BBA - Bioenergetics 1866 (2025) 149528

Contents lists available at ScienceDirect

BBA - Bioenergetics

journal homepage: www.elsevier.com/locate/bbabio

Inside-out submitochondrial particles affect the mitochondrial permeability transition pore opening under conditions of mitochondrial dysfunction

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ARTICLE INFO

Keywords:
Inside-out submitochondrial particles
Mitochondrial dysfunction
Mitochondrial permeability transition pore
F_1F_O-ATPase

ABSTRACT

The inside-out submitochondrial particles (IO-SMPs) showed a strong protective effect against mitochondrial permeability transition pore (mPTP) opening in mitochondria isolated from swine hearts 3 h after explantation. The latter condition was used to emulate situation of mitochondrial damage. We identified that the protective effect of IO-SMPs cannot be attributed to a functional modulation of the enzymatic complexes involved in mPTP formation. Indeed, oxidative phosphorylation and F_1F_O-ATPase activity were not affected. Conversely, mPTP desensitization might be caused by structural modification. IO-SMP incorporation into the mitochondria can modulate the membrane-bound enzyme complexes' functionality, inducing F_1F_O-ATPase to be unable to carry out the conformational changes useful for mPTP opening. Thus, the data are a valid starting point for IO-SMP application in the treatment of impaired cardiovascular conditions supported by mPTP opening.

1. Introduction

Mitochondria produce energy to sustain the demands of the cell. However, mitochondrial dysfunction, characterized by loss of efficiency of the electron transport chain (ETC) and the reduction of high-energy molecule synthesis as adenosine-5′-triphosphate (ATP), is a feature associated with several pathological conditions. In particular neurodegenerative diseases, diabetes, cancer, cardiomyopathies, ischemia/reperfusion injury, ageing and many chronic diseases are characterized by mitochondrial dysfunction [1,2]. One of the phenomena involved in mitochondrial dysfunction is an abnormal mitochondrial permeability transition, *i.e.* the process of non-selective loss of molecules across the inner mitochondrial membrane (IMM). The mitochondrial permeability transition pore (mPTP) has been described as a calcium-induced calcium release channel [3]. It is a giant nonspecific channel with a diameter of 2–3 nm that passes metabolites with molecular weight <1.5 kD and exhibits multiple subconducting states [4], however, the maximal conduction state is between ~ 1.0 and 1.3 nS [5,6]. Although mPTP has physiological and protective roles under normal circumstances, temporarily opening to act as a "Ca^{2+} release valve", preventing mitochondrial Ca^{2+} overload and regulating mitochondrial function, its prolonged opening is accompanied by depolarization, the release of matrix components, generation of reactive oxygen species (ROS), morphological changes (swelling), decrease in cellular ATP levels, activation of mitophagy and, eventually, cell death [7]. Therefore, knowing the molecular bioarchitecture that participates in the formation of the mPTP represents a potential pharmacological target for the treatment of diseases characterized by increased Ca^{2+} and cell death [8,9]. For a long time, the main candidate for the pore-forming role was the adenine nucleotide translocator (ANT), although its role was ascribed as a regulator of mPTP [10]. However, electrophysiological studies have confirmed that ANT can form channels, although not as large as the "canonical" mPTP megachannel [11]. However, a CSA-sensitive interaction of CypD with the oligomycin sensitivity conferring subunit (OSCP) of the F_1F_O-ATPase introduced the hypothesis that the enzyme may form mPTP [12,13]. F_1F_O-ATPase is an enzyme of the IMM. It is composed of a membrane-embedded F_O domain that dissipates the transmembrane electrochemical gradient of H^+ to synthesize ATP at the level of the hydrophilic F_1 domain [14,15]. The enzyme can also hydrolyze ATP by transferring H^+ in the direction opposite to synthesis, thus wording as an H^+ pump and generating an electrochemical gradient across the IMM. This bifunctional activity is supported by the natural cofactor Mg^{2+} [16] while the replacement of Mg^{2+} by Ca^{2+} only allows oligomycin-sensitive ATP hydrolysis [17]. The burst of Ca^{2+}

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https://doi.org/10.1016/j.bbabio.2024.149528
Received 29 August 2024; Received in revised form 24 November 2024; Accepted 26 November 2024
Available online 29 November 2024

concentration in the mitochondrial matrix activates the catalytic site of the F_1F_O-ATPase, supporting the hydrolysis of ATP and triggering the formation of mPTP [18]. To date there are few known modulators of the mPTP: we associate cyclophilin D (CyPD), a *cis-trans* peptidyl-prolyl isomerase of the mitochondrial matrix, which promotes its opening and cyclosporin A (CSA), or its analogues, which inhibit the mPTP opening in a CyPD-dependent manner [8,19,20]. Therefore, targeted modulation of Ca^{2+}-activated F_1F_O-ATPase is an attractive therapeutic target for the treatment of many pathological conditions caused by dysregulations of mPTP formation and opening.

Oxidative and nonoxidative conditions have been used to study the mPTP on isolated mitochondria by considering also inside-out submitochondrial particles (IO-SMPs) [21]. However, the mPTP on recipient mitochondria of IO-SMPs has never been investigated. Therefore, in this study, we evaluate for the first time the effect of IO-SMPs on mitochondria that emulate the damage induced by events triggering mPTP opening, with the aim of finding an ameliorative bioenergetic effect and a hypothetical therapeutic solution on mitochondrial molecular phenomena that support the dysfunction processes.

