

## **Idebenone-induced recovery of glycerol-3-phosphate and succinate oxidation inhibited by digitonin**

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### **Short title**

Recovery of glycerol-3-phosphate and succinate oxidation

## **Summary**

Digitonin solubilizes mitochondrial membrane, breaks the integrity of the respiratory chain and releases two mobile redox-active components: coenzyme Q (CoQ) and cytochrome *c* (cyt *c*). In the present study we report the inhibition of glycerol-3-phosphate- and succinate-dependent oxygen consumption rates by digitonin treatment. Our results show that the inhibition of oxygen consumption rates is recovered by the addition of exogenous synthetic analog of CoQ idebenone (hydroxydecyl-ubiquinone; IDB) and cyt *c*. Glycerol-3-phosphate oxidation rate is recovered to 148 % of control values, whereas succinate-dependent oxidation rate only to 68 %. We find a similar effect on the activities of glycerol-3-phosphate and succinate cytochrome *c* oxidoreductase. Our results also indicate that succinate-dependent oxidation is less sensitive to digitonin treatment and less activated by IDB in comparison with glycerol-3-phosphate-dependent oxidation. These findings might indicate the different mechanism of the electron transfer from two flavoprotein-dependent dehydrogenases (glycerol-3-phosphate dehydrogenase and succinate dehydrogenase) localized on the outer and inner face of the inner mitochondrial membrane, respectively.

## **Key words**

Hyperthyroid liver mitochondria • Oxygen consumption rate • Coenzyme Q • Cytochrome *c*

## Introduction

Digitonin is a well known and frequently used glycoside that solubilizes mitochondrial membranes by specific binding to cholesterol areas (Geelen 2005). It breaks the integrity of the respiratory chain and consequently releases respiratory complexes. Generally, it is supposed that the inhibition of respiratory chain activity by detergents is accompanied by the release of two mobile redox-active molecules, i.e. cytochrome *c* (cyt *c*) and coenzyme Q (CoQ). A recent investigation on respirasome from mouse liver mitochondria showed that mitochondrial respiratory chain supercomplexes still maintained some residual succinate oxidase activity. The transfer of electrons from Complex II to Complex IV correlated with the finding that the membrane-bound mobile carriers (cyt *c* and CoQ) could be detected in isolated supercomplexes (Acín-Pérez *et al.* 2008).

We aimed to find to what extent the oxidation of succinate and glycerol-3-phosphate is inhibited by digitonin treatment because flavoprotein-dependent glycerol-3-phosphate dehydrogenase (GPDH, EC 1.1.99.5) is localized on the outer face of the inner mitochondrial membrane and flavoprotein-dependent succinate dehydrogenase (SDH, EC 1.3.99.1) on the inner face. Both enzymes feed electrons to the respiratory chain CoQ pool. However, the previous papers suggested the absence of Q-binding protein of GPDH (Cottingham and Ragan 1980, Rauchová *et al.* 1992), which was postulated by Yu and Yu (1982) and Yu *et al.* (1978) for SDH. The content of GPDH (in comparison with other mitochondrial dehydrogenases) largely varies in different mammalian tissues with the highest activity in brown adipose tissue of newborn, hibernating or cold-adapted animals. In liver tissue GPDH is very low; however, its content is markedly increased by thyroid hormones action as was firstly shown by Lee and Lardy (1965) and Lee *et al.* (1959).

Because of the extreme hydrophobicity of natural CoQ (Lenaz and Genova 2009) its less hydrophobic synthetic analog idebenone (hydroxydecyl-ubiquinone, IDB) with a 10-carbon side chain ending by a hydroxyl group was developed (Zs-Nagy 1990). It is used for the therapy of several mitochondrial diseases, e.g. Friedreich's ataxia (Di Prospero *et al.* 2007, Lynch *et al.* 2010), Leigh syndrome (Haginoya *et al.* 2009) or Leber's hereditary optic neuropathy (Mashima *et al.* 2000). IDB facilitates the flux of electrons along the respiratory chain and increase the production of adenosine triphosphate (Sugiyama and Fujita 1985). Moreover, it has potent antioxidant action and can serve as a protective scavenger for reactive oxygen species (Rauchová *et al.* 2006). Our previous data showed that IDB strongly activates glycerol-3-phosphate oxidation inhibited by endogenous or added free fatty acids (Rauchová *et al.* 2008). Therefore we choose IDB as a possible substance for the recovery of glycerol-3-phosphate and succinate oxidase activities inhibited by digitonin. The aim of present study was to assess the concentration dependence of digitonin inhibitory effect and to estimate to which extent IDB and cyt *c* supplements are able to restore the enzyme activities inhibited by digitonin.

