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Age related skeletal muscle atrophy and upregulation of autophagy in dogs

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ABSTRACT

Sarcopenia, the age related loss of muscle mass and strength, is a multifactorial condition that occurs in a variety of species and represents a major healthcare concern for older adults in human medicine. In veterinary medicine, skeletal muscle atrophy is often observed in dogs as they reach old age, but the process is not well understood. Autophagy is a mechanism for degradation and recycling of cellular constituents and is potentially involved in sarcopenia. The aim of the present study was to evaluate the expression of three markers of autophagy, Beclin 1, LC3 and p62, in muscle wasting of geriatric dogs, to establish whether the levels of autophagy change with increasing age.

Muscle biopsies from 25 geriatric dogs were examined and compared with those from five healthy young dogs. Samples from older dogs, assessed by routine histology, histoenzymatic staining and immunohistochemistry, showed evidence of muscle atrophy, sarcoplasmic vacuolisation and mitochondrial alterations. Furthermore, in 80% of the muscle samples from the older dogs, marked intracytoplasmic staining for Beclin 1 and LC3 was observed. Significantly greater expression of LC3 II and Beclin 1, but lower expression of p62, was found by Western blotting, comparing muscle samples from old vs. young dogs. The results of the study suggest that enhanced autophagy might be one of the factors underlying muscle atrophy in dogs as they age.

Introduction

Sarcopenia, the age related loss of muscle mass and strength, is a multifactorial condition that occurs in a variety of species and represents a major healthcare concern for older adults in human medicine (Evans, 1995; Doherty, 2003; Buford et al., 2010). A decline in muscle mass generally indicates a decline in muscle protein content and several studies have demonstrated an age-related reduction in the synthesis of specific muscle proteins (Proctor et al., 1998; Nair, 2005; Augustin and Partridge, 2009) with a concurrent increase in proteolysis. Skeletal muscle has four main proteolytic systems, namely, lysosomal, caspase, calpain and ubiquitin–proteasome, all of which could potentially contribute to age-related muscular atrophy.

Autophagy is a ubiquitous and highly conserved catabolic process, which involves intracellular degradation of cytoplasmic components through a lysosomal pathway. The process is involved in a variety of mechanisms related to ageing, including mitochondrial turnover, removal of misfolded protein aggregates and apoptosis. It has been proposed that age-related alterations in autophagy might play a major role in sarcopenia (Tukaj, 2013).

Three main types of autophagy have been described: (1) microautophagy, in which lysosomes directly take up portions of cytoplasm for degradation; (2) chaperone-mediated autophagy, in which chaperone proteins recognise and transport cytoplasmic proteins to the lysosome; and (3) macroautophagy, where there is non-selective sequestration of cytoplasmic material for lysosomal degradation. The latter process has been the focus of a large body of research and represents the most important type of autophagy (Tukaj, 2013; Wojcik, 2013); it consists of a number of steps, specifically induction or initiation and cargo selection, vesicle nucleation and expansion, lysosomal targeting, lysosome docking and autophagosome–lysosome fusion, vesicle breakdown and recycling (Klionsky and Emr, 2000; Tooze and Schiavo, 2008).

Nucleation is considered to be a crucial event that generates an active phagophore (or isolation membrane), which is the foundation

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of the autophagosome. At the phagophore, the serine/threonine kinases required for autophagy (Ulk1/2) function together with the Class III phosphatidylinositol 3-kinase (PI3K) VPS34 complex, which contains Beclin 1 (Tukaj, 2013). For phagophore expansion, elongation and sequestration into double-membrane vesicles (autophagosomes), recruitment of microtubule-associated protein light chain 3 (LC3)-II is required (Longatti and Tooze, 2009). Sequestration of ubiquitinated cargo within the phagophore is greatly aided by p62/SQSTM-1 (Pankiv et al., 2007). The nascent autophagosomes subsequently fuse with lysosomes to form an autophagolysosome, in which the cytoplasmic cargo is degraded by lysosomal acid hydrolases and where the degradation products are recycled (Tukaj, 2013).

The aims of the present study were to investigate whether autophagy was up-regulated in skeletal muscle from older dogs affected by sarcopenia, compared with similar tissues from younger healthy dogs, and to determine whether the size of the dog influenced this process.

