ABSTRACT: Muscle fiber inexcitability and myosin loss underlie weakness in critical illness myopathy (CIM). Nitric oxide (NO) takes part in the maintenance of muscle fiber resting potential and, in pathological conditions accompanied by oxidative stress, may damage proteins through peroxynitrite generation. Sepsis and other conditions associated with CIM may differentially affect expression of NO synthases (NOSs), so that both down-regulation and upregulation with excessive peroxynitrite production can be hypothesized. In six patients with CIM we studied NOS1, NOS2, and NOS3 protein expression by immunohistochemistry and Western blot. To investigate peroxynitrite production, we performed immunohistochemistry for nitrotyrosine and measured nitrotyrosine levels by enzyme-linked immunosorbent assay. In three patients, sarcolemmal staining for NOS1 was selectively absent. In the others, it was absent in atrophic fibers and absent or reduced in non-atrophic fibers. Total NOS1 protein content was reduced by 41% in patients compared to controls, whereas no significant changes were found in levels and localization of NOS2, NOS3, and nitrotyrosine. Further studies are warranted to determine whether NOS1 loss plays a role in the pathophysiology of CIM, possibly reducing the release of NO at the sarcolemma and affecting muscle fiber excitability.

POSSIBLE ROLE FOR NITRIC OXIDE DYSREGULATION IN CRITICAL ILLNESS MYOPATHY

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Critical illness myopathy (CIM) frequently affects patients in the intensive care unit (ICU), causing delay in weaning from mechanical ventilation and severe muscle weakness. CIM is associated with exposure to intravenous steroids and non-depolarizing neuromuscular blocking agents, systemic inflammatory response syndrome (SIRS), sepsis, and multiorgan failure. Its pathogenesis has not been fully elucidated. Muscle membrane inexcitability, increased proteolysis via calpain and ubiquitin upregulation, thick filament loss, and apoptosis seem to be involved.22

Nitric oxide (NO) acts as a signaling molecule in many biological functions. Its role in muscle physiology and pathology is increasingly recognized and regarded with interest as a possible therapeutic target.4,29 In skeletal muscle fibers, NO synthesis is controlled by neuronal NO synthase (NOS1), which is constitutively expressed at the sarcolemma.24 NOS1 has been specifically found in Duchenne muscular dystrophy, in which it seems to contribute substantially to the pathogenesis, but not in other muscular diseases such as non-sarcoglycan-related limb-girdle dystrophies and inflammatory myopathies.8,18,20 NOS3 is also constitutively expressed in muscle and is localized to endothelial cells, whereas NOS2 is an inducible isoform whose expression in muscle fibers seems to be associated with some pathological conditions, including polymyositis and inclusion-body myositis (IBM).9,24,31

Abbreviations: CIM, critical illness myopathy; CMAP, compound muscle action potential; CK, creatine kinase; ELISA, enzyme-linked immunosorbent assay; IBM, inclusion-body myositis; ICU, intensive care unit; MUP, motor unit potential; NADH-TR, nicotinamide aneendine dinucleotide–tetrazolium reductase; NO, nitric oxide; NOS, nitric oxide synthase; SIRS, systemic inflammatory response syndrome; ULN, upper limit of normal

Key words: critical illness myopathy; nitric oxide; nitric oxide synthases; peroxynitrite; sepsis

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Increased muscle NOS2 expression with peroxynitrite production has been found recently in a series of septic patients, but it was not reported whether these patients developed myopathy.\textsuperscript{20} Peroxynitrite is a free radical resulting from the reaction of NO with superoxide anion and has several harmful properties. Interestingly, these include the ability to induce apoptosis and to readily nitrate tyrosine residues in proteins, which in turn leads to protein dysfunction, loss of structure, and hypercatabolism. However, the effect of sepsis on different NOS isoforms is complex and other factors in CIM patients besides sepsis, including steroid therapy, inactivity, and denervation, could differentially influence regulation of NOSs, NO release, and peroxynitrite generation.\textsuperscript{11,21,24}

We investigated protein expression of NOSs and peroxynitrite production in muscles of six patients with CIM.

