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Disruption of pro-oxidant and antioxidant systems with elevated expression of the ubiquitin proteasome system in the cachectic heart muscle of nude mice

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Abstract

Background Current research into the mechanisms of organ atrophy associated with cancer cachexia have centred on the loss of skeletal muscle, as it is one of the most profound physical changes of the disease. However, many patients with cancer cachexia also experience significant atrophy of the heart. The mechanisms causing cardiac tissue wastage in cancer cachexia are largely unknown. However, it is believed to involve a number of molecular interactions between the tumour and host. Increased levels of oxidative stress have been found in cancer cachectic skeletal muscle and has been linked to the activation of the ubiquitin proteasome system (UPS). The aim of the current study was to examine the role of oxidative stress and the UPS in the hearts of mice with cancer cachexia.

Methods Oxidative damage to DNA (8-OH-2dG), mRNA levels of the ROS-producing enzymes NADPH oxidase (NOX), and xanthine oxidase (XDH), the antioxidant enzyme superoxide dismutase (SOD) and key components of the UPS was measured in the heart of mice with cancer cachexia. Protein expression levels of NOX enzyme subunits and SOD enzyme activity was also measured in the same heart samples.

Results 8-OH-2dG levels were 1.5-fold higher in the heart of mice with cancer cachexia, and this was associated with a 1.7-

fold lower level of NOX2 mRNA and twofold higher XDH mRNA in the same hearts. Cancer cachexia was also associated with a 1.5-fold lower level of SOD activity in the heart. Accompanying these pro- and antioxidant differences was a significantly higher level of mRNA for the key UPS elements MURF-1 (4.3-fold) and MAFbx (3.8-fold) in the hearts of mice with cancer cachexia.

Conclusions The current study demonstrated that cardiac atrophy of cachectic mice is associated with oxidative damage to DNA in the myocardium. The higher levels of XDH mRNA in cachectic hearts suggest that xanthine oxidase may have an important role to play in producing oxidative stress. It appears that the combination of higher XDH expression and lower SOD enzyme activity are key contributors to oxidative stress and cardiac tissue damage in cancer-induced cardiac atrophy. Oxidative stress in the myocardium as with skeletal muscle may also induce increased expression of the E3 ligases MURF-1 and MAFbx as seen in this study.

Keywords Cancer cachexia · Oxidative stress · Xanthine oxidase · NADPH oxidase · Superoxide dismutase · Ubiquitin proteasome system

1 Introduction

Current research into the mechanisms of organ atrophy associated with cancer cachexia (CC) is centred on the loss of skeletal muscle mass as it is one of the most profound physical changes of the disease. However, studies have also established that CC causes significant atrophy of the heart muscle in animal models [1–3]. Decreases in heart weight by as much as 60 % have been reported in a rat model of CC [3]. Clinical research does not currently exist examining the pathologic consequence of cardiac atrophy in cancer cachexia. However, Tian et al. used echocardiography to analyse the

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function of cachectic hearts in a mouse model of CC [4]. The study found that, 14 days after the inoculation with tumour cells, the hearts of cancer cachectic mice had reduced heart rate and fractional shortening.

The mechanisms causing cardiac tissue wastage are largely unknown. However, it is believed to involve a number of molecular interactions between the tumour and host. Elevated cytokines in response to tumour presence and tumour-derived factors such as proteolysis inducing factor (PIF) and lipid mobilising factor together produce tissue wasting [5–7]. Host factors such as cytokines including IL-1, IL-6, TNF- α , INF- γ and PIF derived from the tumour have been shown to create a state of oxidative stress and net protein catabolism in skeletal muscle [8]. Similarly increased oxidative stress has been shown to be present in the cachectic heart [9], but how this is linked to the activation of protein breakdown pathways is currently unknown.