2. Materials

2.1. Chemicals and reagents

Na_2ATP, calcium chloride, magnesium chloride, oligomycin, 2,4-Dinitrophenol (DNP), Atractyloside sodium salt, L-Glutamic acid, L-Malic acid, rotenone, antimycin A and succinic acid were obtained from Sigma–Aldrich (Milan, Italy). Fura-FF was purchased from Vinci-Biochem (Vinci, Italy). All other chemicals were reagent grade and used without purification. Quartz double-distilled water was used for all reagent solutions, except when differently stated.

2.2. Mitochondria isolation

Swine hearts of animals slaughtered in the slaughterhouse were used immediately or after 3 h kept on ice, for the isolation of mitochondria named SHM_A (healthy mitochondria) and SHM_B (damaged mitochondria), respectively. On each, after removing as much fat and blood clots, approximately 30 g of heart tissue was rinsed in ice-cold medium A (0.25 M sucrose, 10 mM Tris (hydroxymethyl)-aminomethane (Tris), pH 7.4 with HCl) and finely chopped into fine pieces with scissors. Subsequently, the tissues were gently dried on absorbent paper, weighed, and homogenized with Ultraturrax T25 in medium B (0.25 M of sucrose, 10 mM of Tris, 1 mM of EDTA (free acid), 0.5 mg/mL of BSA without fatty acid, pH 7.4 with HCl) in a ratio of 10 mL of medium B to 1 g of fresh tissue. The mitochondrial fraction was then obtained by gradual centrifugation (Sorvall RC2-B, SS34 rotor). The homogenate was centrifuged at 1000 ×g for 5 min, thus obtaining a supernatant and a pellet. The pellet was re-homogenized again under the same conditions as the first homogenization, and recentrifuged at 1000 ×*g* for 5 min. The supernatants collected from these two centrifugations, filtered through four cotton gauze layers, were centrifuged at 10,500 ×g for 10 min to produce the raw mitochondrial pellet. The raw pellet was resuspended in medium A and further centrifuged at 10,500 ×g for 10 min to obtain the final mitochondrial pellet. The latter was resuspended by gentle agitation using a Potter Elvejehm Teflon homogenizer in a small volume of medium A, thus obtaining a protein concentration approximately of 30 mg/mL [22]. All steps were performed at 0–4 °C. Protein concentration was determined according to the Bradford colorimetric method using the Bio-Rad Protein Assay kit II with BSA as standard [23]. The mitochondrial preparations were then stored in liquid nitrogen.

2.3. Preparation of inside-out submitochondrial particles (IO-SMPs)

(IO-SMPs were prepared according to our established protocol [22,24,25]. A concentration of 20 mg/mL protein was obtained by diluting mitochondria with medium C (0.25 M sucrose, 2 mM EDTA, 10 mM Tris, pH 7.4 with HCl). The chelation of Mg^{2+} ions by the addition of EDTA enables to form inside-out oriented submitochondrial particles, in which the matrix side of the inner mitochondrial membrane inverts out to be exposed to experimental medium [26]. The mitochondrial suspension was saturated with N_2 and sonicated on ice with MSE Sonicator Soniprep 150 at 210 μm amplitude 5 times for 3 s with 10 s intervals. IO-SMP particles were isolated by stepwise centrifugation [25]. Sonicated preparations were diluted 1:2 with medium C and centrifuged at 16,000 *g* for 10 min at 4 °C (Beckman LE-80 K, SW55Ti rotor). The supernatant obtained was further centrifuged at 150,000 *g* for 45 min at 4 °C. The pellet containing submitochondrial particles was carefully resuspended in medium B and homogenized by gentle agitation using a Potter Elvejehm Teflon homogenizer. The protein concentration was determined according to the Bradford method [23]. The IO-SMPs were stored in liquid nitrogen.

2.4. mPTP evaluation

Immediately after the preparation of swine heart mitochondrial fractions, 1 mg/mL of fresh mitochondrial suspensions of SHM_A or SHM_B were energized in the assay buffer (130 mM KCl, 1 mM KH_2PO_4, 20 mM HEPES and 5 mM succinate, pH 7.2 with TRIS), incubated at 37 °C with 1 μg/mL rotenone. Moreover, to evaluate the mPTP activity on SHM_B treated with IO-SMPs, the mitochondria were incubated for 10 min on ice with IO-SMP at a ratio of 1:10 mg protein of IO-SMP to SHM_B. In regard to the induction of the mPTP opening, this was determined by a $CaCl_2$ solution added at intervals of 1 min to guarantee the presence of low Ca^{2+} concentrations equal to 10 μM. Spectrofluorophotometry analysis carried out in the presence of 0.8 μM Fura-FF, allowed for the evaluation of the calcium retention capacity (CRC), the reduction of which indicates the mPTP opening. The probe used has different spectral characteristics depending on the Ca^{2+} absence or presence. In fact, in the absence of Ca^{2+} (Fura-FF low Ca^{2+}), an excitation/emission spectrum at 365/514 nm is detected; alternatively, when the concentration of Ca^{2+} increases (Fura-FF high Ca^{2+}), the spectrum shifts to 339/507 nm. The increase in the fluorescence intensity ratio (Fura-FF high Ca^{2+})/(Fura-FF low Ca^{2+}) is determined by the reduction in CRC and, therefore, allows for the evaluation of the opening of the mPTP [27]. 1 mM MgADP was used as an mPTP inhibitor [28]. The LabSolutions RF software was used to process these measurements.

