Physiological Research Pre-Press Article

# Mitochondrial phospholipase $A_2$ activated by reactive oxygen species in heart mitochondria induces mild uncoupling

**Jan Ježek, Martin Jabůrek, Jaroslav Zelenka, and Petr Ježek**‡

Department of Membrane Transport Biophysics, No.75, Institute of Physiology, Academy of Sciences, Vídeňská 1083, Prague, 14220 Czech Republic

‡ Address correspondence to: Petr Ježek, PhD, DSc, Department of Membrane Transport Biophysics, No.75, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague, 14220 Czech Republic. Fax: 011-420-296442488; E-mail: jezek@biomed.cas.cz

*Short title:* ***Mitochondrial phospholipase $A_2$ oxidative stress protection***

# Summary

Homeostasis of reactive oxygen species (ROS) in cardiomyocytes is critical for elucidation of normal heart physiology and pathology. Mitochondrial phospholipases $A_2$ (mt-$PLA_2$) have been previously suggested to be activated by ROS. Therefore, we have attempted to elucidate physiological role of such activation. We have found that function of a specific i-isoform of mitochondrial phospholipase $A_2$ (mt-i$PLA_2$) is activated by *tert*-butylhydroperoxide in isolated rat heart mitochondria. Isoform specificity was judged from inhibition by bromoenol lactone (BEL), a specific i$PLA_2$ inhibitor. Concomitant uncoupling has been caused by free fatty acids, since it was inhibited by bovine serum albumin. The uncoupling was manifested as a respiration burst accompanied by a slight decrease in mitochondrial inner membrane potential. Since this uncoupling was sensitive to carboxyatractyloside and purine nucleotide di- and tri-phosphates, we conclude that it originated from the onset of fatty acid cycling mediated by the adenine nucleotide translocase (major contribution) and mitochondrial uncoupling protein(s) (minor contribution), respectively. Such a mild uncoupling may provide a feedback downregulation of oxidative stress, since it can further attenuate mitochondrial production of reactive oxygen species (ROS). In conclusion, ROS-induced function of cardiac mt-i$PLA_2$ may stand on a *pro*-survival side of ischemia-reperfusion injury.

*Key words:* ***heart mitochondrial phospholipase $A_2$; fatty acids; uncoupling of mitochondria; adenine nucleotide translocase; defence against oxidative stress.***

# Introduction

Detailed understanding of homeostasis of reactive oxygen species (ROS) in cardiomyocytes is critical for elucidation of normal heart physiology, understanding of pathological phenomena of ischemia-reperfusion (I/R) injury, as well as for related ischemic preconditioning or "late window" phenomena after I/R (Costa and Garlid 2009; Garlid *et al*. 2009; Ježek *et al*. 2009). Mitochondria and, especially, cardiac mitochondria represent a major cellular ROS source that affects redox equilibrium even in the extra-cellular matrix (Ježek and Hlavatá 2005; Ježek and Plecitá-Hlavatá 2009). Total rates of superoxide ($O_2^{\bullet -}$) formation without detoxification (as evaluated in heart submitochondrial particles) can reach 1 nmol $O_2^{\bullet -}\cdot min^{-1}\cdot$(mg mitochondrial protein)$^{-1}$, *i.e*. an order of 1% of $O_2$ consumption. The *in vivo* net steady-state $O_2^{\bullet -}$ concentrations in the mitochondrial matrix are 100 to 200 pmol.l$^{-1}$ due to the presence of 10 to 40 μmol.l$^{-1}$ Mn-superoxide dismutase (MnSOD; Boveris and Cadenas 1997). The evaluated efflux of undismuted $O_2^{\bullet -}$ *in vivo* might correspond to 40 pmol $O_2^{\bullet -}\cdot min^{-1}\cdot$(mg mitochondrial protein)$^{-1}$, the value measured by Han *et al*. (2003) as an efflux from rat heart mitochondria respiring with glutamate plus malate in non-phosphorylating state-4. Since the majority of $O_2^{\bullet -}$ is dismuted to $H_2O_2$, a rather constant flow of $H_2O_2$ from heart mitochondria occurs, contributing to the 10 nM to 100 nM steady-state cytosolic $H_2O_2$ concentrations, estimated to be at least 96% of the total cardiomyocyte $H_2O_2$ production (Boveris and Cadenas 2000). Among other components that affect redox homeostasis is lipid peroxidation that proceeds non-enzymatically in mitochondria as initiated by the highly reactive radicals, hydroperoxyl radical, $HO_2^{\bullet}$; carbonate radical anion, $CO_3^{\bullet -}$; hydroxyl radical, $^{\bullet}OH$; and by peroxynitrite, $OONO^-$ / OONOH (Ježek and Hlavatá 2005). Importantly, $H_2O_2$ and other ROS, including lipid peroxidation intermediates and products play not only pathological but also a signaling role (Gutierrez *et al*. 2006).

Another signaling role is played by lipids (Huang and Frohman 2009). Lipid signaling is not necessarily related to lipid peroxidation, which is however one of its major arms (Niki 2009; Zmijewski *et al.* 2005). Typical example of lipid signaling species is arachidonic acid, cleaved from phospholipids in plasma membrane by phospholipases $A_2$ ($PLA_2$). Also mitochondrial outer and inner membranes (OMM, IMM, respectively) are subjects of $PLA_2$ reaction. Likewise in plasma membrane, $PLA_2$ cleave the *sn*-2 ester bonds of phospholipids, where side chains are usually composed of unsaturated or polyunsaturated fatty acids (PUFA). The reaction leaves lysophospholipids within the OMM and IMM which may affect their integrity and permeability. Mitochondria have been reported to contain both $Ca^{2+}$-insensitive isoform of $PLA_2$ (Broekemeier *et al*. 2002; Williams and Gottlieb 2002) as well as $Ca^{2+}$-dependent $PLA_2$ isoform, presumably activated by superoxide (Guidarelli and Cantoni 2002). In humans, at least 15 different $PLA_2$ proteins are recognized, grouped in three classes: the secretory, $sPLA_2$ (requiring mM $Ca^{2+}$ levels and lacking specificity for arachidonyl; groups I to III, V, IX to XII); the cytosolic $cPLA_2$, usually $Ca^{2+}$-dependent; arachidonyl specific $PLA_2$ enzymes (group IV); and cytosolic $Ca^{2+}$- independent, iPLA2 enzymes. Apart of this classification, one mitochondrial (mt) $PLA_2$ isoform as a lower $M_w$ enzyme was originally thought to belong to group IIA of $sPLA_2$ (Broekemeier *et al*. 2002; Williams and Gottlieb 2002). This original classification intended to explain its insensitivity to arachidonyl-trifluoromethyl ketone ($AACOCF_3$), a specific $cPLA_2$ inhibitor (Thommesen *et al*. 1998). In turn, the $cPLA_2\gamma$ isoform was reported to be located in endoplasmic reticulum and also in mitochondria and to have lysophospholipase activity beside phospholipase $A_2$ activity (Mancuso *et al*. 2004; Yamashita *et al*. 2009). Activation of mt-$PLA_2$ reportedly by superoxide (which was in turn promoted by peroxynitrite inhibition of Complex III) may be a key feature. Also, liver mt-$PLA_2$ was reported as stimulated by superoxide (Wilkins *et al*. 2008). However, Broekemeier *et al*. (2002) found mt-$PLA_2$ which was $Ca^{2+}$ -independent and was indicated by *anti*-$iPLA_2$ antibodies. One can see that a selection has yet to be determined, which $PLA_2$ isoforms act on OMM (these do not need to be specifically mitochondrial isoforms) and which are imported to the intermembrane space or matrix and act on IMM.

