*Free Radical Research*, Volume 38 Number 6 (June 2004), pp. 591–599

Taylor & Francis
healthsciences

# Do Mitochondria make Nitric Oxide? No?

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Accepted by Professor V. Darley-Usmar

*(Received 21 January 2004; In revised form 29 February 2004)*



Several papers have claimed that mitochondria contain nitric oxide synthase (NOS) and make nitric oxide ($NO^{\cdot}$) in amounts sufficient to affect mitochondrial respiration. However, we found that the addition of L-arginine or the NOS inhibitor L-NMMA to intact rat liver mitochondria did not have any effect on the respiratory rate in both State 3 and State 4. We did not detect mitochondrial $NO^{\cdot}$ production by the oxymyoglobin oxidation assay, or electrochemically using an $NO^{\cdot}$ electrode. An apparent $NO^{\cdot}$ production detected by the Griess assay was identified as an artifact. $NO^{\cdot}$ generated by eNOS added to the mitochondria could easily be detected, although succinate-supplemented mitochondria appeared to consume $NO^{\cdot}$.

Our data show that $NO^{\cdot}$ production by normal rat liver mitochondria cannot be detected in our laboratory, even though the levels of production claimed in the literature should easily have been measured by the techniques used. The implications for the putative mitochondrial NOS are discussed.

*Keywords*: Mitochondria; Nitric oxide; Nitric oxide synthase; Artifact; Griess assay

## INTRODUCTION

The synthesis of the important cellular signalling molecule nitric oxide ($NO^{\cdot}$) is catalyzed by nitric oxide synthase (NOS), a family of enzymes that generate $NO^{\cdot}$ from the terminal guanidino nitrogen of arginine, with the concomitant production of citrulline.[1,2] Three distinct isoforms of NOS have been well characterized: endothelial nitric oxide synthase (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS).[2]

In addition, several papers have suggested that a fourth NOS form exists: mitochondrial NOS (mtNOS).[3–10] mtNOS appears to associate with the mitochondrial inner membrane and regulates mitochondrial oxygen consumption, matrix pH, transmembrane potential and calcium buffering capacity.[4,5] A role for mtNOS in apoptosis has also been described.[6] Intact mitochondria and submitochondrial particles (SMPs) have been reported to produce $NO^{\cdot}$ in an energy-dependent manner[7] and it was suggested that this $NO^{\cdot}$ production, catalyzed by mtNOS, is supported by mitochondrial NADPH, which in turn is maintained by an energy-dependent transhydrogenase. mtNOS is inhibitable by the competitive inhibitor $N^{G}$-methyl-L-arginine (L-NMMA) and the $NO^{\cdot}$ it produces has been proposed as a modulator of cytochrome oxidase activity.[7,9]

Giulivi *et al.*[10] have isolated mtNOS from rat liver mitochondria and claimed it to be a homologue of brain NOSα, as identified by various methods (mass spectrometry, amino acid analysis, molecular weight, pI, PCR fragment analysis). In contrast to brain NOSα, it has two post-translational modifications: acylation with myristic acid and phosphorylation at the C terminus.[10] However, some authors have expressed doubts about the nature of mtNOS,[11,12] and so we thought it worthwhile reporting our attempts to detect mitochondrial $NO^{\cdot}$ production.

## MATERIALS AND METHODS

### Materials

EGTA, sodium succinate, mannitol, Hepes, ADP, NADPH, sodium nitrite, sodium nitrate,

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10715760410001694008

sulphanilamide, *N*-(1-naphthyl)ethylenediamine, myoglobin, catalase, nitrate reductase and BSA were obtained from Sigma Chemical Co., USA. Sucrose was obtained from Bio-Rad. eNOS was obtained from Cayman Chemicals. PD-10 columns were obtained from Amersham Biosciences, UK.

## Methods

### *Isolation and Purification of Mitochondria*

Mitochondria from livers of adult male Sprague–Dawley rats (200–250 g) were isolated by differential centrifugation. Some were purified by Percoll centrifugation as described in Ref. [7]. Briefly, the livers from rats (starved overnight and sacrificed by cervical dislocation) were excised, washed with 0.25 M sucrose and homogenized (1/10, w/v) in MSHE (0.22 M mannitol, 0.07 M sucrose, 0.5 mM EGTA, 0.1% BSA, 2 mM Hepes/KOH, pH 7.4) at 4°C. The homogenate was centrifuged at 1000*g* for 10 min in a JA-20 rotor. Any floating fat was removed with a Pasteur pipette and the supernatant was re-centrifuged at the same speed for 10 min. The second supernatant was transferred into a new tube with a Pasteur pipette and centrifuged at 10,000*g* for 10 min.