2.5. Evaluation of oxidative phosphorylation

On fresh mitochondria that guarantee the coupling of oxidative phosphorylation (OXPHOS), respiratory activity was assessed polarographically using a thermostated Oxytherm system (Hansatech Instruments, King's Lynn, UK) in terms of oxygen consumption at 37 °C in a polarographic chamber. The reaction mixture, kept at a fixed temperature and stirred continuously, contained 0.25 mg/mL of SHM_B suspension in the reaction medium (75 mM sucrose, 5 mM KH_2PO_4, 40 mM KCl, 0.5 mM EDTA, 3 mM $MgCl_2$ and 30 mM Tris, pH 7.4 with HCl). In selected experiments, SHM_B were incubated for 10 min on ice with IO-SMP at a ratio of 1:10 mg protein of IO-SMP to SHM_B. The rate of oxygen consumption was evaluated in the presence of specific substrates, 5 mM glutamate+malate in a 1:1 ratio for complex I (indicated as first phosphorylation site) and 10 mM succinate for complex II (indicated as second phosphorylation site). Non-specific oxygen consumption was evaluated in the presence of 1 μM antimycin A and subtracted the respiration activity of first and second phosphorylation sites. To evaluate the coupling between respiratory activity and ADP phosphorylation, 150 nmol of ADP was added to State 2 of respiratory mitochondria [29]. The respiratory control ratio (RCR) of OXPHOS was evaluated as the ratio between State 3 (when ATP is synthesized) and State 4 (when ATP is not synthesized) (State 3/State 4). Respiratory activities were

expressed as nmoles O_2/mg protein/min. Polarographic tests were performed in triplicate on three mitochondrial preparations.

2.6. F-ATPase activity

After thawing SHM_B and IO-SMPs from liquid nitrogen, several preparations were used to evaluate F-ATPase activity. To measure the hydrolysis capacity of ATP by Mg^{2+}-activated F_1F_O-ATPase, 1 mL of a reaction medium, consisting of 0.15 mg of SHM_B or IO-SMPs protein and 75 mM ethanolamine-HCl buffer at pH 9.0, was used in the presence of 6.0 mM Na_2ATP and 2.0 mM $MgCl_2$; while the same buffer was used to determine the activity of Ca^{2+}-activated F_1F_O-ATPase but at pH 8.8 in the presence of 3.0 mM Na_2ATP and 2.0 mM $CaCl_2$. In selected experiments, to favour the incorporation of IO-SMPs to mitochondrial membranes, enzymatic assays were carried out on mitochondria pre-incubated for 15 min on ice with IO-SMPs at a ratio of 1:10 mg of IO-SMPs and mitochondria proteins respectively. All reaction assays were subjected to 5 min of pre-incubation at 37 °C with the subsequent addition of the Na_2ATP substrate to start the reaction. After 5 min, the reaction was stopped using 1 mL of an ice-cold aqueous solution of 15 % (*w/w*) trichloroacetic acid (TCA). At this point, the samples were centrifuged for 15 min at 3.500 rpm (Eppendorf Centrifuge 5202). The indirect determination of the F-ATPase activity was defined spectrophotometrically [30] by calculating the concentration of inorganic phosphate (P_i) hydrolyzed by known quantities of mitochondrial protein present in the supernatant. Therefore, before the start of the reaction, 1.0 μL of 3.0 mg/mL of oligomycin was added to the mixture, used in F-ATPase tests as it represents a specific inhibitor of F-ATPase capable of selectively blocking the F_O subunit, solubilized in dimethyl sulfoxide (DMSO). For each series of experiments, at the same time as the conditions being tested, the total ATPase activity was calculated by evaluating the P_i in control tubes containing 1.0 μL of DMSO per mL of the reaction system. In the experiments we conducted, a 3.0 μg/mL dose of oligomycin gave the greatest inhibition of F-ATPase [31]. In each experiment, F_1F_O-ATPase activity was obtained as the difference between hydrolyzed P_i in the presence of oligomycin and hydrolyzed P_i by total ATPase activity and expressed as μmol P_i·mg protein^{-1}·min^{-1}. The concentration of P_i hydrolyzed by known amounts of mitochondrial protein, which is an indirect measure of F-ATPase activity was evaluated spectrophotometrically according to Fiske and Subbarow [32].

2.7. Statistical analysis

Statistical analyses were performed by GraphPad Prism software. Analysis of variance followed by the Dunnet and Bonferroni test was applied with *P* values as described by the figure captions. The statistical method used is one-way ANOVA. *P*-value <0.05 was considered significant.