Physiological role of mt-$PLA_2$ may lie in balancing mitochondrial biogenesis on the side of biodegradation. It is suggested that mt-$PLA_2$ role lies in removal of poorly functioning mitochondria by participation in an autolysis process. Likewise the other c-isoforms, mt-$PLA_2$ might cleave preferentially arachidonic acid. At ongoing lipid peroxidation mt-$PLA_2$ would cleave also peroxidized fatty acids (FAOOH) from the phospholipid side chains (PLOOH), to yield free FAOOH. This property might just mimic "activation by ROS". Indeed, distinction between a direct interaction of certain ROS species with the enzyme from the higher probability to cleave off FAOOH molecules as ultimate ROS species should be made. Mitochondrial $iPLA_2$ was also reported to modulate the cytochrome c release from mitochondria and influence the permeability transition (Gadd *et al*. 2006). Since lysophospholipids are known to accumulate in ischemic heart and to induce arrhythmia, the $cPLA_2\gamma$, that is abundant in heart, may have a protective role through clearance of lysophospholipids by its *trans*-acylation activity (Yamashita *et al*. 2009). A protective role of mt-iPLA$_2$ has also been demonstrated by other groups (Kinsey *et al.* 2008; Mancuso *et al*. 2007; Seleznev *et al*. 2006).

Since mt-PLA$_2$-liberated FAOOH or PUFA may induce a mild uncoupling of mitochondria, we have attempted in this work to investigate activation of cardiac mt-PLA$_2$ by ROS and study bioenergetic consequences. We have found that function of a likely specific i-isoform of mtPLA$_2$ is induced by *tert*-butylhydroperoxide in isolated rat heart mitochondria. Moreover, liberated FAOOH or PUFA caused a mild uncoupling, which was partly prevented by carboxyatractyloside and purine nucleotide di- and tri-phosphates. Purine nucleotides inhibited only slightly when added after carboxyatractyloside. Therefore, we conclude that FAOOH or PUFA induce uncoupling caused predominantly due to interaction with the adenine nucleotide translocase and by a minor part due to uncoupling protein(s). Such a mild uncoupling may provide a feedback downregulation of oxidative stress, since it is able to attenuate mitochondrial ROS production (Dlasková *et al*. 2008a,b). Thus ROS-induced function of cardiac mt-PLA$_2$ may stand on a pro-survival side of ischemia-reperfusion injury.

## Methods

### *Animals*

Wistar rats (250 to 275 g) were bred and housed in certified animal houses according to EU rules and according to the Institute of Physiology licensing committee approval, in accordance with the Guide for the Care and Use of Laboratory Animals (1985), NIH, Bethesda, or European Guidelines on Laboratory Animal Care.

### *Isolation of heart mitochondria*

Rat heart mitochondria were isolated by differential centrifugation in ice-cold isolation medium containing 180 mmol.l$^{-1}$ KCl, 5 mmol.l$^{-1}$ MOPS buffer, pH 7.2, 2 mmol.l$^{-1}$ EGTA, and 0.5% BSA according to a published procedure (Vaghy *et al*. 1981). The final mitochondrial pellet was washed by a re-suspension and centrifugation in the isolation medium lacking BSA. Protein was determined by the BCA method (Sigma).

### *High-resolution respirometry*

Simultaneous recording of mitochondrial oxygen concentration and consumption was measured using an Oxygraph 2k high-resolution respirometer (Oroboros, Innsbruck, Austria) supplemented with specifically optimized DatLAb analysis software, which allows smoothing of the time derivative of $O_2$ concentration according to the requirements of time resolution and signal stability. All data were collected using identical smoothing parameters. To assay the mitochondrial oxygen consumption, succinate, rather than pyruvate and malate, was chosen as a respiratory substrate to avoid potential complications due to the proposed pyruvate transport mediated by mitochondrial uncoupling proteins (Ježek and Borecký 1998). Mitochondria were allowed to respire with 10 mmol.l$^{-1}$ succinate plus rotenone (5 µmol.l$^{-1}$) in an assay medium containing 120 mmol.l$^{-1}$ KCl, 5 mmol.l$^{-1}$ K-MOPS, 1 mmol.l$^{-1}$ K-EGTA, 0.5 mmol.l$^{-1}$ K-phosphate, and 0.5 mmol.l$^{-1}$ $MgCl_2$, pH 7.2. Oligomycin (1 µl/ml) was added to set the non-phosphorylating state-4 conditions and avoiding ATPase-dependent changes in respiration when purine nucleotides, including ATP, were used in the assay.

### *Measurement of mitochondrial membrane potential*

Changes in the inner membrane potential, $\Delta\Psi_m$, were determined fluorometrically using 2 µmol.l$^{-1}$ TMRE, *i.e.* tetramethylrhodamine ethyl ester (Molecular Probes), at the excitation wavelength of 556 nm, while collecting emission wavelength at 577 nm (Scaduto and Grotyohann 1999) on a Shimadzu RF 5301 PC spectrofluorometer in the assay medium for respiration.

### *Determination of free fatty acids*

Free fatty acids from isolated mitochondria were determined using gas chromatography-mass spectrometry (GC-MS). Reaction mixture was mixed with 3 volumes of 2-propanol/n-heptane/2M phosphoric acid (40/10/1), and treated according to previously published procedure (Puttman *et al.* 1993). The resulting methylesters of free fatty acids were reconstituted in 100 µl of n-heptane and injected into GC-MS. The spectrometry was performed with an Agilent 6890 gas chromatography instrument coupled to an Agilent 5973 mass spectrometer and Agilent ChemStation software (Agilent Technologies, Palo Alto, CA) using parameters according to previously published procedure (Yang *et al*. 2009). Fatty acids were identified and quantified using purified standards (Sigma).

