

Creatine kinase function in mitochondria isolated from gravid and non-gravid guinea-pig uteri

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Abstract

Mitochondria from gravid and non-gravid guinea pig uteri were isolated and respiratory rates examined to determine the responses to ATP, ADP and creatine. It was found that mitochondria isolated from gravid uterus had (i) a markedly higher respiration rate in state 3; (ii) a greater activation of respiration by creatine in the presence of 0.1 mM ATP and (iii) an elevated specific activity of mitochondrial creatine kinase. It was shown by a competitive enzyme method, using pyruvate kinase to trap ADP, that despite the presence of creatine kinase in the mitochondria, there is no functional coupling between mitochondrial creatine kinase and oxidative phosphorylation as has been shown for striated muscle. It is suggested that the function of uterine Mi-CK is to favour high energy phosphate turnover in conditions of increased metabolic demand in gestating uterine smooth muscle.

Key words: Respiration; Creatine kinase; ADP; Uterine mitochondrion; Pregnancy; Energetics

1. Introduction

We have recently reported that the maximal rate of respiration of saponin-skinned uterine fibers was significantly increased in the gravid guinea pig uterus, indicating a higher respiratory activity and/or a greater content of mitochondria in the myometrium [1]. Our previous study was the first attempt to utilize saponin-skinned uterine fibers in a study of uterine energetics. Although creatine stimulated respiration of saponin-skinned fibers has been well demonstrated and characterized in mammalian heart and skeletal muscle [2–5], it was absent in the uterus under our experimental conditions [1] in spite of the presence of active mitochondrial creatine kinase (Mi-CK). Creatine stimulated respiration (in skinned fibers) occurs when the creatine kinase reaction is functionally coupled to ATP production with direct channeling of nascent ATP to Mi-CK in exchange for ADP produced after creatine phosphorylation and an increase in the local concentration of ADP in the vicinity of the ATP–ADP translocase [6–10]. In skinned cardiac and skeletal muscle fibers, as well as in permeabilized cardiomyocytes, the stimulation of respiration by creatine due to a significant decrease in the apparent K_m for ADP, resulting in an amplification of the respiratory signal of

cellular [ADP] [2,3,11]. This decreased K_m and functional coupling is the result of restricted diffusion of ADP in the mitochondria.

Detachment of Mi-CK from the inner mitochondrial membrane by KCl or phosphate abolishes the functional coupling of creatine stimulated respiration. This is seen as an absence in the K_m shift for ADP, despite the presence of active Mi-CK within the intermembrane space [4,12]. So the presence of Mi-CK in the mitochondria does not ensure creatine stimulated respiration in skinned fibers.

In light of the significant increase in Mi-CK content in the gestating uterus [1], one might predict functional coupling and a correlation between Mi-CK activity and creatine stimulated respiration similar to what is seen in heart mitochondria during development [13,14]. It was therefore surprising to find no creatine stimulated respiration in the saponin permeabilized uterine fibers. Clark et al. [1] suggested that this may be due to a specific sensitivity of the coupled CK system in smooth muscle to saponin treatment. An alternative explanation may be that it is caused by the absence of coupling between Mi-CK and oxidative phosphorylation in uterine mitochondria.

This study was performed (i) to investigate the respiratory properties of mitochondria isolated from gravid and non-gravid uteri; (ii) to determine if there is functional coupling of Mi-CK to oxidative phosphorylation; and (iii) to determine what, if any, effect saponin has on the Mi-CK system of uterine mitochondria.

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Abbreviations: CK, creatine kinase; Mi-CK, mitochondrial creatine kinase; CAT, carboxyatractyloside; Cr, Creatine; DTT, dithiothreitol.

2. Materials and methods

2.1. Isolation of mitochondria

Uterine mitochondria were prepared as described in [1]. Adult female guinea pigs (about 1 year old) were anesthetized with an intraperitoneal injection of 100 mg/kg b.wt. pentobarbital less than 2 weeks before term. The uteri were quickly removed, rinsed, and the guinea-pigs exsanguinated. The myometrium was debried of adventitia as well as placental tissue, and minced in an isolation medium containing 0.25 M sucrose, 10 mM Na-HEPES, 0.2 mM EDTA, and pH 7.2 at 4°C. The uterine mince was gently homogenized with a glass-Teflon homogenizer and centrifuged at $600 \times g$ for 10 min. The resulting supernatant was filtered through polyester gauze and centrifuged at $8,000 \times g$ for 15 min, the pellet was resuspended in isolation medium and centrifuged again. The mitochondria in the resulting pellet were resuspended with a minimum volume of isolation medium containing 1 mg/ml BSA.