## **Material and Methods**

### *Materials*

Digitonin was obtained from Merck. A stock solution of digitonin (50 mg/ml) was prepared in dimethylsulfoxide (DMSO). Idebenone was kindly provided by Takeda (Osaka, Japan). Stock solution of IDB was prepared in absolute ethanol. Tris(hydroxymethyl)aminomethane, EDTA, glycerol-3-phosphate, succinate, ADP, cytochrome *c* (horse), 3,3',5-triiodo-L-thyronine (sodium salt, T<sub>3</sub>) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Co (USA). All other reagents were of the purest grade commercially available.

### *Animals*

Experiments were performed on adult male Wistar rats weighting 300-350 g from the authorized rat-breeding unit at our institute housed at  $23 \pm 1$  °C and 12-h light-dark cycle periods (6:00 AM to 6:00 PM) with *ad libitum* access to water and a complete laboratory diet. The maintenance and handling of experimental animals were in accordance with the EU Council Directive 86/609EEC and the investigation was approved by the Expert Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic.

### *Alteration of thyroid status*

Hyperthyroid status was induced in rats and maintained 3-4 weeks. Rats received intraperitoneal injection of 3,3',5-triiodo-L-thyronine 3 times a week (0.15 mg/kg body weight). Hyperthyroid status was confirmed by the increased total serum T<sub>3</sub> levels, increased relative heart weight (heart weight/body weight), decreased relative thyroid gland weight (thyroid gland weight/body weight) and increased activity of GPDH as we showed previously (Rauchová *et al.* 2004, Rauchová *et al.* 2011). The control (euthyroid) rats were age-matched littermates of the hyperthyroid rats.

### *Mitochondria isolation*

Immediately after sacrifice, excised liver were properly cleaned, freed of connective tissue and placed in ice-cold isolation medium (250 mM sucrose, 10 mM Tris-HCl and 1 mM

EDTA, pH 7.4). Liver was gently homogenized in a glass Teflon homogenizer with above medium and isolated by differential centrifugation according to Johnson and Lardy (1967). All procedures were carried out at 4 °C. Experiments were performed on both fresh (intact) and frozen-thawed mitochondria stored at -80 °C. Mitochondrial protein concentration was determined according to Folin method using BSA as a standard (Lowry *et al.* 1951).

#### *Mitochondrial oxygen consumption*

Oxygen consumption by mitochondria was measured at 30 °C with a High Resolution Oxygraph (Oroboros, Austria) as we described previously (Rauchová *et al.* 2011). Briefly, 2 ml of incubation medium contained 100 mM KCl, 10 mM Tris-HCl, 5 mM K-phosphate, 3 mM MgCl<sub>2</sub>, 1 mM EDTA (pH 7.4) and 0.12-0.16 mg protein/ml. Glycerol-3-phosphate or succinate were 10 mM, ADP 1.5 mM. Oxygen uptake was expressed as pmol oxygen/s/mg mitochondrial protein.

#### *Determination of enzyme activities*

Glycerol-3-phosphate and succinate cyt *c* oxidoreductase were determined spectrophotometrically at room temperature following the cyt *c* reduction at 550 nm in 1 ml of medium containing 50 mM KCl, 20 mM Tris-HCl, 2 mM KCN, 1 mM EDTA and 0.05 mM cyt *c* (pH 7.4) using mitochondrial protein 0.9-1.3 mg/ml. The reaction was started by addition of 25 mM glycerol-3-phosphate or succinate. The results were expressed as nmol cyt *c*/min/mg protein using the extinction coefficient of 19 mmol<sup>-1</sup>cm<sup>-1</sup> for cyt *c*.

### *Statistical analysis*

The data were expressed as means  $\pm$  SEM and significance of the differences was evaluated by ANOVA. Only values of  $p \leq 0.05$  were considered statistically significant.