### *Quantification of lipid peroxidation*

Immediately after the high-resolution respirometry assay, mitochondria were frozen in liquid nitrogen and stored at -80$^0$C. Direct estimation of total lipid peroxides was provided by a commercial Lipid

Hydroperoxide assay kit (LPO assay kit, Cayman). Briefly, total lipid hydroperoxides were extracted into chloroform and detected by oxidation of chromogen Fe (II) thiocyanate. Absorbance of resulting Fe (III) thiocyanate, when absorbance was measured at 500 nm and compared with calibration curve prepared using purified 13-hydroperoxy-octadecadienoic acid. Therefore, results of this assay are not confounded by residual $H_2O_2$ or free iron. However, this assay was obscured by TBHP, hence $H_2O_2$ was also used in a parallel assay. Alternatively, mitochondria were treated in the assay medium with 5 µM $C_{11}$-BODIPY$^{581/591}$ and after the fluorescence signal was stabile, a real-time production of lipid peroxidation was studied, using a fluorometric assay based upon quenching of $C_{11}$-BODIPY$^{581/591}$ fluorescence (Drummen *et al*. 2002), excited at 570 nm (slit width 5 nm) and collected at 600 nm (slit width 10 nm) on a Shimadzu RF 5301 PC spectrofluorometer. The experimental conditions paralleled exactly those used during the respirometry assays.

## Results

*Tert-butylhydroperoxide increases respiration in rat heart mitochondria*

In order to mimic mitochondrial oxidative stress, we attempted to simulate ROS-induction of the cardiac mt-PLA$_2$ activity. We have used a classic, widely used, hydrophobic and more stable $H_2O_2$ derivative, *tert*-butylhydroperoxide (TBHP), which might induce mt-PLA$_2$ activity directly, thus simulating $H_2O_2$ as the most probable physiological candidate for activation, similarly as for extracellular matrix metalloproteinases (Nelson and Melendez 2004). Our first test has studied an effect of TBHP on mitochondrial state-4 (non-phosphorylating) respiration. Addition of TBHP to isolated rat heart mitochondria induced an increase in state-4 respiration from 43 ± 2 to 46 ± 1 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ (n=8), which corresponded to an increase of 3.0% ± 0.4% extent with regard to the maximum (FCCP-uncoupled respiration, Fig.1A). In the absence of bovine serum albumin (BSA, required for maximum coupling of heart mitochondria), respiratory control ratios, estimated as ratios of maximum to state-4 respiration, were 3.5 ± 0.1 (n=8). In the presence of 2.5 $\mu mol.l^{-1}$ BSA it was >4. Alternatively, traces of free $Fe^{2+}$ or other transition metals, present in isolated mitochondria, might initiate Fenton reaction yielding $^{\bullet}OH$, and hence initiate lipid peroxidation. However, under our experimental conditions, no significant changes in TBHP-induced respiration were observed when 1 $mmol.l^{-1}$ deferoxamine mesylate, an iron chelator, has been added (Fig.1B) or when Fenton reaction was promoted exogenously by $FeSO_4$ and ascorbate (Fig.1B), indicating that the observed effect is not dependent on lipid peroxidation. We have also attempted to measure the lipid peroxidation directly using the LPO and $C_{11}$-BODIPY$^{581/591}$ assays, respectively (not shown). When using the $C_{11}$-BODIPY$^{581/591}$ assay, we could not detect any lipid peroxidation using the $H_2O_2$ and TBHP in the absence of $FeSO_4$. Changes in $C_{11}$-BODIPY$^{581/591}$ fluorescence could not be detected upon the addition of concentration of $FeSO_4$ lower than 5 $\mu$mol.l$^{-1}$. However, higher concentration of $FeSO_4$ leading to detectable changes in fluorescence were not compatible with mitochondrial integrity, as judged from respiratory rates increasing up to the maximal uncoupled respiration and not sensitive to any of the inhibitors used during this study. When using the LPO assay, we also could not detect any lipid peroxides following the TBHP and $FeSO_4$ treatments. In fact, we were able to detect low nmol.l$^{-1}$concentrations of externally added hydroperoxy linoleic acid as a standard in the absence of mitochondria, but were unable to detect the externally added hydroperoxy linoleic acid in the presence of mitochondria. These results indicate fast mitochondria-dependent decomposition of lipid hydroperoxides that are potentially produced during our assay. In conclusion, our results cannot exclude the production of lipid hydroperoxides, but indicate that lipid peroxidation is not necessary for the observed TBHP-induced uncoupling.

### *Tert-butylhydroperoxide activates iPLA$_2$ in rat heart mitochondria*

Surprisingly, the respiratory increase has been largely prevented by the addition of bromoenol lactone (BEL), a specific $iPLA_2$ inhibitor (Fig.2A), but not by $AACOCF_3$. The inhibitors were added to the assay immediately after the mitochondria, thus allowing about 10 minutes of incubation time before the addition of TBHP. The sensitivity to BEL indicates a possible participation of the specific i-isoform of mt-$PLA_2$, which is not analogous to the $cPLA_2$, since $AACOCF_3$ does not affect it (Thommesen *et al.* 1998). The presumed liberation of free fatty acids (such as unsaturated, PUFA, or even FAOOH) has been further supported by the effect of BSA, which decreased the extent of respiratory acceleration in rat heart mitochondria (Fig.2B). In this experiment, BSA was titrated prior to the TBHP addition to eliminate endogenous free fatty acids. Thus when BSA reached 1.5 $\mu$mol.l$^{-1}$, no uncoupling by the endogenous free fatty acids was detected (not shown). The subsequent addition of TBHP still resulted in the respiration increase, which was reversed by another subsequent addition of 0.25 μmol.l$^{-1}$ BSA (Fig. 2B). To support our hypothesis, that the TBHP-induced effect is due to the release of free fatty acids, we have analyzed the samples obtained from the respiration assays, using gas chromatography-mass spectrometry (Fig. 2C). The data show significant ($p < 0.001$), TBHP-dependent increase in the relative concentration of free linoleic acid, which was prevented by BEL. The estimated absolute levels of free linoleic acid were 36 ± 3 nmol (mg mitochondrial protein)$^{-1}$ prior to the addition of TBHP, and 79 ± 6 nmol (mg mitochondrial protein)$^{-1}$ following the addition of TBHP. The results verify that the TBHP-dependent increase in respiration is caused by the release of free unsaturated fatty acids and further support the indication that this is an iPLA2-dependent process.

