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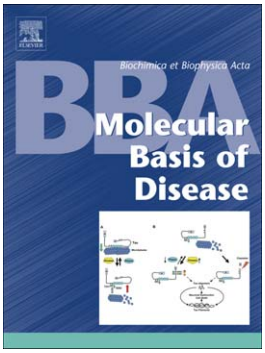

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Antimycin A and lipopolysaccharide cause the leakage of superoxide radicals from rat liver mitochondria

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Key words: Mitochondria, Superoxide radical, Electron spin resonance, Spin probes, Endotoxic shock, Apoptosis

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Summary

Here we show that both Antimycin A, a respiratory chain inhibitor inducing apoptosis, and endotoxic shock, a syndrome accompanied by both necrosis and apoptosis, cause not only an increase but also the leakage of superoxide radicals ($O_2^{\bullet-}$) from rat heart mitochondria (RHM), while $O_2^{\bullet-}$ generated in intact RHM do not escape from mitochondria. This was shown by a set of $O_2^{\bullet-}$-sensitive spin probes with varying hydrophobicity. The levels of $O_2^{\bullet-}$ detected in intact RHM gradually increase as the hydrophobicity of spin probes increases and were not sensitive to superoxide dismutase (SOD) added to the incubation medium. Both Antimycin A and endotoxic shock elevated $O_2^{\bullet-}$ levels. Elevated $O_2^{\bullet-}$ levels became sensitive to SOD but in a different manner. The determination of $O_2^{\bullet-}$ with water-soluble PPH was fully sensitive to SOD, while the determination of $O_2^{\bullet-}$ with the more hydrophobic CMH and CPH was only partially sensitive to SOD, suggesting the release of a portion of $O_2^{\bullet-}$ into the surrounding medium.

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Introduction

The mitochondrial respiratory chain is the major source of superoxide radicals ($O_2^{\bullet-}$) in cells. $O_2^{\bullet-}$ gives rise to other reactive oxygen and nitrogen species (ROS/RNS), such as hydrogen peroxide, hydroxyl radical and peroxynitrite [1-3]. ROS/RNS act as physiological modulators for some cellular functions, but may also cause apoptosis and necrosis, thus contributing to tissue damage [4-7]. It has been well documented that mitochondrial $O_2^{\bullet-}$ can induce cellular death via apoptosis and necrosis [8-11], but the exact mechanism initiating these processes is not yet known. An increase in intracellular $O_2^{\bullet-}$ formation has been suggested as a possible mechanism [4,12-14]. Increased production of ROS/RNS has been shown to induce mitochondrial permeability transition [15], causing apoptosis. However, induction of apoptosis and necrosis involves multiple processes in subcellular organelles other than mitochondria, such as the nucleus, endoplasmatic reticulum and lysosomes. Therefore, one can expect that the translocation of $O_2^{\bullet-}$ from mitochondria to cytoplasm is important to the induction of cell death/dysfunction.

There are two major sites of $O_2^{\bullet-}$ release in the respiratory chain; the first being Complex I, the second Complex III. It has been shown that under certain conditions, e.g. aging, the release of $O_2^{\bullet-}$ from Complex III increases [16], while a number of reports on degenerative diseases suggest that $O_2^{\bullet-}$ from Complex I increases. It has been shown that radicals from Complex III are generated preferentially on the cytoplasmic side of the membrane and Complex I preferentially the matrix side [17].

There are three main approaches in detecting mitochondrial ROS, namely detection of H_2O_2 using visible spectroscopy [17,18], fluorescence probes [19], and direct measurement of $O_2^{\bullet-}$ using electron spin resonance spectroscopy combined with trapping technique [16,17,20]. Over the last 10 years, a new family of spin probes has been developed and has been used successfully for the detection of $O_2^{\bullet-}$ and $ONOO^-$ for *in vitro* and *in vivo* experiments [21-23]. The cyclic hydroxylamines CPH [24], CMH [25], and PPH [22,23] are sensitive and quantitative spin probes that very efficiently scavenge $O_2^{\bullet-}$ radicals in *in vitro* and *in vivo* experimental models. It has been shown that these spin probes have varying permeability and are expected to trap free radicals inside and outside of membrane structures, such as mitochondria (Fig. 1). However, to our knowledge there are very few publications on the application of one of these spin probes to detect ROS generation in mitochondria [16,26].