The subsequent pellet was resuspended in 5 ml of MSHE supplemented with 20 ml of 30% Percoll in MHE (225 mM mannitol, 1 mM EGTA, 25 mM Hepes, 0.1% BSA, pH 7.4) and spun at 95,000*g* in a Beckman Ti70 rotor for 30 min. The fraction with a density of 1.052–1.075 g/ml (lowest band, creamy brown in color) was collected and washed twice with MSHE at 10,000*g* for 10 min to remove the Percoll. Purified mitochondria were then washed twice with 150 mM KCl followed by one wash with MSHE.

### *SMP Preparation*

For the preparation of SMPs, the final mitochondrial pellet was stored frozen at −80°C. After 12 h, the pellet was thawed at room temperature and diluted with MSHE to 10 mg/ml. The suspension was sonicated three times for 1 min with an interval of 2 min between sonications, using an MSE 150-W ultrasonic disintegrator with a 0.75-in titanium probe at high energy setting. Intact mitochondria were removed by centrifugation at 10,000*g* for 15 min. SMPs were collected by further centrifugation at 140,000*g* for 2 h at 4°C. The pellet was washed with MSHE buffer and centrifuged again.

### *Oxygen Consumption*

Oxygen uptake was measured polarographically with a Clark-type electrode calibrated with air-saturated water at 22°C. Sodium dithionite was added to obtain an experimental zero $O_2$ concentration. The reaction medium used consisted of 0.225 M sucrose, 5 mM $MgCl_2$, 20 mM KCl, 10 mM potassium phosphate, 20 mM Hepes/KOH, pH 7.4. Succinate 10 mM, ADP 0.3 mM, L-arg 0.1 mM or L-NMMA 0.1 mM were added as indicated.

### *Detection of $NO^{\cdot}$ Production: Oxymyoglobin Assay*

One milliliter of myoglobin solution (20 mg/ml) was reduced with a slight molar excess of sodium dithionite. The bright red oxymyoglobin produced was passed through a Sephadex G25 column (PD-10 or NAD-25) to eliminate excess sodium dithionite. Potassium phosphate buffer (100 mM; pH 7.4) was used as the eluent. The concentration of oxymyoglobin was determined by measuring absorbance at 417, 542 and/or 580 nm ($\varepsilon_{417}$: 128/mM/cm, $\varepsilon_{542}$: 13.9/mM/cm and $\varepsilon_{580}$: 14.4/mM/cm).[13]

The reaction medium used with intact mitochondria consisted of 0.225 M sucrose, 5 mM $MgCl_2$, 20 mM KCl, 10 mM potassium phosphate, 20 mM Hepes/KOH, pH 7.4 supplemented with 50 μM oxymyoglobin. The reaction was started with the mitochondrial material. L-arg 0.2 mM, succinate 10 mM and L-NMMA 0.1 mM were added as indicated. Oxidation of oxymyoglobin to metmyoglobin was monitored by obtaining, at 2–3 min intervals, a wavelength scan from 500 to 600 nm, as well as specific absorbances at 525, 545, 565 and 572 nm. Metmyoglobin formation was calculated according to Ref. [13].

### *Electrochemical Measurement of $NO^{\cdot}$: $NO^{\cdot}$ Electrode*

The $NO^{\cdot}$ standards and electrode were prepared as described in Ref. [14]. Briefly, phosphate-buffered saline (PBS) at pH 7.0 was bubbled with pure $N_2$ for 30 min to remove $O_2$. The solution was then bubbled with $NO^{\cdot}$ gas for 30 min and kept under an $NO^{\cdot}$ atmosphere until use. Standards were made fresh for each experiment and kept in a glass flask with a rubber septum. Uniform polymerized *o*-aminophenol (POAP) film was electrodeposited on the surface of a glassy carbon electrode (GC, $\phi$ 2 mm) by potential cycles between 0.0 and +0.90 V (vs. SCE) at a scan rate of 50 mV/s in a neutral solution (0.025 M PBS plus 0.1 M KCl) containing 1.0 mM OAP monomer. The supporting electrolyte was bubbled with pure nitrogen for ~10 min to remove oxygen before the addition of the OAP monomer. The deposition of palladium (Pd) nanoparticles into POAP film was achieved by cycling the potential at the POAP/GC electrode between +0.40 and −0.30 V (vs. SCE) at a rate of 50 mV/s in a solution containing 2.0 mM $K_2PdCl_6$ and 0.5 M $H_2SO_4$. The resulting electrode is denoted as Pd@POAP/GC.