**METHODS**

**Patients.** Four men and two women, aged 43–74 years, were admitted to the ICU because of respiratory failure in the course of bacterial pneumonia and sepsis (four patients), viral pneumonia, and anoxic encephalopathy with coma caused by hemorrhagic shock during abdominal surgery. In the last case, sepsis developed in the ICU. The presence of sepsis was evaluated according to published criteria.\textsuperscript{3}

All patients received intravenous steroids (dexamethasone, methylprednisolone, or hydrocortisone for 6–9 days) and non-depolarizing neuromuscular blocking agents (pancuronium or atracurium for 2–8 days). Severe muscle weakness (grade 0–3, Medical Research Council scale) was recognized 9–26 days after ICU admittance and 14–27 days after sepsis onset. In all patients, nerve conduction study showed reduced compound muscle action potential (CMAP) amplitude with normal motor conduction velocity in at least two nerves. Two patients also had abnormal sensory conduction studies, but one of them had type 2 diabetes mellitus. Electromyography showed fibrillation potentials in at least two muscles in all patients. Evaluation of voluntary motor unit potentials (MUPs) was possible in four patients and revealed small, short-duration, polyphasic MUPs with early recruitment. All patients had increased serum creatine kinase (two to six times the upper limit of normal). Muscle biopsy was performed in biceps brachii (five patients) or vastus lateralis (one patient) 4–12 days after recognition of weakness. At the time of biopsy, four patients still had sepsis.

**Histological and Ultrastructural Studies.** Muscle samples were frozen in isopentane cooled in liquid nitrogen and stored at \(-80°C\) until use. Cryostat serial sections were studied by staining with hematoxylin–eosin, modified Gomori trichrome, adenosine triphosphatase (pH 4.3, 4.6, and 9.4), nicotinamide adenine dinucleotide–tetrazolium reductase (NADH-TR), succinate dehydrogenase, cytochrome oxidase, oil-red O, and periodic acid–Schiff.

For electron microscopy, muscle samples were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in Epon resin. Longitudinal and transverse sections were collected on 200-mesh nickel grids, stained with uranyl acetate and lead citrate, and examined using an electron microscope (Morgagni 268 D; FEI Co., Eindhoven, The Netherlands).

**Immunohistochemistry for NOS Isoforms.** Indirect immunoperoxidase for NOS1, NOS2, and NOS3 was performed on cryostat serial sections. Seven samples (five biceps brachii, two vastus lateralis) from gender- and age-matched subjects were studied as normal controls for the constitutive isoforms NOS1 and NOS3. Muscle biopsies from two patients with inclusion-body myositis (IBM) or polymyositis were studied as positive controls for NOS2, as this inducible isoform is upregulated in muscle fibers in both diseases, whereas it is variably expressed in normal muscle.\textsuperscript{9,33} Muscle tissue sections were either fixed in cold acetone or unfixed. The following primary antibodies were incubated overnight at 4°C: mouse monoclonal antibody anti-NOS1 (clone A-11; Santa Cruz Biotechnology, Santa Cruz, California); rabbit polyclonal antibody anti-NOS1 (code 610310; BD Transduction Laboratories, Lexington, Kentucky); mouse monoclonal antibody anti-NOS2 (clone C-11; Santa Cruz Biotechnology, Santa Cruz, California); mouse monoclonal antibody anti-NOS2 (clone 6; BD Transduction Laboratories, Lexington, Kentucky); and mouse monoclonal antibody anti-NOS3 (clone 33; BD Transduction Laboratories, Lexington, Kentucky). For each antibody, we tested multiple dilutions from 1:10 to 1:200. In control slides, primary antibodies were omitted or replaced with isotype-matched antibodies.

In a second set of experiments, immunohistochemistry for NOS1, dystrophin (four primary antibodies directed to N-terminal, C-terminal, and the middle part of the protein), α, β, γ and δ-sarcoglycans, caveolin-3, dysferlin, and α-syntrophin was performed on serial sections of muscle samples from all patients with CIM.
Immunohistochemistry for Nitrotyrosine. Peroxynitrite production was investigated in muscles from all patients with CIM by nitrotyrosine residue detection. Indirect immunoperoxidase for nitrotyrosine was performed on both fixed and unfixed cryostat sections. Primary antibodies (9–36 μg/ml of rabbit polyclonal and mouse monoclonal anti-nitrotyrosine antibodies; Upstate Biotechnology, Lake Placid, New York) were incubated overnight at 4°C. We studied four normal samples (two biceps brachii, two vastus lateralis) as negative controls. Positive controls were muscle biopsies from two patients with IBM, as nitrotyrosine-reactive inclusions within muscle fibers have been described previously in this condition. According to antibodies’ manufacturer’s instructions, muscle tissue sections incubated for 20 minutes at room temperature in a solution inducing nitrotyrosine production (1 mM sodium nitrite and 1 mM hydrogen peroxide in 100 mM acetate buffer, pH 5.0) were also used as positive controls.

