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Bidirectional control of mitochondrial respiration and permeability transition pore by nitric oxide donors and L-arginine. Implication of mitochondrial NO/cGMP/PKG - signaling system

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Abstract

Background/Aims: Numerous studies have shown that cytosolic second messengers nitric oxide (NO) and cGMP and protein kinase G (PKG1) play important role in regulating cell viability by modulating mitochondrial permeability transition pore (MPTP) opening, necrotic and apoptotic cell death. The involvement of mitochondrial calcium-dependent NO-synthase (mtNOS) in the control of MPTP is much less explored. Moreover, possible functioning of mitochondrial mtNOS/guanylate cyclase (GC)/PKG-signaling system (mtNOS/PKG-SS) and its impact on mitochondrial respiration and MPTP have not been analyzed yet. **Methods:** To address the issue we performed the experiments on isolated rat liver mitochondria with the application of specific inhibitors of NOS, GC and PKG. Mitochondrial respiration was supported by pyruvate and glutamate or succinate plus rotenone in the presence of hexokinase and ADP. **Results:** Obtained results indicate that L-arginine and NO-donors (SNP, SNAP) produce bidirectional concentration-dependent effects on mitochondrial respiration and on MPTP opening evoked by calcium ions or palmitoylcarnitine excess. In low concentrations L-arginine (10 to 200 μM) and NO-donors (10 to 50 μM) activate the respiration and raise threshold concentrations of calcium and palmitoylcarnitine, required for dissipation of mitochondrial membrane potential and pore opening. The inhibitors of NOS, GC and PKG eliminate both effects, what disclose the involvement of mtNOS/PKG-SS in the activation of respiration and deceleration of MPTP opening. In high concentrations L-arginine and NO-

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donors inhibit the respiration and promote MPTP opening. Observed alteration in the direction of L-arginine and NO-donors effects suggests that the inhibition evoked by mitochondrial NO excess may dominate over the protection afforded by mtNOS/PKG-SS. **Conclusions:** These findings demonstrate that the functioning of calcium-dependent mtNOS/PKG-SS, involved in complex calcium and NO interplay, might provide feedforward activation of the respiration and lowering the sensitivity of MPTP to calcium and palmitoylcarnitine excess.

Introduction

It is well appreciated that exogenous nitric oxide (NO) suppresses mitochondrial respiration by inhibiting cytochrome c oxidase (COX) and complexes I and II of the electron transport chain [1-5]. Activation of calcium-dependent mitochondrial NO-synthase (mtNOS) by its substrate L-arginine [6, 7] or by Ca^{++} and L-arginine [8] also causes sharp rise of mitochondrial NO coupled with the inhibition of oxygen consumption [6, 7]. However, in contrast to these unidirectional effects, contradictory effects of NO donors and mitochondrial NO on mitochondrial calcium retention capacity (CRC), mitochondrial permeability transition pore (MPTP) opening, and cytochrome c (CytC) release have been observed over last two decades.

As early as 1999, it was shown that over activation of mtNOS by Ca^{++} and L- arginine induces CytC release, while the inhibition of mtNOS diminishes CytC release, raises mitochondrial potential ($\Delta\Psi m$) and CRC [9]. In 2000, it was

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demonstrated that NO evokes concentration-dependent effects [10]. It was shown that, being added in very low or high (supraphysiological) concentrations, NO donor SpermineNONOate promoted calcium evoked mitochondrial swelling, CytC release and MPTP opening, whereas in intermediate concentrations it caused protective effects. Protective and adverse effects of NO donors were attributed to possible action of S-nitrosothiols and peroxynitrite, correspondingly [9, 10].

Concentration-dependent effects of NO-donors were also demonstrated in the investigations performed on permeabilized cells, which have shown that NO-donors dose-dependently diminish mitochondrial Ca^{++} accumulation and, being applied in high doses, promote MPTP opening [11]. In these experiments added L-arginine prevented MPTP-opening and inhibited mitochondrial Ca^{++} uptake [11, 12]. It was assumed that the inhibition of Ca^{++} uptake by intramitochondrial NO may represent negative feedback, which could prevent Ca^{++} overload and MPTP opening [11]. According to another point of view, NO induced mitochondrial depolarization was considered as possible mechanism preventing mitochondrial Ca^{++} accumulation [13].

Ensuing studies confirmed important role of functional state of mtNOS in the determination of optimal range of NO concentrations, required for efficient MPTP control. However, in contrast to earlier results [9], it was shown that the inhibitors of mtNOS promote, while NO donors prevent the dissipation of $\Delta\Psi m$ and mitochondrial swelling evoked by Ca^{++} in isolated mitochondria [14].

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Prevention of MPTP opening, provided by intramitochondrial NO, was explained by the action of accumulated S-nitrosothiols [14].

Present time experiments also demonstrate that moderate doses of NO donor nitroglycerine rise CRC and prevent Ca^{++}-dependent MPTP opening. The authors suppose that NO or reactive nitrogen species may be directly involved in the mechanism of mitochondrial calcium handling and MPTP blockade [15]. Dose-dependent effects of NO donor SNAP on the dissipation of $\Delta\Psi m$ and MPTP opening, evoked by Ca^{++} load or palmitoylcarnitine, have been observed in our preliminary experiments on liver mitochondria [16].

Considered above inconsistent effects of NO donors and L-arginine on mitochondrial respiration, $\Delta\Psi m$ and MPTP cannot be explained by the mechanisms based only on redox regulation of mitochondrial processes, resulting in S-nitrosylation [17, 18] and S-glutationylation [19-22] of numerous proteins. Potential intramitochondrial mechanisms of protection should implicate signaling chains, which are sensing intramitochondrial calcium level and involved in calcium and NO interplay. In favor of this hypothesis may be evidence of some data.

Recently Seya et al have found that cardiac mitochondrial protein fraction possess cGMP-dependent kinase (PKG) activity [23]. Mitochondrial cGMP production by undiscovered yet mitochondrial guanylate cyclase (GC) was also demonstrated by this group [24]. It was shown that SNAP or 8-Bromo-cGMP promote calcium-dependent CytC release implicating voltage-dependent anion channel (VDAC) as final target of this signaling pathway. Observed effects were

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prevented by the application of the inhibitors of NOS, GC, PKG and VDAC. Hydrolysis of cGMP by mitochondrial cyclic nucleotide phosphodiesterase PDE2A was demonstrated in brain and liver mitochondria in independent experiments [25].

These data indicate that all elements of an autonomous Ca^{++}-dependent mtNOS/GC/PKG-signaling system (mtNOS/PKG-SS) may operate in mitochondria. The functioning of this system in mitochondria may look like redundant and duplicating mechanism with respect to cytosolic one. Really, cytosolic NO-synthases (cNOS), NO, GC, cGMP, and PKG1,2 are important signaling molecules involved in the regulation of multiple cytosolic and mitochondrial metabolic pathways and signaling systems [26-30]. Master kinase PKG1, downstream mediator of NO, is considered as last step of cytosolic cNOS/GC/PKG1-signaling system, being implicated in the control of wide range of physiological and pathological processes functioning at different time scales [27, 28]. It is well known that cytosolic PKG1 operates as final mediator of protection targeted to mitochondrial kinases, involved in MPTP control [27-32]. However, listed above inconsistent effects of L-arginine and intramitochondrial NO on mitochondrial respiration and MPTP control are not related to the functioning of cytosolic signaling system and this issue requires further investigation. To investigate whether mitochondrial

Ca^{++}→mtNOS→NO→GC→cGMP→PKG-

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125 signaling system (mtNOS/PKG-SS) might be involved in the control of
126 mitochondrial respiration and MPTP opening, we have performed the experiments
127 on isolated rat liver mitochondria. Specific agonists and inhibitors of this signaling
128 system were applied to evaluate its positive and negative impact on mitochondrial
129 respiration and dissipation of $\Delta\Psi m$ and MPTP opening, evoked by Ca^{++} or
130 palmitoylcarnitine excess. Preliminary results were published earlier [16].
131

132 Materials and methods

133 All animal procedures were performed in accordance with EU directive
134 86/609/EEC and have been approved by the Ethics committee of the Institute of
135 Theoretical and Experimental Biophysics, RAS, Russia. protocol № 03-16-03-18.
136 Male 6-8 week old Wistar rats were kept under the same conditions in air-
137 conditioned and ventilated rooms at 20-22° C and a 12 h/12 h light-dark cycle. All
138 experiments were performed at 26° C. Rats were euthanized with CO_2 Liver
139 mitochondria were isolated using standard techniques of differential centrifugation
140 in the medium containing 300 mM sucrose, 1 mM EGTA, and 10 mM Tris-HCl
141 (pH 7.4). Mitochondrial preparations were washed twice with the release medium
142 not containing EGTA, resuspended in the medium of the same composition, and
143 stored on ice as described earlier [16]. Mitochondria incubation medium contained:
144 125 mM KCl, 3 mM KH_2PO_4, 10 mM HEPES (pH 7.4), 0.5 mM $MgCl_2$.
145 Mitochondrial protein content was determined by Lowry method with bovine
146 serum albumin as standard. Oxygen consumption in the mitochondrial suspension

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147 was determined by a polarographic method with a Clark type O_2 electrode in
148 closed chamber of 1mL, containing 1.0-1.2 mg mit. protein, under continuous
149 stirring. The electric potential difference (ΔΨm) on the inner mitochondrial
150 membrane was measured by determining the redistribution of lipophilic cation
151 tetraphenylphosphonium (TPP^+) between incubation medium and mitochondria.
152 Fall in the concentration of TPP^+ reflect the rise in ΔΨm. Concentration of TPP^+
153 [TPP^+] in the mitochondrial incubation medium was recorded with TPP^+ selective
154 electrode. Changes in calcium ion concentration in the incubation medium were
155 recorded by the Ca^{++} selective electrode (Nico, Moscow, Russia). Simultaneous
156 registration of ΔΨm and Ca^{++} were carried out in open chamber of 1 ml, containing
157 1.0-1.2 mg mit protein, under continuous stirring. Mitochondrial swelling was
158 monitored as a decrease in the optical density of a mitochondrial suspension
159 (0.35mg/mL) in open chamber of 2 mL. Absorbance at 540 nm was measured
160 using Ocean Optics USB4000 spectrophotometer.
161

162 Background of the protocol of experiments

163 L-glutamate (10 mM) and pyruvate (0.5-1 mM) were used as substrates to
164 keep turnover of both spans of the Krebs cycle running, provide sufficient NADPH
165 production, and preserve substrate-level phosphorylation in the Cycle. In some
166 experiments mitochondrial respiration was supported by succinate (5mM) and
167 rotenone (2 μM) or pyruvate (1 mM) and L-malate (5mM). All experiments were
168 performed in the presence of 10 IU hexokinase, 10 mM glucose and 0.5 mM

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MgCl. Subsequent addition of 0,75mM ADP provided high steady state respiration rate (VO_2ss), which was close to State 3 respiration rate (80-90 % of VO_2max). All values of $VO_2ss = d[O_2]/dt$ were calculated on linear parts of polarographic tracks at appropriate values of pO_2 (as indicated in corresponding legends).