2.2. Measurement of respiratory parameters

The respiratory rates were determined at 30°C with a Clark oxygen electrode in an oxygraph cell containing 0.6–1.2 mg mitochondrial protein in 3 ml of respiration solution. The solution contained: 10 mM Ca-EGTA buffer, pCa 7.0, 3 mM free Mg^{2+} , 20 mM taurine, 0.5 mM dithiothreitol, 20 mM imidazole, 5 mM glutamate, 2 mM malate, 3 mM phosphate, 2 mg/ml fatty acid-free bovine serum albumin and potassium 2-(*N*-morpholino) ethanesulfonate to an osmolarity of 0.16 M, pH 7.0 [1,5,6]. In experiments using pyruvate kinase, the medium also contained 2.0 mM phosphoenolpyruvate. In the experiments performed in the presence of creatine, solid creatine was added at the appropriate time and allowed to dissolve completely. The solubility of oxygen at 30°C was assumed to be 400 ng atoms oxygen/ml [12]. Rates of respiration were expressed in ng atoms of oxygen/min/mg of mitochondrial protein (determined using the Bradford protein assay from Bio-Rad).

2.3. Creatine kinase activity and isoenzymes

Total creatine kinase activity was determined after homogenization of the tissue by Polytron and incubation for 30 min for complete extraction of CK in solution containing 100 mM KH_2PO_4 , 1 mM EGTA, 0.6 mM DTT or 15 mM *N*-acetyl cysteine, pH 8.5; 0°C. The homogenate was then centrifuged at $20,000 \times g$ for 30 min at 0°C and the supernatant was used for analysis. Creatine kinase activity was assayed at 30°C as described earlier [1,2,15]. Isoenzyme fractionation was performed using cellulose acetate electrophoresis at 200 V and 4°C for 1 h. Creatine kinase isoenzymes were visualized using an isoenzyme determination kit from Gelman UV [15]. The electrophoretograms were scanned fluorometrically (at 375 nm) using a CS9000 Shimadzu dual wavelength scanning densitometer [16] and creatine kinase isoenzyme distribution was calculated as a percentage of the total area of the creatine kinase enzyme profile. All materials used were reagent grade. Saponin and lyophilized pyruvate kinase (500 U/mg) were purchased from Sigma.

2.4. Statistics

All results are expressed as the mean \pm S.E.M. Differences between groups were compared using Student's *t*-test for unpaired data. *P* values of less than 0.05 were considered significant.

3. Results and discussion

Fig. 1 shows two oxygen consumption traces of mitochondria isolated from gravid and non-gravid uteri. This experiment was designed to assess respiratory characteristics of mitochondria together with Mi-CK function. Addition of 25 mM creatine during state 4 respiration (V_{iv}), (when ADP was consumed) resulted in increased respiration (V_{Cr}) (more distinct for mitochondria isolated from gravid uteri) because of Mi-CK acting as an ADP regenerating system. Addition of 1 mM ADP resulted in the maximal rate of respiration (V_{iii}) which was inhibited by carboxyatractylsides, an inhibitor of ATP-ADP

translocase (V_{cat}). Table 1 shows the characteristics of mitochondria isolated from gravid and non-gravid uteri. As seen in the gestating uterine mitochondria, there is a greater maximal rate of ADP stimulated respiration per mg of protein (V_{iii}), and a higher respiratory control index (V_{iii}/V_{iv} ratio). This indicates a significant increase in oxidative capacity of the isolated mitochondria per mg mitochondrial protein during gestation. Therefore the oxygen consuming capacity of these mitochondria increase to a greater extent than other protein constituents of the mitochondria. Thus, during gestation there is a three-fold increase in maximal respiration as well as an increase in maximal force along with other energetic adaptations [1,17,18]. Consistent with the previously reported increase in Mi-CK [1], the mitochondria from gestating guinea-pig also exhibited a 3-fold increase in CK specific activity and a concomitant increased response to 25 mM creatine (see Table 1 and Fig. 1).

