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n-3 PUFA dietary lipid replacement normalizes muscle mitochondrial function and oxidative stress through enhanced tissue mitophagy and protects from muscle wasting in experimental kidney disease

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ABSTRACT

Introduction and methods: Skeletal muscle mitochondrial dysfunction may cause tissue oxidative stress and consequent catabolism in chronic kidney disease (CKD), contributing to patient mortality. We investigated in 5/6-nephrectomized (Nx) rats the impact of n3-polyunsaturated fatty-acids (n3-PUFA) isocaloric partial dietary replacement on gastrocnemius muscle (Gm) mitochondrial master-regulators, ATP production, ROS generation and related muscle-catabolic derangements.

Results: Nx had low Gm mitochondrial nuclear respiratory factor-2 and peroxisome proliferator-activated receptor gamma coactivator-1alpha, low ATP production and higher mitochondrial fission-fusion protein ratio with ROS overproduction. n3-PUFA normalized all mitochondrial derangements and pro-oxidative tissue redox state (oxydized to total glutathione ratio). n3-PUFA also normalized Nx-induced muscle-catabolic proinflammatory cytokines, insulin resistance and low muscle weight. Human uremic serum reproduced mitochondrial derangements in C2C12 myotubes, while n3-PUFA coincubation prevented all effects. n3-PUFA also enhanced muscle mitophagy in-vivo and siRNA-mediated autophagy inhibition selectively blocked n3-PUFA-induced normalization of C2C12 mitochondrial ROS production.

Conclusions: In conclusion, dietary n3-PUFA normalize mitochondrial master-regulators, ATP production and dynamics in experimental CKD. These effects occur directly in muscle cells and they normalize ROS production through enhanced mitophagy. Dietary n3-PUFA mitochondrial effects result in normalized catabolic derangements and protection from muscle wasting, with potential positive impact on patient survival.

1. Introduction

Skeletal muscle catabolism and loss of muscle mass are common and negatively affect patient survival in chronic kidney disease (CKD) [1,2]. Mitochondrial dysfunction with low energy production and excess mitochondrial reactive oxygen species (ROS) generation are emerging CKD-induced muscle metabolic derangement with a potentially causal role in muscle catabolism [3,4]. Uremic toxins such as indoxyl sulfate are reported to directly induce mitochondrial dysfunction by reducing expression of master regulators of mitochondrial biogenesis and redox balance such as peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α) [5], potentially resulting in reduced energy metabolism and altered mitochondrial dynamics [6,7] through excess mitochondrial fission [8,9], with direct enhancement of mitochondrial ROS production, reduction of antioxidant enzyme activities [6] and oxidative stress [10]. Impaired or inadequate removal of fragmented or

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depolarized mitochondria through tissue mitophagy is also a potential pivotal mechanism which we recently showed to be involved in onset of oxidative stress in experimental CKD [4]. Importantly, robust evidence indicates that tissue oxidative stress may cause clustered protein-catabolic derangements including inflammation and insulin resistance [3,11], directly leading to excess protein degradation and muscle loss [12,13]. Strategies to prevent or reverse CKD-associated muscle mitochondrial dysfunction and pro-oxidative derangements are therefore an unmet clinical need and an urgent research priority [14].

In recent years, metabolic activities of n-3 polyunsaturated fatty acids (n-3 PUFA) have been increasingly hypothesized to primarily involve mitochondria [15–17]. In experimental conditions in non-muscle tissues or cells, n-3 PUFA notably resulted in upregulated PGC1α protein expression and activity [18], with potential to coordinate clustered enhancement of mitochondrial mass, oxidative capacity and anti-oxidative patterns of ROS production and antioxidant enzyme activities [6,7]. Recent evidence suggests that n-3 PUFA may also upregulate the nuclear respiratory factor-2 (NRF2) [19], an additional mitochondrial master regulator [20] interacting with PGC1α [21], as well as a major positive modulator of cellular redox homeostatic systems [22,23]. Anti-oxidative n-3 PUFA activities have been accordingly reported in physiological conditions as well as in obesity and healthy aging models in various tissues, with reduced mitochondrial ROS emission [17,24–26] and enhancement of antioxidant enzyme activities [27,28]. Regulation of mitochondrial dynamics by n-3 PUFA with enhanced mitochondrial fusion proteins [29] was also reported in obesity in non-muscle tissues [26]. n-3 PUFA are reported to inhibit expression and activation of p38/stress-activated protein kinase (p38/SAPK) [27], a driver of metabolic adaptation in skeletal muscle with potential negative impact on mitochondrial homeostasis through negative PGC1α modulation [30] in the presence of stress-related stimuli including inflammation and increased ROS, also in CKD [31]. However, mitochondrial and anti-oxidative activities of n-3 PUFA are only partially understood, with discrepancies in available literature and models [16–18,24,25,32,33]. Most importantly, no studies are available for skeletal muscle in muscle wasting models with high risk for mitochondrial dysfunction. While PUFA may positively contribute to muscle trophism [34], whether potential metabolic effects of n-3 PUFA translate into reduced disease-induced catabolism and preserved muscle mass is unknown.

We tested the hypothesis that n-3 PUFA improve muscle mitochondrial homeostasis in vivo in a rodent CKD model, through improved expression of p38/SAPK, mitochondrial master regulators and reduced mitochondrial ROS production, with lower tissue protein catabolism and wasting. Higher n-3 PUFA intake was provided through isocaloric, isolipidic partial dietary fat replacement in order to avoid excess calorie intake and its potential confounding metabolic impact. We also aimed at demonstrating whether potential mitochondrial effects of n-3 PUFA directly occur in muscle cells and whether they involve enhanced tissue mitophagy, which may improve mitochondrial pro-oxidative derangements by removing dysfunctional fragmented organelles [4].

2. Materials and methods

2.1. Experimental design - in vivo uremic model

Animal study design, experimental procedures and surgical technique were approved by the Italian Ministry of Health Animal Experimentation Authority (DM 274/2013-B 07/11/2013), and were performed in adherence with EU Directive 2010/63/EU and National Institutes of Health guidelines. Thirty male 12 wk. old healthy, clean-conventional Wistar rats (Envigo) were randomly (https://www.randomizer.org/) assigned to 5/6 nephrectomy (Nx; $n = 20$) via a single-step laparotomic approach or sham surgery ($n = 10$) as previously described [4,35]. Animals and samples were only identified by blind number until analysis. Ten days after nephrectomy, all animals were free of any complication, surgical related medication or treatment and were randomly assigned to standard diet (Nx, $n = 10$: 18 % total calories from fat, 58 % carbohydrates, 24 % proteins; Teklad global diet 2018, 3.1 kcal/g, Harlan) or modified diet, with isocaloric isolipidic replacement of soybean-derived fat with highly refined n-3 PUFA preparation (Nx-PUFA, n = 10: 27 % of total dietary lipids; Harlan, Italy; n-3 PUFA 600 mg/g by EPAX, Norway), leaving all other diet macro- and micronutrients unchanged as described elsewhere [35]. Sham operated animals were kept on standard diet. At all times, rats were housed in individual cages, with a 12-h light–dark cycle and ad libitum access to water and food. Body weight and food intake were monitored every three days. Caloric intake was calculated from the difference in weight between the amount of chow provided at the start and that remaining at the end of each three-day interval, basing on manufacturer's reported caloric content. After thirty days of dietary treatment (i.e. 40 days after surgery), following 3 h fasting and anesthesia (thiobutabarbital 100 mg/kg, tiletamine/zolazepam[1:1] 40 mg/kg, i.p.), gastrocnemius, soleus and extensor digitorum longus muscles were surgically isolated and removed, were placed in iced saline for immediate ex-vivo processing or snap frozen for further analyses [36]. Blood was collected by heart puncture, plasma separated by centrifugation and stored at −80 °C.

2.2. In vitro studies

Cell incubation with uremic or control human plasma was used as an in vitro model of uremia. Four days after differentiation of C2C12 myoblasts (ATCC CRL-1772) into myotubes [36] and synchronization by starvation, cells were treated for 48 h with 10 % (*v*/v) human uremic (HUP) or control plasma added with heparin (final concentration 3 U/ml) [4]. The following day, equimolar amounts (500 μM) of soybean derived monounsaturated (Intralipid, Fresenius Kabi) or fish oil derived n-3 PUFA enriched (Omegaven, Fresenius Kabi) lipid emulsions were added for 24 h.

To assess the role of autophagy, siRNA-mediated genomic silencing of autophagy related protein 5 (ATG5) was used in comparison to non-targeting control siRNA #4 (both from Dharmacon) [4,36].

2.3. Plasma measurements

Plasma levels of glucose, creatinine, and urea were assessed by standard enzymatic–colorimetric assays [4,37].

2.4. Protein analyses

High throughput xMAP technology (Magpix; Luminex Corporation) was used to asses cytokine profile and protein phosphorylation at insulin receptor ($IR^{Y1162/Y1163}$), insulin receptor substrate-1 ($IRS—1^{S312}$), protein kinase-B (AKT^{S473}), glycogen synthase kinase-3β ($GSK3\beta^{S9}$), mammalian target of rapamycin ($mTOR^{S2448}$), proline-rich AKT substrate protein ($PRAS40^{T246}$), ribosomal protein-S6 kinase ($P70S6K^{T421/S424}$), $p38/SAPK^{Thr180/Tyr182}$ and mitogen-activated protein kinase/extracellular signal-regulated kinase-1/2 ($MAPK/ERK1/2^{Thr185/Tyr187}$) levels, using commercial kits (Life Technologies, USA) [4]. Protein phosphorylation levels are expressed as phospho-protein units per total pg of the same protein. Cytokine levels were normalized by protein content. Protein levels of NRF2 and PGC1α (SAB4501984 and SAB21087, Sigma-Aldrich), mitochondrial dynamin like GTPase (OPA1) and dynamin-related protein-1 (DRP1; 5391 and 80471, Cell Signaling Technology), superoxide dismutases $_{Mn}$SOD and $_{Cu/Zn}$SOD (SOD-110 and SOD-101, Stressgen), were measured by western blot.

