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Short title: ***Selenoprotein T and post-conditioning cardioprotection***

A selenoprotein T-derived peptide protects the heart against ischemia/reperfusion injury through inhibition of apoptosis and oxidative stress

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Abstract

Aim: Selenoprotein T (SelT or SELENOT) is a novel thioredoxin-like enzyme whose genetic ablation in mice results in early embryonic lethality. SelT exerts an essential cytoprotective action during development and after injury through its redox-active catalytic site. The present study aims to determine the expression and regulation of SelT in the mammalian heart in normal and pathological conditions, and to evaluate the cardioprotective effect of a SelT-derived peptide, SelT43-52(PSELT) encompassing the redox motif which is key to its function, against ischemia/reperfusion(I/R) injury.

Methods: We used the isolated Langendorff rat heart model, and different analyses by immunohistochemistry, western blot and ELISA.

Results: We found that SelT expression is very abundant in embryo but is undetectable in adult heart. However, SelT expression was tremendously increased after I/R. PSELT (5 nM) was able to induce pharmacological post-conditioning cardioprotection as evidenced by a significant recovery of contractility (dLVP) and reduction of infarct size (IS), without changes in cardiac contracture (LVEDP). In contrast, a control peptide lacking the redox site did not confer cardioprotection. Immunoblot analysis showed that PSELT-dependent cardioprotection is accompanied by a significant increase of phosphorylated Akt, Erk1/2 and Gsk3α-β, and a decrement of p38MAPK. PSELT inhibited the pro-apoptotic factors Bax, caspase 3 and cytochrome c, and stimulated the anti-apoptotic factor Bcl-2. Furthermore, PSELT significantly reduced several markers of I/R-induced oxidative and nitrosative stress.

Conclusion: These results unravel the role of SelT as a cardiac modulator and identify PSELT as an effective pharmacological post-conditioning agent able to protect the heart after ischemic injury.

Introduction

Selenium (Se) is an essential micronutrient, the benefits of which to human health as an antioxidant are widely recognized. Selenium deficiency has been implicated in a number of disorders, including infertility, increased cancer incidence, susceptibility to viral infection, mental development retardation and accelerated aging.[1] Accumulating evidence suggests that selenium is also important for optimal functioning of the cardiovascular system.[2]

The majority of Se beneficial effects are actually mediated by the biological activity of a particular class of proteins in which it is incorporated, the selenoproteins,[3] where the oligoelement is inserted as a selenocystein (Sec), the 21st natural amino acid.[4]

The genes encoding selenoproteins harbor, at their 3'-untranslated region (UTR), a specific hairpin motif, designated the Sec insertion sequence (SECIS), which is responsible for the recognition and decoding of the in frame UGA stop codon as a signal for the incorporation of the Sec residue.[4] In mammals, 25 selenoprotein-encoding genes have been identified[5] whose entire invalidation through Sec tRNA gene knockout leads to early embryonic lethality in rodents.[6] Likewise, mutations in human Sec tRNA or SECIS binding protein lead to complex disorders.[7]

Due to the presence of Sec in their catalytic site, selenoproteins exert important oxidoreductase activities and include several major enzymes, such as glutathione peroxidases (GPx), thioredoxin reductases (TrxR) and iodothyronine deiodinases which protect cells from oxidative stress and catalyze key redox reactions.[8] In addition to their role in detoxification of free radicals, diverse essential functions were attributed to selenoproteins, such as the implication of deiodinases in thyroid hormone metabolism,[9] the selenoproteins N, M or K in Ca^{2+} regulation[10] or TrxR in signal transduction and transcription regulation.[8] However, the role of many selenoproteins and their mechanisms of action are still elusive.

Several selenoproteins are highly expressed during development[11-13] and their loss has been associated with major anatomical alterations and functional defects in rodents.[14,15] In particular, selenoprotein T (SelT or SELENOT) is a key thioredoxin-like enzyme present in the endoplasmic reticulum,[16,17] which is essential at early stage of mouse development.[18,19] SelT is highly expressed in most embryonic organs, but declines gradually as the organs develop, to disappear in most adult tissues except the endocrine glands.[17,13,20] Targeted inactivation of SelT in pancreatic β-cells resulted in a reduction in pancreatic islet size and impaired glucose tolerance, thus indicating a possible defect in cell commitment to β-cell lineage. Similarly, SelT depletion in other neuroendocrine and endocrine cells provoked Ca^{2+} store depletion, ER stress, misfolded protein accumulation and hormone production and secretion defects.[16,17]

Although a large body of evidence has now accumulated showing that SelT affects the survival, differentiation and function of nervous and endocrine cells[16-20] via its redox activity, nothing is known about the expression and function of SelT in other vital tissues such as the heart, a tissue which is highly exposed to oxidative stress in particular after ischemia/reperfusion (I/R). The present study was undertaken to elucidate: *i*) the expression of SelT during rat heart ontogenesis and after *ex-vivo* induced ischemia in adult rat heart, *ii)* whether the exogenously administrated SelT-derived peptide SelT43-52 (PSELT), which encompasses the active redox site CXXSec is able to protect the heart in pharmacological post-conditioning after I/R damage, and *iii*) the underlying intracellular mechanisms activated by PSELT to confer cardioprotection.

Results

SelT expression during heart ontogenesis

We analyzed by immunofluorescence and western blot the cardiac expression of SelT at different stages of heart development and in the adult. For this, we used embryonic (E7), newborn (P14) and adult (3-month old) rat cardiac tissue. A strong SelT immunoreactivity was observed in the embryonic tissue, which co-localized with calsequestrin-2 (Fig. 1A, B), used as a marker of cardiac sarcoplasmic reticulum staining, indicating that cardiomyocytes express high levels of SelT during heart development (Fig. 1A, B). In newborn heart, only some areas of the cardiac tissue, mostly in the periphery was labeled for SelT whereas the remaining tissue exhibited only calsequestrin-2 staining (Fig. 1A, B), perhaps reflecting still ongoing postnatal differentiation of heart in this region. In the adult, cardiac tissue was devoid of SelT labeling (Fig. 1A, B), thus indicating that SelT expression could be linked to cardiac tissue development and remodeling, and is turned off in mature adult tissue. This ontogenetic pattern of SelT expression was confirmed by western blot analysis (Fig. 1C).

SelT expression during cardiac ischemia

To determine whether SelT expression could be induced in adult heart after injury, immunofluorescence histochemical analysis of cardiac tissue and western blot analysis of cardiac extracts were carried out in order to evaluate SelT levels after I/R compared to controls (Fig. 2). Immunofluorescence analysis revealed that I/R triggers a tremendous increase in SelT immunoreactivity in cardiac tissue (Fig. 2A). In contrast, no change was observed for calsequestrin-2 in this condition (Fig. 2A). In accordance, western blot analysis showed that SelT is not expressed in control hearts as seen by immunohistochemistry. However, I/R induced a large increase in SelT expression compared to control (Fig. 2B).

