Iron deficiency-induced loss of skeletal muscle mitochondrial proteins and respiratory capacity; the role of mitophagy and secretion of mitochondria-containing vesicles

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Abstract
Iron homeostasis is essential for mitochondrial function, and iron deficiency has been associated with skeletal muscle weakness and decreased exercise capacity in patients with different chronic disorders. We hypothesized that iron deficiency-induced loss of skeletal muscle mitochondria is caused by increased mitochondrial clearance. To study this, C2C12 myotubes were subjected to the iron chelator deferiprone. Mitochondrial parameters and key constituents of mitophagy pathways were studied in presence or absence of pharmacological autophagy inhibition or knockdown of mitophagy-related proteins. Furthermore, it was explored if mitochondria were present in extracellular vesicles (EV). Iron chelation resulted in an increase in BCL2/Adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and BNIP3-like gene and protein levels, and the appearance of mitochondria encapsulated by lysosome-like vesicular structures in myotubes. Moreover, mitochondria were secreted via EV. These changes were associated with cellular mitochondrial impairments. These impairments were unaltered by autophagy inhibition, knockdown of mitophagy-related proteins BNIP3 and BNIP3L, or knockdown of their upstream regulator hypoxia-inducible factor 1 alpha. In conclusion, mitophagy is not essential for development of iron deficiency-induced reductions in mitochondrial proteins or respiratory capacity. The secretion of mitochondria-containing EV could present an additional pathway via which mitochondria can be cleared from iron chelation-exposed myotubes.

KEYWORDS
extracellular vesicles, mitochondrial clearance, myotubes, iron depletion

Abbreviations: 3-MA, 3-methyladenine; BAFA1, Bafilomycin A1; BNIP3, BCL2/Adenovirus E1B 19 kDa protein-interacting protein 3; BNIP3L, BNIP3-like; CS, citrate synthase; DFP, deferiprone; DNM1L, dynamin 1 like protein; EV, extracellular vesicles; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; FIS1, mitochondrial fission 1 protein; FLOT1, Flotillin-1; FUNDC1, FUN14 domain-containing protein 1; GABARAPL1, GABA type A receptor associated protein like 1; HADH, β-hydroxyacyl-CoA dehydrogenase; HIF, hypoxia-inducible factor; HSP70, Heatshock protein 70; LC3B, microtubule-associated protein light chain 3 B; MTPF1, fission protein mitochondrial protein 18 kDa; MFN, mitofusin; OPA1, dynamin-like 120 kDa protein, mitochondrial; OXPHOS, Oxidative phosphorylation; PARK2, Parkin; PINK1, PTEN-induced putative kinase 1; RLU, Relative luciferase units.

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1 | INTRODUCTION

Mitochondria are highly dynamic, cytoplasmic organelles that play a key role in a variety of cellular functions, of which the oxidative phosphorylation-mediated synthesis of ATP from ADP is the best known. Due to its high energy requirements, skeletal muscle is a metabolically active tissue and, especially the type I fibers, contain a high amount of mitochondria. Illustrative of the plasticity of the mitochondrial network in skeletal muscle, the number of mitochondria can vary greatly depending on physiological requirements (eg, during physical activity or inactivity) and resource availability (eg, during starvation).1 Even in mature skeletal muscle, where mitochondria are placed in a highly organized environment and are subject to contractile forces, mitochondria undergo dynamic fusion events which are essential for normal contractile function.2 Iron homeostasis is essential for proper mitochondrial function as iron is an essential component for several of the protein complexes that make up the electron transport chain, and hence is essential for cellular respiration and subsequent ATP production. Indeed, it is well established that iron deficiency in vivo results in decreased skeletal muscle mitochondrial quantity and function culminating in impaired oxidative substrate metabolism.3-8 Illustrative of its clinical significance, iron deficiency has been associated with muscle weakness and decreased exercise capacity in patients with congestive heart failure and chronic obstructive pulmonary disease.9,10 However, the intracellular mechanisms of how iron deficiency drives this loss of skeletal muscle mitochondrial quantity and quality remain to be elucidated.

Mitochondrial clearance can be achieved via mitochondrial breakdown1 or via secretion of mitochondria in the extracellular environment.11-13 There are several routes of mitochondrial breakdown, including mitophagy (ie, mitochondrial autophagy), mitochondrial-derived vesicles and proteasome-mediated mitochondrial protein degradation.1,14 Mitophagy has the largest breakdown capacity, as it is able to clear complete mitochondria, and it is best described in literature. During mitophagy, mitochondria are engulfed by an autophagosomal membrane which subsequently fuses with a lysosomal structure in which the mitochondria are degraded. Mitophagy can roughly be divided into receptor-mediated and ubiquitin-mediated mitophagy. Receptor-mediated mitophagy is initiated by activation of mitophagy receptors like Parkin (PARK2).18-20 Although iron deficiency has been shown to result in increased mitophagy in several models including Caenorhabditis elegans, yeast, osteosarcoma, and neuroblastoma cells, evidence of iron deficiency-induced mitophagy in skeletal muscle is still lacking.21-23 Recently, the secretion of mitochondria via extracellular vesicles (EV) has been described as an alternative mechanism for mitochondrial clearance.11,12 However, there is currently no data reporting secretion of mitochondria in EV from skeletal muscle, nor in relation to alterations in iron metabolism.