A key catabolic pathway known to contribute to both skeletal and cardiac muscle atrophy is the ubiquitin proteasome system (UPS). The UPS is the principle mechanism by which muscle proteins are degraded. The UPS has been found to be upregulated in both experimental models and patients with CC [10, 11]. Enzymes which allow ubiquitin to tag proteins for destruction are termed E3 ligases. Currently, only three of the discovered several hundred E3 ligases have been found to be important in the process of muscle atrophy. These are termed E3 alpha, muscle-specific F-box (MAFbx), and muscle specific ring finger 1 (MURF-1) [12]. Examining a model of congestive heart failure, Adams et al. found a positive correlation between TNF- α and the E3 ligases MAFbx and MURF-1 [13]. Furthermore, the study found that TNF- α was able to bring about troponin I ubiquitinylation leading to impaired contractility of the heart.

Oxidative stress in a CC patient population may be an important link between the loss of tissue and initiation of key catabolic pathways [14]. A study by Kaynar et al. found that patients with either non-small cell or small cell lung cancer have elevated levels of xanthine oxidase (XO) activity compared with controls [15]. In the heart, XO is an important enzyme capable of catalyzing the oxidation of hypoxanthine and xanthine to uric acid whilst reducing O_2 to O_2^- and hydrogen peroxide (H_2O_2). Traditionally, increases in XO in the failing heart are associated with ventricular remodelling, fibrosis, and hypertrophy [16]. The contribution of XO in cancer-induced cardiac atrophy has not been investigated, despite the enzyme being implicated in myocardial remodelling and dysfunction [17, 18]. In terms of atrophy of the myocardium, like that of skeletal muscle, cardiomyocyte size is dependent on the relative rates of protein synthesis and degradation [19]. Increased levels of oxidative stress have been found in cancer cachectic skeletal muscle and has been linked to the activation of the UPS [20]. However, no such studies have examined the role of oxidative stress in the cancer

cachectic heart. The current study aimed to test the hypothesis that increased levels of oxidative stress in the heart muscle of mice with cancer cachexia is associated with and up regulation of the UPS.

2 Materials and methods

2.1 Animals

All experimental procedures were carried out with approval from the Victoria University animal ethics committee (AEETH 07/05). Six-week-old BALB/c nu/nu nude mice purchased from the Animal Resource Centre, Western Australia, and were acclimatised for 1 week prior to injection of the cancer cell lines. Animals were maintained under controlled conditions of a 12-h light/dark cycle at 21 °C. Food and water were provided ad libitum. Food and water were weighed before and after the experimental period to ensure weight loss in the CC group was not due to a decrease in caloric intake. Animals were randomised into three weight-matched groups consisting of a no cancer (control, $n=8$), cancer without cachexia control (cancer, $n=12$) and cancer with cachexia (cachexia, $n=16$).

2.2 Tumour model

Cancer and cancer cachexia were induced as previously described [25]. In brief, the murine adeno-carcinoma cell lines 13 (MAC13) and 16 (MAC16) were cultured in RPMI with 10 % FBS and 0.5 % penicillin/streptomycin (Invitrogen). Mice inoculated with the MAC13 cell line develop a malignant tumour without cachexia and are used as a cancer control (cancer). The MAC16 cell line produces a both solid tumour and cachexia (cachexia). Cells were kept in a humidified incubator at 37 °C in 10 % CO_2 . Cells were grown to 80 % confluence and removed from cell culture flasks via trypsinisation. These cells were centrifuged at 500 $\times g$ for 5 min at 4 °C and isolated from the growth media. Cells were then resuspended in sterile PBS pre-incubated to 37 °C and drawn up into a sterile syringe with a 25-gauge needle for subsequent injection into mice. Mouse body weights were recorded daily, and tumour size was measured using calipers. Animals were euthanised before weight loss in the cachexia group reached 25 % of their initial weight or before tumour growth exceeded 1,000 mm³; this occurred at 30 \pm 3 days. Tissues were harvested and immediately placed in liquid nitrogen before storing in an –80 °C freezer for later analysis. In the case of the heart, it was removed intact, blotted to remove any blood, and weighed. The atria and any remaining portions of the great vessels were then removed prior to the ventricles being frozen.

