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Mitochondrial Nitric Oxide Synthase: A Ubiquitous Regulator of Oxidative Phosphorylation?

T. E. Bates, A. Loesch,* G. Burnstock,* and J. B. Clark

*Department of Neurochemistry, Institute of Neurology, University of London, Queen Square, London, WC1N 3BG, England; and *Department of Anatomy and Developmental Biology, University College London, Gower Street, London, WC1E 6BT, England*

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In this article we demonstrate the immunocytochemical localization of nitric oxide synthase in mitochondria isolated from heart, skeletal muscle, and kidney, using a monoclonal antibody directed against the endothelial form of nitric oxide synthase. The possibility that mitochondrially located nitric oxide synthase is a ubiquitous regulator of mitochondrial oxidative phosphorylation in mammalian cells is discussed.

In a recent paper we presented immunocytochemical evidence for the presence of nitric oxide synthase (NOS) in non-synaptic brain mitochondria and liver mitochondria (1). We also suggested that mitochondrially located NOS (mtNOS) might be involved in the regulation of oxidative phosphorylation, because of the ability of nitric oxide (NO) (a product of NOS activity) to bind to cytochrome oxidase and inhibit electron transport (2). If mtNOS is an intrinsic regulator of mitochondrial electron transport, then one would predict that it would be present in mitochondria from other mammalian tissues. Therefore in this study we have investigated the mitochondrial localization of NOS within mitochondria isolated from heart, skeletal muscle and kidney utilizing a postembedding immunogold procedure with silver enhancement.

MATERIALS AND METHODS

Isolation of mitochondria. Preparations of heart, skeletal muscle and kidney mitochondria were isolated from adult male Wistar rats using minor modifications of established (non-enzymatic) methods (3-5). Isolated mitochondria were incubated in respiration medium with 2.5 mM malate and 10 mM glutamate at 37°C prior to pelleting in a centrifuge at 16,000g.

Immunocytochemistry. Isolated mitochondria were fixed for 1h at 4°C with 2% paraformaldehyde and 0.05% glutaraldehyde in 100 mM phosphate buffer pH 7.4, dehydrated through graded ethanol and embedded in Unicryl resin (Agar, U.K.). Ultrathin sections (on uncoated nickel grids) were processed for post-embedding colloidal gold immunocytochemistry as previously reported (1,6). Mouse anti-eNOS monoclonal antibody (Affiniti, isotype: IgG1, specificity: human and rat) was used at a dilution of 1:25-1:50; gold-labelled goat anti-mouse IgG: 10nm (BioCell, U.K.) was used at a dilution of 1:25. Signal was intensified with silver using a silver enhancing kit (Biocell, U.K.). After the immunoprocedure, the ultrathin sections were stained with uranyl acetate and lead citrate and subsequently examined with a JEM-1010 electron microscope. The specificity of the immunoprocedure was investigated routinely, including the omission of the primary antibody step, resulting in elimination of positive labelling. The monoclonal anti-eNOS antibody used was raised in mouse using a 20.4 kDa protein fragment corresponding to amino acids 1030 to 1209 of human eNOS as an antigen.

In order to establish the percentage of mitochondria positive (and negative) for eNOS, mitochondria were counted upon examination of electron micrographs. Data is expressed as mean ± S.E.M. (n = number of microscopic fields studied).

RESULTS

Silver enhanced gold immunolabelling for eNOS was seen within mitochondrial preparations from heart, skeletal muscle and kidney (Fig 1a–c). A high percentage of mitochondria from all three tissues were positive for eNOS. However, the labelling signal varied from mitochondrion to mitochondrion. Approximately 86.4% ± 3.3% of mitochondria in heart (154 mitochondria out of a total of 176 examined; n = 6), 70.3% ± 3.8% in skeletal muscle (132 mitochondria out of a total of 184 examined; n = 6) and 63.7% ± 4.4% in kidney (234 mitochondria out of a total of 351 examined; n = 8) displayed the silver-gold signal/immunodeposit. The immunodeposit was seen