In this study, we hypothesized that loss of skeletal muscle mitochondrial content and respiratory capacity induced by iron deficiency is caused by increased mitochondrial clearance. Differentiated murine skeletal muscle cells were subjected to an iron chelating compound. Subsequently, parameters related to mitochondrial content and respiratory capacity, as well as abundance of key constituents of mitophagy pathways were studied in the presence or absence of inhibition of general autophagy or specific knockdown of several important mitophagy-related proteins. Furthermore, we explored if mitochondria were packaged into extracellular vesicles in response to iron chelation.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Subjects include murine skeletal muscle cells (C2C12) (Cat# CRL-1772, RRID:CVCL_0188) (ATCC, Manassas, VA, USA). Cells were cultured (37°C, 5% CO₂) and seeded in matrigel-coated dishes (#354234, BD Biosciences, Breda, NB, the Netherlands) at 85% confluence, in low-glucose (1 g/L) Dulbecco's modified Eagle medium (DMEM; #22320-022) containing 50 U/ml penicillin and 50 µg/mL streptomycin (#15140-122) and 9% fetal bovine serum (all from Gibco, Bleiswijk, ZH, the Netherlands). Cells were subsequently differentiated for 5 days in high-glucose DMEM (4.5 g/L) (#41966-029) containing 50 U/mL penicillin and 50 µg/mL streptomycin (#15140-122, all from Gibco) and 1% heat-inactivated fetal bovine serum (differentiation medium: DM). For indicated experiments, a C2C12 cell line stably expressing a hypoxia-inducible factor (HIF) reporter plasmid, which expresses the luciferase gene controlled by a promoter bearing five consecutive HIF-responsive elements from the human vascular endothelial growth factor A promoter (5HRE/hCMVmp-luc), was used. Subsequently, cells were exposed to 300 µM DFP (Deferiprone, #379409, Sigma-Aldrich, Zwijndrecht, ZH, the Netherlands), or 10 nM BAFA1 (Bafilomycin A1, #B1793, Sigma-Aldrich).

For indicated experiments, transfection of 2 nM of target-mRNA-specific silencer select siRNA (BNIP3 (#s63059), BNIP3L (#s63062), GABA Type A Receptor Associated Protein Like 1 (GABARAPL1) (#s81311)) or silencer select
scrambled control siRNA (#4390843) (all from Thermo Scientific, Landsmeer, NH, the Netherlands) was initiated in presence of 0.12% RNAiMAX (#13778150, Thermo Scientific). Similarly, transfection of 5 nM of HIF1A-specific stealth siRNA (#MSS205126) or low GC scrambled control siRNA (#12935200) (Thermo Scientific) was initiated in presence of 0.2% RNAiMAX. All transfections were performed in DM 24 hours prior to DFP exposure.

2.2 | Isolation of extracellular vesicles

Cells were cultured for 24 hours in EV-depleted DM, generated by overnight centrifugation of DM in polyallomer tubes (Beckman Coulter, Woerden, ZH, the Netherlands) at 100 000g in a SW32 rotor in a XPN-90 Ultracentrifuge (Beckman Coulter) at 4°C. Conditioned medium was collected from 150-cm² differentiated myotubes, centrifuged at 200g for 10 minutes at 4°C, and transferred to a new tube and centrifuged at 2000g for 10 minutes at 4°C to discard dead cells and debris. Medium was transferred to polyallomer tubes and centrifuged at 16 000g for 30 minutes at 4°C and subsequently at 100 000g for 65 minutes at 4°C. The pellets from the 16 000g and 100 000g centrifugation steps were resuspended in phosphate-buffered saline (PBS) and overlayed by a 2.5-0.4 M sucrose gradient in polyallomer tubes and centrifuged overnight at 188 000g in an SW41 rotor at 4°C.24,25 EV-containing sucrose fractions were collected from both gradients based on EV density characteristics (1.12-1.19 g/mL), and were subsequently diluted in PBS and centrifuged at 100 000g in an SW32 rotor for 65 minutes at 4°C. Pellets were dissolved in 30-µL PBS and were used for subsequent analyses. We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV190019).26

2.3 | RNA extraction and qPCR

Cells were lysed in 350-µL RLT lysis buffer containing 1% β-mercaptoethanol by running the sample through a 20-gauge needle. RNA was extracted using the RNeasy plus mini kit (#74134, Qiagen, Venlo, LB, the Netherlands) according to the manufacturer’s protocol. 400 ng of RNA was used for cDNA synthesis using the Tetro cDNA synthesis kit (#BIO-65043, Bioline, Alphen aan de Rijn, SH, the Netherlands) according to manufacturer’s protocol.

