Review Article

Annals of Behavioral Neuroscience

Over Expression of NOS2 in Ragged-red Fibers from Patients with Mitochondrial Disorders due to Mutations in mtDNA

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Received: Jan 07, 2018; Accepted: Feb 01, 2018; Published: Feb 04, 2018

Abstract

Mitochondrial diseases (MDs) are a group of heterogeneous disorders due to impaired oxidative phosphorylation causing defective ATP production. The histopathological hallmark is the presence of ragged-red fibers (RRFs), muscle fibers with excessive mitochondrial proliferation. Nitric oxide synthases (NOSs) are enzymes responsible of the synthesis of nitric oxide (NO), a ubiquitous signaling molecule involved in many physio-pathological processes. Three NOS isoenzymes have been identified so far including neuronal NOS (NOS1), inducible NOS (NOS2) and endothelial NOS (NOS3). Despite the expression and the subcellular localization of NOS1 and NOS3 have been previously investigated, a possible involvement of NOS2 in MDs has never been assessed. We evaluated the expression of NOS2 in muscle biopsies from 17 patients with mitochondrial respiratory chain dysfunction. Our data demonstrate that NOS2 is overexpressed in RRFs and the correspondence between NOS2 immunoreactivity and SDH staining suggests that the protein localizes to the mitochondria. Together with previous studies from the literature, these findings indicate a possible role NOSs in the pathogenic events leading to MDs.

Keywords: Nitric oxide synthases; Ragged-red fibers; Mitochondrial disorders

Abbreviations: NOSs: Nitric Oxide Synthases; nNOS: neuronal Nitric Oxide Synthase; iNOS: inducible Nitric Oxide Synthase; eNOS: endothelial Nitric Oxide Synthase; TNF- α : Tumor Necrosis Factor α ; IFN- γ : Interferon γ ; LPS: lipo Poly Saccharide; NADPH: Nicotinamide Adenine Dinucleotide Phosphate; H4B: tetrahydrobiopterin; FAD: Flavin Adenine Dinucleotide; FMN: Flavin Mononucleotide; MDs: Mitochondrial Disorders (MDs); nDNA: nuclear DNA; mtDNA: mitochondrial DNA; COX: Cytochrome C Oxidase; SDH: Succinate Dehydrogenase; MERRF: Myoclonic Epilepsy with Ragged-Red Fibres; MELAS: Mitochondrial Encephalomyopathy with Lactic Acidosis and Stroke-like episodes; CPEO: Chronic Progressive External Ophthalmoplegia; NADPHd: Nicotinamide Adenine Dinucleotide Phosphate Diaphorase; sIBM: sporadic Inclusion Body Myositis; PGC-1 α : Peroxisome proliferator-activated receptor-Gamma Co-activator 1 α

Introduction

Nitric oxide synthases (NOSs) constitute a family of isoenzymes responsible of the synthesis of nitric oxide

(NO), a highly reactive gaseous molecule and a ubiquitous signaling factor with a key role in many physiological processes [1]. Up to now, three different isoforms of NOS are known and named after the tissue or cell types

in which they have been identified; neuronal NOS (nNOS or NOS1), macrophage or inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3) [2].

The term constitutive NOSs has been used to indicate NOS1 and NOS3 whereas NOS2 has been named inducible because its expression is triggered by specific stimuli such as inflammatory mediators (i.e. TNF- α , IFN- γ) or microbial products (i.e. LPS) [3]. Recently the aforementioned definition has been reevaluated in light of evidence of a more wide tissue and cell types distribution of NOSs. NOS1 and NOS3 are expressed not only in neuronal and endothelial cells, respectively, but have been also detected in keratinocytes, smooth muscle fibroblasts, hepatocytes, lymphocytes and many other cells [1]. Similarly, the expression of NOS2 is not only confined to macrophages but occurs in different cell types [1]. The classification of constitutive vs inducible NOSs has been also revised based on the observation that the activity of NOS1 and NOS3 can be triggered by specific stimuli such as spinal cord injury and shear stress, respectively and that NOS2 is constitutively active in some cells under physiological conditions [4-6].