All electrochemical measurements were performed on a BAS 100 B/W workstation (Bioanalytical Systems, West Lafayette, USA) controlled

by the BAS 100 B/W software from a Gateway 2000 personal computer. The three-electrode system consisted of a Pd@POAP/GC as the working electrode, a SCE reference electrode and a platinum wire auxiliary electrode. All potentials were quoted with reference to the SCE. Prior to each measurement, the working electrode (Pd@POAP/GC) was inserted in a solution containing 5.0 ml respiratory buffer (0.225 M sucrose, 5 mM $MgCl_2$, 20 mM KCl, 10 mM potassium phosphate, 20 mM Hepes/KOH, pH 7.4) and its potential was cycled between 0.0 and +0.80 V at a rate of 50 mV/s until a steady response was obtained. The detection of $NO^{\cdot}$ was conducted in amperometric mode and the solution was stirred mechanically during the experiment. The currents from the electrocatalytic oxidation of $NO^{\cdot}$ were recorded at the applied potential of +700 mV for $NO^{\cdot}$. The currents generated at this potential have been shown to be directly proportional to the concentrations of $NO^{\cdot}$.[14]

### *Nitrate and Nitrite Determination: Griess Assay*

Intact mitochondria (4 mg/ml) were incubated with reaction medium (0.225 M sucrose, 5 mM $MgCl_2$, 20 mM KCl, 10 mM potassium phosphate, 20 mM Hepes/KOH, pH 7.4) for 30 min, while SMPs (2 mg/ml) were incubated with reaction medium plus 0.5 mM NADPH, 5 μM tetrahydrobiopterin and 50 nM FMN for 30 min. $Ca^{2+}$ 1 mM, Calmodulin 5 μg/ml, Succinate 10 mM, L-arg 0.2 mM and/or L-NMMA 0.1 mM were added as indicated. Suspensions were then centrifuged at 12,000*g* for 10 min at 22°C. Supernatants were recovered and incubated with 0.1 mM NADPH and 10 units/ml nitrate reductase for 30 min at 22°C. The $NO_2^-$ formed was incubated for 10 min with 1% (v/v) sulphanilamide in 30% (v/v) acetic acid and 0.1% (w/v) *N*-(1-naphthyl)ethylenediamine in 60% (v/v) acetic acid in a 1:1 ratio.[7] Absorbance was measured at 540 nm and the amount of nitrate and/or nitrite produced was determined using calibration curves of standard $NO_2^-$ and $NO_3^-$ solutions.

### *Protein Determination*

Protein concentrations were determined using the Bio-Rad DC assay kit (Sigma): 5 μl of sample was incubated at room temperature with 25 μl of Reagent A and 200 μl of Reagent B for 15 min. Absorbance was read at 750 nm. Bovine serum albumin was used as a standard.

### *Data Evaluation and Analysis*

Data are presented as mean ± standard deviation. Statistical analysis was done using the Student's *t*-test.

## RESULTS

### Oxygen Consumption of Intact Mitochondria

Intact coupled rat-liver mitochondria [respiratory control ratio (RCR) of $5 \pm 0.5$, $n = 6$] were isolated by differential centrifugation from rat liver and purified by Percoll centrifugation, to decrease contamination by other subcellular organelles.[7] To determine the respiratory rate of mitochondria in State 4, oxygen consumption was observed upon supplementation with succinate. Neither L-arg nor L-NMMA (each tested up to 10 mM final concentration) had any effect on mitochondrial respiration in State 4 (Fig. 1 shows a representative example of more than 10 separate experiments using different mitochondrial preparations).

To observe the respiratory rate of mitochondria in State 3, oxygen consumption was observed upon supplementation with succinate and ADP in the presence of L-arg or L-NMMA. Again no effect of either was observed (Fig. 1). These results indicate that mitochondrial respiration in States 3 and 4 is not affected by the addition of L-arg or L-NMMA, in contrast to previous reports[7] that the endogenous production of $NO^{\cdot}$ by mtNOS can inhibit mitochondrial respiration.

### Effect of Succinate on Nitrate and Nitrite Production by Intact Mitochondria and Submitochondrial Particles

It has been claimed[7] that mitochondria and SMPs produce $NO^{\cdot}$ in an energy-dependent manner, which is inhibited by the NOS inhibitor $N^G$-methyl-L-arginine (L-NMMA). However, in our laboratory, the addition of 10 mM arginine plus succinate to 2 mg/ml SMPs in the presence of cofactors for NOS did not result in any increase in the amount of $NO_2^-$ and $NO_3^-$ detected (Fig. 2A). L-NMMA (0.1 mM) had no effect on the amounts of $NO_2^-$ and $NO_3^-$ detected (data not shown).