2.5. Mitochondrial enzyme activity and ex-vivo ATP synthesis

Mitochondrial cytochrome *c* oxidase and citrate synthase enzyme activities were measured in muscle homogenates by kinetic spectrophotometrical methods as referenced [38]. ATP synthesis rate was

measured by luciferin and luciferase luminometric assay with different combinations of respiratory substrates in fresh mitochondria isolated by differential centrifugation [36–38]. ATP synthesis rate was assessed on the linear phase after ADP addition, interpolating light emission readings on a standard ATP curve. Results were normalized by CS activity as for mitochondrial ROS measurements and expressed as μmol (U CS)$^{-1}$ min^{-1}.

2.6. Ex vivo mitochondrial ROS generation

ROS production in isolated intact mitochondria during incubation with different respiratory substrates was measured by H_2O_2 generation detection using the Amplex Red – horseradish peroxidase method as referenced [4,36,37]. Results were normalized by citrate synthase activity, measured in the same mitochondrial preparation.

2.7. Ex-vivo superoxide anion production

Skeletal muscle ex vivo superoxide anion production from mitochondrial and non-mitochondrial sources was assessed by the lucigenin chemiluminescent method in gastrocnemius whole tissue homogenate [4,39]. Samples were incubated with the mitochondrial respiratory substrate succinate (10 mM) alone or with the addition of the mitochondrial uncoupling agent carbonyl cyanide chlorophenylhydrazone (5 mM) and the difference in light emission was considered as mitochondrial-specific superoxide production. Similarly, superoxide generation from reduced nicotinamide adenine dinucleotide phosphate (NADPH) and xanthine oxidases was assessed as the difference in light emission induced by the addition of specific enzyme inhibitors to production rates obtained in the presence of specific substrates for each source (200 mM diphenyleneiodonium on 1 mM NADPH-stimulated production for NADPH oxidase; 200 mM oxypurinol on 500 mM xanthine-induced generation for xanthine oxidase). Sample protein content as measured by the bicinchoninic acid (BCA) assay (Pierce) was used to normalize results.

2.8. Mitophagy

Mitophagy was assessed by analysis of cytochrome *c* oxidase (COX IV) and microtubule-associated protein-1A/1B-light chain-3 (LC3) co-localization in skeletal muscle transversal sections as referenced [4]. Five fields per tissue were randomly chosen and the degree of co-localization of COX IV and LC3 quantified, using Pearson's coefficient method (JACoP plugin in for ImageJ; NIH Image).

2.9. Tissue redox state and enzyme activities

Gastrocnemius muscle total and oxidized glutathione (GSSG) were measured on tissue samples as described [37]. Reduced fraction was calculated by subtracting GSSG from total glutathione. Catalase and glutathione peroxidase activities were measured using commercially available and validated spectrophotometrical kits, following manufacturer's recommendations (Invitrogen and Abcam). Results were normalized by protein content.

2.10. Systemic insulin resistance and tissue glucose uptake

Systemic insulin resistance was assessed by insulin tolerance test (ITT) [36], performed at day 30 after surgery. Briefly, insulin (Humulin R 0.75 U/kg; Eli Lilly) was injected intraperitoneally after 4-h fasting. Glucose in tail blood was measured (ACCU-CHEK Active; Roche) twice, immediately before injection and then at 15, 30, 45, and 60 min and analyzed as percent of basal levels. Measurement of tissue glucose uptake was performed using the ex vivo nonradioactive 2-deoxyglucose method as detailed elsewhere [36] and expressed as 2-deoxyglucose [μmol]/ total sample protein[mg] per 30 min.

2.11. Protein degradation

Muscle proteolysis was assessed by western blot, measuring actin cleavage as reflected by the relative ratio of 14-kDa actin fragment over β-actin expression [4].

2.12. Tissue water content

Skeletal muscle water content was measured by assessing the net weight difference of tissue samples (≈100 mg) before and after complete lyophilisation. The mean of three independent measurements taken using a calibrated analytical scale was used.

2.13. Fiber type

Changes in muscle fiber types were assessed using the established method of relative quantification of types I and II of myosin heavy chain (MHC), following separation by electrophoresis in 6 % Glycerol SDS-gel, staining, and identification by molecular weight and migration distance [40]. Total MHC was also used to calculate myosin-to-actin ratio. Quantification was performed by peak integration on lane optical density profile (ImageJ). To confirm muscle fiber changes in gastrocnemius muscle by a second independent method, we also assessed the ratio of slow/fast fibers using Troponin I (4002, Cell Signalling) isoforms quantification by western blot [40].

2.14. Statistical analysis

Sample size was decided by power analysis, assuming expected differences in muscle mass to be comparable in magnitude to those previously observed in the same nephrectomy model [4]. Groups were compared by unpaired ANOVA (GraphPad) followed by post-hoc pairwise *t*-test with Bonferroni's correction for multiple comparisons. Corrected p was considered statistically significant if <0.05, and indicated by groups not sharing the same letter. Data are presented as mean ± mean standard error.

3. Results

3.1. Animal characteristics and phenotype

As expected, Nx resulted in higher plasma creatinine and urea concentration, and n-3 PUFA dietary replacement did not modify these parameters at 40 days from surgery [4,35]. Average caloric intake measured during the whole study period was comparable in all groups and no difference in caloric intake was observed at any point during PUFA vs. standard diet treatment (Table 1, Supplementary Fig. 1). Physiologic increments in body weight and body mass index during the 40 d study period were impaired in both Nx groups compared to the sham-surgery group. Plasma glucose concentrations were comparable among Nx groups (Table 1).

Skeletal muscle mitochondrial master regulators, function, dynamics, ROS generation, antioxidant enzyme activities and redox state

Compared to sham-operated animals, untreated Nx had higher activating phosphorylation of p38/SAPK [31], low gastrocnemius levels of master regulators of mitochondrial biogenesis and activity PGC1α and NRF2, with lower tissue mitochondrial protein content (a surrogate marker of tissue mitochondrial content) as well as lower activity of the rate-limiting Krebs cycle enzyme citrate synthase and lower mitochondrial ATP production both under basal and ADP-stimulated conditions (Fig. 1).

Tissue balance of mitochondrial fission and fusion proteins was also altered in Nx through higher fission DRP1 and lower fusion OPA1 levels (Fig. 2A–C). Higher potential mitochondrial fragmentation was however not associated with activated tissue mitophagy which was only moderately and non-significantly enhanced in Nx (Fig. 2D–E). Mitochondrial

Table 1
Animal characteristics. Effects of 30 day isocaloric, isolipidic n-3 PUFA enriched diet in 5/6 nephrectomized rats (Nx) on body weight, body mass index (BMI), waist circumference at tissue collection (T40) and their variation (Δ) over time from day of surgery (T0); average daily food intake after surgery and during UnAG or vehicle treatment; plasma glucose, urea and creatinine levels at tissue collection. Feeding with PUFA enriched diet started at day 10, after full recovery from surgery. Sham were fed with standard diet throughout the study. $p < 0.05$ between groups not sharing the same letter, mean ± SEM, $n = 10$/group.

		Sham	Nx	Nx-PUFA
Body weight [g]	T0	346 ± 5 [a]	352 ± 8 [a]	349 ± 3 [a]
Δ Body weight [g]	T0-T40	88 ± 6 [a]	57 ± 4 [b]	53 ± 10 [b]
BMI [g/cm^2]	T0	0.68 ± 0.03 [a]	0.62 ± 0.07 [a]	0.64 ± 0.01 [a]
Δ BMI [g/cm^2]	T0-T40	0.14 ± 0.04 [a]	−0.02 ± 0.02 [b]	0.02 ± 0.01 [b]
WC [cm]	T0	18.6 ± 0.9 [a]	18.5 ± 1.1 [a]	18.4 ± 0.3 [a]
Δ WC [cm]	T0-T40	3.3 ± 0.2 [a]	0.3 ± 0.4 [b]	0.5 ± 0.3 [b]
Average daily caloric intake [kcal/d]	T0-T40	61.9 ± 1.5 [a]	59.3 ± 1.9 [a]	63.0 ± 2.8 [a]
Plasma glucose [mg/dl]	T40	125 ± 7[a]	95 ± 8 [b]	105 ± 3 [b]
Plasma urea [mg/dL]	T40	15.2 ± 1.4 [a]	35.2 ± 4.0 [b]	32.9 ± 2.1 [b]
Plasma creatinine [μmol/L]	T40	12.9 ± 3.2 [a]	29.3 ± 3.1 [b]	27.7 ± 0.9 [b]

superoxide and H_2O_2 emission were higher in Nx compared to sham, while activities of antioxidant enzymes superoxide dismutase (in both its mitochondrial and cytoplasmic isoforms), glutathione peroxidase and catalase were lower (Fig. 3A–F). Clustered pro-oxidative mitochondrial derangements in Nx were further accordingly associated with altered gastrocnemius redox state as reflected by higher GSSG-to-total glutatione ratio, a validated marker whose elevation indicates redox imbalance and oxidative stress (Fig. 3G–H).

Compared to Nx, isocaloric partial n-3 PUFA dietary lipid replacement completely normalized p38/SAPK activation and all mitochondrial and redox parameters, including mitochondrial ATP production, ROS emission, antioxidant enzyme activities and GSSG-to-GSH + GSSG ratio (Figs. 1–3). These effects were associated with substantial enhancement of tissue mitophagy (Fig. 2D–E).

