行政院國家科學委員會補助專題研究計畫成果報告

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※Increased Cardiac Cytochrome-c-oxidase Gene Expression ※
※and Activity in an Accelerated Cardiac-hypertrophy Model ※

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執行期間：89 年 08 月 01 日至 90 年 07 月 31 日

計畫主持人：黃志揚 助教授

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□赴國外出差或研習心得報告一份

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□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

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行政院國家科學委員會專題研究計畫成果報告

Preparation of NSC Project Reports

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一、中文摘要

病理性心肌肥厚是引起心肌狹死的主要危險因子，但引發的分子轉並不清楚。在心肌細胞肥大過程中，粒腺體是主要能量供應來源。就目前所知，在心肌肥厚末期常因無充份的氧化能力，而導致心肌衰竭。另外證據顯示，細胞死亡蛋白（Bax）會由細胞質轉移至粒腺體表層，引發細胞色素－c－氧化酵素（COX）的流失而釋放至細胞質，進而活化染色體切割酵素（Caspases），切割 DNA 並導致心肌細胞死亡。

我們曾採用新的大白鼠腹動脈完全結紮方式，來誘發心肌肥厚快速形成。手術後第二天，即可見心肌肥大及細胞色素－c－氧化酵素活性增加。但細胞色素－c－氧化酵素活性卻也在手術後第七天，隨著心肌纖維化的逐漸形成而漸漸減低。因此我們更進一步來檢測 COX Vb 的基因表現及 COX protein 量的變化，以了解 COX 基因表現及 COX 蛋白活性是否與 COX 蛋白量有絕對性的關係。藉此了解在心肌肥大轉惡為心肌纖維化及衰竭過程中，COX 基因表現的角色。以 dot-blotting 及 in situ hybridization 的技術來探討 COX Vb 的基因表現差異，發現基因表現變化與 COX 蛋白活性及心肌細胞肥大及衰竭有直接的關連性。未來並希望以先天性高血壓鼠(SHR)及重風性高血壓鼠(SHRSP)與此腹動脈完全結紮動物模形(coarctation)作對照。

關鍵詞：專題計畫、報告格式、國科會

Abstract

Chronic pressure overload leads to cardiac hypertrophy, the mechanism of which are not understood. The contribution of the mitochondrial compartment, the main energy source for cardiac hypertrophic growth, is especially little known. Here cardiac hypertrophy resulting from chronic of the rat abdominal aorta between the origins of the renal arteries. The study focused on the early (1 to 7 days) postsurgery peripd. Mean carotid systolic blood pressure remained at about l38 mmHg the 7 day, in the shams but increased rapidly in coarcted rats (CR) to 175 mm Hg by day 3 and to 207 by day 7. CR heart weights increased over shams by l6% by day 3 (P<.00l) and remained increased to day 7. Heart weight: body weight ratios were 3.39 and 4.49 (P<.00l) at day 3 for sham and CR animals, respectively, and 3.32 and 4.81 (P<.001) at day 7. The CR heart weight increase was paralleled by an increase in cytochrome c oxidase (CYTOX) activity in isolated heart mitochondria. CR CYTOX activity was increased 44, 43 and 31% (P<.005, .00l and .005, respectively) over the shams at days3, 5 and 7, respectively. Further, by day 3, the steady-state level of CYTOX subunit Vb mRNA, measured by dot blotting and IN SITU hybridation, in the CR hearts was the higher by 62% and 51% (P<. 005), individually, than in the same day shams. Results suggest that the rapid cardiac hypertrophy observed here is mediated by an increase in mitochondrial energy-producing activity associated with a positive change in CYTOX gene expession. Supported by this grant.

Keywords:

cytochrome-c-oxidase, cardiac hypertrophy.

二、緣由與目的

The heart has a remarkable capacity to enlarge its mass in response to an increased work load (e.g. pressure overload) induced either pathologically or experimentally (l0) The mechanisms leading to cardiac muscle cell (myocyte) enlargement during cardiac hypertrophy are not well understood. The mitochondrial compartment is the main source of energy in the myocyte for its hypertrophic growth. A recent review of the relevant literature (11), however, shows many inconsistencies in reports regarding the behavior of the mitochondrial compartment during the onset and progression of cardiac hypertrophy. In addition, Investigateots on the molecular events involved in mitochondrial biogenesis and function during the hypertrophy process are few. This is surprising since it is well-known that insufficient oxidative capacity often contributes to the cardiac failure frequently observed at least in late stages of hypertrophic cardiomyopathies(6) .