## **Results**

### *Glycerol-3-phosphate and succinate oxygen consumption rates in euthyroid and hyperthyroid rat liver mitochondria*

We compared oxygen consumption rates from fresh (intact) and frozen-thawed liver mitochondria isolated from euthyroid (control) and hyperthyroid rats (Table 1). In both fresh and frozen-thawed mitochondria from hyperthyroid rats glycerol-3-phosphate oxidation was about three fold higher than in control animals. Similarly, succinate oxidation was also significantly increased in triiodothyronine-treated rats, however, to a lower extent, by 67 % and 38 % in fresh and frozen-thawed mitochondria, respectively.

### *The effect of cytochrome c supplement on glycerol-3-phosphate and succinate oxidation rate in frozen-thawed mitochondria*

We evaluated the role of cyt *c* in activation of glycerol-3-phosphate and succinate oxidation in frozen-thawed mitochondria, where cyt *c* was released from the membrane. Using frozen-thawed mitochondria isolated from hyperthyroid rats we could increase glycerol-3-phosphate oxidation nearly 2 times and succinate oxidation more than 3 times by 20  $\mu$ M cyt *c* supplements (Fig. 1A, B). In agreement with our previous findings on brown adipose tissue mitochondria, the significant additional increase of glycerol-3-phosphate oxidation was also

reached by BSA (Rauchová *et al.* 2003). The rate of succinate oxidation by BSA was not changed.

#### *The digitonin inhibition of glycerol-3-phosphate and succinate oxidation*

We measured the inhibitory effect of digitonin on the glycerol-3-phosphate and succinate oxygen consumption rates in the presence of 40  $\mu$ M cyt *c* (Fig. 2A, B). In the presence of 5 mg digitonin/mg mitochondrial protein both enzyme activities were inhibited to 15 and 25 % of original values of glycerol-3-phosphate and succinate oxygen consumption rate, respectively. However, oxygen consumption rate of succinate indicated more resistance at low digitonin concentrations (1-2 mg digitonin/mg mitochondrial protein).

#### *The idebenone-induced recovery of digitonin-inhibited glycerol-3-phosphate and succinate oxygen consumption rates*

In further experiments we tested to what extent of glycerol-3-phosphate and succinate oxygen consumption rates inhibited by digitonin could be recovered by supplement of IDB (Fig. 3A). Glycerol-3-phosphate oxygen consumption rate was increased after IDB supplement to 148 % of control values. Inhibition of succinate oxygen consumption rate was also increased by IDB; however, the restoration was not complete and reached only 68 % of control values (Fig. 3B). Similarly, the oxygen consumption rate of glycerol-3-phosphate could be restored by an addition of CoQ<sub>1</sub> (Rauchová *et al.* 2011).



### *The idebenone-induced recovery of digitonin-inhibited glycerol-3-phosphate and succinate cytochrome c oxidoreductases*

In the last part of our experiments we evaluated the activating effect of IDB on glycerol-3-phosphate and succinate cyt *c* oxidoreductase activities inhibited by digitonin (Fig. 4A, B). In agreement with previous data on oxygen consumption rate, glycerol-3-phosphate cyt *c* oxidoreductase was more sensitive to digitonin action in comparison with succinate cyt *c* oxidoreductase. Similarly, IDB recovery was better in the case of glycerol-3-phosphate cyt *c* oxidoreductase (Fig. 4A) in comparison with succinate cyt *c* oxidoreductase (Fig. 4B).

### **Discussion**

In the present study we showed the inhibition of glycerol-3-phosphate- and succinate-dependent oxygen consumption rates by digitonin and the recovery by cyt *c* and IDB supplements.

