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Oral resveratrol therapy inhibits cancer-induced skeletal muscle and cardiac atrophy in vivo

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Abstract

The mechanism by which cancer mediates muscle atrophy has been delineated in the past 3 decades, and includes a prominent role of tumor-derived cytokines, such as IL-6, TNFα and IL-1. These cytokines interact with their cognate receptors on muscle to activate the downstream transcription factor NF-κB and induce sarcomere proteolysis. Experimentally, inhibiting NF-κB signaling largely prevents cancer-induced muscle wasting, indicating its prominent role in muscle atrophy. Resveratrol, a natural phytoalexin found in the skin of grapes, has recently been shown to inhibit NF-κB in cancer cells, which led us to hypothesize that it might have a protective role in cancer cachexia. Therefore, we investigated if daily oral resveratrol could protect against skeletal muscle loss and cardiac atrophy in an established mouse model. We demonstrate resveratrol inhibits skeletal muscle and cardiac atrophy induced by C26 adenocarcinoma tumors through its inhibition of NF-κB (p65) activity in the skeletal muscle and heart. These studies demonstrate for the first time the utility of oral resveratrol therapy to provide clinical benefit in cancer-induced atrophy through the inhibition of NF-κB in muscle. These findings may have application in the treatment of diseases with parallel pathophysiologies such as muscular dystrophy and heart failure.

Keywords

resveratrol; cancer cachexia; skeletal muscle; cardiac; atrophy; NF-κB inhibition

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Conflict of interest

S.S., M.C., K.M., L.W., X.Y., J.R., D.G., M.W. declare no conflicts of interest.

Introduction

Cancer cachexia is a wasting syndrome that affects as many as 80% of cancer patients with advanced disease [1–3]. While cancer cachexia is estimated to be the cause of death in more than 20% of all cancer patients, the morbidity and collapse in the quality of life affects nearly all patients [2, 4, 5]. Clinically, the manifestations of cancer cachexia are well established and include loss of skeletal muscle mass and adipose tissue, anorexia, asthenia, and anemia with considerable alterations in lipid, carbohydrate and protein metabolism [2, 5, 6]. Patients remain in a hypermetabolic state as the tumor burden increases, due to the tumor production of catabolic pro-inflammatory cytokines [5]

Cancer cachexia is a complex metabolic disorder which diminishes lean body mass in response to increases in tumor-released inflammatory cytokines, including TNFα, IL1, and IL-6 [7, 8]. These tumor derived cytokines interact with their receptors on striated muscle to activate the transcription factor NF-κB to enhance muscle atrophy, by enhancing the degradation of the sarcomere[9–12]. The accelerated protein degradation and proteolysis of the contractile apparatus is mediated by the ubiquitin proteasome system, including specific ubiquitin ligases in muscle such as muscle ring finger-1 (MuRF1)[7, 13]. Inflammatory cytokine-driven NF-κB activation is central in muscle atrophy found in a number of diseases including muscular dystrophy, heart failure, sepsis, and cancer [14–16]. Pharmacologic and genetic inhibition of NF-κB prevents ubiquitin-dependent muscle wasting [10, 17] and can lead to retention of muscle mass, strength, and can promote muscle regeneration [18–20]. Consequently, inhibition of NF-κB activity has enormous potential for the prevention and treatment of muscle atrophy.

Resveratrol, a natural phytoalexin found in the skin of grapes, has recently been shown to have beneficial effects on a variety of disease processes, including hepatocellular carcinoma[21] and neurodegenerative disease [22]. Resveratrol's activity in these diseases has been due, in part, to its ability to inhibit NF-κB nuclear translocation and accumulation. It does this by primarily inhibiting IκB kinase (IKK), whose activation is necessary for NF-κB activity. In the present study, we test the hypothesis that resveratrol treatment inhibits cancer-induced cachexia by inhibiting NF-κB to protect against cancer-induced cardiac atrophy *in vivo*.