3.2. Skeletal muscle inflammation, insulin signalling and glucose metabolism

Compared to sham-operated animals, oxidative stress in untreated Nx resulted in altered tissue cytokine patterns with a proinflammatory profile characterized by higher IL-1α, IL-1β and TNFα, and lower anti-inflammatory IL-10 (Fig. 4). Nx also decreased activating phosphorylation of insulin signalling proteins involved in glucose uptake activation (including pAKT^{S473} and pGSK3β^{S9}; Fig. 5A) and low insulin action was confirmed by low insulin-stimulated ex vivo muscle glucose uptake (Fig. 5B). Nx rats accordingly showed increased systemic insulin resistance at insulin tolerance test (Fig. 5C–D). n-3 PUFA completely normalized all of the above alterations (Figs. 4–5).

3.3. Skeletal muscle protein-anabolic and –catabolic signalling, protein degradation, fiber type and muscle mass

Compared to sham, Nx also had inhibited insulin-dependent protein anabolic signalling (pmTOR^{S2448}, pPRAS40^{T246}, and pP70S6K$^{T421/S424}$) in gastrocnemius muscle (Fig. 6A), directly supporting inhibition of the protein synthetic machinery. Enhanced protein degradation was indicated by higher tissue content of 14-kDa actin fragment, an established marker of muscle protein breakdown [4,41] (Fig. 6B). A more specific impact on the contractile component of muscle protein was confirmed by reduced myosin-to-actin ratio, a marker of lower contractile potential observed in clinical settings and models characterized by muscle wasting [42,43] (Fig. 6C). Consistent with the above observations, gastrocnemius muscle dry weight was lower in Nx than in sham-operated animals, which was notably not due to changes in muscle water content (Fig. 6D–E). Moreover, fiber composition assessed through myosin heavy chain types and troponin isoforms was also altered in Nx with derangement of the ratio between more oxidative type 1 and more glycolytic type 2 fibers due to marked reduction of the type 1 component (Fig. 6F–H). Conversely, n-3 PUFA completely normalized all Nx-induced alterations in protein metabolism regulatory signalling and markers, contractile protein content, muscle weight as well as fiber composition (Fig. 6). In order to confirm a potential direct, specific impact of CKD and n-3 PUFA on more oxidative type 1 muscle fibers, fiber type content were also assessed in the eminently oxidative type 1 soleus muscle along with key oxidative enzyme activities. In soleus muscle, CKD lowered myosin-to-actin ratio, with a marked reduction of the predominant type 1 fiber. These changes were associated with consistent lower citrate synthase as well as cytochrome *c* oxidase enzyme activities. N-3 PUFA notably completely normalized oxidative fiber content and mitochondrial alterations also in soleus muscle (Supplementary Fig. 3).

3.4. In vitro C2C12 myotubes CKD model

To demonstrate direct muscle activities of n-3 PUFA, we performed experiments in C2C12 myotubes incubated with human uremic plasma (HUP), using an established in vitro uremia model [31,44]. In excellent agreement with in vivo findings [4,44], HUP incubation resulted in higher activation of p38-SAPK while not of MAPK/ERK, low PGC1α and NRF2, pro-fission changes in fission-fusion protein balance and higher mitochondrial ROS generation. Potential downstream metabolic derangements were further confirmed by reduced activation of insulin signalling. Co-incubation with a mixed triglyceride emulsion including mono-unsaturated fatty acids and n-6 PUFA had no effects on HUP-induced mitochondrial and metabolic derangements. In contrast, in agreement with in vivo results, co-incubation with an n-3 PUFA-enriched emulsion normalized mitochondrial derangements, p38-SAPK phosphorylation and insulin signalling (Fig. 7).

3.5. C2C12 mitophagy inhibition

In order to further characterize mitochondrial events upstream of mitophagy activation and its potential mechanistic involvement in anti-oxidative and metabolic effects of n-3 PUFA, we also conducted in vitro experiments with or without ATG5-dependent mitophagy inhibition via mRNA silencing. Mitophagy inhibition had no impact on n-3 PUFA-induced normalization of p38-SAPK activation, PGC1α and NRF2 levels and fission-fusion protein balance. On the other hand, in the absence of mitophagy n-3 PUFA were unable to modify high mitochondrial ROS production and low insulin signalling (Fig. 7).

4. Discussion

The current study demonstrated in a rodent CKD model that partial isocaloric replacement of dietary lipids with n-3 PUFA: 1) normalizes mitochondrial master regulators, mitochondrial mass and ATP production, altered mitochondrial dynamics and pro-oxidative mitochondrial changes including high mitochondrial ROS generation and low antioxidant enzyme activities in skeletal muscle; 2) mitochondrial activities of n-3 PUFA occur directly in muscle cells, involve inhibition of p38 SAPK and require enhanced tissue mitophagy to normalize ROS production; 3) anti-oxidative PUFA activities result in normalized tissue inflammation and insulin resistance with complete protection from muscle wasting. The current findings therefore indicate a novel role of dietary n-3 PUFA to prevent CKD-associated muscle mitochondrial dysfunction,

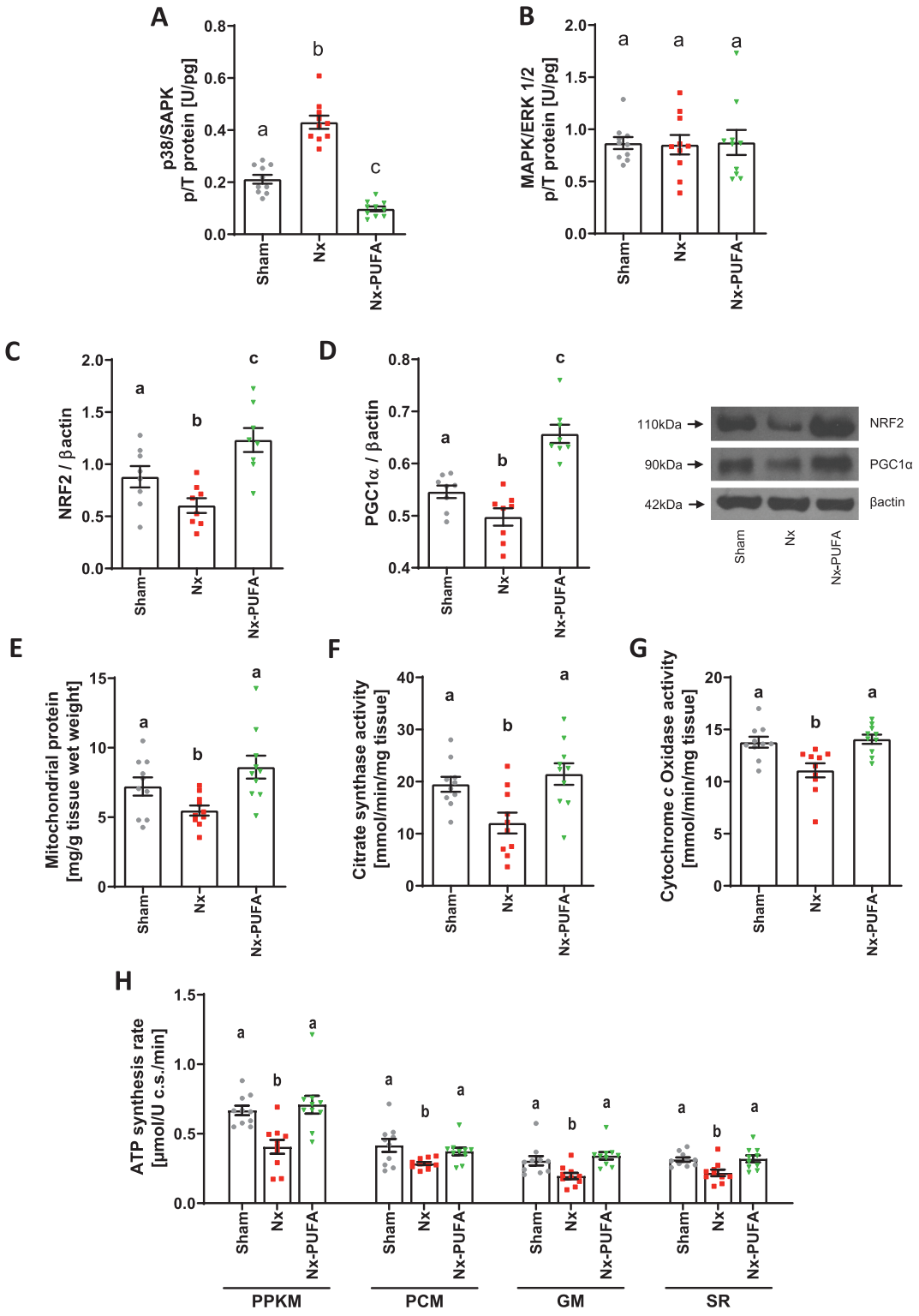

Fig. 1. n-3 PUFA and skeletal muscle mitochondrial biogenesis master regulators and function in 5/6 nephrectomized rats. Effects of 30 day isocaloric, isolipidic n-3 PUFA enriched diet in 5/6 nephrectomized rats (Nx) on phosphorylation of p38/SAPK $^{T180/Y182}$ (A) and MAPK/ERK 1/2$^{T185/Y187}$ (B), Nrf2 (C) and PGC1α (D) protein expression with representative blots, mitochondrial superoxide generation in gastrocnemius whole muscle tissue homogenate (E), mitochondria citrate synthase (F) and cytochrome *c* oxidase (G) activities and ATP synthesis rate in intact isolated mitochondria with different respiratory substrates (H; PPKM: 0.25 mmol/l Pyruvate, 12.5 µmol/l Palmitoyl-L-Carnintine, 2.5 mmol/l α-Ketoglutarate, 0.25 mmol/l Malate; PCM: 25 µmol/l Palmitoyl-L-Carnintine, 0.5 mmol/l Malate; GM: 10 mmol/l Glutamate, 5 mmol/l Malate; SR: 20 mmol/l Succinate, 0.1 mmol/l Rotenone) in rat gastrocnemius muscle. U CS: units of citrate synthase. $p < 0.05$ between groups not sharing the same letter, mean ± SEM, $n = 8–10$/group.