The aim of this study was to define both quantity of mitochondrial ROS/RNS, and whether they can escape from mitochondria under normal conditions and with stimuli known to block the respiratory chain and induce apoptosis.

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Materials and methods

Animals

The study was approved by the local Committee on Animal Experiments of Vienna, Austria, and all experiments were performed under the conditions described in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (publication NIH 86-23; revised 1985). Adult male Sprague-Dawley rats weighing 280 ± 21g (Animal Research Laboratories, Himberg, Austria) were divided into two groups: control group receiving saline i.p. (n = 6), group receiving 20mg lipopolysaccharide/kg i.p. (LPS; E. coli 026:B6, Difco, Detroit, MI) (n = 6). Rats were euthanized 16h after injection; hearts were quickly extracted and stored in ice-cold preparation buffer. The animals used in this experiment were well characterized by blood analysis and histological examination and recently published [27].

Chemicals

Methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH), 1-hydroxy-3-carboxy-2,2,5,-tetramethyl-pyrrolidine hydrochloride (CPH), 4-phosphono-oxy-2,2,6,6-tetramethyl-piperidine-N-hydroxyl (PPH), were obtained from NOxygen Science Transfer & Diagnostics GmbH (Germany); superoxide dismutase, Antimycin A were purchased from Sigma-Aldrich (Germany). The chemical structure of spin probes used and their tentative location in mitochondria is presented in Fig. 1.

Preparation of mitochondria and determination of respiratory function

Rat heart mitochondria (RHM) and rat liver mitochondria (RLM) were isolated from adult male Sprague-Dawley rats as described [18]. Rat hearts were excised, washed, minced and homogenized in preparation buffer containing 0.25M sucrose, 10mM Tris, 0.5mM EDTA, 0.05% BSA, pH 7.2. The homogenate was centrifuged at 800g and 4°C for 10 min; the supernatant was centrifuged at 11400g for 10 min. The resulting pellet was refilled with preparation buffer and centrifuged at 11400g for 10 min. This procedure was repeated twice. Isolated mitochondria were resuspended in preparation buffer and stored on ice for the duration of the experiment.

Respiratory parameters of RHM were determined with a high-resolution respirometer Oxygraph-2k (OROBOROS Instruments, Austria). The parameters of mitochondrial respiration (Respiration rate, respiratory control, P/O ratio) of RHM (0.5mg/ml) were measured in buffer containing 80mM KCl, 5mM KH_2PO_4, 20mM Tris-HCl, 1mM

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diethylenetriaminepentaacetic acid (DETAPAC), 0.1% bovine serum albumin, pH 7.4, and succinate (10 mM) or glutamate / malate (5mM respectively).

Octanol/Water partition

Typically 400µM of the spin probe dissolved in distilled water was mixed with varying volumes of octanol. The mixture was vortexed 5 min and let sit for 5 min to achieve phase separation. 50µL of water phase was carefully aspirated, transferred to another tube and mixed with 450µL of 100µM Phosphate buffer. Then 15µL of freshly prepared 85mM ferricyanide was added; the mixture was vortexed and subjected to EPR measurements as described below. The molar extinction coefficient of ferricyanide was determined spectrophotometrically at 420nm. The octanol/water partition was calculated by the slope of octanol/water ratio vs. intensity of EPR signals.

Mitochondria/medium partition

0.23mM spin trap (CMH, CPH, or PPH) was added to RLM and vortexed. Ferricyanide was then added as an oxidizing agent as described above. The mixture was centrifuged for 5 minutes at 10100rpm at 4°C in a capillary tube. Sample was frozen in liquid nitrogen 11 min after ferricyanide addition. The frozen sample was cleaved at the division of pellet and supernatant. The two fractions were then stored separately in liquid nitrogen and subjected to EPR measurement.

EPR spectra were recorded on a Bruker EMX EPR spectrometer (BioSpin GmbH Rheinstetten/Karsruhe, Germany) using a flat cell FZK 200 (Magnttech GmbH, Berlin, Germany) under the following settings: microwave frequency 9.77 GHz, microwave power 1 mW, modulation amplitude 2 G, gain 50, time constant 0ms, sweep time 60s at liquid nitrogen temperature.

