Attenuation of proteolysis and muscle wasting by curcumin c3 complex in MAC16 colon tumour-bearing mice

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Muscle wasting or cachexia is caused by accelerated muscle protein breakdown via the ubiquitin–proteasome complex. We investigated the effect of curcumin c3 complex (curcumin c3) on attenuation of muscle proteolysis using *in vitro* and *in vivo* models. Our *in vitro* data indicate that curcumin c3 as low as $0.50 \mu g/ml$ was very effective in significantly inhibiting (30%; $P < 0.05$) tyrosine release from human skeletal muscle cells, which reached a maximum level of inhibition of 60% ($P < 0.05$) at $2.5 \mu g/ml$. Curcumin c3 at $2.5 \mu g/ml$ also inhibited chymotrypsin-like 20S proteasome activity in these cells by 25% ($P < 0.05$). For *in vivo* studies, we induced progressive muscle wasting in mice by implanting the MAC16 colon tumour. The *in vivo* data indicate that low doses of curcumin c3 (100 mg/kg body weight) was able to prevent weight loss in mice bearing MAC16 tumours whereas higher doses of curcumin c3 (250 mg/kg body weight) resulted in approximately 25% ($P < 0.05$) weight gain as compared with the placebo-treated animals. Additionally, the effect of curcumin c3 on preventing and/or reversing cachexia was also evident by gains in the weight of the gastrocnemius muscle (30–58%; $P < 0.05$) and with the increased size of the muscle fibres (30–65%; $P < 0.05$). Furthermore, curcumin inhibited proteasome complex activity and variably reduced expression of muscle-specific ubiquitin ligases: atrogin-1/muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MURF-1). In conclusion, oral curcumin c3 results in the prevention and reversal of weight loss. The data imply that curcumin c3 may be an effective adjuvant therapy against cachexia.

MAC16 tumours: Cachexia: Curcumin: Proteasomes: Muscle wasting

Cachexia is defined as the progressive wasting of body tissues that primarily affects muscle and adipose tissue[1–3]. Patients suffering from sepsis, trauma, AIDS and many types of cancer exhibit cachexia[4–10]. All cancer patients exhibit some degree of cachexia, and it is one of the most important factors leading to early morbidity and mortality, accounting for up to 30% of all deaths[11]. It is particularly more pronounced in pancreatic and head–neck cancers[6,12–14]. Studies during the last 10 years have concluded that muscle wasting is primarily caused by accelerated muscle protein breakdown via the ubiquitin–proteasome complex[15]. Expression in muscle of two ubiquitin ligases, namely mouse atrophy gene-1 (atrogin-1) (also described as the muscle atrophy F-box; MAFbx) and the muscle RING finger 1 (MURF-1)[16,17], is up-regulated in various animal models of muscle atrophy, including fasting, cancer, sepsis, disuse, denervation, diabetes and uraemia[16–20]. Similarly, muscle wasting situations in humans, including immobilisation[21], acute quadriplegic myopathy and neurogenic atrophy[22], are also accompanied with up-regulated MAFbx/atrogin-1 and MURF-1 expression.

Various inflammatory cytokines, including TNFα, interferon γ, IL-6, leukaemia inhibitory factor, and mediators, including proteolysis-inducing factor and lipid mobilising factor, are known to play a role in the development of cachexia[23]. Currently, therapies aimed at neutralising these cytokines or mediators have had only limited success[24]. Furthermore, anorexia is often accompanied by cachexia; however, refeeding a balanced diet does not reverse the progression of cachexia[25]. Recently, attempts have been made to supplement diets with nutrients that specifically inhibit muscle proteolysis[26]. One such promising supplement is curcumin (1,7-bis(4-hydroxy-3-methoxyphenil)1, 6-heptadiene-3,5-dione), which is present in turmeric. Curcumin has anti-inflammatory, antioxidant, anticarcinogenic, antidiabetogenic, antibacterial, antiviral and free radical-scavenging properties[27–31]. The pharmacology and putative anticancer properties of curcumin have been extensively reviewed[32]. Recent studies indicate that curcumin may also possess antiproteolytic properties. For example, curcumin is reported to inhibit proteasome activity in HeLa cells[33], as well as attenuate the proteolysis-inducing factor-induced increase in proteasome activity in the muscle tissues of tumour-bearing mice[34]. The intraperitoneal administration of curcumin daily for four consecutive days before a lipopolysaccharide

(LPS) injection in mice blunted LPS stimulation of atrogin-1/MAFbx mRNA expression in gastrocnemius and extensor digitorum longus muscle of mice[35]. Curcumin has been shown to increase the rate and extent of muscle regeneration after trauma[36]. In contrast to these studies, intraperitoneal administration of curcumin (20 µg/kg body weight) has been ineffective in preventing muscle wasting or changes in the body weight of rats bearing the highly cachectic Yoshida AH-130 ascites hepatoma[37]. Similarly, curcumin treatment by mouth at higher dose levels (150 and 300 mg/kg body weight) was shown to be ineffective in preventing loss of body weight in mice bearing the MAC16 colon tumour[34]. It is, therefore, not clear why a compound that has potential inhibitory activities against protein degradation *in vitro* failed to reverse cachexia in experimental models. Low curcumin absorption and hence bioavailability have been suggested as the primary reasons for the failure of curcumin[34,38]. We hypothesised that this failure of curcumin in those studies was due to using an animal model that exhibited a drastic body-weight loss (20 % over 5 d) as well as short treatment duration (4–5 d). To test our hypothesis, we used a standardised patented curcumin extract, and reinvestigated its effect on proteolysis in human skeletal muscle cells, and also treated cachectic animals that were gradually losing body weight (20 % over 21 d) for a longer duration (21 d).