Measurement of the parameters characterizing MPTP opening in isolated mitochondria

Opening of the MPTP was registered as: loss of calcium buffering capacity (characterized by steep rise of calcium in the incubation medium); dissipation of mitochondrial $\Delta\Psi m$; mitochondrial swelling. Induction of MPTP was achieved by sequential loading of the incubation medium with 20 µM of Ca^{++} ($CaCl_2$) or 20 µM D,L-palmitoylcarnitine (PC). Total concentrations of added Ca^{++} or PC, required for pore opening, determined mitochondrial CRC and threshold concentration of PC (PC*). Maximal mitochondrial swelling rate (Vsw) was determined as Vsw = Δ o.d./min. The impact of mtNOS/PKG-SS on mitochondrial steady state respiration or on MPTP opening was evaluated by the determination of the values of VO_2ss or of CRC, PC*, and Vsw, respectively. Activation of mtNOS-SS was attained by the application of L-arginine, NO-donors, and Ca^{++}. 7-NI, ODQ, and KT5823 were used to inhibit mtNOS, GC and PKG, correspondingly.

Materials

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All reagents were purchased from Tocris (UK) and Sigma (USA). S-nitrosoacetylpenacillamine (SNAP), 7- Nitroindasole (7-NI) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) were dissolved in ethanol and KT5823-in dimethyl sulfoxide (DMSO). Final concentration of DMSO was 0.1-0.2%. Sodium nitroprusside (SNP) was dissolved in the water.

Statistics

Statistical analysis of the experimental data was carried out by applying ANOVA One Way Tukey's Test. The columns depicted on the Figures represent mean of four values, which were registered in separate experiments carried out on mitochondrial preparations. Data presented as mean ± S.E.M. of 4 independent experiments. $P < 0.05$ was taken as the level of significance.

Results

Bidirectional concentration-dependent effects of NO-donors and L-arginine on mitochondrial respiration. Impact of mtNOS/PKG-SS

Coupled rat liver mitochondria, respiring on 10 mM L-glutamate and 0.5 mM pyruvate as substrates, have mean resting state respiration rate about 19 ±

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1.4ng-at O/min/mg prot. Addition of 0.75 mM ADP increased oxygen consumption rate to steady state values VO2ss = 54.5 ± 1.7 ng-at O/min/mg prot. Representative polarographic traces (O_2 traces) are depicted at Fig 1. Black traces correspond to control experiments, while coloured traces characterize the influence of low and high concentrations of NO donor SNP (Fig 1A) and L-arginine (Fig 1B) on mitochondrial respiration.

NO-donors and L-arginine evoke activation or inhibition of mitochondrial respiration in dependence of the concentrations used

Preincubation of mitochondria with low concentrations of SNP (20 μM; blue trace at Fig 1A) or L-arginine (20 μM; blue trace at Fig. 1B) resulted in the increase of oxygen consumption rate (rise of the trace slope). High concentrations of SNP (400 μM; red trace, Fig 1A) and L-arginine (1000 μM; red trace, Fig 1B) evoked the inhibition of mitochondrial respiration.

To evaluate statistical significance of obtained results, steady state values of VO_2ss were measured at [O2] = 300-320 ng-atO/mL on linear parts of corresponding polarographic traces in the series of four experiments. Calculated average values of VO_2ss are depicted at Fig 2 in the form of columns. Grey columns correspond to control experiments with VO_2ss = 54.5 ± 1.7 ng-at O/min/mg prot. This value of VO_2ss was taken as 100%. Two sets of black columns characterize the impact of different concentrations of SNP (Fig 2A) and L-arginine (Fig 2B) on mean values of VO_2ss. Presented data show that the application of 5-50 μM SNP produces marked activation of steady state

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mitochondrial respiration rate. Maximal increase of VO2ss by 38% was observed at 20μM SNP (Fig 2A, second black vs. gray columns). In a like manner, 20μM of L-arginine raises steady state respiration rate by 33% (Fig 2B, second black vs. gray columns).

Higher concentrations of SNP (50-400 μM) and L-arginine (200-1000 μM) dose-dependently diminish mean values of mitochondrial steady state respiration rate. Moreover, high concentrations of SNP (400 μM) and L-arginine (1000 μM) suppress respiration rate by 20-22 % compared to control state respiration (right black vs. gray columns at Fig 2A, 2B respectively). Registered inhibition of mitochondrial respiration by SNP and L-arginine represents well known phenomenon [5-7]. It may be explained, for example, by known competitive (with respect to oxygen) inhibition of COX by the excess of NO released from SNP or produced by mtNOS from L-arginine [1, 6]. Red trace at Fig 1A demonstrates that mitochondria cannot maintain steady state respiration at the excess of SNP (400 μM). The decrease of O_2 content in the medium is accompanied by progressive suppression of the respiration.

Discovered activation of the respiration by low concentrations of L-arginine or SNP was statistically significant in all presented above experiments ($p< 0.05$). It may be also evoked by low doses of NO-donor SNAP, which stimulates VO_2ss in the range of concentrations from 10 to 100 μM (not shown). Besides, the effects of NO-donors and L-arginine do not depend on the substrates used and may be reproduced on mitochondria respiring with pyruvate and malate or succinate (5

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mM) plus rotenone (2 μM). In the last case, the preincubation of mitochondria with 10 μM SNP or 20 μM L-arginine raised steady state respiration rate by 24% and 19%, correspondingly, in comparison with control state respiration rate (93.5 ± 3.4 ng-at O/min/mg prot).

Fig 1. Activation and inhibition of mitochondrial respiration by NO-donor SNP (left) and L-arginine (right). Concentration- dependent effects of SNP (A) and L-arginine (B) on mitochondrial respiration are depicted by representative polarographic traces (O_2 traces). Mitochondria (1.0-1.2 mg) were incubated in closed chamber of 1 mL. Incubation medium included 0.5 mM pyruvate, 10 mM L-glutamate and hexokinase. Steady state respiration was evoked by 0.75 mM ADP. Black traces correspond to control experiments. Concentration- dependent effects of SNP (A) and L-arginine (B) on mitochondrial respiration. Blue traces fit to low concentrations of SNP (A; 20 μM) and L-arginine (B; 20 μM) in the medium. Red traces match to high concentrations of SNP (A; 400 μM) and L-arginine (B, 1000 μM). All explanations are given in the text.

Fig 2. Bidirectional concentration-dependent effects of SNP (A) and L-arginine (B) on mean steady state respiration rate (VO_2ss) and involvement of mtNOS/PKG-SS in the activation mitochondrial respiration by low concentrations of SNP and L-arginine. All conditions of the experiments correspond to Figure 1. All columns represent mean values of steady state

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respiration rates VO_2ss. Grey columns correspond to control experiments. Black columns describe the experiments performed with different concentrations of SNP (A) and L-arginine (B) in the medium. Hatched columns depict the impact of the inhibitors of NOS, GC, and PKG (7-NI, ODQ, and KT5823, respectively) on mean values of VO_2ss. Individual values of VO_2ss were calculated on linear parts of corresponding polarographic traces at the concentrations of $[O_2]$ = 300-320 ng-atom O/mL. Data represent means ± S.E. of 4 separate experiments for each column (n = 4). Concentrations of SNP, L-arginine (L-Arg), 7-NI, ODQ, and KT5823 (KT) are given in μM. Control value of VO_2ss = 54.5±1.7 ng-at O/min/mg prot was taken as 100%. * = $p < 0.05$ compared to control value of VO_2ss (gray columns) or values of the experiments with 20 μM of SNP or L-arginine (second black columns).

Involvement of mtNOS/PKG-SS in the activation of mitochondrial respiration

Analyzing observed activation of the respiration by low concentrations of SNP, SNAP and L-arginine, we have supposed that mtNOS/PKG-SS may be involved in the regulation of the respiration and/or energy metabolism. To investigate whether the inhibition of this signaling system could abrogate activation of mitochondrial respiration by NO-donors and L-arginine, we performed the experiments with the application of respective inhibitors of mtNOS,

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GC, and PKG. The results presented at Fig 2 by hatched columns demonstrate the impact of 7-NI, ODQ, and KT5823 on steady state respiration rate. Fig 2A shows that ODQ and KT5823 prevent activating effect of SNP by lowering mean VO_2ss (two hatched vs. first black columns). In a like manner, the activation of the respiration by 20 μM L-arginine was not observed after the incubation of mitochondria with 7-NI, ODQ, and KT5823 (Fig 2B, three hatched vs. first black columns). Similar effects of the inhibitors were observed in mitochondria respiring on succinate plus rotenone (not shown).

These results led us to suggest that PKG might be involved in the regulation of oxidative phosphorylation by phosphorylating some proteins, like to the mechanism provided by mitochondrial adenylate cyclase (AC)/protein kinase A (PKA) cascade [33-36]. If this is the case, than activation of mtNOS/PKG-SS by L-arginine may provide bidirectional control of the respiration, which includes the activation of respiration with the involvement of PKG and direct inhibition of cytochromes by NO excess (and/or its derivatives). Soluble GC has about ten times higher affinity to NO than COX [7]. Assuming comparable sensitivity to NO of mitochondrial and cytosolic GC, we might speculate that at low concentrations of L-arginine (or NO) the activation of respiration by PKG will dominate over well known inhibitory effect of NO [1-6].

Activation of cyclosporine A-dependent MPTP opening by palmitoylcarnitine. Impact of low concentrations of L-arginine

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Previously we have shown that in rat liver mitochondria, respiring on pyruvate and L-glutamate, added D, L-palmitoylcarnitine (PC) induces cyclosporine A (CsA) sensitive MPTP opening. Activation of mitochondrial respiration by ADP resulted in lowering of critical concentration of PC (PC*) required for pore opening [16].