For quantitative PCR amplification, 4.4 µL of 1/50 diluted cDNA was used with target- and species-specific primers (Table 1) and 2X Sensimix SYBR® & Fluorescein mix (#QT615-20, Bioline) on a LightCycler480 384-wells PCR machine (Roche, Almere, FL, the Netherlands). Specificity of PCR amplification was tested with melt curve analysis, and expression levels of genes of interest were corrected using a normalization factor calculated based on the expression of four different housekeepers (B2m, Ppia, Rplp0, and Rpl13A) using the GeNorm software (Primerdesign, Southampton, NY, USA).

| Sense primer | Antisense primer | Ensembl identifier |
|--------------|------------------|--------------------|
| B2m          | CTTCTGTGGTCTTGTCCTCAGTA | GTATGGTCCGGCTCCATCTC | ENSMUSG00000060802 |
| Ppia         | TCCCTCCTTTCAGAATTATCCA | CCGGGAATGCTATATGGG | ENSMUSG00000071866 |
| Rpl13A       | CAGCTCGGAGGAGAAGGAGAAGG | GCAGGGCATGAGGCAAACATG | ENSMUSG00000074129 |
| Rplp0        | GGAGCGAGAAGGACGCTCCTT | GGACATCACTGAAATATGGGA | ENSMUSG00000067274 |
| Bnip3        | AGTTTTTTCCTCATCTCGTACTG | TGTTGACAGAAGGATGAGGAA | ENSMUSG00000066632 |
| Opa1         | GGGGAAACAGTGTGTGAGAGA | AACAAGGCGACATGATGAGGA | ENSMUSG00000038084 |
| Mfn1         | GCTGGCTCGCTTGCTCTGATGT | TCCAGCTGTGGTGCACATCTG | ENSMUSG00000027668 |
| Mfn2         | CGAGGGATAGGAGGAGGAGG | ACCAATCCAGATGGGAGGA | ENSMUSG00000029020 |
| Fis1         | GGCAAATACGCGGCTCAAGA | GCCATGCTACGGGATCCATC | ENSMUSG00000019054 |
| Mtfp1        | CCACACACGCTGGAGGCTGCTG | GGCCTGTTCACTGGAGGCTG | ENSMUSG0000004748 |
| Bnip3        | AGTCTGGCAACGACGCTCAAG | TCCAAACATGATGTTCCCATCTT | ENSMUSG00000022051 |
| Fundc1       | CGATGTATTTGGCCACAGTTC | CCACCTGAGCTGGCAACCTG | ENSMUSG00000025040 |
| Pink1        | GTCTGAGGAGGAGGACGAGG | TTAAGATGCTGCGTGGGAGG | ENSMUSG00000028756 |
| Park2        | CTCGGCTGTCCTCACTCCTC | CCTCGGCGCCTACTGG | ENSMUSG00000028382 |
| mt-Cox2      | CACATCCAAGCAGCAGTACTG | ATTTAGGAGGAGGCTGCTG | ENSMUSG00000064354 |
| Hif1a        | AATGAAGTGCACCTGTCACAA | TGGGCCGTGACTGAGG | ENSMUSG00000021109 |
2.4 Mitochondrial DNA copy number

Cells were lysed in 350-µL DNA lysis buffer (0.1 M Tris/HCl pH 8.5, 5 mM EDTA pH 8.0, 0.2% SDS, and 0.2 M NaCl) or 5 µL of EV in PBS was added to 350-µL DNA lysis buffer. Proteinase K was added to a final concentration of 0.2 mg/mL and the lysates were incubated overnight at 55°C. Lysates were centrifuged at 20 000×g for 5 minutes and DNA from the supernatants was precipitated with isopropanol. DNA pellets were washed twice with 70% ethanol and air-dried. DNA pellets were centrifuged at 20 000×g for 5 minutes and DNA from the supernatants was precipitated with isopropanol. DNA pellets were centrifuged at 20 000×g and the lysates were incubated overnight at 55°C. Lysates were centrifuged at 20 000×g for 5 minutes and DNA from the supernatants was precipitated with isopropanol. DNA pellets were washed twice with 70% ethanol and air-dried. DNA pellets were dissolved in elution buffer (10 mM Tris/HCl, pH 8.0) and heated to 55°C for 2 hours followed by overnight incubation at 4°C. For DNA isolated from cells, 4.4 µL 1/100 diluted DNA was used for qPCR as described above using mitochondrial DNA (mtDNA) specific mt-Cox2 or genomic DNA (gDNA)-specific Ppia primers (Table 1) and the mtDNA/gDNA ratio was calculated. For DNA isolated from EV, 4.4 µL of 1/20 diluted DNA was used for qPCR as described above, using mtDNA-specific mt-Cox2 primers (Table 1).

2.5 Protein extraction and Western Blotting

Cells were lysed in 100-µL whole cell lysis buffer (150 mM NaCl, 1% Nonidet, 1 mM DTT, 1x complete protease inhibitor cocktail (#11697498001, Sigma-Aldrich), 20 mM tris, pH 7.4). Lysates were incubated while rotating for 30 minutes at 4°C and subsequently centrifuged at 20 000g for 30 minutes at 4°C. Protein concentration was determined using the Pierce BCA Protein Assay Kit (#23225, Thermo Scientific) according to the manufacturer’s protocol. Lysates (1 µg/µL) were aliquoted in sample buffer (0.25 M Tris-HCl, 8% (w/v) SDS, 40% (v/v) glycerol, 0.4 M DTT, 0.04% (w/v) Bromophenol Blue, pH 6.8) and boiled for 5 minutes at 95°C. Protein extraction and Western Blotting