Materials and Methods

Cell Lines

The transplantable C-26 adenocarcinoma cells were maintained as previously described [23, 24]

Animals and Tumor Implantation

Ninety-six female CD2F1 mice aged 45 to 70 days (weight 19 to 22 grams) were obtained from the Charles River Laboratories (Wilmington, MA) and randomly divided into four groups: 1) control mice, 2) control mice treated with resveratrol (100 mg/kg, 200 mg/kg, or 500 mg/kg), 3) tumor-bearing mice, and 4) tumor-bearing mice treated with resveratrol (100 mg/kg, 200 mg/kg, or 500 mg/kg). On day 0, the mice selected to go in the tumor-bearing groups were injected subcutaneously in the right flank with 150 μL (approximately 750,000 cells) of C-26 adenocarcinoma cells.

Treatment with Resveratrol

Trans-resveratrol (Cayman Chemical, Ann Arbor, MI, Catalog #70675) was solubulized in 1.5% methylcellulose./ 0.2% Tween-20 with vigorous vortexing. Beginning on day 6, mice

received daily single oral treatment delivered intragastrically using a 20 g gavage needle. Resveratrol treated mice received daily single gavage treatment of 100 mg/kg, 200mg/kg, or 500mg/kg resveratrol, while controls received a comparable treatment of 250 μl of vehicle alone.

Body Composition Analysis

Prior to tumor injection, all animals underwent body composition analysis using quantitative magnetic resonance systems (QMR) echo MRI (EchoMRI-100, Echo Medical Systems, Houston TX).

Echocardiography

Echocardiography was performed on conscious mice using a Visual Sonics Vevo 770 ultrasound biomicroscopy system as previously described by our laboratory [25].

Total RNA isolation/Real Time PCR determination of mRNA Expression

Total RNA was isolated from flash frozen tissue using Trizol (Sigma Chemical, St. Louis, MO), cDNA made using High Capacity cDNA Archive kit (Applied Biosystems). Semi-quantitative RT-PCR was performed using an MJ Research Inc. Thermo Cycler (Waltham, MA) and New England Biolab (Ipswich, MA) reagents using the primers detailed in supplemental data.

NF-κB activity assay

Nuclear extracts were isolated from heart and gastrocnemius muscle immediately after harvest and NF-κB activity determined according to the manufacturer's protocol (Cayman Chemical, Ann Arbor MI).

Additional materials and methods can be found in the supplemental data section.

Results

Resveratrol showed no evidence of drug induced toxicity at the levels used in this study

Pharmacological studies detailing the metabolism of resveratrol both in animal and humans have been carried out, and all indicate rapid absorption, distribution, metabolism and excretion of resveratrol [26, 27]. After oral administration, wide tissue distribution in rats has been observed after 24 hours and significantly decreases after 72 hours.[28] Toxicological studies have also been completed with tolerance of dosages as high as 700 mg/kg in rats [28] without toxic effects, and several Phase I clinical trials are underway at oral doses as high as 100 mg/kg/day in humans[26, 28]. We therefore dosed resveratrol orally at 100–500 mg/kg/day in the present study. We monitored toxicity by histology and lethality throughout the study. No events surrounding drug delivery were noted, including any obvious toxicity or lethality caused by daily oral gavage of resveratrol 100–500 mg/kg/day. Additionally, we did not observe any drug-induced hepatotoxicity or nephrotoxicity across all doses of resveratrol delivery, as confirmed by a blinded, independent pathologist on histological examination (Supplemental Figure 1).

Resveratrol protects against C26 adenocarcinoma-induced weight loss in mice

We assayed resveratrol's ability to prevent cancer-induced skeletal and cardiac muscle atrophy in the C26-adenocarcinoma mouse model[29] previously described by our laboratory [24]. Cultured C26 adenocarcinoma cells were injected into the back flank where it develops into a tumor and induces muscle wasting in CD2F1 mice within 2 weeks. In the present study, tumors were palpable by day 6 after inoculation in the tumor bearing subset