Superoxide radical determination

RHM (0.5 mg/ml) were incubated in buffer containing 80mM KCl, 5mM KH_2PO_4, 20mM Tris-HCl, 1mM diethylenetriaminepentaacetic acid (DETAPAC), 0.1% bovine serum albumin, pH 7.4, succinate (10 mM) or glutamate /malate (5mM respectively) and different spin probes CMH, CPH, and PPH for 20 min at room temperature. A shaking table was used to facilitate oxygen diffusion in the mitochondrial suspension. Additionally, the

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concentrations of spin probe radicals were determined in the presence of Antimycin A (2µg/ml) and Antimycin A plus SOD (28.500 U/ml).

EPR spectra were recorded on a Bruker EMX EPR spectrometer (BioSpin GmbH Rheinstetten/Karsruhe, Germany) at room temperature (22°C) using a flat cell FZK 200 (Magnttech GmbH, Berlin, Germany) under the following settings: microwave frequency 9,777 GHz, microwave power 1mW, modulation frequency 100 kHz, modulation amplitude 2 G, gain 5 x 10^5, time constant 40ms, sweep time 41.9s. Upon reaction with $O_2^{\bullet-}$, CPH is transformed into a stable $CP^{\bullet}$ radical (3-carboxy-proxyl). Therefore standard solutions of 3-carboxy-proxyl were used to quantify the levels of radical forms of spin probes.

Statistics

All data are presented as mean ± SEM. Statistical analysis was performed by one way ANOVA test followed by post hoc test for least significant difference (LSD). Significance was based on a value of $p<0.05$. The calculations were made with the software SPSS 11.5 for Windows (SPSS Inc.) and MS Excel (Microsoft Corp.).

Results

Fig. 2 shows that in intact control mitochondria, a well-detectable signal of spin probes can be seen with CPH; the greatest intensity observed in the presence of CMH both with succinate (A,B) and glutamate/malate (D,E). In contrast, control mitochondria do not produce detectable levels of PPH-radicals with either succinate (C) or glutamate/malate (F). Addition of Antimycin A resulted in the appearance of a clear signal in the presence of PPH and a significant escalation in CMH and CPH signal intensities. CMH and CPH signals increased approx. 6-fold in the presence of succinate; 2-fold in the presence of glutamate/malate. Addition of SOD completely abolished PPH signals and partially decreased CPH and CMH intensities. The effect of SOD was more pronounced in CPH.

Fig. 3A shows that there is a good correlation between hydrophobicity of spin probes and the intensities of their radical signals determined in intact mitochondria. The octanol/water partition expressed as percent spin probe found in octanol, as well as the level of corresponding radicals generated by intact mitochondria, was highest for CMH (80%) and

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gradually declined to 20% for CPH and nearly 0% for PPH. Fig. 3B shows the distribution of spin probes between mitochondria and medium. Here we see that PPH, similar to the octanol/water partition, is present preferentially in medium; CMH and CPH in mitochondria.

Fig. 4A illustrates the effects of spin probes tested on the respiratory function of mitochondria. No significant differences in State 4 and State 3 respiration rates were found. However, we observed a decreasing trend in respiration rates with the increase in spin probe hydrophobicity. Fig. 4B shows that none of the spin probes significantly influenced respiratory control values or P/O ratio in heart mitochondria.

Fig. 5 shows that LPS challenge did not influence ROS production in the presence of succinate, but increased ROS production in the presence of glutamate/malate. A small increase (25%) was observed in the presence of CMH. A 4-fold increase in $O_2^{\bullet-}$ generation was found in the presence of the water-soluble spin probe PPH. CPH was not used in this experiment as it resembles CMH affinity.

Discussion

It is commonly accepted that mitochondrial ROS potentially cause damage to all cellular components and activate cytosolic stress pathways, DNA damage, and upregulation of JNK, p38, and p53 [28]. However it is not known if mitochondrial ROS escape from mitochondria to induce these processes.

