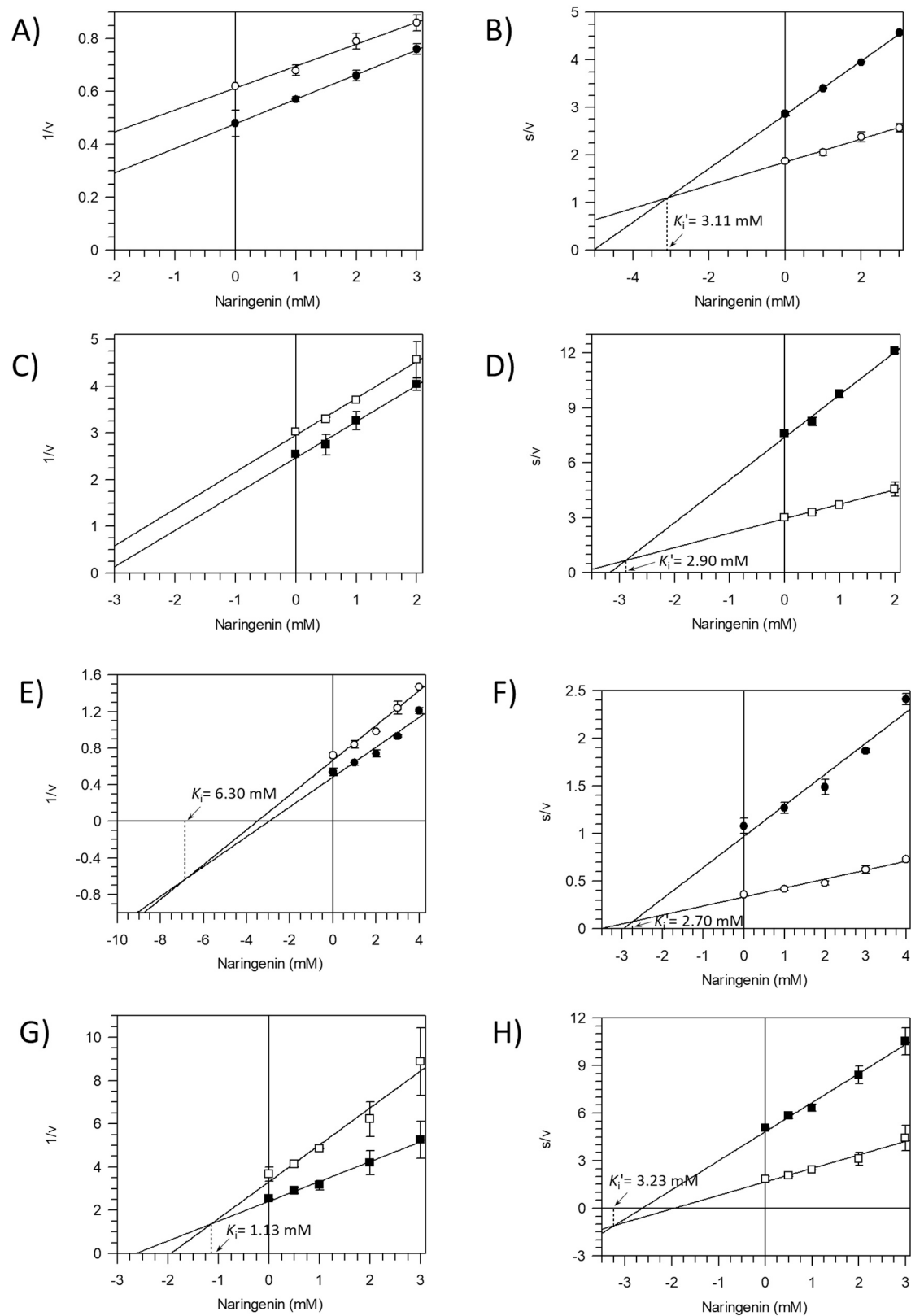


Fig. 4. Inhibition kinetics of the mitochondrial Mg^{2+} - and Ca^{2+} -activated F_1F_0 -ATPase activities by naringenin. Dixon (A and C) and Cornish-Bowden (B and D) plots of Mg^{2+} -activated F_1F_0 -ATPase at 3 mM ATP (\circ) or 6 mM ATP (\bullet); and Ca^{2+} -activated F_1F_0 -ATPase at 1 mM ATP (\square) or 3 mM ATP (\blacksquare). Dixon (E and G) and Cornish-Bowden (F and H) plots of Mg^{2+} -activated F_1F_0 -ATPase at 0.5 mM Mg^{2+} (\circ) or 2 mM Mg^{2+} (\bullet); and Ca^{2+} -activated F_1F_0 -ATPase at 0.5 mM Ca^{2+} (\square) or 2 mM Ca^{2+} (\blacksquare). K_i and K_i' values were obtained as detailed in the kinetic analyses section of Materials and Methods. Each point represents the mean \pm SD from five experiments on distinct mitochondrial preparations.