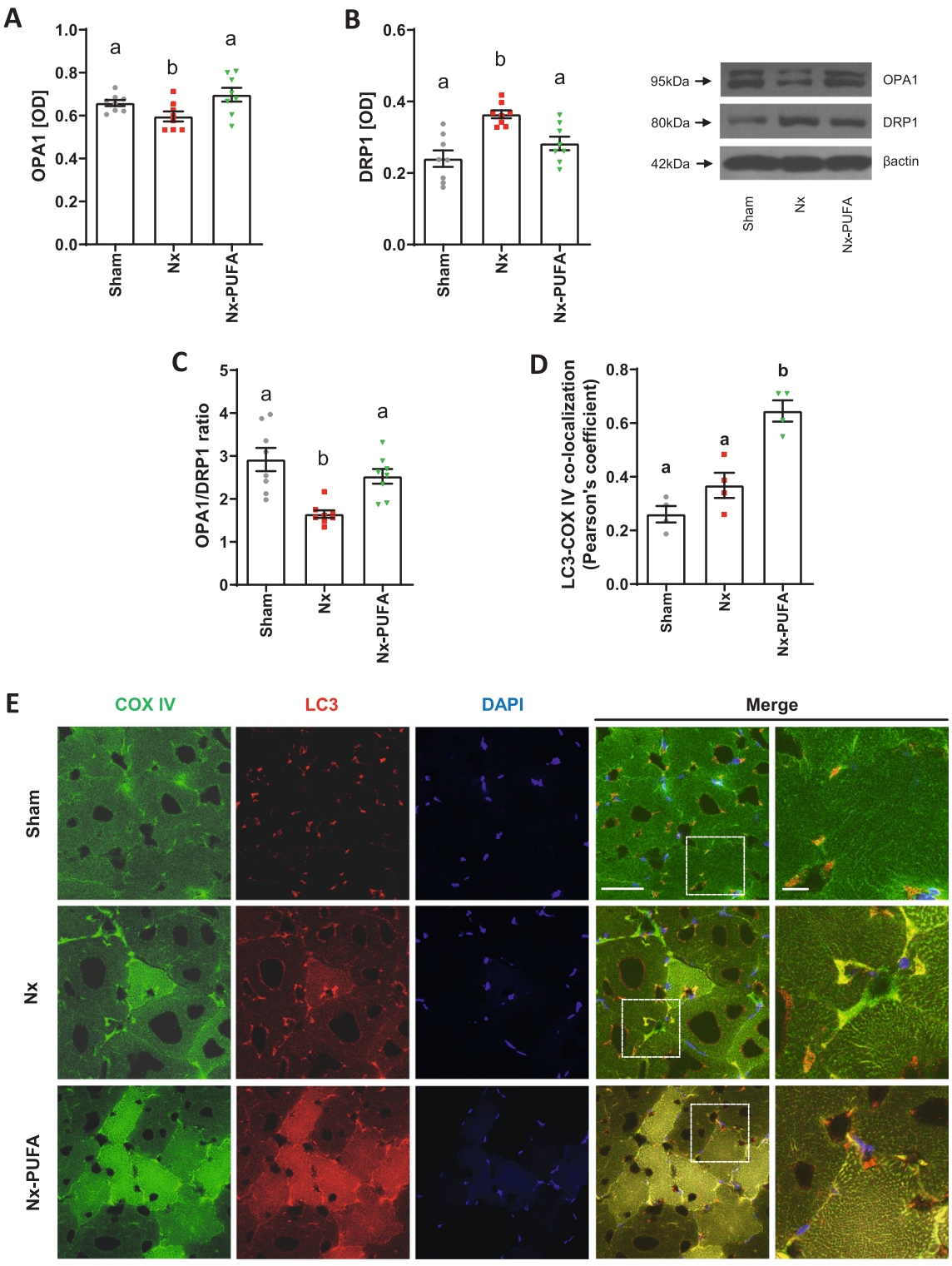

Fig. 2. n-3 PUFA and skeletal muscle mitochondrial dynamics and mitophagy in 5/6 nephrectomized rats. Effects of 30 day isocaloric, isolipidic n-3 PUFA enriched diet in 5/6 nephrectomized rats (Nx) on OPA1 (A) and Drp1 (B) protein levels with representative blots, OPA1/DRP1 ratio (C) and co-localization analysis of mitochondrial marker COX IV (green), LC3 (red) and nuclei (blue) in samples (D; scale bars = 50 μm and 10 μm) of rat gastrocnemius muscle with representative images (E). Contrast and brightness settings are equal in all images. OD: optical density. $p < 0.05$ between groups not sharing the same letter, mean ± SEM, $n = 4–8$/group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mitochondrial-induced oxidative stress and muscle wasting, with potential to improve related clinical outcomes [45].

4.1. Mitochondrial n-3 PUFA activities

Strategies to prevent or limit muscle catabolic derangements in CKD represent an unmet clinical need and a research priority. Adequate calorie and protein intake should be primarily ensured for all patients to

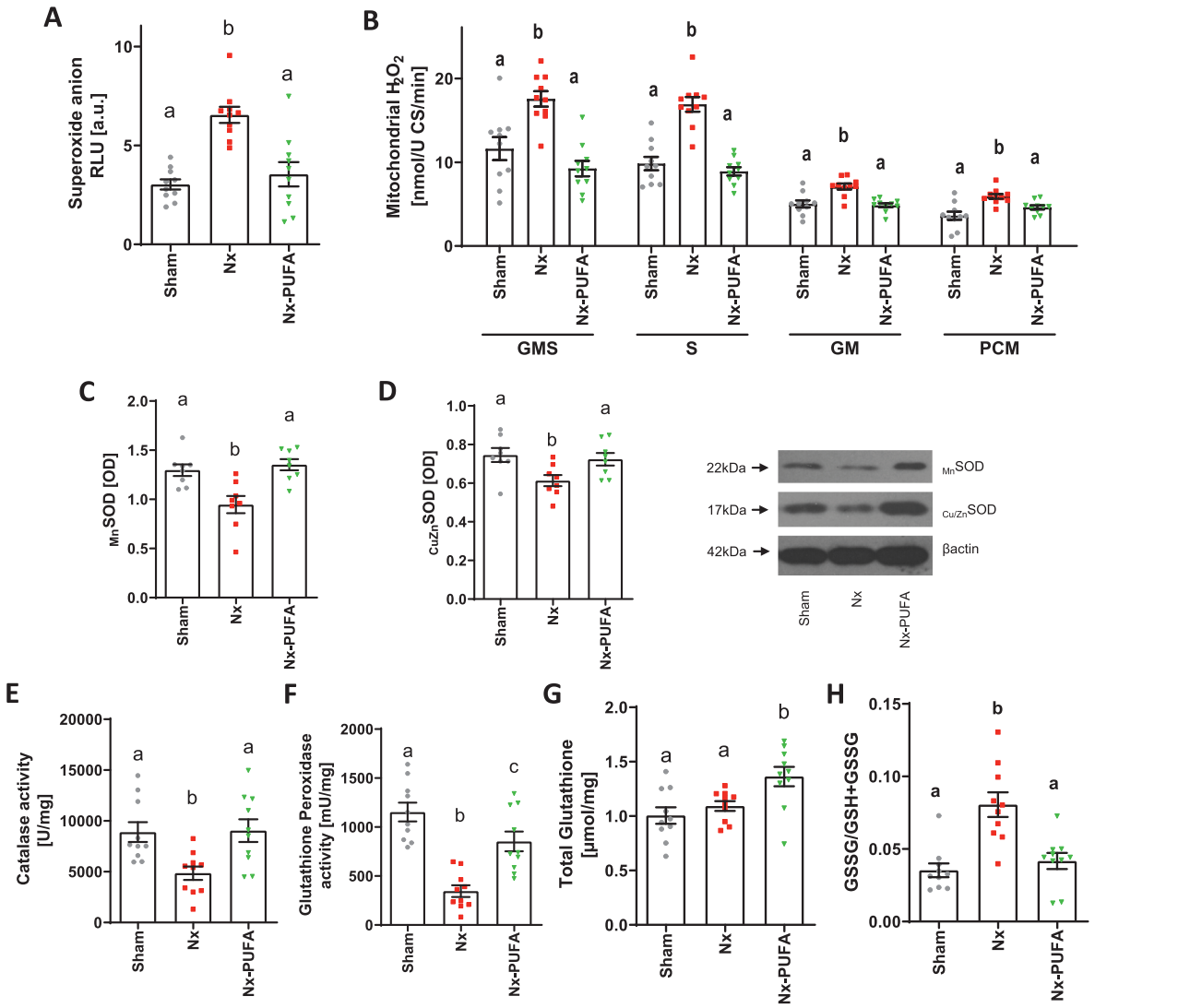

Fig. 3. n-3 PUFA and skeletal muscle redox state in 5/6 nephrectomized rats. Effects of 30 day isocaloric, isolipidic n-3 PUFA enriched diet in 5/6 nephrectomized rats (Nx) on mitochondrial-derived superoxide production (A), H_2O_2 production in intact isolated mitochondria with different respiratory substrates (B, GMS: 4 mmol/l glutamate, 2 mmol/l malate, 10 mmol/l succinate; S: 10 mmol/l succinate; GM: 8 mmol/l glutamate, 4 mmol/l malate; PCM: 50 μmol/l palmitoyl-L-carnitine, 2 mmol/l malate), $_{Mn}$SOD (C) and $_{Cu/Zn}$SOD (D) protein expression with representative blots, catalase (E) and glutathione peroxidase activities (F) and total (G) and oxidized (GSSG) over total (H, GSH: reduced) tissue glutathione in gastrocnemius muscle. RLU: Relative Light Units; a.u.: arbitrary units; OD: optical density; U: catalase units. $p < 0.05$ between groups not sharing the same letter, mean ± SEM, $n = 10$/group.