### *Tert-butylhydroperoxide via iPLA$_2$ induces uncoupling of rat heart mitochondria*

Monitoring of the inner membrane potential, $\Delta\Psi_m$, indicated a slight potential decrease upon the TBHP addition to rat heart mitochondria (Fig.3A, B). Likewise respiration, the $\Delta\Psi_m$ drop was prevented by BSA and by BEL. Since the increased respiration at diminishing $\Delta\Psi_m$ in parallel strictly defines the uncoupled respiration, we can conclude that TBHP-induced mt-iPLA$_2$ activation liberates free fatty acids, including unsaturated fatty acids, PUFA, or possibly FAOOH, which concomitantly cause a mild uncoupling of mitochondria (Fig. 4A, B).

### *Tert-butylhydroperoxide via mt-iPLA$_2$ induces uncoupling due to adenine nucleotide translocase and uncoupling protein function*

The addition of carboxyatractyloside (CAT) after (Fig.4A, 5A) or prior to TBHP prevented partly the observed uncoupling (Fig.4A) as well as the $\Delta\Psi_m$ drop (Fig.5A). Similarly, GDP and GTP added after TBHP partially decreased the TBHP-elevated respiration (Fig.4.B) and raised $\Delta\Psi_m$ back to the original level (Fig.5B). These results suggest participation of free fatty acids inducing uncoupling in either adenine nucleotide translocase (termed also the ADP/ATP carrier), and certain isoforms of uncoupling proteins, such as UCP2, likely present in rat heart (Alán *et al.* 2009). This uncoupling may result from providing free fatty acids (by mt-PLA$_2$-mediated liberation) for fatty acid cycling mediated by these two members of the SLC25 gene family of mitochondrial anion carriers. Fatty acid-induced uncoupling mediated by the adenine nucleotide translocase has already been shown to be inhibited by carboxyatractyloside (Skulachev 1991), whereas if mediated by the uncoupling proteins, the reported fatty acid cycling is inhibited by purine nucleotide di- and triphosphates (Beck *et al*. 2007; Jabůrek *et al*. 1999; 2004; Ježek *et al*. 2004; Žáčková *et al*. 2003). In rat heart mitochondria the CAT-sensitive adenine nucleotide translocase component was much greater than the putative UCP2 contribution as documented by only a slight GTP effect after previously added CAT (Fig.4B).

## Discussion

Dealing with isolated rat heart mitochondria and still observing $PLA_2$ activity indicates that the participating phospholipase is indeed a specific mitochondrial $PLA_2$ isoform. It either tightly sticks to OMM and cannot be washed out during isolation of mitochondria or it must be located in the intermembrane space, from which it acts on the inner OMM leaflet and the outer IMM leaflet. It might be even located in the matrix. Such a matrix mt-$PLA_2$ would act on the inner IMM leaflet. This distinction is, however, out of scope of this paper. Here, we rather focused onto the consequences of mt-$PLA_2$ –catalyzed reaction. Nevertheless, due to the observation of inhibition by BEL, the i$PLA_2$-specific inhibitor, we can classify the heart mt-$PLA_2$ activated by TBHP as an iPLA2, in agreement with previously published findings (Williams and Gottlieb 2002; Mancuso *et al.* 2007).

Our observation of increased uncoupling, sensitive to BEL, suggests that our experiments reflect the mt-i$PLA_2$ reaction affecting IMM. Alternatively, the action of iPLA2 on OMM may lead to the release of free fatty acids (PUFA, FAOOH) that would be subsequently redistributed into IMM. The ability of tested inhibitors to restore the mitochondrial coupling upon the TBHP treatment has demonstrated that neither membrane has been severely damaged or affected by a potential lipoperoxidation. Theoretically, the observed uncoupling might originate from the lysophospholipids disturbing the IMM integrity. However, in this case the uncoupling could not be prevented by BSA, as observed. Consequently, fatty acid species are likely to be cleaved off the phospholipids due to mt-i$PLA_2$ reaction, an event that is supported by our data. Our results show that the addition of TBHP leads to a significant BEL-sensitive release of free linoleic acid, which is consistent with iPLA2-catalysed cleavage of cardiolipin, a phospholipid that is unique to mitochondrial inner membrane and that contains 90-95% linoleic acid (Lesnefsky *et al.* 2001).Why such a reaction is initiated upon the TBHP treatment? Since our data indicate that TBHP-induced lipid peroxidation is not necessary for the observed process, we speculate that TBHP acts as an $H_2O_2$ analogue directly activating mt-iPLA$_2$ function. $H_2O_2$ activation of mt-PLA$_2$ might be similar to the well described activation of extra-cellular matrix metalloproteinases (Nelson and Melendez 2004).

Once fatty acid, PUFA, or even FAOOH are released, they may cause uncoupling of mitochondria. The phenomenon of fatty acid-induced uncoupling has already been described in 1950s and has not yet been completely understood. The most plausible explanation, preferred by the authors, is based on the fatty acid cycling hypothesis. This hypothesis suggested by Skulachev (1991) predicts that certain IMM carriers, namely those belonging to the SLC25 gene family, are able to carry negatively charged (*i.e.* dissociated) fatty acid molecules. Consequently, since neutral (*i.e.* protonated) fatty acids readily flip-flop across the lipid bilayer membranes, the cycling is possible. In such cycling, protonated fatty acids translocate protons across the membrane (hence as protonophores do uncouple mitochondria), whereas upon deprotonation fatty acid anions are expelled from respiring mitochondria due to their negatively charged inner leaflet of IMM. One of the most convincing evidences of the fatty acid cycling hypothesis is based on the existence of so-called inactive fatty acid which do not flip-flop in a protonated form across the membrane and in parallel are unable to induce uncoupling (Ježek *et al.* 1997a,b). Most importantly, fatty acid cycling mediated by the adenine nucleotide translocase has been shown to be inhibited by its specific inhibitor carboxyatractyloside (Skulachev 1991), whereas purine nucleotide di- and tri-phosphates inhibit the presumed fatty acid cycling mediated by mitochondrial uncoupling protein UCP2 (Beck *et al.* 2007; Jabůrek *et al.* 1999; 2004; Ježek *et al.* 2004; Žáčková *et al.* 2003).

Since the observed TBHP-induced mt-iPLA$_2$-assisted release of free fatty acids has caused uncoupling that was sensitive to carboxyatractyloside and to GTP, we conclude that the adenine nucleotide translocase and UCP2 are major proteins enabling such an uncoupling in rat heart mitochondria. Involvement of the first one can be understood when we recall that this is the most abundant carrier of the SCL25 gene family in IMM. Carriers with higher abundance can naturally out-compete the other carriers for fatty acid anion binding followed by the uniport. The role of UCP2 in the heart has recently been emphasized by McLeod *et al.* (2005) as potential attenuator of mitochondria superoxide production. Our transcript screening for various UCP isoforms have reflected this finding (Alán *et al.* 2009). Surprisingly, UCP2 mRNA in the heart has been the third abundant after lung and spleen among the studied tissues.

