Nitric oxide stress in sporadic inclusion body myositis muscle fibres: inhibition of inducible nitric oxide synthase prevents interleukin-1β-induced accumulation of β-amyloid and cell death

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Sporadic inclusion body myositis is a severely disabling myopathy. The design of effective treatment strategies is hampered by insufficient understanding of the complex disease pathology. Particularly, the nature of interrelationships between inflammatory and degenerative pathomechanisms in sporadic inclusion body myositis has remained elusive. In Alzheimer's dementia, accumulation of β-amyloid has been shown to be associated with upregulation of nitric oxide. Using quantitative polymerase chain reaction, an overexpression of inducible nitric oxide synthase was observed in five out of ten patients with sporadic inclusion body myositis, two of eleven with dermatomyositis, three of eight with polymyositis, two of nine with muscular dystrophy and two of ten non-myopathic controls. Immunohistochemistry confirmed protein expression of inducible nitric oxide synthase and demonstrated intracellular nitration of tyrosine, an indicator for intra-fibre production of nitric oxide, in sporadic inclusion body myositis muscle samples, but much less in dermatomyositis or polymyositis, hardly in dystrophic muscle and not in non-myopathic controls. Using fluorescent double-labelling immunohistochemistry, a significant co-localization was observed in sporadic inclusion body myositis muscle between β-amyloid, thioflavine-S and nitrotyrosine. In primary cultures of human myotubes and in myoblasts, exposure to interleukin-1β in combination with interferon-γ induced a robust upregulation of inducible nitric oxide synthase messenger RNA. Using fluorescent detectors of reactive oxygen species and nitric oxide, dichlorofluorescein and diaminofluorescein, respectively, flow cytometry revealed that interleukin-1β combined with interferon-γ induced intracellular production of nitric oxide, which was associated with necrotic cell death in muscle cells. Intracellular nitration of tyrosine was noted, which partly co-localized with amyloid precursor protein, but not with desmin. Pharmacological

inhibition of inducible nitric oxide synthase by 1400W reduced intracellular production of nitric oxide and prevented accumulation of β-amyloid, nitration of tyrosine as well as cell death inflicted by interleukin-1β combined with interferon-γ. Collectively, these data suggest that, in skeletal muscle, inducible nitric oxide synthase is a central component of interactions between interleukin-1β and β-amyloid, two of the most relevant molecules in sporadic inclusion body myositis. The data further our understanding of the pathology of sporadic inclusion body myositis and may point to novel treatment strategies.

Introduction

Sporadic inclusion body myositis (IBM) is a common acquired myopathy that leads to severe disability and for which no effective treatment is available (Schmidt and Dalakas, 2010). The complex disease pathology includes accumulation of aberrant molecules such as β-amyloid and a specific inflammation with an attack of muscle fibres by auto-aggressive cytotoxic T cells (Schmidt and Dalakas, 2010). Different pathways of protein degradation seem to be impaired and may contribute to the aggregation of aberrant molecules in the fibres (Askanas et al., 2009). It is known that muscle cells, under pro-inflammatory conditions, can produce a range of cytokines and chemokines (De Paepe et al., 2009) as well as co-stimulatory molecules (Wiendl et al., 2003; Schmidt et al., 2004). Thus, it has been proposed that muscle fibres may directly contribute to the recruitment and local activation of T cells in the lesion (Wiendl et al., 2005).

Recent data from our own group suggest that there is a distinct interrelationship between inflammation and degeneration in that interleukin (IL)-1β can induce accumulation of β-amyloid in muscle cells (Schmidt et al., 2008). This is particularly enhanced by interferon (IFN)-γ, which upregulates the receptor for IL-1β. Thus, this cytokine combination typically resembles the inflammatory environment of chronic myositis. However, so far it is unclear how interactions between inflammation and degeneration could occur. One possible mediator that may play a central role in this network of mechanisms is nitric oxide, since it has been shown in sporadic IBM that nitric oxide and the inducible nitric oxide synthase (iNOS), the enzyme that generates high levels of nitric oxide in the micromolar range, as well as other oxidative stress-related molecules have been associated with intracellular protein aggregates, mostly in the form of β-amyloid depositions within the vacuolated fibres (Yang et al., 1998; Tateyama et al., 2003).

With respect to the pathomechanisms of sporadic IBM, we here study the role of iNOS in muscle cells under pro-inflammatory conditions and how this relates to accumulations of β-amyloid.

Materials and methods

Patients and muscle cell culture

Ten skeletal muscle biopsies of patients with histologically confirmed diagnosis of sporadic IBM were used and compared with samples from patients with dermatomyositis ($n = 11$), polymyositis ($n = 8$), adults with muscular dystrophy ($n = 9$, including facioscapulohumeral, Becker's and limb girdle muscular dystrophy), and diagnostic muscle biopsies without myopathic changes ($n = 10$). Muscle samples for primary cell cultures were taken after informed consent from non-myopathic patients that required surgery. All samples were used in accordance with the guidelines of the institutional review board. Muscle cell progenitors (satellite cells) were grown using a recently established protocol (Schmidt et al., 2008). Briefly, the muscle was thoroughly minced and digested with trypsin. All fragments were collected and seeded in a 75 cm² flask in Skeletal Muscle Cell Growth Medium (Provitro). After 2–3 subcultures over 2–3 weeks, a 95–100% pure CD56+ myoblast culture was obtained by magnetic cell sorting with anti-CD56 (neural cell adhesion molecule) specific magnetic beads, following the supplier's protocol (Miltenyi Biotec). At 80% confluence, fusion was induced by switching to Dulbecco's Modified Eagle Medium supplemented with 2% horse serum (Gibco/Invitrogen), penicillin and streptomycin. After 3–5 days, multinucleated myotubes had formed that typically reached 95% purity as revealed by immunohistochemical staining for desmin. Muscle cells were exposed to the cytokines IL-1β (20 ng/ml), IFN-γ (300 U/ml) and tumour necrosis factor (TNF)-α (10 ng/ml) (all from Chemicon/Merck Millipore), alone or in combinations, or exogenous nitric oxide released from the synthetic donor diethylenetriamine NONOate (Cayman Chemicals) at 50–500 μM for 12–72 h in serum-free medium X-vivo 15 (Cambrex Bio Science). Controls were kept in X-vivo 15 only. After washing in pre-warmed phosphate-buffered saline, the cells were fixed and stored at −80°C until staining (see below). The iNOS-specific inhibitor 1400W (N-3-aminomethyl-benzyl-acetamidine) was used at 10 μM, the non-specific NOS-inhibitor L-NMMA (L-N^G-monomethyl arginine) was used at 100 μM (both from Calbiochem/Merck Millipore), and the general caspase-inhibitor Q-VD-OPh was used at 20 μM (R&D Systems); all concentrations were recommended by the suppliers. Experiments for RNA analysis or flow cytometry were performed in 24-well plates and for immunocytochemistry in 8-well chamber slides (both from Nunc).

Extraction of messenger RNA and quantitative polymerase chain reaction

Extraction of messenger RNA (RNeasy®Kit, Qiagen) was followed by reverse transcription (SuperScript® II, Invitrogen) following the supplier's instructions. The resulting complementary DNA was stored at −20°C. Quantitative (real-time) polymerase chain reaction was performed as previously described (Schmidt et al., 2008) on a 7500 real-time polymerase chain reaction system by 6-carboxyfluorescein (FAM)-labelled specific primer/probe pairs for: glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs99999905_m1) and iNOS (Hs00167257_m1)

(from Applied Biosystems). Target messenger RNA expression was quantified using the $2^{-\Delta c(t)}$ method in relation to expression of GAPDH messenger RNA and multiplied by 1000.