Fig. 2A shows the oxygen consumption of isolated mitochondria from gravid myometrium and the effect of saponin on respiration. As can be seen, the addition of ATP (100 μ M) resulted in an increase in oxygen consumption due to the presence of endogenous mitochondrial ATPases. There was a significant increase in respiration caused by 25 mM creatine because of the presence of active Mi-CK producing ADP from ATP. To check for possible effects of saponin on the Mi-CK function and mitochondrial respiration, 50 μ g/ml saponin was added. Saponin did not influence the rate of respiration, which was activated by creatine or ADP. The same result was obtained when saponin was added to the medium before suspending the mitochondria (data not shown). It

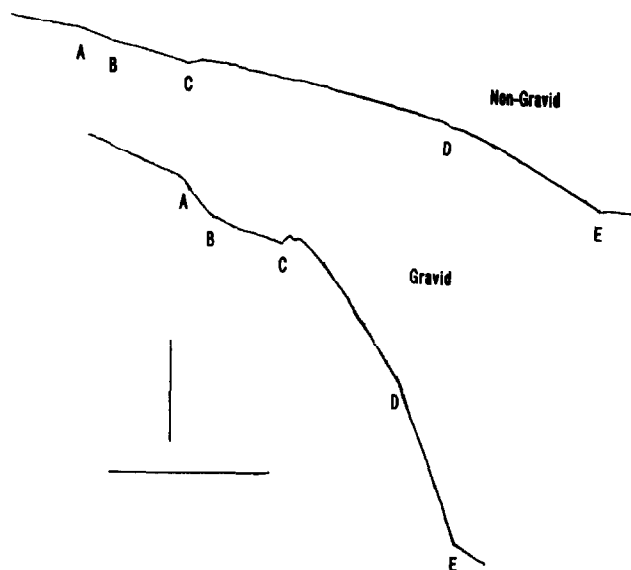


Fig. 1. Oxygraph traces of respiratory activities of gravid and non-gravid uterine mitochondria (0.31 mg protein/ml). The respiration seen prior to addition of ADP is V_o . Additions: A, 100 μ M ADP; B, state IV respiration; C, 25 mM creatine; D, 1 mM ADP; E, 35 μ M carboxyatractylsides. Vertical bar is 50 ng atom oxygen/ml and horizontal bar is 5 min.

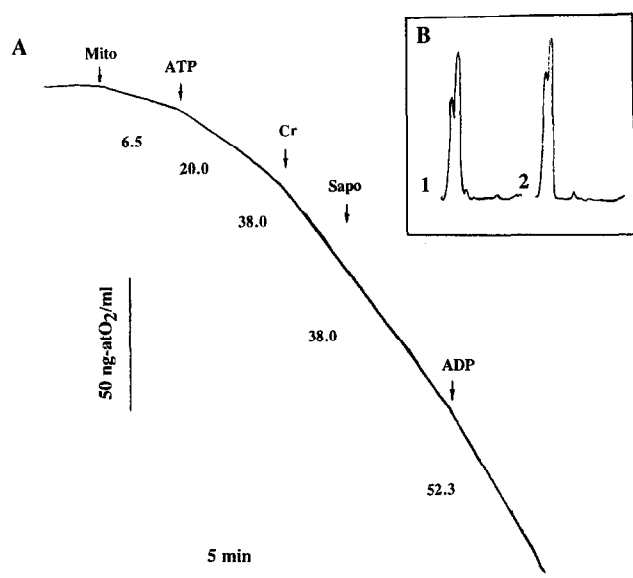


Fig. 2. Effect of saponin on uterine mitochondria. (A) Oxygraph trace of respiratory activities of mitochondria isolated from gravid uterus 0.4 mg protein/ml (mito). Additions: ATP, 0.1 mM; creatine (Cr), 25 mM; saponin (Sapo), 50 μ g/ml; ADP, 0.5 mM. The numbers indicate the respiration rates in ng atoms of oxygen per min per mg of protein. (B) Electrophoretograms of the creatine kinase isoenzymes from saponin treated (1) and untreated (2) uterine gravid mitochondria. Before and after saponin treatment the specific activity of Mi-CK is not different than control values for CK (Table 1).