Experimental methods

Cell cultures

Proliferating human skeletal myoblast cells (Cambrex Bio Science, Walkersville, MA, USA) were cultured in Clonetics® Skeletal Muscle Cell Basal Medium (SkBM®; Cambrex Bio Science) supplemented with bovine serum albumin, bovine fetuin, insulin, dexamethasone, recombinant human epidermal growth factor and gentamycin sulfate with amphotericin B (all supplied with SkBM® as SingleQuots®), according to the manufacturer's instructions. Cells were not differentiated into myotubules under these conditions. Cells were subcultured when they were approximately 70 % confluence.

Validation of curcumin c3 complex

The present study was performed using curcumin c3 complex, a standardised preparation (Sabinsa, Piscataway, NJ, USA) which has been used extensively in human safety trials[39–41]. For validation, the composition of curcumin c3 complex was analysed by a reversed-phase HPLC method using an Agilent 1100 HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with an Agilent 1100 diode-array detector, an Agilent 1100 autosampler system and an Agilent Zorbax Eclipse XDB-C18 column (4·6 × 150 mm; 5 µm). The mobile phase consisted of A (15 % acetonitrile in 0·05 M-KH$_2$PO$_4$; pH 2·9) and B (80 % acetonitrile in distilled water) solvents as follows: 0 % solvent B from 0–5 min; 0–80 % solvent B from 5–30 min (linear gradient); 100 % solvent B from 30·1–35 min; 0 % solvent B from 35·1–45 min (end) at a flow rate of 0·5 ml/min. The detection wavelengths were 260 and 428 nm. Chromatographic peaks were identified by comparing retention times of samples with those of standards (curcumin, bisdemethoxycurcumin, demethoxycurcumin) as described[42].

Cytotoxic effects of curcumin

Cells (1 × 10^4 per well) were seeded in a ninety-six-well plate overnight and then treated with varying concentrations of curcumin c3 in serum-free medium for 24 h. Curcumin c3 was dissolved in dimethyl sulfoxide (5 mg/ml) as a stock solution. A sample of curcumin c3 was diluted in media before treatment. The final concentration of dimethyl sulfoxide was kept at 0·1 %. The control cells were treated with vehicle only (0·1 % dimethyl sulfoxide). The effect of the curcumin c3 on skeletal muscle cell viability was determined with a water-soluble tetrazolium salt-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay in accordance with the manufacturer's instructions (Roche Biosciences, Indianapolis, IN, USA). This assay is based on mitochondrial dehydrogenase activity, which is present only in the respiring viable cells.

In vitro *protein degradation*

Protein degradation in response to serum starvation was assayed as described[43]. Briefly, skeletal muscle cells were plated in twenty-four-well tissue culture plates for 24 h, then rinsed with serum-free SkBM® culture media, and finally labelled under serum-free conditions with L-[3, 5 ^{3}H]tyrosine (1905·3 GBq (51·50 Ci)/mmol; 0·036996 MBq (1 µCi)/ml in each well) for another 24 h. Labelled monolayers were washed three times with serum–free SkBM® containing 50 µM–cycloheximide and 2 mM–unlabelled tyrosine and then incubated in the same media (2 ml) for 48 h in the presence or absence of curcumin c3. A sample of the media was then removed (750 µl) for determining radioactive tyrosine release, mixed with 5 ml of ScintiVerse (Fisher Scientific, Hanover Park, IL, USA) and the radioactivity was quantified using a Beckman L6000 liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA, USA). Inhibition of tyrosine release in the media by curcumin c3 was calculated from tyrosine release in the absence of curcumin (control, 100 %) after correcting for subtraction of background counts.

Animal model of cachexia

The murine MAC16 colon tumour model for inducing cachexia in mice was established as previously described[44]. Murine MAC16 tumour cells originally derived in Dr Michael Tisdale's laboratory (Aston University, Birmingham, UK) were kindly provided by Dr Constance Monitto (John Hopkins Hospital, Baltimore, MD, USA). Cells were initially maintained in culture in Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine (GIBCO BRL; Life Technologies, Rockville, MD, USA) containing 12 % fetal bovine serum (HyClone, Logan, UT, USA) and penicillin–streptomycin (100 U/ml and 100 µg/ml, respectively) in a humidified atmosphere with 5 % CO$_2$ at 37°C. For tumour induction, 200 µl of MAC16 cells (5 × 10^6/ml in PBS) were injected subcutaneously in the lower back of Hsd:Athymic nude–*nu* male mice (aged 6–7 weeks, average body weight 26·71 (SD 1·31) g; Harlan Laboratories, Indianapolis, IN, USA).