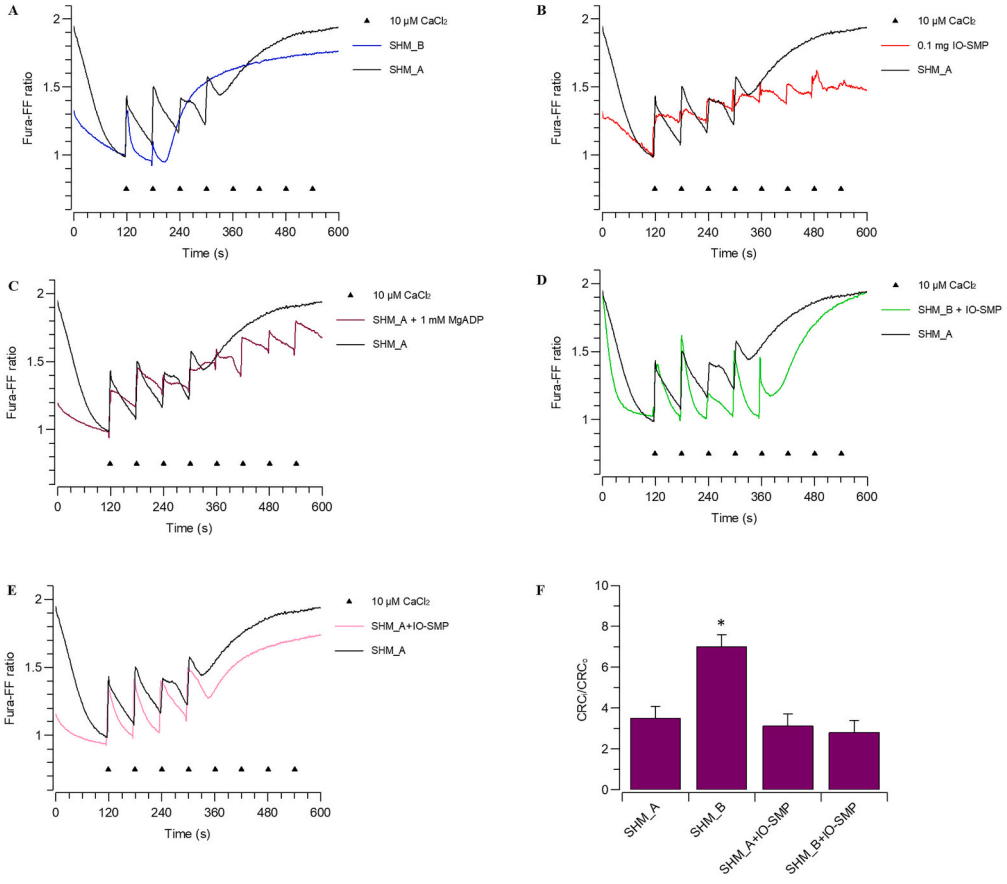

Fig. 1. Evaluation of mPTP opening. Representative curves (A,B,C,D,E) of the CRC expressed as the Fura-FF ratio (Fura-FF high Ca^{2+})/(Fura-FF low Ca^{2+}), evaluated in response to subsequent 10 μM $CaCl_2$ pulses (shown by the triangles), as detailed in Materials and methods, in the control condition (black line, SHM_A), and mitochondria isolated 3 h after explantation (blue line, SHM_B) (A), in IO-SMP (red line, 0.1 mg IO-SMP) (B), in presence of MgADP (purple line, SHM_A + 1 mM MgADP) (C), in SHM_B incubated with IO-SMP (green line, SHM_B + IO-SMP) (D), and in SHM_A incubated with IO-SMP (purple line, SHM_A + IO-SMP) (E). Quantitation of the mPTP is expressed as the ratio of the number of calcium pulses required to induce mPTP in MgADP-inhibited (CRC_i) and untreated (CRC_0) mitochondria in different conditions (F). Data represent the mean ± SD from three independent experiments carried out on three different mitochondrial preparations. Analysis of variance was performed by Bonferroni test among all groups. * Indicate significantly different values between treatment (* $P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. mPTP modulation by IO-SMPs

The study of the mPTP opening was performed by evaluating the levels of mitochondrial Ca^{2+}. the mPTP formation caused an uncontrolled release of mitochondrial calcium (calcium retention capacity, CRC) once the threshold value of mitochondrial calcium accumulation was reached [33]. Therefore, CRC decrease was induced by adding different pulses of 10 μM Ca^{2+} to evaluate the mPTP opening. CRC was significantly reduced in SHM_B (mitochondria obtained from heart tissue stored for 3 h at 4 °C after explantation) compared to SHM_A, mitochondria obtained immediately after heart explantation (Fig. 1A). mPTP opening evaluated on IO-SMPs (Fig. 1B) showed the same well-known mPTP profile depicted in the presence of MgADP inhibitor (Fig. 1C). Conversely, the effect of IO-SMPs incubated with SHM_B caused an increase in CRC on damaged mitochondria, improving the CRC ability as well as in SHM_A (Fig. 1D). Interesting, SHM_A in presence of IO-SMP did not chance the CRC activity (Fig. 1E). The mPTP opening desensitization to Ca^{2+} could be appreciated by the increase in CRC, as the increase in the ratio (Fura-FF high Ca^{2+})/(Fura-FF low Ca^{2+}), highlighted by more Ca^{2+} additions required to promote mPTP opening on SHM_B incubated with IO-SMPs. Indeed, mPTP formation expressed as the ratio of the number of Ca^{2+} pulses required to induce the mPTP in the presence (CRCi) and the absence (CRCo) of MgADP inhibitor, was doubled in the SHM_B, while no differences were showed between SHM_A, SHM_A + IO-SMP, and SHM_B + IO-SMP (Fig. 1F).

3.2. IO-SMPs effect on oxidative phosphorylation

To evaluate the protective effect induced by IO-SMPs on SHM_B we have carried out studies on the enzymatic activity of OXPHOS. We detected State 3 and State 4 of SHM_A, SHM_B, and SHM_B + IO-SMP energized with Glutamate+Malate (first phosphorylation site) or succinate (second phosphorylation site). The data showed significant differences for SHM_A in oxygen consumption in State 3 independently of the site of phosphorylation (Fig. 2A,D). Conversely, State 4 of SHM_A was doubled in respiratory activity only at the first phosphorylation site while no difference was recorded for the second phosphorylation site (Fig. 2B,E). As a result, the State 3/State4 ratio at the first site (Fig. 2B) or second site (Fig. 2D) of phosphorylation in the presence or the absence of IO-SMPs were the same, whereas SHM_A had a significantly high value (Fig. 2C,F).