However, there was nearly zero UCP2 transcripts present in mouse heart, indicating important difference between these two rodent models (Alán *et al*. 2009). This fact excludes the use of the UCP2-KO mice for confirmation of our interpretation ascribing the GTP-sensitive uncoupling to UCP2. In spite of the fact that even adenine nucleotide translocase binds also GTP with much lower affinity and the GTP effect on TBHP-induced uncoupling might be ascribed predominantly to the adenine nucleotide translocase, we conclude that both UCP2 and the adenine nucleotide translocase participate in the observed effect, while the latter has major contribution.

Our principal finding has shown that these two proteins may act in concert with the mitochondrial phospholipase-$A_2$, mt-iPL$A_2$, and may provide a protective role in attenuation of the mitochondrial superoxide production due to mild uncoupling. The fact that mild uncoupling attenuates superoxide production even formed within the Complex I has been recently reported by our group (Dlasková *et al*. 2008a, b). The mild translocase- and UCP2- mediated uncoupling is enabled by mt-iPL$A_2$-assisted release of free fatty acids, PUFA and possibly FAOOH which are cleaved off phospholipids, namely cardiolipin. We show that elevated concentrations of TBHP (corresponding to $H_2O_2$ *in vivo*) lead to activation of mt-iPL$A_2$. Thus we suggest a protective role of mt-iPL$A_2$ in all situations when oxidative stress is elevated in the heart. For example ischemic preconditioning has been shown to enhance fatty acid-dependent mitochondrial uncoupling (Carreira *et al*. 2007) and we may speculate that also mt-iPL$A_2$-assisted release of free fatty acids participates in this phenomenon.

## Acknowledgements

Supported by grants 303/07/0105 from the Grant Agency of the Czech Republic; ME09018 from the Czech Ministry of Education, and KJB500110902 (to JJ) and AV0Z50110509 from the Academy of Sciences. We would like to thank Prof. Libor Vítek from the Institute of Clinical Biochemistry and Laboratory Diagnostics, 1st Faculty of Medicine, Charles University, Prague, for generously providing us with GC-MS.

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## FIGURE LEGENDS

Fig. 1. ***Tert*****-butylhydroperoxide increases respiration in rat heart mitochondria (A) independently of lipid peroxidation (B)**. Time courses of $O_2$ consumption rates are illustrated, as directly calculated by the Oroboros oxygraph 2k. At the end of each run 50 nmol.$l^{-1}$ FCCP was added to reach maximum respiration. **Panel A**: Induction of respiratory increase by 25 μmol.$l^{-1}$ TBHP (**a**) and control measurement in the absence of TBHP (**b**). **Panel B**: Tests, whether traces of free $Fe^{2+}$ or other transition metals potentiate the TBHP-induced respiratory increase using chelator, 1 mmol.$l^{-1}$ deferoxamine mesylate, were negative (**a**) as well as testing, whether artificial Fenton reaction (induced by 1.25 μmol.$l^{-1}$ $FeSO_4$ and 10 μmol.$l^{-1}$ ascorbate) affects the increase (**b**) .

Fig. 2. ***Tert*****-butylhydroperoxide activates mt-iPLA$_2$ in rat heart mitochondria.**

**Panel A**: Time courses of $O_2$ consumption rates are illustrated analogously to Fig.1.The iPLA$_2$-specific inhibitor BEL (5 μmol.$l^{-1}$; "+BEL") added before 25 μmol.$l^{-1}$ TBHP prevented the respiration increase, whereas the cPLA$_2$-specific inhibitor AACOF$_3$ was without the effect. **Panel B**: Time courses of $O_2$ consumption rates are illustrated analogously to Fig.1. The TBHP-induced respiration increase was reversed by consecutive additions of 0.25 μmol.$l^{-1}$ BSA. In this experiment, BSA was titrated prior to the TBHP addition to eliminate endogenous free fatty acids. Thus when BSA reached 1.5 μmol.$l^{-1}$, no uncoupling by the endogenous free fatty acids was detected (not shown). The subsequent addition of TBHP still resulted in the respiration increase, which was reversed by the subsequent addition of 0.25 μmol.$l^{-1}$ BSA. **Panel C**: Relative changes in the concentrations of selected free fatty acids are plotted as a mean ± S.D. ($n = 3$). While the levels of saturated fatty acids remained invariable, the treatment with TBHP (25 μmol.$l^{-1}$; "+TBHP") caused significant increase in the relative concentration of free linoleic acid ($p < 0.001$). The iPLA$_2$-specific inhibitor BEL (5 μmol.$l^{-1}$; "+BEL") added before TBHP prevented the linoleic acid release.

Fig. 3. ***Tert*-butylhydroperoxide induces decline in membrane potential of rat heart mitochondria**. In all assays 20 nmol.l$^{-1}$ FCCP has been added at the end of each run. **Panel A**: Monitoring of the inner membrane potential, $\Delta\Psi_m$, indicated a slight potential decrease upon the addition of TBHP (25 μmol.l$^{-1}$) to rat heart mitochondria, prevented by iPLA$_2$-specific inhibitor BEL (10 μmol.l$^{-1}$; "+BEL"). **Panel B**: Likewise respiration, the $\Delta\Psi_m$ drop was reversed by BSA (6.25 μmol.l$^{-1}$); compare to the bottom trace where TBHP was omitted ("no addition").

Fig. 4. ***Tert*-butylhydroperoxide-induced respiration is reversed by inhibitors of adenine nucleotide transclocase (A) and uncoupling proteins (B)**. Time courses of $O_2$ consumption rates are illustrated analogously to Fig.1. **Panel A**: 2 μmol.l$^{-1}$ carboxyatractyloside (CAT), a specific translocase inhibitor was added after 1 mmol.l$^{-1}$ GTP in the absence or presence of 5 μmol.l$^{-1}$ BEL ("+BEL"). **Panel B**: 1 mM GTP was added after 2 μmol.l$^{-1}$ CAT.

Fig. 5. ***Tert*-butylhydroperoxide-induced decline in membrane potential is reversed by inhibitors of adenine nucleotide transclocase (A) and uncoupling proteins (B)**. In all assays 20 nmol.l$^{-1}$ FCCP has been added at the end of each run. **Panel A**: Monitoring of the inner membrane potential, $\Delta\Psi_m$, indicated a slight potential decrease upon the addition of TBHP (25 μmol.l$^{-1}$) to rat heart mitochondria, reversed by 1 μmol.l$^{-1}$ CAT and prevented by iPLA$_2$-specific inhibitor BEL (10 μmol.l$^{-1}$; "+BEL"). **Panel B**: Likewise respiration, the $\Delta\Psi_m$ drop was reversed by 1 mmol.l$^{-1}$ GTP; compare to the bottom trace where TBHP was omitted ("no addition").