2.3 SOD enzyme activity

Heart tissues were washed in heparinised PBS, and 15 mg of tissue was used for enzyme activity assays. Heart tissue was homogenized in 5 ml of 20 mM HEPES buffer (pH 7.2) containing 1 mM EGTA, 210 mM mannitol and 70 mM sucrose per gram of tissue. Samples were standardised so that 10 µl of sample with 10 mg of protein was added to each well on the 96-well assay plate. The assay kit detecting total SOD activity was undertaken as per the manufacturer's instructions (Cayman Chemical). The amount of activity was calculated and standardised for protein using the Bradford method (Bio-Rad).

2.4 DNA oxidation assay

An ELISA kit was used to measure the DNA oxidation byproduct 8-hydroxy-2-deoxy guanosine (StressMarq Biosciences). DNA was extracted from 15 mg of tissue using a DNA isolation kit (Promega). Each sample was then diluted so that 50 µg of DNA was used in the 8-hydroxy-2-deoxy guanosine assay. The competitive immunoassay involves the binding of free 8-hydroxy-2-deoxy guanosine to an antibody coated 96-well plate. The assay and sample concentration of 8-hydroxy-2-deoxy guanosine was carried out as per the manufacturer's instructions.

2.5 RT-PCR

RNA was extracted from frozen heart tissue samples using tri-reagent (Molecular Research Centre). Total RNA concentration yielded per sample was quantified spectrophotometrically at 260 nm. All samples were reverse-transcribed to cDNA using reverse transcription kit (Marligen). cDNA was stored at −20 °C until further analysis. All primer sequences were designed using Primer 3 (primer sequences shown in Table 1). Real-time PCR was performed using a IQ 5 PCR

detection system (Bio Rad). A supermix (Bio Rad) was prepared containing 10 ng of sample cDNA, forward and reverse primers diluted to a final concentration of 300 nM, in a total volume of 25 µl. Quantification of mRNA expression was achieved by running samples in duplicate and over a period of 40 cycles. Fluorescence data were tabulated and mRNA levels quantified using threshold cycle values. 18S was used as a housekeeping gene to compensate for variations in input RNA amounts by normalizing values to this gene.

2.6 Protein quantification

Heart tissue was homogenized in 20 mM HEPES buffer. The homogenate was centrifuged, and the supernatant was collected and placed on ice. Protein content was assayed using the BCA Protein Assay Kit (Bio Rad). Fifty micrograms of total cellular protein was then loaded on 12 % polyacrylamide gels (NuSep) and separated by standard SDS-PAGE. Gels were then transferred to polyvinylidene fluoride membranes (Bio Rad). Blots were blocked with Tris-buffered saline including 0.1 % Tween 20 and 5 % nonfat dried milk. Primary antibody (Santa-Cruz) against p47^{phox}, NOX2, and GAPDH at a dilution of 1 in 2,000 was added to the membrane overnight at 4 °C. Secondary donkey–antigoat antibodies (Santa-Cruz) conjugated to HRP at a 1 in 10,000 dilution was added to the membrane for 1 h at room temperature. Antigen–antibody complexes were analysed by chemiluminescence using the immune-star HRP detection kit (Bio Rad). Subsequent development and detection of the membrane was achieved with the Chemidoc system (Bio Rad). Protein expression data were then analysed using the Quantity One software (Bio Rad).

2.7 Statistical analysis

The reported values are expressed as mean±SEM. Data were analyzed using two-way ANOVA, with a Tukey's test post hoc analysis performed to determine differences between