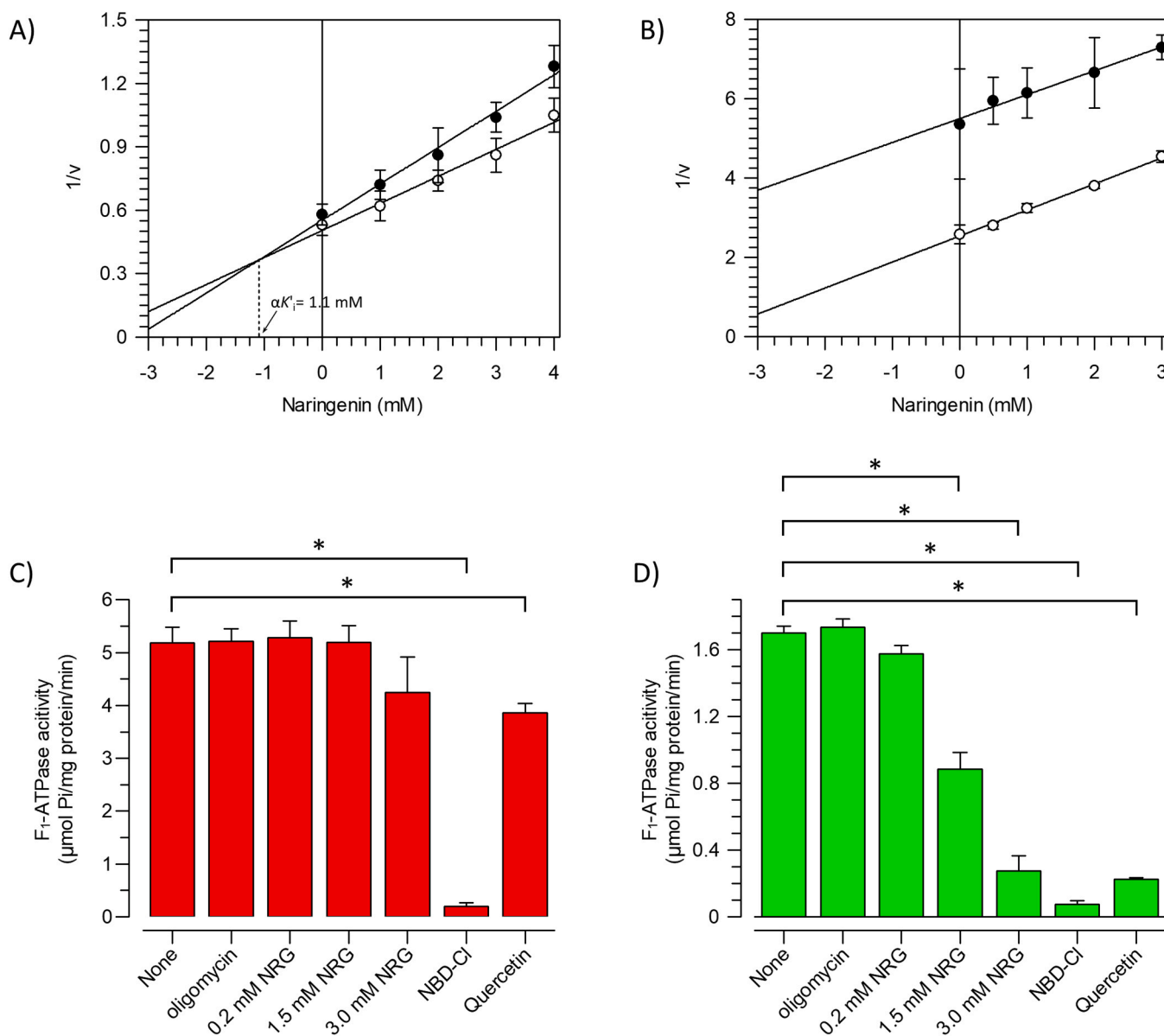


Fig. 5. Effect of naringenin on the F_1 domain. Multiple inhibitor analysis by Dixon plots for Mg^{2+} -activated F_1F_0 -ATPase in the presence of 2 mM Mg^{2+} plus 6 mM ATP (A) or Ca^{2+} -activated F_1F_0 -ATPase 2 mM Ca^{2+} plus 3 mM ATP (B) inhibition by naringenin in the absence (\circ) or in the presence of 0.4 mM quercetin (\bullet) as detailed in the kinetic analyses section of Materials and Methods 2.4. C) Mg^{2+} - and (D) Ca^{2+} -activated F_1 -ATPase activities were evaluated in the absence or in the presence of the inhibitors: 3 μ g/mL oligomycin; 0.2 – 1.5 – 3.0 mM naringenin (NRG); 75 μ M NBD-Cl; and 0.75 mM quercetin. Each value represents the mean \pm SD from three independent experiments carried out on distinct F_1 preparations. The asterisk (*) indicates significantly different enzyme activity values ($P < 0.05$) in the absence and in the presence of inhibitors.

of high salt-induced injury.

4. Discussion

An impairment of mitochondrial function is linked to CVDs. We investigated the possibility that NRG can resolve the mitochondrial dysfunction caused by the mPTP opening. By acting at the mitochondrial level, NRG may protect from CVDs. In the specific, it may prevent the development of target organ damage in hypertension, a prevalent pathological condition linked to severe cardiac and vascular damage increasing the risk of developing heart failure, myocardial infarction, stroke, and kidney failure (Mollace et al., 2021; Nesci et al., 2023; Rubattu et al., 2015).