prevent and treat malnutrition and wasting [2,14], but CKD-induced muscle abnormalities may cause resistance to nutrient-induced anabolism, while low-protein diets may be needed to slow CKD progression, thereby limiting nutritional options [13]. Skeletal muscle mitochondria represent an alternative treatment target, since a cluster of excess ROS emission and impaired antioxidant defenses has been reported in CKD models [3,4] and may cause tissue oxidative stress and muscle-catabolic derangements, including inflammation and insulin resistance [3]. Most importantly, these alterations may be primarily caused by impaired expression and activity of mitochondrial master regulators NRF2 and PGC1α which may directly down-regulate not only mitochondrial biogenesis, but also mitochondrial function and dynamics [20,46–48], thereby further promoting and sustaining excess ROS production and impair antioxidant defenses [20,22,23,49]. Our study demonstrates that elevating the n-3 PUFA dietary lipid fraction with identical total calorie and lipid intake results in complete normalization of CKD-associated derangements of skeletal muscle mitochondrial biogenesis and function as indicated by mitochondrial protein content, enzyme activities and ATP production. n-3 PUFA also normalized mitochondrial dynamics with normal fission-fusion protein ratio and ROS emission as well as antioxidant enzyme activities, resulting in completely normalized tissue redox state. The current results therefore support a novel role of n-3 PUFA in therapeutic strategies to prevent or treat muscle mitochondrial dysfunction and oxidative stress in CKD. The study also notably demonstrated direct n-3 PUFA mitochondrial activities in muscle cells, with in vitro experiments confirming that n-3 PUFA directly enhance mitochondrial master regulators PGC1α and NRF2 activities [18,19] as well as dynamics regulators and ROS production. Our study is further consistent with a primary role of mitochondrial derangements in the onset of CKD-induced muscle oxidative stress, since oxidized-to-total glutathione ratio, a validated marker of tissue redox balance, was normalized in n-3 PUFA-treated animals despite residual ROS overproduction from alternative sources, with particular regard to NADPH oxidase while excess CKD-induced ROS overproduction at the level of xanthine oxidase was interestingly also normalized by n-3 PUFA treatment (Supplementary Fig. 2).

The current findings are globally consistent with, and expand the recent hypothesis that n-3 PUFA activities may converge on

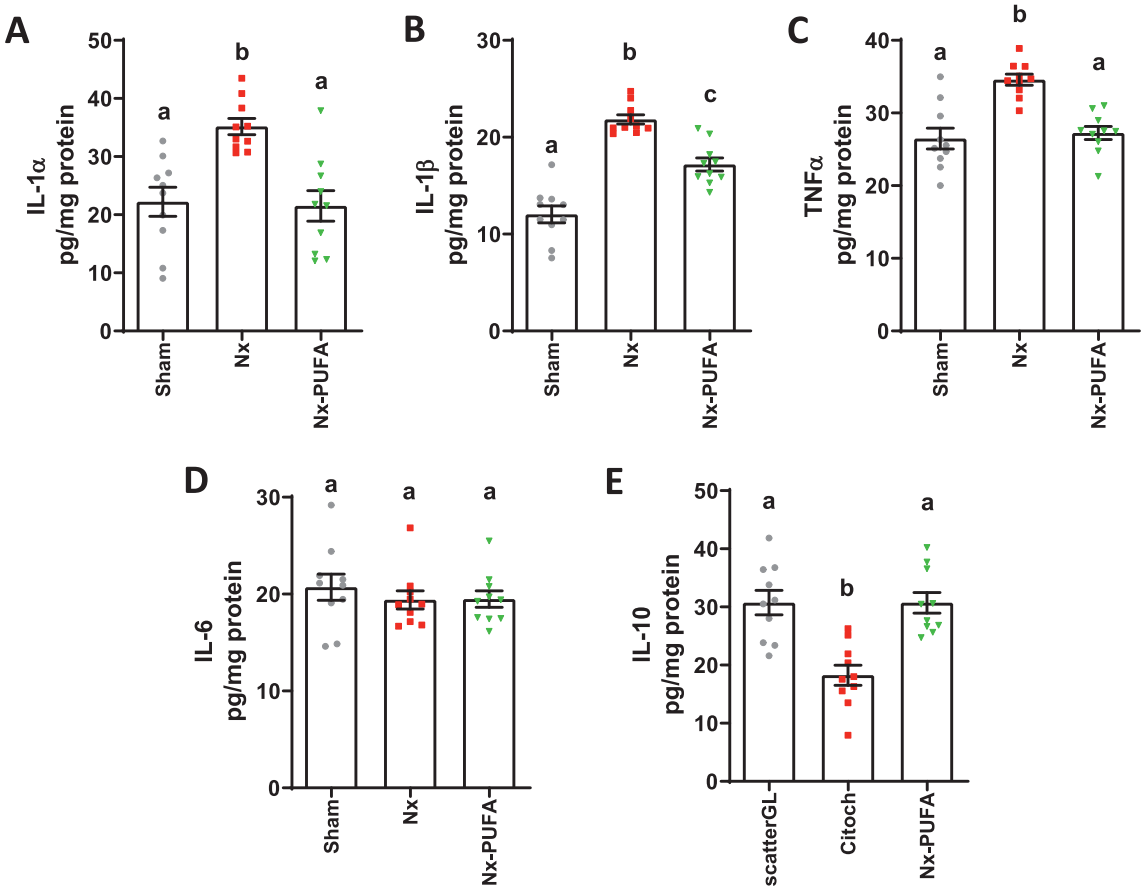

Fig. 4. n-3 PUFA and skeletal muscle inflammation in 5/6 nephrectomized rats. Effects of 30 day isocaloric, isolipidic n-3 PUFA enriched diet in 5/6 nephrectomized rats (Nx) on protein expression of IL-1α (A), IL-1β (B), TNFα (C), IL-6 (D) and IL-10 (E) measured by xMAP technology in gastrocnemius muscle. $p < 0.05$ between groups not sharing the same letter, $n = 10$/group.

mitochondria, with reports of reduced ROS emission and higher oxidative capacity in some, although not all available studies from aging and obesity models [17,24,25]. Recent evidence also supports potential involvement of n-3 PUFA in the regulation of mitochondrial dynamics in non-muscle tissues, with increased fusion protein levels [26]. n-3 PUFA effects to normalize antioxidant enzyme activities are also suggested by preliminary findings in cardiac and, partly, skeletal muscle [17,27]. Most importantly, the current results are consistent with emerging evidence for a fundamental role of NRF2 and PGC1α not only in enhancing mitochondrial biogenesis and function, but also in maintaining mitochondrial dynamics by promoting fusion over fission and ultimately regulating mitochondrial and tissue ROS production and antioxidant defenses [20,22,23,46–49]. Normalization of NRF2 and PGC1α expression could therefore provide a potential unifying mechanism underlying clustered n-3 PUFA mitochondrial activities, both in vivo and in vitro, in the current CKD model.

Since major muscle stress responder p38/SAPK may be specifically activated by uremic toxin-driven inflammation and ROS increase [50] and may in turn negatively influence mitochondrial homeostasis by impairing mitochondrial biogenesis and enhancing fragmentation with potential further negative impact on tissue redox state, we investigated p38/SAPK activation as a potential mediator of both CKD-induced derangements and PUFA activities [5,27,31]. Both in vivo and in vitro experiments confirmed muscle p38/SAPK activation in CKD, which was normalized by n-3 PUFA in both settings in parallel with decreased inflammation and oxidative stress; the current findings therefore support the involvement of p38/SAPK-mediated stress-response homeostasis in mitochondrial CKD-induced muscle derangements and its counteraction by n-3 PUFA.

4.2. Role of mitophagy

Effects of n-3 PUFA on mitochondrial ROS production were abolished in vitro by co-incubation with silencing RNA inhibiting the autophagosome component ATG5. Despite a moderate non-significant increase in untreated nephrectomized animals, only dietary n-3 PUFA replacement lead to substantial enhancement of in vivo mitophagy. Indeed the role of autophagy in the maintenance of muscle mass remains controversial [51], with whole-tissue autophagy activation potentially contributing to skeletal muscle loss in acute and chronic disease conditions [1,52]. The current findings conversely demonstrate that selective activation of skeletal muscle mitophagy mediates at least in part anti-oxidative effects of n-3 PUFA, likely by reducing ROS overproduction by damaged and fragmented mitochondria. The current results therefore support the concept that the impact of autophagic activities on tissue oxidative stress and catabolic changes is substantially modulated by their localization and target organelles. Most importantly, they indicate a selective anti-oxidative and potentially anti-catabolic impact of mitophagy activation, whereas enhanced autophagy at non-mitochondrial level might directly contribute to muscle depletion in various experimental and disease conditions [51–53].