Table 1 PCR primer sequences

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
18 s	GTAACCCGTTGAACCCCAT	ATCCAATCGGTAGTAGCG
MURF-1	AGGAGCAAGTAGGCACCTCA	GTCCATGTCTGGAGGTCGTT
MAFbx	AAGATCAAACGCTTGCGAAT	GAACATCATGCAGAGGCTGA
NOX1	TGGTCATGCAGATTAACTTTG	CATTGTCCCACATTGGTCTCC
NOX2	TTGGGTCAGCACTGGCTCTG	TGGCGGTGTGCAGTGCTATC
NOX4	TGTTGGGCTAGGATTGTGTT	AAAAGGATGAGGCTGCAGTTG
p67 ^{phox}	CTGGCTGAGGCCATCAGACT	AGGCCACTGCAAGAGTGCTTG
p40 ^{phox}	GCCGCTATCGCCAGTTCTAC	GCAGGCTCAGGAGGTTCTTC
CuZn SOD	TGAACAGTTGTGTTGTCAG	TCCATCACTGGTCACTAGCC
Mn SOD	TGGCTTGGCTTCAATAAGGA	AAGGTAGTAAGCGTGCTCCCACAC
XDH	ATCTGGAGACCCACTGCACC	TGTGCTCACGAAGAGCTCCAT

groups where appropriate. Significance was set at a P value equal to or <0.05

3 Results

The animals in this study were randomised to one of three groups being a no-tumour control, cancer or cancer cachexia. Animals in the cachexia group displayed all of the hallmarks of cancer cachexia including the loss of body weight ($P=0.003$), skeletal ($P=0.003$) and cardiac ($P=0.002$) muscle with no significant differences in food and water intake between the groups (Table 2). An analysis was then carried out to ascertain whether key oxidative and proteolytic pathways were upregulated in cardiac tissue.

Oxidative damage in the heart muscle was assessed by measuring 8-hydroxy-2-deoxyguanosine (8-OH-2dG), a biomarker of DNA oxidation (Fig. 1). The hearts of cachectic mice displayed a significant 1.5-fold higher 8-OH-2dG (71 ± 26 pg/ml) concentration compared with control (45 ± 18 pg/ml, $P=0.02$) and cancer (43 ± 24 pg/ml, $P=0.015$) groups. Further studies were then carried out to assess which pathways may be contributing to the oxidative damage in the cancer cachectic hearts.

To investigate the potential source of oxidative stress contributing to the oxidative DNA damage found in the cachectic hearts, we investigated the ROS producing enzymes, NADPH oxidase and XO. RT-PCR was performed on the key regulatory subunits (p40^{phox} and p67^{phox}) and the catalytic core units (NOX1, NOX2 and NOX4) of NADPH oxidase. There were no changes in the key regulatory or catalytic core units of NADPH oxidase in the heart muscle samples except for NOX2 (Table 3). The current experiment found a significant 1.7-fold lower level of gene expression for NOX2 in cachectic hearts ($P=0.03$) compared with the cancer group (Table 3). To assess the possibility of posttranslational modification to the NADPH oxidase protein components, a protein expression analysis was undertaken on p47^{phox} and NOX2. There was no significant difference in either p47^{phox} and NOX2 protein

Table 2 Body weight, organ weights, food and water intake

	Control ($n=8$)	Cancer ($n=12$)	Cachexia ($n=16$)
Body weight (g)	21.7 \pm 0.4	22.3 \pm 0.3	17.6 \pm 0.5 ^{##}
Heart weight (mg)	121.0 \pm 0.01	112.6 \pm 0.01	92.0 \pm 0.01 ^{##}
Quadriceps (mg)	122.9 \pm 2.6	117.0 \pm 4.2	90.1 \pm 2.1 ^{##}
Food (g)	3.84 \pm 0.55	3.58 \pm 0.34	3.64 \pm 0.47
Water (ml)	3.92 \pm 0.13	3.61 \pm 0.13	3.80 \pm 0.21