3.3. IO-SMPs effect on F_1F_O-ATPase activity

Considering the hypothesis of mitochondrial F_1F_O-ATPase as the molecular structure that characterizes the mPTP, it was evaluated whether the ameliorative effect of IO-SMPs on mPTP inhibition was correlated with the activity of mitochondrial F_1F_O-ATPase in the presence of the natural cofactor Mg^{2+} or by Ca^{2+}, the latter responsible of mPTP formation. Mg^{2+}-dependent F_1F_O-ATPase assays were performed on SHM_B (Fig. 3A), IO-SMP (Fig. 3B), or SHM_B + IO-SMP (Fig. 3C) and evaluated the effects of 150 μM atractyloside (ATR), a known competitive inhibitor of the transporter of the mitochondrial nucleotide, or the action of uncoupler dinitrophenol (DNP) to evaluate the orientation of IO-SMPs. Indeed, IO-SMPs were refractory to ATR and stimulated by DNP. On SHM_B (Fig. 3A) and IO-SMP (Fig. 3B) DNP induced activation on Mg^{2+}-dependent F_1F_O-ATPase, whereas the inhibitory effect of ATR was detected on SHM_B but abolished on IO-SMP. Although on SHM_B treated with IO-SMs there were no significant effects induced by ATR or DNP (Fig. 3C), Mg^{2+}-dependent F_1F_O-ATPase activity on IO-SMP was higher than SHM_B or SHM_B + IO-SMP (Fig. 3D).

Ca^{2+}-dependent F_1F_O-ATPase activity on SHM_B was responsive to ATR inhibition but not stimulated by DNP (Fig. 4A). Contrariwise, opposite effect was detected on IO-SMP (Fig. 4B). Ca^{2+}-dependent F_1F_O-ATPase activity detected on SHM_B + IO-SMP only showed the inhibitory effect by ATR (Fig. 4C). In the same way, as Mg^{2+}-dependent F_1F_O-ATPase activity, Ca^{2+}-dependent F_1F_O-ATPase activity in IO-SMPs was greater than SHM_B. Moreover, SHM_B + IO-SMP did not show a synergic effect on Ca^{2+}-dependent F_1F_O-ATPase activity when SHM_B were treated with IO-SMP (Fig. 4D).

4. Discussion

Mitochondrial dysfunction plays a significant role in tissue degeneration and loss of function occurring in several pathophysiological conditions such as ageing, diabetes, neurodegenerative, and cardiovascular diseases. In the context of cardiovascular dysfunction, a primary form of damage to the myocardium following cardiac arrest is cell death mediated by mitochondria-dependent apoptosis happening during warm ischemia of tissues [34]. In response to ischemic insult, pro-

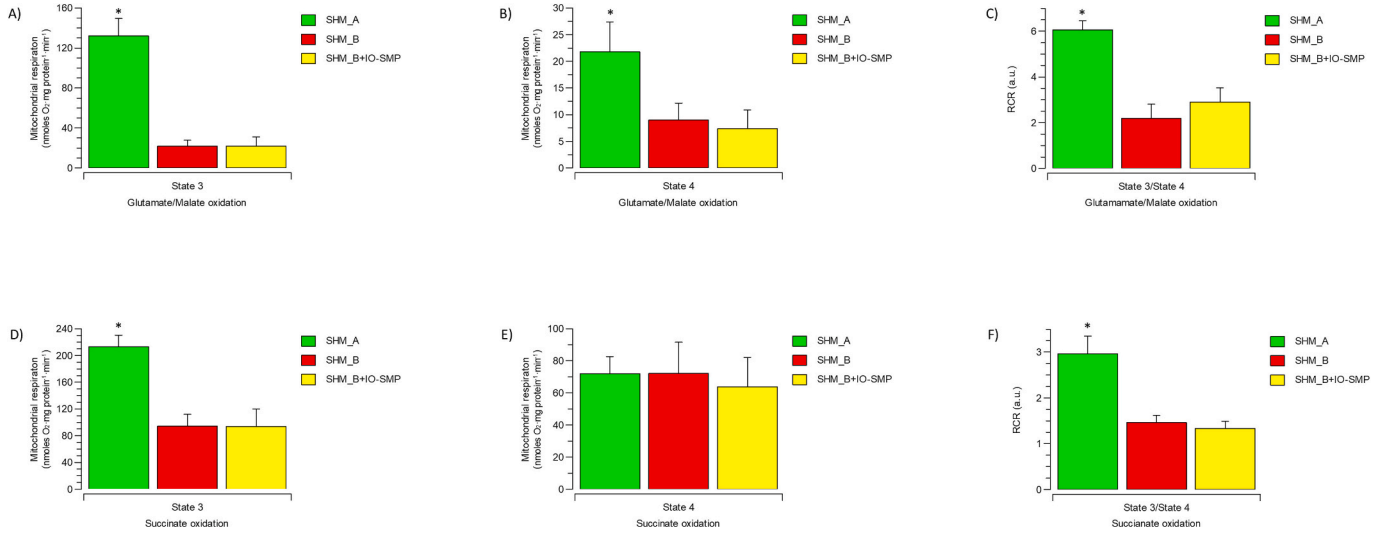

Fig. 2. Evaluation of oxidative phosphorylation function. IO-SMP effects on State 3, State 4, and State 3/State 4. Glutamate/Malate (A-C) and succinate (D-E) stimulated SHM_A (greenbar), SHM_B (red bar) SHM_B + IO-SMP (yellow bar). Data expressed as columns represent the mean ± SD from three independent experiments carried out on three different mitochondrial preparations. * Indicate significantly different (* $P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