Dissipation of mitochondrial potential, inhibition of respiration, and mitochondrial swelling induced by PC. Protection provided by CsA

Preincubation of mitochondria with pyruvate, L-glutamate and low concentrations of PC (20-25 μM) raised ΔΨm and accelerated steady state mitochondrial respiration rate by 35- 40% in comparison with the values attained with pyruvate and L-glutamate (not shown). On the contrary, high doses of PC (above 50 μM) evoked steep dissipation of ΔΨm (rise of [TPP^+]) and progressive inhibition of the respiration. This process is developed soon after addition of ADP (Fig 3A; black traces). Added CsA prevented the dissipation of ΔΨm, in spite of profound suppression of the respiration (Fig 3A; blue traces).

Besides dissipation of ΔΨm and suppression of the respiration, PC induced mitochondrial swelling. As shown at Fig 3B, low concentrations of PC (20 μM) evoked mitochondrial swelling, which was characterized by swelling rate Vsw = 0.26 a.u./min (black trace). Preincubation of mitochondria with CsA (2 μM) prevented this effect (blue trace). Second addition of 20 μM PC resulted in deep mitochondrial swelling, characterized by Vsw = 2.4 a.u./min (black trace). However, in this case CsA produced limited protective effect diminishing Vsw

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four times to 0.57 a.u./min and having no effect on the magnitude of mitochondrial swelling (blue trace).

Fig 3. PC excess evokes dissipation of ΔΨm, suppression of respiration and mitochondrial swelling. Impact of Cyclosporine A. Representative experiments are depicted. (A): Impact of PC excess (50 μM) and CsA (1.5-2 μM) on mitochondrial respiration (O_2 traces) and ΔΨm (TPP^+ traces). Mitochondrial incubation medium included 10 mM of L-glutamate and 1mM pyruvate. Mitochondria were preincubated with PC (50 μM; black traces) or with PC and CsA (1.5 μM; blue traces). All other conditions as at Fig 1. (B): Mitochondrial swelling evoked by PC in the absence (bottom trace) and in the presence of CsA (2 μM, top trace) in the media. PC (20 μM) was added two times as indicated. Maximal values of the rate of swelling (Vsw, expressed in a.u./min) are indicated on the traces. The experimets were carried out in open chamber of 2 mL, containing 0.35 mg prot/mL of mitochondria. 0.75 mM ADP was added 1 min before first application of PC. Incubation medium included 5 mM of succinate and 2 μM rotenone. All other conditions as at Fig 1.

Protective effect of L-arginine on the dissipation of ΔΨm and inhibition of mitochondrial respiration evoked by PC

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Profound fall of ΔΨm and suppression of the respiration may be also evoked by PC added in the phase of active steady state respiration induced by ADP. Fig 4A shows that the application of 50 μM PC (PC*) produced the dissipation of ΔΨm and suppression of the respiration (black traces). Preincubation of mitochondria with 200 μM L-arginine prevented the dissipation of ΔΨm and restored active steady state respiration (green traces). PKG inhibitor KT5823 abrogated protective effects of L-arginine by restoring the dissipation of ΔΨm and inhibition of the respiration evoked by PC (red traces). It should to mention that, in comparison with L-arginine, CsA prevents only the dissipation of ΔΨm (pore opening) but cannot reactivate mitochondrial respiration (Fig 3A; blue traces).

Based on these results, we may suppose that two separate protective mechanisms may be realized with the implication of mtNOS/PKG-SS. First mechanism might be directed to the regulation of mitochondrial respiration. This mechanism may implicate the same signaling events, which provide the activation of the respiration by L-arginine in the absence of PC (Fig 1 and 2). Second mechanism of protection may be directed on MPTP control and based on the phosphorylation of some key proteins by PKG.

Interestingly, protective effect of L-arginine is not realized in mitochondria respiring on succinate plus rotenone (Fig 4B). In this case, added 50 μM PC also evoked dissipation of ΔΨm and suppression of the respiration (black traces), however L-arginine did not protect mitochondria against deleterious effect of PC (green traces). Observed dissimilarity of L-arginine effects (Fig 4A and 4B) may

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386 be explained, assuming that mitochondria respiring on succinate plus rotenone (at
387 PC excess) cannot provide substrate-level phosphorylation in the Krebs cycle to
388 keep appropriate level of GTP, required for cGMP production by GC and
389 subsequent activation of PKG.

390

391 **Fig 4. L-arginine prevents dissipation of ΔΨm and suppression of respiration**
392 **evoked by PC excess (A). Absence of protective effect of L-arginine in**
393 **mitochondria respiring on succinate plus rotenone (B).** Representative
394 experiments are depicted. (A): Impact of PC excess (50 µM) and L-arginine (200
395 µM) on mitochondrial respiration (O_2 traces) and ΔΨm (TPP^+ traces).
396 Mitochondrial incubation medium included 10 mM of L-glutamate and 1mM
397 pyruvate. PC was added 1 min after activation of the respiration by 0.75 mM ADP.
398 Mitochondria were preincubated: without (black traces), with L-arginine (green
399 traces), and with L-arginine plus 1.5 µM KT5823 (red traces). All other conditions
400 as at Fig 3A. (B): All conditions of the experiment corresponds to those of Panel
401 A, except that incubation medium included 5 mM succinate plus 2 µM rotenone.
402 Abbreviations: L-Arg = L-arginine; KT = KT5823.

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Impact of mtNOS/PKG-SS on the values of threshold concentrations of PC required for dissipation of ΔΨm

To evaluate critical (threshold) concentrations of PC*, providing the dissipation of ΔΨm (and activation of MPTP opening), we used sequential loading of the incubation medium with 20 μM PC till the time point characterized by fast fall of ΔΨm. This procedure is shown at Figure 5A. Brown line corresponds to control mitochondria, in which third addition of PC (PC* = 60 μM) evokes steep fall of ΔΨm (rise of $[TPP^+]$in medium). Green line describes the influence of 100 μM SNAP on ΔΨm. SNAP provides partial protection by raising total concentration of added PC to new critical value of PC* = 100 μM. Two diagrams presented at Fig 5B and 5C characterize the involvement of mtNOS/PKG-SS in the regulation of MPTP sensitivity to PC.

Concentration-dependent effect of NO-donor SNAP on MPTP opening by PC.

Added SNAP raises PC* level in concentration-dependent manner (Fig 5B, blue and violet vs. black columns), being most effective at 100 μM (violet column). This optimal concentration of SNAP provides effective protection by lowering MPTP protein complex sensitivity to PC in comparison with control state. Preincubation of mitochondria with 100 μM SNAP increases critical PC* level from 50.0 ± 5.8 μM to 95.0 ± 6.2 μM (violet vs. black columns). High concentrations of SNAP (300 μM; grey column) were ineffective.

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Observed protection, afforded by low concentrations of SNAP (to100 μM), was eliminated by the application of GC and PKG inhibitors ODQ and KT5823 (Fig 5B; red and green columns). As a result, the sensitivity of ΔΨm (and MPTP) to PC excess increased about two times. Critical PC* values, providing the dissipation of ΔΨm, diminished from maximal values of 95.0 ± 6.2 μM (violet column) to 45.0 ± 5.0 μM and 55.0 ± 5.0 μM (green and red columns), which were close to control values of PC* = 50.0 ± 5.8 μM (black column).

Fig 5. Impact of mtNOS/PKG-SS on threshold concentrations of D, L-palmitoylcarnitine (PC*) required for the dissipation of ΔΨm and MPTP opening. (A): An example of threshold PC* concentrations determination. Mitochondria (1.0-1.1 mg) were incubated in open chamber of 1 mL without (control, brown trace) or with 100 μM SNAP (green trace). Conditions of the experiment correspond to those of Fig 1. Ca^{++} (20 μM) was added before the stimulation of mitochondrial respiration by 750 μM ADP. Third addition of 20 μM PC evoked fall of ΔΨm (rise of TPP^{+}) and pore opening, what fits to threshold value of PC*=60 μM. Preincubation of mitochondria with SNAP diminished probability of pore opening by raising PC* to 100 μM; (B): Diagram incorporates mean threshold values of PC* measured in control experiments (black columns) and in the experiments with mitochondria preincubated with SNAP (blue and violet columns), SNAP + ODQ and SNAP + KT5823 (red and green columns); *p< 0.05 compared to control PC* value (black) or to the value of experiment with

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449 SNAP (violet column). (C): Diagram describes the impact of L-arginine (L-Arg)
450 on mean threshold values of PC* and elimination of protective effect of L-arginine
451 by 7-NI, ODQ, and KT5823 (brown, red and green columns). KT = KT5823. All
452 concentrations are given in μM. Data represent means ± S.E. n = 4. *p< 0.05
453 compared to PC* value of gray column or to PC* value of experiment with L-
454 arginine (violet column).

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458 **Impact of L-arginine on the dissipation of ΔΨm by PC**

459 Calcium and PC act synergistically by reinforcing effects each other [16].
460 This statement may be illustrated by black and grey columns at Figure 5C. In
461 control experiments mean PC* value was equal to 55.0 ± 5.0 μM (black column).
462 Preincubation of mitochondria with 20 μM Ca^{++} resulted in lowering of PC* to
463 45.0 ± 5.0 μM (gray column). Higher concentrations of Ca^{++} provided profound
464 lowering of PC*. Taking into account multiple positive and negative effects of
465 calcium on mitochondrial energy metabolism, mtNOS and MPTP [8, 37], we used
466 this boundary concentration of Ca^{++} (20 μM) to analyze protective effect of L-
467 arginine on the dissipation of ΔΨm by PC. Optimal concentration of mtNOS
468 substrate L-arginine (500 μM) was applied to avoid adverse effects of higher doses
469 of L-arginine (above 1000 μM) on mitochondrial respiration (Fig 1B and 2B) and

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threshold PC* concentrations (Fig 5C; light blue column). In the experiments presented at Fig 5C preincubation of mitochondria with 500 μM L-arginine substantially raised critical PC* level from 45.0 ± 5.0 μM to 80.0 ± 8.2 μM (violet vs. gray columns) by strengthening protective mechanisms. Observed effect of L-arginine was eliminated by the inhibitors of NOS, GC and PKG (brown, green and red columns), which returned critical PC* values to the range of control values of PC* = 45-50 ± 5.0 μM.

Like SNAP, high concentrations of L-arginine (above 2000 μM) did not provide protective effect and even promoted pore opening by diminishing threshold PC* concentrations to control values (Fig 5C; light blue vs. gray columns).

Thus, results presented at Fig 5 denote that the activation of mtNOS/PKG-SS, evoked by optimal concentrations of L-arginine or SNAP, may implicate new protective mechanisms, which may provide the rise of threshold concentration of PC* required for the dissipation of ΔΨm and MPTP opening. Studied protective effects have not been observed at the concentrations of L-arginine and SNAP higher than 1000 μM and 200 μM, respectively. Apparently, observed adverse effects of high concentrations of L-arginine and SNAP may be based on the inhibition of the respiratory chain and MPTP complex proteins by NO and/or its derivatives. Likely, this inhibition may dominate over protective effect of mtNOS/PKG-SS at the excess of intramitochondrial NO.