Values are expressed as mean \pm SEM

* $P<0.05$, significantly different from control group

[#] $P<0.05$, significantly different from cancer group

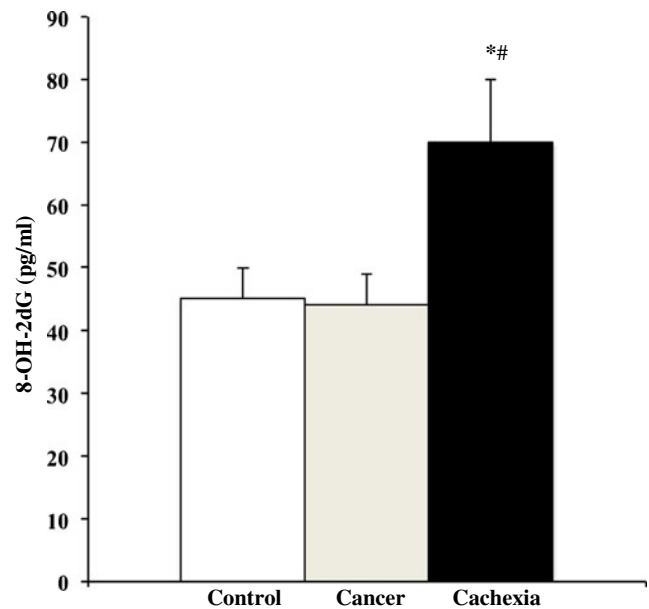


Fig. 1 DNA oxidation by-product 8-hydroxy-2-deoxyguanosine concentration in control, cancer and cachexia. Values are expressed as mean \pm SEM. Significantly different from control group ($P<0.02$). #Significantly different from cancer group ($P<0.05$)

abundance in the cancer cachectic hearts compared with both the control and cancer groups (Table 3).

Subsequently, the XDH enzymatic pathway known to produce ROS was investigated. The RT-PCR results showed a significant twofold higher level of XDH mRNA ($P=0.04$) in the heart of cachexia mice in comparison to control and cancer groups (Table 3). Other possible contributors to the state of

Table 3 Gene and protein expression data

Gene	Control ($n=8$)	Cancer ($n=12$)	Cachexia ($n=16$)
MURF-1	2.5 \pm 1.0	1.8 \pm 0.5	4.0 \pm 1.0 [#]
MAFbx	1.4 \pm 0.8	1.6 \pm 0.9	6.3 \pm 2.0 ^{##}
NOX1	482 \pm 117	440 \pm 50	343 \pm 52
NOX2	176 \pm 48	177 \pm 32	99 \pm 21 [#]
NOX4	16 \pm 7	8 \pm 3	9 \pm 2
P67	73.1 \pm 12.3	87.4 \pm 15.2	89.6 \pm 9.3
P40	865 \pm 125	452 \pm 23	263 \pm 49
CuZn SOD	490 \pm 106	587 \pm 168	458 \pm 115
Mn SOD	412 \pm 73	431 \pm 73	666 \pm 215
XDH	453 \pm 69	469 \pm 69	1421 \pm 69 ^{##}
Protein expression			
p47 ^{phox}	1.6 \pm 0.5	2.0 \pm 0.4	2.2 \pm 0.6
NOX2	0.9 \pm 0.1	0.6 \pm 0.1	1.0 \pm 0.3

Gene expression values are expressed as arbitrary units, mean \pm SEM. Protein expression values are expressed as, band intensity (square millimetres) adjusted for GAPDH, mean \pm SEM

* $P<0.05$, significantly different from control group

[#] $P<0.05$, significantly different from cancer group

oxidative stress observed in the cachectic hearts may have been the suppression of the antioxidant enzyme, superoxide dismutase (SOD). Mitochondrial and cytoplasmic SOD displayed no change in mRNA levels (Table 3). However, further investigation of total SOD enzyme activity showed a significant 1.5-fold lower activity in mice with cachexia compared with control ($P=0.02$) and cancer ($P=0.001$, Fig. 2).

Accompanying a state of oxidative stress in cancer cachectic hearts were higher levels of expression of the major muscle proteolytic pathway, the UPS. MURF-1 and MAFbx mRNA expression was significantly higher, 4.3-fold ($P=0.04$) and 3.8-fold ($P=0.04$), respectively, in the hearts of the cachexia mice compared with the cancer group (Table 3).

4 Discussion

The cardiac atrophy secondary to CC observed in this study is associated with a pro-oxidative shift and consequential oxidative myocardial tissue damage. Furthermore, upregulation of the key ubiquitin ligases in cachectic hearts suggests induction of the UPS may be in response to oxidative stress and subsequent protein damage.