The F_1F_0 -ATPase has been studied for its features to form the mPTP (Alavian et al., 2014; Bonora et al., 2013). The cryo-EM structure of the

enzyme (Pinke et al., 2020) has corroborated the proposed “bent-pull” model in pore formation (Mnatsakanyan and Jonas, 2020). Moreover, the substitution of natural cofactor Mg^{2+} in the catalytic site on β subunits of F_1 domain with Ca^{2+} can trigger a conformational change signal to the membrane-embedded F_0 domain to form the mPTP (Giorgio et al., 2017). The link between F_1F_0 -ATPase, Ca^{2+} and mPTP (Nesci, 2017) has led to the identification of Ca^{2+} -activated F_1F_0 -ATPase as a molecular marker to block mPTP formation (Nesci, 2020). Different small compounds and ions were discovered to be insensitive to Mg^{2+} -activated F_1F_0 -ATPase activity but they selectively inhibited Ca^{2+} -activated F_1F_0 -ATPase (Algieri et al., 2020, 2019; C. Algieri et al., 2023, 2021; V. Algieri et al., 2023, 2021; Nesci et al., 2021). The phenomenon has been suggested to be linked with the mPTP event (Nesci, 2022; Nesci and Pagliarani, 2021a, 2021b). However, cyclosporine A-sensitive Ca^{2+} -induced permeability transition pore opening is dependent on at least two