In control slides, the specificity of the nitrotyrosine staining was verified by omitting or replacing primary antibodies with isotype-matched antibodies and by treating muscle tissue sections with 100 mM sodium hydrosulfite in 10 mM NaHCO₃ (pH 9–10) for 20 minutes, to reduce nitrotyrosine to aminotyrosine.

Detection of NOS Protein Expression by Western Blot. For Western blot analysis of NOS isoforms, as well as for measurement of nitrotyrosine levels by enzyme-linked immunosorbent assay, we employed biceps brachii samples from five CIM patients and five normal controls. Frozen tissues were homogenized and resuspended in cold lysis buffer. Total proteins were spectrophotometrically quantified. For Western blot, 30 μg of proteins were separated by 8% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to Hybond enhanced chemiluminescence nitrocellulose membranes, and blocked with 5% non-fat milk in Tris-buffer saline (TBS–Tween 20) (0.1%, v/v). Membranes were incubated overnight at 4°C with mouse monoclonal antibodies anti-NOS1 (1:200; Santa Cruz Biotechnology), anti-NOS2, or anti-NOS3 (1:2500; BD Transduction Laboratories). After washing in TBS–Tween 20 and incubation with anti-mouse IgG secondary antibody (1:10,000 for 1 hour), WBs were developed by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, New Jersey) and quantified by a computerized densitometric system (Bio-Rad Image Processing and Analysis System; Bio-Rad, Hercules, California). The amount of loaded protein was checked by Red Ponceau staining. The quantity of NOSs proteins was normalized for total protein loaded and expressed as arbitrary units.

Measurement of Nitrotyrosine Muscular Content by Enzyme-Linked Immunosorbent Assay (ELISA). For nitrotyrosine quantification, 50 μg of proteins diluted in carbonate buffer (pH 9.6) were plated in an ELISA multi-well plate and incubated overnight at 4°C. A standard curve was constructed by incubating serial dilutions of nitro-ovine serum albumin (Sigma-Aldrich, Milan, Italy). After blocking of non-specific binding sites and washing, wells were incubated with mouse monoclonal anti-nitrotyrosine antibody (1:250 for 1 hour at 37°C; Upstate Biotechnology) and subsequently with secondary anti-mouse antibody (1:10,000, 45 min at 37°C). After washing, results were visualized by adding 3,3′,5,5′-tetramethylbenzidine, the reaction was stopped by adding sulfuric acid solution, and optical density was read at 450 nm in a microplate reader (Spectra Max 190; Molecular Devices, Sunnyvale, California).

RESULTS

Histological and Ultrastructural Studies. In all patients with CIM, histopathological examination showed myofiber atrophy and focal or diffuse reduction of myosin–ATPase reactivity within muscle fibers. Muscle fiber necrosis was occasional and inflammatory infiltrates were scarce. Ultrastructural examination revealed disruption of sarcomeric architecture with myosin loss and relative preservation of thin filaments and Z-lines (Fig. 1A).

Immunohistochemistry for NOS1. In normal muscles, antibodies against NOS1 stained the sarcolemma of all muscle fibers (Fig. 1B). Sarcolemmal NOS1 immunoreactivity was absent in three patients with CIM. In the others, it was absent in atrophic fibers and absent or reduced in non-atrophic fibers (Fig. 1C). In further experiments performed on serial sections from CIM muscles, we found that immunostaining for dystrophin, sarcoglycans, caveolin-3, dysferlin, and α-syntrophin was preserved in muscle fibers lacking NOS1 (Fig. 1D and E).

NOS1 immunostaining was negative in control slides where primary antibodies were omitted or replaced.