Recently, this laboratory began to work on anew model of cardiac hypertrophy (l6,4). Our studies showed that the heart rapidly hypertrophies with in 2-3 days postsurgery in this model that uses a complete coarctation of the abdominal aorta in the rat as the inducer of a pressure overload to the heart (2). In the present study, we have began as investigaters into the biochemical and molecular events underlying the adaptation of the mitochondrial compartment to meet the increased energy demands of the cardiomyocyte as they undergo this rapid hypertrophic growth. We have used cytochrome c oxidase (GOX), which catalyzes the rate-limiting reaction of the mitochondrial respiratory chain (3), as a model protein for investigating the underlying adaptive mechanism. We have studied COX activity and examined COX gene expression by determining mRNA for one of the nuclear-encoded subunits of COX enzyme during cardiac hypertrophy in the new model system using complete coarctation of the abdominal aorta.

三、研究方法

All experiments were performed in accordance with university and federal regulations governing the care and use of animals in research.

Coarctation of abdominal aorta (Male Sprague-Dawley rats weighing between 240 and 300 g were used to induce cardiac hypertrophy by complete coarctation of the abdominal aorta between the origins of the renal arteries (16,4). After a rat was anesthetized with pentobarbital sodium (45.5 mg/kg; 0.07 ml per l00 g body weight), its left side was shaven and a horizontal incision 3 cm. in length was made to the epidermis. Using curved hemostats, the superficial fascia were separated and an incision of the same size was made to the abdominal musculature. The viscera were exposed, and adipose tissue was separated from the left kidney which was moved to obtain maximal clearance to the abdominal aorta. The abdominal aorta was isolated and completely coarcted with a ligature (Coats, Dual Duty Plus, carpet thread) Placed between the origins of the renal arteries. The left kidney and adipose tissue were returned to their normal positions, the abdominal musculature was rejoined using coated vicryl sutures (Ethicon, Johnson & Johnson, FS-2 cutting), and the epidermis was rejoined using michel wound clips. Next, a solution of l% iodine in 70% ethanol was used to clean the wound. The rats were returned to the animal care room (one per cage) and allowed to recover. All animals were fed a standard rat chow and water supplemented with tetracycline (22 mg/kg body weight) to ward Off infection. Sham rats were subjected to the same procedure, but no ligature was placed . *Catheterization of carotid artery.* Blood pressures of coarcted (CR) and sham animals were measured at 1, 3, 5, and 7 days postsurgery and of animals not subjected to the above surgery (0 day controls) after catheterization of the carotid artery as follows: a rat was anesthetized with pentobarbital sodium (32.5 mg/kg; 0.05 ml per l00 g body weight), the ventral side of its neck was shaven, and a midsagittal incision was made from the top of the rib cage to the underside of the chin. The fascia were separated, and an incision was made to the underlying

musculature. The carotid artery was isolated from underneath the neck muscles. The attached vagus nerve was moved aside and three silk threads (Coats, Dual Duty Plus, carpet thread; approx. l0 cm) were placed underthe artery. One thread was used to constrict the carotid artery at the rostral end. The second thread was used to block off circulation at the caudal end while a nick was made to the artery. A polyethylene catheter was inserted 0.5 cm into the artery and the third thread was used to tie the catheter in place. The caudal-end thread then was released, the catheter was flushed with l0% heparin to prevent blood clotting, and the blood pressure was measured using a Micro-Med Blood Pressure Analyzer (Louisvill, KY). *Henrt isolation.* Once the blood pressure measurements were taken, the animals were sacrificed. The thoracic cavity was opened, and the heart was removed. Excess adipose and connective tissue were removed from the heart which was drained of blood and weighed .