was also shown that saponin left the mitochondrial creatine kinase isoenzyme practically unchanged (Fig. 2B). Moreover, the specific activity of creatine kinase in the saponin treated mitochondria was not different from control values seen in Table 1. After exposing the mitochondria to saponin and separating the mitochondrial pellet from the supernatant there was neither a difference in the specific activity of the pellet nor any detectable creatine kinase activity in the supernatant. Thus, Mi-CK remained in the mitochondrial fraction and was not lost during or due to the saponin treatment. Therefore, one can conclude that saponin does not change the respiratory or CK activities of uterine mitochondria.

To check if Mi-CK in the uterus is coupled to oxidative phosphorylation or if it is working as a simple ADP regenerating system, we applied a competitive enzyme method which has been described for heart mitochondria [12,19,20]. In this method, pyruvate kinase plus phosphoenolpyruvate almost completely abolished respiration activated by ADP due to rapid rephosphorylation of nascent ADP into ATP (Fig. 3A,B). In heart mitochondria when Mi-CK is coupled to oxidative phosphorylation the addition of creatine activates the CK reaction. The ADP produced is preferentially taken up by ATP-ADP translocase, resulting in diffusion limitations for ADP and causes a decreased accessibility of ADP to pyruvate kinase (Fig. 3A). In uterine mitochondria, however, ADP is not restricted within the mito-

chondria, but rather is free to mix with the medium. Therefore, functional coupling of Mi-CK to the adenine nucleotide translocase is not possible. Fig. 4A shows the response of heart mitochondria (with coupled Mi-CK) after the addition of creatine. There is a striking inhibition of ADP-stimulated respiration by pyruvate kinase plus phosphoenolpyruvate in myometrial mitochondria which is unchanged by the addition of creatine. In heart mitochondria, however, there was a significant activation of respiration when creatine was added. When Mi-CK is not coupled to ATP production (for example after detachment of the enzyme from the inner mitochondrial membrane [4,12,20]) creatine loses its ability to stimulate respiration due to rapid removal of ADP by external pyruvate kinase (Fig. 3B). It can be seen in Fig. 4B that the respiratory behavior of uterine mitochondria is different from that of heart mitochondria because of coupled Mi-CK. There is no creatine stimulated respiration due to competition by the pyruvate kinase reaction (Fig. 4B). This observation shows that in spite of the remarkable activity of Mi-CK in uterine mitochondria (Fig. 1), functional coupling of Mi-CK in uterine mitochondria is absent.

The presence of Mi-CK in the uterus, but lack of coupling to respiration, may be caused by one or a combination of reasons. In smooth muscle little information is available concerning the precise location of Mi-CK. One explanation, therefore, could be that Mi-CK in uterine mitochondria is not closely associated with the translocase because it is free in the intermembrane space (Ishida et al., personal communication). Ishida et al. (personal communication) demonstrated, using electron microscopy, that the Mi-CK in the guinea pig smooth muscle is distributed throughout the intermembrane

Table 1

General characteristics of mitochondria isolated from gravid and non-gravid guinea-pig uteri

	Non-gestating (n = 4)	Gestating (n = 7)
Rate of respiration (ng atoms of oxygen per min per mg protein)		
V_o	7.05 \pm 1.4	6.1 \pm 1.36
V_{iv}	7.25 \pm 0.4	12.9 \pm 5.2
V_{cr}	20.6 \pm 5.6	51.8 \pm 10.6*
V_{iii}	33.8 \pm 1.8	93.3 \pm 19.7*
V_{cat}	6.5 \pm 1.3	12.6 \pm 3.2
Relative respiratory parameters		
RCI	4.7 \pm 0.3	7.0 \pm 1.7
%CAT	80.7 \pm 3.9	85.7 \pm 1.9
Specific CK activity (IU/mg mitochondrial protein)		
Mi-CK	0.11 \pm 0.007	0.338 \pm 0.11*

* $P < 0.05$ compared to gestating.

* $P < 0.01$ compared to gestating.