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Involvement of mtNOS/PKG)-SS in the control of calcium retention capacity

Calcium retention capacity (CRC), i.e. critical concentration of Ca^{++} required for pore opening, was measured by standard procedure of sequential loading of the medium with 20 μM of Ca^{++} ($CaCl_2$). This procedure is depicted at Fig 6A, which demonstrates that the incubation of mitochondria with the inhibitor of PKG KT5823 (2 μM) diminishes CRC from control value of 140 μM to 100 μM (red vs, blue lines). The fifth addition of 20 μM Ca^{++} is accompanied by steep decrease of $\Delta\Psi m$ (rise of TPP^{+}) and rise of Ca^{++} in the medium (red lines). Two diagrams presented at Fig 6B and 6C characterize the involvement of mtNOS/PKG-SS in the control of CRC.

Control of Mitochondrial Calcium Retention Capacity by NO-donors

Preincubation of mitochondria with low concentrations of PC (20 μM) diminished CRC from control value of 135.0 ± 5.0 to 110.0 ± 5.7 μM (Fig 6B, grey vs. black columns). This lowering of CRC by 19% demonstrates synergistic action of Ca^{++} and PC and might indicate on the involvement of long chain AcylCoA's in the regulation of intramitochondrial Ca^{++} threshold for pore opening. Application of low concentrations of NO-donors SNP and SNAP (up to 50 μM) caused protective effect by raising mean CRC on 50-70% (Fig 6B; violet vs. gray columns). This protection was eliminated by the inhibitors of the enzymes of mtNOS/PKG-SS. Preincubation of mitochondria with GC inhibitor ODQ resulted

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513 in significant shortage of CRC values for SNP and SNAP nearly to control values
514 (Fig 6B, green vs. violet columns). Inhibitor of PKG (KT5823, 2 μM) produced
515 similar effect (Fig 6B, red vs. violet columns). Higher concentrations of SNP (100
516 and 200 μM) evoked opposite effect by diminishing CRC two to three times (Fig
517 6B, blue and light-blue vs. violet columns). Observed promotion of pore opening
518 by SNP excess may be based on inhibition of the respiratory chain and MPTP
519 complex proteins by NO, nitrosothiols, etc.
520
521 **Fig 6. Involvement of mtNOS/PKG-SS in the regulation of mitochondrial**
522 **calcium retention capacity (CRC).** (A): Determination of CRC values by
523 registering steep alterations of ΔΨm (TPP^+, top traces) and Ca^{++} concentration in
524 medium (bottom traces). Mitochondria (1.0 mg) were incubated in open chamber
525 of 1 mL without (control) or with 2 μM KT5823 (KT). Conditions of the
526 experiment correspond to those of Fig 1. PC (20 μM) was added after the
527 stimulation of respiration by 750 μM ADP. Seventh addition of 20 μM Ca^{++}
528 evoked an extrusion of Ca^{++} from the matrix, dissipation of ΔΨm, and pore
529 opening in control mitochondria, what corresponds to CRC = 135 μM. Inhibitor of
530 PKG KT5823 diminished CRC to 100 μM (B): Diagram describes protective
531 effects of SNP and SNAP on pore opening by Ca^{++} overload. Presented mean
532 values of CRC for control mitochondria (black columns) and for mitochondria
533 preincubated with SNP, SNAP and inhibitors of GC (ODQ) and PKG (KT5823);
534 Control value of CRC = 135.0 ± 5.0 μM was taken as 100%. (C): Diagram

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describing positive impact of L-arginine on mean CRC values and elimination of L-arginine effect by 7-NI, ODQ and KT5823 (KT). All other conditions as at Fig 5. All concentrations are given in μM. Control value of CRC = 145.0 ± 5.0 μM was taken as 100%. Data represent mean ± S.E. of 4 separate experiments. *p< 0.05 compared to control + PC columns (grey) or to SNP, SNAP or L-arginine columns (violet).

Control of Mitochondrial Calcium Retention Capacity by L-Arginine

Like to previous example, preincubation of mitochondria with 20 μM PC diminished CRC by 20% from control value of 145.0 ± 5.0 to 125.0 ± 5.5 μM (Fig 6B, grey vs. black columns). Preincubation of mitochondria with 500 μM L-arginine raised CRC value by 56% from 125 ± 5.0 μM to 195.0 ± 9.6 μM (Fig 6C, violet vs. gray columns). The inhibitor of mtNOS 7-NI (100 μM) evoked marked decrease of CRC, which was about two times lower than control value of CRC (Fig 6C, brown vs. gray columns). The inhibitors of GC and PKG (Fig 6C, red and green columns) also eliminated observed protective effect of L-arginine. High concentrations of L-arginine (2000 μM), like to NO donors, promoted pore opening by lowering CRC to control values (Fig 6C, light blue vs. gray columns).

The data presented at Fig. 6 suggest that low concentrations of L-arginine and NO-donors produce protective effects, providing marked rise of CRC in

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comparison with control values. Substantial fall of CRC, observed in the presence of the inhibitors of mtNOS/PKG-SS, may indicate on the involvement of this signaling system in the control of MPTP and mitochondrial calcium handling.

Discussion

Analyzing known contradictory effects of NO-donors and intramitochondrial NO on the induction of MPTP by Ca^{++} [9-16], we hypothesized that potential mechanisms of protection cannot be limited only by redox-based mechanisms. We supposed that calcium-dependent mtNOS/PKG-SS may represent important part of multi-level system involved in mitochondrial Ca^{++} and NO interplay and MPTP control. Signal transduction in mitochondrial signaling chain: **Ca^{++}→mtNOS→NO→mtGC→cGMP→mtPKG**, provides functional dependence of mitochondrial PKG (mtPKG) activity on intramitochondrial Ca^{++} concentration. Transmission of calcium signal to final mediator mtPKG may create at least two feedforward loops, which might involve phosphorylation of some key proteins by mtPKG. Positive feedforward loop may ensure the activation of mitochondrial oxidative phosphorylation, while negative feedforward loop might oppose MPTP opening induced by Ca^{++} load.

MPTP is considered as common death pathway to the development of apoptosis and necrosis observed at ischemia-reperfusion [37-42], acute

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steatohepatits [31, 39, 43], action of various drugs and toxins [39, 44], etc. Calcium and reactive oxygen species are recognized as key mediators involved in MPTP opening and cell death [39, 40]. However, besides rise of Ca^{++} and reactive oxygen species, ischemia/reperfusion and some other pathological processes are characterized by the accumulation and efflux from damaged cells of long chain fatty acids (LCFA) [45-47] and their carnitine derivatives [45]. The accumulation of these primary "toxins" is considered as one of triggering events evoking mitochondrial calcium overload and oxidative stress [39, 47, 48]. LCFA and long chain acyl carnitines are oxidized in form of long chain acyl-CoA's, which known to inhibit various NAD(P)H-dependent dehydrogenases [49-51]. However, molecular mechanisms providing the involvement of long chain acyl-CoA's in the induction of MPTP are far from being clear and require further investigations. Our preliminary results indicate that PC induces CsA-dependent dissipation of $\Delta\Psi m$, which may be prevented by NO-donor SNAP [16]. Therefore, in the present study we used calcium and PC overload to induce MPTP opening.

To investigate quantitatively the impact of mtNOS/PKG1-SS on MPTP opening, we selected mean threshold values for Ca^{++} (CRC) and PC (PC*) as regulatory parameters, characterizing the sensitivity of MPTP to Ca^{++} and PC overload. Besides that, to evaluate the involvement of mtNOS/PKG-SS in the regulation of mitochondrial respiration, we determined mean values of steady state respiration rates VO2ss. Hexokinase plus ADP were applied to set VO2ss at 80-90% of State3 respiration rate. Pyruvate and L-glutamate were selected as

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601 substrates to keep balanced turnover of both spans of the Krebs cycle and provide
602 sufficient production of NADPH and GTP, the cofactors required for
603 mtNOS/PKG-SS operation.

604 Obtained results indicate that NO-donors (SNP, SNAP) and L-arginine
605 produce bidirectional concentration-dependent effects on mitochondrial respiration
606 and on MPTP opening evoked by calcium and PC excess. In low concentrations
607 NO-donors (10-50 μM) and L-arginine (10- 200 μM) cause moderate activation of
608 steady state respiration by increasing VO2ss on 30-40 % (Fig 2A and 2B; black vs.
609 gray columns). Besides that, NO-donors and L-arginine suppress the dissipation of
610 ΔΨm and MPTP opening by raising the values of two regulatory parameters (CRC
611 and PC*), characterizing MPTP sensitivity to calcium and PC load. Low
612 concentrations of NO-donors (to 50 μM) and L-arginine (to 500 μM) enlarge
613 threshold values of PC* and CRC by 60-100% (Fig 5B and 5C, 6B and 6C; violet
614 vs. gray columns).

615 Application of the inhibitors of NOS, GC and PKG eliminated both effects,
616 what reveal the involvement of mtNOS/PKG-SS in the activation of respiration
617 (Fig 2, hatched vs. first black columns) and deceleration of MPTP opening (Fig 5
618 and 6, green and red vs. violet columns). Mitochondrial PKG may act as final
619 mediator involved in the activation of respiration and MPTP control. PKG may
620 operate like PKA, which is known to activate mitochondrial oxidative
621 phosphorylation by phosphorylating the cytochromes of respiratory chain [33- 36].
622 However, in comparison to PKA action, the activation of mitochondrial respiration

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623 by PKG could reinforce the regulation of oxidative phosphorylation by calcium, by
624 balancing the effect of direct activation of key dehydrogenases of the Krebs cycle
625 by calcium ions [8].

626 In high concentrations L-arginine (above 500 μM) and NO-donors (above
627 100 μM) produce opposite action, by inhibiting mitochondrial respiration (Fig 1A
628 and 2) and promoting MPTP opening (Fig 5B and 5C, 6B and 6C; blue vs. violet
629 columns). This alteration in the direction of their effects may indicate on opposite
630 impact of NO and mtNOS/PKG-SS on both processes. Apparently, overproduction
631 of mitochondrial NO, resulting in subsequent inhibition of mitochondrial
632 respiratory chain and of other related systems, may overcome positive effects
633 provided by mtNOS/PKGSS (i.e. by mtPKG).