Staining of muscle tissue and cultured cells

Frozen sections (5 μm) of muscle biopsy specimens or cultured muscle cells were fixed with 4% paraformaldehyde at room temperature for 10 min followed by methanol at $-20°C$ for 10 min. Non-specific binding was reduced by 30 min incubation with 5% bovine serum albumin and 3% goat serum (all from Jackson ImmunoResearch) in phosphate-buffered saline (0.05 M, pH 7.4, 0.15 M saline). The following primary anti-human antibodies were used: mouse anti-β-amyloid (clone 6E10 from Covance/HISS Diagnostics) at 10 μg/ml for 24 h at 4°C (Vattemi *et al.*, 2004; Rajendran *et al.*, 2006; Schmidt *et al.*, 2008); polyclonal rabbit anti-iNOS (AB5384 from Chemicon/Merck Millipore) at 1/500 for 1 h at room temperature; polyclonal rabbit anti-nitrotyrosine (no. 06–284 from Upstate/Merck Millipore) at 2 μg/ml for 2 h at room temperature; mouse anti-nitrotyrosine (clone 1A6 from Upstate/Merck Millipore) at 10 μg/ml overnight at 4°C. Specificity of anti-nitrotyrosine antibodies was demonstrated in two ways: (i) using a standard preadsorption protocol (Lunemann *et al.*, 2007), the primary mouse or rabbit antibodies (at 1/40 pre-dilution) were blocked with 5 mM 3-nitro-L-tyrosine (Sigma) for 2 h at 37°C, followed by centrifugation at ~18 000*g* for 30 min; upon final dilution of the supernatant, each antibody was subsequently used for immunocytochemistry; and (ii) as a positive control, *in situ* tyrosine nitration was induced in fixed myoblasts by incubation with 5 mM peroxynitrite for 10 min and compared with degraded peroxynitrite as negative control (both from Merck Millipore); the cells were washed in phosphate-buffered saline and used for immunocytochemical detection by anti-nitrotyrosine antibodies as described above.

Immunoreactivity was detected using Alexa 488 or Alexa 594-conjugated highly preadsorbed secondary antibodies from goat (all from Molecular Probes/Invitrogen). Staining of amyloid aggregation was achieved by thioflavin-S (T1892-25G from Sigma) at 1% for 5 min at room temperature. In unfixed cells, cell viability was assessed by propidium iodide (Molecular Probes/Invitrogen) at 2 μg/ml for 20 min at 37°C.

Nuclear counterstaining in fixed cells or tissue was performed by 4′,6-diamidino-2-phenylindole (DAPI, Molecular probes/Invitrogen) at 1/50 000 for 30 s, followed by mounting in Fluoromount G (Electron Microscopy Sciences). Immunofluorescent microscopy and digital photography were performed on a Zeiss Axiophot microscope, using appropriate filters for green (488 nm), red (594 nm) and blue (350 nm) fluorescence and a cooled charge-coupled device (CCD) digital camera (Retiga 1300, Qimaging) using the Image-Pro software (Media Cybernetics, Inc). For quantification, grey scale analysis was performed from digital images using Scion Image (Scion Corporation) and the value was expressed as arbitrary units.

Flow cytometry

For detection of intracellular free radicals including reactive oxygen species and nitric oxide, the fluorescent detectors dichlorofluorescein diacetate (20 μM) and diaminofluorescein-2 (DAF-2; 20 μM) were added to each well 2 h before the end of the experiment (both from Calbiochem). Myoblasts (rhabdomyosarcoma cell line CCL-136 from the American Type Culture Collection) were washed twice with warm phosphate-buffered saline before detachment with trypsin (Invitrogen).

Cells were collected in FACS (fluorescence-activated cell sorting) tubes, kept on 4°C throughout staining procedures and washed twice in annexin binding buffer (10 mM HEPES pH 7.4; 150 mM NaCl; 5 mM KCl; 2 mM $CaCl_2$). Cells were stained with 4 μl AnnexinV-APC (Bender MedSystems) for 15 min at 4°C, resuspended in 100 μl buffer and stained with 7-aminoactinomycin at 4 μg/ml (Molecular Probes/Invitrogen). Flow cytometric analysis of 50 000 events was performed on a FACS Calibur with Cell Quest Pro (Becton Dickinson) and analysed with the WinMDI 2.8 software (The Scripps Institute).

Statistics

Unpaired *t*-test, ANOVA with Tukey's *post hoc* test, Pearson correlation, and Grubbs' test for exclusion of outliers were calculated using GraphPad Prism V4.0 (GraphPad Software) with *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ as significant values.

Results

Overexpression of inducible nitric oxide synthase and co-localization with β-amyloid in sporadic inclusion body myositis muscle