Cells were lysed in 100-µL whole cell lysis buffer (150 mM NaCl, 1% Nonidet, 1 mM DTT, 1x complete protease inhibitor cocktail (#11697498001, Sigma-Aldrich), 20 mM tris, pH 7.4). Lysates were incubated while rotating for 30 minutes at 4°C and subsequently centrifuged at 20 000g for 30 minutes at 4°C. Protein concentration was determined using the Pierce BCA Protein Assay Kit (#23225, Thermo Scientific) according to the manufacturer’s protocol. Lysates (1 µg/µL) were aliquoted in sample buffer (0.25 M Tris-HCl, 8% (w/v) SDS, 40% (v/v) glycerol, 0.4 M DTT, 0.04% (w/v) Bromophenol Blue, pH 6.8) and boiled for 5 minutes at 95°C. Ten micrograms of protein per sample were run through a Criterion 26-wells 12% precast gel (#3450119, Bio-Rad Laboratories BV, Veenendaal, UT, the Netherlands) in 1x MES buffer (#1610796, Bio-Rad Laboratories BV) at 100 volts and was subsequently blotted on a nitrocellulose membrane by electroblotting. At least two protein ladders were loaded on each gel (#1610373, Precision Plus Protein All Blue Standards, Bio-Rad Laboratories BV). Twenty microliters of EV in PBS were combined with 20-µL 2× sample buffer and boiled for 5 minutes at 95°C. Twenty microliters of EV in sample buffer were run through Criterion gels as described above.

Membranes were washed, blocked with 3% nonfat, dried milk (Campina, Amersfoort, UT, the Netherlands) in TBS-Tween-20 (0.05%) for 1 hour, washed, and incubated overnight at 4°C with different protein-specific primary antibodies against: BNI3P (#3769S, RRID:AB_2259284, Cell Signaling Technology), BNI3P3L (#12396, RRID:AB_2688036, Cell Signaling Technology), DNM1L (#8570, RRID:AB_10950498, Cell Signaling Technology), FUNDC1 (#sc-133597, RRID:AB_10609242, Santa Cruz Biotechnology), microtubule-associated protein light chain 3 (LC3)B (#2775, RRID:AB_915950, Cell Signaling Technology), Heatshock protein (HSP)70 (#NB110-96425, RRID:AB_1262360, Novus), Flotillin-1 (FLOT1) (#610821, RRID:AB_398140, BD Biosciences), and oxidative phosphorylation (OXPHOS) complex subunits (#MS604, RRID:AB_2629281, MitoScience LLC) all diluted in 3% nonfat, dried milk or 5% bovine serum albumin in TBS-Tween-20. Membranes were washed and incubated with horseradish peroxidase-labeled, primary antibody-specific, secondary antibody (#BA-9200, #BA-1000, Vector Laboratories, Amsterdam, NH, the Netherlands) (1:10 000 diluted in 3% nonfat, dried milk in TBS-Tween-20) for 1 hour at room temperature.

Membranes were washed and incubated with either 0.5× SuperSignal West Pico Chemiluminescent Substrate or 0.25× SuperSignal West Femto Chemiluminescent Substrate (#34578, #34095, Thermo Scientific) for 5 minutes, depending on the expected signal strength. Photographs were taken with the LAS-3000 or Amaresham Imager 600 and analyzed with ImageQuant TL software (GE Healthcare Life Sciences). Analyses were performed on original, unaltered images. After analyses, the contrast of the depicted representative images was enhanced when needed and applied for whole photograph equally.

2.6 Enzyme activity assays

Cells were lysed in 100-µL SET buffer (250 mM sucrose, 2 mM EDTA, 10 mM Tris, pH 7.4) and snap-frozen in liquid nitrogen. Subsequently, samples were thawed, incubated on ice for 30 minutes, and centrifuged at 20 000g for 10 minutes at 4°C. A final concentration of 1% BSA was added to the 60 µL of sample. Protein concentration was determined by the Pierce BCA Protein Assay Kit in the remaining supernatant. Enzyme activities were measured spectrophotometrically (Multiskan Spectrum, Thermo Lab Systems, Landsmeer, NH, the Netherlands) as described previously for both citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (HADH).27

2.7 Respirometry

A Seahorse XF96 sensor cartridge with Seahorse calibrant (pH 7.4) was incubated in a CO₂-free incubator at 37°C.
24 hours prior to the measurement (#101085-004, Agilent, Santa Clara, USA). Cells were washed twice with XF assay media (DMEM with 1x Glutamax, 1 mM sodium pyruvate, 0.9 g/L NaCl, 4.5 g/L glucose, phenol red, pH 7.35 ± 0.05) and incubated for 1 hour in assay media in a CO₂-free incubator at 37°C. A mitochondrial stress test was performed using final concentrations of 1 µM oligomycin, 2 µM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and 1 µM rotenone/antimycin-A. Cells were lysed in 0.05% SDS in Hanks’ balanced salt solution (HBSS), and protein concentration was determined with the Pierce BCA Protein Assay Kit to correct respirometry data. Basal respiration was determined by subtracting the nonmitochondrial respiration (ie, oxygen consumption rate (OCR) measured after rotenone/antimycin-A) from the OCR measured at baseline. Proton leak was determined by subtracting the nonmitochondrial respiration from the OCR measured after oligomycin. Coupled respiration was determined by subtracting the proton leak from the basal respiration. Maximal respiration was determined by subtracting OCR measured after rotenone/antimycin-A from the OCR measured after FCCP.