Materials and methods

Animals and tissue samples

Twenty-five dogs (10 male, 15 female) of various breeds, aged between 15 and 22 years of age, were recruited into the study (Table 1). The dogs had died of natural causes or had been euthanased with informed owner consent by means of intravenous injection of 10 mg/kg Pentothal (Abbott Laboratories) and 70 mg/kg Tanax (MSD Animal Health). None of the study dogs showed any clinical evidence of neuromuscular disease or metabolic diseases including hypothyroidism, hyperadrenocorticism, renal disease, diabetes mellitus or neoplasia. None of the study dogs had a history of inappetance prior to entry into the study or had recently received glucocorticoid therapy, or any other drugs known to have an effect on muscle tissue.

Each owner consented to necropsy and use of tissues for research purposes, according to the ethical guidelines of the Diagnostic Service of the Department of Pathology and Animal Health of the University of Naples Federico II. Immediately post-mortem, muscle biopsies of approximately 1–2 cm diameter were obtained from the biceps femoris muscle. All samples were frozen in isopentane pre-cooled in liquid nitrogen, and stored at –80 °C until further processed. Biopsies of biceps femoris muscle, obtained from five healthy young dogs, aged between 2 and 5 years (Table 1), euthanased for reasons unrelated to the study, were used as control tissue for histological, immunohistochemical and Western blotting analyses, again with owner consent.

Table 1
Study population.

Dog ID number	Breed	Sex	Weight	Age (years)	Muscle atrophy present?
1	Rottweiler	F	>10 kg	15	Yes
2	Mix breed	M	<10 kg	17	Yes
3	Pit bull terrier	F	<10 kg	16	Yes
4	Crossbreed	M	<10 kg	16	Yes
5	Spitz	FN	<10 kg	18	Yes
6	Crossbreed	FN	<10 kg	16	Yes
7	German shepherd dog	M	>10 kg	15	Yes
8	Labrador retriever	M	>10 kg	19	Yes
9	Crossbreed	M	>10 kg	16	Yes
10	Miniature poodle	FN	<10 kg	18	Yes
11	Crossbreed	M	>10 kg	16	Yes
12	Crossbreed	F	<10 kg	18	Yes
13	German shepherd dog	F	>10 kg	16	Yes
14	Crossbreed	F	<10 kg	19	Yes
15	Crossbreed	FN	<10 kg	21	Yes
16	German shepherd dog	FN	>10 kg	17	Yes
17	Boxer	M	>10 kg	16	Yes
18	Yorkshire terrier	FN	<10 kg	17	Yes
19	Yorkshire terrier	F	<10 kg	18	Yes
20	Irish setter	F	>10 kg	17	Yes
21	Crossbreed	M	<10 kg	19	Yes
22	Crossbreed	FN	>10 kg	18	Yes
23	Doberman	M	>10 kg	15	Yes
24	Labrador retriever	M	>10 kg	16	Yes
25	Yorkshire terrier	F	<10 kg	20	Yes
c1	Crossbreed	M	<10 kg	3	No
c2	German shepherd dog	F	>10 kg	5	No
c3	Crossbreed	FN	<10 kg	4	No
c4	Labrador retriever	M	>10 kg	2	No
c5	Yorkshire terrier	M	<10 kg	3	No

M, male; F, female; FN, female neutered.

Histological examination

Tissue sections of 10 μm were cut in a transverse plane with a cryostat (–20 °C) and were stained according to the methods detailed previously (Paciello and Papparella, 2009). Briefly, haematoxylin and eosin (H&E) and Engel trichrome were used for a basic morphological examination of muscle fibres; NADH-tetrazolium reductase (NADH-TR), succinic dehydrogenase (SDH) and cytochrome oxidase (CYOX) were used to indicate activity and distribution of mitochondria; ATPase at pH 9.4 and pH 4.3 was employed for histochemical fibre subtyping; and esterase staining was used to evaluate lipofuscine aggregates inside muscle fibres.