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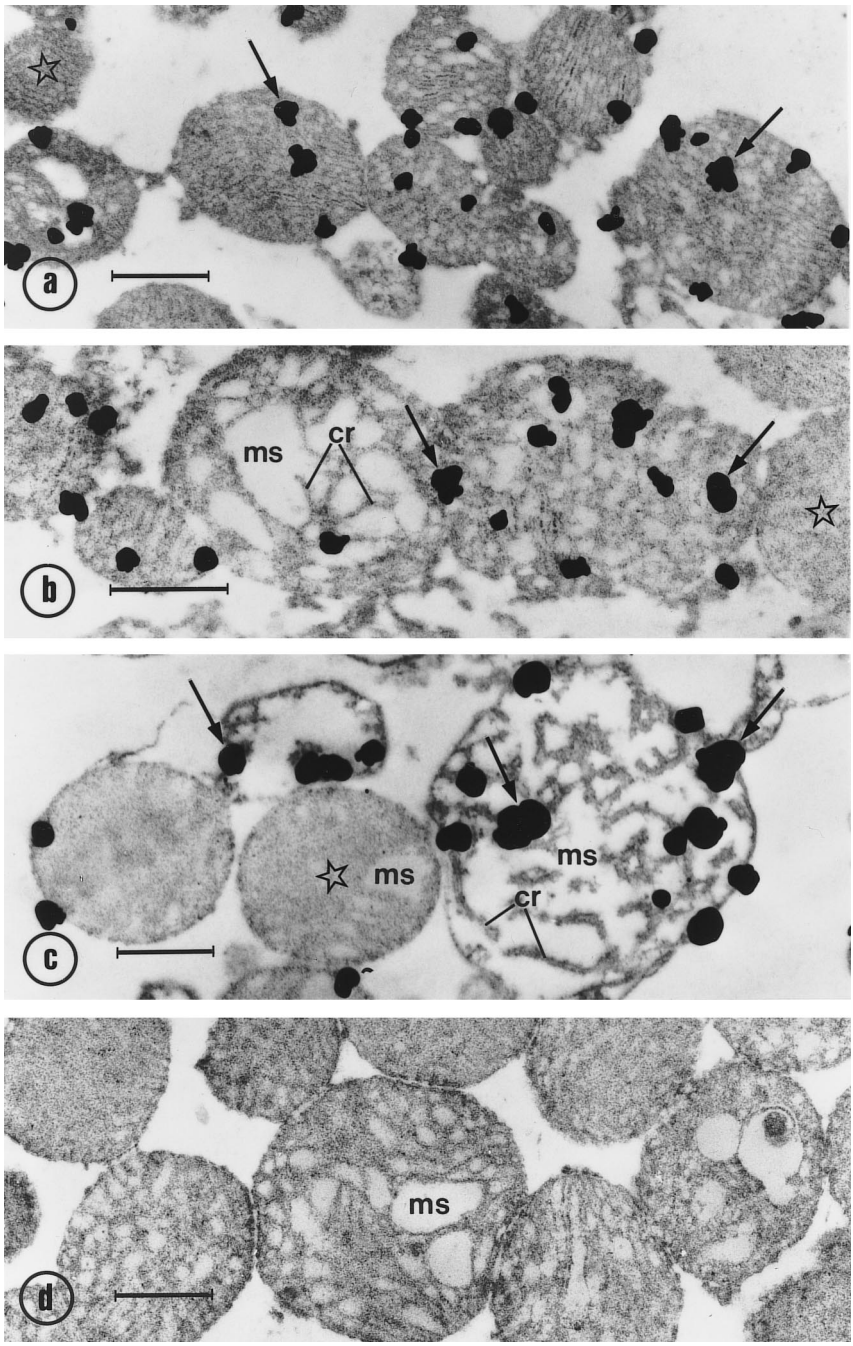

FIG. 1. Electron micrographs of mitochondria from (a) heart, (b) skeletal muscle, and (c) kidney of the rat labeled for eNOS by the silver enhanced immunogold method and (d) a control specimen from heart. In a, b, and c note the irregular, silver–immunogold label (arrows) in the mitochondria; unlabeled mitochondria can also be seen (stars); ms, matrix space; cr, cristae. In d, note the unlabeled mitochondria in the control preparation (obtained on omission of eNOS antibody). Bars: 0.5μm.

over the mitochondrial membranes and the matrix space, but was not observed in all mitochondria. A possible explanation could be that in some mitochondria the isolation/immunoprocedure caused a loss of the mtNOS protein or change in the conformation of the presenting epitope. No immunolabelling was observed in "procedural" control mitochondria, where the primary antibody was omitted from the immunoprocedure (Fig 1d).

DISCUSSION

The finding that there is eNOS immunoreactivity localized in mitochondria from heart, skeletal muscle and kidney, as well as in non-synaptic brain mitochondria and liver mitochondria (1), prompts the speculation that eNOS (or a protein with significant homology to eNOS) is present in the mitochondria of most (if not all) mammalian tissues. To be able to produce nitric oxide, NOS must have an adequate supply of arginine. The concentration of arginine in brain tissue is approximately 100 μM in rats and 70 μM in humans (7), although little is known about its subcellular distribution. Arginine uptake has been demonstrated in mitochondria isolated from brain (8), liver (9) and kidney (10,11), and intramitochondrial arginine concentration has been estimated to be 120-300 μM (9), which is well in excess of the K_m of eNOS for arginine (12). Previous investigators using liver mitochondria found low or zero measured rates of mitochondrial arginine uptake (13). This is probably due to the mitochondrial preparations used being contaminated with arginase from the cytosol (14,15).