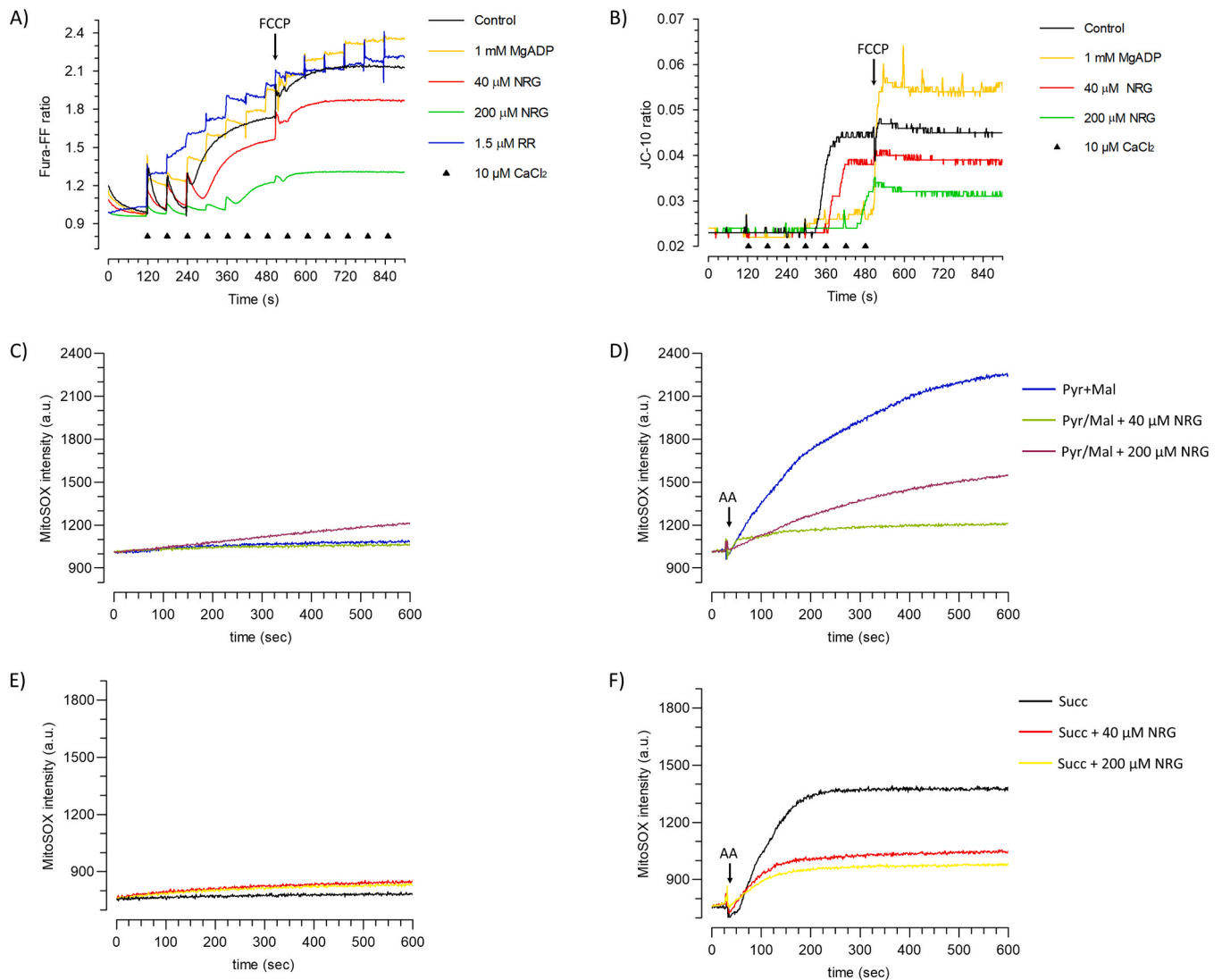


Fig. 6. mPTP opening and ROS production in the presence of naringenin. Representative curves of (A) calcium retention capacity (CRC) expressed as Fura-FF ratio and (B) of the membrane potential expressed as JC-10 ratio to detect the mPTP opening. Evaluation of the anion superoxide production in mitochondria energized with pyruvate plus malate (C and D), substrates for the first site of oxidative phosphorylation (complex I) and with succinate (E and F), the substrate for the second site of oxidative phosphorylation (complex II). The arrow indicates the injection of 0.5 μM FCCP or 1.0 μM antimycin A (AA), an inhibitor of mitochondrial respiration triggering the stimulation of anion superoxide. The experiments were carried out in triplicates on three distinct mitochondrial preparations.

pore-forming components, a core part formed either the F_1F_0 -ATPase (Bonora et al., 2013; Mnatsakanyan et al., 2019; Urbani et al., 2019) and the adenine nucleotide translocator (ANT) (Karch et al., 2019; Neginskaya et al., 2019), both of which could be transformed into the high-conductance pore in response to higher mitochondrial matrix Ca^{2+} level (Neginskaya et al., 2022). Accordingly, it is possible that the mPTP consists of at least two pore-forming components. Moreover, the supramolecular organization of F_1F_0 -ATPase with ANT plus phosphate carrier (PiC) in the ATP synthasome supercomplex is inversely related to mPTP formation. Cyclophilin D (CypD), a well-established positive regulator of the mPTP, appears to interact with all the components of the ATP synthasome destabilizing the supramolecular organization of the complexes and promoting the mPTP opening (Beutner et al., 2017). Conversely, HAP1 cell lines with vestigial ATP synthase (lacking of g and F6 subunit) are more sensitive to mPTP opening asserting that the F_1F_0 -ATPase is not a component of mPTP but instead inhibits the pore opening (Pekson et al., 2023).