2.8 | Luciferase activity

Cells were lysed in 1× Reporter Lysis Buffer (#E397A, Promega, Madison, USA) and snap frozen in liquid nitrogen. After thawing the lysate was centrifuged at 20,000g for 2 minutes and the soluble fraction was used for determination of luciferase activity. Samples were analyzed with a single-tube luminometer with injector (#LB9507, Berthold Technologies, Bad Wildbad, BW, Germany) by measurement of relative luciferase units (RLU) after addition of luciferase reagent (20 mM Tricine, 1.07 mM [(MgCO₃)₄Mg(OH)₂H₂O], 2.67 mM MgSO₄, 0.1 mM EDTA, 33.3 mM DTT, 270 µM coenzyme A, 470 µM beetle luciferin potassium salt, and 580 µM ATP, pH 7.8) to the sample. Protein concentration was determined with the Pierce BCA Protein Assay Kit, which was used to correct luciferase data.

2.9 | Electron microscopy

Cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer in the cell culture dish and kept in the plate until the polymerization was completed. The cells were kept in the fixative during 24 hours at 4°C. Subsequently, cells were washed with 0.1 M cacodylate buffer and post-fixed with 1% osmium tetroxide in the same buffer containing 1.5% potassium ferricyanide during 1 hour in darkness at 4°C. Then the samples were dehydrated in ethanol, infiltrated with Epon resin during 2 days, embedded in the same resin and polymerized at 60°C during 48 hours. Ultrathin sections were obtained using a Leica Ultracut UCT ultramicrotome and mounting on Formvar-coated copper grids. They were stained with 2% uranyl acetate in water and lead citrate. Sections were observed in a Tecnai T12 electron microscope equipped with an Eagle 4kx4k CCD camera (Thermo Fisher Scientific).

Ultrastructural features resembling a second membrane encapsulating mitochondria (indicative of autophagosomal-vesicle formation) or structures resembling lysosomes containing mitochondria (indicative of lysosomal breakdown) were identified. EV were defined as separate membrane-enclosed structures near the plasma membrane of approximately 100-1000 nm in diameter. Quantification was performed by selecting 10 random cells and counting EV across the length of approximately 50 µm of plasma membrane and presenting the average number of vesicles per 10 µm plasma membrane. This quantification was performed in three independent samples.

2.10 | Statistics

Data were analyzed using an unpaired Student’s t-test in case of one comparison over two groups, or an one-way ANOVA with Bonferroni as a post hoc test in case of multiple comparisons over multiple groups, all executed with the GraphPad Prism 5.0 software. Data are expressed as mean ± SEM, with the number of samples (n), reflecting the biological replicates within one experiment, depicted for each graph. Depicted representative western blots had equally applied brightness and contrast adjustments. All western blot quantifications were performed on original and unadjusted photographs.

3 | RESULTS

3.1 | Iron chelation results in impaired mitochondrial respiratory capacity and decreased levels of mitochondrial constituents

Myotubes subjected to iron chelation, that is, exposure to 300 µM DFP, had a lower basal, coupled, and maximal respiration (ie, oxygen consumption rate (OCR)), decreased mtDNA copy number, and lower protein levels of OXPHOS complex subunits NDUFB8 (complex I) and SDHB (complex II). No changes in proton leak, HADH activity, CS activity or protein levels of OXPHOS complex subunits CQCRC2 (complex III) and ATP5A (complex V) were observed (Figure 1A-E). No changes in protein levels of mitochondrial fission master regulator Dynamin 1 Like protein (DNM1L), or in mRNA levels of fusion proteins Mitofusin
(MFN1) and Dynamin-like 120-kDa protein, mitochondrial (OPA1), or fission protein Mitochondrial fission 1 protein (FIS1) were found in iron chelation subjected myotubes. Fusion protein MFN2 and fission protein Mitochondrial protein 18 kDa (MTPF1) mRNA expression was lower in these cells (Figure 1F-G). To demonstrate that the decrease in mitochondrial constituents resulted specifically from the iron chelation and were not mediated by any other nonspecific DFP-related effect, cells were exposed to the presaturated iron chelator (300 μM DFP + 100 μM FeCl3). Exposure to presaturated DFP resulted in a rescue of the DFP-induced reductions of mitochondrial constituents (Figure 2A-C). These experiments indicate that the DFP-induced reductions in mitochondrial constituents did indeed result specifically from myotube iron chelation.

3.2 | Iron chelation results in mitochondria engulfed in double-layered membranes, mitochondrial-lysosomal co-localization, and increased BNIP3 and BNIP3L levels

Mitochondria surrounded by double-layered membranes and mitochondria in lysosomal-like structures were observed in myotubes subjected to iron chelation, while these were not detected in untreated cells (Figure 3A). In line with this, mRNA expression of mitophagy receptors BNIP3 and BNIP3L was higher in myotubes exposed to iron chelation compared to control myotubes (Figure 3B). However, PINK1, PARK2, and FUNDC1 mRNA expression was not significantly different in exposed myotubes (Figure 3B). Moreover, both BNIP3 and BNIP3L protein levels were higher in myotubes exposed...
to iron chelation (Figures 2C and 3C), while FUNDC1 protein levels were lower (Figure 3C). Together, these data points toward increased mitophagy initiation in response to iron chelation.