Our results demonstrate that the combination of permeable and non-permeable spin probes allows us to distinguish between $O_2^{\bullet-}$ that is formed and remains in mitochondria and that which is released into the surrounding medium. We failed to detect any $O_2^{\bullet-}$-levels in the medium of intact rat heart mitochondria using the non-permeable spin probe PPH. In contrast, hydrophobic spin probes, CPH and CMH, were able to detect $O_2^{\bullet-}$ in intact mitochondria. This suggests that normal rat heart mitochondria produce certain levels of $O_2^{\bullet-}$ which, however, do not exit mitochondria in the form of $O_2^{\bullet-}$. Antimycin A, an inhibitor of the respiratory chain, inducing apoptosis [29,30], is known to stimulate $O_2^{\bullet-}$ production [15] in Complex III both in the matrix and intermembrane space [17], but it was not known if this agent induces the release of $O_2^{\bullet-}$ into the surrounding medium through the outer membrane [31].

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In our experiments, the addition of Antimycin A drastically increased $O_2^{\bullet-}$-production determined by CPH and CMH. In contrast to intact mitochondria, a remarkable amount of $O_2^{\bullet-}$ was also detected in the presence of PPH in incubation medium. The effects were more pronounced in the presence of succinate, stimulating $O_2^{\bullet-}$-production exclusively in Complex III, which is in line with the fact that Antimycin A stimulates $O_2^{\bullet-}$ production in Complex III[32]. The fact that PPH detects $O_2^{\bullet-}$ in the incubation medium indicates that the outer mitochondrial membrane is permeable to $O_2^{\bullet-}$. A portion of $O_2^{\bullet-}$ diffused from mitochondria into the medium is expected to be sensitive to SOD. This was confirmed by experiments presented in Fig. 2, as SOD completely abolished the formation of PPH-radicals and significantly decreased CMH and CPH radical levels. The latter is likely a reflection of the population of radicals formed in the medium since a portion of CMH and CPH remains in the medium in accordance with their partition coefficients. This is illustrated by the correlation presented in Fig. 3. Similar data of PPH-radical sensitivity to SOD has been demonstrated in brain mitochondria [26]. The data obtained from the mitochondrial suspension confirm that PPH is located preferentially in medium. However we did not see a significant difference in the distribution of either CPH or CMH as in the octanol/water system. This discrepancy probably has two causes. The first being that one cannot avoid a small portion of medium being present in the mitochondrial pellet; consequently we detect not only the mitochondrial but also a slight medium component of the CPH signal (as well as for PPH, this being why the data on PPH are more pronounced in the octanol/water system). The medium component of CPH should be higher than CMH based on their hydrophobicity. The second reason may be that CMH and CPH have dissimilar distribution inside mitochondria, e.g. between mitochondrial matrix and intramembrane space. The latter may also explain why CMH detects more ROS than CPH. However the aim of this study was not to estimate the distribution of ROS inside the mitochondria but rather to examine the conditions introducing mitochondrial $O_2^{\bullet-}$ into the intracellular network, allowing the interaction of mitochondrial ROS with other subcellular organelles.

It has been shown that mitochondrial ROS are involved in the development of apoptosis. Our data suggests that mitochondrial ROS may damage the endoplasmic reticulum. Therefore we tested two models with known increased ROS production, both leading to apoptosis. The main question was whether these two quite different models would result in a release of $O_2^{\bullet-}$ from mitochondria. If so, then one can assume that the release of $O_2^{\bullet-}$ from mitochondria is an important event for induction of apoptosis. To be sure that our spin probes do not disturb

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mitochondria, we examined the effect of spin probes on the respiratory function of mitochondria.

The data presented in Fig. 4 show that there is no significant influence of spin probes on mitochondrial function. However, the question of whether the respiratory chain influences the formation of spin probe radicals remains unanswered. Sensitivity to SOD is the commonly accepted proof to indicate that only $O_2^{\bullet-}$ contribute to the formation of the radical form of a spin probe. However, SOD is a large protein which is unable to diffuse into mitochondria, therefore this examination is not possible. An interesting, but not significant, trend was observed between hydrophobicity of spin probes and respiration rates observed in their presence (Fig. 4). The mean rate of respiration decreased as the hydrophobicity of spin probes increased, suggesting that spin probes entering mitochondria can accept electrons from the respiratory chain. Unfortunately there is no proof whether or not $O_2^{\bullet-}$ mediates an electron transfer from the respiratory chain to spin probes. The alternative explanation may be that spin probes alter the availability of Ca2+ or ADP to mitochondria [33]. Therefore, the question addressing the origin of spin probe radicals formed in mitochondria remains open. This is probably a mixed population of $O_2^{\bullet-}$ and other species. In contrast, SOD abolishes the PPH signal, demonstrating that it originates from $O_2^{\bullet-}$.