ATG5 inhibition also allowed to identify mitophagy-independent effects of n-3 PUFA to stimulate p38/SAPK as well as NRF2, PGC1α and mitochondrial fission-fusion protein ratio. These observations directly indicate that n-3 PUFA activities to inhibit p38/SAPK activation and to stimulate NRF2 and PGC1α expression may directly lead to normalized fission-fusion protein balance [46,47], but potential improvement of mitochondrial biogenesis and dynamics per se are not sufficient to normalize mitochondrial ROS production. This finding is in

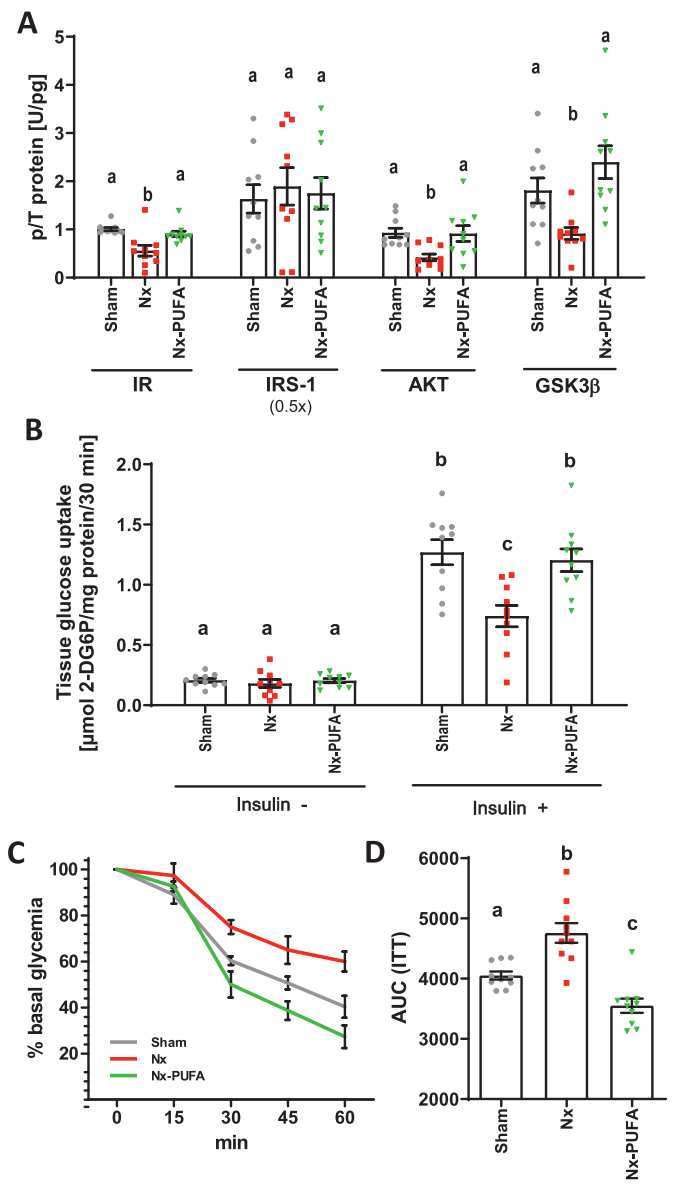

Fig. 5. n-3 PUFA and skeletal muscle insulin signalling and action in 5/6 nephrectomized rats. Effects of 30 day isocaloric, isolipidic n-3 PUFA enriched diet in 5/6 nephrectomized rats (Nx) on gastrocnemius muscle relative phosphorylation of $IR^{Y1162/Y1163}$, IRS—1^{S312}, AKT^{S473}, $GSK3\beta^{S9}$ (A) and on extensor digitorum longus muscle glucose uptake in the presence or absence of insulin co-incubation (B). Effects of n-3 PUFA enriched diet on systemic insulin resistance measured by insulin tolerance test (ITT), as shown by relative glycemia over time after insulin injection (C) and by related area under the curve (AUC; D). Results for IRS-1 have been scaled by multiplication for the indicated factor to improve figure readability. p/T:phosphoprotein to total protein ratio, $p < 0.05$ between groups not sharing the same letter, mean ± SEM, $n = 10$/group.

good agreement with available knowledge that mitochondrial morphology and dynamics are not the only regulators of ROS production, that also depends on altered organelle polarization [49,54], which may therefore not be normalized in the absence of sustained mitophagy-mediated removal of damaged organelles. Further studies should be designed to directly clarify relationships between mitochondrial dynamics, organelle polarization and mitophagy.

4.3. Anti-catabolic n-3 PUFA activities

The study also aimed at verifying whether metabolic and mitochondrial activities of n-3 PUFA result in prevention of skeletal muscle catabolic derangements and muscle loss. Muscle pro-inflammatory cytokine patterns and insulin resistance in untreated CKD animals were indeed consistently normalized by dietary n-3 PUFA. In particular, PUFA treatment prevented impairment of protein-anabolic signalling involving master regulators of both glucose utilization and protein synthesis such as AKT, mTOR, PRAS40 and P70S6k [11,36,37]. The validated marker 14 kDa actin fragment also demonstrated normalized protein breakdown [4,41], and normalized muscle mass directly confirmed prevention of muscle wasting. Additional experiments in both mixed gastrocnemius and eminently oxidative soleus muscles further indicated that anabolic effects of n-3 PUFA appear to primarily involve oxidative type 1 fibers across different muscle groups with heterogeneous fiber content and metabolic characteristics. Importantly, in vitro experiments confirmed that blockade of anti-oxidative effects by mitophagy inhibition prevented normalization of insulin signalling, an important component of protein-anabolic machinery. The above observations overall confirm the hypothesis that mitochondrial-induced oxidative stress plays a key role in activating muscle catabolism and wasting in CKD and that n-3 PUFA mitochondrial activities also contribute to normalized catabolism. These findings accordingly support the key permissive role of mitophagy in n-3 PUFA-induced prevention of muscle wasting. While indicating a potential pivotal role for p38/SAPK activation in mitochondrial activities, our results also show that the MAPK-ERK pathway, potentially involved in muscle protein catabolism regulation, is not modified by CKD or n-3 PUFA in this setting, and this observation is in line with reports showing that MAPK-ERK does not play a direct and independent role in wasting models [55].

4.4. Dietary n-3 PUFA administration

We administered n-3 PUFA as partial dietary replacement of the lipid dietary fraction, to avoid any changes in total calorie and protein dietary intake. Indeed calorie and protein imbalances may have contributed at least in part to inconsistent results in previous studies, with n-3 PUFA being commonly administered as dietary supplements in vivo, resulting in potential confounding from higher total calorie and lipid intake [16–18,25,32,33] which might per se profoundly affect skeletal muscle energy-protein metabolism. PUFA diet composition in the current study included ~1.6 % *w*/w of n-3 PUFA-enriched fish oil, corresponding to ~1 % of energy intake as EPA and DHA and to a human equivalent dose of ~2.5 g/day 50. This notably corresponds to the average test dose used by other authors investigating effects of n-3 PUFA in healthy humans (range 0.8–4.5 g/day) [34], thereby optimizing potential clinical translation of our findings. The current results provide proof of principle that nutritional therapeutic intervention to enhance the n-3 PUFA fraction in the diet of CKD patients, with unchanged calorie or protein intake, has potential to prevent CKD-associated muscle mitochondrial derangements, metabolic abnormalities and muscle loss.

It should also be noted that direct assessment of muscle function, including muscle strength and exercise capacity, was not included in the study protocol for technical reasons. Muscle function is also an important determinant of patient prognosis, directly associated with relevant outcomes including patient autonomy and quality of life [12,14,45]. Potential increments in exercise capacity and strength are however suggested by combined improvements in mitochondrial function and muscle mass. Also importantly, loss of muscle mass in gastrocnemius was primarily due to loss of myosin heavy chain, i.e. the main contractile protein, also confirmed by reduction of its ratio to actin, a clinically validated marker predicting loss of muscle function in clinical setting [42,43,56]. These findings collectively support the functional relevance of CKD-induced derangements and the potential clinical impact of normalized MHC content following n-3 PUFA dietary replacement. In addition, it should be considered that skeletal muscle mass preservation has independent clinical relevance, as previous studies show that muscle