Preparation of 'heart mitochondria. The isolated heart was washed in cold (0-4℃) SNTE buffer (0.20 M sucrose, 0.13 NaCl, l mM Tns · HCl, pH 7.4 [5], and l mM EGTA neutralized with This to pH 7.4 [9]) and minced with scissors, SNTE buffer was added (l.5 ml per g tissue) and the minced heart homogenized at ice temperature with a Model PT l0/35 Polytron homogenizer (setting 11, Brinkmann lnstruments, Westburg, NY) for 2 cycles of l0 seconds each. The homogenate was centrifuged at 500g for l0 min in a Beckman J20.l centrifuge, the pellet discarded, and the supernatant centrifuged at 8000g for l5 min. The mitochondrial pellet was resuspended to 30 mg Biuret protein per ml SNTE buffer. All steps were done at 0-4℃. Biuret protein was measured according to Layne (7).

Preparation of reduced cytochrome c. Cytochrome c (horse heart, Sigma Chemical Co. no. C-7752, TypeⅢ, prepared without using TCA) was reduced with dithionite as described (14) in 50 mM phosphate (K_2HPO_4-KH_2PO_4) buffer at pH 7.0 (13). Reduced cytochrome c was made fresh for each experiment and stored in an air-tight container on ice for the assays. *Cytochrome c oxidase activity.* COX activity was measured spectrophometricallly by following the oxidation of reduced cytochrome c at 550 nm (13) in 50 mM phosphate buffer (PH 7.0) containing 0.5% Tween 80 (5). The final concentration of reduced cvtochrome c was 60 uM and that of mitochondrial protein was 0.013 mg/ml. Rates are expressed as umol cytochrome c oxidized per min per mg mitochondrial protein. *RNA extraction.* Total RNA was extracted using the Ultraspec RNA Isolation System (Biotecx Laboratories, lnc.) according to directions supplied by the manufacturer. Each heart was thoroughly homogenized (1ml Ultraspec reagent per 100 mg tissue) with a Polytron homogenizer. The RNA precipitate was washed twice by gentle vortexing with 70% ethanol, collected by centrifugation at l2,000g, dried under vacuum for 5-l0 min., dissolved in 50-l00 ul of diethylpyrocarbonate-treated water, and incubated for l0-l5 min. at 55-60℃.

Measurement of heart mRNA levels. A 500 bp mouse kidney cDNA encoding COX subunit Vb inserted in the vector pGEM-7Zf(+) was supplied by Dr. N. Avadhani (Univ. of Pennsylvania, Philadelphia). Vb probes were synthesized in an "anti-sense" orientation using digoxigenin (DlC)-labeled UTP and a Genius 4 transcription kit (Boehringer-Mannheim, lndianapolis, IN) according to the manufacturer's protocol. Heart mRNA encoding subunit Vb was detected chemiluminescently on dot blots by hybndization to DlG-labeled probes using nylon membranes, the chemiluminescent CDP-star reagent and Lumi-film (all supplied by Boehringer-Mannheim) was used as a standard to control for any variations in amounts of heart RNA added to the dots for hybridization. Levels of Vb mRNA were measured by densitometry.

四、研究報告應含的內容

Results:

Male Sprague-Dawley rats were subjected to complete coarctation of the abdominal aorta between the origins of the

renal arteries and followed up to 7 days postsurgery. The body weight of sham-operated animals remained nearly constant through most of the postsurgery period but, on the average, increased a small, but not significant, extent by day 7 (Table 1). In contrast, the body weight of the CR decreased by day 3 and remained lower than the control sham-operated animals through the remaining postsurgery period. On day 7 postsurgery, the CR weighed, on average, 19% less than the sham controls (Table 1). This result result correlates with the previous study from this laboratory (3) that showed a 22 % decrease in body weight for CR compared to sham animals on day 7 postsurgery. Blood pressure measurements on randomly chosen animals clearly dernonstrated the hypertensive effects of the aortic coarctation. On days 1 and 7 postsurgery, the carotid arterial systolic blood pressure of sham animals was 152 and 148 mm Hg, respectively, while that of the CR was 167 and 233, respectively.