634 Another example, illustrating the involvement of mtNOS/PKG-SS in the
635 control of MPTP, is presented at Fig 4. This example demonstrates that protection
636 afforded by L-arginine depends on the types of substrates used. Fig 4A shows that
637 200 μM L-arginine prevents the dissipation of $\Delta\Psi m$ by PC excess in mitochondria
638 respiring on L-glutamate and pyruvate (green vs. black lines). This effect is
639 abrogated by PKG inhibitor KT5823 (red lines). However, in mitochondria
640 respiring on succinate plus rotenone at PC excess, protection provided by L-
641 arginine cannot be realized (Fig. 4B, green vs. black lines). Observed difference of
642 L-arginine effects may be explained by the fact that in the last case the Krebs cycle
643 and substrate-level phosphorylation are not functioning and mitochondrial

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644 oxidative phosphorylation cannot keep appropriate level of GTP required for
645 cGMP production and activation of PKG.

646 It is worth to note that mtNOS/PKG-SS cannot be considered as redundant
647 element in multi level control of oxidative phosphorylation and MPTP. Being
648 calcium-dependent, this signaling system may be involved in the functioning of
649 several feedback and feedforward regulatory mechanisms, two of which are more
650 obvious and directed on the activation of mitochondrial respiration and MPTP
651 control. In this case mitochondrial PKG, along with cytosolic PKG1, might be
652 considered as final mediator of protection involved in MPTP control.

653 It is well known that dozens of kinases and phosphatases are translocated to
654 and localized in mitochondria [52, 53] and part of them are having as the targets
655 structural (or functional) components of MPTP complex [31, 41]. However, direct
656 evidence of PKG and GC translocation into mitochondria remains to be open
657 question.

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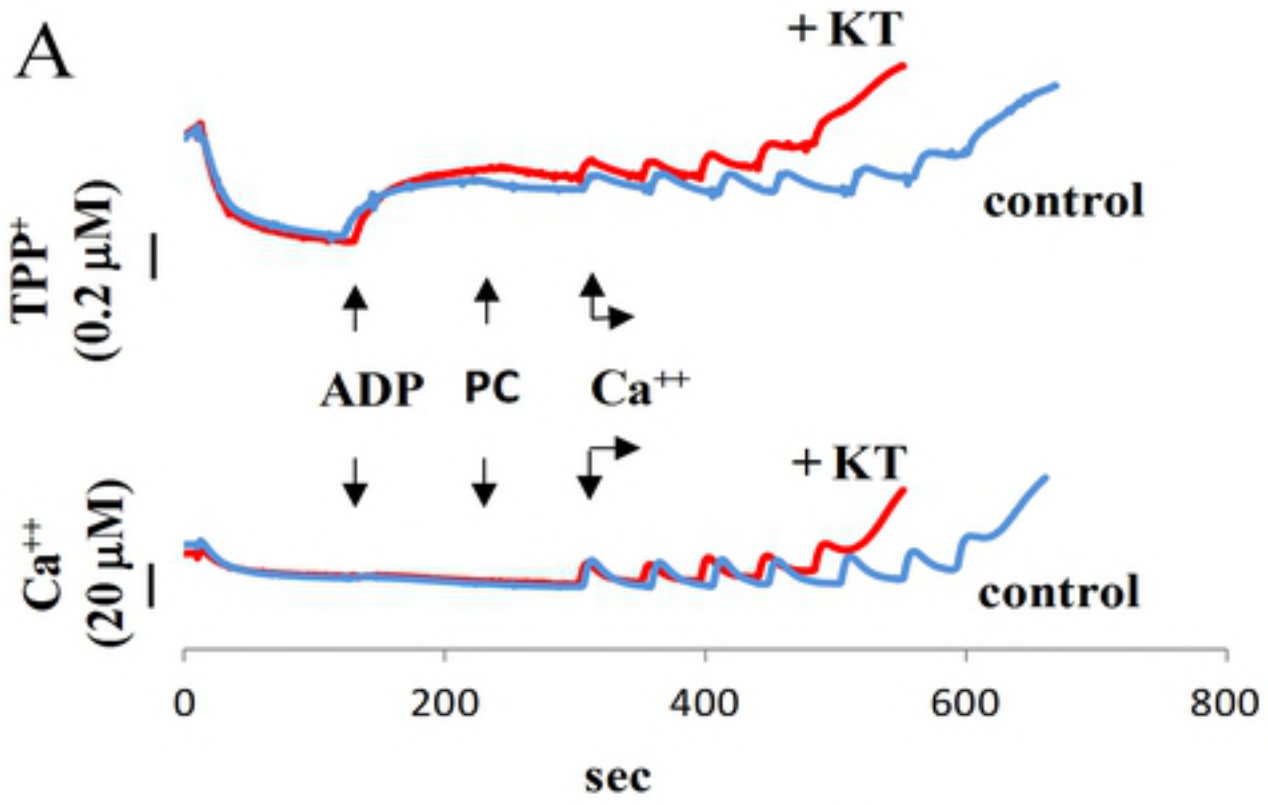