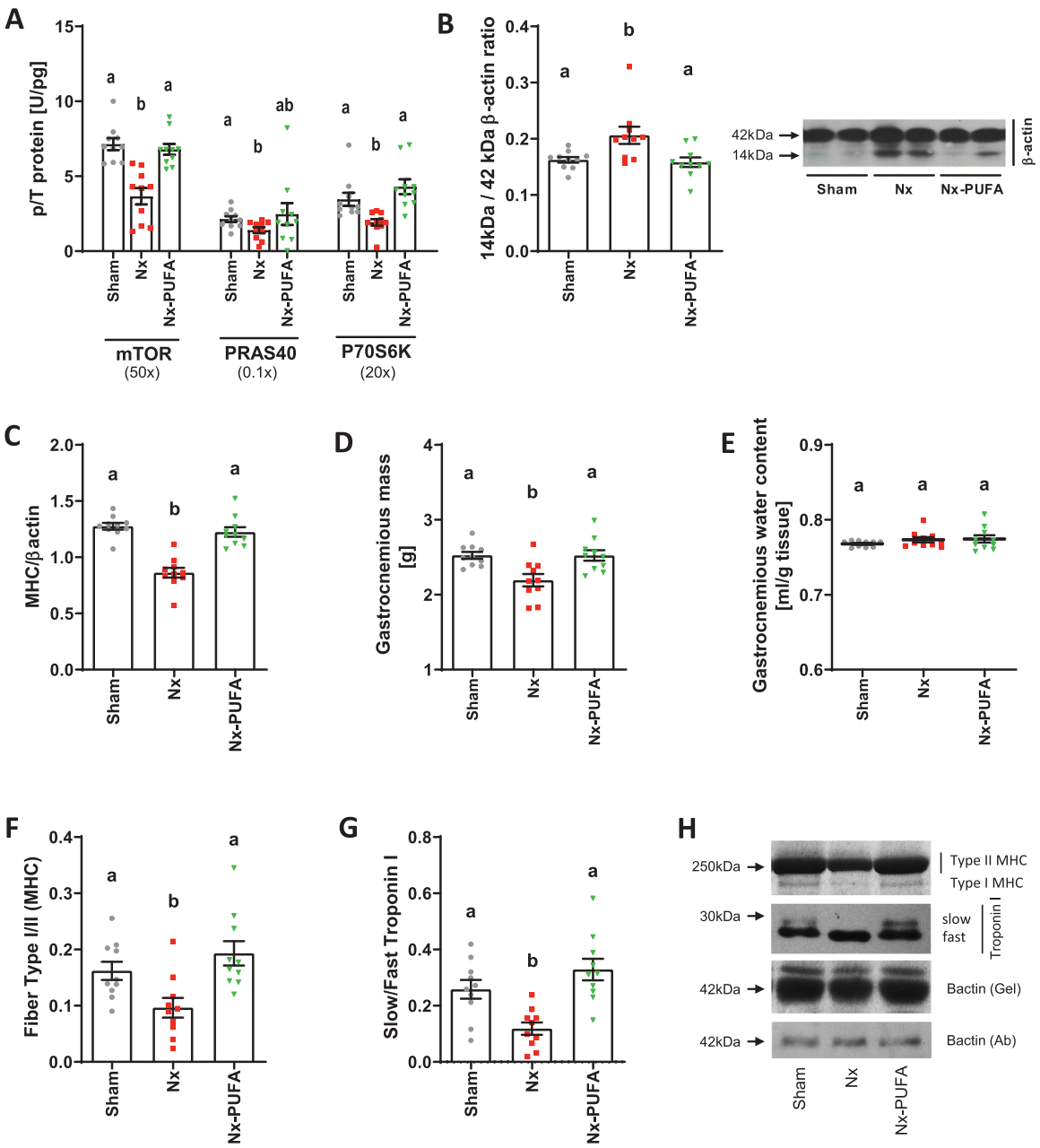

Fig. 6. n-3 PUFA and skeletal muscle protein anabolic and catabolic pathways, fiber type and mass in 5/6 nephrectomized rats. Effects of 30 day isocaloric, isolipidic n-3 PUFA enriched diet in 5/6 nephrectomized rats (Nx) on gastrocnemius muscle relative phosphorylation of $mTOR^{S2448}$, $PRAS40^{T246}$, $P70S6K^{T421/S424}$ (A), on muscle 14 kDa actin fragment content, expressed as relative optical density over 42 kDa β-actin expression with representative blot (B), on gastrocnemius myosin-to-actin ratio (C), muscle mass (D) and water content (E), fiber type I over II ratio according to Myosin Heavy Chain type content (F) and to Low/Fast Troponin I protein expression ratio (G) with representative blots (H). Results in panel A have been scaled by multiplication for the reported factor to improve figure readability. p/T: phosphoprotein to total protein ratio, $p < 0.05$ between groups not sharing the same letter, mean ± SEM, n = 10/group.

mass loss per se is associated with negative outcomes in CKD patients [45] as well as other disease conditions [45,57,58]. Measurement of spontaneous animal activity was also not included in the study protocol; we however suggest that any changes in activity patterns were unlikely to have determined the observed changes in muscle mitochondrial and metabolic parameters for various reasons. Firstly, available evidence does not report any primary impact of n-3 PUFA dietary increase on spontaneous activity in unrestraint, ad libitum fed rats [59,60]. Most importantly, in vitro experiments clearly indicate that PUFA activities on various mitochondrial master regulators and functional parameters were at least partly directly occurring in skeletal muscle and completely independent of physical activity that is of course not affecting in vitro experiments. The current results therefore demonstrated novel relevant PUFA muscle activities, at least partly independent of any changes in physical activity levels.

5. Conclusions

In conclusion, n3-PUFA isocaloric partial dietary lipid replacement normalizes mitochondrial master regulators NRF2 and PGC1α, mitochondrial mass and ATP production and mitochondrial dynamics in experimental CKD. These effects occur directly in muscle cells and result in normalized ROS production through enhanced tissue mitophagy. Anti-oxidative PUFA activities result in normalized tissue inflammation

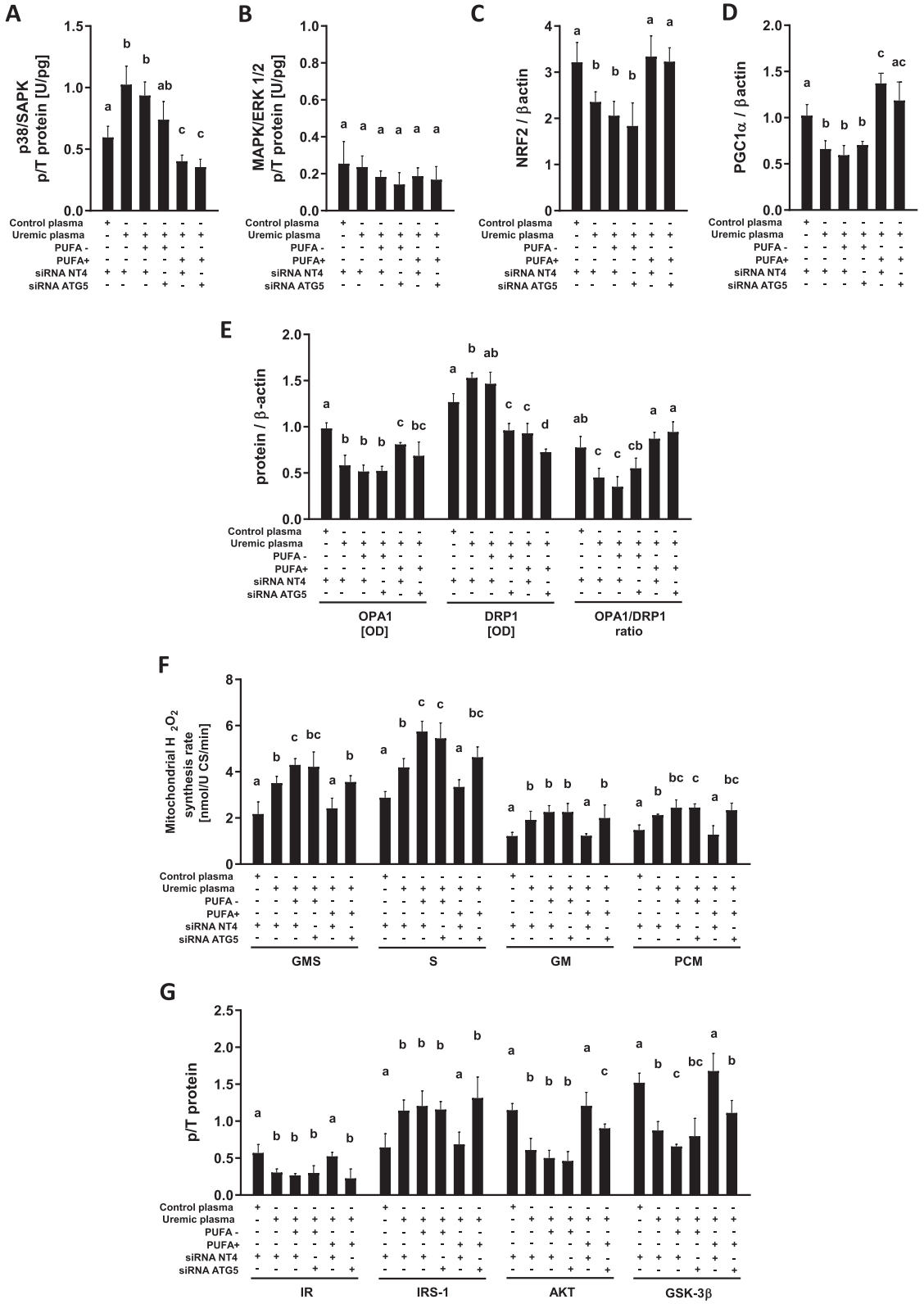

Fig. 7. Impact of n-3 PUFA on myotubes in an in vitro model of uremia. Effects of 24 h 500 μM n-3 PUFA (PUFA+) vs. MUFA (PUFA-) treatment on p38/SAPK $^{T180/Y182}$ (A) and MAPK/ERK 1/2$^{T185/Y187}$ (B), NRF2 (C), PGC1α (D), OPA1 and DRP1 protein levels and OPA1/DRP1 ratio (E), isolated mitochondria H_2O_2 synthesis rate with different respiratory substrates (F; GMS: Glutamate+Succinate+Malate; S: Succinate; GM: Glutamate+Malate; PCM: Palmitoyl-L-Carnintine+Malate), on relative phosphorylation of IR$^{Y1162/Y1163}$, IRS—1^{S312}, AKT^{S473} and GSK3β^{S9} (G) in C2C12 myotubes incubated for 48 h with 10 % (vol/vol) plasma from uremic or control subjects. U CS: units of citrate synthase; p/T:phosphoprotein to total protein ratio; TP: total protein content. $p < 0.05$ between groups not sharing the same letter, mean ± SEM, $n = 3$ independent experiments for each condition.

and insulin resistance with complete protection from muscle wasting. Our results indicate a novel role of dietary n3-PUFA to enhance mitophagy to prevent muscle mitochondrial oxidative stress and protect from muscle catabolism and wasting in CKD, thereby potentially improving patient morbidity and mortality.

Author contributions

GGC contributed to study design, set up and performed surgery and experiments, analyzed data and contributed to manuscript writing, AS, FB and GR performed experiments and contributed to data analysis and discussion, DB performed anesthesia and guided perioperative therapy, care and pain control and contributed to data discussion, GG and MG provided knowledge and materials for in vitro experiments and contributed to data discussion, RHM MZ and PV contributed to data discussion. RB designed the study, reviewed data and wrote the manuscript and acts as guarantor. All authors critically reviewed and gave final approval to the manuscript.

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CRediT authorship contribution statement

Gianluca Gortan Cappellari: Conceptualization, Methodology, Formal analysis, Investigation, Validation, Writing – original draft. **Annamaria Semolic:** Methodology, Formal analysis, Investigation. **Giulia Ruozi:** Conceptualization, Methodology, Investigation. **Davide Barbetta:** Conceptualization, Methodology, Investigation. **Francesca Bortolotti:** Methodology, Investigation. **Pierandrea Vinci:** Writing – review & editing. **Michela Zanetti:** Conceptualization, Writing – review & editing. **Robert H. Mak:** Conceptualization, Writing – review & editing. **Giacomo Garibotto:** Conceptualization, Resources, Writing – review & editing. **Mauro Giacca:** Conceptualization, Resources, Writing – review & editing, Supervision. **Rocco Barazzoni:** Conceptualization, Methodology, Validation, Writing – original draft, Supervision.