Once the tumour was palpable the body weight of the animals and progression of tumour growth were recorded every day post−tumour implantation (PI) using a digital caliper (Fisher Scientific, Pittsburgh, PA, USA). Animals that exhibited a loss of 5–7 % of initial body weight (10–12 d PI) were randomised into treatment groups (five animals per group). Mice were orally administered daily with placebo vehicle (200 μl olive oil) or 100 mg/kg body weight or 250 mg/kg body weight of curcumin c3 (in 200 μl olive oil) as described[45]. Animals were given a standard laboratory non−purified diet (LabDiet, catalogue no. 5001; Ted's Feed, Indianapolis, IN, USA) and water *ad libitum*. The diet consists of 24 % proteins, 10·7 % fats, 48 % carbohydrates and 5 % fibres. Upon completion of the study, mice were euthanised by inhalation of the anaesthetic gas isoflurane. Mice were skinned and tumours removed to measure the carcass body weight. Hindquarters were removed and weighed. Gastrocnemius muscles from both legs were removed, weighed and quickly frozen in liquid N_2. Thigh muscles were used for tissue sections. Tissue specimens were stored at −80°C for biochemical analysis. The protocol for these studies was approved by the Methodist Research Institute's Animal Research Committee and strictly followed the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication no. 85-23, revised 1996).

Muscle histology

Transverse serial sections of quadriceps muscle (10 μm) were prepared using a cryostat (Leica CM1900; Leica Microsystems, Bannockburn, IL, USA). The sections were stained using haematoxylin and eosin staining (Sigma Chemical Co., St Louis, MO, USA). Images of rectus femoris muscle sections were recorded using a digital camera (mounted on a microscope) as described[46]. The surface area of individual muscle bundles (representing mixed fibre types) were measured using ImagePro software (Cybernetics, Silver Spring, MD, USA).

Proteasome assay

The effect of curcumin c3 on proteasome activity was assayed in both muscle extracts of MAC16 tumour-bearing mice as well as in serum-starved human skeletal muscle cells. Gastrocnemius muscles of mice were homogenised using a polytron homogeniser in 20 mM-2-amino-2-hydroxymethyl-propane-1,3-diol (Tris)-HCl (pH 7·5) containing 2 mM-ATP, 5 mM-$MgCl_2$ and 1 mM-dithiothreitol. The homogenate was centrifuged at 800 *g* and the supernatant fraction was used for determining the chymotrypsin-like activity of the 20S proteasome using a kit (20S Proteasome Activity kit; Boston Biochem, Cambridge, MA, USA). The activities were adjusted for protein concentrations. Other protease-like activities of the proteasome complex were not determined.

Western blot analysis

Muscle tissues from animals were homogenised in a homogenising buffer (0·25 M-sucrose, 50 mM-HEPES (pH 7·4), 2 mM-ethylene glycol tetraacetic acid) using a polytron homogeniser. The homogenate was solubilised in 1:1 ratios with lysis buffer (20 mM-Tris-HCl (pH 7·4), 137 mM-NaCl, 100 mM-NaF, 2 mM-Na_3VO_4, 10 % glycerol, 1 % nonidet P-40, 2 mM-phenylmethanesulfonylfluoride, leupeptin (1 μg/ml), aprotinin (0·15 units/ml) and 2·5 mM-diisofluorophosphate) for 10 min on ice. The detergent-solubilised extracts were centrifuged to remove insoluble matter. After evaluating the protein content using a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA), 15 μg of protein solubilised in Laemmli sample loading buffer was loaded onto each lane of a 4–12 % gradient SDS–polyacrylamide gel. Proteins were electrophoretically separated and transferred onto nitrocellulose membranes (Millipore Corporation, Bedford, MA, USA) for immuno-Western blot analysis. Blots were then incubated with anti-MAFbx (Oncogene Research Products, Calbiochem, San Diego, CA, USA) and anti-MURF-1 (Oncogene Research Products) primary antibodies (1:1000 dilution in Tris-buffered saline with Tween-20) according to the manufacturer's specifications and proteins were detected using a peroxidase-conjugated secondary antibody (1:5000 dilution in Tris-buffered saline with Tween-20 containing 1 % bovine serum albumin) with an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotechnology, Piscataway, NJ, USA). For reprobing, membranes were stripped in buffer consisting of 62·5 mM-Tris-HCl (pH 6·8), 2 % SDS and 100 mM-β-mercaptoethanol for 30 min at 50°C followed by six washes in Tris-buffered saline (pH 7·4) with 0·1 % Tween 20. To verify an equal distribution of protein loading, blots were reprobed with a peroxidase-conjugated glyceraldehyde 3-phosphate dehydrogenase antibody (1:1000 dilution in Tris-buffered saline with Tween-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The resolved proteins were quantified by densitometry using a Kodak Image Station (MM2000; Kodak, Rochester, NY, USA).