Besides an adequate supply of arginine, NOS needs a variety of co-factors. The presence of cofactors such as FMN and FAD within mitochondria is well established as they are essential components of complexes I and II respectively (16). Protoporphyrin IX haem is an essential substrate for mitochondrial heme biosynthesis (17), and NADPH is also present within mitochondria in millimolar concentrations (18). There is also evidence for the mitochondrial uptake of tetrahydrobiopterin (BH4) (19) and the presence of calmodulin within mitochondria (20).

However, the mitochondrial synthesis or assembly of these co-factors may be less important than for other enzymes within mitochondria as several of the cofactors needed for full NOS activity are tightly bound to NOS (12), and it may be that there is no requirement for their separate importation into mitochondria, as they may be imported already bound to mtNOS. It would appear that mtNOS (like all the sub-units of complex II) is wholly encoded for by the nuclear genome, as it is known that mitochondrial DNA is highly conserved, (there are few non-coding base sequences and no introns) and codes for 13 polypeptide subunits of the respiratory chain complexes I, III, IV and V and the RNA that is needed for their synthesis (21).

A recent study by Kobzik and co-workers (22) demonstrated an NOS activity associated with diaphragm muscle mitochondrial homogenates where exogenous cofactors were added in excess. The same study demonstrated a decrease in oxygen uptake in submitochondrial particles (inside-out mitochondria), in the presence of L-arginine, but not with D-Arginine. These data support the work of Maina (23), who demonstrated that the addition of arginine to isolated monkey liver mitochondria blocked both state 3 (+ ADP) respiration and uncoupled respiration. In addition Watrous and Frangipane (24), demonstrated that the addition of 1-40 mM L-arginine to isolated rat brain mitochondria decreased oxygen consumption in the presence of dinitrophenol (an uncoupler) and succinate. In the latter study, D-arginine also did not affect oxygen consumption. Both of these studies (22,24) provide evidence that the arginine-induced decrease in mitochondrial oxygen consumption is stereospecific, and the three NOS isoforms thus far identified have a stereospecifity for L-arginine as opposed to D-Arginine (12). If there is a sufficient supply of arginine and all essential co-factors then NOS will make NO. However, if the supply of arginine or BH4 is limited then superoxide, H_2O_2 and other NO species may be produced (12). However, in the case of superoxide, mitochondria have a high activity of the manganese form of superoxide dismutase, which converts superoxide to H_2O_2. It is known that physiological levels of H_2O_2 are not harmful to mitochondrial

function except in the presence of divalent cations such as Cu^{2+} where the hydroxyl radical can be formed (25). This may be particularly important in pathological situations as excessive NO production by mtNOS may lead to reduction of Fe^{3+} to Fe^{2+} (26), which is a requirement for release of iron from proteins such as ferritin (27).

Superoxide and NO rapidly react to form the peroxynitrite anion, a potent oxidising agent (12). Therefore it may be that mtNOS is in part a peroxynitrite synthase and that a proportion of NO produced by mtNOS does not bind to cytochrome oxidase but reacts with superoxide produced by the electron transport chain (25) to form peroxynitrite.

A recent review (28) suggests that nitric oxide (produced within the cytosol) controls oxidative phosphorylation by adjusting the K_m of cytochrome oxidase for oxygen. However, the half-life of nitric oxide is relatively short in vivo (27), and NO (produced by mtNOS) in close proximity to cytochrome oxidase would be more likely to compete with oxygen and inhibit oxidative phosphorylation.

The data presented in this study in combination with our previous data (1) suggest that mtNOS is present in many of the major tissue types in mammalian systems, and in addition offers the prospect that oxidative phosphorylation may be regulated (at least in part) by the NO that is produced by this protein. Following the localisation of this protein to mitochondria, much work needs to be performed in order to determine its precise role in mitochondrial metabolism. For example, it may be that mtNOS has a more profound affect on oxidative phosphorylation in developing animals (29) and in ageing (30), where lower activities of cytochrome oxidase have been measured compared to the mature adult.

Mitochondrial NOS may also be of importance in pathological conditions, as it has been demonstrated that there is a decrease in state 3 respiration (+ ADP) in mitochondria isolated from gerbil brain following ischaemia (31) which may be partially due to an increase in the activity of mtNOS, as mitochondrial calcium increases during ischaemia (32) and eNOS is known to be calcium dependant (12). In addition, reperfusion following ischaemia has been shown to cause a decrease in cytochrome oxidase activity (33), which may be due to activation of mtNOS and the consequent increased formation of hydroxyl radicals from peroxynitrite breakdown.

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