As revealed by quantitative (real-time) polymerase chain reaction, iNOS messenger RNA expression was upregulated in skeletal muscle biopsies from patients with sporadic IBM compared with non-myopathic controls (Fig. 1A). Detectable levels of iNOS were present in five out of ten patients with sporadic IBM, two of eleven with dermatomyositis, three of eight with polymyositis, two of nine with muscular dystrophy and two of ten controls. The messenger RNA expression of iNOS was significantly higher in sporadic IBM compared with all other groups (Fig. 1A). The severity of pathological changes such as inflammatory infiltrates was comparable among the samples of one group and representative of the respective disease. By immunohistochemistry, protein expression of iNOS was detectable in muscle fibres of patients with sporadic IBM, whereas the signal remained much lower in dermatomyositis and usually remained restricted to areas of perifascicular atrophy or blood vessels and inflammatory cells (Fig. 1B). The signal remained lower in polymyositis compared to sporadic IBM. In muscular dystrophy, only some fibres were positive for iNOS and almost no signal was noted in control patients. In many muscle fibres, a staining of the sarcolemma was observed in addition to the predominant staining of the cytoplasm, which is in line with reports by others (Tews and Goebel, 1998; De Paepe *et al.*, 2004). Intracellular production of nitric oxide was reflected by staining for nitrated tyrosine residues and specificity of the respective antibodies is demonstrated in Supplementary Fig. 1. Nitrotyrosine was present in sporadic IBM muscle fibres, but much less in dermatomyositis muscle, where it was more localized to capillaries and connective tissue (Fig. 1B). In polymyositis, a lower signal was noted in few fibres and immune cells. In muscular dystrophies and non-myopathic controls, no relevant staining of nitrotyrosine was observed. As revealed by double-staining of sporadic IBM muscle samples, a co-localization
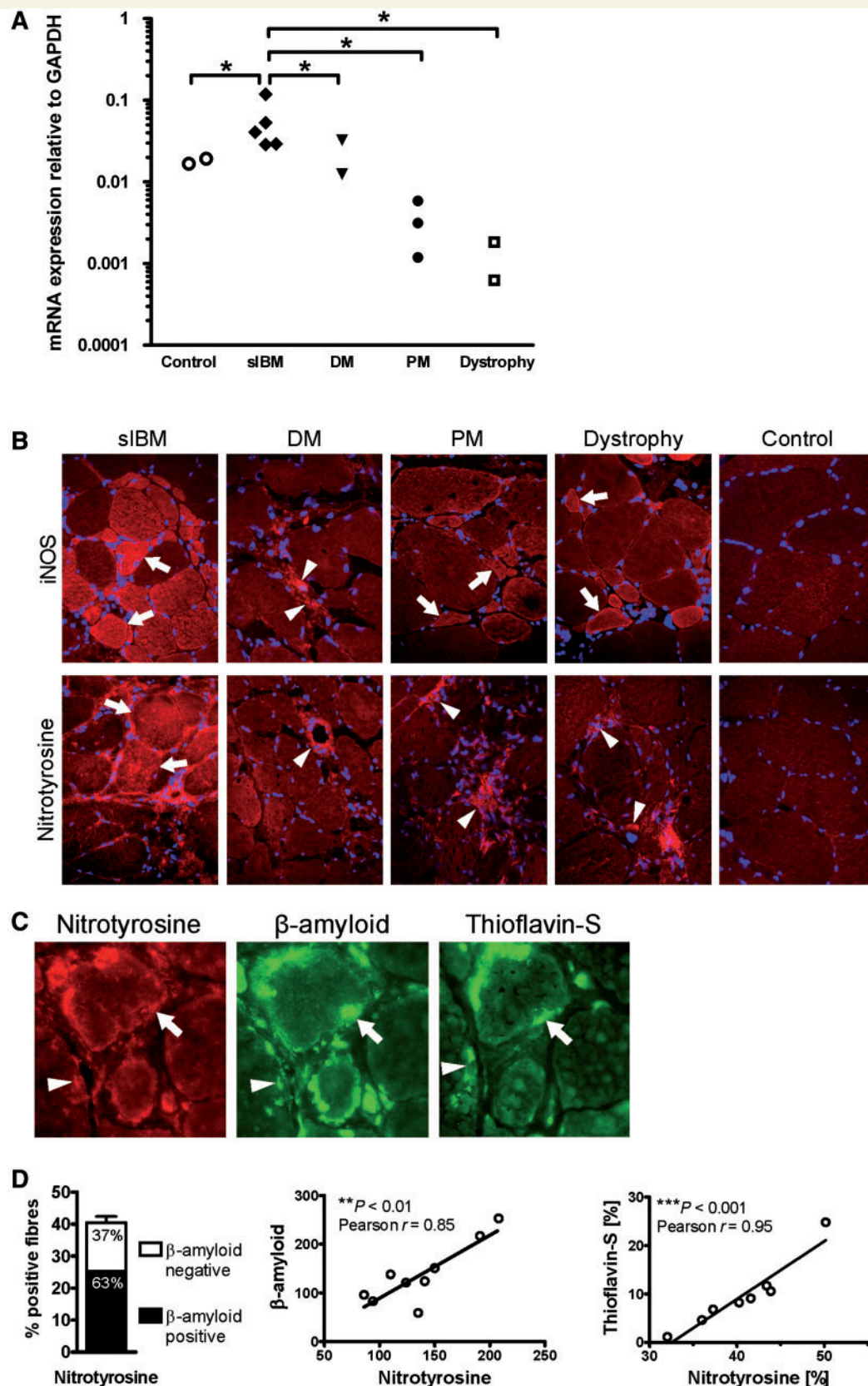
Figure 1 Quantification of iNOS messenger RNA expression and immunohistochemical staining in patients with sporadic IBM (sIBM) compared with controls. (**A**) GAPDH-normalized expression of iNOS messenger RNA amplified by quantitative (real-time) polymerase chain reaction from skeletal muscle biopsy samples of patients with sporadic IBM ($n = 10$, closed diamonds) compared with non-myopathic control patients ($n = 10$, open circles), dermatomyositis (DM; $n = 11$, closed triangles), polymyositis (PM; $n = 8$, filled

(continued)

of nitrotyrosine and β-amyloid was noted in muscle fibres (Fig. 1C). Thioflavin-S staining of a serial section demonstrated that not all of the immunohistochemically detected β-amyloid was present in form of aggregates, although it cannot be ruled out that small structures have been missed by this approach. Quantitative analysis of immunohistochemical staining in sporadic IBM samples revealed that 40% of all fibres were positive for nitrotyrosine and a majority of 63% of these were double-positive for β-amyloid (Fig. 1D). The majority of all β-amyloid-positive fibres displayed labelling by nitrotyrosine, yet $35.8 \pm 12.3\%$ of the fibres remained negative (data not shown). A correlation analysis demonstrated a statistically significant association between muscle fibres positive for nitrotyrosine and β-amyloid as revealed by immunohistochemistry as well as thioflavin-S staining (Fig. 1D).

These data are in line with previous reports in IBM and Alzheimer's dementia and suggest that, in sporadic IBM muscle, upregulation of iNOS with subsequent production of intracellular nitric oxide is associated with accumulation of β-amyloid.

In vitro upregulation of inducible nitric oxide synthase and intracellular production of nitric oxide in muscle cells

Inducible nitric oxide synthase messenger RNA was not detectable in primary myotube cultures from non-pathological skeletal muscle. Upon IFN-γ-induced upregulation of the IL-1β receptor (Adams *et al.*, 2002), the amyloidogenic cytokine IL-1β led to a profound upregulation of the messenger RNA for iNOS in myotubes within 12 h (Fig. 2A); cytokine exposure for 36 h revealed similar results (data not shown). The effect of TNF-α, IL-1β or IFN-γ alone remained much lower and TNF-α failed to show an additive effect together with IL-1β or IFN-γ. Therefore, IL-1β and

IFN-γ were used for all subsequent experiments. Protein expression of iNOS was demonstrated by immunocytochemical staining, which revealed a significantly higher signal in myotubes upon 72 h exposure to IL-1β and IFN-γ compared with controls (Fig. 2B); an exposure for 40 h yielded similar results (not shown). Intracellular production of nitric oxide was evidenced by immunocytochemical staining for nitrotyrosine, which was significantly upregulated upon IL-1β and IFN-γ compared with controls. Immunohistochemical double labelling revealed a co-localization of the signals for iNOS and nitrotyrosine in muscle cells exposed to the cytokines (Supplementary Fig. 2). Using flow cytometric analysis of dichlorofluorescein as detector dye, intracellular production of reactive oxygen species and nitric oxide was quantified (Swindle *et al.*, 2004). Exposure to IFN-γ and IL-1β upregulated the production of reactive oxygen species/nitric oxide in myoblasts after 48 h and even more after 72 h (Fig. 2C).

These data demonstrate that IL-1β together with IFN-γ can induce expression of iNOS and lead to intracellular production of nitric oxide in cultured muscle cells.

Intracellular accumulation of β-amyloid, IL-1β + IFN-γ-induced nitric oxide and necrotic cell death in muscle cells

We have previously demonstrated that IL-1β + IFN-γ induces intracellular accumulation of β-amyloid in muscle cells (Schmidt *et al.*, 2008). To test the hypothesis that nitric oxide would augment the amount of intracellular β-amyloid, myotubes were exposed to the synthetic nitric oxide-donor diethylenetriamine-NONOate. After 24 h, the β-amyloid signal as revealed by fluorescent immunocytochemistry was clearly enhanced and reached statistical significance as reflected by grey scale analysis (Fig. 3A). Using flow cytometric