The activity of NOS1 and NOS3 is dependent on Ca²⁺/calmodulin; indeed, the increase in intracellular Ca²⁺ triggers calmodulin binding to NOS1 and NOS3 and their consequent activation [7]. On the contrary, NOS2 functions independently from Ca²⁺/calmodulin because calmodulin binds to NOS2 also at resting intracellular Ca²⁺ concentrations [7]. Despite this difference, the three NOS isoenzymes catalyze the same reaction, namely the conversion of L-arginine and molecular oxygen (O₂) to NO and L-citrulline [8]. The reaction requires the cofactors/coenzymes nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (H4B), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and iron protoporphyrin IX [8].

Mitochondrial disorders (MDs) are a group of clinically and genetically heterogeneous diseases characterized by a deficit in mitochondrial oxidative phosphorylation [9]. Being the electron transport chain under the dual genetic control of nuclear (nDNA) and mitochondrial (mtDNA), MDs have been associated to mutations in either of the two genomes [9]. As regard the pathogenic effects of mtDNA mutations, the peculiar rules of the mitochondrial genetics namely, the presence of several mtDNA molecules in a cell, the heteroplasmy of mtDNA occurring in the majority of diseased conditions, the threshold effect and the maternal inheritance of mitochondrial genomes

dictate the phenotypic expression of a mtDNA defect, especially in high energy requiring tissues such as central nervous system and skeletal muscle [9]. Even if they are considered rare disorders, the prevalence of MDs due to mtDNA mutations is about 1 in 5000 and 1 in 200 individuals have potentially pathogenic mutations that could result in a disease phenotype in the offspring of female carriers [10]. The histopathological feature of MDs is the presence in skeletal muscle of *ragged-red fibers* (RRFs), namely muscle fibers with remarkable proliferation of dysfunctional mitochondria visible with modified Gomori trichrome staining [11]. The histoenzymatic double staining for cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) is used to highlight muscle fibers with abnormal mitochondrial proliferation (i.e. increased subsarcolemmal SDH staining) and defective oxidative phosphorylation (i.e. reduced or absent COX reactivity) [11].

The abnormal proliferation of mitochondria observed in RRFs has been viewed as an attempt to increase the oxidative capacity in mitochondria with defective respiratory chain [11]. The impairment of oxidative phosphorylation and oxidative stress are considered to play a major role in the pathogenesis of MDs even if the exact molecular events leading to the disease are not completely understood [12].

In the present study we evaluated muscle biopsy specimens from 17 patients with mitochondrial respiratory chain dysfunction. Clinical, morphological, biochemical and genetic data have been previously reported [13]. Briefly, two patients had MERRF (myoclonic epilepsy with ragged-red fibres) and the m.8344A>G mutation; five patients had MELAS (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes) and the m.3243A>G mutation; six patients had CPEO (chronic progressive external ophthalmoplegia) and a single deletion; and one patient had CPEO and multiple deletions. Two patients had histological (i.e., RRFs and COX deficiency) and biochemical (complex IV deficiency) signs of mitochondrial myopathy, and one patient had morphological signs of mitochondrial myopathy (5% RRFs and COX deficiency in 20% of the fibres). Three patients were affected by proximal muscle weakness but mtDNA mutations have not yet been identified. Controls were 10 muscle biopsies from subjects who were ultimately deemed to be free from muscle diseases.

Serial 8- μ m-thick cryosections were stained with standard histochemical and histoenzymatic methods

including modified Gomori trichrome, succinate dehydrogenase (SDH), cytochrome c oxidase (COX) and COX/SDH [13].

Immunohistochemistry was performed on 6.5-um-thick transverse muscle sections using immunofluorescence techniques [14]. The following well-characterized antibodies against NOS2 were used: two rabbit polyclonal (BD Transduction Laboratories, 610332, and Thermo Fisher, PA1-036) and a mouse monoclonal (BD Transduction Laboratories, 610328). Immunostaining was assessed in a blind manner by two independent investigators. In a subset of evaluated muscle biopsies including one patient with MERRF, one with MELAS and one with CPEO, the total number of fibers *per* section, the number of RRFs and of RRFs positive to mouse monoclonal and rabbit polyclonal NOS2 antibodies (BD Transduction Laboratories) were counted and quantitatively analyzed.

Immunoreactivity to NOS2 antibodies was detected in RRFs of all 17 patients with MDs (Figure 1).