V_o , basal respiration; V_{iv} , respiration after 100 μ M ADP was consumed; V_{cr} , respiration with 25 mM creatine and 100 μ M ATP; V_{iii} , respiration in the presence of 1 mM ADP; V_{cat} , respiration in the presence of carboxyatractylolide (35 μ M); RCI, respiratory control index; %CAT, percentage of inhibition of the respiration rate by carboxyatractylolide.

space, and not at contact sites or bound to the inner membrane. It has been shown in intact cardiac mitochondria that when Mi-CK is detached and free in the intermembrane space, functional coupling is lost [12]. Therefore, the scheme shown in Fig. 3B is probable for uterine mitochondria. Indeed, Mi-CK from smooth muscle is not the same protein as in sarcomeric tissue since it is the product of a different gene [21–23]. This protein may not have the properties needed to associate with the mitochondrial membrane. Alternatively, it may be readily detached from the membrane due to specific sensitivity to the ionic environment. In striated muscle, Mi-CK is bound to the outer surface of the inner mitochondrial membrane due to interaction with cardiolipin [24]. The connection of cardiolipin to adenine nucleotide translocase was shown in cardiac mitochondria [25], resulting in close association of adenine nucleotide translocase and Mi-CK [6]. One can suggest that the cardiolipin content in smooth muscle mitochondria is lower or its distribution is different and therefore does not allow the

mitochondrion

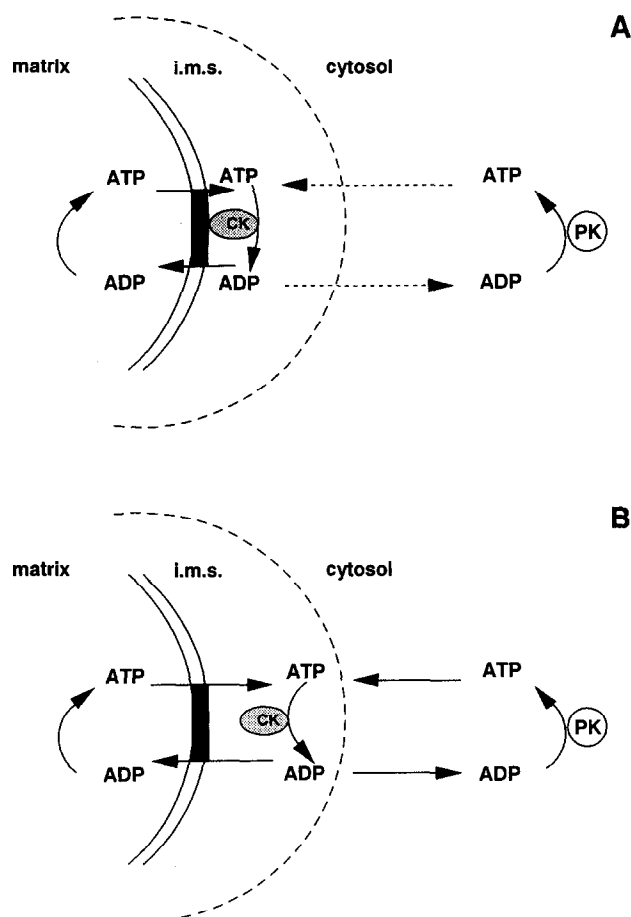


Fig. 3. Schematic representation of coupled (A) and uncoupled (B) Mi-CK systems. Pyruvate kinase (PK) and ATP-ADP translocase (T) compete for ADP produced by the CK reaction. i.m.s., intermembrane space. (A) CK is bound to the mitochondrial membrane and coupled to oxidative phosphorylation; accessibility of ADP to PK is limited. (B) CK is not bound or coupled; ADP is completely accessible to external PK.