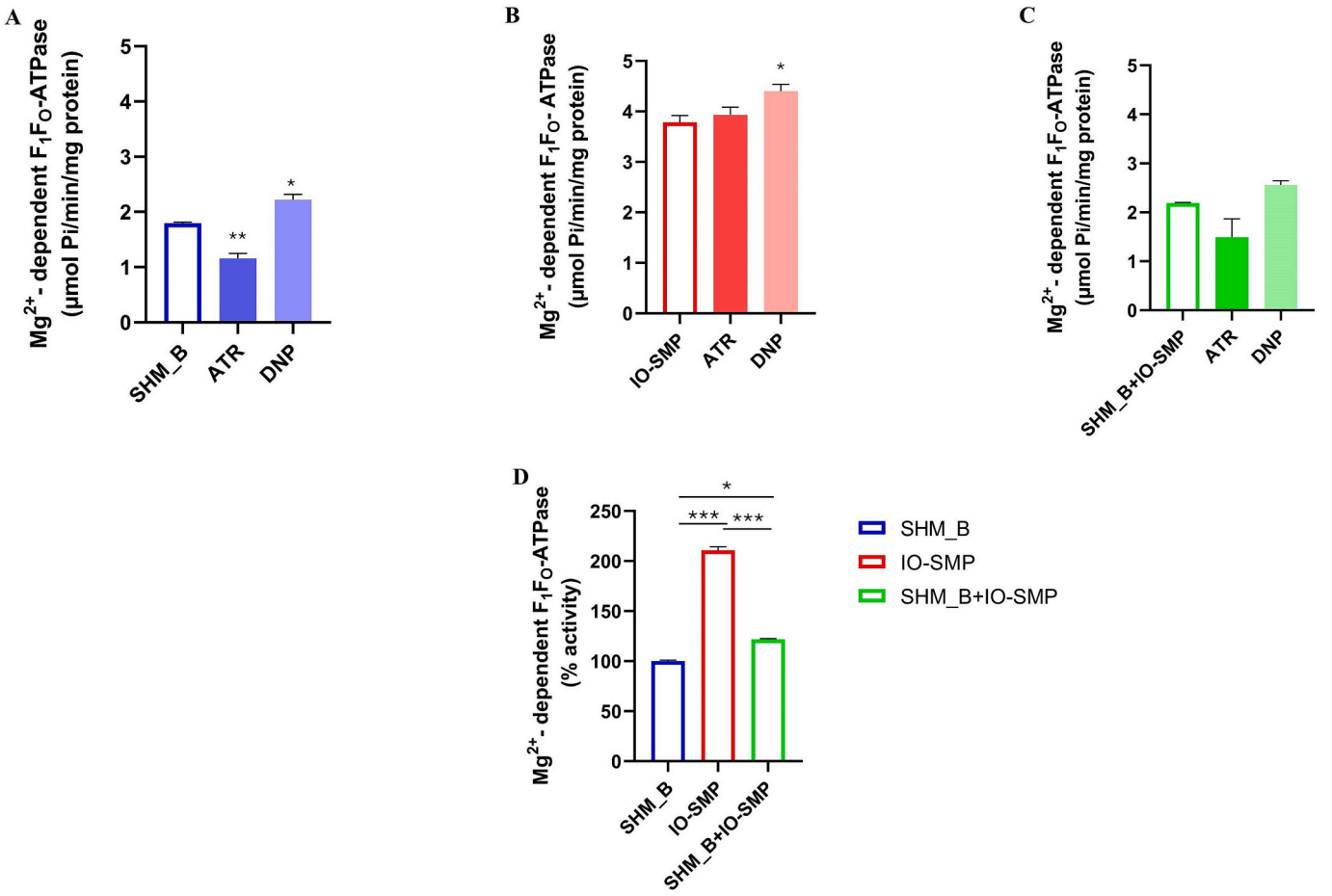

Fig. 3. Characterization and F-ATPase activities in freeze–thawed mitochondria and in IO-SMP. Mg^{2+}-activated F_1F_O-ATPase activity in SHM_B (A), IO-SMP (B), and SHM_B + IO-SMP (C) without (CTR) and with 150 μM ATR or 450 μM DNP. D) Comparison of Mg^{2+}-activated F_1F_O-ATPase activity reported as a percentage (%) in different groups. Data represent the mean ± SD from three independent experiments carried out on three different mitochondrial preparations. Statistical analysis was performed by Dunnet test on each group compared to the control (CTR) (A, B and C) and by Bonferroni test among all groups (D). * Indicate significantly different (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