The staining was bulky in the subsarcolemmal region whereas it had a granular uneven distribution in the sarcoplasm (Figure 1). The pattern and the intensity of reactivity resembled those of SDH histochemical reaction. RRFs were positive independently of their COX activity (COX positive or COX deficient/negative) (Figure 1). The pattern of immunoreactivity was similar with all three NOS2 antibodies (Figure 1).

No abnormal immunostaining was observed in muscle biopsies from control subjects. Quantitative analysis of NOS2 positive fibers is reported in table 1.

In the present study we document the over expression of NOS2 in RRFs of patients with MDs regardless of the clinical phenotype and the genetic mutation. Data regarding the expression, fiber type distribution and subcellular localization of NOSs in skeletal muscle from different species have been reported in the literature [2,15-20]. NOS1 has been detected on the sarcolemma and, to a lesser extent, in the sarcoplasm of muscle fibers whereas NOS2 and NOS3 have been localized in the cytosol, sarcoplasmic reticulum, mitochondria and along contractive myofibrils [21]. Contrarily to the inducible expression of NOS2 in macrophages, NOS2 is constitutively expressed under physiological conditions in skeletal muscles of different species including guinea pigs, rats, mice and humans [18,19,22-24]. A granular distribution of NOS2 in the sarcoplasm of type I fibers

has been observed in guinea pig and Ca^{2+} -independent NOS activity accounting for about 50% of the total NOS activity has been detected in particulate fraction of muscle [18]. NOS activity has been also found in mouse skeletal muscle and an increase in NOS2 activity, mRNA and protein expression has been reported in the mice after treatment with LPS and in murine C2C12 myoblasts after incubation with LPS/IFN γ [22].

The expression and localization of NOS1 and NOS3 have been previously investigated in skeletal muscle of patients with MDs [25-27]. NOS1 was detected on the sarcolemma of muscle fibers with abnormal mitochondria proliferation as well as of normal muscle fibers, contrarily to previous observations reporting a stronger staining of NOS1 in the sarcolemma region of RRFs [25,26]. A remarkable immunoreactivity to NOS3 was reported in RRFs of patients with mitochondrial respiratory chain dysfunction [25,26]. NOS3 was observed on myofibrils, in the sarcoplasm and in the subsarcolemmal region of RRFs with a distribution corresponding to SDH histochemical reactivity and therefore compatible with a mitochondrial localization of the protein [25,26]. Reduced NOS activity, detected as NADPH diaphorase (NADPHd) activity, was observed in the sarcoplasm of COX deficient fibers, irrespective of their mitochondrial content [26,27]. On the other hand, NADPHd histochemical staining was increased on the sarcolemma in fibers with abnormal mitochondrial proliferation (RRFs) and in COX negative fibers, and was higher in COX deficient fibers with mitochondrial proliferation (RRFs/COX-) compared with COX deficient fibers with a normal mitochondrial content (COX-) [27]. These data suggested a positive correlation between NOS and COX sarcoplasmic activity and between NOS1 and NOS3 expression and mitochondrial content of myofibers [25,27]. Despite the interesting findings described in these studies, the presence and the distribution of NOS2 in skeletal muscle of patients with MDs were not addressed up to now. In the literature, the upregulation of NOS2 has been observed in skeletal muscle of patients with other pathological conditions including sporadic inclusion body myositis (sIBM), dermatomyositis, polymyositis and muscular dystrophy and in patients with chronic heart failure [24,28,29]. According to these studies, NOS2 localizes to sarcolemma, along myofibrils and in the sarcoplasm [24,28,29].

Here we report the increased expression of NOS2 in the sarcoplasm of muscle fiber with abnormal mitochondrial proliferation (RRFs), regardless of their COX activity, of patients with mitochondrial respiratory chain disorders.

Figure 1: Immunoreactivity to NOS2 antibodies in ragged-red fibers.