PSELT effect on post-ischemic cardiac function

In basal condition after 40 min of equilibration, the cardiac parameters for all groups were: dLVP=72±2 mmHg; LVEDP=5–8 mmHg; CP=68±7 mmHg; HR=220±11 mmHg. Endurance and stability of the preparations were assessed by measuring the performance variables every 10 min. These parameters were stable up to 190 min.

The possibility that PSELT elicits cardioprotection was investigated by comparing the effects induced by the I/R maneuver with those elicited by the peptide administered after I/R for post-conditioning (PostC). The effects of PSELT on both systolic and diastolic functions were analyzed at EC_{50} dose of 5 nM obtained by preliminary dose-response curves (data not shown). Systolic function was evaluated by measuring the developed left ventricular pressure (dLVP), an index of inotropic activity. A limited contractility recovery was observed in the I/R group (dLVP at the end of reperfusion: 27±4 mmHg; baseline values: 75±2 mmHg) (Fig. 3A). PSELT (5 nM) induced an important improvement of dLVP recovery during reperfusion (dLVP at the end of reperfusion: 56± 6 mmHg; baseline values: 74±4 mmHg) (Fig. 3A). The inert peptide without Sec had no effect, indicating that PSELT exerts a selective action via an intact CXXSec site. It is known that the contracture, evaluated as LVEDP is an index of diastolic function. It has been reported in the rat heart that 4 mmHg or more above the baseline level of LVEDP indicates an important cardiac

damage.[21] Our results showed that in I/R group, LVEDP markedly increased compared to baseline (4±4 mmHg at baseline; 24±4 mmHg at the end of reperfusion) (Fig. 3B). A similar effect was observed in the presence of the inert PSELT (5 ± 4 mmHg at baseline; 20±2 mmHg at the end of reperfusion). In contrast, PSELT (5 nM) added during reperfusion abolished contracture, LVEDP being 9±2 mmHg at the end of reperfusion (Fig. 3B), indicating that this peptide significantly reduces heart damage after I/R. The IS was also evaluated and was expressed as a percentage of LV mass. The IS was 75±3% after I/R, which was strongly reduced (38±1%) in the presence of PSELT at 5 nM (Fig. 3C). The inert PSELT yielded an IS of 59±9% (Fig. 3C).

PSELT affects cardioprotective pathways

The mechanism of action underlying the cardioprotective effect of PSELT (5 nM) was studied by using selective inhibitors of intracellular pathways involved in cardioprotection, such as MDL12,330A, a specific inhibitor of adenylate cyclase, wortmannin, a specific inhibitor of PI3K, PD098059, a selective inhibitor of Erk-1/2, and 5-hydroxy-decanoate, a specific inhibitor of mitoKATP-channels. The Infarct Size in the presence of selective inhibitors was compared with I/R and PSELT 5 nM groups showed in Fig. 3C. The results indicated that PSELT-dependent reduction of IS was abolished in hearts co-treated with the above inhibitors since the IS was 68±2% in the presence of MDL12,330A; 83±3% in the presence of wortmannin; 73±3% in the presence of PD098059; and 67±10% in the presence of 5-hydroxy-decanoate (Fig. 4A). A similar trend was observed in the presence of the inhibitors for PSELT-dependent improved recovery of dLVP and contracture (LVEDP) (data not shown).

The involvement of the kinases Akt (*protein kinase B*), Erk-1/2 (*extracellular signal–regulated kinases 1/2*), p38MAPK (*mitogen-activated protein kinase*) and GSK3β (*glycogen synthase kinase 3-beta*) in the PSELT-induced cardioprotection was evaluated by western blot analysis of the phosphorylated and not phosphorylated forms of these enzymes. Representative bands and densitometric analyses for the different kinases are shown in Fig. 4. In homogenates from hearts exposed to 5 nM PSELT at the reperfusion, the levels of phosphorylated Akt (Fig. 4B), Erk-1/2 (Fig. 4C) and GSK3α-β (Fig. 4D) were higher, while those of phosphorylated p-38MAPK (Fig. 4E) were lower as compared to I/R.

PSELT influence on apoptotic indexes

The effect of PSELT (5 nM) administration at the reperfusion on the expression of the pro- and anti-apoptotic proteins, Bax, Bcl-2, active caspase3 and Cyt c are reported in Fig. 5. The peptide PSELT induced a significant reduction of the expression of the pro-apoptotic factors Bax (Fig. 5A), active caspase 3 (Fig. 5B) and Cyt c (Fig. 5C), and a significant increase of expression of the anti-apoptotic factor Bcl-2 (Fig., 5D) compared to I/R.

As shown in Fig. 5E, mitochondria exhibited a significant decrease of Cyt c expression in I/R group compared to the Sham group, while a significant recovery was observed in the mitochondria of PSELT group. In accordance, PSELT treatment caused a significant reduction of Cyt c in the cytosol, compared to I/R group.

PSELT influence on cellular redox balance control

The control of the redox balance by PSELT was evaluated after I/R by analyzing 3-nitrotyrosine by immunohistochemistry, by measuring intracardiac ROS production by ELISA assay and by assessing the expression of specific markers involved in the production of free radicals, such as XO and AOX-1. As shown in Fig. 6A, I/R induced a burst of 3-nitrotyrosine, an indicator of nitrosative stress, which was reversed in the presence of 5 nM PSELT. Similary, I/R provoked an increase in intracardiac ROS production, which was significantly reduced in the presence of 5 nM PSELT (Fig. 6B). In addition, XO and AOX-1 expression was significantly increased under I/R as compared to Sham, while post-conditioning with 5 nM PSELT abolished the expression of these oxidative stress markers (Fig. 6C, D).

Discussion

In the present work, we described for the first time the ontogenetic expression of SelT in the rat heart. We observed that the protein is highly expressed in the embryonic heart, but decreases in newborn heart, and is absent in adult heart. Interestingly, the adult rat heart exposed to I/R injury re-expressed SelT, thus arguing for a role of SelT in adult heart under pathological conditions. We showed that administration of a SelT-derived peptide (PSELT) containing the active CysValSerSec motif to the isolated and Langendorff-perfused adult heart after ischemia, elicited a strong cardioprotective effect. PSELT-dependent cardioprotection is accompanied by activation of the RISK pathway, inhibition of the apoptotic pathway, counteraction of intracellular ROS increase, and regulation of oxidative and nitrosative stress markers. These observations indicate that SelT is induced in adult heart after I/R likely to protect the cardiac tissue from harmful free radicals, and that a short peptide carrying the active catalytic site of SelT is able to protect the heart against I/R-induced oxidative stress and apoptosis.