In apparent contrast, our mitochondrial preparations appeared to show a significant increase in nitrate and nitrite ($NO_2^-$ and $NO_3^-$) production upon the addition of 10 mM succinate alone or in combination with 0.2 mM L-arg (Fig. 2B), based on an absorbance change in the Griess reaction. However, this was not inhibitable by L-NMMA (Fig. 2C). We performed a wavelength scan to investigate this apparent discrepancy (Fig. 3). In contrast to the authentic $NO_2^-/NO_3^-$ standards which gave an absorbance peak at 540 nm as expected (Fig. 3A), the mitochondrial or SMP samples did not have an obvious peak (spectrum for mitochondria depicted in Fig. 3B, similar graph obtained for SMPs). The apparent increase in absorbance in the Griess

reaction appears due to a chromogen which is not that generated by $NO_2^-$ or $NO_3^-$ and may be a mitochondria-related artifact in the Griess assay.

When samples were spiked with varying amounts of $NO_2^-$ and $NO_3^-$, the correct amounts were detected, with the correct spectrum (Fig. 3C). Hence there is no significant interference with the assay from any of the mitochondrial or SMP components or chemicals added.

We assayed at mitochondrial and SMP concentrations (4 and 2 mg/ml, respectively) up to 4–5 times those used by Giulivi *et al.* (1 and 0.4 mg/ml, respectively),[9] and were still unable to detect $NO_2^-$ and $NO_3^-$ formation. Even at 20 mg/ml we were still unable to detect anything, although there were significant problems with turbidity in the assay at such high levels.

### Measurement of Mitochondrial NO· Production by the Spectrophotometric Oxymyoglobin Assay

We attempted to detect NO· using the oxymyoglobin assay, which is based on the reaction of NO· with oxymyoglobin to form metmyoglobin. Figure 4 depicts wavelength scans (500–600 nm) of a 0.1 mg/ml mitochondrial sample to which substrates were added at the time points indicated. Although changes in absorbance were observed, the general shape of the spectral curve remained constant; there was no spectral shift characteristic of authentic NO· formation. When NO solution was