The current study demonstrated the importance of cardiac atrophy as a hallmark of the disease with an average 20 % decrease in heart muscle weight compared with controls.

The consequence of such cardiac atrophy was demonstrated by Tian et al. who used echocardiography to analyse the function of cachectic hearts in a mouse model of cancer cachexia [4]. Their study found that, 14 days after the inoculation

with tumour cells, the hearts of cancer cachectic mice had reduced heart rate and fractional shortening [4]. To date, the mechanisms influencing cardiac tissue wasting in a state of CC are largely unknown. However, previous studies have shown that patients and animals with CC all show degrees of oxidative stress [21, 22]. To elucidate whether a state of oxidative stress exists in the heart of mice afflicted with CC, the current study measured levels of 8-OH-2dG and found a significantly higher levels in the cachexia group hearts. The higher 8-OH-2dG suggests that the activation of pro-oxidant pathways in atrophied hearts of cachectic mice. Importantly, both the release of cytokines such as TNF- α and tumour-derived factors such as PIF have been shown to have pro-oxidant effects [20, 21]. Gomes-Marcondes and Tisdale used myotubes in cell culture exposed to FeSO_4 and H_2O_2 which showed that key components of the ubiquitin proteasome system were upregulated by oxidative stress [20]. NOX2 mRNA expression was lower in cachexia hearts compared with the control group, while the Western blot data were unchanged. The results suggest a decreased role for NADPH oxidase in contributing to cellular oxidative stress in cachectic hearts. The results are also in keeping with a previous study by Sullivan-Gunn showing a decrease in NOX2 levels in skeletal muscles of cancer cachectic mice [23]. The animals in this study were allowed to lose up to 25 % of their initial body weight, thus constituting late-stage cachexia. Considering that NADPH oxidase enzyme activity has been shown to degrade cultured myotubes in vitro [23], a time course study would be beneficial in understanding whether the current results are specific to cardiac atrophy in late cachexia. Another key pathway of ROS formation in the myocardium is XDH that was measured using RT-PCR. The results showed a twofold higher level of XDH in the cachexia group hearts. The activation of XDH has been implicated in a number of disease states involving the myocardium such as heart failure and myocardial dysfunction [16, 17]. A study by Ekelund et al. highlighted the importance of XO in cardiac disease by showing that inhibition of the enzyme by allopurinol is capable of increasing left ventricular function and decreasing myocardial oxygen consumption in a dog model of heart failure [24].

In evaluating the potential contribution of antioxidant systems in the cachectic heart, SOD was analysed both at a gene and enzyme activity level. The SOD enzyme is a key endogenous antioxidant in the myocardium for the elimination of superoxide and has been implicated in the pathogenesis of CC [25]. Interestingly, while the cytosolic and mitochondrial SOD mRNA levels were unchanged in cachectic hearts, total SOD enzyme activity was significantly lower compared with controls. This suggests that the SOD enzyme is not compensating for increased levels of ROS. Thus, the concurrent higher levels of XDH and lower SOD activity may significantly contribute to a disruption in the balance between pro-oxidant and antioxidant mechanisms, leading to a state of oxidative stress as evidenced by greater oxidative damage to DNA (8-