Immunohistochemistry for NOS2, NOS3, and Nitrotyrosine. In CIM patients as well as in normal controls, muscle fibers did not show any immunoreactivity for NOS2. Nevertheless, anti-NOS2 antibodies stained scattered interstitial cells in two controls and
several infiltrating cells in CIM patients (Fig. 1F). As previously reported, in patients with IBM or polymyositis, we found many positive cells in infiltrates and a discontinuous sarcolemmal reactivity for NOS2 in some muscle fibers surrounded or invaded by inflammatory cells (data not shown).9

In both patients and controls, anti-NOS3 antibody immunostained capillaries and the walls of larger vessels but no muscle fibers (Fig. 1G). Control slides, where primary antibodies were omitted or replaced, were negative for NOS2 and NOS3.

In CIM patients as well as in normal controls, immunoreactivity for nitrotyrosine was absent from muscle fibers (Fig. 1H and I). In CIM patients, a faint staining for nitrotyrosine was rarely seen in infiltrating cells invading or surrounding necrotic fibers (Fig. 1I). As previously reported in IBM patients, anti-nitrotyrosine antibody faintly stained several infiltrating cells and some little sarcoplasmic areas surrounding or close to rimmed vacuoles.31 Muscle tissue sections from both normal and diseased muscles incubated in the nitrotyrosine-inducing solution showed a strong immunoreactivity. This was abolished by omitting or replacing primary antibodies with isotype-matched antibodies and markedly diminished by reducing nitrotyrosine to aminotyrosine. Under these conditions, muscle tissue sections from CIM and IBM patients were negative for any nitrotyrosine immunostaining.

**Evaluation of NOS Protein Isoforms and Nitrotyrosine Content.** In CIM patients, mean total NOS1 content was reduced by 41% compared to controls (Mann–Whitney test, P < 0.05; Fig. 2). In patients showing no sarcolemmal staining for NOS1 at immunohistochemistry, Western blot indicated that total NOS1 content was 0.8–0.94 AU and was reduced by 66%–71% compared to controls.

NOS2 was undetectable both in CIM patients and normal controls. NOS3 protein levels were 0.29 ± 0.05 AU in patients and 0.3 ± 0.1 AU in controls (mean ± SEM; Mann–Whitney test, P > 0.05).
Nitrotyrosine levels were 5.9 ± 0.7 nmol/L in patients and 6.5 ± 0.1 nmol/L in controls (Mann–Whitney test, \( P < 0.05 \)).

**DISCUSSION**

In six patients with ICU-acquired neuromuscular weakness, we diagnosed critical illness myopathy on the basis of clinical, electrophysiological, and pathological examinations. In these patients, sarcolemmal NOS1 immunostaining was absent or reduced, whereas NOS2 and NOS3 protein expression and nitrotyrosine levels were substantially unchanged compared to controls.

In muscle fibers of septic patients, Lanone and colleagues reported NOS2 upregulation with increased peroxynitrite production.\(^7\) In patients with CIM, we found NOS2 and nitrotyrosine, a footprint of peroxynitrite generation, in infiltrating cells but not in muscle fibers. NOS2 expression and peroxynitrite production occur during activation of inflammatory cells and have been reported within infiltrates in inflammatory myopathies and other dysimmune diseases.\(^6,7,8,9,18\) The absence of NOS2 and nitrotyrosine in muscle fibers may appear conflicting, as five of our patients had sepsis and four still had sepsis at the time of biopsy, but this finding may have several explanations. In septic patients, Lanone and colleagues performed biopsies of rectus abdominis within an average of 12 hours from the onset of sepsis, whereas we sampled biceps brachii or vastus lateralis 14–27 days after sepsis onset.

In animal models of sepsis, NOS2 was found to be expressed in muscle fibers 12 hours after injection of lipopolysaccharides (LPS), but it disappeared within 24–48 hours.\(^7,10\) Moreover, NOS2 induction was found in diaphragm but not in limb muscles of septic rats and was prevented by dexamethasone administration.\(^7\) It is possible as well that, in septic patients with CIM, NOS2 expression does not occur in limb muscles, is inhibited by concomitant steroid therapy, or is an early and transient event. In this last case, peroxynitrite production subsequent to NOS2 induction may also occur, but it might be unrecognized in muscle biopsies performed several days after sepsis onset. In fact, in a septic experimental paradigm, the half-life of nitrotyrosine bonds in plasma was measured to be about 1 week and correlated with plasma protein turnover.\(^19\) In skeletal muscle, the half-life of myofibrillar proteins is 1–2 weeks and the half-life of sarcoplasmic proteins is even shorter.\(^15\) Moreover, increased proteolysis occurs in muscle tissue during sepsis and degradation of nitrated proteins is specifically accelerated.\(^5,17\) Overall, although we did not found NOS2 induction and peroxynitrite production in muscle biopsies of CIM patients, we cannot exclude the possibility that these occur and play a pathogenic role in early phases of CIM, possibly contributing to protein breakdown and apoptosis.