Recently, the existence of supramolecular associations of the respiratory complexes was confirmed (Eubel *et al.* 2004, Lenaz and Genova 2010, Lenaz *et al.* 2010, Schägger and Pfeiffer 2000, Vonck and Schäfer 2009). However, the integrity of the respiratory chain is also dependent on two mobile carriers, i.e. CoQ that transfers electrons between NADH and flavoprotein-dependent dehydrogenases and Complex III (Lenaz and Genova 2009) and cyt *c* that transfers electrons between Complex III and Complex IV (cytochrome *c* oxidase) (Hüttemann *et al.* 2011). A lipophilic CoQ embedded in the membrane lipid bilayer can be a limiting factor for the respiratory chain function when its biogenesis is depressed by altered activity of enzymes participating on its synthesis. Several mitochondrial diseases due to the primary or secondary CoQ deficiency were described up to now (Quinzii and Hirano 2010, Rötig *et al.* 2007). A hydrophilic heme-protein cyt *c* localized on the external surface of the

inner membrane can be easily released by a disruption of the outer mitochondrial membrane albeit a very small portion ( $11.2 \pm 2.1$  %) of cyt *c* still persist bound on the inner mitochondrial membrane (Cortese *et al.* 1995). Recently, Benard *et al.* (2008) described three different mobile pools of both cyt *c* and CoQ in rat liver and muscle mitochondria what have implications for understanding the function of oxidative phosphorylation, cell physiology and pathology of mitochondrial diseases.

We tried to find experimental model conditions under which CoQ and cyt *c* deficiency can be repaired and the electron flux restored. We used a disintegration of the mitochondrial membrane by a mild non-ionic detergent digitonin because under these conditions both CoQ and cyt *c* are not able to maintain connection between particular complexes and participate in electron transfer to Complex IV. Several studies describe that an application of CoQ or its less hydrophobic derivative IDB has beneficial effects in mitochondrial diseases (Geromel *et al.* 2002, Kerr 2010).

Our data showed that exogenous IDB together with cyt *c* can restore the electron transport between two flavoprotein-dependent enzymes and Complex IV, when the integrity of the respiratory chain was damaged by a detergent action and the oxidase activity strongly depressed. Obtained results also support the idea that the mechanism of the electron transport between two flavoprotein-dependent enzymes (GPDH and SDH) is not identical. Glycerol-3-phosphate-dependent electron transport is more sensitive to detergent damage than succinate-dependent one. The difference could be due to the presence of Q-binding protein on succinate dehydrogenase complex that was not identified on GPDH. In addition, different localization of SDH and GPDH on the inner mitochondrial membrane could play a role.

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**Table 1.** Glycerol-3-phosphate and succinate oxygen consumption rates in fresh and frozen-thawed mitochondria isolated from euthyroid and hyperthyroid rat liver

Mitochondria	Oxygen consumption rate	
	Glycerol-3-phosphate	Succinate
<b>Fresh mitochondria</b>		
Euthyroid liver	71 ± 7	430 ± 42
Hyperthyroid liver	196 ± 11 *	718 ± 35 *
<b>Frozen-thawed mitochondria</b>		
Euthyroid liver	34 ± 3	86 ± 21
Hyperthyroid liver	91 ± 5 *	119 ± 6 *

In incubation medium glycerol-3-phosphate or succinate was 10 mM and ADP 1.5 mM (in the fresh mitochondria assay), glycerol-3-phosphate or succinate was 10 mM (in frozen-thawed mitochondria assay). Oxygen uptake is expressed as pmol oxygen/s/mg protein. Data are means ± S.E.M. from at least five measurements, \*  $p \leq 0.05$  compared with euthyroid liver.



### **Legends to figures:**

**Fig. 1A** The effect of cytochrome *c* and serum albumin in glycerol-3-phosphate oxygen consumption rate in frozen-thawed hyperthyroid rat liver mitochondria, **B** The role of cytochrome *c* and serum albumin in succinate oxygen consumption rate in frozen-thawed hyperthyroid rat liver mitochondria

In incubation medium glycerol-3-phosphate (GP) or succinate (SUC) was 10 mM, cytochrome *c* (CYT) 20  $\mu$ M and bovine serum albumin (BSA) 0.5 mg/ml. Oxygen uptake is expressed as % of control values. Data are expressed as means  $\pm$  SEM from at least five measurements and the significance of the differences was evaluated by ANOVA, \*  $p \leq 0.05$  compared with GP/SUC, #  $p \leq 0.05$  compared with GP/SUC + CYT

**Fig. 2A** Digitonin inhibition of glycerol-3-phosphate oxygen consumption rate, **B** Digitonin inhibition of succinate oxygen consumption rate

In incubation medium glycerol-3-phosphate or succinate was 10 mM, cytochrome *c* 20  $\mu$ M.