Figure 1 Continued

circles) and muscular dystrophy ($n = 9$, open squares). A statistically significant elevated expression of iNOS messenger RNA was noted in five of ten patients with sporadic IBM (*$P < 0.05$ versus all other groups) compared with two of ten controls, two of eleven with dermatomyositis, three of eight with polymyositis and two of nine with muscular dystrophies. Each dot represents one patient (arbitrary units). (**B**) Fluorescent immunohistochemistry of iNOS (red, Alexa-594) or nitrotyrosine (red, Alexa 594) in representative muscle biopsy specimens of sporadic IBM compared to dermatomyositis, polymyositis and muscular dystrophy, which all had a similar grade of pathological changes, as well as non-myopathic controls. Large areas of the sporadic IBM muscle are positive for iNOS (*top row*), whereas the signal remains much lower and restricted to perifascicular fibres in dermatomyositis and lower in polymyositis; only few fibres are positive in muscular dystrophy and basically no staining is noted in the control muscle. A profound nitration of tyrosine is noted in sporadic IBM muscle (*bottom*), whereas mostly the connective tissue and capillaries are positive in dermatomyositis and few fibres and immune cells in polymyositis; no relevant staining is noted in muscular dystrophy and controls. Arrows depict iNOS-positive fibres in sporadic IBM, polymyositis and dystrophy as well as staining signals for nitrotyrosine in fibres of patients with sporadic IBM. Arrow heads indicate vessels or immune cells that are positive for iNOS or nitrotyrosine in dermatomyositis, polymyositis and dystrophy. (**C**) Double immunofluorescent labelling for nitrotyrosine (*left*: red, Alexa-594) and β-amyloid (*middle*: green, Alexa-488) and detection of amyloid by thioflavin-S histochemistry in a serial section (*right*: green) in a representative sample of sporadic IBM. A co-localization between nitrotyrosine and β-amyloid is observed and thioflavin-S demonstrates that not all of the immunolabel for β-amyloid represents protein aggregation. Arrows and arrow heads point to protein aggregations within muscle fibres that display co-localization of all three markers. Photos were taken by a CCD camera using a conventional fluorescent microscope with a $\times 40$ (**B**) or $\times 63$ (**C**) objective. All photomicrographs of one panel are shown at the same total magnification. Freezing artefacts are visible in several panels, but this did not affect the overall detection of the respective markers or their quantitative assessment. (**D**) Quantitative analysis by manual photo-microscopy of nine patients with sporadic IBM stained by immunohistochemical double labelling for β-amyloid and nitrotyrosine and thioflavin-S histochemistry as in (**C**). Forty per cent of the fibres are positive for nitrotyrosine and a majority of 63% of these is double-positive for β-amyloid (*left*). Linear regression analysis of the total number of fibres positive for each marker reveals a statistically significant correlation between nitrotyrosine and β-amyloid (*middle*: **$P < 0.01$, Pearson $r = 0.85$) as well as between nitrotyrosine and thioflavin-S (*right*: ***$P < 0.001$, Pearson $r = 0.95$).
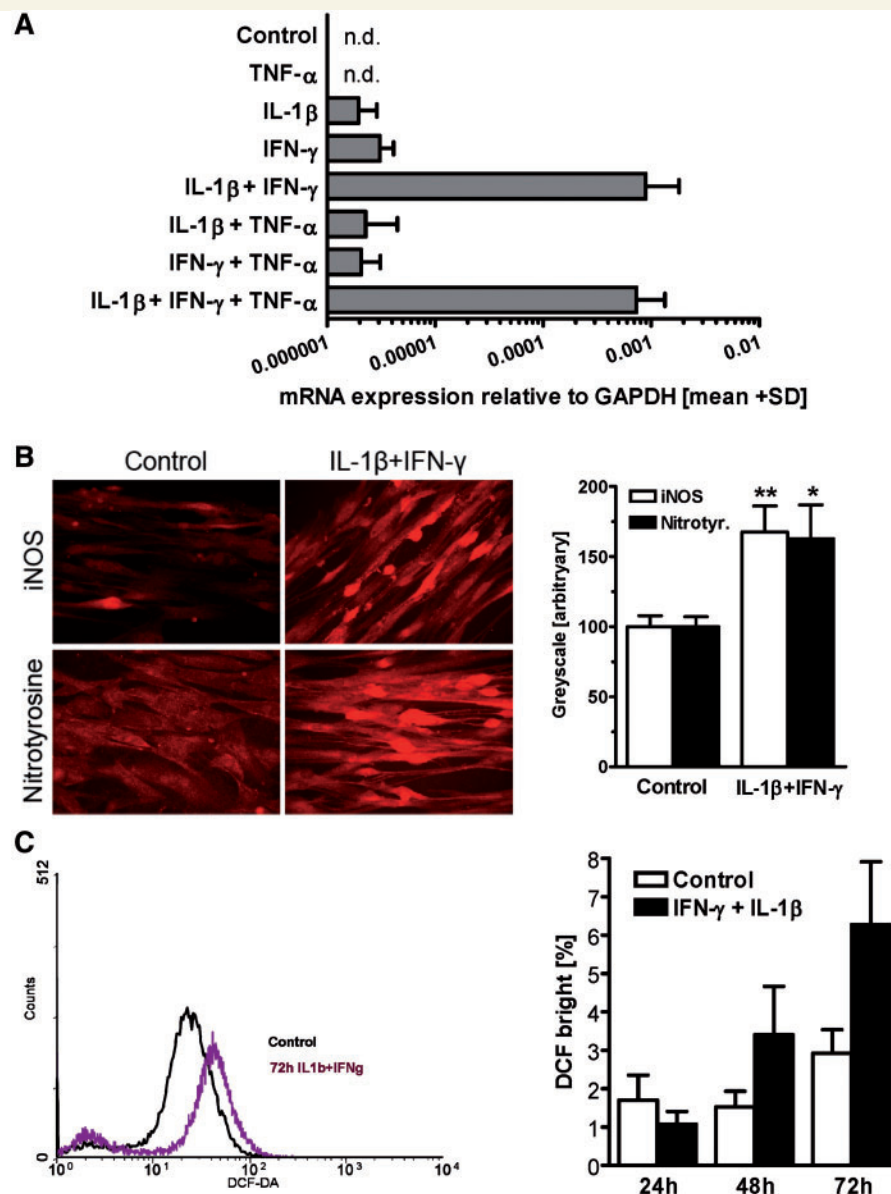
Figure 2 *In vitro* analysis of expression of iNOS, production of nitric oxide and nitrotyrosine staining in cultured muscle cells. (**A**) Twelve hours after exposure to pro-inflammatory cytokines IFN-γ (300 U/ml), TNF-α (5 ng/ml) or IL-1β (20 ng/ml), alone or in combination, the messenger RNA expression of iNOS relative to GAPDH was quantified. Only combinations with IL-1β and IFN-γ induced a robust upregulation of the messenger RNA of iNOS. Data are given as mean + SD of three wells per condition from one representative experiment of three with similar results. n.d. = not detectable. (**B**) Immunohistochemical staining for iNOS and nitrotyrosine in primary myotube cultures exposed to IL-1β (20 ng/ml) and IFN-γ (300 U/ml) compared to medium alone for 72 h (red, Alexa-594, *left*). Compared with controls, a much higher staining intensity is revealed after cytokine exposure, which is statistically significant by grey scale analysis (*right*). One representative experiment of three with similar results is shown. Photo-microscopy with a $\times 40$ objective. (**C**) Flow cytometry analysis of human myoblasts (rhabdomyosarcoma cell cline CCL-136) using the fluorescent detector dichlorofluorescein diacetate to visualize production of reactive oxygen species and nitric oxide. The histogram (*left*) demonstrates a marked shift of the staining intensity upon 72 h of exposure to IL-1β + IFN-γ (purple) compared with controls (black). Quantitative quadrant analysis of four experiments with similar results reveals an elevated percentage of dichlorofluorescein (DCF)$_{bright}$ cells (*right*, data indicated as mean + SEM, not statistically significant). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