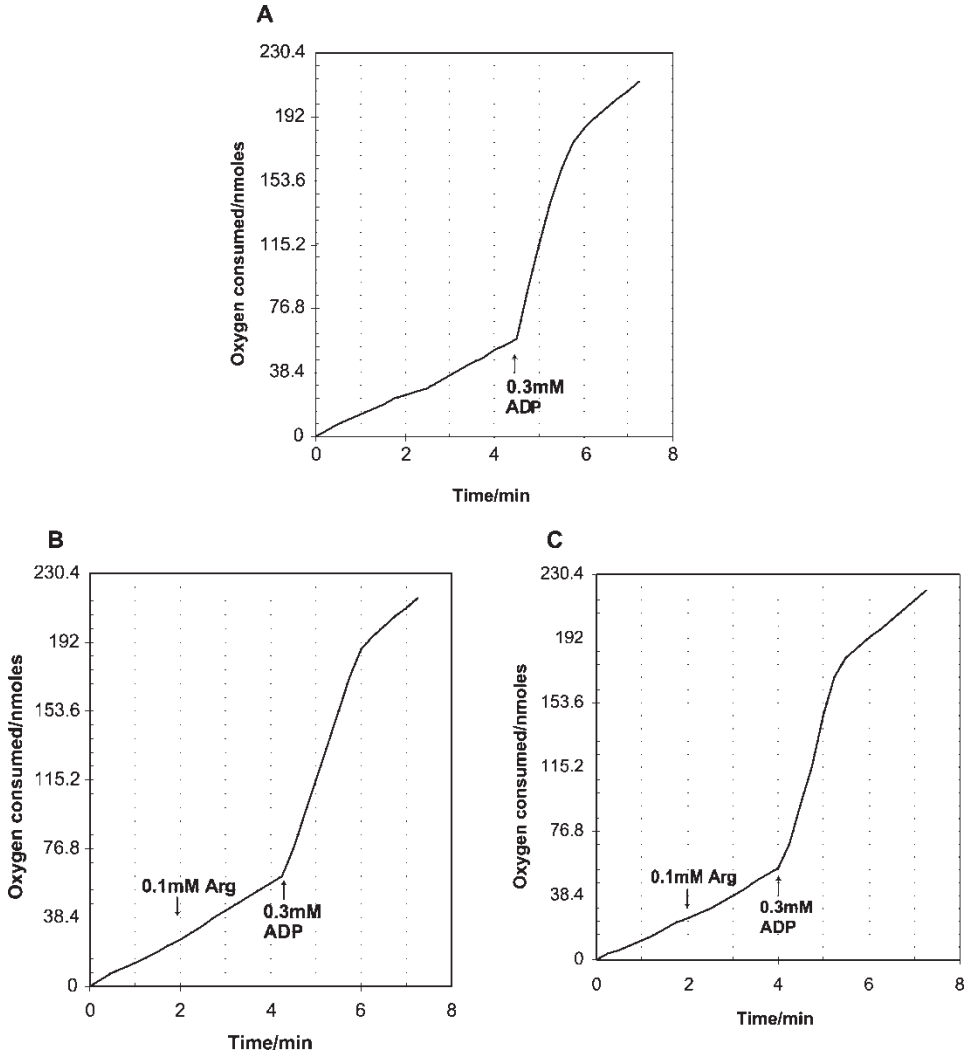


FIGURE 1 Oxygen consumption of isolated intact mitochondria in the presence of various substrates. The oxygen uptake of 1 mg/ml mitochondria suspended in reaction medium was measured as described in the "Materials and methods" section. All samples were supplemented with 10 mM succinate. Initial oxygen uptake represents mitochondrial respiratory rate in State 4, while the respiratory rate in State 3 is represented by oxygen uptake after the addition of ADP. (A) Depicts the oxygen consumption of mitochondria. (B) Shows mitochondrial oxygen consumption in the presence of 0.1 mM L-arg, while (C) shows mitochondrial oxygen consumption in the presence of 0.1 mM L-NMMA.