Cardioexpression of SelT during ontogenesis and ischemic condition

Selenoproteins are increasingly recognized as essential for the development and function of nervous, endocrine and metabolic tissues.[11-13] Alteration of their synthesis is associated with major disorders, including muscular dystrophy, diabetes and thyroid disease.[22] A first interesting finding of our study is that SelT is very abundant in calsequestrin-positive cardiomyocytes during embryonic ontogenesis of rat heart, while its expression was reduced in newborn and was undetectable in the adult heart. It has been shown that proliferative growth of the myocytes for cardiac development is observed during the entire embryonic and fetal period until birth and that myocytes are in principle only able to increase their volume after birth[23] suggesting that SelT is required during early hyperplastic growth of cardiomyocytes. The ontogenetic expression pattern of SelT in heart is similar to that observed in the brain and other organs, such as kidney, liver, lung, skeletal muscle and adrenal gland. In fact, SelT expression is drastically reduced after cell differentiation in most tissues, as observed in chromaffin cells and Purkinje cells.[13,18,17] In contrast, its expression is maintained in several adult endocrine tissues, such as pituitary, thyroid or testis and is highly induced in metabolically active proliferating cells and in cells that are endowed with some plasticity and regenerative capacity.[13,20] Together, these observations suggest that SelT expression is associated with cell plasticity, a process which requires increased metabolism and energy but which generates free radicals. Our recent studies performed in neuronal cells showed that SelT is required for oxidative stress tolerance and that its biosynthesis is coupled to mitochondrial function during

differentiation.[19] Therefore, SelT expression during cardiac tissue ontogenesis is likely involved in cardiomyocyte protection and differentiation. However, in the maturing and adult heart, SelT expression becomes dispensable unless it is submitted to a noxious condition like I/R which triggers its re-expression in cardiomyocytes. This finding is reminiscent of the elevated expression of several selenoproteins in the nervous system (i.e. SelN, SelW, SelT and SelP and GPx) under stressful conditions, which is associated with protection against oxidative stress.[11,24-26]

Cardioprotective effect of PSELT

It has been reported that enzymes belonging to the selenoprotein family, e.g. GPx and TrxR in conjunction with Trx play a cardioprotective role after an ischemic injury.[27] For instance, GPx1 inhibits I/R-induced apoptosis of cardiac myocytes in mice,[28] and its deletion causes heart and vascular dysfunction.[29] The TrxR/Trx system exerts a protective effect against I/R injury by reducing infarct size and improving ventricular function recovery.[30] Furthermore, Nakamura[31] showed that, in patients subjected to bypass surgery, Trx inactivation was deleterious in I/R injury. To date, there are no indications about the role exerted by endogenous or exogenous SelT in cardioprotection. In the present study, we found that pharmacological post-conditioning with PSELT which encompasses SelT active site protects the Langendorff perfused rat hearts exposed to I/R injury. We found that, compared to the IS detected in hearts exposed to I/R (~75%), hearts post-conditioned with PSELT showed a remarkably reduced IS (~40%). IS reduction correlates with systolic function recovery and with the absence of contracture development. These effects raise the question of the site of action of PSELT. Although no definitive answer can be provided at this point for cardiomyocytes, we have recently shown using neuroblastoma cells that a fluorescent PSELT crosses the plasma membrane and may thus act intracellularly (manuscript in preparation). Whether PSELT acts at the level of the sarcoplasmic reticulum where SelT is localized remains to be determined.

Of note, PSELT cardioprotection is abolished when a control peptide where the Sec residue was replaced by a Ser, was administrated in post-ischemic condition, highlighting the essential role of Sec for the cardioprotective effect.

PSELT triggers various intracellular signaling mechanisms to provide cardioprotection

It is known that post-conditioning protection involves components of the RISK cascade, such as PI3K-Akt and Erk-1/2, and requires mitoKATP-channel opening.[32] We here evaluated whether these intracellular mediators are involved in PSELT protective effects on the ischemic-reperfused heart. We found that PSELT protection was abolished by exposing the hearts to specific inhibitors of adenylate cyclase, PI3K, Erk-1/2 and mitoKATP-channels, suggesting the involvement of these pathways. Consistent with these results, we observed an increased phosphorylation of Akt, Erk-1/2, and GSK3α-β after reperfusion with PSELT. Our data suggest that PSELT-induced protection takes place if the pathways involving adenylate cyclase, PI3K and Erk-1/2, as well as mitoKATP-channels are simultaneously activated during early reperfusion. Accordingly, mitochondria appear as the terminal effector of PSELT-induced pharmacological post-conditioning protection. In addition, a decrease in p-38MAPK phosphorylation was observed. This kinase is known for its role in myocyte apoptosis.[33] In cardiomyocytes, activation of p-38MAPK results in a rapid onset of lethal cardiomyopathy associated to cardiomyocyte hypertrophy.[34] Conversely, p-38MAPK inhibition may reduce cardiac hypertrophy, inhibit apoptosis and prevent the progression of heart failure,[34] suggesting that PSELT could exert similar effects as seen in the present study for its anti-apoptotic action which is involved in the cardioprotective effect. It is

known that changes in anti- and pro-apoptotic protein ratios result in inhibition or promotion of cell death.[35] For instance, the Bcl-2 family, which includes anti- and pro-apoptotic mediators of proteins is a key regulator of apoptosis. Blc-2 prevents Cyt c release and caspase activation, while Bax promotes these processes.[35] Activated caspase-3 is one of the main apoptosis mediators that acts by cleaving other caspases and the anti-apoptotic Bcl-2.[36] In agreement with the pharmacological post-conditioning protection induced by PSELT, we observed indeed that exposure of the ischemic heart to the peptide during the early reperfusion increased Bcl-2 on the one hand and decreased Bax, activated caspase-3 and cytosolic release of Cyt c on the other hand.