assessment of myoblasts, an enhanced intracellular staining for β-amyloid was confirmed upon increasing concentrations of exogenous nitric oxide (Fig. 3B). A similar staining result was seen in myoblasts upon exposure to a combination of IFN-γ and IL-1β (data not shown).
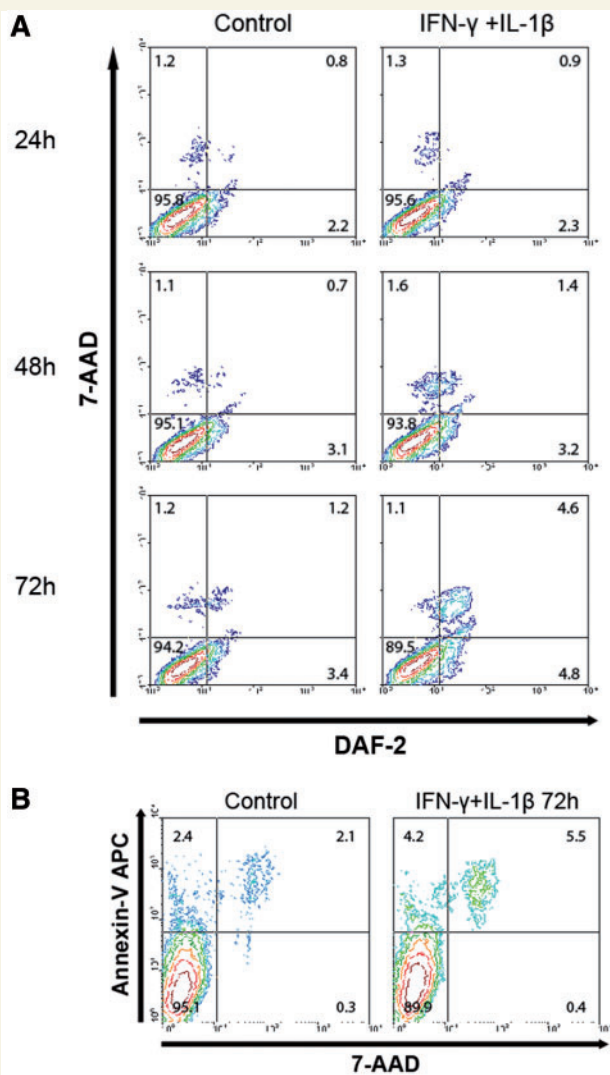
Flow cytometric analysis of myoblasts labelled by 7-aminoactinomycin and the intracellular nitric oxide detector dye DAF-2 demonstrated that the viability was impaired in muscle cells that displayed an elevated intracellular production of nitric oxide after 72 h of exposure to IFN-γ + IL-1β (Fig. 4A).
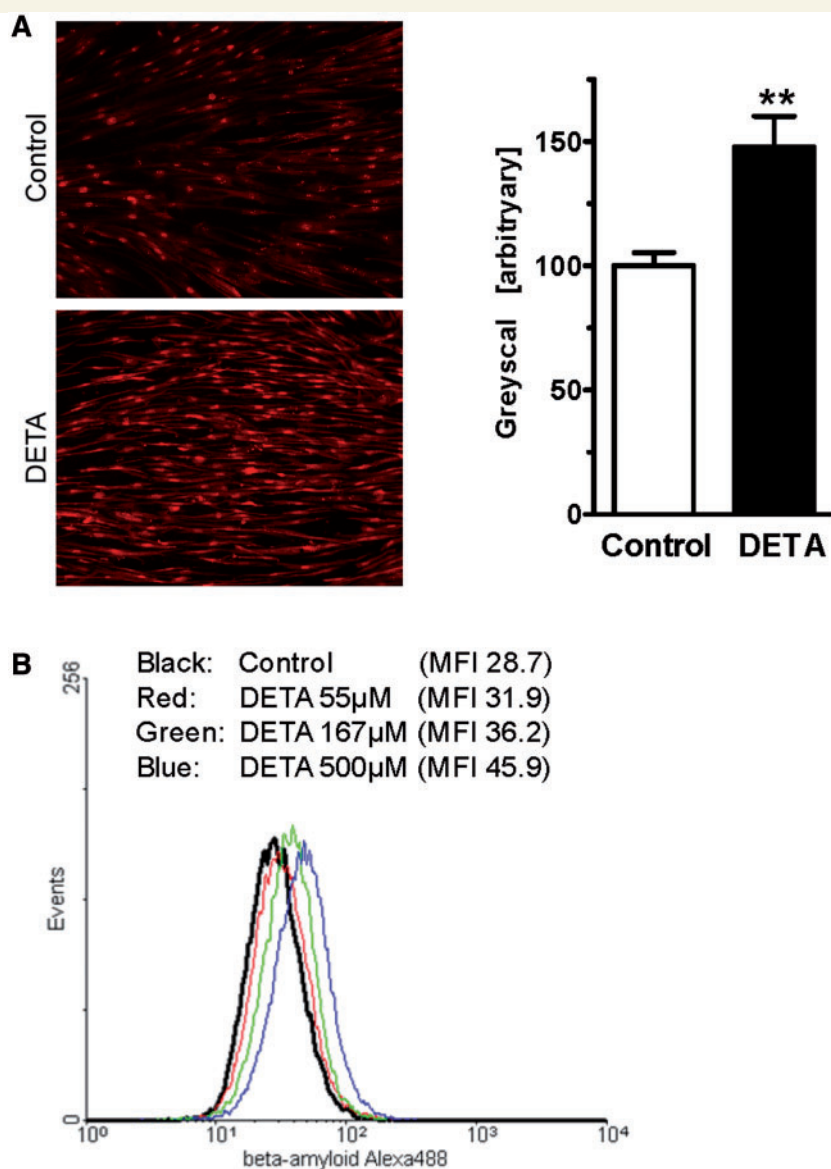
Figure 3 *In vitro* assessment of intracellular β-amyloid. Immunocytochemical staining revealed intracellular accumulation of β-amyloid in primary muscle cell cultures (**A**) and myoblasts (**B**) after 24 h exposure to the synthetic nitric oxide-donor diethylenetriamine (DETA). As reflected by microscopy and digital image analysis by grey scale densitometry (**A**), the staining signal for intracellular β-amyloid was enhanced by exposure of myotubes to 300 μM diethylenetriamine as a synthetic nitric oxide donor. Comparable results were observed by FACS-analysis of myoblast cells (human rhabdomyosarcoma cell line CCL136): 24 h exposure to increasing concentrations of diethyle-netriamine induced a gradual enhancement of the mean fluorescence intensity (MFI) of the intracellular staining signal for β-amyloid (**B**). Data in (**A**) are given as mean + SEM. Photos in (**A**) were taken by a conventional fluorescent microscope with a charge-coupled device camera using a ×20 objective. Cell culture experiments were performed in duplicate and one representative of at least three experiments with similar results is displayed. **$P < 0.01$.

Double labelling for 7-aminoactinomycin and annexin-V revealed a total of 5.9% dead cells upon exposure to IL-1β + IFN-γ com-pared with 2.4% in controls and 4.2 versus 2.4% early apoptotic cells (Fig. 4B). Cellular damage by intracellular nitric oxide can be caused by nitration of tyrosine residues of relevant proteins. Upon exposure to IFN-γ + IL-1β, immunohistochemical double labelling revealed that nitrotyrosine partly co-localized to amyloid precursor protein, but not to desmin (Fig. 5). This suggests that, under con-ditions of intracellular nitration, amyloid precursor protein is part of the muscular proteins that can be impaired in function. Furthermore, IFN-γ + IL-1β induced an upregulation of the pro-tein expression of amyloid precursor protein but not desmin (Fig. 5), which is in line with our previous observations (Schmidt *et al.*, 2008). To address the pathway of induction of cell death, a caspase inhibitor was used. Whereas muscle cell death induced by staurosporin was completely abolished by the caspase inhibitor [$82.8 \pm 7.5\%$ versus $10.7 \pm 2.5\%$ (mean ± SD), **$P < 0.01$], cell death after IL-1β + IFN-γ remained basically unchanged.