mice. On Day 17, at completion of the study, there were no significant differences in tumor weights among all tumor bearing mice (Figure 1A). Tumor bearing mice had a mean weight loss of 8.8±1.7% from their baseline weights, while control mice gained 2.1±1.8% over the experimental period (Figure 1B), despite all groups having an equivalent food intake (Figure 1C). In contrast, mice challenged with tumors and treated with 200 mg/kg of resveratrol gained weight similar to the control group. Mice treated with 500 mg/kg and challenged with tumor also maintained their weight compared to tumor bearing mice (Figure 1B). Mice with tumors treated with 100 mg/kg of resveratrol were not protected against the tumor-induced cachexia, losing 4.6 ± 2.9% of their body weight from baseline. These findings demonstrate that resveratrol at doses >/= 200 mg/kg protects against tumor-induced weight loss in this mouse model.

To delineate if resveratrol had any independent affects on muscle mass, control mice without tumor were treated with 100–500 mg/kg in parallel with treated mice challenged with tumor. We found that non-tumor control mice treated with resveratrol alone exhibited body weights equal to control mice at lower doses (100 mg/kg, 200 mg/kg). At the highest dose tested (500 mg/kg), mice lost weight compared to age-matched controls (Figure 1B). These findings demonstrate that 500 mg/kg resveratrol may cause some weight loss in the absence of cachexia, while the lower levels (100–200 mg/kg) do not affect body weight. None of the resveratrol doses tested cause an increase in muscle mass, suggesting that the protective effects of resveratrol were not due to independent increases in muscle mass.

Resveratrol protects against cachexia-induced lean body and fat mass loss

Cancer cachexia is characterized by the loss of both skeletal muscle mass and adipose tissue resulting from alterations in protein and lipid metabolism. [2, 5, 6] We next determined the body composition of mice challenged with tumors in the presence or absence of resveratrol by MRI body composition analysis to determine lean body mass (LBM) and fat mass (FM). We identified that 200 and 500 mg/kg of resveratrol significantly protected against tumor-induced loss of LBM and FM composition in tumor-bearing mice (Figure 2A and 2B). Compared to controls, untreated tumor bearing mice lost 5.3% LBM (16.1 ± 0.3 vs. 17.1 ± 0.1 g) in contrast to resveratrol-treated tumor bearing mice, which were completely protected (LBM (200 mg/kg)=17.4 ± 0.19 g ; LBM (500 mg/kg)=17.3 ± 0.3 g). Untreated tumor bearing mice lost 33.3% of their limited FM, compared to control mice (1.80 ± 0.15 vs. 3.1 ± 0.10 g). Tumor-challenged mice treated with 200 and 500 mg/kg were significantly protected (2.7 ± 0.36 g and 2.4 ± 0.17 g, respectively). Resveratrol treatment given to control mice without tumors did not affect LBM or FM. Since resveratrol did not cause increases in total body mass (including LBM and FM) in the absence of tumor, these studies indicate that resveratrol protection against tumor induced weight loss is not due to its ability to increase muscle weight, LBM, or FM by itself.

Resveratrol inhibits tumor-induced gastrocnemius and cardiac atrophy

Isolated quadriceps and the heart were collected and weighed immediately after harvest. Tumor bearing mouse quadriceps weighted significantly less (~23%) than corresponding quadriceps muscle from control mice (Figure 3A). Isolated quadriceps muscle from control mice treated with 100 mg/kg, 200 mg /kg and 500 mg/kg resveratrol were not significantly different from control mice (Figure 3A). In contrast, quadriceps muscle from tumor-challenged mice treated with 200 mg /kg and 500 mg/kg resveratrol were significantly higher (16.6 ± 6.5%, 10.3 ± 4.9%, respectively) than untreated tumor-challenged mice (Figure 3A), suggesting that >= 200 mg/kg resveratrol protected against tumor-induced skeletal muscle atrophy. Tumor-bearing mice demonstrated significant hind limb atrophy as evidenced by a 12.2% loss of mass compared to non-tumor-bearing controls (Figure 3B). To determine a more general effect on skeletal muscle, we also weighed hind limb mass. Hind

limb muscle mass was preserved in resveratrol treated (200 and 500 mg/kg) tumor-bearing animals as evidenced by significant differences in treated tumor-bearing mice having a 12.4 ± 5.7% and 9.2 ± 4.3% higher mass, respectively, than untreated tumor challenged mice. Resveratrol treatment in control mice did not significantly differ from control mice at all 3 doses tested.