Statistical analysis

One-way ANOVA was used for overall comparisons across all treatment groups. *Post hoc* pairwise comparisons were performed using Tukey's multiple-comparison test. Statistical analysis was completed using Minitab 14.2 (Minitab Inc., State College, PA, USA). Data were summarised by treatment group using mean and standard error. The Student's *t* test was also used (as mentioned elsewhere) to determine differences between individual groups compared with control. A value of $P < 0.05$ was considered statistically significant.

Results

Curcumin c3 composition

We tested the purity of curcumin c3 complex and found it to contain 73 % curcumin, 22 % desmethoxycurcumin and 4 % bis-desmethoxycurcumin as reported by the manufacturer. Treatment of curcumin c3 at 100 mg/kg body weight therefore contained 73 mg curcumin, 22 mg desmethoxycurcumin and 4 mg bis-desmethoxycurcumin/kg body weight, whereas treatments with 250 mg curcumin c3/kg body weight contained 182·5 mg curcumin, 55 mg desmethoxycurcumin and 10 mg bis-desmethoxycurcumin/kg body weight.

Curcumin c3 inhibits protein breakdown and chymotrysin-like 20S proteasome activity in skeletal muscle cells

In an initial screen, we investigated cytotoxic concentrations of curcumin c3 for human skeletal muscle cells. Curcumin c3 was well tolerated up to a concentration of 2·5 μg/ml. Concentrations over 2·5 μg/ml appeared to be toxic, causing total cell death at 5 μg/ml (data not shown). The subsequent experiments were therefore performed under non-toxic concentrations. Data shown in Fig. 1(a) demonstrate that curcumin c3 has a dose-dependent effect on protein degradation as assayed by tyrosine release during serum starvation. It is clear from the data that a curcumin c3 concentration as low as 0·50 μg/ml was very effective in significantly inhibiting (30 %; $P<0·05$) tyrosine release, which reached a maximum level of inhibition of (60 %; $P<0·05$) at 2·5 μg/ml. Consistent with this result, our data in Fig. 1(b) demonstrate that curcumin c3 at 2·5 μg/ml was able to inhibit chymotrysin-like proteasome 20S activity by 25 % ($P<0·05$).

Curcumin c3 treatment prevents body-weight loss

Data presented in Fig. 2 indicate that placebo-treated tumour-bearing animals progressively lost body weight, reaching a total loss of approximately 18–20 % at day 21. When treated at a lower dose of curcumin c3 (100 mg/kg body weight), MAC16-tumour-bearing animals effectively maintained their initial loss of 4–7 % of body weight ($P<0·05$ compared with placebo). However, tumour-bearing animals treated with a higher dose of curcumin c3 (250 mg/kg body weight) initially resisted any loss of body weight during the first 10 d treatment and then started gaining weight and were able to increase their weight by 8–10 % from their initial body weight by day 21 ($P<0·05$) and by 25 % ($P<0·05$) compared with placebo-treated tumour-bearing control. These animals achieved comparable body-weight gains to those of non-tumour-bearing placebo-treated animals (Fig. 2). Subtraction of the tumour's weight from the animal's weight at the end of the study (day 21 post-treatment) indicated that low and high doses of curcumin c3 treatment caused a net increase in body weight by 24 % ($P<0·05$) and 35 % ($P<0·05$), respectively, compared with that of placebo-treated animals (Table 1). The animals in all three groups did not exhibit significant differences in tumour weights at day 21 (Table 1). Moreover, the animals did not exhibit significant differences in average daily food intake before and after tumour implantation (Table 1).

Curcumin improves muscle characteristics

We next examined whether the increase in body weight in tumour-bearing animals on curcumin c3 treatment was due
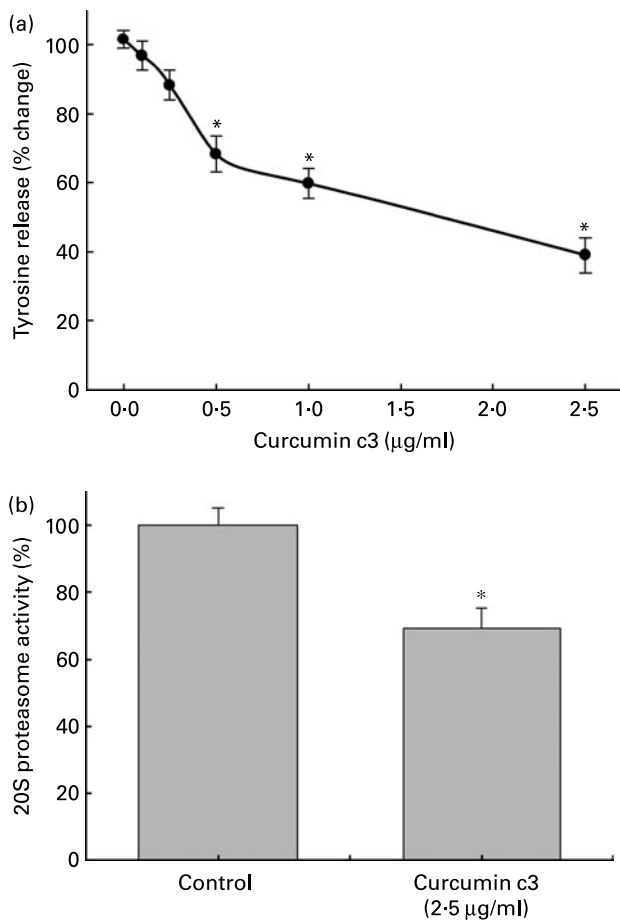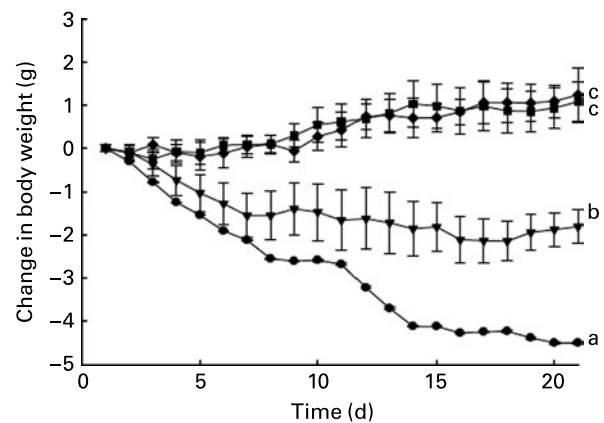