PSELT inhibits oxidative stress

In the ischemic heart, a notable increase in mitochondrial superoxide radical anion and hydrogen peroxide production leads to cell damage during reperfusion.[37] The contribution of ROS to cardiomyocyte cell death and apoptosis, typical of I/R injury, is well established.[37] To maintain a redox balance, cells engage several reducing enzymes including members of the selenoprotein family, such as GPx or TrxR, which play instrumental roles in cell survival and homeostasis.[38] These enzymes exert antioxidant activities that impact diverse cellular functions including redox equilibrium, protein folding and Ca^{2+} homeostasis.[38] On the basis of these observations, we investigated the influence of PSELT on the myocardial redox balance by analyzing the intracardiac oxidative and nitrosative stress[39] and by evaluating enzymes and factors involved in free radical production such as XO[40] and AOX-1.[41] Interestingly, compared to I/R condition, hearts perfused with PSELT after ischemia showed a significant reduction of all the oxidative/nitrosative markers used. It is known that XO is important in ischemic conditions. In fact, ATP depletion and the subsequent loss of membrane Ca^{2+} gradient increases Ca^{2+} levels and activates Ca^{2+}-dependent proteases which cause selective proteolysis of the dehydrogenase into XO. This in turn acts on both hypoxanthine and xanthine at the expense of molecular oxygen to produce superoxide ion.[40] Accordingly, in the ischemic heart, as well as in myocardial infarction, XO may importantly contribute to free radical-mediated damage.[40] AOX-1 is a member of the molybdo-flavoenzyme family of proteins, which catalyzes the oxidation of a variety of aldehydes, leading to the production of hydrogen peroxide. Under certain conditions, AOX1 can catalyze the formation of the superoxide free radical, and this suggests its involvement in the I/R heart damage.[41] It is of interest that exposure of the reperfused heart to PSELT is accompanied by a decrease of XO and AOX-1. These results agree with those reported by Boukhzar et al. which showed that SelT silencing affects oxidative/nitrosative stress and survival of dopaminergic neurons.[18] In fact, thanks to its thioredoxin-like motif, SelT was effective in catalyzing the reduction of oxidized substrates.[18] Our findings on the effect of PSELT on components of the redox state in the ischemic heart suggests that SelT is a novel essential effector of the intracardiac antioxidant system able to counteract free radical damage responsible for cell death during I/R.

Conclusion

This is the first study describing the expression of SelT during heart development and after I/R, and showing that its thioredoxin-like activity modelled by PSELT is cardioprotective. By decreasing infarct size and improving post-ischemic cardiac function through the control of various signalling effectors of apoptosis and oxidative stress, PSELT counteracted the damages induced by myocardial reperfusion. This protective action required the activation of

prosurvival kinases and the mitoKATP channel. Our observations on the beneficial effects of PSELT, a peptide which encompasses the active redox site CysValSerSec of SelT, here reported for the first time, provide new information on the biological significance of this protein for cardiac function and protection. They also pave the way for future studies aimed to investigate the possible clinical relevance of PSELT, which might represent a new class of drugs to be tested for reducing cardiac I/R injury. This is important in a medical context since it could allow the development of new adjunctive therapies to be coupled with the reperfusion to reduce morbidity and mortality.[48]

Material and Methods

Animals

Male Wistar rats (Harlan Laboratories Srl, Udine, Italy), weighing 250-300 g, were housed (three per cage) in a ventilated cage rack system under standard conditions. The animals had access to food and water *ad libitum*. The investigation conforms to Italian law (DL. 26/14) and to the Guide for the Care and Use of Laboratory Animals, according to National Institutes of Health (2011). The project was approved by the Italian Ministry of Health, Rome, and by the Ethics Review Board of the University of Calabria.

Peptides and drugs

The SelT-derived peptide 43-52 (PSELT) corresponding to the sequence FQICVSUGYR in its reduced form, and an inactive peptide without Sec used as a control, were chemically synthesized with the method of solid phase on a Fmoc resin as previously described[42] using an Applied Biosystems model 433A peptide synthesizer (AB Sciex, Courtaboeuf, France). MDL-12,330A (MDL), a specific inhibitor of adenylate cyclase, wortmannin (WT), a potent phosphatidylinositol 3-kinase (PI3K) inhibitor, PD-98059 (PD), a specific inhibitor of Erk-1/2 and 5-hydroxydecanoate (5HD), a mitoKATP channel blocker, were purchased from Sigma Aldrich. All drug-containing solutions were freshly prepared just before the experiments.

Isolated heart perfusion

Rats were heparinized (2.500 U i.m.) and anesthetized with ethyl carbamate (2 g/kg rat, i.p.) 10 min later. Hearts were rapidly excised and transferred in ice-cold buffered Krebs-Henseleit solution (KHs) and weighed. The performance of the rat heart was evaluated according to the Langendorff technique. The aorta was immediately cannulated with a glass cannula and connected with the Langendorff apparatus to start the retrograde perfusion[43] at a constant flow-rate of 12 mL/min with oxygenated KHs, containing 4.7 mM KCl, 113 mM NaCl, 25 mM NaHCO3, 1.8 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 1.1 mM mannitol, 11 mM glucose, 5 mM Na-pyruvate (Sigma Aldrich) (pH 7.4; 37 °C; 95% O2 and 5% CO2). The perfusion pressure was set to 100 mmHg and kept constant throughout the experiments. The hearts were kept in a temperature-controlled chamber (37 °C). To avoid fluid accumulation the apex of the left ventricle (LV) was pierced. A water-filled latex balloon, connected to a pressure transducer (BLPR; WRI, Inc., Sarasota, FL, USA), was inserted into the left ventricle through the mitral valve, to allow isovolumic contractions and to

continuously record cardiac mechanical parameters. Another pressure transducer was located above the aorta to measure coronary pressure (CP). The developed left ventricular pressure (dLVP, an index of contractile activity) and the left ventricular end-diastolic pressure (LVEDP, an index of contracture) were measured to evaluate inotropism.[44] The endurance of the preparations was stable up to 190 min. The performance variables were measured every 10 min. Parameters were recorded by using the PowerLab data acquisition system (AD Instruments, Oxford, UK) as previously reported.[44]

Experimental protocols

Ischemia/Reperfusion (I/R) studies: each heart was stabilized for 40 min during which the baseline parameters were recorded. After stabilization, hearts were randomly assigned to one of the groups described below and then subjected to 30 min of global, no-flow ischemia followed by 120 min of reperfusion (I/R). The concentration of PSELT (5 nM), corresponding to the EC_{50} dose, was chosen on the basis of preliminary dose-response curves (data not shown).

Experimental groups

1. In the first group (**Sham group**), hearts were stabilized and perfused for 190 min.
2. In the second group (**I/R group**), hearts were stabilized and subjected to I/R protocol.
3. In the third group (**PSELT 5 nM group**), PSELT at the EC_{50} dose (**5 nM**) was infused for 20 min at the beginning of 120 min of reperfusion.
4. In the fourth group (**Inert PSELT group**), inert PSELT, at the same concentration of PSELT EC_{50} **(5 nM)** was infused for 20 min at the beginning of 120 min of reperfusion.
5. In the groups 5-8 (**PSELT + inhibitors groups**) hearts were perfused with PSELT 5 nM plus one of the following inhibitors: MDL (100 nM), WT (100 nM), PD (10 nM) or 5HD (10 µM); perfusion with each inhibitor was started 5 min before ischemia (inhibitor alone) and during the early 20 min of reperfusion in the presence of PSELT 5 nM.