Tumor bearing mice exhibited a significant loss of total heart weight compared to control mice. Treatment with 200 and 500 mg/kg was protective against this 8.6% total heart weight loss identified after 17 days of tumor burden (Figure 3C). Treatment of control mice with 100 mg/kg, 200 mg/kg, 500 mg/kg of resveratrol did not affect total heart weight (Figure 3C). To further characterize the effects of resveratrol on the heart, we performed in vivo trans-thoracic echocardiography on conscious mice from control, tumor-challenged with and without 500 mg/kg resveratrol (Figure 4A, 4B, Table 1). We identified that 17 days of tumor burden induced a decrease in anterior and posterior wall of the left ventricle, reflecting the cardiac atrophy identified by a decrease in heart weight / body weight ratio and relative wall thickness (Figure 4). In our first set of studies, mice challenged with tumor and assayed by echocardiography lost approximately 8.8% of their body weight after 17 days of tumor (Figure 1B). However, in our echocardiography studies, they lost only 6.8% (=21.8–20.4/21.8) (tumor vs. control, Table 1). This may be due to the sample size and tumor size variation we seen in individual mice. Treatment of tumor challenged mice with 500 mg/kg resveratrol completely prevented the decrease in anterior and posterior wall thickness (Figure 4A, 4B, Table 1). Treatment of control mice with resveratrol did not affect baseline LV wall thickness or heart weight (Figure 4A, Table 1), suggesting that protection resveratrol afforded the heart was not drug induced increases in muscle mass, but more likely due to an inhibition of the signaling pathways mediating cardiac atrophy.

Resveratrol inhibits NF-κB transcription factor activity in tumor induced skeletal muscle and heart atrophy

The C-26 adenocarcinoma tumor induces cachexia through its release of TNFα, IL-1, IL-6, which interact with their cognate receptors on muscle to activate the downstream transcription factor NF-κB[30–34], to induce cachexia and muscle atrophy. Recent studies have demonstrated experimentally the dominant role of NF-κB in muscle atrophy [18–20, 35, 36] and resveratrol's ability to inhibit NF-κB in cancer cells [37]. Therefore, we investigated if resveratrol's inhibition of tumor-induced gastrocnemius and heart atrophy was due in part to its inhibition of NF-κB (Figure 5). We identified that nuclear bound NF-κB (p65 subunit) activity was significantly elevated in tumor-induced gastrocnemius and heart, and completely reversed in mice treated with the highest levels of resveratrol (200 mg/kg and 500 mg/kg) (Figure 5A, 5B). Gastrocnemius muscle from untreated tumor bearing mice had 30.5% higher mean NF-κB activity compared to control mice (Figure5A). A significant decrease in NF-κB (p65) activity was seen in tumor bearing mice given 200 and 500 mg/kg of resveratrol (decreased 39% and 51%, respectively) (Figure5A). Treated control mice also had a significant decrease in NF-κB (p65) activity after treatment with 200 and 500 mg/kg dosage (decreased 34%, and 41%, respectively) compared to untreated tumor challenged mice, indicating that resveratrol decreased endogenous NF-κB activity induced by tumor in skeletal muscle.