The development of cardic hypertrophy after complete aortic coarctation is illustrated in Table 2. Heart weights of sham-operated animals remained unchanged throughout the postsurgery period. In contrast, by day 3 after coarctation, hearts were significantly enlarged and remained so through the rest of the observation period of 7 days. This was not due to tissue edema because the wet weight-to-dry weight ratio remained the same during the same postsurgery period. As expected from the data in Table 1 and the heart weight data in Table 2, the heart weight-to-body weight ratios were increased significantly from days 3 through 7 postsurgery in the CR compared to the 0-day controls (Table 2). These ratios essentially were unchanged compared to the 0-day controls during the same postsurgery period in sham animals

Table 3 shows the results for enzymatic activity of COX following surgery. The effects of the stress of the surgical procedure were apparent and especially in sham animals, i.e., a general decrease in cardiac COX activity after day 1 postsurgery. Also, some variability in COX activity was noted between different CR or sham animals used on each day of postsurgery. This variability is likely due to the high speed grinding that not easily controlled but is needed to homogenize the tough cardiac tissue in order to isolate useful quantities of mitochondria. In any case by day 3, COX activity per mg mitochondnal protein of coarcted hearts routinely exceeded that of sham hearts. The increased COX –specific activity correlated with the increased heart weight in CR that also occurs by day 3 postsurgery (Table2). In spite of the variability of COX activity from one mitochondrial preparation to another, cardiac COX-specific activity in CR was significantly different that of shams (Fig. 1) when the results were examined statistically by the method of "paired comparisons" (l). In order to make this comparison, each experiment was run with a pair of animals, one CR and one sham, treated identically except for the placement of the ligature (seen Methods). At day 1 postsurgery, cardiac COX activity of the CR showed a small, but sinificant, decrease when compared to that of the sham animals (Fig. l). By day 3 when the hearts of CR have hypertrophied (Table 2), however, cardiac COX-specific activity is significantly higher that in the nonhypertrophied hearts of sham animals (Fig. l). COX-specific activity in the heart then remains significantly higher in the CR compared to the sham animals through the remaining period of observation.

COX subunit Vb mRNA levels were determined by dot blotting using hearts isolated from 3 pairs of CR and sham animals at3 days postsurgery (Fig. 2) when CR hearts are hypertrophied. Levels of β-actin mRNA, used as a control for RNA addition, did not vary in any case among the hearts from the different animals used. Density (arbitrary units) of each Vb mRNA dot (Fig. 2 top) was measured and divided by the density of the corresponding β-actin mRNA dot. Results obtained for the CR and sham animals are show in Figure 2 (bottom). Clearly, the levels of COX Vb mRNA pe rmg total heart RNA are significantly higher in the CR compared to the sham animals.

Discussion:

Cardiac hypertrophy resulting from chronic pressure overload is rapidly induced in the present model system: complete coarctation of the rat abdominal aorta between the origins of the renal artery. In the first study from this laboratory on this new model system, blood pressure was found to increase significantly by l day following surgery and peak at and remain constant above 200 mm Hg after3 days (2). Blood pressures of sham controls remained between l40-150 mm Hg during the same period postsurgery. Cardiac hypertrophy was observed at 2-3 days postsurgery in CR animals. The present study verifies the earlier study and supports the view that the present model system using complete coarctation is an accelerated model of cardiac hypertrophy compared to other rat models ‹ for example, those that use partial coarctation (11) or treatment with excess thyroid hormone ()5) to inducec cardiac hypertrophy.

For tissue viability and adequate funetion to be maintained during hypertrophy, the cellular enlargement that occurs must by accompanied by proportional increases in the volume of cellular organelles. In order to provide the energy needed for the hypertrophic growth of cardiac muscle cells (myocytes) and for the functional maintenance of the enlarged cardiac myocytes, increases in the volume of the mitochondrial compartment can be expected. A review of the relevant literature (l1), however, reveals discrepancies regarding the behavior of the mitochondnal compartment during the onset and progression of cardiac hypertrophy even when induced by the same stimulus. Some sthidies on cardiac hypertrophy induced by partial aortic coarctation find an increase in mitochondrial content, i.e.,mitochondrial biogenesis, but an increase is not always found in other studies. Similarly, the mitochondrial responses to renovascular hypertension or thyroid hormone-induced cardiac hypertrophy also are variable. Two recent reports, however, indicate changes in COX activity and the levels of mRNAs encoding COX subunits remain constant per mg total heart protein during adaptive cardiac growth under conditions in which cardiac hypertrophy is induced by partial aortic coarctation (l5,11) or by excess thyroid hormone (l5), This suggests that mitochondrial biogenesis is balanced with the adaptive cellular growth of the whole myocardium for 7 days (l5) or even up to 28 days (11) following partial aortic coarctation or for at least 5 days of excess thyroid- hormone treatment (l5).