PPH detects more $O_2^{\bullet-}$ in LPS-treated animals in the presence of glutamate/malate, demonstrating that LPS-shock, similar to Antimycin A stimulus, induces the release of $O_2^{\bullet-}$ into the surrounding medium. However, in contrast to Antimycin A, $O_2^{\bullet-}$ is released from Complex I. Interestingly enough, Complex I has already been shown to increase ROS production in liver, but not heart, mitochondria as shown in our recent study [27]. This study was done using the permeable spin probes, CMH and CPH, and non-permeable PPH. Here we show that $O_2^{\bullet-}$ generation is also increased in heart mitochondria, but only that amount of $O_2^{\bullet-}$ which is detected in the medium.

Mitochondrial $O_2^{\bullet-}$ participates in intramitochondrial and extramitochondrial signaling pathways as well as causing necrosis and apoptosis. Our data show that two stimuli well known to induce apoptosis and necrosis cause the release of $O_2^{\bullet-}$ from mitochondria into the surrounding medium, which is the cytoplasm in living cells. Since induction of apoptosis and necrosis involves many processes in subcellular organelles other than mitochondria, such as the nucleus, endoplasmic reticulum and lysosomes, we expect that the translocation of $O_2^{\bullet-}$

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from mitochondria to cytoplasm is important for the induction of those processes leading to cell dysfunction and cell death.

The following conclusions are the result of our study: (i) All spin probes can be used for detection of mitochondrial $O_2^{\bullet-}$, but their sensitivity is a linear function of hydrophobicity (octanol/water coefficient); (ii) mitochondrial $O_2^{\bullet-}$ generated by intact rat heart mitochondria remain inside the matrix or/and intermembrane space; (iii) the pro-apoptotic agent Antimycin A and endotoxic shock induce the leakage of $O_2^{\bullet-}$ into the surrounding medium from Complex III and I, respectively.

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Figure legends

Fig. 1 Chemical formulas of spin probes used in this study and expected distribution between incubation medium and mitochondria.

CMH - methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine

CPH - 1-hydroxy-3-carboxy-2,2,5,-tetramethyl-pyrrolidine

PPH - 4-phosphono-oxy-2,2,6,6-tetramethyl-piperidine-N-hydroxyl

Circles indicate the group important to spin probe permeability.

Fig. 2 Effect of Antimycin A (AA) and superoxide dismutase (SOD) on superoxide production in rat heart mitochondria (RHM). Heart mitochondria were incubated in a buffer containing 80mM KCl, 5mM KH_2PO_4, 20mM Tris, 1mM DETAPAC, 0.5% BSA, pH 7.4 at 25°C for 20 minutes. **A, B, C** show levels of spin probe radicals in the presence of succinate (10mM) and **D, E, F** in the presence of glutamate and malate (5mM respectively).

Significance:

* - $p < 0.05$ to RHM

** - $p < 0.005$ to RHM

§ - $p < 0.005$ to RHM+AA

§§ - $p < 0.005$ to RHM+AA

Fig. 3A - Correlation between the octanol/water partition coefficients of spin probes and intensities of spin probe signals detected in intact mitochondria for CMH, CPH and PPH.

B – Mitochondria/medium partition of spin probes. Measurements were performed in RLM suspension as described in the Methods section.

Fig. 4A – Spin probe effect on oxygen uptake in State 3 and State 4 in heart mitochondria.

B – Spin probe effect on respiratory control in heart mitochondria. After isolation, RHM were incubated in buffer containing 80mM KCl, 5mM KH_2PO_4, 20mM Tris, 1mM DETAPAC, 0.5% BSA, pH 7.4 at 25°C for 20 minutes and with one of the tested spin probes. Absence of a spin probe was used as control.

Fig. 5 Effect of LPS challenge on the levels of $O_2^{\bullet-}$ generated by heart mitochondria isolated from sham rats and rats receiving 20mg LPS/ kg and euthanized after 16 hours. RHM were isolated as described in the Methods section. CMH (**A**) and PPH (**B**) were used to detect $O_2^{\bullet-}$.