Analysis of the heart revealed that tumor challenge induced a 19% increase in NF-κB activity compared to control hearts (Figure 5B). Treatment of tumor bearing mice with 200 and 500 mg/kg resveratrol significantly decreased NF-κB (p65) activity compared to tumor challenged mice (21.6%, and 31.2%, respectively) (Figure 5B). Treatment with 200 and 500 mg/kg resveratrol induced a significant reduction in NF-κB (p65) activity in control mice (18.7% and 33.8%, respectively) compared to untreated tumor challenged mice. Recent studies have identified that NF-κB induction of E3 ubiquitin ligases regulate atrophy is

skeletal muscle[10, 11]. Furthermore, our laboratory has determined a critical role of the cardiac ubiquitin ligase muscle ring finger-1 (MuRF1) in cardiac atrophy [25]. Specifically, we identified that mice lacking MuRF1 were essentially unable to atrophy in response to dexamethasone [25]. This contrasts with skeletal muscle, where both MuRF1 and Atrogin-1 play a partial role in inhibiting skeletal muscle atrophy [13]. We next focused our investigation on how resveratrol's inhibition of NF-κB might affect cardiac MuRF1 expression in vivo.

Resveratrol inhibits cardiac MuRF1 mRNA expression

Tumor challenge induced MuRF1 mRNA expression in the heart significantly (~20%) after 17 days of tumor (Figure 5C). While resveratrol treatment did not affect MuRF1 expression in control animals, 200 and 500 mg/kg resveratrol significantly inhibited the tumor-induced MuRF1 compared to tumor challenge alone (~25% and ~75%, respectively), reaching MuRF1 mRNA levels significantly less than that found in control animals (Figure 5C). Since MuRF1 plays a pivotal role in cardiac atrophy in vivo [25], and MuRF1 is regulated in part by NF-κB [10, 11], these findings suggest one mechanism by which resveratrol inhibits tumor-induced cardiac atrophy by the inhibition of NF-κB-induced MuRF1 expression in vivo.

Discussion

The C26-adenocarcinoma model of cancer cachexia has been used over the last 3 decades for research on the natural history of carcinomas and anti-tumor therapy, as recently reviewed by Aulino, et al., 2010 [29]. Nearly 190 papers can be found by searching PubMed for C26 cancer, many focusing on C26-induced cachexia [29]. The C26-adenocarcinoma model was established in 1975 in an effort to create models which could be used to test biological- and chemo-therapies [38]. It can be established in nude and SCID mice, as well as wild type mice, suggesting that lymphocytes play little, if any, role in the pathophysiology of tumor progression [31]. The predominant mechanism by which the C26-adenocarcinoma cell line induces cachexia is through its release of inflammatory cytokines [31], paralleling the mechanism by which cancer causes cachexia in humans [8].

Initial studies of the C26-adenocarcinoma mouse model, as used in the present study, identified a prominent role of IL-6 in the induction of muscle atrophy. In these studies, inhibiting IL-6 using cytokine specific reagents prevented muscle atrophy induced in C26-adenocarcinoma bearing mice. Additional studies identified that IL-6 was not the sole mechanism for the induction of muscle cachexia [34, 39]. The C26-adenocarcinoma cells were also found to secrete TNFα, IL-1, and IL-6 in vivo, along with yet to be identified cachexia factors that induce skeletal muscle atrophy in vivo [31, 33]. These pro-inflammatory cytokines bind to their cognate receptors found on skeletal muscle and heart, to activate the transcription factor NF-κB [40], which mediates their downstream effects. The activation of NF-κB is essential to the process of atrophy in striated muscle. This has been proven convincing by using dominant negative inhibitory NF-κB complexes (IKK), which effectively inhibits NF-κB. Over-expression of a dominant negative IKKα/β-EGFP fusion protein, which inhibits NF-κB markedly inhibited disuse induced atrophy up to 70% in vivo [17]. Other studies have also identified that pharmacologic or genetic inhibition of IKK/NF-κB similarly inhibits ubiquitin dependent muscle wasting (proteolysis) [10]. In these studies, NF-κB inhibition inhibited the expression of the ubiquitin ligase MuRF1, one of two ubiquitin ligases that are necessary for skeletal muscle atrophy to occur [10].