apoptotic proteins from the intermembrane space of mitochondria are released to the cytosol [35]. The pathological process culminates in the activation of caspases and degradation of cellular components [36] causing cell death. Specifically, respiratory chain complexes can exploit an important role [37–39] to regulate the opening of the mPTP, the molecular event considered the "point of no return" [40–42]. Then, the cell is destined to die [35]. ROS are responsible for inducing mitochondrial dysfunctions and damage to macromolecules within the mitochondria, including DNA, proteins, and lipids [43–45]. ROS overproduction and Ca^{2+} overload in mitochondria are associated with cell death and tissue damage linked to mPTP formation [46,47]. In light of these considerations, the results obtained in this study may be highly valuable in preventing these detrimental phenomena. The molecular processes that might occur in our experimental model of SHM_B might depend on the long time interval between heart explantation and mitochondria isolation [35] triggering mitochondrial dysfunctions. The mPTP opening occurred earlier in SHM_B than in control SHM_A, which were isolated immediately after explantation and preventing mitochondrial insult. Although mPTP was not detectable in IO-SMPs, SHM_B interaction with IO-SMPs induced a protective effect. CRC capability increased leading to the phenomenon of mPTP opening to conditions comparable to the control. Indeed, it is interesting to assume that IO-SMPs might be able to accumulate calcium although IO-SMPs did not exhibit calcium-induced calcium release (Fig. 1B). The addition of IO-SMPs to SHM_B may cause a reduction of external Ca^{2+} concentration responsible to trigger mPTP. Accordingly, CRC could apparently increase. However, this effect can be excluded. CRC of SHM_A was not affected by the presence of IO-SMP (Fig. 1E). By assuming that IO-SMPs can reestablish the mPTP in the closed state, the IO-SMPs effect on CRC was not evident on healthy SHM_A but improved in impaired mitochondria, *i.e.* SHM_B.

Considering the lack of effect on OXPHOS and Mg^{2+}- or Ca^{2+}-dependent F_1F_O-ATPase activities, could be deduced that the action of IO-SMPs was not functional on the mitochondrial F_1F_O-ATPase. Moreover, State 3 and State 4 evaluated starting from complex I (glutamate/malate oxidation) or complex II (succinate oxidation) of the ETC did not undergo significant modulations. The OXPHOS activity was not affected by IO-SMPs although in turn were rich in enzymatic complexes of the ETC [22]. The functional characterization in terms of the hydrolytic activity of the F_1F_O-ATPase allowed us to provide more information on the IO-SMP. Indeed the results of Figs. 3 and 4 confirmed their correct "inside-out" folding. In detail, the lack of inhibition by ATR on the activity of the F_1F_O-ATPase in IO-SMP demonstrated that the catalytic domain of F_1 was facing the external side of the vesicles and was not dependent on ATR-sensitive adenine nucleotide transport inside the SMP [48]. The results were corroborated by the DNP that stimulated the ATP hydrolysis driving proton flux inside the vesicles of F_1F_O-ATPase dissipating the protonmotive force created by enzyme activity [25]. The only effect induced by IO-SMPs on mPTP opening was probably due to a structural action, induced by the integration of the particles in the mitochondria. It is also known that greater membrane permeability is associated with an increase in its fluidity [49,50], therefore we