Immunofluorescence micrographs showing a dense subsarcolemmal and granular sarcoplasmic staining to tested antibodies in muscle fibers with abnormal mitochondrial proliferation (RRFs). (A, B) On serial muscle sections two RRFs were immunopositive for NOS2 rabbit polyclonal antibodies (BD and ThermoFisher, respectively). (C, D) On serial sections, a RRF shows immunoreactivity to NOS2 rabbit polyclonal and mouse monoclonal antibodies (BD), respectively. (E, F) Immunostaining for NOS2 mouse monoclonal (BD) in a cytochrome c oxidase deficient ragged-red fiber and in a muscle fiber with excessive mitochondrial proliferation and normal cytochrome c oxidase activity. (G, H) A RRF with absent cytochrome c oxidase reactivity reacts for NOS2 rabbit polyclonal antibody (BD). A partial correspondence between NOS reactivity (E, G) and succinate dehydrogenase staining is visible in the subsarcolemmal region (F, H).

Table 1: Quantification of ragged-red fibers immunopositive for NOS2.

Patient#	Clinicalphenotype	RRFs*	Rabbitpolyclonal anti-iNOS (BD) [§]	Mouse monoclonal anti-iNOS (BD) [§]
P1	MERRF	0,7	66,7	83,3
P2	MELAS	1,3	20,0	12,0
P3	PEO	4,4	35,1	49,7

#P1, P2 and P3 were previously indicated as P1, P3 and P8 (13).

*percentage of fibers with increased SDH staining (ragged-red fibers) relative to total number of fibers in a muscle section

§percentage of RRFs positive for the reported antibody

Similarly to the previously reported pattern of NOS3 immunoreactivity, the partial correspondence between NOS2 distribution and SDH staining pointed to a mitochondrial localization of the protein [26]. Although previous studies showed the localization of NOSs to the mitochondria in skeletal muscle, further investigations are needed to confirm whether NOS2 is located to the mitochondrial compartment in RRFs [21,30].

It is well documented that NO is a key modulator of several processes which are regulated at the level of mitochondria [31]. NO takes part to the control of mitochondrial biogenesis through the activation of peroxisome proliferator-activated receptor- γ co-activator 1 α (PGC-1 α) [32]. Therefore, the increased expression of NOS2 in RRFs might suggest the involvement of the enzyme in signaling pathways leading to the abnormal mitochondria proliferation in RRFs, as previously hypothesized for NOS1 and NOS3 [26].

Our observations and data reported in the literature suggest that NO is likely to be produced close to or even within mitochondria in RRFs [25-27]. Besides its regulatory functions in many physiological processes, NO exerts also cytotoxic effects some of which occurs at the level of mitochondria: NO competes with molecular oxygen for oxygen-binding site on cytochrome c oxidase (complex IV) with consequent inhibition of the electron transport chain, oxygen consumption and ATP production [31]. NO can also target reactive cysteines on proteins which undergo S-nitrosylation or react with superoxide (O_2^-) produced during oxidative phosphorylation to generate peroxynitrite (ONOO $^-$), a highly reactive nitrogen species, which is responsible of protein nitration through the conversion of tyrosine residues to 3-nitrotyrosines [33,34]. When the antioxidant responses are overwhelmed, these events lead to nitrosative/nitrative stress, compromised cell functions and eventually to disease [35]. Published data reported a role of NO also in triggering apoptosis through the opening of mitochondria permeability transition pore [36]. In previous study we detected skeletal muscle proteins that undergo tyrosine nitration in MDs and identified an upregulated eNOS located on vessels walls as a possible source of NO in these disorders [14]. We also documented the occurrence of a caspase-dependent programmed cell death in muscle of patients with MDs and reported a remarkable immunoreactivity of RRFs to proteins involved in the mitochondrial-mediated apoptosis pathway [13]. The overexpression of NOS2 in RRFs suggests that an increased amount of NO could be directly produced in the muscle fibers and not only at

the level of vascular elements and that it may have a role in the establishment of nitrative stress and in triggering programmed cell death [13,14,37].

The mechanisms of NOS2 upregulation in RRFs remains to be elucidated. It has been hypothesized a connection between TNF α and the presence of NOS2 in normal skeletal muscle tissue [24]. Our previous finding of an increased expression of TNF α and TNFR2 in RRFs of patients with MDs suggests a link between the expression of NOS2, possibly in the mitochondria, and TNF α [37].

Conclusions

In summary, this study demonstrates the overexpression of NOS2 in RRFs of patients with mitochondrial respiratory chain dysfunction adding a piece to the complex puzzle of MD pathogenesis.

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