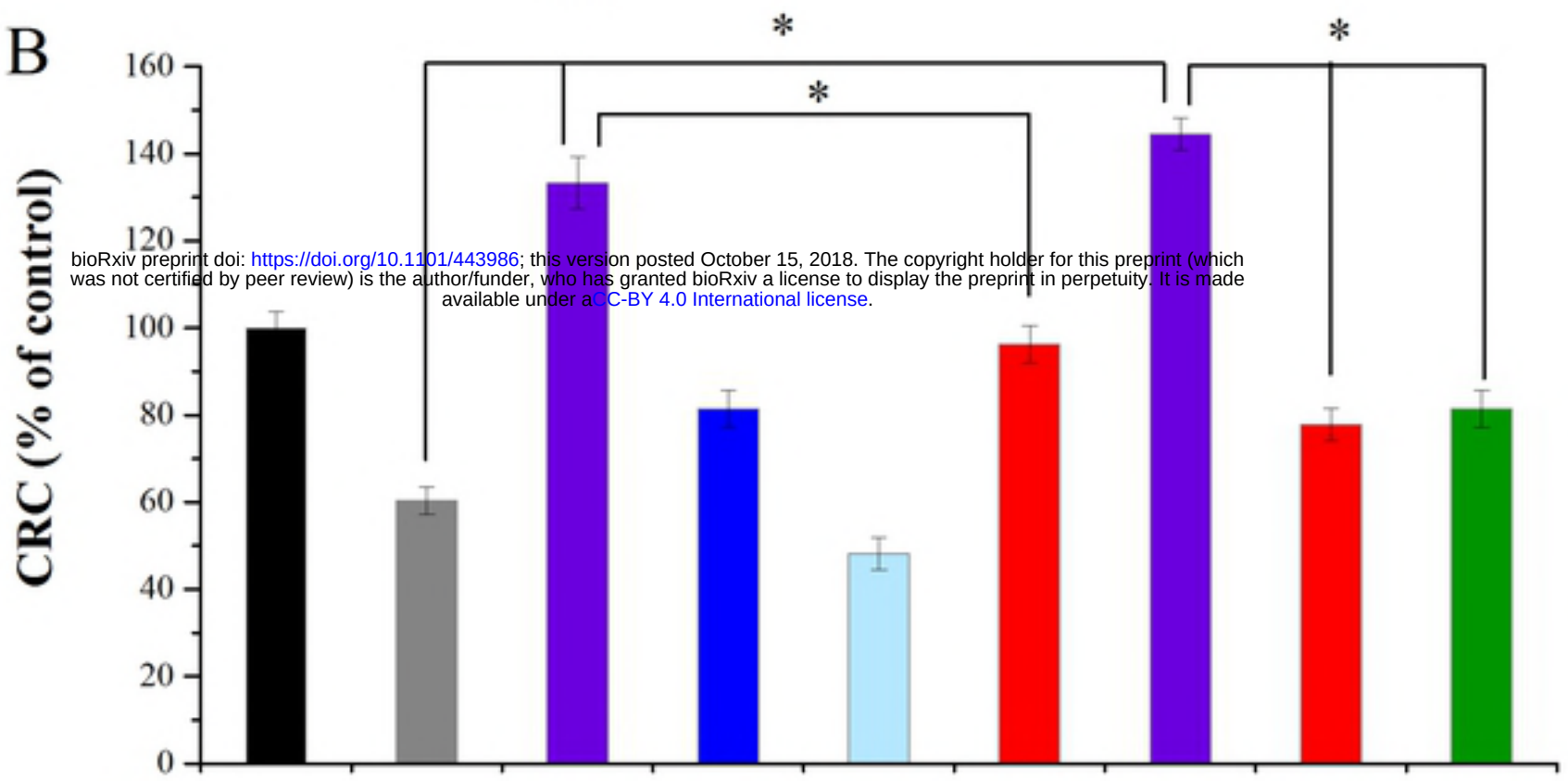

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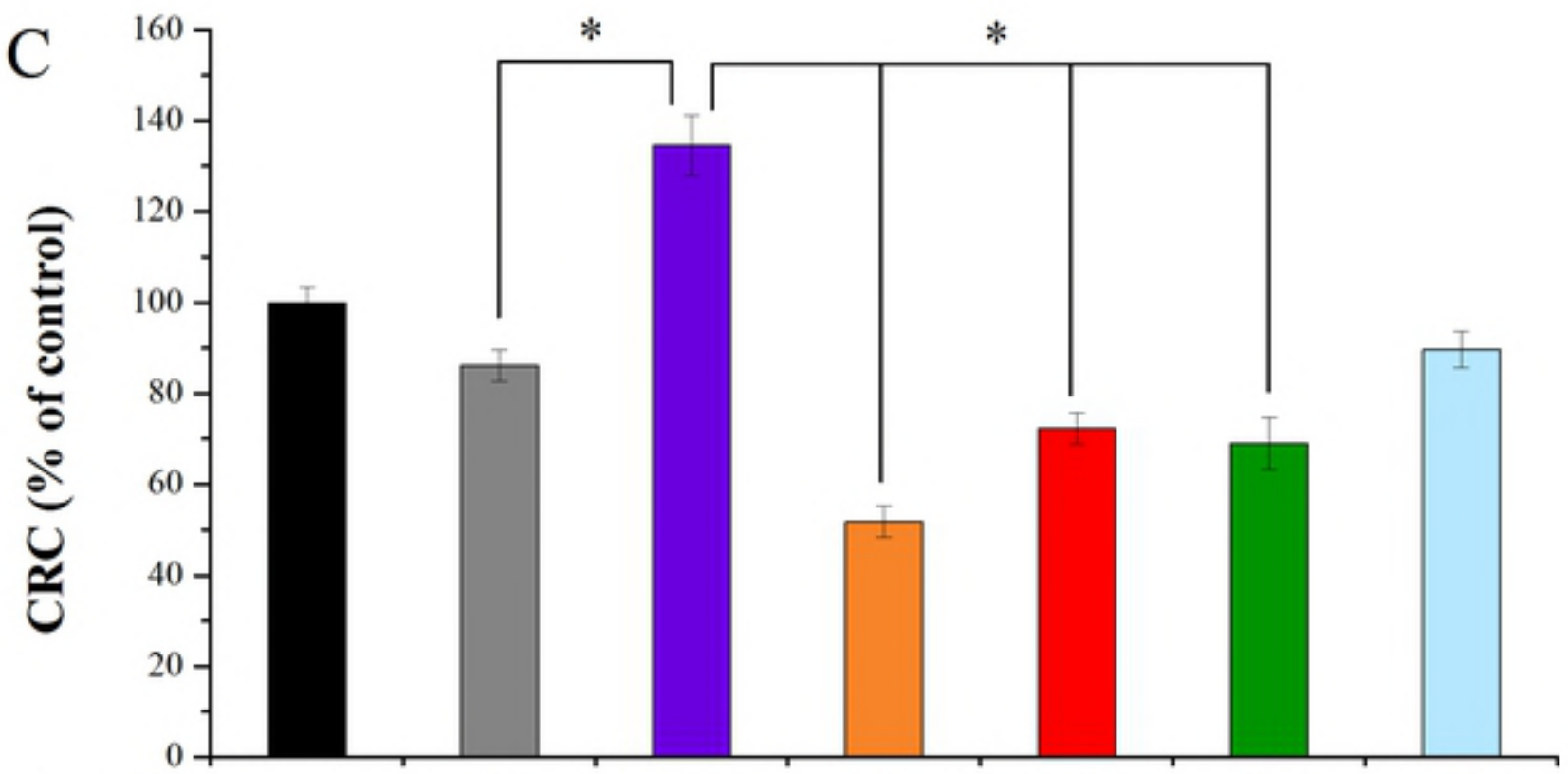
PC	-	20	20	20	20	20	20	20	20
SNP	-	-	50	100	200	50	-	-	-
ODQ	-	-	-	-	-	100	-	100	-
SNAP	-	-	-	-	-	-	50	50	-
KT	-	-	-	-	-	-	-	-	2



PC	-	20	20	20	20	20	20
L-Arg	-	-	500	500	500	500	2000
7NI	-	-	-	100	-	-	-
ODQ	-	-	-	-	100	-	-
KT	-	-	-	-	-	2	-

Fig6

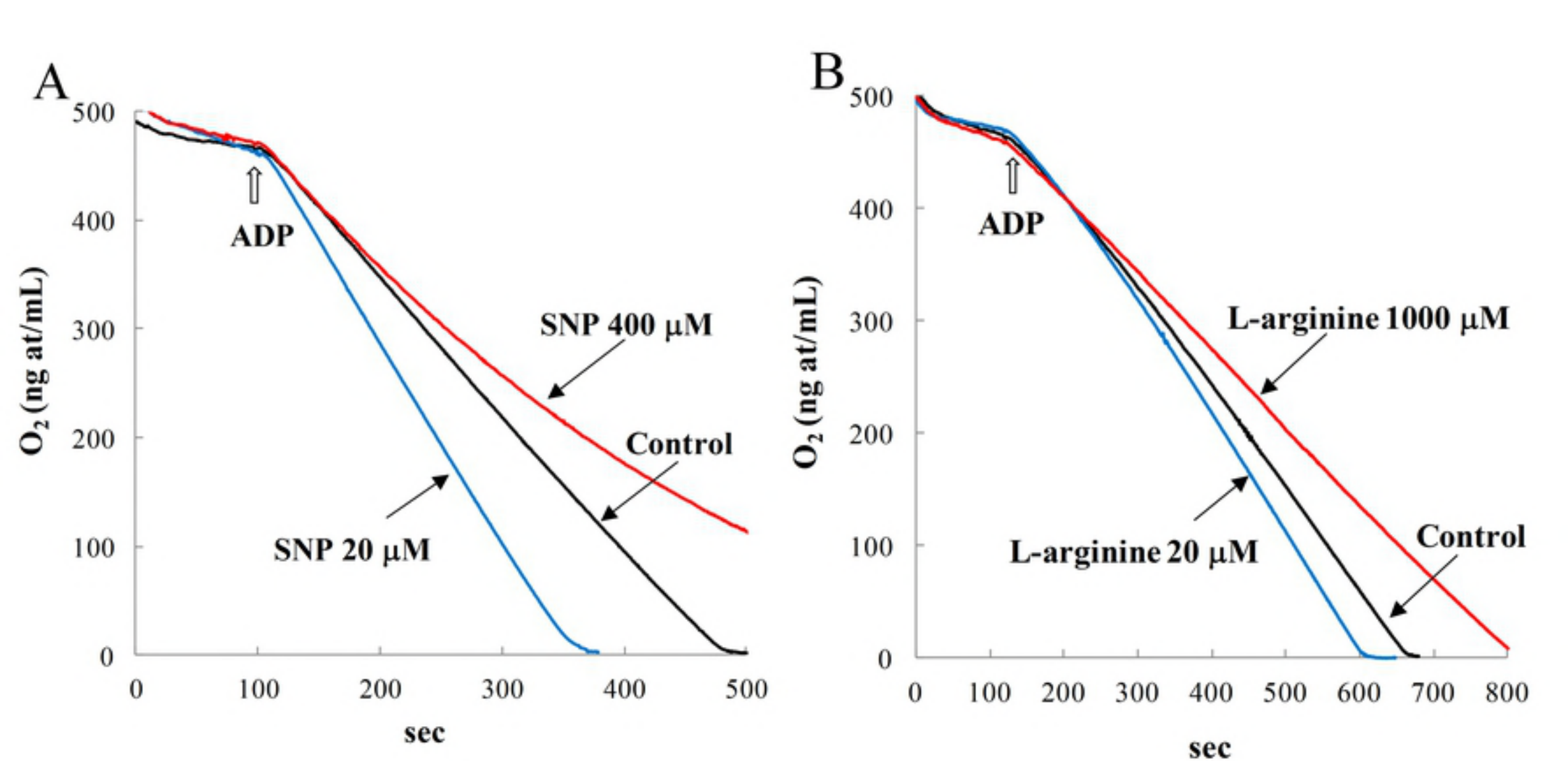
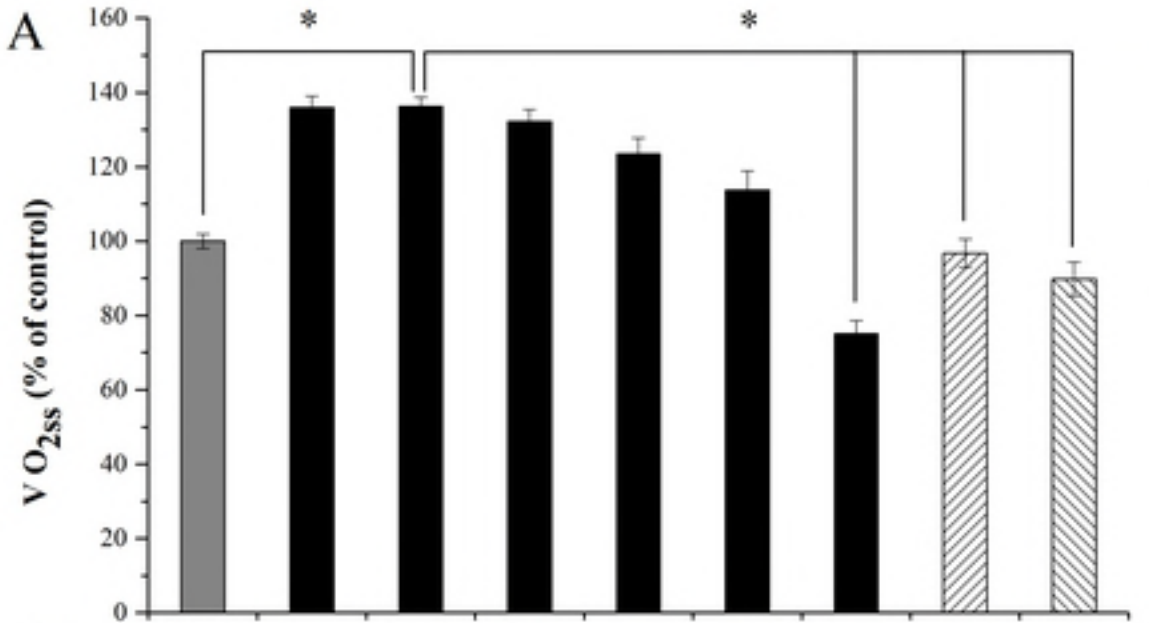
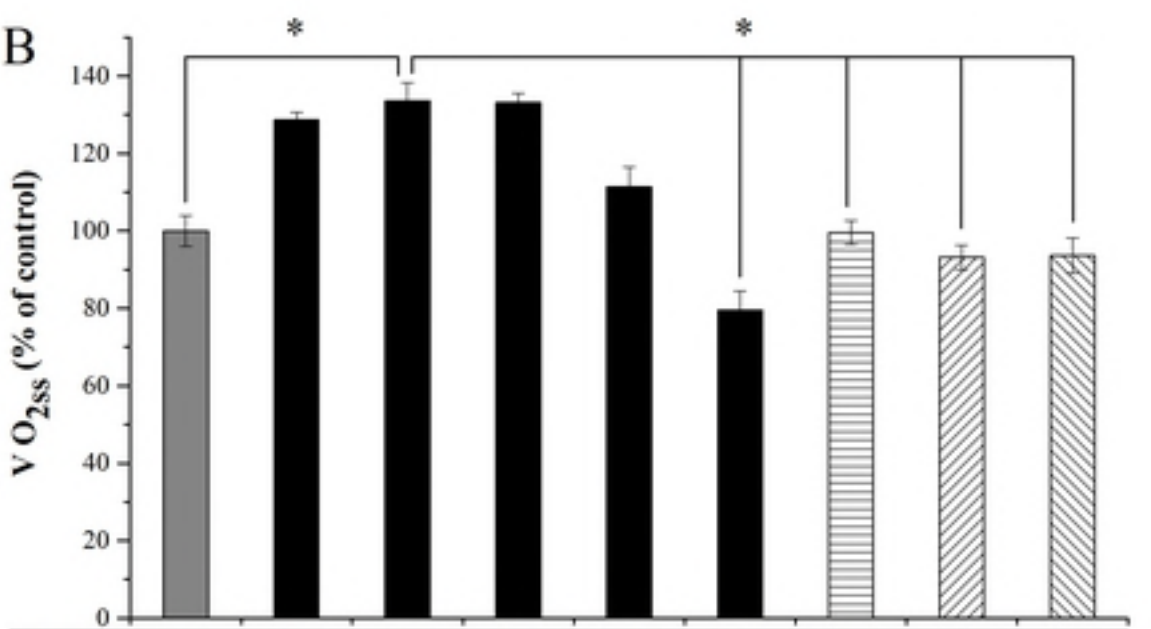