In all experiments, the inhibitor concentration was selected on the basis of previous reports.[45] Previous data showed that in the hearts perfused with inhibitors alone, the dLVP recovery, the LVEDP and the infarct size were similar to I/R group (data not shown).

Assessment of myocardial injury

To measure the infarct area, hearts were rapidly removed from the perfusion apparatus at the end of reperfusion. The left ventricles were dissected transversely into 2-3 mm slices. After 20 min of incubation at 37°C in 0.1% nitro blue tetrazolium in phosphate buffer (59.8 mM NaH_2PO_4, 484.9 mM Na_2HPO_4, pH: 7.4), unstained necrotic tissues were carefully separated from stained viable tissues by an independent observer who was not aware of the nature of the intervention. The weights of the necrotic and non-necrotic tissues were then determined, and the necrotic mass was expressed as a percentage of total left ventricular mass (% IS/LV), including septum.[46]

Western blot

Apex of cardiac ventricles were homogenized in ice-cold RIPA lysis buffer (Sigma-Aldrich) containing a mixture of protease inhibitors (1 mM aprotinin, 20 mM phenylmethylsulfonyl fluoride, and 200 mM sodium orthovanadate). Then homogenates were centrifuged at 15000 x g for 20 min at 4 °C for debris removal. Protein concentration was determined using a Bradford reagent according to the manufacturer's procedure (Sigma-Aldrich). Equal amounts of proteins (30 µg) were separated on 12% SDS-PAGE gels [(for SelT, β-tubulin, Bax, Bcl-2, Active Caspase 3, cytochrome c, (Cyt c), and cytochrome oxidase subunit IV, (COX-4)] or on 10% SDS-PAGE gels (for p-Akt, Akt, p-Erk-1/2, Erk1/2, p-GSK3α/β, GSK3α/β, p-p38MAPK, and p38MAPK), or on 8% SDS-PAGE gels [(for xanthine oxidase, (XO) and aldehyde oxidase-1, (AOX-1)], subjected to electrophoresis and transferred to polyvinyl difluoride membranes. The membranes were blocked with non-fat dried milk, and incubated overnight at 4 °C with different antibodies including polyclonal rabbit antibodies against SelT (Acris antibodies, USA), p-Akt, Akt, GSK3α/β, Erk-1/2, monoclonal rabbit against GAPDH, monoclonal mouse antibodies against p-Erk1/2, AOX-1, Bax, Bcl-2, Cyt c, COX-IV, polyclonal goat antibody against XO and β-tubulin (Santa Cruz Biotechnology, USA), Active Caspase 3, p-GSK3α/β (Sigma Aldrich), p-p38MAPK, p38MAPK (Cell Signaling Technology, USA), diluted 1:1000 in Tris-buffered saline and 0.2% Tween 20 containing 5% non-fat dry milk (TBSTM). Antibodies against Akt, Erk-1/2, p38MAPK, GSK3α/β, β-tubulin, GAPDH and COX-IV were used as loading controls. Anti-rabbit and anti-mouse peroxidase-linked secondary antibodies (Santa Cruz Biotechnology, California, USA) were diluted 1:2000 in TBSTM. Immunodetection was performed using the ECL PLUS enhanced chemiluminescence kit (Amersham). Autoradiographs were obtained by membrane exposure to X-ray films (Hyperfilm ECL, Amersham). Immunoblots were digitalized; densitometric analyses of the bands were performed evaluating the areas and the pixel intensity represented by 256 Gray values (0=white; 256=black) and the background was subtracted. The analyses were carried out using NIH IMAGE 1.6 (National Institutes of Health, Bethesda, Maryland).

Enzyme-linked immunosorbent assay (ELISA)

Detection of reactive oxygen species (ROS) in heart samples was performed by ELISA using a commercial kit (ROS, Sunred Biological Technology, Shanghai, China) as follows: the left ventricles (n=3 for each group) of Sham, I/R or with PSELT (5 nM) groups were homogenized using Ultra-Turrax® in phosphate buffered saline, PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4; pH 7.4) plus a mixture of protease inhibitors (1 mM aprotinin, 20 mM phenylmethylsulfonyl fluoride, and 200 mM sodium orthovanadate) and centrifuged at 15 000 x g for 20 min (4 °C). The supernatants were then assayed with the ELISA kit.

Mitochondrial isolation

Mitochondria were isolated from the ventricles as previously described.[47] At the end of perfusion, ventricle samples of Sham, I/R and PSELT 5 nM groups were harvested and homogenized in mitochondrial isolation buffer [(IBc): 0.1 M Tris-MOPS, 0.1 M EGTA-Tris and 1 M sucrose, pH 7.4]. The homogenates were centrifuged at 2000 x g for 10 min at 4 °C and the supernatants were collected and centrifuged again at 5000 x g for 10 min at 4 °C. The sedimented

mitochondrial pellets were washed twice and resuspended in 50 µL of IBc buffer. The 5000 x g supernatant represented the cytosolic fraction. All manipulations were carried out at 4 °C. To confirm the presence of mitochondria in the pellets, the monoclonal mouse antibody against COX-IV was used as mitochondrial loading control.

Immunofluorescence

For immunohistochemistry, rats were anesthetized with sodium pentobarbital (120 mg/kg; Ceva Santé Animale, Libourne, France) and heparinized and perfused through an intracardiac cannula with 0.9% NaCl in 0.1 M phosphate buffer (pH 7.4), followed by 4% paraformaldehyde (PFA) in PBS. Hearts were excised and post-fixed in the same fixative at 4°C, which was changed to PBS azide after 24 h. Tissues were sectioned into 50-µm or 10-µm slices with a vibratome. The sections were incubated with 1% donkey serum diluted in 1% bovine serum albumin (BSA) and 0.3% Triton X-100 in PBS for 2 h at room temperature, and then exposed overnight at 4 °C to primary antibodies against SelT[16] diluted 1:200, anti-nitrotyrosine (NT) used as a marker of nitrosative stress (Merck Millipore, France) diluted 1:200 and calsequestrin-2, used as a marker of cardiac sarcoplasmic reticulum staining (Santa Cruz Biotechnology, USA) diluted 1:200. Immunostaining was visualized using Alexa Fluor 488 or 594-conjugated secondary antibodies diluted 1:200 (Invitrogen, France). Counterstaining with 1 µg/mL 4,6- diamino-2-phenylindole (DAPI, Sigma-Aldrich) in PBS for 1 min was performed prior to mounting the slides with PBS/glycerol 50/50. Samples were analyzed with a Leica SP2 confocal laser scanning microscope (DMRAX- UV) equipped with the Acousto-Optical Beam Splitter system (Leica Microsystems, France). The microscopic observations were made on The Cell imaging platform PRIMACEN (www.primacen-crihan.fr).