Fig. 1. Inhibition of proteolysis (a) and chymotrysin-like 20S proteasome activity (b) in muscle cells by curcumin c3. (a) Inhibition of proteolysis was determined by monitoring the release of radioactive tyrosine from pre-labelled human skeletal muscle cells in the presence or absence of curcumin (control, 100 %). The radioactivity was quantified using a Beckman L6000 liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA, USA). (b) 20S chymotrysin-like proteasome activity was determined in the supernatant fraction of cell homogenate using a kit (20S Proteasome Activity Kit; Boston Biochem, Cambridge, MA, USA). The activities were adjusted for protein concentrations. Values are the means for at least three experiments, with standard errors represented by vertical bars. * Mean value was significantly different from that of the control treatment ($P<0·05$).

Fig. 2. Effect of curcumin c3 on body weight of MAC-16 tumour-bearing mice. Animal body weights were recorded every day post-tumour implantation (PI). Animals (n 5) that exhibited a loss of 5–7 % of initial body weight (10–12 d PI) were randomised into treatment groups. Mice were orally administered daily with placebo vehicle (200 μl olive oil; −●−) or 100 mg (−▼−) or 250 mg (−■−) curcumin c3/kg body weight (in 200 μl olive oil). Non-tumour bearing mice (n 3) of comparable weights were also treated with vehicle (200 μl olive oil) for comparison (−♦−). Values are means, with standard errors represented by vertical bars. The results were analysed using ANOVA. [a,b,c] Mean values at day 21 with unlike letters were significantly different ($P<0·001$). Using Tukey's multiple-comparison test, significant differences were found between groups ($P<0·05$).

Table 1. Effect of curcumin c3 treatment on MAC-16 tumour-bearing mice

(Mean values with their standard errors)

	Control (no treatment)		Curcumin c3 (100 mg/kg body weight)		Curcumin c3 (250 mg/kg body weight)		
	Mean	SE	Mean	SE	Mean	SE	P*
Daily food intake (g)†	2·17	0·18	2·24	0·14	2·45	0·12	0·427
Tumour weight (g)	3·35	0·72	2·59	0·33	2·85	0·68	0·647
Body weight – tumour weight (g)	17·53[a]	1·28	21·81[b]	1·55	23·85[b,c]	2·02	<0·05
Hindquarters (g)	2·29	0·12	2·63	0·16	3·17	0·30	0·071
Gastrocnemius muscle weight (g)	0·12[a]	0·01	0·15[b]	0·01	0·19[c]	0·02	<0·05

[a,b,c] Mean values within a row with unlike superscript letters were significantly different (P<0·05).
* Analysed by ANOVA for at least five animals per group.
† The daily food intake of the non-tumour-bearing animals was 2·47 (SE 0·09) g.

to improvements in the animals' muscle characteristics. Weights of gastrocnemius muscle were increased by 30 % (P<0·05) and 58 % (P<0·05) in low- and high-curcumin c3-treated animals, respectively, compared with those of placebo-treated animals (Table 1). Consistent with these observations, muscle fibre size was also increased, as depicted in Fig. 3(a). Quantification of these muscle fibres indicated that their size increased by 30 % (P<0·05) and 65 % (P<0·05) in low- and high-curcumin c3-treated animals, respectively, compared with that of placebo-treated animals (Fig. 3(b)). The weight of hindquarters was statistically not different in

(a)