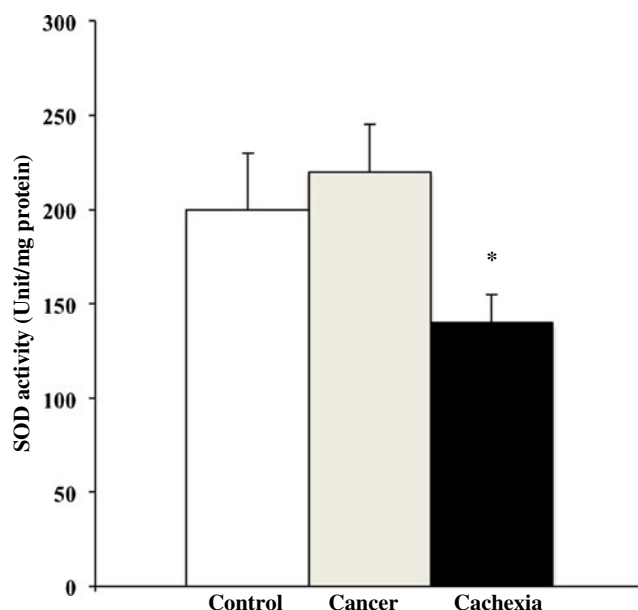


Fig. 2 Total SOD activity in control, cancer and cachexia. Values are expressed as mean \pm SEM. *Significantly different from control group ($P < 0.05$). #Significantly different from cancer group ($P < 0.05$)

Oh-2dG) in the cachectic heart. With respect to how oxidative stress may contribute to cardiac atrophy in CC, it is currently unknown; however, evidence suggests induction of the UPS may be an important link between oxidative stress and atrophy in cachectic muscle [20]. Otsuka et al. found that in patients' hearts undergoing dilated cardiomyopathy, oxidative stress and the UPS activation are colocalised [26]. Increases in levels of XO and altered SOD enzyme function in the myocardium are traditionally linked to cardiac fibrosis and cardiomyopathies [16, 27]. However, the current study shows an association between oxidative stress and cardiac atrophy, suggesting an alternative pathologic role for oxidants in the myocardium. In elucidating the mechanisms by which ROS contributes to the loss of lean body mass, Gomes-Marcondes and Tisdale attempted to replicate the oxidative stress seen in cancer cachexia by using murine myotubes in cell culture [20]. The myotube cells were exposed to H₂O₂ which induced oxidative stress. Their results showed that the induction of oxidative stress caused an increase in the expression of important UPS elements including the 20S proteasome and the E2_{14k} conjugating enzyme [20].

The UPS E3 ligases MURF-1 and MAFbx are expressed in cardiomyocytes and are an important component of cardiac protein turnover [28]. Increased mRNA expression of these ligases is linked to the degradation of cardiac troponin I, α -actin-2 and MyoD leading to impaired contractility [13, 29]. This study shows that both MURF-1 and MAFbx are upregulated at the mRNA level (Table 3) which is in keeping with results seen in studies analysing cachectic heart muscle tissue [30]. Interestingly, Cosper and Leinwand using CD2F1 hybrid mouse model of CC did not see any change in the UPS but found an upregulation in autophagy-related protein degradation [2]. However, the study by Tian et al. using a mouse model of CC similarly to this study found that mRNA of UPS E3 enzymes to be increased in cachectic hearts [30]. Furthermore, Tian and colleagues found that there was increased protein ubiquitinylation in the hearts of cancer cachexia mice compared with controls suggesting that indeed the UPS has an important role to play in cardiac atrophy secondary to CC [30]. A limitation of the current study is the changes in MURF-1 and MAFbx that are at the mRNA level and so correlative in nature. Future investigations would include the evaluation of UPS activation, the state of activation of other proteolytic systems and the phosphorylation levels of Akt and Foxo proteins upstream of ubiquitin ligase activation.

5 Conclusion

The current study used a mouse model of CC producing significant body weight loss and a reduction in heart mass by 20 %. The experimental data demonstrated that cardiac

atrophy of cachectic mice is related to a state of oxidative DNA damage in the myocardium. Levels of XDH mRNA were higher in CC hearts, suggesting that XO may have an important role to play in producing oxidative stress. Most evidently, it appears that the combination of higher XDH expression and lower SOD enzyme activity are key contributors to oxidative stress and cardiac tissue damage in cancer-induced cardiac atrophy. Oxidative stress in the myocardium as with skeletal muscle may also induce increased expression of the E3 ligases MURF-1 and MAFbx as seen in higher mRNA levels of both in this study. Further research is needed in a time-dependent manner to ascertain whether these changes are seen in early cachexia and are indeed initiating mechanisms.

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Conflict of interest E. Hinch, M. Sullivan-Gunn, V. Vaughan, M. McGlynn and P. Lewandowski declare that they have no conflict of interest.

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