In contrast ‹ in the present accelerated model of cardiac hypertrophy, COX –specific activity per mg mitochondrial protein is increased by 31-44% during the period of 3 to 7 days postsurgery. This result correlates with the ultrastructural study of Legato et al. (8) who showed that during cardiac hypertrophy mitochondrial DNA content per mg mitochondrial protein in cardiac myocytes is increased and that the mitochondria themselves become smaller and more densely packed with cristae and inner membrane proteins. The imposition of a more severe aortic constriction in the present study than in the other studies (l5,l1) combined with a resultant greater pressure overload may lead to disproportionate changes in mitochondrial activity. Lt has been known for sometime (l2) that mitochondrial responses during cardiac hypertrophy appear related to the abruptness with which the work overload is applied as well as the magnitude of the overload stimulus. In any case, an increased level of mRNA for the nudear-encoded subunit Vb of COX also was observed in the present study as the heart hypertrophied and the COX activity reached a maximum. Followup studies will examine additional COX subunit mRNAS encoded either in the nuclear genome or in the mitochondrial genonme to determine if any change in one subunit mRNA is balanced with similar changes in other subunit mRNAs. Also to be examined are levels of the COX protein during cardiac hypertrophy to determine whether any changes in COX mRNA leveel are balanced by corresponding changes in COX protein-levels. Such studies will help define

controls, transcriptional or posttranscriptional, on COX gene expression during cardiachypertrophy ·

Otherthan the two reportscited above(l5,1l), studies on the molecular events involved in mitochondrial biogenesis during the process of cardiac hypertrophy are lacking in the literature.

In summary, the rapid cardiac hypertrophy induced by complete coarctation of the abdominal aorta in the rat is accompanied by a disproportionate increase in mitochondrial energy-producing activity, a result shown here for the first time. Insufficient oxidative capacity contributes to the cardiac failure frequently observed in hypertrophy cardiomyopathies induced by a variety of conditions (5). The rapid cardiac hypertrophy that develops in the present model provides a unique system to study the molecular basis underlying the adaptation of the oxidative system in cells in response to the demands of hypertrophic growth.

四、計畫成果自評

實驗進行順利，成果顯著。

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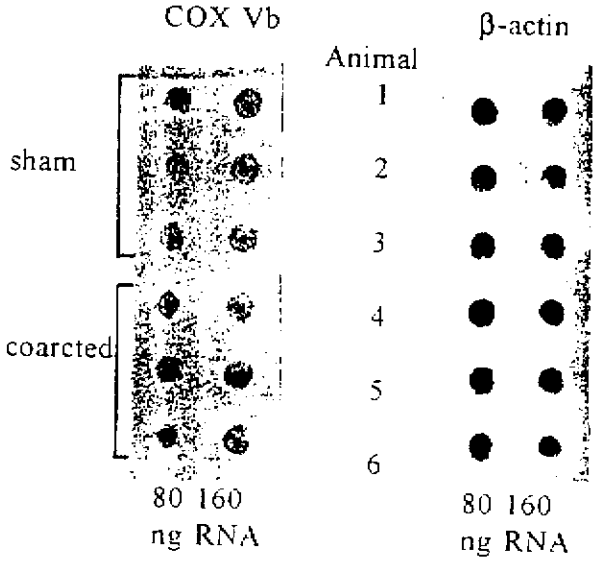
Figure 1. Figure showing the cardiac COX-specific activity in coarcted and sham animals 1, 3, 5, and 7 days postsurgery when the results were examined statistically by the method of "paired comparisons"(1). Number of animals measured at each day postsurgery are given in Table 2. *P<.01, ~P<.005, ΔP<.001 when compared to the relative levels.