Scoring of the major morphological alterations, observed in the biopsies, was performed by light microscopy at 20× magnification. The proportions of atrophic fibres, necrotic fibres, ragged and pre-ragged red fibres, rimmed vacuoles, CYOX negative fibres and accumulation of lipofuscines were scored as follows: 0 (none); 1–25% of affected fibres (classified as mild); 26–50% of affected fibres (classified as moderate); >50% of affected fibres (classified as severe). Approximately 20 fields at 20× magnification were evaluated for each section by two independent pathologists (TBP, OP) with a concordance rate of 95%.

Immunohistochemical examination

Immunohistochemical analysis was performed according to the method previously described (Paciello and Papparella, 2009). Briefly, 10 μm sections of muscle tissue were obtained using a cryostat. Slides were air dried for 1 h at room temperature, washed in phosphate-buffered saline (0.01 M PBS, pH 7.2) and fixed in acetone at 4 °C for 3 min. Endogenous peroxide activity was blocked using 0.3% hydrogen peroxide in methanol (Sigma–Aldrich), applied for 15 min at room temperature. After two washes in PBS, sections were incubated for 30 min at room temperature with Background Sniper (Biocare Medical). Sections were incubated overnight at 4 °C with the primary antibodies against Beclin-1 (Santa Cruz Biotechnology) diluted 1:300; LC3 (Abcam), diluted 1:3000; and p62 (Santa Cruz Biotechnology), diluted 1:200 in PBS. The immunoblot confirmed the specificity of these antibodies in canine tissues in accordance with guidelines published by Kurien et al. (2011).

After two washes with PBS, the MACH 1 Universal HPR-Polymer Detection Kit (Biocare Medical) was used according to the manufacturer's instructions. The sections were subsequently counterstained in haematoxylin, dehydrated in alcohol, clarified in xylene and mounted in aqueous mounting medium. In the corresponding negative control sections, the primary antibody was either omitted or replaced with a 1:20 dilution of mouse serum (for p62 antibody) or rabbit serum (for Beclin-1 and LC3) (Jackson ImmunoResearch). The degree of immunoreactivity was categorised as follows: 0, negative staining observed in muscle fibres; 1–25% positively stained fibres; 26–50% positively stained fibres; > 50% positively stained fibres.

Western blotting

Muscle tissues from geriatric dogs and young control dogs were sectioned at 20 μm by cryostat and lysed at 4 °C in 200 μL of lysis buffer (TBS, 20 mM Tris-HCl pH 7.6, 140 mM NaCl, 30 mM sodium pyrophosphate, 5 mM EDTA, 0.55% Nonident P40, 1% Triton X-100, 50 mM NaF, 0.1 mM Na_3VO_4, 1 mM PMSF, 1 mM benzamidine, 1 mM iodoacetamide, 1 mM phenantroline). Protein concentrations were determined by BCA protein assay (Pierce), and lysates were adjusted to equivalent concentrations with lysis buffer. Aliquots of muscle lysate (30 μg protein per lane) were then separated by SDS-PAGE. Proteins were transferred to PVDF membranes and blocked overnight at 4 °C with 5% non-fat dried skimmed milk (Marvel, Premier Beverages) in Tris-buffered saline, supplemented with 0.05% Tween 20 (TTBS). Membranes were incubated with primary antibodies against Beclin-1 (Santa Cruz Biotechnology) at 1:500 dilution, LC3 (Abcam) at 1:3000 dilution or p62 (Santa Cruz Biotechnology) at 1:500 dilution. Horseradish peroxidase-conjugated secondary antibodies were applied in blocking solution for 1 h at room temperature. Immunoreactive bands were visualised by autoradiography using Super Signal West Pico Chemiluminescent Substrate Kit (Pierce). Membranes were subsequently stripped and re-probed using anti-β-actin monoclonal antibody (Sigma-Aldrich) to confirm equal loading of proteins in all lanes. Band intensities were determined on scanned images using Image J software[1] to determine average pixel intensity.

Statistical methods

Data obtained from Western blotting were analysed with Statview software (Abacus Concepts) by one-factor analysis of variance (single-factor ANOVA). Data comparing aged vs. young dogs from assessment of immunohistochemistry and

[1] See: http://rsb.info.nih.gov/ij/ (accessed 01 July 2015).