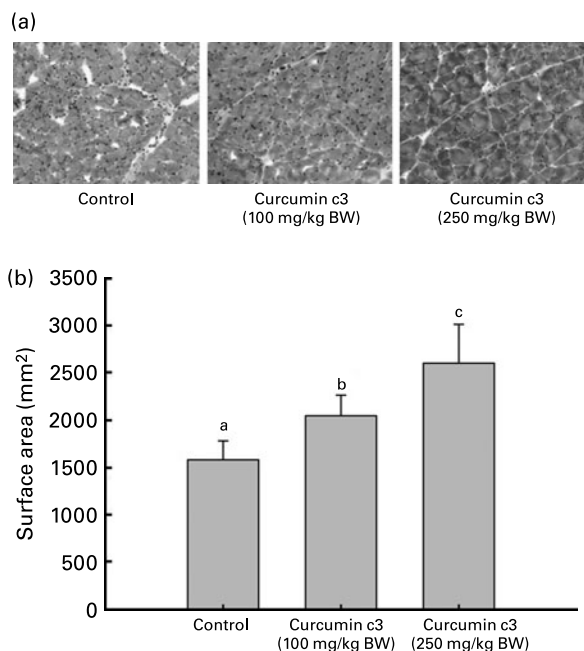Control   Curcumin c3 (100 mg/kg BW)   Curcumin c3 (250 mg/kg BW)

(b)

Fig. 3. Effect of curcumin c3 on muscle fibre size. (a) Transverse serial sections of quadricep muscles (10 μm) were prepared. The sections were stained using haematoxylin and eosin staining and pictures of rectus femoris muscle section were recorded using a digital camera. (b) Surface area of individual muscle bundles (ten mixed fibre types from four different viewing sites at random in each group) was measured using ImagePro software (Cybernetics, Silver Spring, MD, USA). Values are means for five animals per group, with standard errors represented by vertical bars. The results were analysed using ANOVA. [a,b,c] Mean values at day 21 with unlike letters were significantly different (P<0·001). Using Tukey's multiple-comparison test, significant differences were found between all groups (P<0·05). BW, body weight.

animals treated with low (P<0·12) and high (P<0·06) curcumin c3, compared with placebo-treated animals (Table 1).

Curcumin c3 inhibits muscle proteolysis

We further examined whether improvements in muscle characteristics after curcumin c3 treatment was a result of curcumin c3's effect on the proteasome pathway. Curcumin c3 treatment resulted in the inhibition of chymotrypsin-like proteasome 20S activity by 22–25 % (P<0·05) as compared with placebo (Fig. 4(a)). However, there was no significant difference between low v. high curcumin c3 treatment. We further examined the expression of atrogin-1/MAFbx and MURF-1 expression through Western blot analysis (Fig. 4(b)). Expression of atrogin-1/MAFbx was reduced by 20–25 % (P=0·11) in isolated gastrocnemius muscle on curcumin c3 treatment compared with that of controls (placebo treatment); however, expression of MURF-1 was reduced by 40–75 % (P=0·06) on treatment with curcumin c3 (Fig. 4(c)). It is interesting to note that a significant difference (P<0·05) in the inhibition of MURF-1 expression was observed when animals treated with high doses of curcumin were directly compared with the control group using Student's t test.