Fig.1.

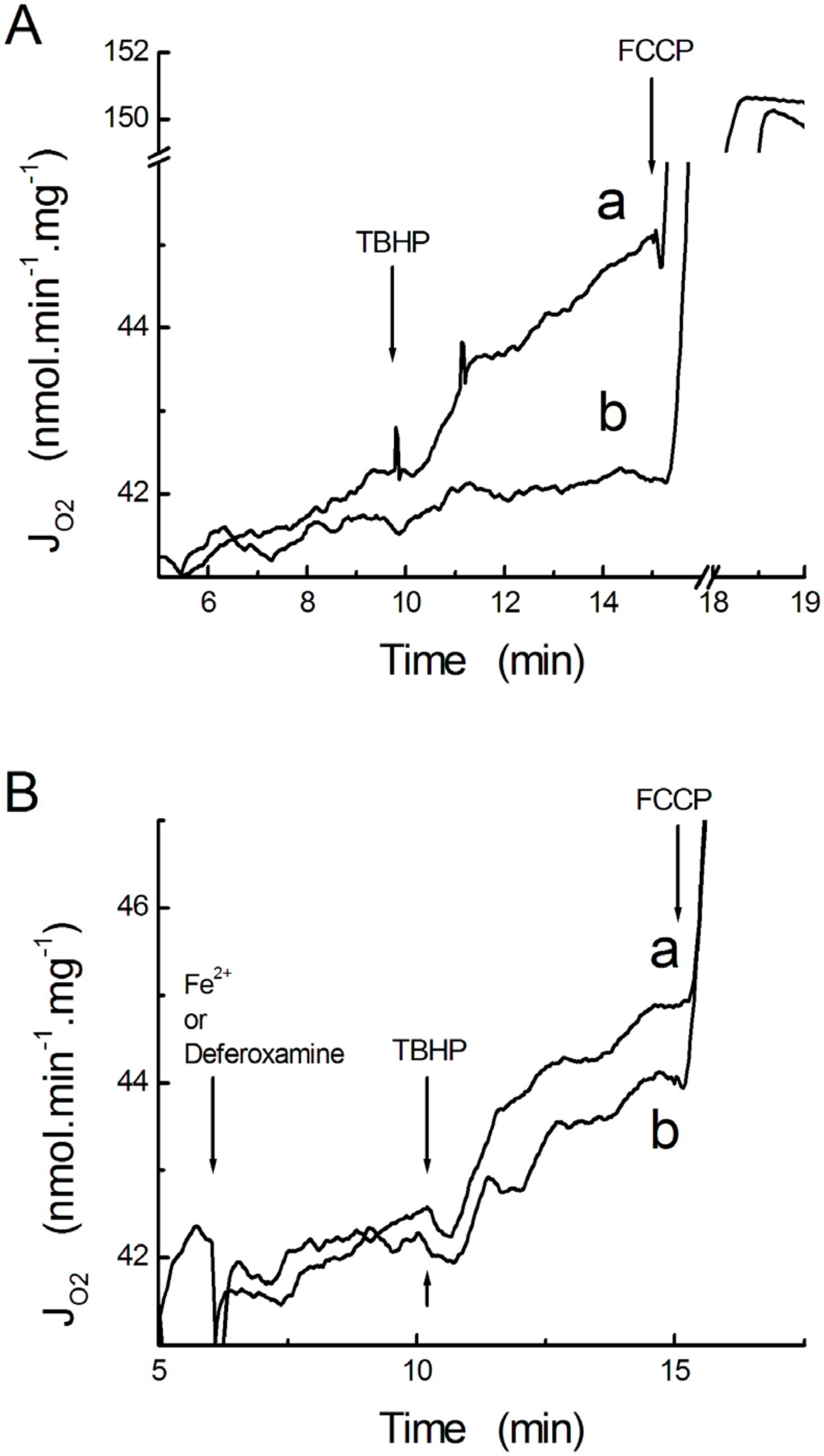

A
152
150
44
42
$J_{O2}$ (nmol.min$^{-1}$.mg$^{-1}$)
TBHP
FCCP
a
b
6
8
10
12
14
18
19
Time (min)
B
46
44
42
$J_{O2}$ (nmol.min$^{-1}$.mg$^{-1}$)
Fe$^{2+}$
or
Deferoxamine
TBHP
FCCP
a
b
5
10
15
Time (min)

Fig.2.