In our patients, changes in sarcolemmal NOS1 immunoreactivity cannot be ascribed to some non-specific damage of the muscle membrane, as other dystrophin-complex proteins were expressed properly. NOS1 loss cannot be considered as a generic sign of muscle injury, as it has been found in dystrophinopathy but not in many other muscle diseases.\(^8,9,18\) Thus, we believe that this finding represents a feature of critical illness myopathy. Western blot revealed that NOS1 protein levels were significantly reduced by 41% in the group of CIM patients compared to controls and by 66%–71% in patients who displayed a complete absence of sarcolemmal NOS1 at immunohistochemistry. This apparent discrepancy is likely due to a higher sensitivity of Western blot in detecting residual protein. An additional explanation may be that Western blot measures total protein levels in muscle tissue lysates, thus including a minor quota of the enzyme expressed in subcellular compartments of muscle fibers and by intramuscular nerve terminals.

Several conditions associated with CIM may affect regulation of NOSs and determine muscle NOS1 loss. Systemic inflammatory response during...
skeletal and SIRS may play a role in combination with steroids, inactivity, and denervation. In animal models of sepsis, muscular NOS1 may be upregulated or downregulated in different species and changes in the expression of this isoform seem to persist after NOS2 induction has terminated.\textsuperscript{10,13} In muscle biopsies (rectus abdominis) from septic patients, NOS1 mRNA and NOS1 protein contents were found to be reduced.\textsuperscript{21} Steroids downregulate NOS1 transcription in neuroblastoma cells and in rat brain and, although not specifically investigated, it is possible that this also occurs in muscle cells.\textsuperscript{23,28} Disuse reduces NOS1 expression in muscle.

In a recent study on 10 volunteers after 55 days of bed rest, sarcolemmal immunofluorescence for NOS1 was reduced by 30\% in type 2 fibers of vastus lateralis.\textsuperscript{6} However, only minor changes were found in type 1 fibers and in soleus muscle. Moreover, the effect of shorter periods of bed rest and the response of upper-limb muscles to inactivity were not evaluated. Finally, denervation influences sarcolemmal NOS1 expression, which is reduced or absent in neurogenic muscle atrophy. In acute cases, it has been shown that NOS1 disappears from sarcolemma shortly after denervation, and before the appearance of atrophy.\textsuperscript{16} It is possible that functional denervation caused by neuromuscular blocking agents and nerve-terminal degeneration due to coexistent critical illness polyneuropathy contribute to disruption of sarcolemmal NOS1 in CIM patients.

Further studies, possibly in an animal model of CIM, may help to determine whether NOS1 loss plays a role in the pathophysiology of this myopathy. Muscle membrane inexcitability is one of the pathophysiological events underlying weakness in patients with CIM. Studies in animal models have shown that inexcitability is due to both depolarization of resting potential and dysregulation of Nav1.4 sodium channels.\textsuperscript{25} One hypothesis to be examined is that loss of sarcolemmal NOS1, which is expected to reduce NO release at the muscle membrane, may contribute to muscle inexcitability. In fact, NO has been found to take part in maintenance of the resting potential of muscle fibers.\textsuperscript{26,27} Decreased NOS1 activity and reduced NO production seem to mediate the early development of muscle fiber depolarization after denervation.\textsuperscript{26,27} Moreover, NO exerts a regulatory role on sodium channels in some neuronal populations and in cardiac myocytes.\textsuperscript{1,2,22} In skeletal muscle fibers, both NOS1 and Nav1.4 sodium channels bind to α-syntrophin at the sarcolemma, and it is likely that NO also influences muscular sodium currents.\textsuperscript{14}

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