Phenolic phytochemicals, and in particular polyphenols, can prevent ATP hydrolysis by F_1 domain interaction without affecting the catalytic sites of the enzyme. F_1 -ATPase is a motor protein that couples the

rotation of its rotary γ subunit within a globular hexamer formed by non-catalytic sites on three α subunits alternate with three β subunits accommodating three catalytic sites. These compounds bind to distinct and unrelated regions in the hydrophobic area of the enzyme, which is where the C-terminal tip of the γ subunit contacts with the “bearing”, a loop region, below the “crown region” of the α and β subunits (Gledhill et al., 2007). NRG, as a flavone belonging to the class of polyphenols, can inhibit the catalysis of ATP hydrolysis with higher selectivity towards Ca^{2+} -activated F_1F_0 -ATPase than the enzyme in the presence of natural cofactor Mg^{2+} .

The mechanism of inhibition of F_1F_0 -ATPase might be explained by NRG binding with F_1 domain. The NRG binding site is different from those of ATP and divalent cation cofactor (Mg^{2+} or Ca^{2+}). Interestingly, the F_1F_0 -ATPase link to ATP is necessary to allow the interaction of NRG with the enzyme. Likely, the ATP binding change mechanism of F_1F_0 -ATPase (Boyer, 2002) causes the appropriate conformational modification in the enzyme to guarantee the NRG binding. Otherwise, with respect to divalent cation, NRG has an affinity higher with F_1F_0 -ATPase complexed with Mg^{2+} than with the enzyme-free, whereas NRG can displace F_1F_0 -ATPase from binding to Ca^{2+} cofactor. Accordingly, the