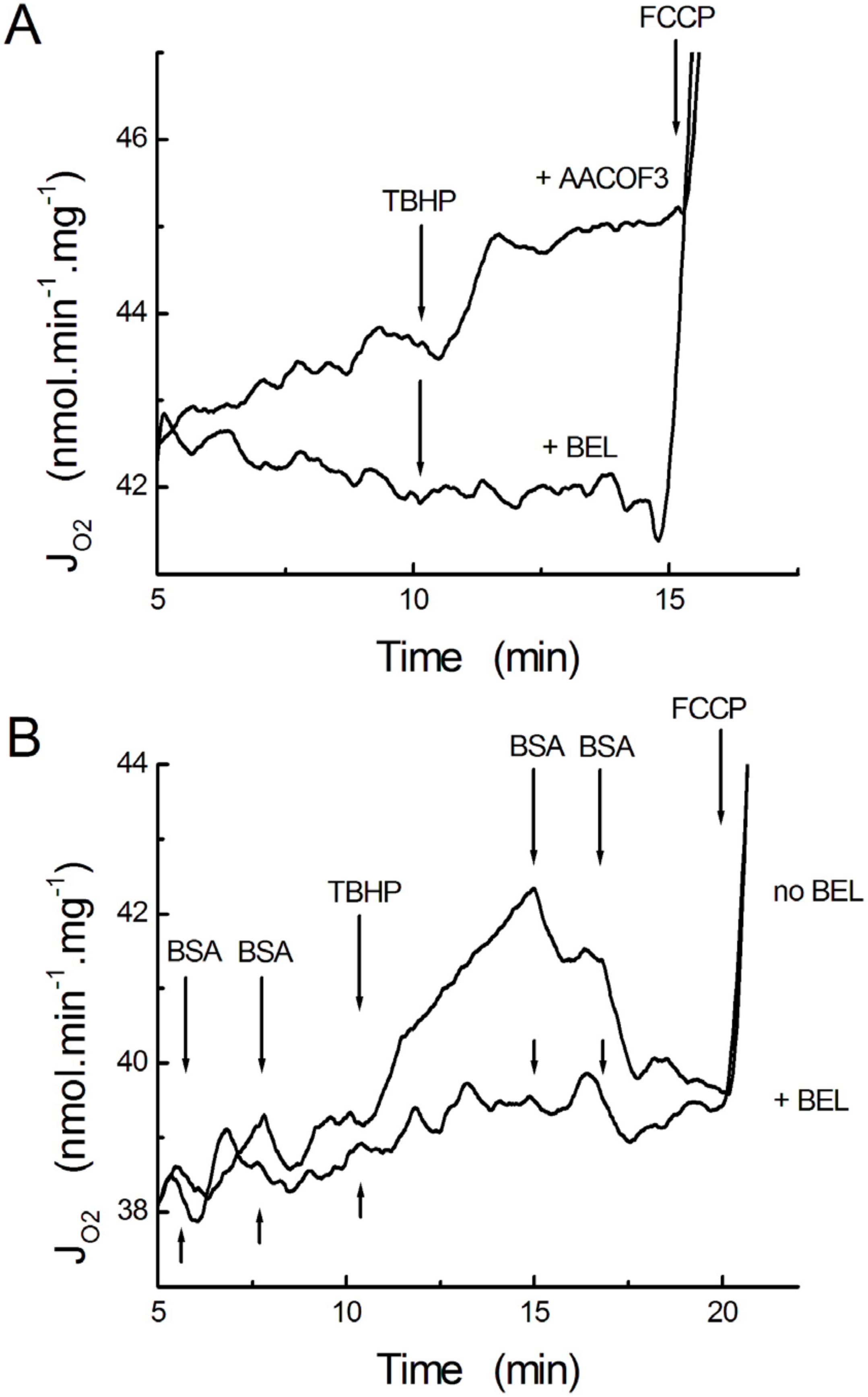

A
FCCP
46
44
42
+ AACOF3
TBHP
+ BEL
$J_{O2}$ (nmol.min$^{-1}$.mg$^{-1}$)
5
10
15
Time (min)
B
44
42
40
38
BSA
BSA
TBHP
BSA
BSA
FCCP
no BEL
+ BEL
$J_{O2}$ (nmol.min$^{-1}$.mg$^{-1}$)
5
10
15
20
Time (min)

Fig.2C

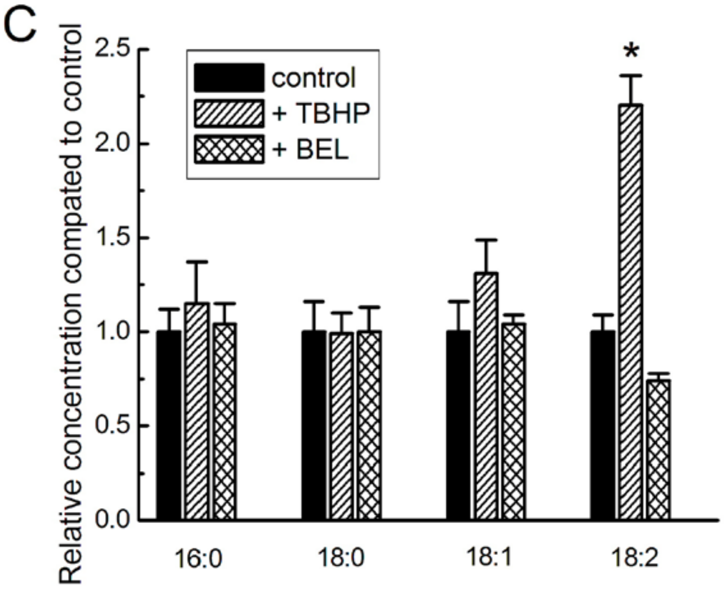

C
Relative concentration compated to control
2.5
2.0
1.5
1.0
0.5
0.0
control
+ TBHP
+ BEL
*
16:0
18:0
18:1
18:2

Fig.3.

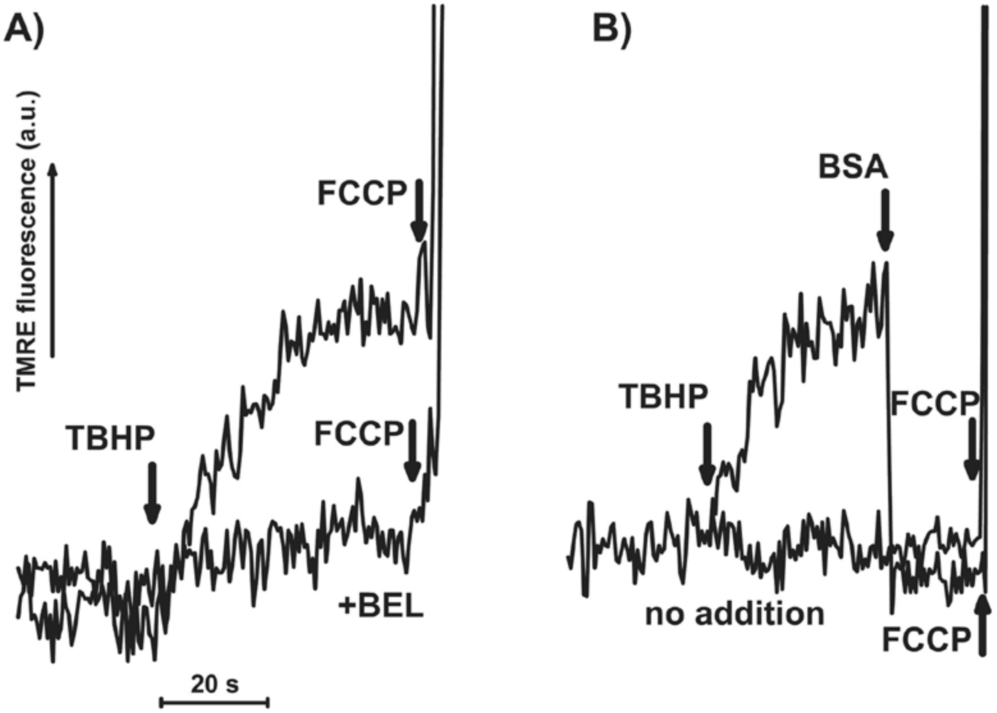

A)
TMRE fluorescence (a.u.)
TBHP
FCCP
FCCP
+BEL
20 s
B)
BSA
TBHP
FCCP
no addition
FCCP

Fig.4.