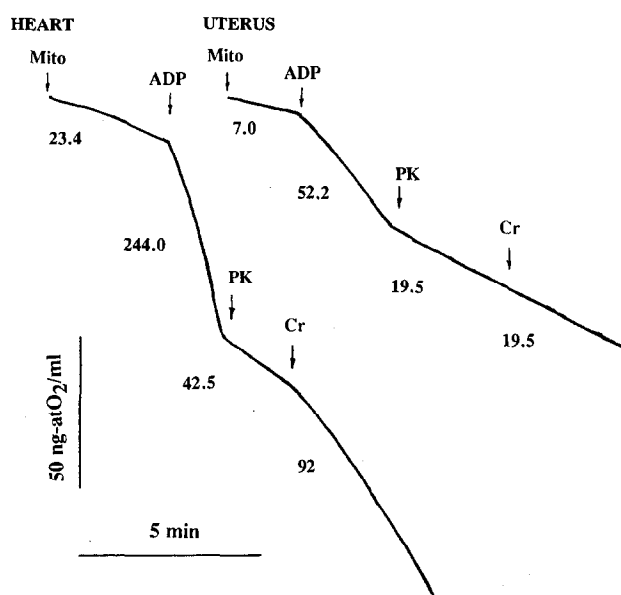


Fig. 4. Effect of creatine (25 mM) on the rates of ADP stimulated respiration inhibited by the pyruvate kinase reaction. Oxygraph traces of respiration of mitochondria from heart and uterus of pregnant guinea-pig. In addition, the reaction medium contained 2.0 mM phosphoenolpyruvate. Additions: mitochondrial suspension (mito) ADP, 0.7 mM; pyruvate kinase (PK), 130 IU/ml; creatine (Cr), 25 mM. Numbers show the respiration rates (ng atoms oxygen per min per mg of protein).

close spatial relationship between translocase and Mi-CK. Additionally, it has been shown that a specific adenine nucleotide translocase is preferentially expressed in heart and skeletal muscle [26]. Therefore, it may be that a specific Mi-CK isoenzyme (the sarcomeric form), a specific translocase and a cardiolipin environment may all be prerequisites for structural and functional coupling between translocase and Mi-CK.

Our previous work has shown two significant changes in uterine mitochondria during gestation. The first is an increase in maximal respiratory capacity [1]. The second change is an increase in the specific activity of mitochondrial creatine kinase, probably due to an increase in Mi-CK per mitochondrion [1]. One of the possible functions of the creatine kinase system in the uterus may be to keep ADP levels low for uterine function. ADP has been shown to have striking inhibitory effects on the relaxation of smooth muscle [27–29]. Therefore, a system such as Mi-CK, cytoplasmic creatine kinase and myokinase could function to prevent high ADP concentrations and thus preserve normal uterine contractile function. A similar mechanism has been proposed for the heart during anemic hypertrophy [30]. Another role for the Mi-CK system may be to enable efficacious coupling of mitochondrial energetics and contractile function [31]. Such coupling would, however, require (or at least be aided by) restricted diffusion of ADP within the mitochondria and creatine stimulated respiration. One im-

portant parameter yet to be determined is the affinity for ADP by uterine mitochondria. Degani et al. [32] estimated the free ADP in the myometrium using NMR to be 40 μM . This concentration of ADP would stimulate maximal respiration, assuming that the K_m for ADP is about 10 μM as is seen in heart mitochondria [2,3]. It seems logical, therefore, that the kinetics of isolated uterine mitochondria be found to better characterize the role of the mitochondria in nucleotide regulation. If the mitochondria are not able to respond to creatine, then it is likely that it is responding to the ADP produced by the contractile proteins. This would be consistent with the results of Lynch et al. [33] where oxygen consumption was increased with tension generation.

Apparently, the changes observed in gestating uterine mitochondria are due to a process of adaptation in preparation for the impending increase in metabolic demands of delivery. In this study we found that there is no coupling between uterine Mi-CK and oxidative phosphorylation in the presence of an ADP trap. However, the significant increase in Mi-CK activity in gravid uterus allows us to conclude that the Mi-CK system appears to be intimately involved in the metabolic changes associated with gestation.

The results of the present study show that (i) the oxidative capacity of uterine mitochondria is increased during gestation; (ii) uterine mitochondria are capable of the ADP regenerating reaction due to the presence of active Mi-CK in intermembrane space; (iii) in uterine mitochondria there is no restricted diffusion of ADP as evidenced by a lack of functional coupling between Mi-CK and oxidative phosphorylation; (iv) respiration in the presence of creatine and the specific activity of Mi-CK are increased with gestation.

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