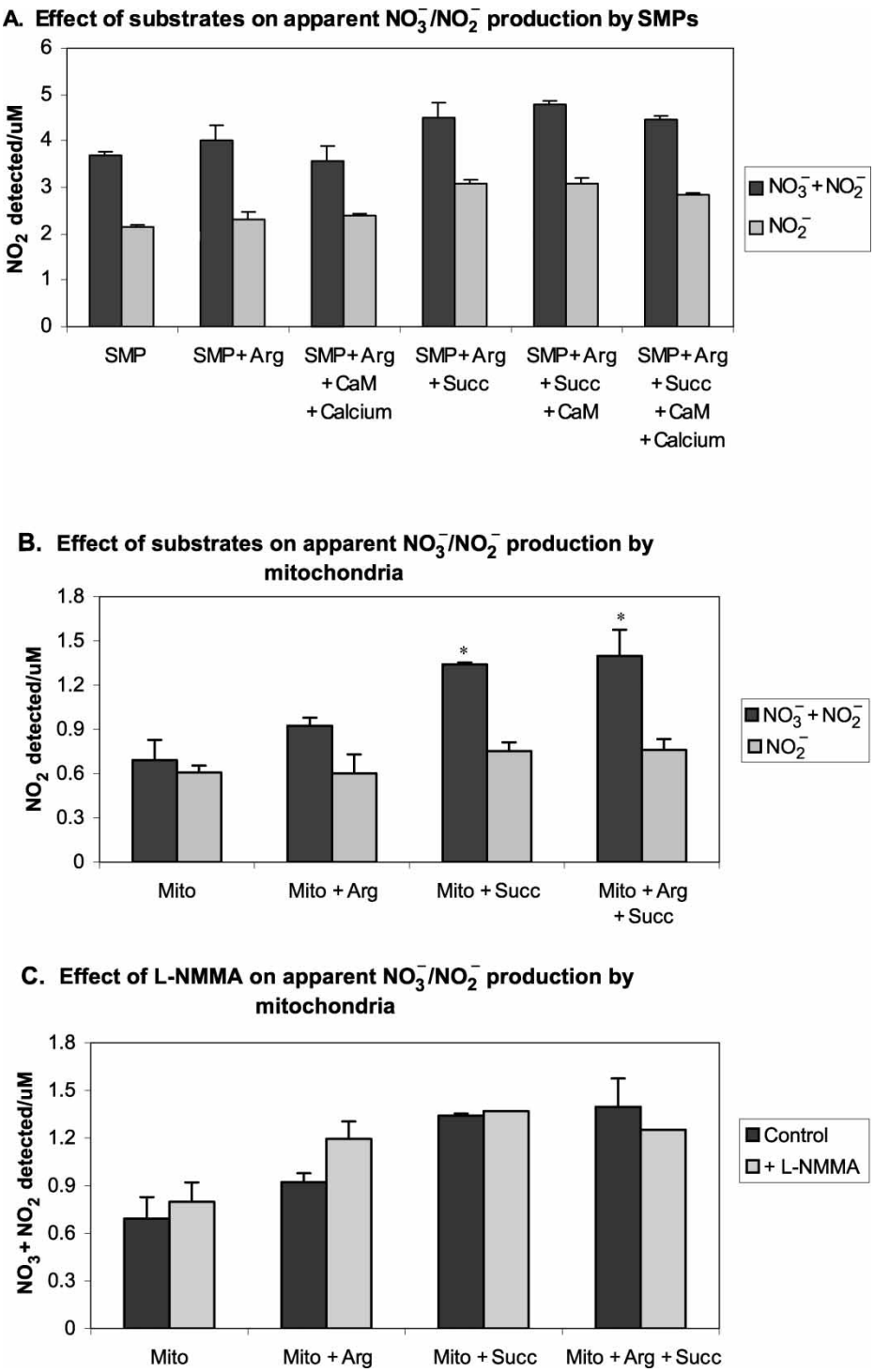


FIGURE 2 Apparent nitrate and nitrite production by SMPs and intact mitochondria. (A) 2 mg/ml SMPs or (B, C) 4 mg/ml intact mitochondria were incubated as indicated above. Concentrations of reactants and the method of nitrate and nitrite measurement are described in the "Materials and methods" section. The basal levels of $NO_2^-/NO_3^-$ are due to its presence in the organelle fractions and in the reaction medium. Statistical analysis was done using the Student's *t*-test ($*P < 0.01$ for mitochondria incubated with succinate and mitochondria incubated with succinate and L-arg).

added to the mitochondrial samples containing substrates, the correct spectrum was obtained (data not shown) indicating that none of the reaction components themselves interfere with the assay. When measurements were made only at specific wavelengths rather than running the whole spectrum an apparent increase in metmyoglobin formation is observed immediately after the addition of mitochondria to oxymyoglobin preparations, but it was not inhibited by L-NMMA. It is most likely due to the absorbance of the mitochondria (Fig. 4). Brookes[12] has commented