**Fig. 3A** Digitonin inhibition and idebenone release of glycerol-3-phosphate oxygen consumption rate, **B** Digitonin inhibition and idebenone release of succinate oxygen consumption rate

In incubation medium glycerol-3-phosphate (GP) or succinate (SUC) was 10 mM, cytochrome *c* (CYT) 40  $\mu$ M, digitonin (DIG) 5 mg/mg protein, idebenone (IDB) 20  $\mu$ M and KCN 2 mM.

**Fig. 4A** Digitonin inhibition (DIG) and idebenone release (DIG+IDB) of glycerol-3-phosphate cyt *c* oxidoreductase, **B** Digitonin inhibition (DIG) and idebenone release (DIG+IDB) of succinate cyt *c* oxidoreductase

In incubation medium glycerol-3-phosphate or succinate was 25 mM, cytochrome *c* (CYT) 20  $\mu$ M, idebenone 20  $\mu$ M and KCN 2 mM.

Figure 1

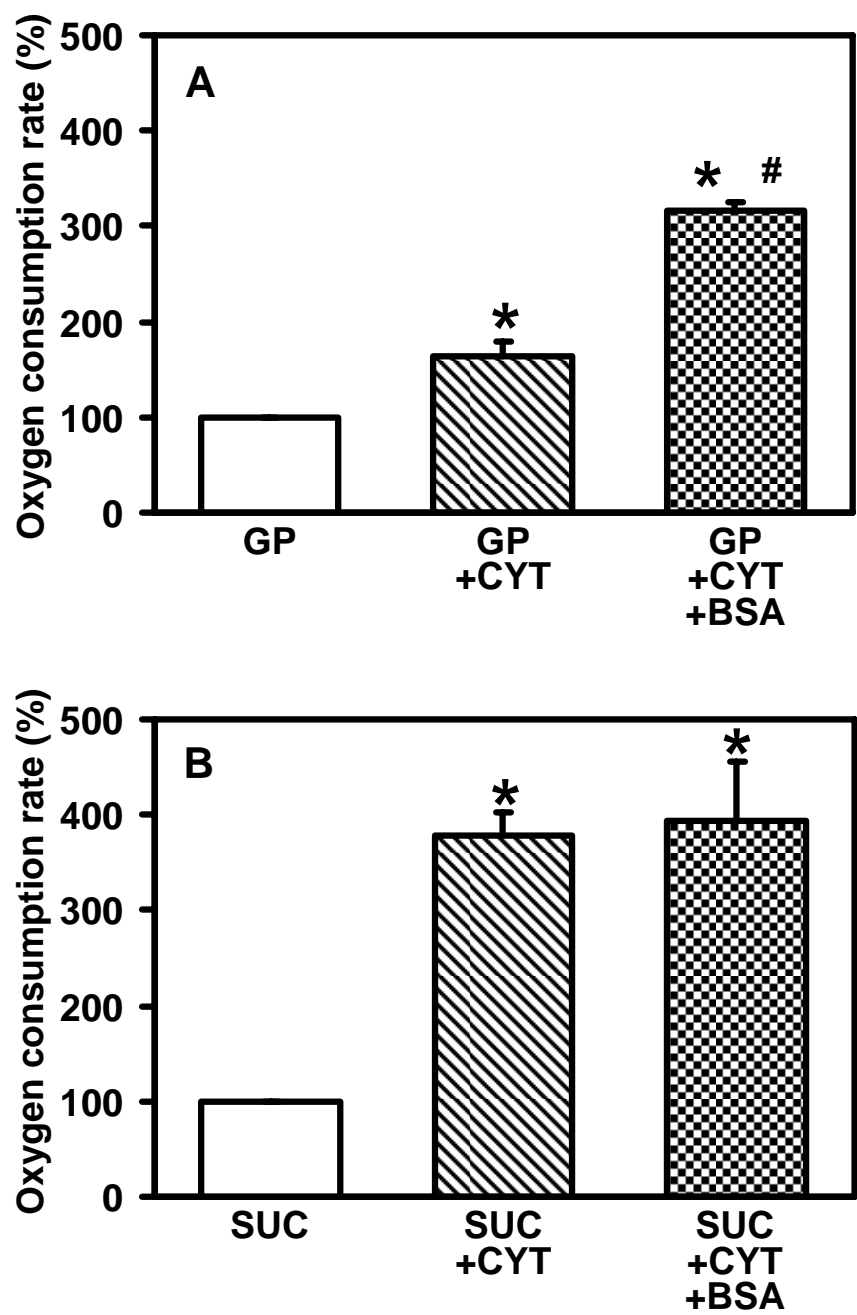


Figure 2

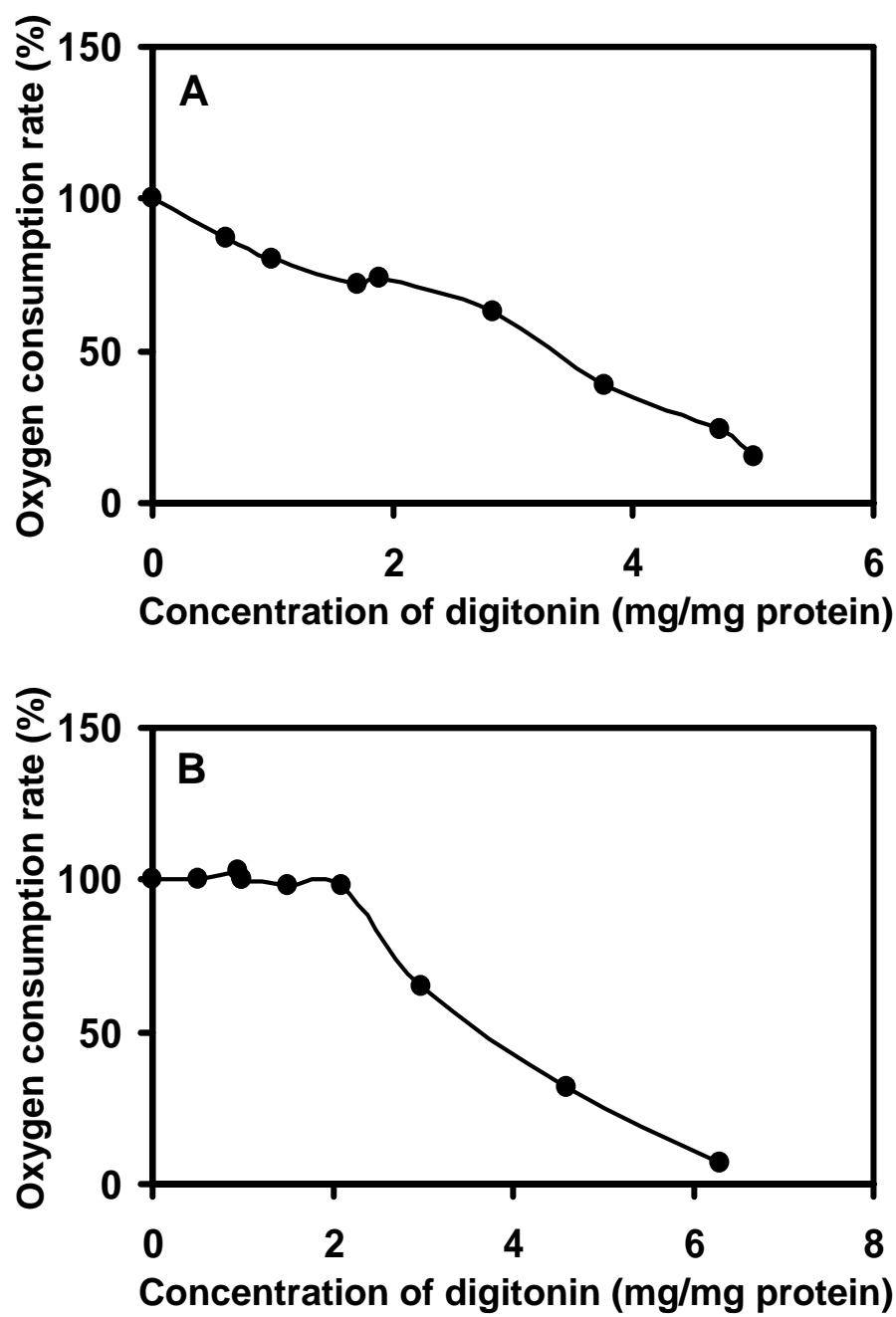


Figure 3

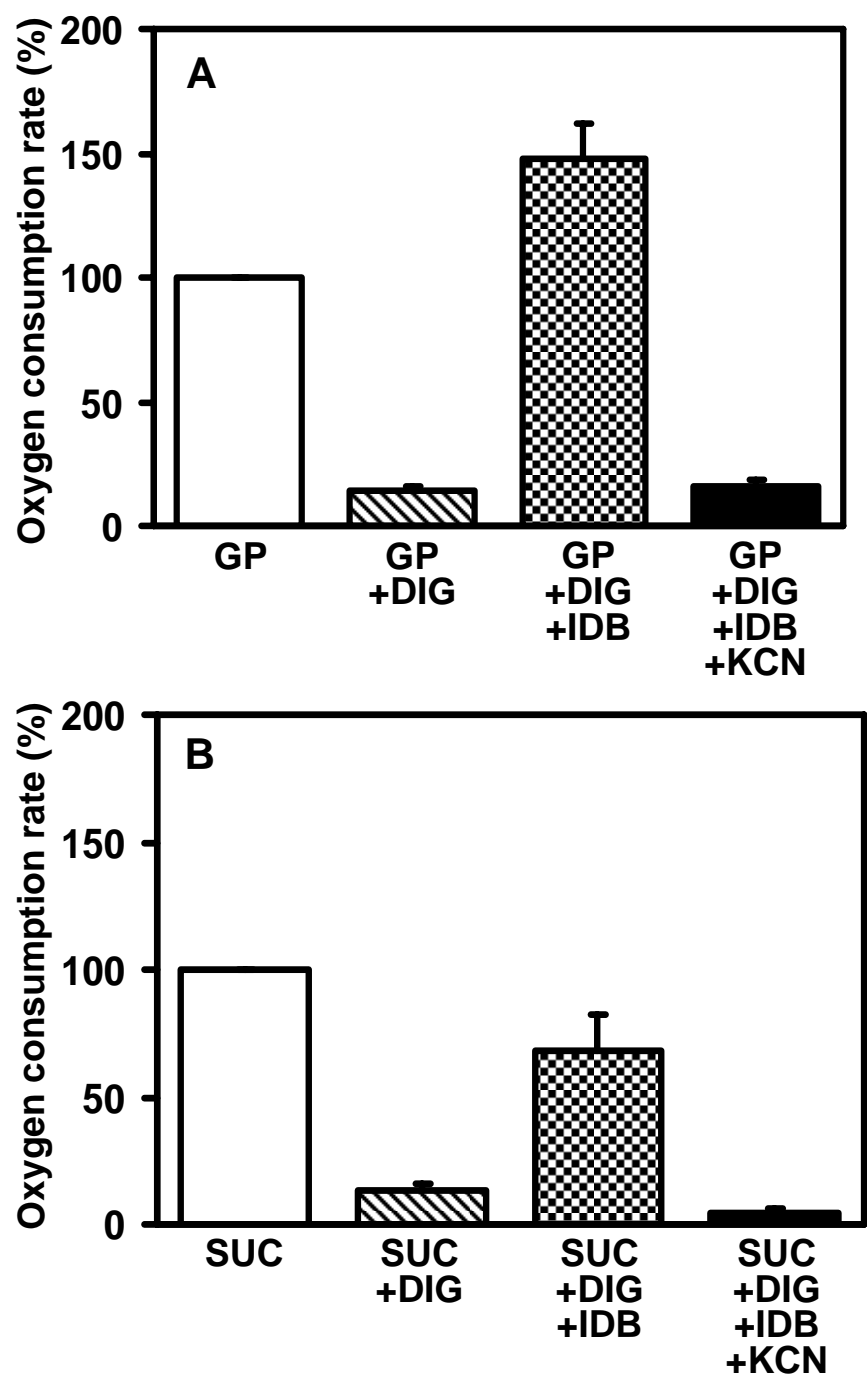


Figure 4