Figure 4 Flow cytometric analysis for detection of intracellular nitric oxide and cell death in myoblasts. (**A**) Myoblasts (rhabdomyosarcoma cell line CCL-136) were exposed to IL-1β (20 g/ml) and IFN-γ (300 U/ml) compared to medium alone for 24, 48 or 72 h compared to controls. Two hours prior to the end of the incubation time, DAF-2 was applied to the cells for specific intracellular detection of nitric oxide. Shortly before analysis by flow cytometry, cells were carefully collected, protected from light and stained with 7-aminoactinomycin D (7-AAD) for detection of cell death. Note the distinct increase of the fractions of dead cells (*top quadrants*) and DAF-2$_{bright}$ cells, which harbour nitric oxide (*right quadrants*). A substantial increase of the rate of cells that contain nitric oxide and display a reduced cell viability is observed. (**B**) Myoblasts were exposed to IL-1β + IFN-γ compared to control for 72 h. Cell death detection staining was achieved by staining for annexin-V APC and 7-aminoactinomycin. Only some non-specific staining for annexin-V was noted, but no evidence of induction of apoptosis. All data are shown as contour-plots and the numbers in each quadrant represent the percentage of cells that are positive for the respective marker(s).

Moreover, exogenous nitric oxide from diethylenetriamine induced a necrotic morphology in muscle cells as revealed by bright-field microscopy (data not shown).

Collectively, this demonstrates that exogenous as well as intracellular nitric oxide induces intracellular accumulation of β-amyloid and subsequent cell death of muscle cells.

Prevention of accumulation of β-amyloid and cell death by pharmacological inhibition of inducible nitric oxide synthase

Using the nitric oxide-specific reporter dye DAF-2, flow cytometry demonstrated that intracellular production of nitric oxide inflicted by 72 h of exposure to IL-1β + IFN-γ was almost completely abrogated by the iNOS-specific inhibitor 1400W (Fig. 6A). The non-specific NOS-inhibitor L-NMMA was not sufficiently effective in myoblasts (data not shown). Accordingly, immunolabelling for nitrated tyrosine showed that the cytokine-induced production of intracellular nitric oxide was significantly decreased upon iNOS-inhibition by 1400W (Fig. 6B). We have previously shown that exposure to IL-1β, particularly together with IFN-γ, induces intracellular accumulation of β-amyloid (Schmidt et al., 2008). As evidenced by thioflavin-S histochemistry, accumulation of β-amyloid inflicted by IL-1β + IFN-γ was significantly diminished by 1400W (Fig. 6C). Inhibition of iNOS by 1400W and subsequent reduction of accumulation of β-amyloid completely prevented cytokine-induced cell death as revealed by propidium-iodide staining in myotubes (Fig. 6D).

These data suggest that intracellular production of nitric oxide inflicted by IL-1β together with IFN-γ is a key mechanism in skeletal muscle that leads to intracellular accumulation of β-amyloid and cell death.

Discussion

Here we demonstrate that iNOS was overexpressed in skeletal muscle of patients with sporadic IBM compared with dermatomyositis, polymyositis, muscular dystrophy and non-myopathic biopsies and that signs of nitric oxide stress co-localized to accumulations of β-amyloid. In muscle cells, IL-1β in combination with IFN-γ induced upregulation of iNOS. Intracellular production of nitric oxide was followed by accumulation of β-amyloid and subsequent cell death by necrosis, which was all prevented by pharmacological inhibition of iNOS. Collectively, the findings reveal a distinct interplay between IL-1β, iNOS, nitric oxide, accumulation of β-amyloid and cell death in skeletal muscle (Fig. 7).

Inducible nitric oxide synthase is the major enzyme that, compared to the neuronal nitric oxide synthase and endothelial nitric oxide synthase isoforms, can produce large and sustained amounts of nitric oxide under inflammatory conditions (Kleinert et al., 2004). Cell stress associated with upregulation of iNOS and production of nitric oxide has been demonstrated in inflammatory myopathies including sporadic IBM (Tews and Goebel, 1998; Wanchu et al., 1999; De Paepe et al., 2004). In the present
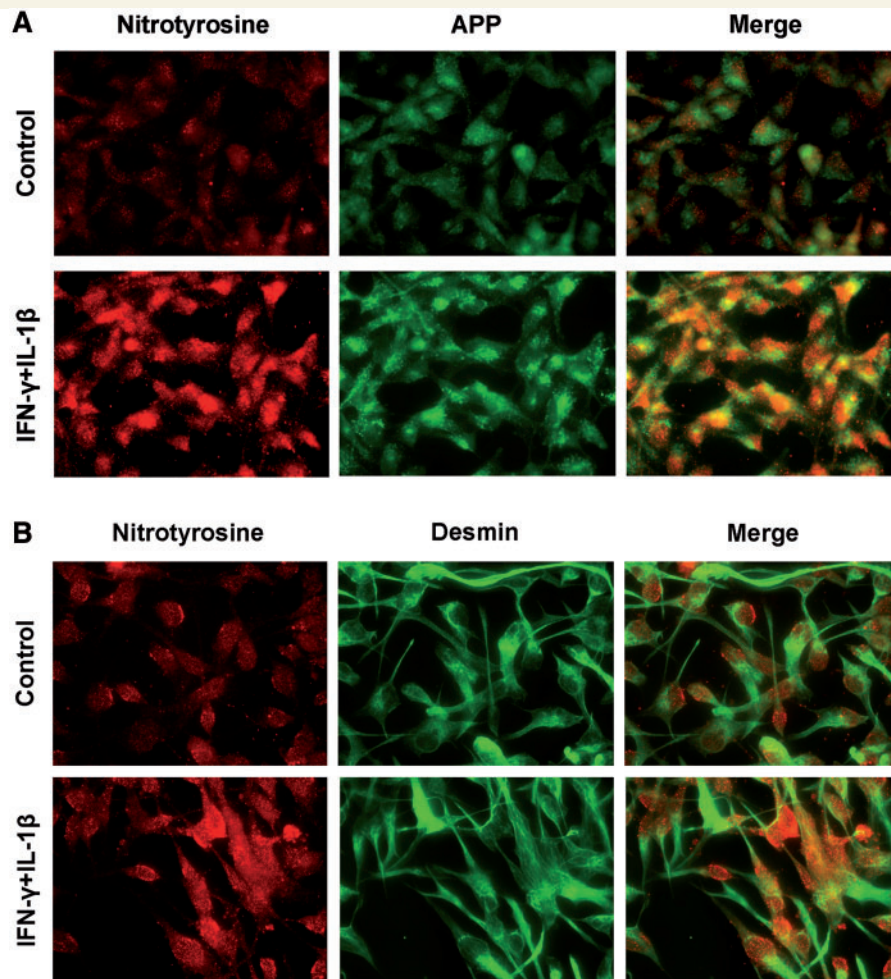
Figure 5 Co-localization analysis between nitrotyrosine and amyloid precursor protein (APP) or desmin in cultured muscle cells. Seventy-two hours after exposure of myoblasts (human rhabdomyosarcoma cell line CCL 136) to the pro-inflammatory cytokines IFN-γ (300 U/ml) and IL-1β (20 ng/ml), immunohistochemical staining for nitrotyrosine (red, Alexa-594 in **A** and **B**) displays an upregulation compared with controls (similar to Fig. 2B). Amyloid precursor protein (green, Alexa-488 in **A**) is upregulated upon IFN-γ + IL-1β compared with controls, whereas the expression of desmin remained unchanged (green, Alexa-488 in **B**). A partial co-localization between amyloid precursor protein and nitrotyrosine is noted (yellow in merged image, **A**), whereas no overlap between nitrotyrosine and desmin could be observed (merged image, **B**). One representative experiment of three with similar results is shown. Photo-microscopy with a ×63 objective.