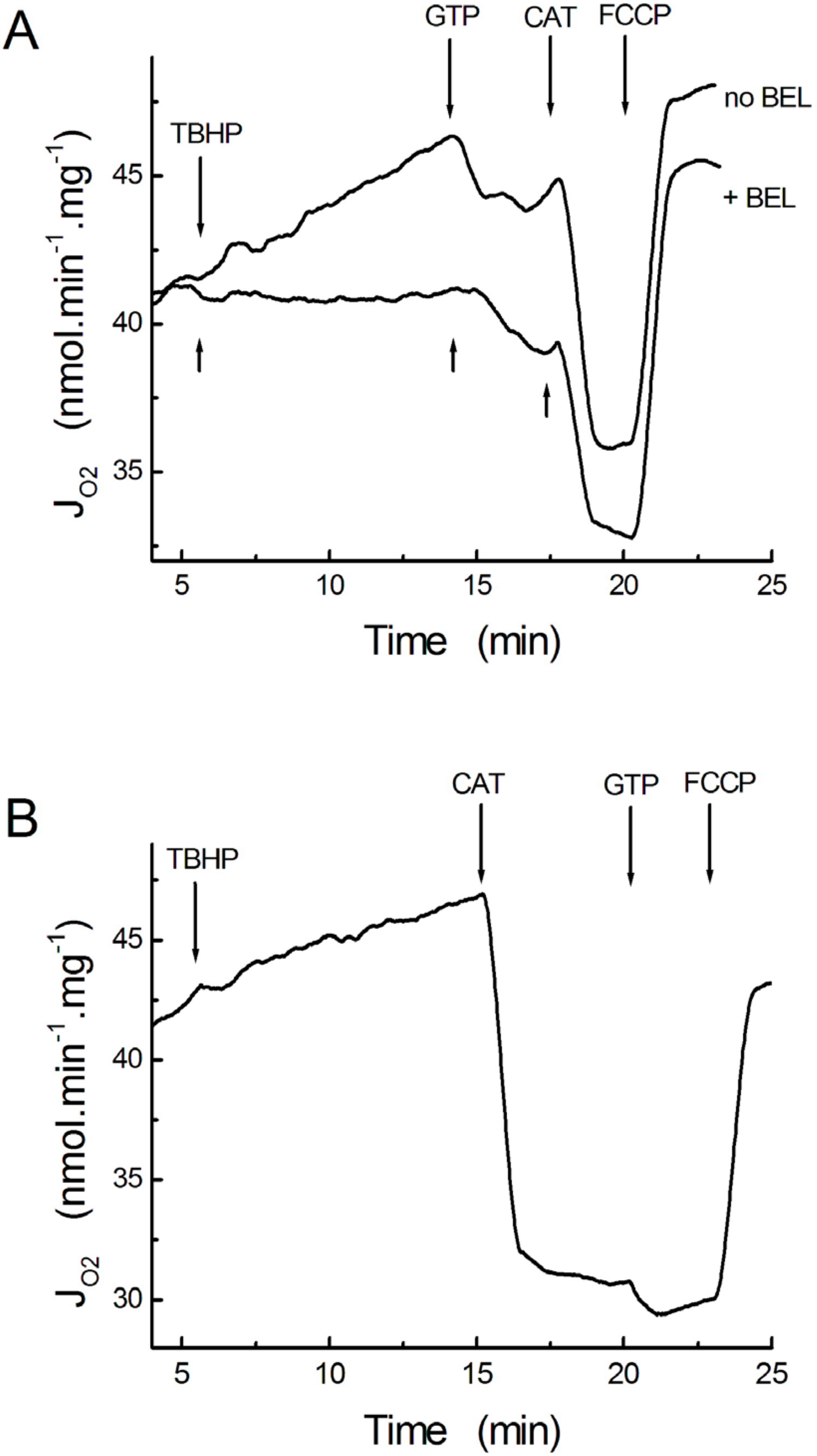
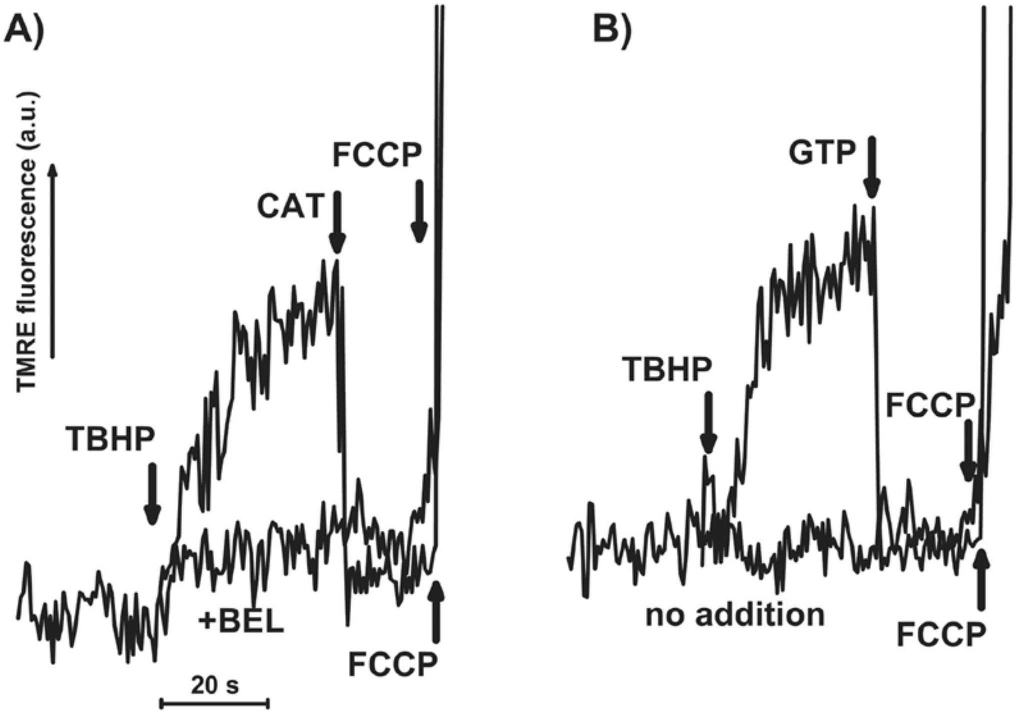

A
GTP
CAT
FCCP
TBHP
no BEL
+ BEL
$J_{O2}$ (nmol.min$^{-1}$.mg$^{-1}$)
45
40
35
5
10
15
20
25
Time (min)
B
CAT
GTP
FCCP
TBHP
$J_{O2}$ (nmol.min$^{-1}$.mg$^{-1}$)
45
40
35
30
5
10
15
20
25
Time (min)

Fig. 5.


A)
TMRE fluorescence (a.u.)
FCCP
CAT
TBHP
+BEL
FCCP
20 s
B)
GTP
TBHP
FCCP
no addition
FCCP