### 3.3 Iron chelation-induced decreased mitochondrial constituents are not autophagy dependent

To test if the iron chelation-mediated decrease in mitochondrial constituents were autophagy dependent, autophagy initiation and lysosomal breakdown were inhibited using the type III Phosphatidylinositol 3-kinases (PI-3K) inhibitor 3-MA and a vacuolar type H⁺-ATPase inhibitor BAFA1, respectively. DFP-induced decreases in mitochondrial constituents were not influenced by the presence of either 3-MA or BAFA1 (Figure 4A,B,D,E,G). Accumulation of BNIP3, BNIP3L, and LC3B was not observed during inhibition of autophagy initiation with 3-MA (Figure 4C), while protein levels of BNIP3, BNIP3L, and LC3B were accumulating after inhibition of lysosomal breakdown with BAFA1 (Figure 4F,G). Autophagic flux, calculated by subtracting the group-average fold change value without BAFA1 from the individual fold change values with BAFA1 (ie, thereby representing the BAFA1-specific accumulation), was not different for any marker between the conditions (Figure 4G). Since SDHB showed the largest decrease after DFP exposure (Figure 1E), only SDHB protein levels were reported as a marker of the DFP-induced decreased mitochondrial constituents. Together, these data indicate that the iron chelation-induced decrease in mitochondrial constituents are not autophagy dependent, and that iron chelation does not increase autophagic flux.

### 3.4 Iron chelation-induced decreases in mitochondrial constituents and respiratory capacity are not BNIP3/BNIP3L dependent

To test if the iron chelation-mediated decrease in mitochondrial constituents and respiratory capacity were dependent on activation of the mitophagy receptors BNIP3 and BNIP3L, we performed a siRNA-mediated knockdown of BNIP3 and BNIP3L and subsequently investigated SDHB protein levels, mtDNA copy number, and mitochondrial respiration and proton leak in response to DFP treatment. The knockdown of BNIP3/BNIP3L resulted in abolishment of the basal as well as DFP-induced increase in BNIP3 and BNIP3L and subsequently investigated SDHB protein levels, mtDNA copy number, and mitochondrial respiration and proton leak in response to DFP treatment. The knockdown of BNIP3/BNIP3L resulted in abolishment of the basal as well as DFP-induced increase in BNIP3 and BNIP3L levels (Figure 5A). However, in line with our findings after pharmacological inhibition of autophagy, the knockdown of BNIP3/BNIP3L did not result in a rescue of either the loss of mitochondrial constituents or mitochondrial respiratory capacity in myotubes exposed to DFP (Figure 5B-G), suggesting that the iron chelation-induced decrease in mitochondrial constituents and respiratory capacity are not BNIP3/BNIP3L-dependent. Separate siRNA-mediated knockdown experiments of BNIP3 or BNIP3L were performed as well, which also showed no rescue of mitochondrial parameters (data not shown).
Iron chelation-induced decreases in mitochondrial constituents and respiratory capacity are not HIF1A dependent

It is known that shortage of free iron results in HIF1A stabilization in several cell types, and HIF1A has been positioned as a major upstream regulator of BNIP3 and BNIP3L expression.\textsuperscript{21,28,29} To test if HIF1A plays a role in iron chelation-induced loss of mitochondrial constituents and respiratory capacity independently of BNIP3 and BNIP3L, we studied if iron chelation induced HIF transcriptional activity, and we subsequently studied whether DFP-induced reductions in mitochondrial constituents and respiratory capacity were dependent on HIF1A in our model.

HIF transcriptional activity was potently increased in myotubes exposed to iron chelation (Figure S1A). Moreover, the siRNA-mediated knockdown of HIF1A (Figure S1B) resulted in the abolishment of the increase in BNIP3 and BNIP3L protein levels in myotubes exposed to DFP (Figure S1C). Although a significantly higher maximal mitochondrial respiration was found in HIF1A knockdown myotubes exposed to iron chelation compared with scrambled control myotubes exposed to iron chelation, and the iron chelation-induced decreases in basal and coupled mitochondrial respiration that were present in scrambled control myotubes were no longer present in HIF1A knockdown myotubes, HIF1A knockdown did not rescue the loss of mitochondrial constituents nor maximal respiration in myotubes exposed to DFP (Figure S1D-I).

Extracellular vesicles containing mitochondrial cargo appear after iron chelation

Since our data showed that iron chelation-induced mitochondrial clearance was not dependent on autophagy, lysosomal breakdown, BNIP3/BNIP3L or HIF1A signaling, we subsequently investigated if iron chelation-induced mitochondrial clearance could also be executed via secretion of mitochondria-containing EV. Myotubes exposed to iron chelation were found to secrete large amounts of EV in their extracellular milieu, while unexposed myotubes were not. These EV varied in size from <100 nm to ± 500 nm (Figure 6A-C).
Inhibition of general autophagy initiation or lysosomal breakdown during iron chelation does not rescue loss of mitochondrial constituents. mtDNA/gDNA ratio (A), SDHB protein levels (B), BNIP3, BNIP3L, LC3BI, and LC3BII protein levels (C), are depicted of myotubes exposed for 24 hours to control (white bars), 300 μM DFP (black bars), control + 3-MA (light gray bars), or 300 μM DFP + 3-MA (dark gray bars). mtDNA/gDNA ratio (D), SDHB protein levels (E), BNIP3, BNIP3L, LC3BI, and LC3BII protein levels (F) are depicted of myotubes exposed for 24 hours to control (white bars), 300 μM DFP (black bars), control + BAFA1 (light gray bars), or 300 μM DFP + BAFA1 (dark gray bars). Autophagic flux (group-average fold change value without BAFA1 subtracted from the individual fold change values with BAFA1) is depicted for control (white bars) and 300 μM DFP (black bars) conditions (G). Data are depicted as mean ± SEM (n = 3), and significant differences are depicted compared with control or indicated bars *P < .05, **P < .01, ***P < .001
Neither knockdown of GABARAPL1, which has been implicated in both the intracellular trafficking of vesicles to the plasma membrane during hypoxia, and in mitochondrial breakdown,30,31 nor knockdown of BNIP3/BNIP3L, which are both highly transcriptionally upregulated during iron chelation,32 prevented DFP-induced secretion of these EV as analyzed with electron microscopy (Figure 6B-C). Interestingly, clear mitochondrial structures were detected in several of the larger EV by electron microscopy (Figure 6A). To verify this observation, EV were purified from 16 000 and 100 000 g pellets of DFP-exposed and unexposed myotubes, and both EV and mitochondrial content markers were quantified. Indeed, a higher expression of the EV markers HSP70 and Flotillin-1, as well as higher levels of mitochondrial proteins and a higher mtDNA copy number were detected in the 16 000 g EV fraction derived from DFP-exposed myotubes compared with unexposed myotubes (Figure 7A-B). These mitochondrial proteins were mainly detected in the 16 000 g, and not in the 100 000 g pellet (Figure 8).