ACCEPTED MANUSCRIPT

* - $p < 0.05$ to control

** - $p < 0.005$ to control

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

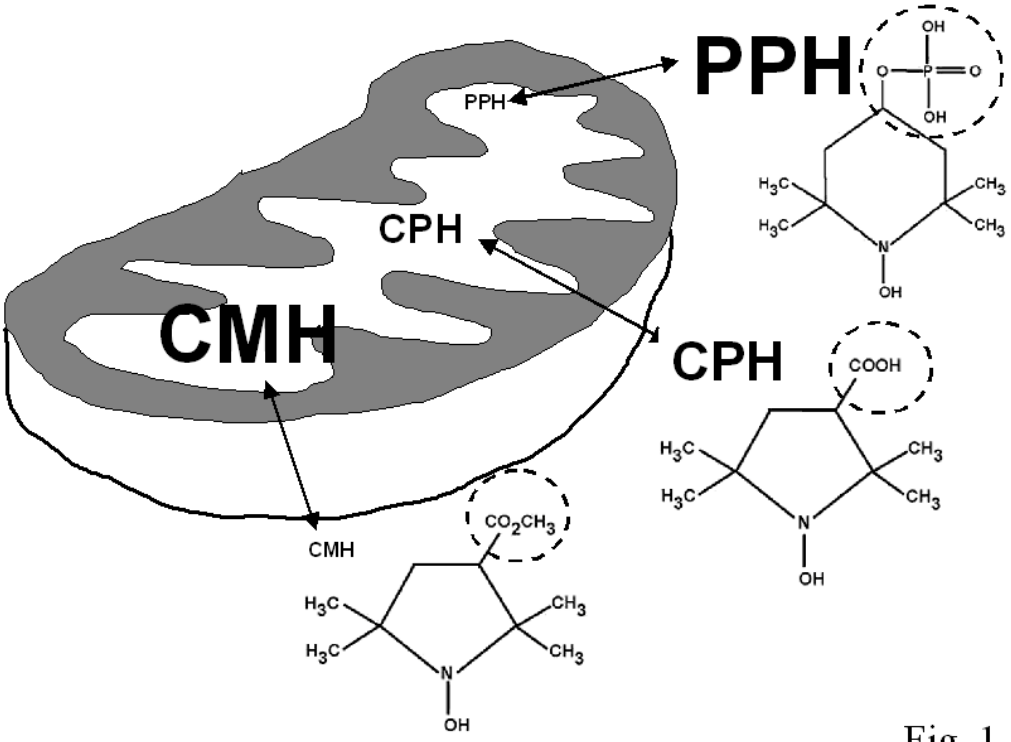

Fig. 1.

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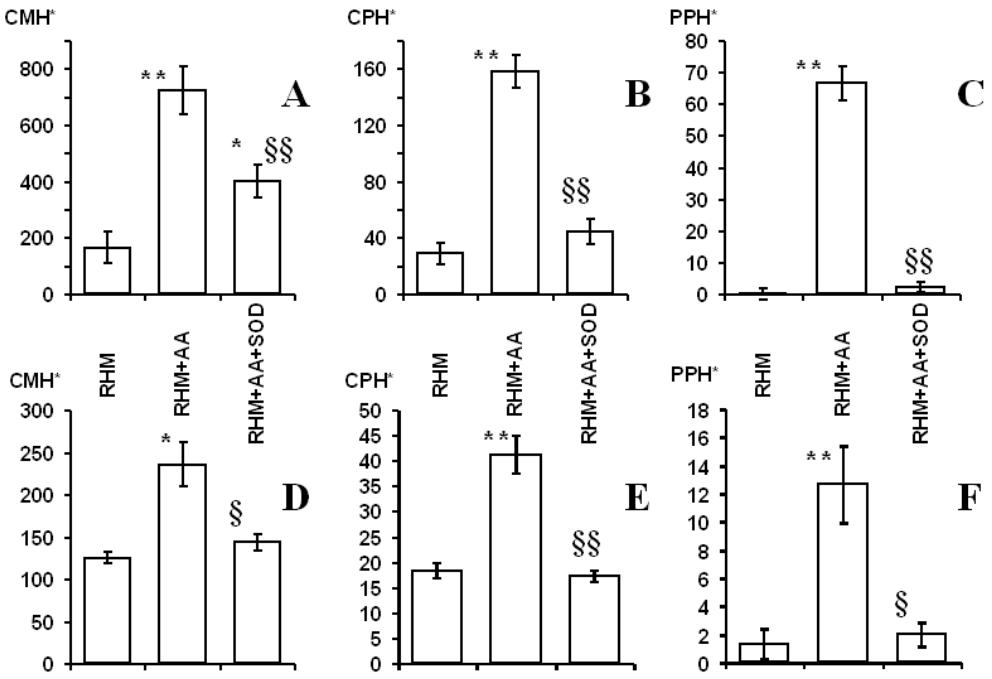