study, we have demonstrated an overexpression of iNOS in sporadic IBM muscle at the levels of messenger RNA and protein. The protein expression was higher than expected from the respective messenger RNA expression, which could be explained by post-transcriptional regulation and possible splice variants that may not have been detected by our polymerase chain reaction assay. A decay of messenger RNA due to its general half-life and other non-specific as well as specific mechanisms including endogenous small interfering RNA may contribute to and further explain this difference. Intracellular cell stress by nitric oxide in iNOS-overexpressing muscle fibres in sporadic IBM was demonstrated by staining for nitrotyrosine, which co-localized and significantly correlated with intra-fibre depositions of β-amyloid. This confirms and extends previous observations of a co-localization of oxidative stress and β-amyloid in patients with myopathies

characterized by rimmed vacuoles (Tateyama *et al.*, 2003) as well as co-localization of iNOS/nitrotyrosine and filamentous inclusions/vacuoles in fibres from patients with hereditary IBM (Yang *et al.*, 1998). Although the intracellular detectors for nitric oxide and reactive oxygen species, DAF-2 and dichlorofluorescein, respectively, are generally accepted and widely used in various model systems (Kim *et al.*, 2007; Dedkova and Blatter, 2009) including skeletal muscle (Silveira *et al.*, 2003), the sensitivity and specificity of DAF-2 has previously been questioned (Broillet *et al.*, 2001; Roychowdhury *et al.*, 2002). But owing to the demonstration in our study of a co-localization of nitrotyrosine and iNOS and an efficient blocking of all functional effects in muscle cells by the iNOS-inhibitor 1400W, we suggest that indeed nitric oxide-mediated mechanisms play an important role during inflammation-mediated cell stress in skeletal muscle. However, we
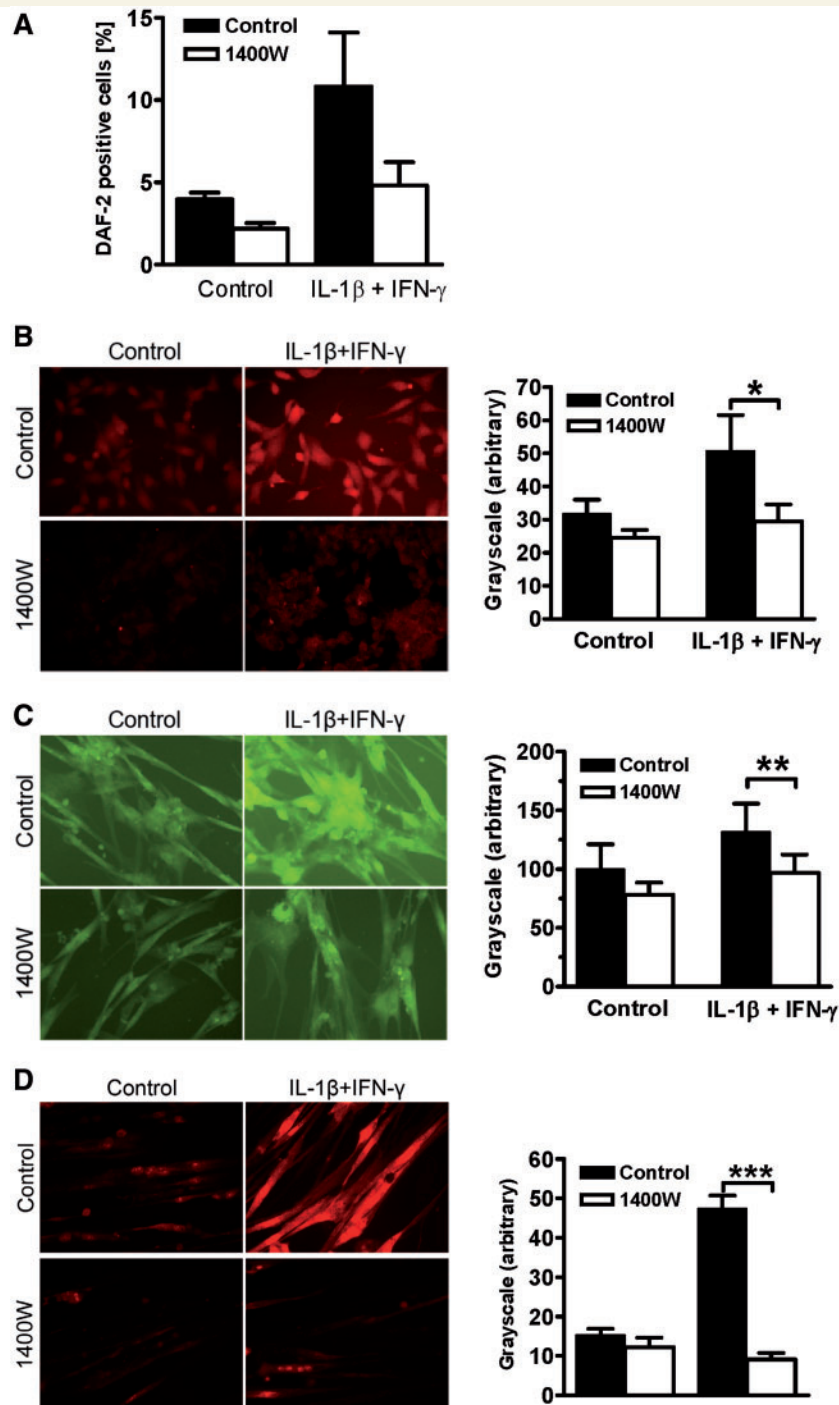
Figure 6 Pharmacological block of iNOS by 1400W. Myoblasts (**A** and **B**) or primary human myotubes (**C** and **D**) were exposed to IL-1β (10 ng/ml) and IFN-γ (300 U/ml) for 72 h in presence of 1400W, a specific iNOS inhibitor. (**A**) Intracellular detection of nitric oxide was achieved by the fluorescent detector DAF-2 and flow cytometry (similar to Fig. 4A). IL-1β + IFN-γ led to an increase of the rate of DAF-2 positive cells, which was attenuated by 1400W, but did not reach statistical significance. Data are depicted as mean + SEM from three independent experiments with similar results. (**B–D**) Muscle cells were cultured in chamber slides and stained for nitrotyrosine (Alexa-495, red, **B**), thioflavin-S (green, **C**) or propidium iodide (red, **D**). IL-1β + IFN-γ strongly elevated the staining signals for nitrated tyrosine as indicator of intracellular nitric oxide (**B**), intracellular accumulation of amyloid (**C**) and cell death (**D**). Incubation with 1400W completely abolished the signals for nitric oxide, amyloid and cell death, which were all statistically significant as indicated by grey scale analysis (*right* panels in **B–D**; data shown as mean + SD). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Photo-microscopy with a x 40 objective (**B–D**).
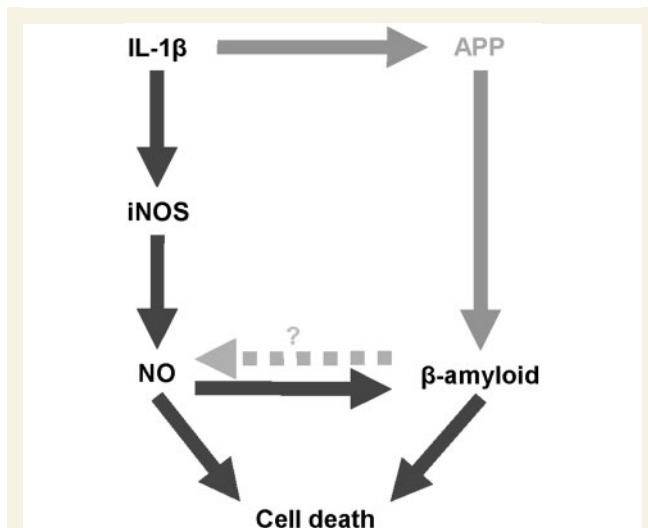
Figure 7 Model of interactions between nitric oxide (NO) and β-amyloid in sporadic IBM pathology. Findings from this article are indicated in dark grey, previous observations on induction of amyloid precursor protein (APP) by IL-1β are shown in light grey (Schmidt *et al.*, 2008) and a hypothetical pathway is depicted as dashed light grey arrow. IL-1β leads to upregulation of APP as well as iNOS. iNOS leads to intracellular production of nitric oxide, which contributes to accumulation of β-amyloid as well as direct induction of cell death. Upregulation of APP augments production of β-amyloid, which also causes cell death. In a bidirectional fashion, accumulation of β-amyloid could upregulate expression of iNOS/nitric oxide.