A
500
400
300
200
100
0
O2 (ng at/mL)
ADP
SNP 400 μM
Control
SNP 20 μM
0
100
200
300
400
500
sec
B
500
400
300
200
100
0
O2 (ng at/mL)
ADP
L-arginine 1000 μM
L-arginine 20 μM
Control
0
100
200
300
400
500
600
700
800
sec

Fig1



SNP	0	10	20	50	100	200	400	20	20
ODQ	-	-	-	-	-	-	-	100	-
KT	-	-	-	-	-	-	-	-	1,5



L-Arg	0	10	20	100	500	1000	20	20	20
7-NI	-	-	-	-	-	-	-	100	-
ODQ	-	-	-	-	-	-	-	100	-
KT	-	-	-	-	-	-	-	-	1,5

Fig2

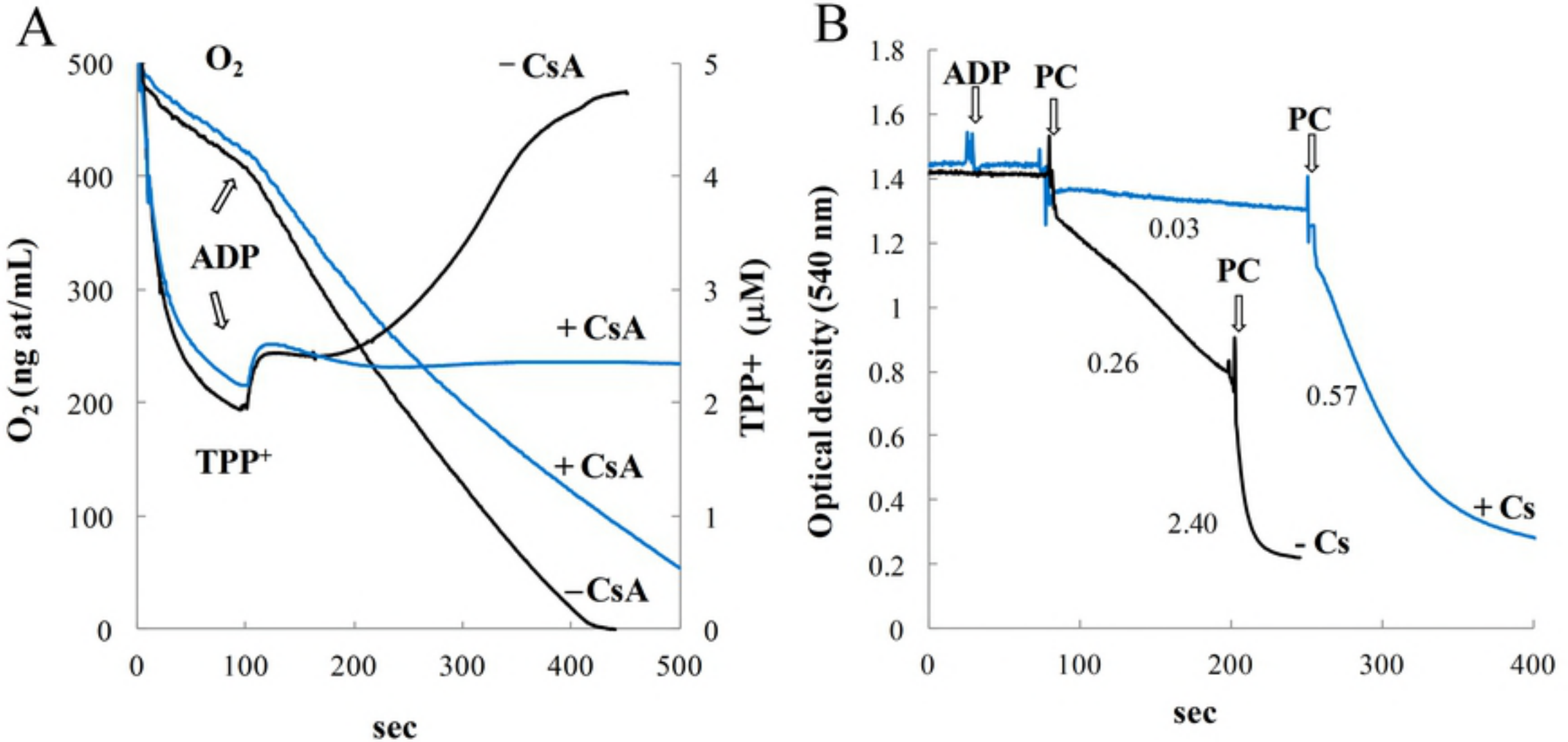

A
O2
−CsA
ADP
+CsA
TPP+
+CsA
−CsA
O2 (ng at/mL)
TPP+ (μM)
sec
B
ADP
PC
PC
0.03
PC
0.26
0.57
+Cs
2.40
- Cs
Optical density (540 nm)
sec