Statistics

All data were expressed as mean ± SEM. One-way ANOVA, non-parametric Newman-Keuls multiple comparison test (for post-ANOVA comparisons) and t-test were used for western blot and ELISA analyses. Differences at $*p =<0.05$, $**p =<0.01$, $***p =<0.001$ were considered statistically significant. Two-way ANOVA, non-parametric Bonferroni's multiple comparison test (for post-ANOVA comparisons) was used for the time course of hemodynamic analysis. The statistical analyses were carried out using Graphpad Prism5.

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Figure legends

Fig. 1 SelT expression during heart ontogenesis.

(A) SelT and calsequestrin-2 immunoreactivity in the embryo, newborn and adult rat hearts. Anti- calsequestrin-2 was used as a marker of cardiac sarcoplasmic reticulum. Nuclei were stained in blue by DAPI. **(B)** Larger views of the images shown in **(A)**. **(C)** Western blot analysis of embryo, newborn and adult rat heart tissues (n=3 hearts/group) with SelT antibody. Histograms represent the ratio of densitometric analysis of protein/loading control: $p<0.05$ (*), $p<0.01$ (**), by One-Way ANOVA/Newman-Keuls Multiple Comparison Test.

Fig. 2 SelT expression in the rat heart subjected to ischemia.

(A) Immunoreactivity of SelT and calsequestrin-2 in Sham and I/R-treated rat hearts (n=3 hearts/group). Nuclei are stained in blue by DAPI. **(B)** Western blot analysis of SelT in hearts (n=3 hearts/group) from Sham and I/R groups. Histograms represent the ratio of densitometric analysis of protein/loading control: $p<0.05$ (*) by t-test.

Fig. 3 Systolic and diastolic function and infarct size.

(A) dLVP and **(B)** LVEDP variations. Data are expressed as changes of dLVP and LVEDP values (mmHg) from the stabilization to the end of the 120-min of reperfusion with respect to the baseline values for I/R (n=5) or I/R in the presence of PSELT 5 nM (n=5) and Inert PSELT (n=4). Grey boxes indicate the ischemic period (Bonferroni Multiple Comparison test, dLVP=40.21% of total variation between groups ($p <0.001$); LVEDP=44.05 % of total variation between groups ($p <0.001$). Inset graph shows the dLVP and LVEDP at the end of reperfusion (One-way ANOVA/Newman-Keuls Multiple Comparison Test, *=$p <0.05$; **=$p<0.01$). **(C)** Infarct size (n=5 hearts for I/R, PSELT 5 nM, n=4 hearts for Inert PSELT groups). The amount of necrotic tissue measured after 30-min global ischemia and 120-min reperfusion is expressed as percent of the left ventricle mass (LV) (% IS/LV). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), by One-Way ANOVA/Newman-Keuls Multiple Comparison Test.

Fig. 4 Mechanism of action of PSELT in cardioprotection.

(A) Infarct size for PSELT+inhibitor groups (MDL; WT; PD; 5HD) (n=4) (for comparison, IS values of I/R and PSELT 5 nM groups showed in Fig. 3C have been added). The amount of necrotic tissue measured after 30-min global ischemia and 120-min reperfusion is expressed as percent of the left ventricle mass (LV) (% IS/LV). $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), by One-Way ANOVA/Newman-Keuls Multiple Comparison Test. Western blot analysis of phosphorylated **(B)** Akt, **(C)** Erk-1/2, **(D)** GSK3α/β, **(E)** p38MAPK in heart tissues from Sham, I/R and PSELT 5 nM groups (n=3 hearts/group). Histograms represent the ratio of densitometric analysis of protein/loading control: $p<0.05$ (*), $p<0.01$ (**), by One-Way ANOVA/Newman-Keuls Multiple Comparison Test.

Fig. 5 Effect of PSELT on apoptotic markers.

Western blot analysis of **(A)** Bax, **(B)** Bcl-2, **(C)** active caspase 3 and **(D)** Cyt c expression in heart tissues of Sham, I/R and PSELT 5 nM groups (n=3 hearts/group). Histograms represent the ratio of densitometric analysis of protein/loading control: $p<0.05$ (*), $p<0.01$ (**), by One-Way ANOVA/Newman-Keuls Multiple Comparison Test. **(E)** Western blot analysis of Cyt c expression in heart isolated cytosolic and mitochondrial fractions of Sham, I/R and PSELT 5 nM groups (n=3 hearts/group). Anti-COX-IV was used as mitochondrial loading control. Histograms represent the densitometric analysis of the bands: bars represent the area of each band expressed as a percentage of the total area of the same band in the 3 groups for cytosolic and mitochondrial fractions. $p<0.05$ (*), by One-Way ANOVA/Newman-Keuls Multiple Comparison Test.

Fig. 6 Effect of PSELT on redox balance control markers.

(A) Immunoreactivity of nitrotyrosine (NT) in Sham, I/R and PSELT 5 nM groups (n=3 hearts/group). **(B)** Intracardiac levels of reactive oxygen species (ROS) production (n=3 hearts/group) in Sham, I/R and PSELT 5 nM groups. Western blot analysis of **(C)** XO, and **(D)** AOX-1 in heart tissues from Sham, I/R and PSELT 5 nM groups (n=3 hearts/group). Histograms represent the ratio of densitometric analysis of protein/loading control: $p<0.05$ (*), by One-Way ANOVA/Newman-Keuls Multiple Comparison Test.