cannot exclude that reactive oxygen species or other free radicals could contribute to muscle damage as demonstrated in this study.

In a recently established muscle cell culture model, we have shown that IL-1β induces accumulation of β-amyloid (Schmidt *et al.*, 2008), but the underlying mechanisms had so far remained elusive. Using this model, we have now shown that in an inflammatory environment with presence of a combination of IL-1β and IFN-γ, human muscle cells upregulate the expression of iNOS, which confirms previous observations (Okuda *et al.*, 1997; Adams *et al.*, 2002). Further, intracellular signs of nitration of tyrosine were detectable while long-term cytokine exposure induced production of nitric oxide and reactive oxygen species in human muscle cells as determined by sensitive intracellular reporter dyes and flow cytometry. Augmentation of intracellular nitric oxide was followed by intracellular accumulation of β-amyloid and death of the muscle cells. Because pharmacological inhibition of iNOS efficiently blocked intracellular nitric oxide and completely prevented accumulation of β-amyloid and induction of cell death, the findings confirm and extend previous reports which demonstrated that muscle cell death can be inflicted directly by nitric oxide (Tews and Goebel, 1998) or via β-amyloid itself (Querfurth *et al.*, 2001). This synergistic toxicity of cytokines and β-amyloid via upregulation of iNOS expression is also in line with a previous report on muscle cells exposed to IFN-γ and β-amyloid (Baron *et al.*, 2000).

It is possible that nitration of amyloid precursor protein enhances subsequent accumulation of β-amyloid. At the same time, our data confirm that pro-inflammatory cytokines such as IL-1β and + IFN-γ can upregulate protein expression of amyloid precursor protein, which is in line with our previous observations (Schmidt *et al.*, 2008). Therefore, two pathways may contribute to accumulation of β-amyloid in muscle cells exposed to pro-inflammatory cell stress: a direct effect of the cytokines as well as an indirect effect via nitric oxide (Fig. 7). We demonstrate that a large fraction of >20% of the fibres is immunohistochemically positive for β-amyloid, which is well in accord with previous observations (Muth *et al.*, 2009; Vattemi *et al.*, 2009). An even bigger fraction of 37% of the fibres has been demonstrated to be positive for amyloid precursor protein (Muth *et al.*, 2009). Although it can be expected that muscle fibres with a strong accumulation of β-amyloid will eventually die, a large fraction of the fibres that are positive for β-amyloid by immunohistochemistry appear morphologically normal (Muth *et al.*, 2009). This implies that counter-regulatory mechanisms such as macroautophagic processing of unwanted proteins and cell-stress pathways may help the muscle fibres to cope with the protein overload, at least for a limited time (Schmidt, 2010). However, the rate of necrosis and ultimate fate of β-amyloid positive fibres has yet remained elusive. Although not supported by convincing evidence, it is possible that β-amyloid, in a bidirectional manner, may contribute to the overexpression of iNOS and production of nitric oxide (Fig. 7).

High concentrations of cytokines and chemokines can be directly detrimental to muscle cells as observed for IL-1β (Broussard *et al.*, 2004) and TNF-α (Li *et al.*, 2005). In this study, the cell death in muscle cells upon IL-1β + IFN-γ exposure did show morphological signs of necrosis and was not blocked by caspase inhibitors, which suggests that mechanisms of apoptosis are not predominant in this experimental setting. This is consistent with our recent observation that muscle cells fail to undergo apoptosis after exposure to TNF-α (Keller *et al.*, 2011) and the fact that apoptosis is absent in inflammatory myopathies including sporadic IBM (Schneider *et al.*, 1996; Behrens *et al.*, 1997; Olive *et al.*, 1997; Tews and Goebel, 1998; Nagaraju *et al.*, 2000). We have previously demonstrated that two major differences discriminate the inflammation in the muscle of sporadic IBM when compared to polymyositis or dermatomyositis (Schmidt *et al.*, 2008): first, the local expression of pro-inflammatory mediators as cytokines and chemokines is much higher in sporadic IBM and most likely begins many years before the clinical onset of symptoms. Whereas in polymyositis and dermatomyositis the level of tissue inflammation usually declines throughout the course of the disease, a chronic inflammatory environment continues to be present in sporadic IBM, mostly even at late stages of the disease. Secondly, only in sporadic IBM, a large fraction of muscle fibres themselves produce cytokines and chemokines. These fundamental differences suggest that there is a prolonged and high local concentration of pro-inflammatory mediators in the sporadic IBM muscle, which may ultimately lead to elevation of iNOS and other cell-stress molecules as well as accumulation of β-amyloid. It is possible that yet unknown underlying conditions like ageing processes, certain HLA-subtypes, impaired protein degradation and insufficient

counter-regulation of cell stress may sustain or even initiate the vicious circle of pathomechanisms in sporadic IBM (Needham and Mastaglia, 2008; Schmidt, 2010).

Our data suggest that a primary inflammatory trigger in skeletal muscle can cause a toxic accumulation of β-amyloid. This is in line with our recent observations in a muscle cell culture model (Schmidt *et al.*, 2008; Muth *et al.*, 2009) and a study by others in a mouse model of IBM with lipopolysaccharides (LPS)-induced local inflammation in the muscle (Kitazawa *et al.*, 2008). A similar interaction between iNOS and β-amyloid has previously been demonstrated in the animal model of Alzheimer's dementia, where knock-out of iNOS reduced accumulation of β-amyloid and mortality (Nathan *et al.*, 2005). Other cell stress events including heat shock proteins as αβ-crystallin (Muth *et al.*, 2009) and endoplasmic reticulum overload (Nagaraju *et al.*, 2005) are also relevant to skeletal muscle and could be closely related to each other as suggested for endoplasmic reticulum-stress and nitric oxide (Gotoh and Mori, 2006). Such a network of mechanism may be of particular relevance to sporadic IBM which occurs in older individuals, because the capacity to cope with oxidative stress is significantly reduced with ageing (Ferrington *et al.*, 2005).

In summary, our data underscore the relevance of a specific interplay between inflammatory and β-amyloid-associated patho-mechanisms in sporadic IBM. Upregulation of iNOS with subsequent production of nitric oxide may be a key event in this pathological network (Fig. 7) and act in concert with endoplasmic reticulum overload and other cell stress pathways to trigger and augment accumulation of β-amyloid (Schmidt, 2010; Schmidt and Dalakas, 2010). The data profoundly further our understanding of the pathology of sporadic IBM and identify regulation of iNOS as a promising target for future therapies.

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Supplementary material

Supplementary material is available at *Brain* online.

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