Figure 2. Figure showing density of COX subunit Vb mRNA dot blots divided by the density of the corresponding β-actin mRNA dot. Hearts were used from 3 pairs of CR and sham animals 3 days postsurgery when CR hearts are hypertrophied.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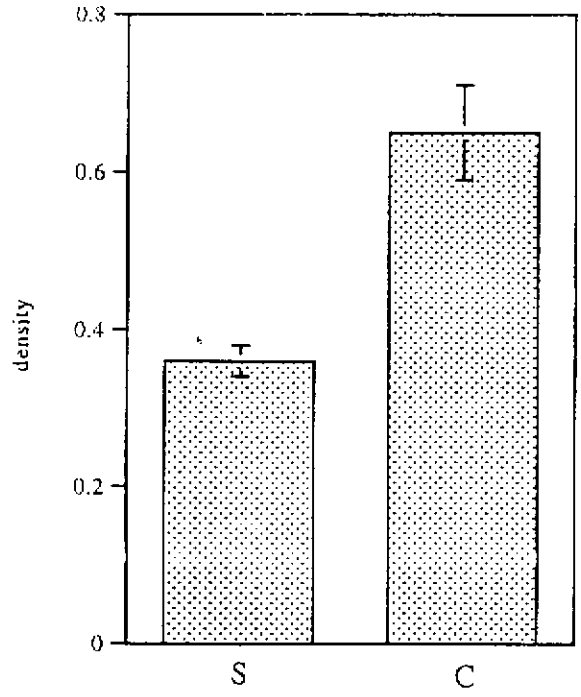
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Figure 2

Table 1. *Body weights of sham-operated and coarcted rats*

Days Postsurgery	Sham	Coarcted
0	264.88±3.37 (n=8)	-----
1	271.75±13.33 (n=4)	274.50±10.14 (n=4)
3	273.14±6.30 (n=7)	248.50±5.19 (n=6)
5	266.00±4.93 (n=3)	213.33±4.98 (n=3)
7	289.75±17.04 (n=4)	234.00±7.60 (n=4)

Data are mean ± SEM of the body weights (g) following surgery; n, number of animals. Zero day values represent data from animals saerificed on the day of surgery.

Table 2. *Heart weights and heart weight to body weight ratios of sham-operated and coarcted rats*

Days Postsurgery	HW, g		HW/BW, mg/g	
	Sham	Coarcted	Sham	Coarcted
0	0.96±0.02 (n=8)	-----	3.63±0.08 (n=8)	-----
1	0.92±0.03 (n=4)	0.97±0.02 (n=4)	3.38±0.08[□] (n=4)	3.54±0.14 (n=4)
3	0.95±0.03 (n=7)	1.13±0.03[*] (n=6)	3.47±0.07 (n=7)	4.56±0.13[*] (n=6)
5	0.93±0.01 (n=3)	1.06±0.02[□] (n=3)	3.51±0.08 (n=3)	4.98±0.18[*] (n=3)
7	1.00±0.07 (n=4)	1.08±0.04[□] (n=4)	3.44±0.08 (n=4)	4.62±0.30[○] (n=4)

Data are mean ± SEM. HW and HW/BW are the heart weights (g) and heart weight to body weight ratios (mg/g), respectively, following surgery; n, number of animals. Zero day values represent baseline data from animals sacrificed on the day of surgery. [□]P<0.05, [○]P<0.025, [*]P<0.001 compared to zero-day animals.

Table 3. *COX activity in coarcted (CR) and sham (S) rats on days 1 through 7 postsurgery.*

Days Postsurgery	Activity		CR:S ratio
	CR	S	
1	0.56±0.10 (n=4)	0.64±0.09 (n=4)	0.90±0.10
3	0.42±0.06 (n=3)	0.30±0.06 (n=3)	1.44±0.10
5	0.47±0.02 (n=3)	0.33±0.02 (n=3)	1.43±0.05
7	0.47±0.12 (n=4)	0.35±0.07 (n=3)	1.31±0.13

Activity values are umoles cytochrome c oxidized/min/mg mitochondrial protein (mean ± SEM).