Discussion

Our data indicate that a low dosage of curcumin c3 (100 mg/kg body weight) was able to prevent weight loss in mice bearing cachexia-inducing MAC16 tumours whereas a higher dosage at 250 mg/kg body weight resulted in weight gains compared with that of placebo-treated animals. These animals maintained body weight similar to normal non-tumour-bearing mice despite the presence of tumour. It is noticeable that tumour burdens in both curcumin- and placebo-treated animals were not statistically different (P<0·67); therefore, the effect of curcumin on body weight is independent of tumour burden. The effect of curcumin c3 on body-weight gain was also independent of food intake as there was no statistically significant difference between the food intake of control and curcumin c3-treated animals. Furthermore, the effects of curcumin c3 treatment on inhibiting and/or reversing cachexia in MAC16 tumour-bearing mice are also evident by weight gain in gasctrocnemic muscle (30–58 %), and increased size of muscle fibres (30–65 %). It is interesting to note that the present
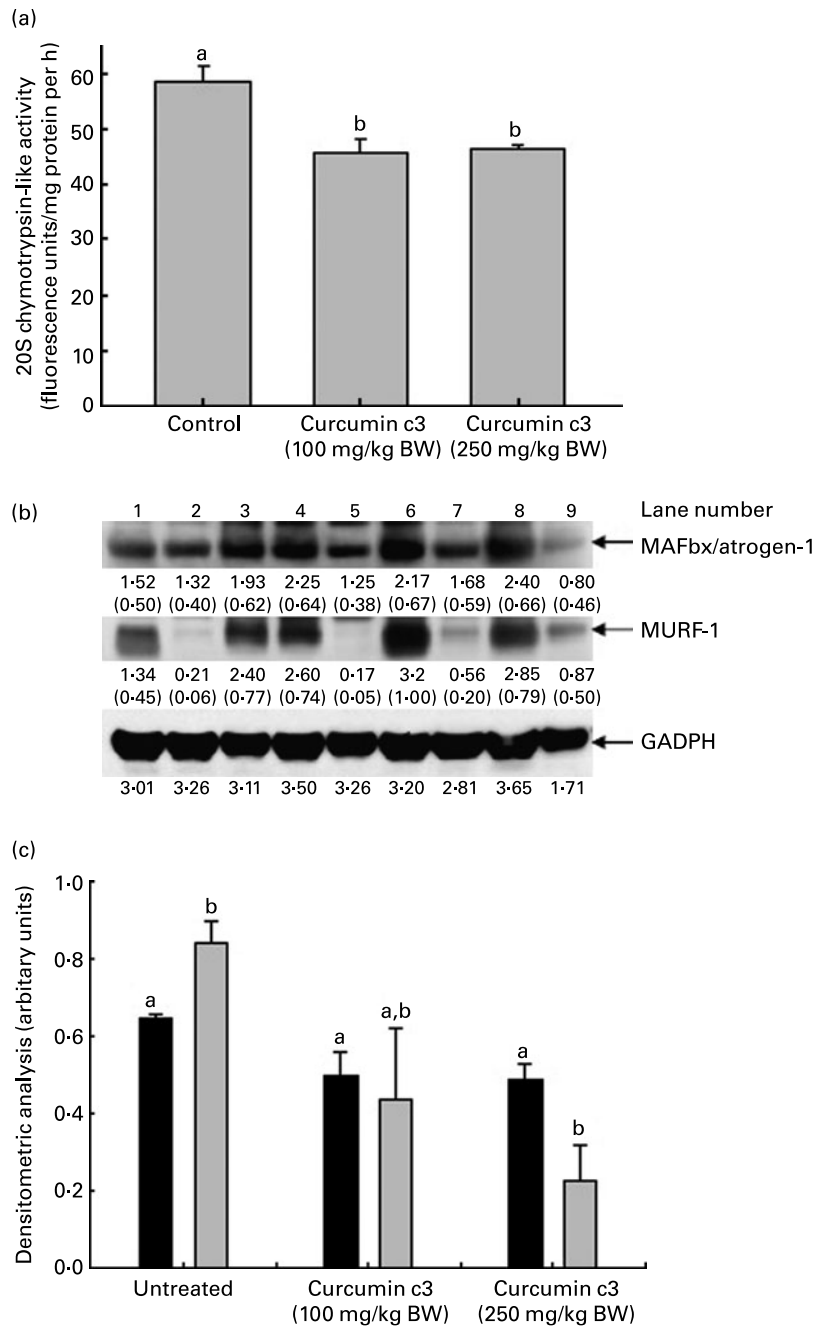
Fig. 4. Effect of curcumin c3 on 20S chymotrypsin-like proteasome activity and muscle atrophy F-box (MAFbx)/atrogen-1 and muscle RING finger 1 (MURF-1) expression in muscle. Gastrocnemius muscle homogenate was used to determine 20S chymotrysin-like activity and MAFbx/atrogen-1 and MURF-1 expression at the end of the study (day 21 on curcumin treatment). (a) Chymotrysin-like 20S proteasome activity in the supernatant fraction of muscle homogenates was determined using a kit (20S Proteasome Activity Kit; Boston Biochem, Cambridge, MA, USA). (b) The solubilised protein extracts of muscle homogenates were used to determine MAFbx/atrogen-1 and MURF-1 expression by Western blot analysis. Lanes 1, 2 and 7 represent MAC16−curcumin c3-treated mice (250 mg/kg body weight (BW)); lanes 3, 5 and 9 represent MAC16−curcumin c3-treated mice (100 mg/kg BW); lanes 4, 6 and 8 represents MAC16-untreated mice. (c) Quantification of protein expression (■, MAFbx/atrogen-1; □, MURF-1) was determined by densitometry analysis. Densities (arbitrary units) are shown underneath each band whereas the numbers in parentheses refer to densities normalised to the loading control (glyceraldehyde 3-phosphate dehydrogenase; GADPH). Values are the means for three experiments, with standard errors represented by vertical bars. The results were analysed using ANOVA. [a,b,c] Mean values with unlike letters were significantly different ($P < 0.05$).

results are not in agreement with previous studies on the effect of curcumin on the cachectic MAC16 mouse model[34]. However, there are technical differences in the present study compared with previous studies[34]. In previous studies, a drastic loss in muscle mass or body weight was induced by the

tumours in a shorter period of time (20 % over 5 d)[34], while we established a gradual loss in body weight by implanting a smaller load of MAC16 cultured cells. This approach resulted in a slow body-weight loss totalling 18−20 % over 21 d. Furthermore, we used a different source

of curcumin, i.e. curcumin c3 complex, which has been extensively used in human trials[39−41]. The composition of curcumin extracts in the previous study was not reported; it is therefore not clear if there were compositional differences in our curcumin c3 complex from that used in previous studies. It is possible that purity and compositional differences may have contributed to the biological activity of the curcumin c3 effects. Moreover, we suspended curcumin in olive oil, whereas other studies used curcumin dissolved in dimethyl sulfoxide and then diluted (100 ×) in PBS[34]. It is possible that olive oil may serve as a better vehicle for curcumin c3 complex as it is hydrophobic in nature and provides improved bioavailability compared with an aqueous vehicle such as PBS used in the previous study. Lastly, the animals in the present study were on curcumin c3 treatment for 21 d, whereas previous studies used a 4–5 d treatment[34,37]. Treatment over a longer period of time with curcumin c3 might have overcome the low bioavailability of curcumin in general. Several investigators have reported low plasma levels of curcumin and its metabolites[38,42]. Recent studies demonstrate that curcumin and its metabolites are cleared from plasma in rats within 2 h of oral treatment[42]. During the present investigation, the concentration of curcumin was not measured because it was not possible to withdraw blood from these mice on a daily basis during the 21 d treatment. In our opinion, these factors, including the rate of body wasting, the unique patented composition of curcumin c3 complex, its suspension in olive oil and the duration of treatment might have facilitated the efficacy of curcumin c3 complex.