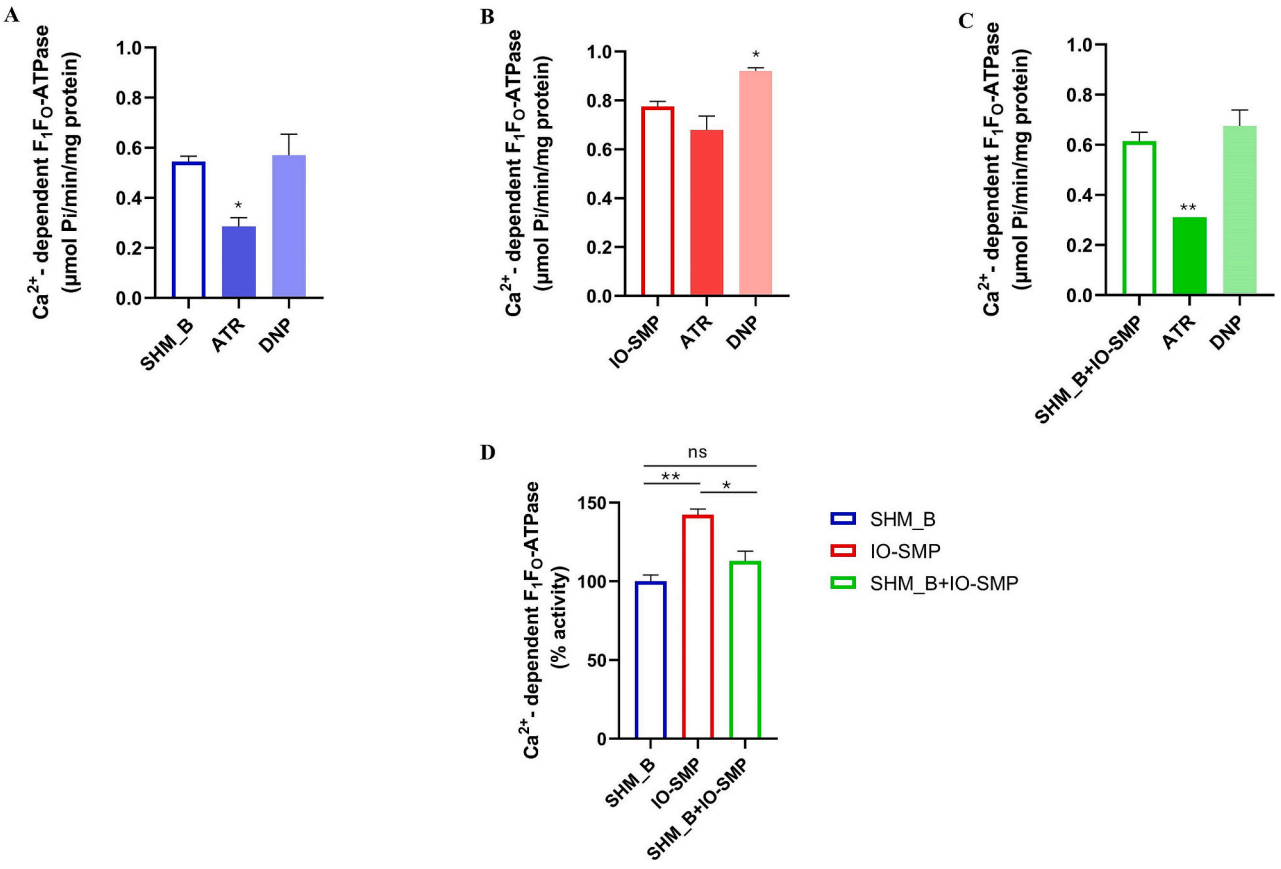

Fig. 4. Characterization and F-ATPase activities in freeze–thawed mitochondria and in IO-SMP. Ca^{2+}-activated F_1F_O-ATPase activity in SHM_B (A), IO-SMP (B), and SHM_B + IO-SMP (C) without (CTR) and with 150 µM ATR or 450 µM DNP. D) Comparison of Ca^{2+}-activated F_1F_O-ATPase activity was reported as a percentage (%) in different groups. Data represent the mean ± SD from three independent experiments carried out on three different mitochondrial preparations. Statistical analysis was performed by Dunnet test on each group compared to the control (CTR) (A, B and C) and by Bonferroni test among all groups (D). * Indicate significantly different (* $P < 0.05$; ** $P < 0.01$).

hypothesize that the co-incubation of IO-SMP led to integration into the membrane modulating the membrane-bound enzyme complexes functionality. Considering the hypothesis on the "bent-pull-twist" mechanism [51], the F_1F_O-ATPase would find itself in a surrounding environment that did not facilitate the flexibility necessary to carry out the conformational changes useful for mPTP opening [52]. In addition to this, the integration of lipids such as cardiolipin has a stabilizing role of membrane-embedded enzymes, such as cytochrome *c*. It is conceivable that the integration of IO-SMPs in the mitochondria could have a protective effect on the direct phenomenon of mPTP opening. Furthermore, IO-SMPs may contain structurally/functionally active components of the F_1F_O-ATPase to compensate for the impaired enzyme in recipient SHM_B mitochondria. We can assert that IO-SMPs mediated SHM_B structural changes on the bioarchitecture of the mPTP. However, these effects could have positive long-term repercussions on the molecular events leading to cell death. Consistently, the release of the pro-apoptotic factor cytochrome *c* could make it difficult for its stabilization in the membrane given by the interaction with the integrated cardiolipin or due to the rescue of mitochondrial swelling [53]. Although our hypotheses based on the current experimental data will certainly require further investigations to shed light on the specific molecular mechanisms underlying such evidence, these findings open new avenues to further research in the field of an innovative therapeutic approach to cardiovascular dysfunction.

In summary, in mitochondria that had suffered damage attributable to events triggering mPTP opening (SHM_B), treatment with IO-SMP strongly reduced the sensitivity to mPTP opening up to levels comparable to SHM_A. The characterization in terms of functionality, both by evaluating OXPHOS and F_1F_O-ATPase activity, allowed us to understand that IO-SMP did not affect the function of the mitochondrial enzymatic complexes under study. Specifically, Ca^{2+}-dependent F_1F_O-ATPase is known to be involved in the bioarchitecture of mPTP [9]. We therefore hypothesize that the desensitizing effect on mPTP opening by IO-SMPs was structural. No evident experimental data are now present to explain the possible mechanism of mPTP inhibition by IO-SMP. Probably the integration of the IO-SMPs into the SHM_B altered the membrane-bound enzyme complexes functionality, reducing the possibilities of F_1F_O-ATPase to carry out the conformational changes in favour of mPTP formation. Since mPTP opening is involved in a variety of pathophysiological events, the results obtained here should provide the basis and offer an interesting stimulus to further research to better clarify the molecular mechanisms underlying the protective effects of IO-SMPs. In particular, their possible use as modulators of the mPTP to protect the damage generated by this phenomenon in multiple pathological conditions could be discovered.

CRediT authorship contribution statement

Cristina Algieri: Writing – original draft, Investigation, Formal analysis. **Antonia Cugliari:** Formal analysis. **Patrycja Anna Glogowski:** Data curation. **Silvia Granata:** Resources. **Micaela Fabbri:** Methodology. **Fabiana Trombetti:** Writing – review & editing. **Maria Laura Bacci:** Funding acquisition. **Salvatore Nesci:** Writing – original draft, Visualization, Validation, Supervision, Funding acquisition, Conceptualization.

Funding sources

The research leading to these results received funding from the University of Bologna, Italy under Grant Agreement No Almaidea 2022 to MLB and SN.

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. Maria Laura Bacci reports financial support was provided by University of Bologna. 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.

Acknowledgements

Danilo Matteuzzi and Roberto Giusti (Department of Veterinary Medical Sciences, University of Bologna) are gratefully acknowledged for kindly conferring swine hearts from a local abattoir to Biochemistry laboratories.

Data availability

Data will be made available on request.

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