4 | DISCUSSION

This study is the first to show that iron deficiency in myotubes not only results in mitochondria surrounded by double membrane structures and mitochondrial-lysosomal co-localization, but also in increased expression levels of key proteins involved in mitophagy. Besides these indications for mitophagy initiation, iron chelation in myotubes in the current study also resulted in EV-mediated mitochondrial secretion in cultured myotubes. These changes were associated with decreased mitochondrial respiratory capacity and reductions in the abundance of specific mitochondrial proteins and mitochondrial DNA copy number, but with only marginal changes in mitochondrial dynamics markers. No signs of excessive cytotoxicity or apoptosis were found in the current study, as assessed by scanning nuclei of iron chelation exposed cells for signs of chromatin condensation with electron microscopy, checking presence of DNA laddering in iron chelation exposed cells, and using ECAR data to check if glycolysis was unaltered in iron chelation exposed cells (data not shown). Moreover, although iron accumulation in the lysosomes is associated with increased chance of lysosomal rupture and impaired function,32 the autophagic flux of LC3BII is comparable between control and iron chelation, which makes it unlikely that lysosomal activity is severely impaired by iron chelation in our model. Experiments using pharmacological inhibitors of autophagy initiation or lysosomal degradation, or knockdown of specific components of the mitophagy machinery, revealed that the reductions...
in mitochondrial protein levels or respiratory capacity were not dependent on general autophagy, BNIP3- and BNIP3L-mediated mitophagy, or HIF1A signaling. Mitochondrial secretion via EV might therefore represent an additional pathway for reducing mitochondrial content during iron deficiency. However, the exact mechanisms underlying the degradation of specific mitochondrial proteins as well as the molecular mechanisms and physiological relevance of the release of mitochondrial-containing vesicles in response to iron chelation in muscle remain to be elucidated.

From animal models, it is well known that iron deficiency reduces mitochondrial function and content.\(^3\)\(^-\)\(^8\) Moreover, iron deficiency has been shown to have negative effects on exercise capacity and skeletal muscle performance in humans (reviewed in Ref. [8]). Collectively, this is in line with our data, as we clearly show that iron chelation induced a decline in mitochondrial respiratory capacity, and reduced mitochondrial DNA copy number in myotubes. However, although protein levels of SDHB and NDUFB8 (ie, subunits of OXPHOS complexes I and II) were reduced in response to iron chelation, HADH activity, CS activity, and protein levels of CQCRC2 and ATP5A (ie, subunits of OXPHOS complexes III and V) remained unaffected. Interestingly, other studies have also found that mitochondrial enzymes\(^33\)\(^-\)\(^35\) and OXPHOS complexes\(^36\)\(^,\)\(^37\) were unequally affected by iron deficiency in a similar manner. Although the simplest explanation for this discrepancy would be that iron chelation specifically targets iron-containing, and not noniron-containing, proteins for breakdown or clearance, this does not completely explain our findings nor previously reported data. Indeed, although SDHB, NDUFB8, and CQCRC2 all contain iron, we observed that only SDHB and NDUFB8 were affected by iron chelation. It was previously proposed that iron sulfur clusters-based complexes are more sensitive to iron deficiency than hemoproteins,\(^37\) which might provide a logical explanation for our observations, since both SDHB
and NDUFB8 contain iron sulfur clusters while CQCRC2 contains a hemoprotein. Moreover, it has been suggested that the Krebs cycle, in which CS plays an essential role, is upregulated in iron deficiency to counteract the decreased electron flux in the electron transport chain, which in our study could have compensated for any loss of CS activity. Combined, our results suggest that iron chelation influences mitochondrial content by selectively targeting mitochondrial proteins for degradation, and hence, changes the mitochondrial composition. However, since this study did not focus on changes in mitochondrial composition, more research is necessary to understand to what extent mitochondrial composition changes after iron chelation, and how this relates to decreased mitochondrial constituents and respiratory capacity.