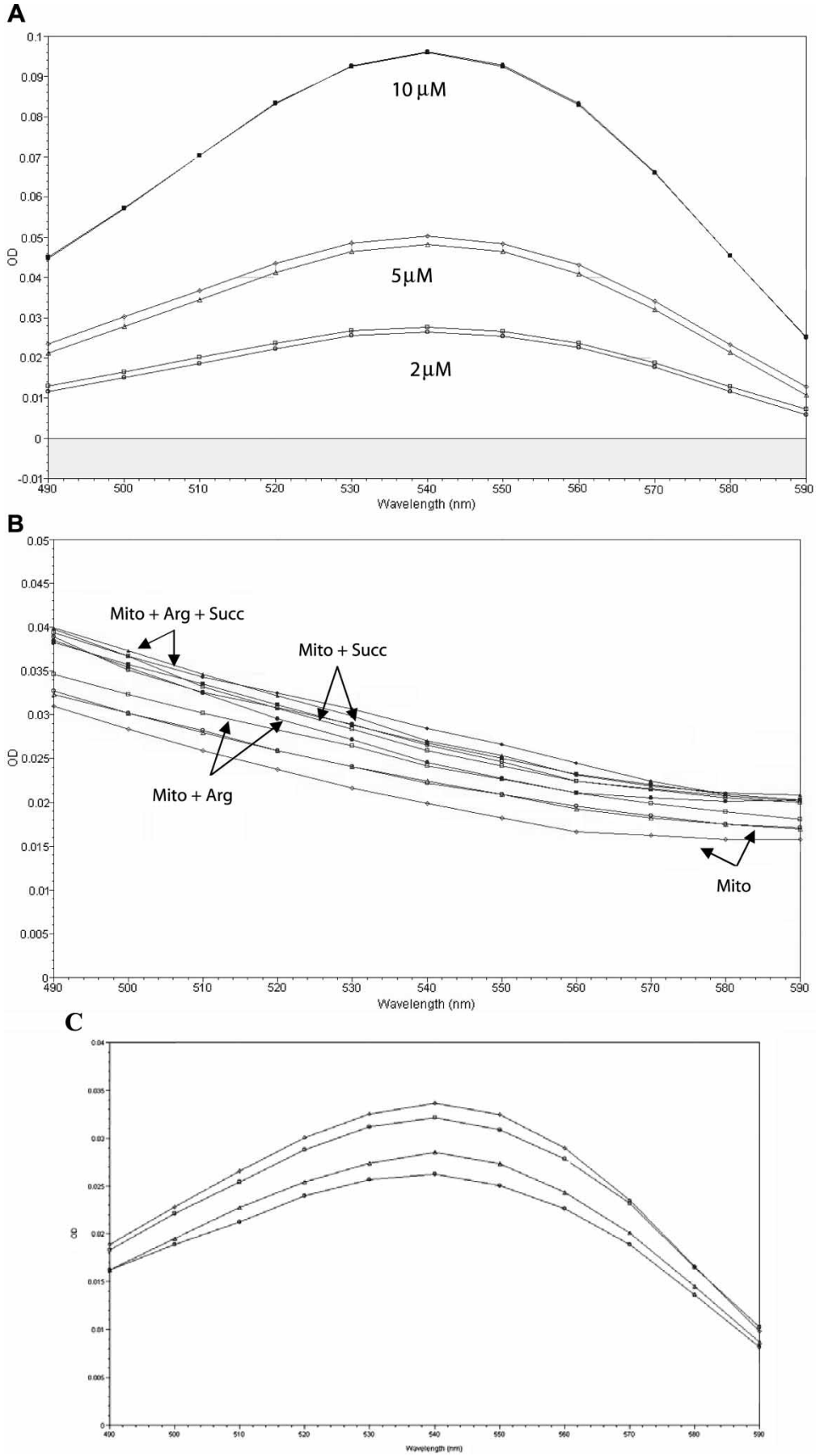


FIGURE 3 Wavelength scans of mitochondrial samples from 490 to 590 nm. (A) Nitrate standards: 2, 5, 10 µM. (B) 4 mg/ml mitochondria ± 0.2 mM L-arginine ± 10 mM succinate. (C) Mitochondrial samples spiked with $NO_2^-$.

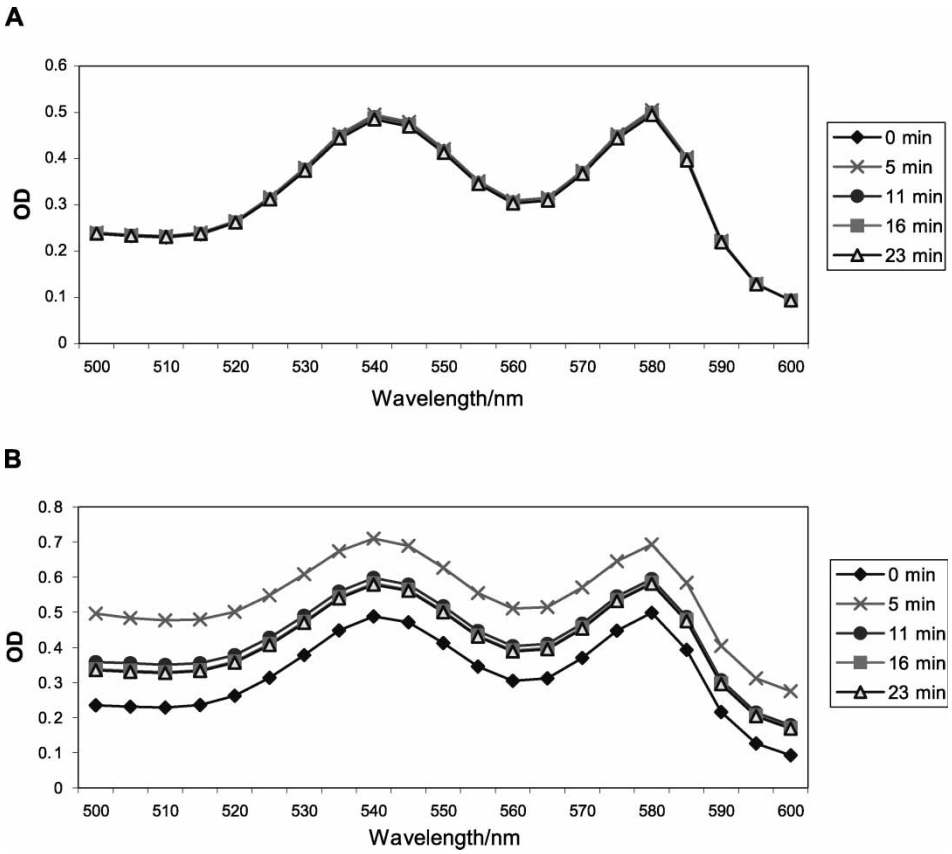


FIGURE 4 Rate of NO· production as measured by the rate of oxymyoglobin oxidation. (A) Control: 10 mM succinate was added at 11 min and 0.2 mM L-arginine was added at 16 min. (B) 0.1 mg/ml mitochondria were added at 5 min, followed by 10 mM succinate at 11 min and 0.2 mM L-arginine at 16 min.



on the artifacts that can be produced by measurement only at specific wavelength in the oxymyoglobin assay.

### Measurement of NO· Production Using an NO· Electrode

The NO· electrode has a high sensitivity in detecting NO· at sub-micromolar levels, the presence of 500 nM pure NO· being revealed by a sharp decrease in current, which then returns gradually to the original level (Fig. 5A). The amount of NO· detected can thus be quantified by comparing the size of the spike with that of a known amount of pure NO· The addition of mitochondria or substrates may momentarily disturb the recording system, resulting in apparent sharp spikes in the current (Fig. 5C,D).