Fig3

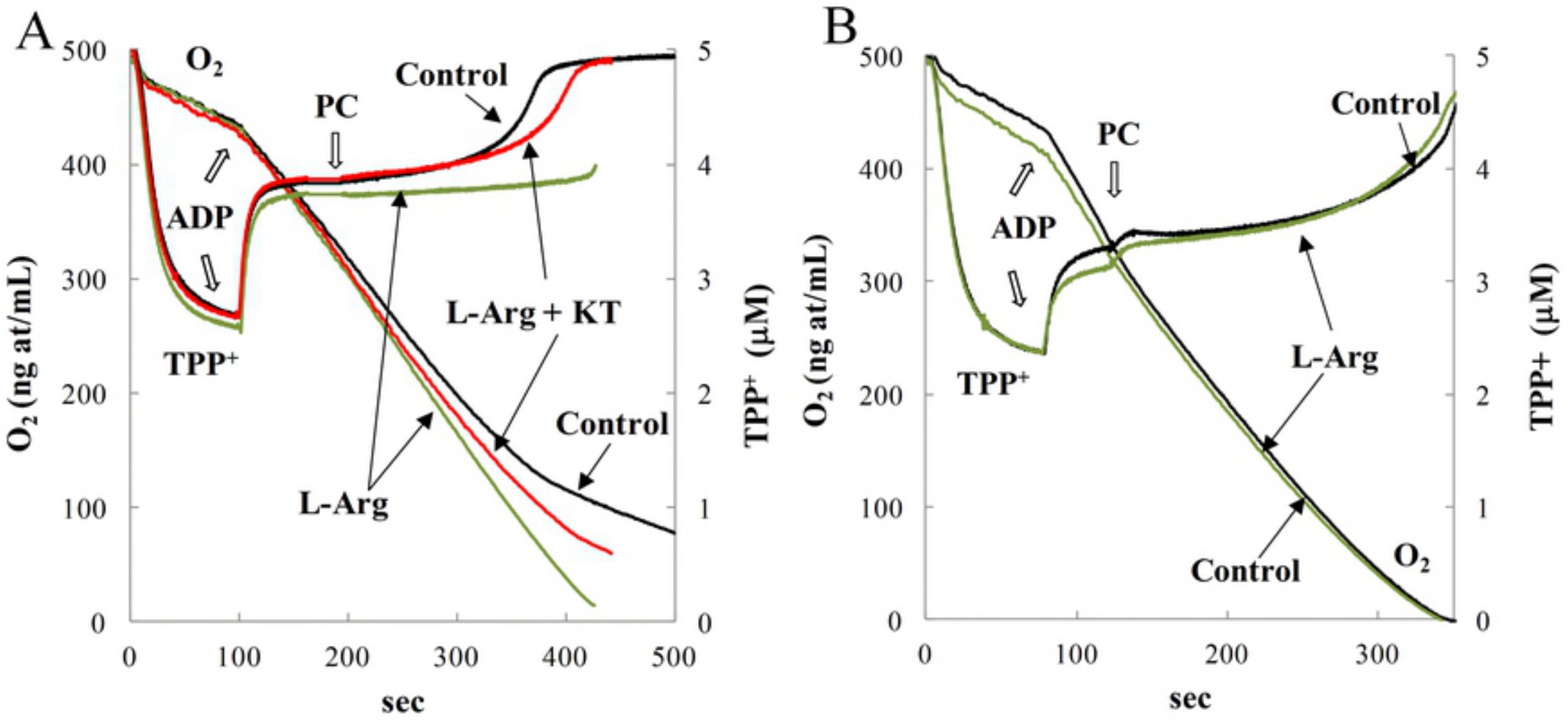
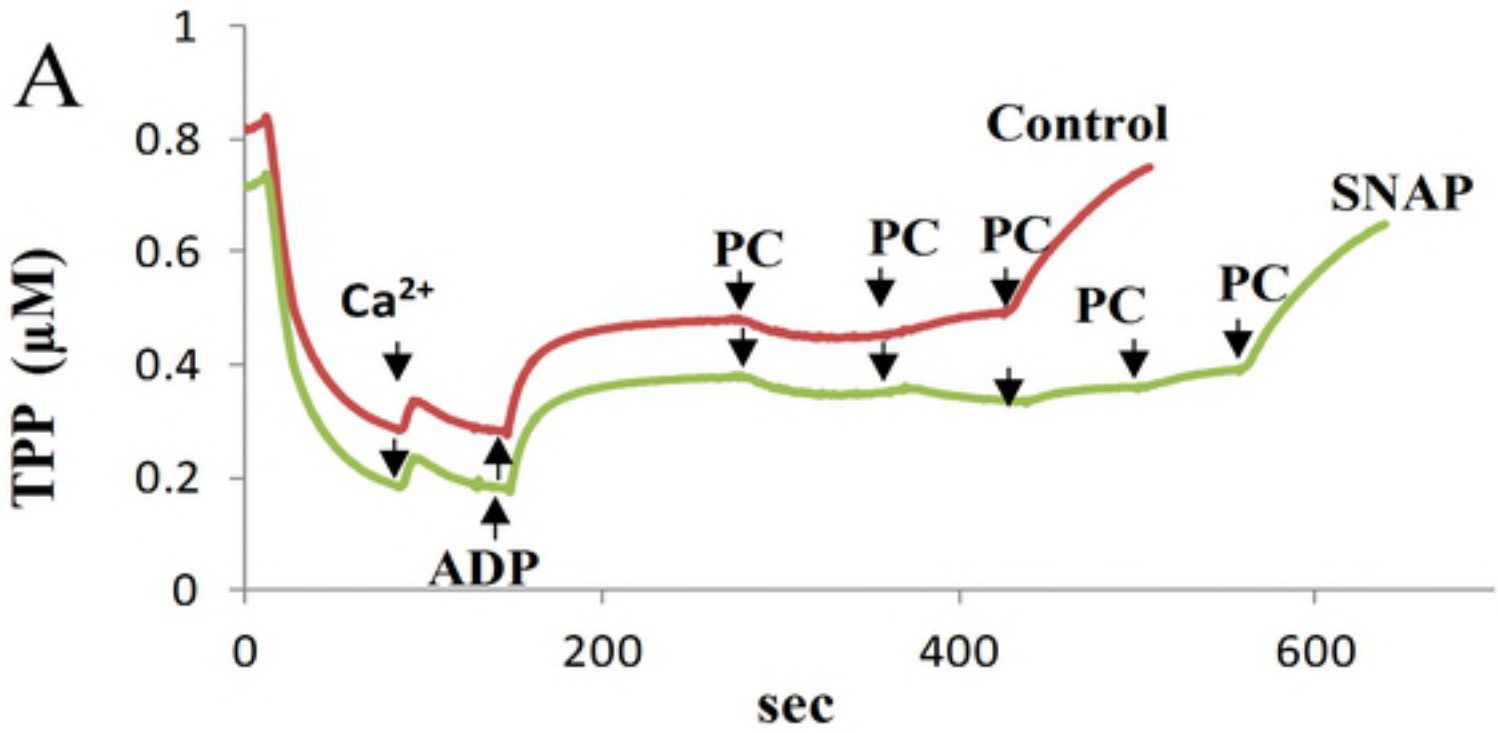
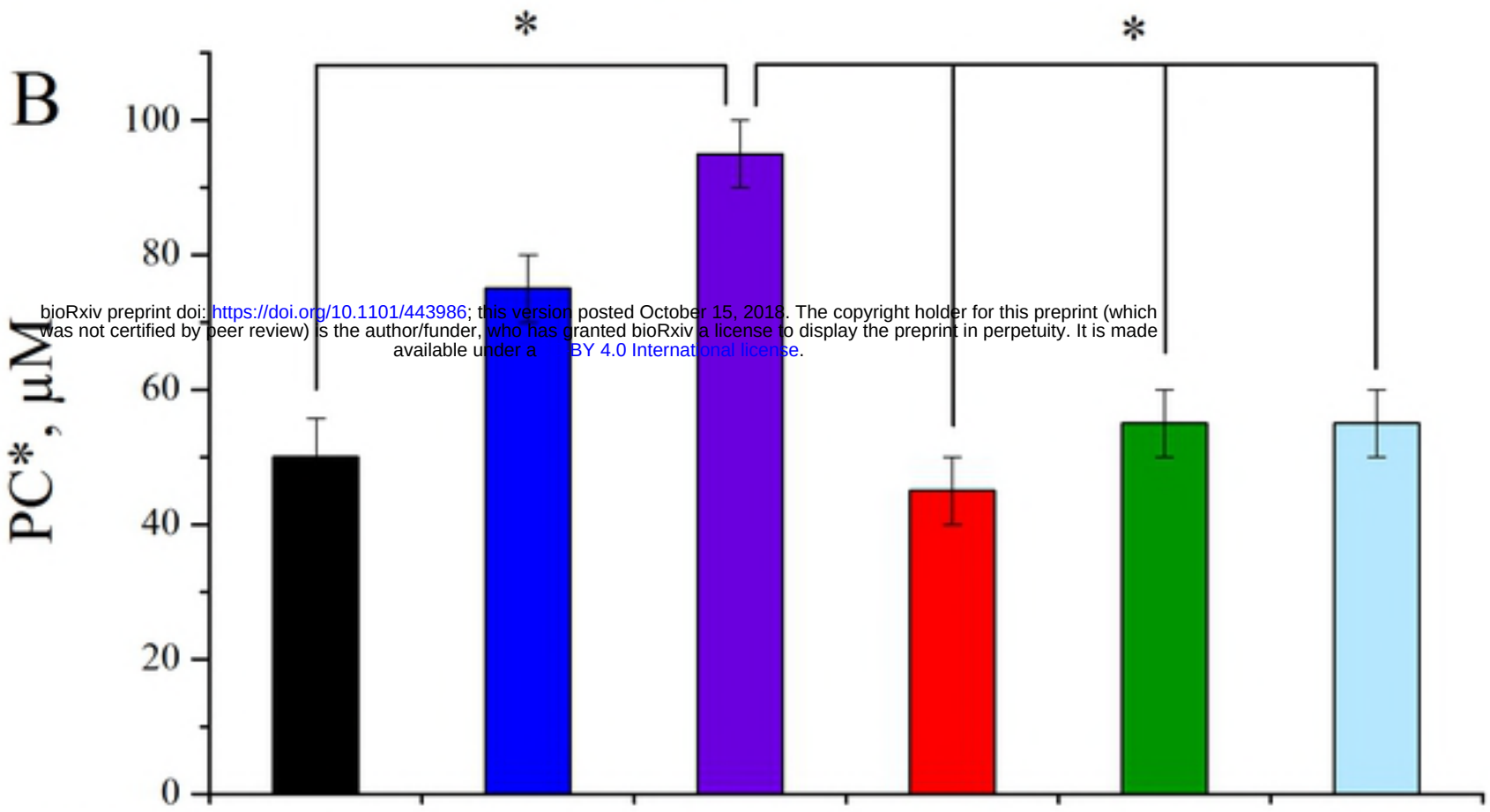

A
O2
PC
Control
ADP
TPP+
L-Arg + KT
L-Arg
Control
O2 (ng at/mL)
TPP+ (μM)
sec
0
100
200
300
400
500
1
2
3
4
5
B
PC
Control
ADP
TPP+
L-Arg
Control
O2
O2 (ng at/mL)
TPP+ (μM)
sec

Fig4





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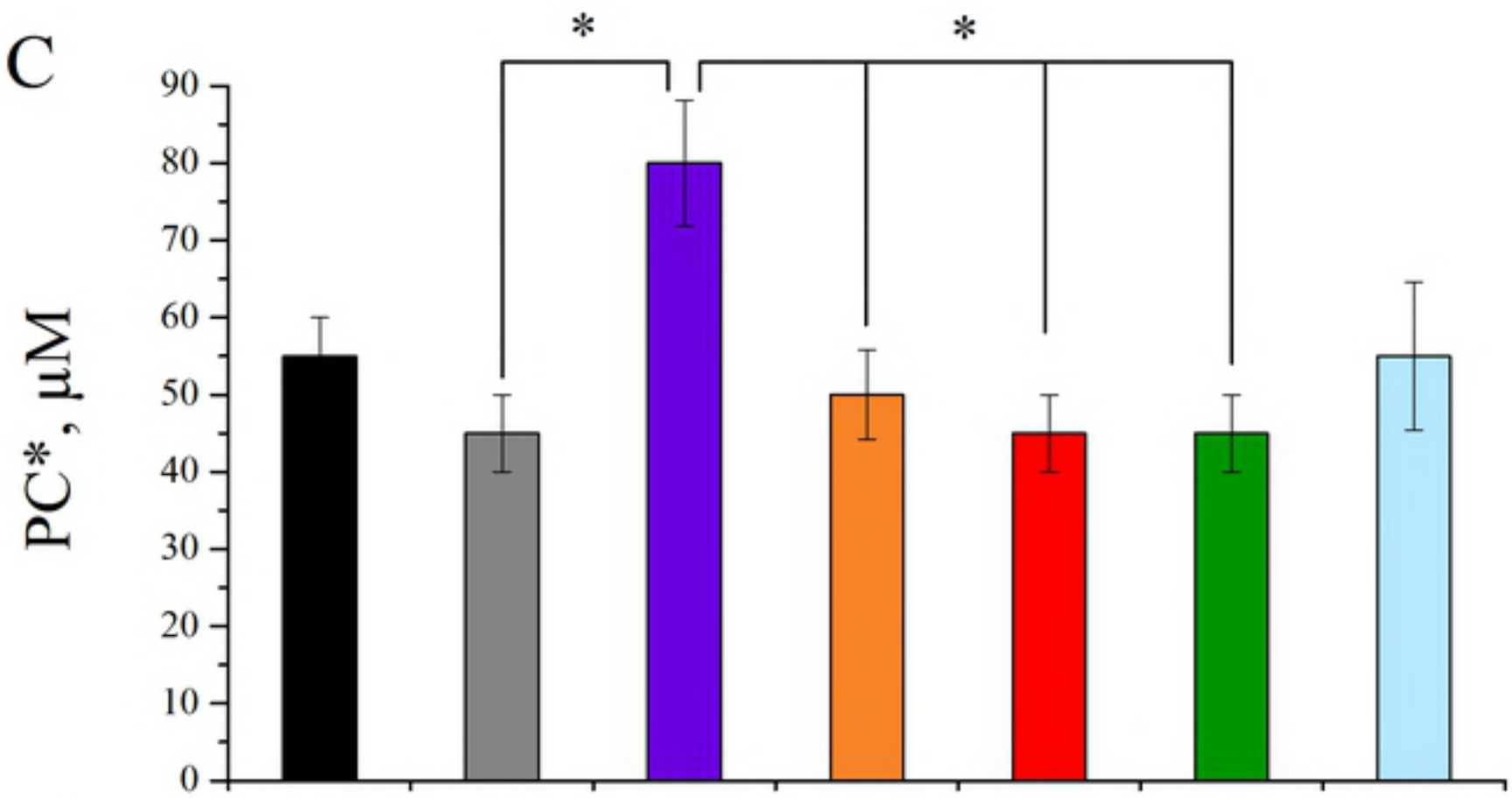
SNAP	-	50	100	100	100	300
ODQ	-	-	-	100	-	-
KT	-	-	-	-	2	-



Ca++	-	20	20	20	20	20	20
L-Arg	-	-	500	500	500	500	2000
7NI	-	-	-	50	-	-	-
ODQ	-	-	-	-	100	-	-
KT	-	-	-	-	-	2	-

Fig5