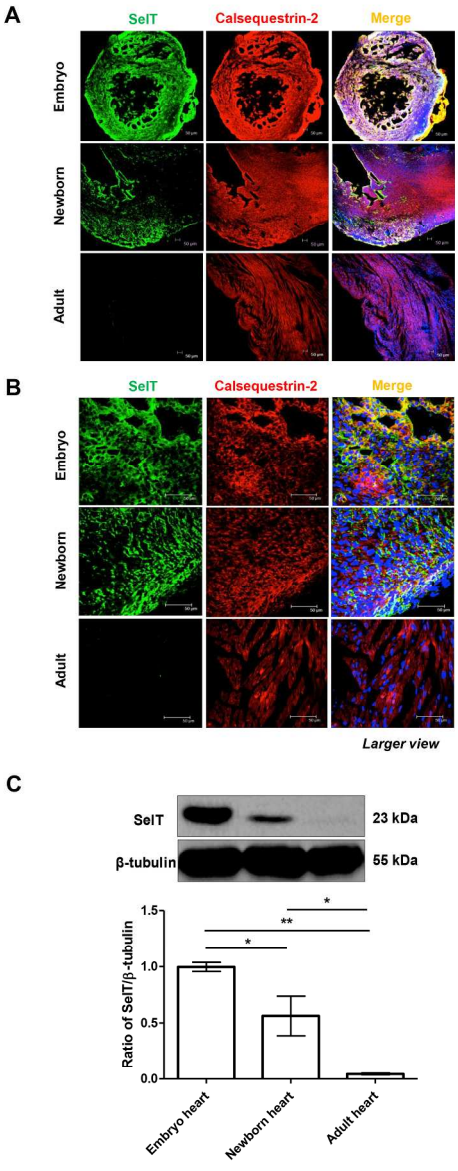

Fig. 1

Figure 1

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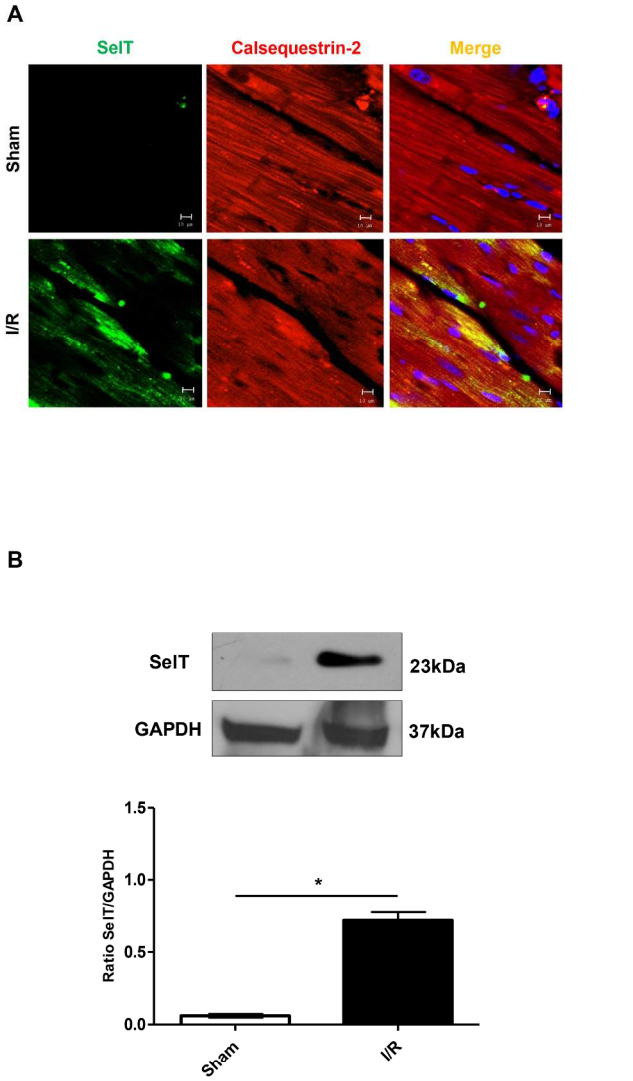

Fig. 2

Figure 2

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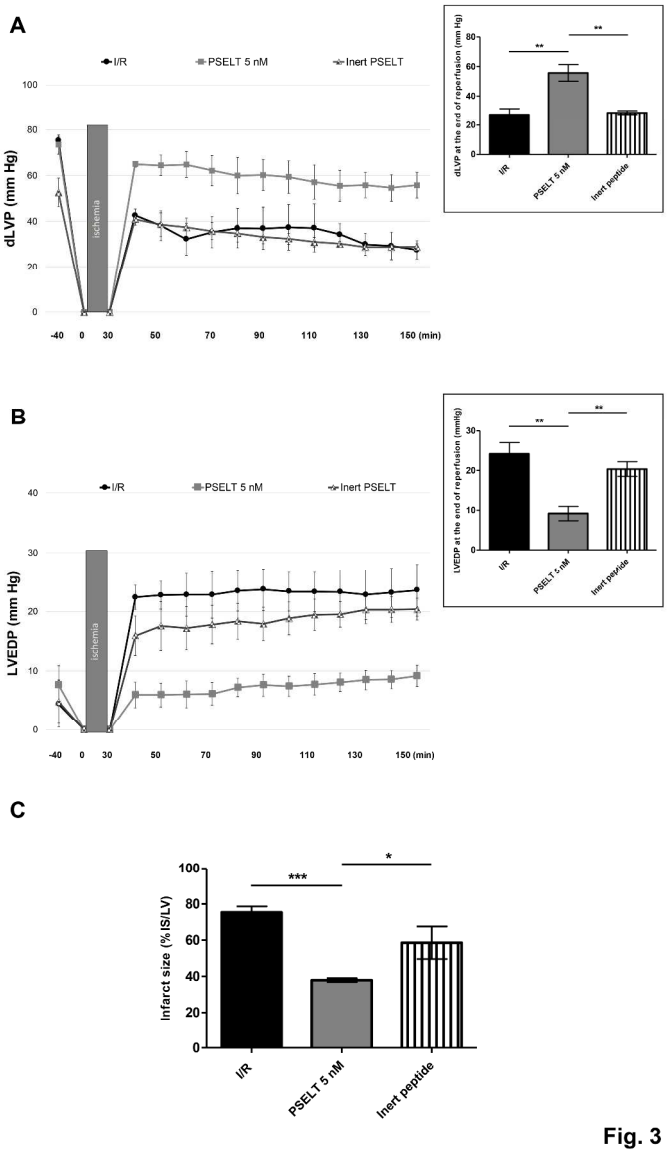