Although the observed current appeared to decrease upon the addition of succinate to mitochondria (Fig. 5C), this phenomenon also occurs in the presence of L-NMMA (Fig. 5D) and when succinate is added to the buffer alone (Fig. 5B). It may thus represent an interaction of succinate with the electrode. Further addition of arginine to succinate-supplemented mitochondria did not cause any detectable NO· production (Fig. 5C).

These results indicate that mitochondrial NO· production does not occur at a level detectable by the NO· electrode.

### Detection of NO· Formation in Mitochondrial Samples Supplied with Exogenous NOS

When eNOS was supplied exogenously to mitochondria or SMP, NO· production could be detected by all the assays used in this paper (data not shown). However, the presence of mitochondria caused a lower level of NO· to be detected, as was also observed when NO· was spiked into mitochondria. This is consistent with many literature reports that mitochondria consume NO· [12,15]

## DISCUSSION

In our laboratory, percoll-purified intact mitochondria and submitochondrial particles did not produce NO· at a level high enough to be detected by any of the assays used, when artifacts were taken into account. Claimed levels of NO· production by mitochondria and SMP in the literature should easily be detectable by any of the assays used here.[12] Moreover, the addition of L-arginine to intact

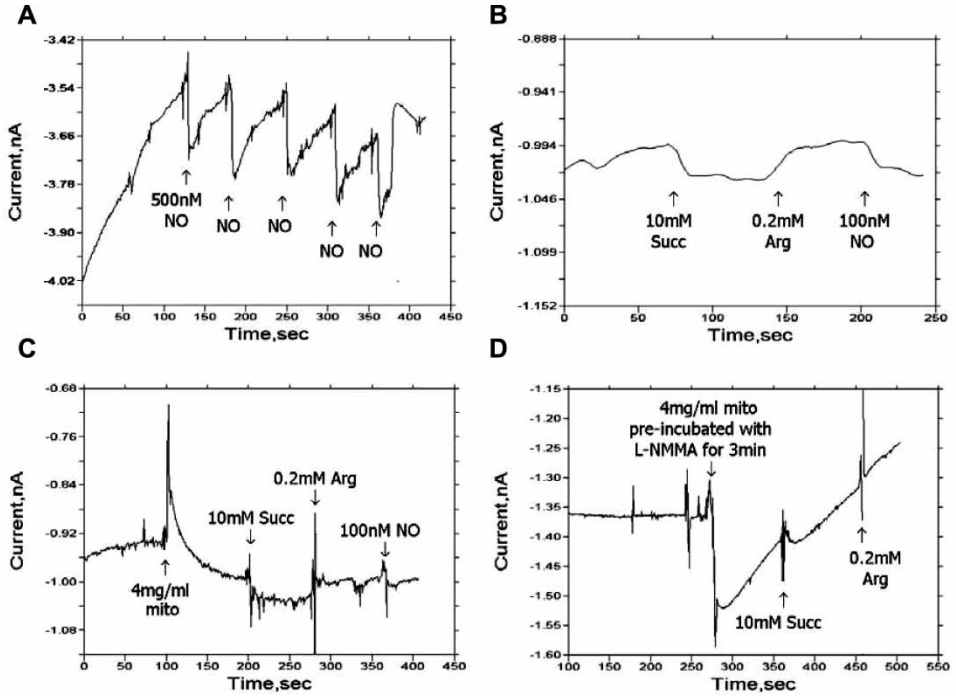


FIGURE 5 Measurement of NO· production using the NO· electrode. (A) Current response of NO· at the Pd@POAP/GC sensor. A typical amperogram is shown as the current response of NO recorded at the applied potential of +700 mV. Arrows indicate injections of 500 nM NO·. (B–D) Mitochondria, succinate, arginine and NO·were injected as indicated and their effect on the current response noted. Representative experiments of at least 5 are shown.



mitochondria did not inhibit the respiratory rate in both States 3 and 4. There was also no increase in mitochondrial oxygen uptake upon the addition of L-NMMA.

There are several possible explanations for this. The simplest is that liver mitochondria from the rats we used do not contain an active NOS. Additionally, low mtNOS activity could not be detected if the mitochondrial removal rate of NO·[12,15] were greater than the maximum rate of NO· production in homogenates. Brookes[12] has argued that the currently-available data are not convincing of the presence of mtNOS. Our data do not address directly the issue of whether a mtNOS is present, but they do suggest that NO· production by any mtNOS that was present is not a significant source of NO· in the rat liver mitochondria used in our laboratory. There was no evidence that NO· from mtNOS affects oxidative phosphorylation under experimental conditions. Of course, regulation of mitochondrial function by NO· arising from other subcellular fractions can probably occur *in vivo*.[15–17]

## *Acknowledgements*

We are grateful to the National Medical Research Council, the Academic Research Fund of the National University of Singapore and the Singapore Totalisator Board for research support.

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