Fig. 2.

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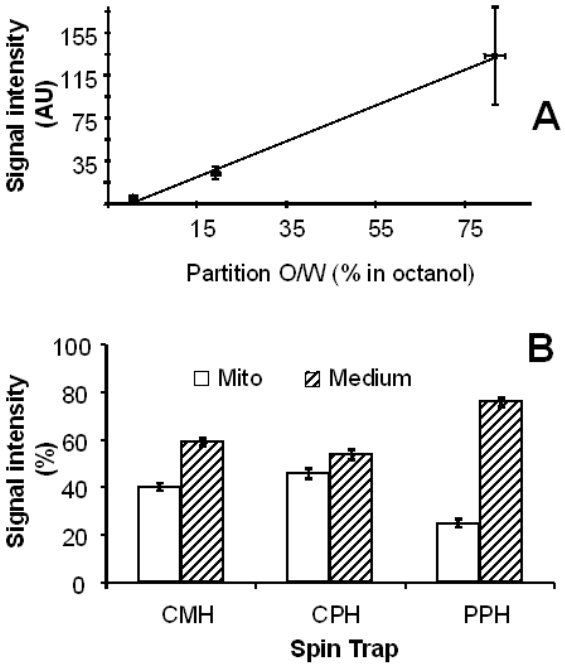

Fig. 3

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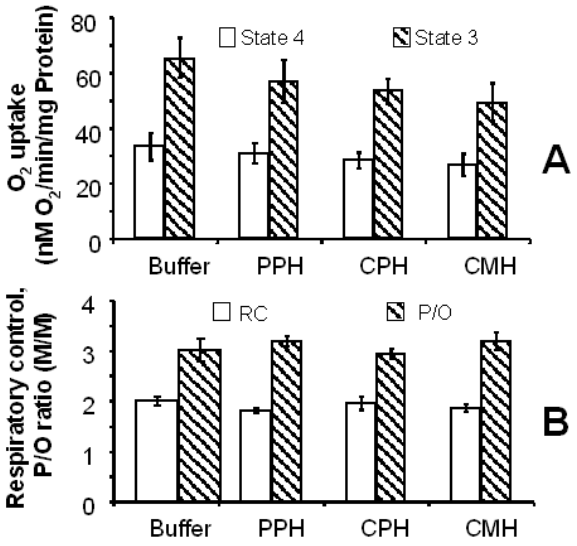

Fig. 4

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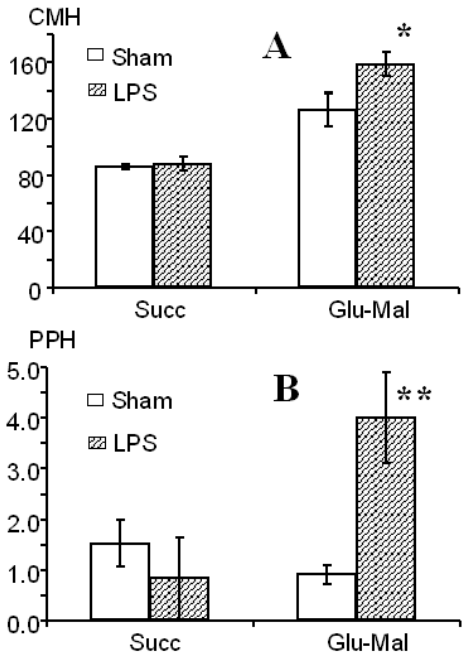

Fig. 5.

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