Declaration of competing interest

Authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2022.155242.

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n-3 PUFA DIETARY LIPID REPLACEMENT NORMALIZES MUSCLE MITOCHONDRIAL FUNCTION AND OXIDATIVE STRESS THROUGH ENHANCED TISSUE MITOPHAGY AND PROTECTS FROM MUSCLE WASTING IN EXPERIMENTAL KIDNEY DISEASE

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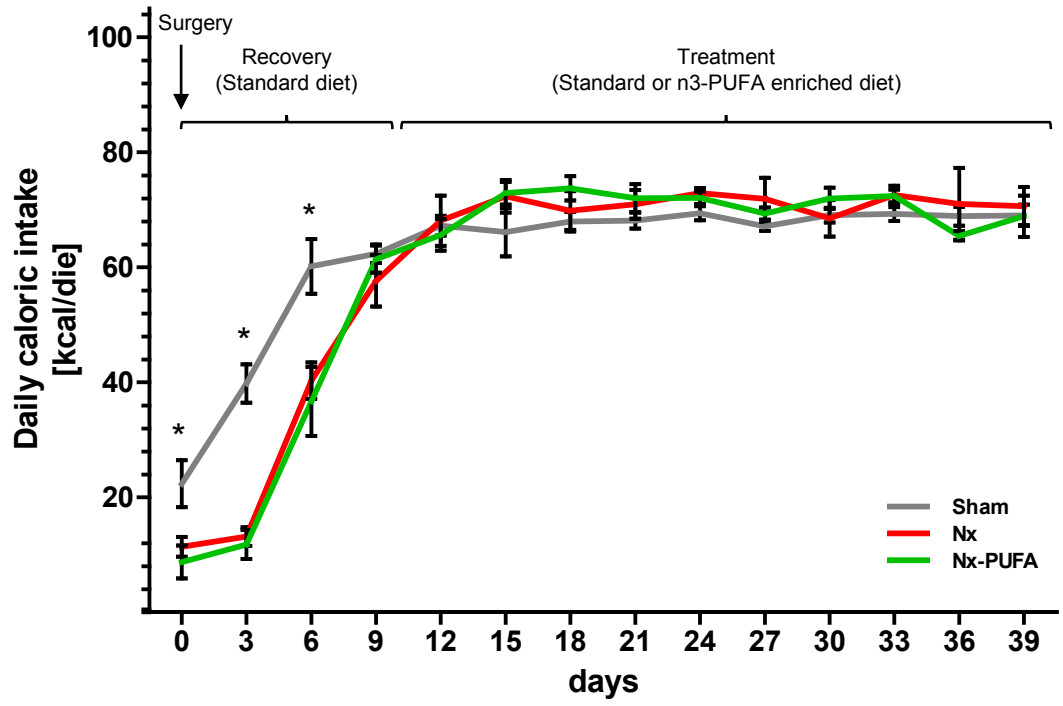
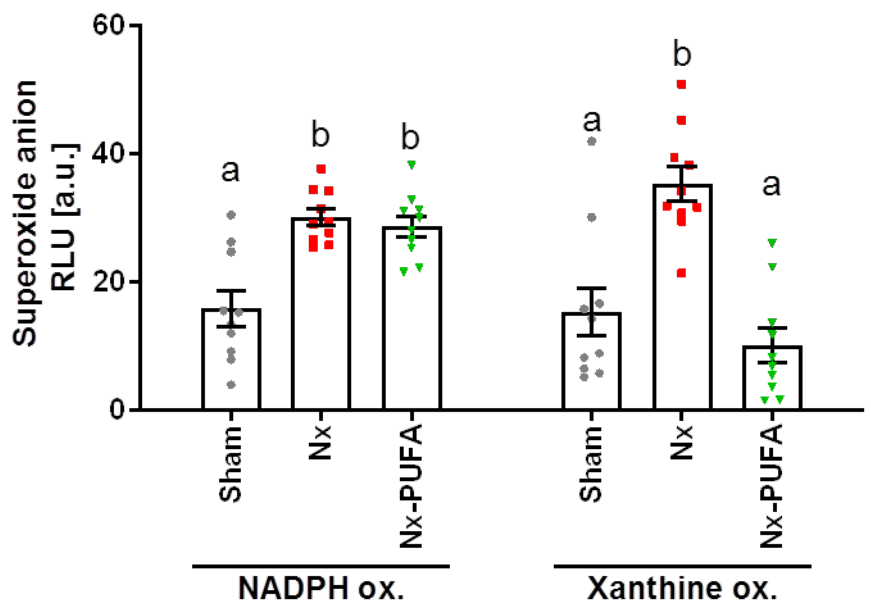
Supplementary Figures

Supplementary Figure 1



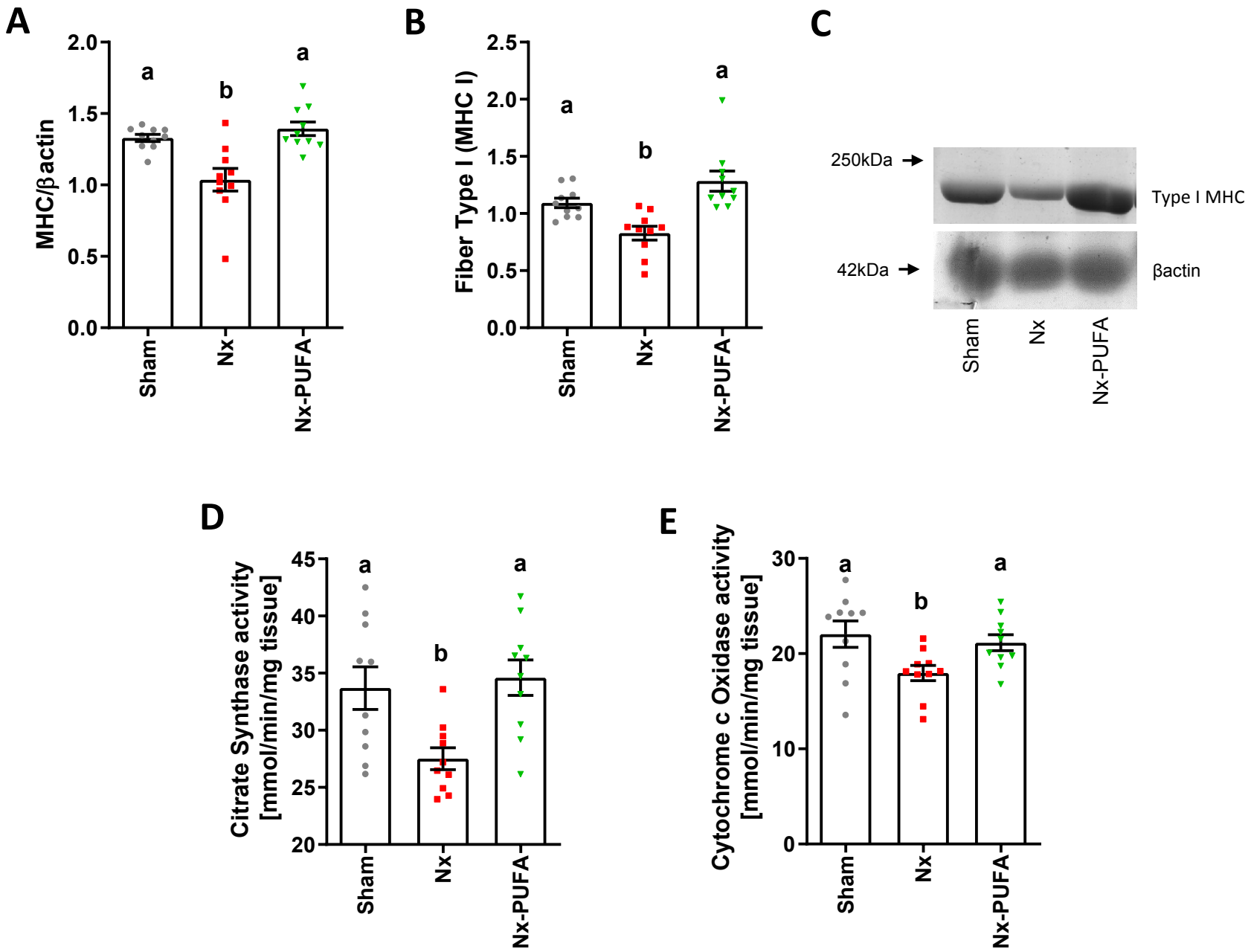
Supplementary Figure 1. Caloric intake in study groups over time. Time course graph of caloric intake for the three study groups, reporting measures taken every three days starting from sham or 5/6 nephrectomy (Nx) surgery. Feeding with PUFA enriched diet started at day 10, after full recovery. *$p<0.05$ sham vs Nx and Nx-PUFA, mean±SEM, n=10/group.

Supplementary Figure 2



Supplementary Figure 2. Other superoxide sources. Effects of 30 day isocaloric, isolipidic n-3 PUFA enriched diet in 5/6 nephrectomized rats (Nx) on NADPH-oxidase- and Xanthine oxidase-derived superoxide RLU: Relative Light Units. Different letters indicate $p<0.05$ between groups, mean±SEM, n=10/group.

Supplementary Figure 3



Supplementary Figure 3. Soleus muscle. Effects of 30 day isocaloric, isolipidic n-3 PUFA enriched diet in 5/6 nephrectomized rats (Nx) on soleus myosin to actin ratio (A), fiber type I in terms of Myosin Heavy Chain (MHC) Type I representation (B) with representative gel (C) and mitochondria citrate synthase (D) and cytochrome c oxidase (E) activities. Different letters indicate $p<0.05$ between groups, mean±SEM, n=10/group.