Fig. 3

Figure 3

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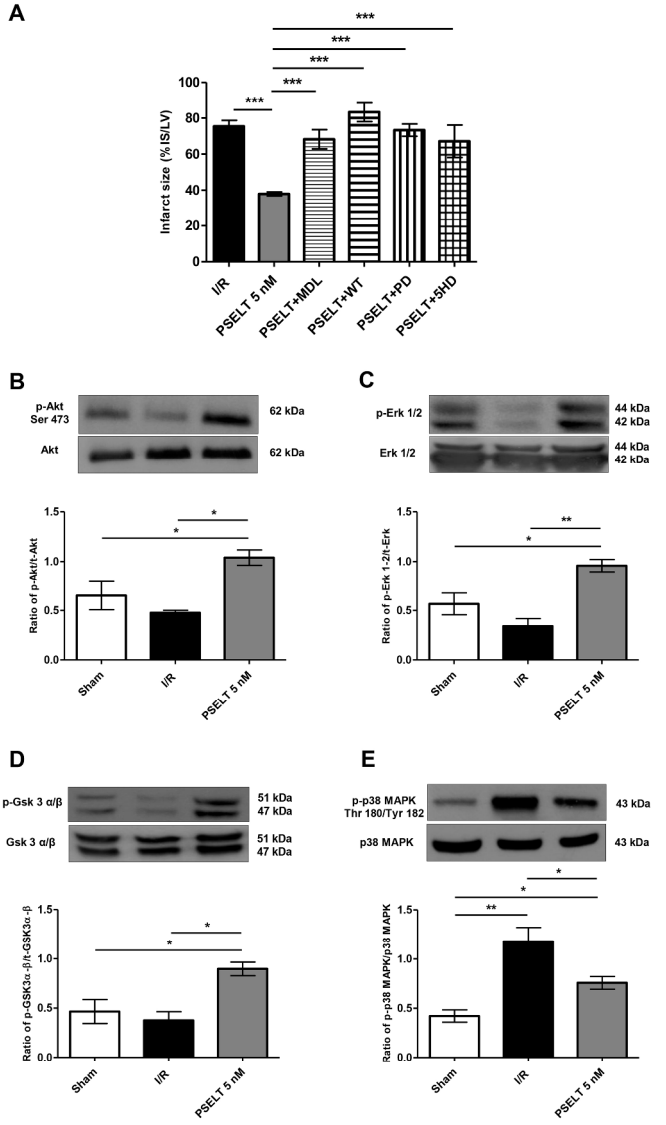

Fig. 4

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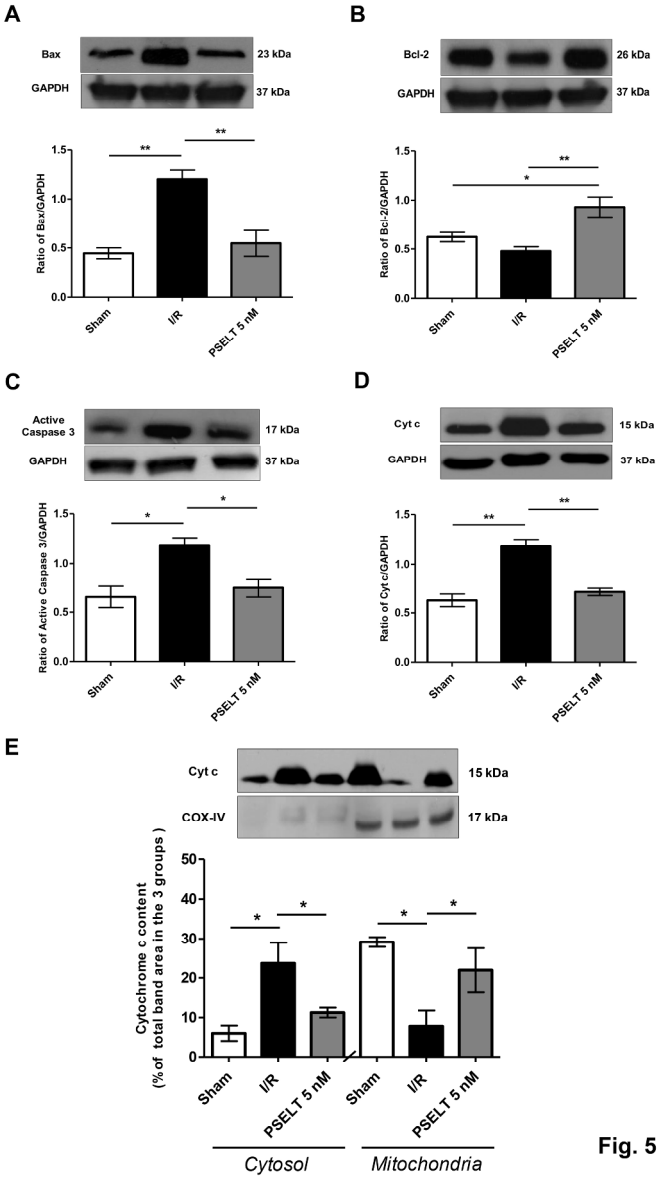

Fig. 5

Figure 5

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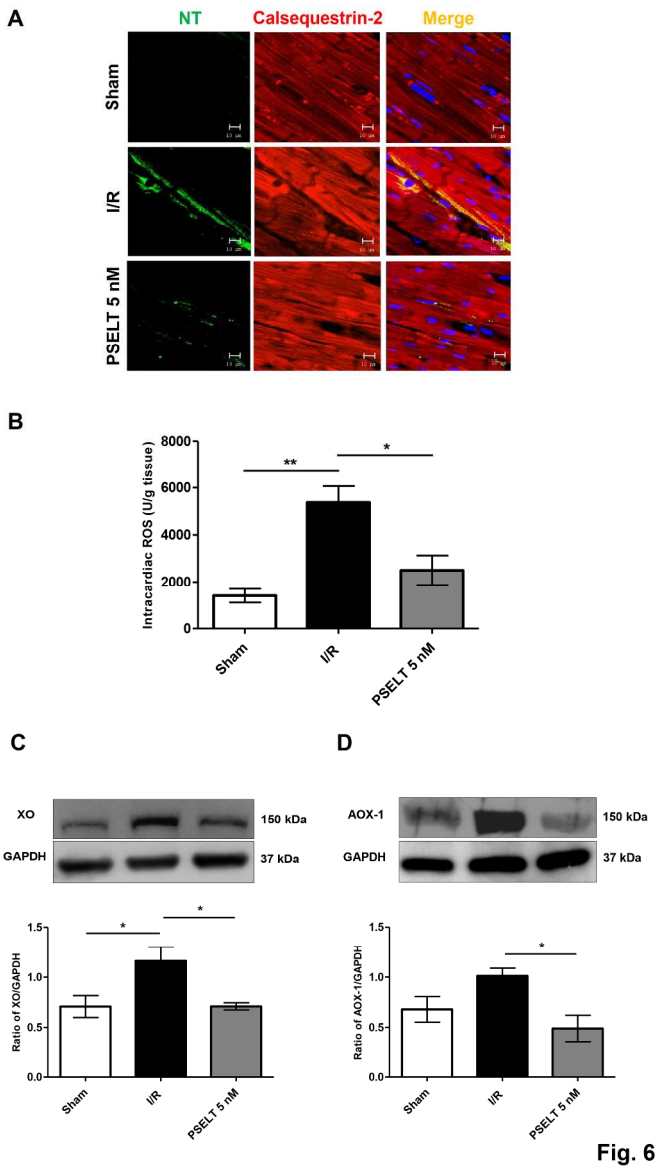

Fig. 6

Figure 6

296x419mm (300 x 300 DPI)