The ubiquitin–proteasome pathway is the primary pathway involved in protein catabolism and is felt to be the major degradation pathway involved in various cachectic conditions[17,47−50]. The ubiquitin–proteasome pathway is stimulated by TNF, IL-1, interferon γ and other pro-inflammatory mediators[51−54]. Pro-inflammatory cytokines and proteolysis-inducing factor activate the ubiquitin–proteasome pathway through the transcription factor NF-κB. We tested 20S chymotypsin-like activity of the ubiquitin–proteasome pathway in muscle specimens of curcumin c3- or placebo-treated animals. The present results indicate that chymotrysin-like activity of the 20S proteasome was significantly suppressed in mice treated with curcumin c3 compared with placebo-treated mice. However, there was no difference on inhibition of chymotrysin-like activity between low and high doses of curcumin c3 treatment. During the present investigation, we did not examine the chymotrysin-like activity of the 20S proteasome in non-tumour-bearing muscle; it is therefore not clear if the inhibition of chymotrypsin-like activity of the 20S proteasome reached a basal level on curcumin treatment. Nevertheless, the present results are consistent with previous findings where curcumin attenuated the proteolysis-inducing factor-induced increase in the 'chymotrypsin-like' enzyme activity of the 20S proteasome[34].

Genes encoding for ligases (i.e. atrogin-1/MAFbx and MURF-1) within the ubiquitin–proteasome pathway are instrumental in the development of muscle atrophy[17]. For example, the development of atrophy and muscle proteolysis during sepsis is blocked by proteasome inhibitors[55]. We tested the expression of atrogin-1/MAFbx and MURF-1 in muscles isolated from curcumin- and placebo-treated animals. Expression of MURF-1 was inhibited in the muscle of animals treated with high doses of curcumin c3, but there was no significant effect on atrogin-1/MAFbx expression. It is not clear from these results if curcumin inhibited proteolysis through atrogin-1/MAFbx- and MURF-1-mediated pathways. It is possible that other cellular pathways, including pathways for protein synthesis, may be involved in mediating curcumin effects on body-weight regulation. Several pathways have been proposed for the effect of curcumin c3 in directly regulating protein degradation and synthesis. For example, expression of MAFbx/atrogin-1 and MURF-1 under stressing conditions is regulated by unphosphorylated forkhead box transcription factors class O (FoxO). Phosphorylation of FoxO is regulated by phosphatidyl inositol 3′-kinase (PI3′K)-dependent protein kinase B (AKT) activity, which inhibits protein degradation by inhibiting MAFbx/atrogin-1 and MURF-1 expression and diverts signals for protein synthesis[56−58]. Atrogin-1/MAFbx expression is also up-regulated via a p38 mitogen-activated protein kinase (MAPK)-dependent mechanism in C2C12 myotubes[59], while MURF-1 expression is stimulated through an NF-κB-dependent mechanism[45,60,61]. Curcumin has been shown to prevent activation of NF-κB and prevent sepsis-induced muscle protein degradation[62]. Furthermore, curcumin has been shown to block phosphorylation and subsequent degradation of inhibitor of NF-κB (IκBα)[63], which results in an increase in the rate and extent of muscle regeneration after trauma[36]. In contrast to these studies, elevated activity of NF-κB in *mdx* mice exhibiting muscular dystrophy was resistant to dietary curcumin treatment[64]. The *mdx* mice have impaired sarcoplasmic calcium ATPase and depressed p38 MAPK activities, which suggests the involvement of these enzymes in the lack of curcumin effect on NF-κB activity[64]. Curcumin also has a p38-inhibiting property[65], which has been shown to obstruct p38-mediated TNF-α up-regulation of atrogin-1/MAFbx in C2C12 myotubes[59]. Similarly, the administration of curcumin daily for four consecutive days before LPS injection blunted LPS stimulation of atrogin-1/MAFbx mRNA expression in mice muscle[35]; however, these experiments failed to show curcumin regulation of atrogin-1/MAFbx expression through mediating AKT activity during LPS stimulation. Based on these data, one could speculate that curcumin regulates both protein degradation and synthesis pathways possibly through regulating AKT, NF-κB and/or p38 MAPK activation. Further experiments are therefore required to address the molecular effects of curcumin on the regulation of these cellular mediators.

In conclusion, treatment of cachectic animals bearing MAC16 tumours with curcumin c3 resulted in the prevention and reversal of cachexia. Curcumin c3 also attenuated 20S proteasome activity but its effects on the inhibition of atrogin-1/MAFbx and MURF-1 expression are not clear. The data presented in the present study imply that curcumin c3 may have an effective therapeutic or an adjuvant therapeutic potential against cachexia.

Acknowledgements

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