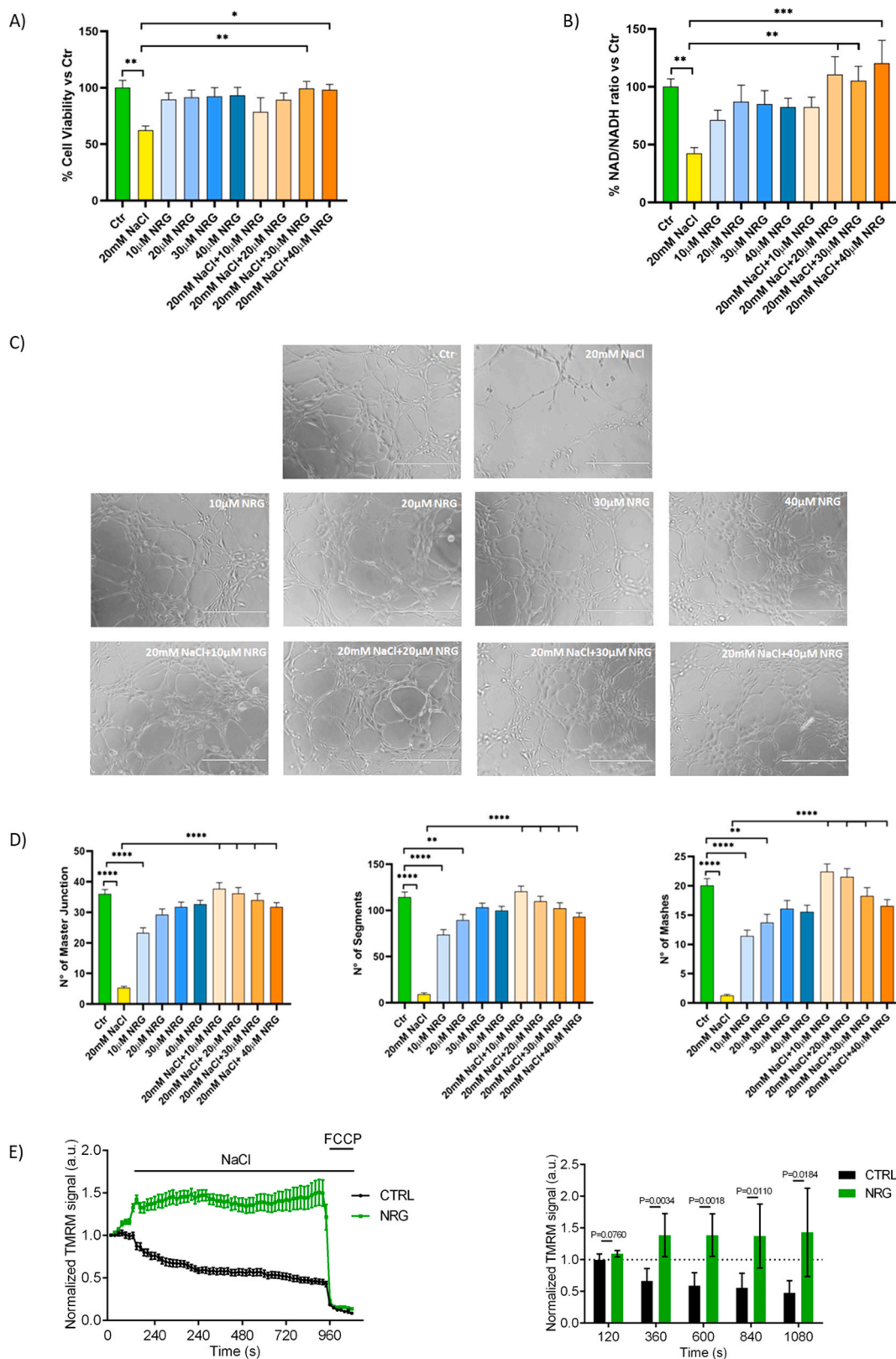


Fig. 7. Naringenin rescues cell viability and exerts beneficial effect on mitochondrial function and angiogenesis in SHRSP cerebral ECs exposed to high salt load. ECs were treated for 72 hrs with 20 mM NaCl either in the absence or in the presence of naringenin (from 10 to 40 µM). A) Evaluation of cell viability; N= 5. B) Evaluation of NAD/NADH ratio (as a measure of complex I activity) N= 4. C) Representative images of Matrigel assay and (D) the quantification of master junctions, segments, and meshes; N= 5. Ctrl indicates untreated cells. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ were obtained using one-way ANOVA followed by Bonferroni post hoc analysis. Data are reported as mean±SEM. D) Kinetics of time-dependent changes in TMRM signal, upon addition of 80 mM NaCl. 10 µM FCCP has been used as reference. Where indicated, cells were pre-treated with 40 µM naringenin (NRG) for 1 h. N = 4 independent experiments; mean±SEM. The bars in the graph (right panel) report the normalized TMRM values at different time points upon addition of NaCl. N = 4 independent experiments; mean±SD.

inhibition of F_1F_0 -ATPase by NRG before the activation by Ca^{2+} might suggest a protective role in the mPTP opening.

In all likelihood, the NRG interacts with the F_1 domain of the enzyme as highlighted by the inhibitory effect revealed on the F_1 -ATPase activity and by the overlap of the binding site with QUE on the Ca^{2+} -activated F_1F_0 -ATPase. The different effect of NRG on the enzyme activated by the two divalent cations has been speculated to be related to the different steric hindrance of ions since the Ca^{2+} has an atomic radius higher than Mg^{2+} (Casadio and Melandri, 1996; Nesci et al., 2018).

Homeostasis of Ca^{2+} and ROS production are regulated in mitochondria for maintaining cellular bioenergetics and metabolic precursor synthesis. However, pathological conditions, environmental conditions or injurious stimuli, causing mitochondrial dysfunction, promote the beginning and/or development of human diseases and the aging process (Harrington et al., 2023). The relevance of the underlying pathways causing mitochondrial impairment may aid in preventing various pathologies. mPTP opening is the biological event in mitochondria triggering different forms of regulated cell death (Izzo et al., 2016). The mPTP opening indicates that the IMM integrity has been lost, and mitochondria no longer maintain ion homeostasis. In all likelihood, mitochondrial dysfunction is counteracted by the NRG effect through two of its action features on mitochondrial biology. The negative role on the kinetics of Ca^{2+} -activated F_1F_0 -ATPase blocks its bioarchitecture mechanism triggering the mPTP formation, whereas the antioxidant character of the molecular structure of NRG could have a scavenger effect on ROS production in mitochondria. NRG may possess the ability to arrest the feedforward loop supporting that an increase in Ca^{2+} and ROS activates the mPTP (Bauer and Murphy, 2020).