In line with previous studies performed in nonskeletal muscle models (eg, *C. elegans* and neuronal cells), we are the first to report that mitochondrial clearance is increased in response to iron chelation in myotubes as well. Using electron microscopy, we found several double membrane-like structures enclosing mitochondria and we identified the presence of mitochondria in lysosomal-like structures in myotubes exposed to iron chelation. Furthermore, in line with a previous study using iron chelators in neuronal cells, protein levels of the transcriptionally regulated mitophagy receptors BNIP3 and BNIP3L were higher in response to iron chelation, while protein levels of the posttranscriptionally regulated mitophagy receptor FUNDC1 were lower. In addition, no changes in ubiquitin-mediated mitophagy-related transcription were observed in response to iron chelation. Together, these results indicate a role for receptor-mediated mitophagy in iron chelation-induced mitochondrial clearance.

Although these observations pointed toward increased activation of receptor-mediated mitophagy after iron chelation, mitochondrial dynamics signaling, which is highly interconnected with mitophagy, was not found to be indicative of increased mitochondrial fission. Moreover, neither inhibition of autophagy initiation or lysosomal breakdown could prevent the loss of SDHB protein levels, and autophagic flux was not different between DFP or control myotubes. Furthermore, the specific knockdown of the mitophagy receptors BNIP3 and BNIP3L, or knockdown of HIF1A (which is known to be stabilized by iron chelation and to induce mitophagy via the BNIP3 pathway), were unable to prevent the complete loss of mitochondrial respiratory capacity or SDHB protein levels as well. These data are in contrast with a previous report showing that the loss of mitochondrial
quantity in SH-SY5Y cells in response to iron chelation is dependent on mitophagy, although these authors were also unable to prevent this with the siRNA-mediated knockdown of BNIP3. In addition, this study also determined that iron chelation-induced mitophagy was PINK1/Parkin independent, which is in contrast to studies in C. elegans, which reported that iron chelation-induced mitophagy is dependent on PINK/Parkin and BNIP3. Our data imply that mitophagy might be present, but is not essential for the observed iron chelation-induced loss of mitochondrial constituents and respiratory capacity in myotubes, suggesting involvement of an alternative mechanism of mitochondrial clearance in the context of iron chelation.

Relevant in this context, we show for the first time that a large amount of EV-like structures is present near the plasma membrane of iron chelated myotubes. Furthermore, some of these vesicles appeared to contain mitochondrial cargo, or sometimes even apparent intact mitochondria. These mitochondria were mainly identified in the larger vesicles on electron microscopy (>500 nm), which was in line with the observation that mitochondria were predominantly found detected in the 16 000g fraction of the vesicle isolation. This suggests that mitochondria are being discarded from iron chelation-exposed myotubes via EV. Although myotubes have been reported to secrete EV, and secretion of mitochondria via EV has previously been described in nonmuscle cells, this is the first report describing mitochondria-containing EV in muscle cells. As stated above, elimination of mitochondria through EV has recently been described as an alternative manner of mitochondrial clearance or horizontal mitochondrial transfer. Since we showed that mitochondrial respiratory capacity is impaired after iron chelation, it could be speculated that, as an alternative to mitophagy, skeletal muscle cells donate impaired mitochondria to their environment where, possibly, cells (eg, macrophages) that are better equipped to perform mitochondrial breakdown can clear them. Moreover, although we did not test if inhibition of autophagy or mitophagy resulted in an increased secretion of mitochondria-containing vesicles, it is even possible that the secretion of mitochondria is part of a pro-survival mechanism able to compensate for impaired mitophagy. Although no literature is present on the intracellular pathway leading toward iron chelation-induced vesicle secretion, we report that this pathway is independent of BNIP3 and BNIP3L, suggesting a dispensable role for receptor-mediated mitophagy signaling in this specific vesicle secretion. Moreover, we also report that it is independent of GABARAPL1, which has previously been positioned as a mediator of intracellular trafficking. Future research is necessary to unravel the exact molecular signaling leading to the secretion of mitochondria-containing vesicles, and more specifically, to determine the interplay between autophagy and vesicular secretion in this context. A suitable target is this context is mTORC1 signaling, which is a known autophagy initiator upon inhibition, and has previously been shown to be suppressed by iron chelation in other cell types. Combined, our current results suggest that the secretion of mitochondrial-containing EV might be an alternative, or additional, route to mitochondrial clearance under iron deprivation.

In conclusion, our results show that in myotubes exposed to iron chelation, mitochondria are packed into vesicles, are present in lysosomal structures, and are present in secreted vesicles in the extracellular space. Moreover, iron chelation results in reductions of mitochondrial constituents and respiratory capacity in myotubes, which are not autophagy dependent. Although there are currently no methods available for specifically inhibiting EV secretion to study the causal relationship between the secreted mitochondria and the observed loss of mitochondrial constituents, our current results suggest a possible interplay between intracellular mitochondrial breakdown and mitochondrial secretion, which likely contribute to the mitochondrial impairments observed upon iron chelation. In order to gain new knowledge about the role of iron in the mitochondrial homeostasis, future studies should focus on elucidating the physiological relevance of EV-secreted mitochondria.

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS
P. Leermakers, A. Remels, A. Schols, and H. Gosker designed research; P. Leermakers and M. Zonneveld performed research; P. Leermakers, A. Remels, M. Zonneveld, and K. Roushop performed data analysis; P. Leermakers, A. Remels, M. Zonneveld, K. Roushop, A. Schols, and H. Gosker provided resources for study; P. Leermakers, A. Remels, M. Zonneveld, K. Roushop, A. Schols, and H. Gosker wrote and reviewed manuscript.

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**SUPPORTING INFORMATION**
Additional Supporting Information may be found online in the Supporting Information section.

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