The ECs used in the present study were obtained from a known animal model of human hypertensive disease and related vascular damage, the SHRSP (Rubattu et al., 2016b). This model accelerates stroke occurrence when fed with a high salt/low potassium dietary regimen (Rubattu et al., 2016b, 1996). A severe mitochondrial complex I dysfunction due to Ndufc2 subunit inhibition was discovered as a major molecular mechanism underlying vascular damage and stroke predisposition in SHRSP (Rubattu et al., 2016a). We recently showed that primary cerebral ECs obtained from SHRSP pups represent a suitable experimental tool. Once exposed to a high salt load, they allow to dissect out the therapeutic effect of both chemical and natural substances able to counteract mitochondrial dysfunction (Algieri et al., 2022a; C. Algieri et al., 2023; Stanzione et al., 2023). In the current study, we provide consistent evidence by showing that NRG is able to counteract in a significant manner the high salt-induced injury in cerebral ECs, by strongly inhibits mPTP formation. Indeed, NRG preserved the mitochondrial membrane potential, and rescued both cell viability and endothelial cell tube formation, with a parallel recovery of complex I activity, in high salt-exposed ECs.

To sum up, the results have provided new knowledge on the mechanisms underlying cardiovascular protection by NRG through its impact on mitochondrial dysfunctions. In cellular models mimicking cardiovascular diseases with doxorubicin treatment, we highlighted a mitochondrial dysfunction counteracted by bergamot polyphenolic fraction (BPF) (Algieri et al., 2022b), which contains NRG. However, we can assert that, on high salt-exposed ECs predisposing to hypertension and onset of cardiovascular damage, BPF has a protective effect (Stanzione et al., 2023). It would be worthwhile to investigate the possibility that NRG, excluding the additive or synergistic action of other BPF polyphenols, could prevent mitochondrial dysfunction associated with cardiovascular diseases. The reduction of mPTP opening and the attenuation of oxidative stress may bring a great contribution in terms of efficacy and safety in the use of natural molecules to fight against major CVDs such as hypertension and its complications, the cardiac consequences of hyperglycemia, and ischemic heart disease, with the possibility of integrating or enhancing existing therapeutic strategies. Moreover, our results suggest that NRG represents a suitable approach to rescue endothelial function, through an improvement of mitochondrial

function, in conditions of high salt-induced injury. Of note, NRG alone exerted a downregulatory effect on both complex I activity and the angiogenic properties of the cells.

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CRediT authorship contribution statement

Salvatore Nesci: Conceptualization; Formal analysis; Funding acquisition; Supervision; Validation; Visualization; Writing - original draft. **Cristina Algieri:** Investigation. **Matteo Antonio Tallarida:** Formal analysis; Writing - Review & Editing. **Rosita Stanzione:** Investigation; Formal analysis. **Donatella Pietrangelo:** Methodology; Resources. **Fabiana Trombetti:** Methodology; Writing - Review & Editing. **Luca D'Ambrosio:** Resources. **Maurizio Forte:** Methodology. **Maria Cotugno:** Data Curation. **Rachele Bigi:** Writing - Review & Editing. **Loredana Maiuolo:** Writing - Review & Editing. **Antonio De Nino:** Writing - Review & Editing. **Saverio Marchi:** Writing - Review & Editing. **Paolo Pinton:** Writing - Review & Editing. **Giovanni Romeo:** Conceptualization; Writing - Review & Editing. **Speranza Rubattu:** Conceptualization; Funding acquisition; Supervision; Validation; Visualization; Writing - original draft.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Salvatore Nesci reports financial support was provided by University of Bologna, grant numbers RFO 2021 and Alma Idea 2022. Speranza Rubattu reports financial support was provided by Italian Ministry of Health Ricerca Corrente. Speranza Rubattu reports financial support was provided by Italian Ministry of University and Research PNRR - M4C2-I1.3 Project PE_00000019 "HEAL ITALIA", CUP B53C22004000006. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ejcb.2024.151398](https://doi.org/10.1016/j.ejcb.2024.151398).

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