Proinflammatory mediators, such as TNFα, are potent inducers of the ubiquitin ligase MuRF1 [13]. Recent studies have shown that MuRF1 expression is regulated by the transcription factor NF-κB [10, 41], which is activated by a number of proinflammatory cytokines, including TNFα. Similarly, the C26-adenocarcinoma tumor in mice induces

protein catabolism by inducing the ubiquitin ligase MuRF1 [42]. C26 burden induces specific loss of thick, but not thin filament sarcomere components [24] and alters myosin isoform expression [43], consistent with the actions of MuRF1 [44]. The dystrophin complex is decreased in C26 adenocarcinoma-bearing mice, a phenomenon essential for muscle degradation [42]. This model has allowed the demonstration of the role of stem cells in skeletal muscle during cachexia[45, 46].

While MuRF1 plays only a partial role in mediating skeletal muscle atrophy in vivo [13], our laboratory has recently identified that MuRF1 activity is necessary to induce ANY cardiac atrophy [25]. Specifically, we identified that the significant dexamethasone-induced atrophy induced in wild type hearts is essentially absent in MuRF1 −/− mice [25]. We therefore focused our present studies on the ubiquitin ligase MuRF1 expression in the heart. We hypothesized that resveratrol's inhibition of cardiac NF-κB would reduce cardiac MuRF1 expression, representing one mechanism by which cardiac atrophy is inhibited in the C26-adenocarcinoma-induced cachexia mouse model. In fact, we did identify that resveratrol inhibited tumor-enhanced MuRF1 expression in vivo (Figure 5C). Therefore, our findings are consistent resveratrol's prevention of cardiac atrophy to be due, in part, to the inhibition of MuRF1, possibly through its inhibition of NF-κB. However, MuRF1 is also regulated by other transcription factors such as FOXO transcription factors [47–49], so it is possible that resveratrol inhibits MuRF1 by mechanisms other than NF-κB in the heart. The findings in the present study demonstrate resveratrol protects against cancer induced cardiac atrophy by inhibiting NF-κB and MuRF1, suggesting one or more mechanisms by which it is cardioprotective.

Cachexia in human cancer patients presents as muscle loss, often with the loss of fat associated with anorexia, inflammation, and insulin resistance [50]. Previous studies have demonstrated that C26-adenocarcinoma induced cachexia induces a loss of both lean muscle and fat, as we identified in the present study [51, 52]. The details of how cachexia causes fat loss has not been completely delineated, but increased expression of hormone-sensitive lipase is a down-stream effect of factors secreted by tumors [50]. While it might be possible inflammatory cytokines and NF-κB may mediate this in muscle, not enough about these complex signaling pathways in adipocytes is currently known to support this hypothesis [53].

One of the strengths of the current study is that resveratrol did not affect the C26-adenocarcinoma tumor size, allowing us to determine the resveratrol's efficacy specifically at the level of skeletal muscle and heart. However, it is important to note that resveratrol has been reported to inhibit tumor growth, including multiple myeloma cells (IM-9 cell line), HELA cells (cervix carcinoma), and K-562 cells (chronic myeloid leukemia)[37, 54, 55]. In fact, the efficacy of NF-κB inhibition by resveratrol to protect against skeletal muscle loss induced by the MAC16 tumor (using 1 mg/kg/day) in a previous study by Wyke et al.[56] could be attributed to its inhibition of tumor growth itself, making the present study unique from a mechanistic point of view. The ability of resveratrol to inhibit tumor growth itself may be of great benefit to patients with cachexia. Along with our present study, these findings indicate that resveratrol protects against cancer cachexia by 2 distinct mechanisms: 1) by inhibiting tumor growth directly, inducing cell death resulting in a reduction of total cytokine release; and 2) by inhibiting cytokine-driven NF-κB signaling at the level of the skeletal muscle and heart. Since resveratrol does not affect the C26-adenocarcinoma tumor in the present study, resveratrol may be even more potent then the results of the present study demonstrate.

The contribution of cardiac complications in human cancer cachexia has not been studied widely. However, many of the inflammatory mediators found cachexia are found in heart failure and directly cause cardiac dysfunction, including IL-6, IL-1, and TNFα. The best

studied is TNFα, which itself can induce heart failure and has been implicated in the pathophysiology of a number of heart diseases [7]. This suggests that cardiac involvement may be common in cardiac cachexia, particularly in severe disease. Experimentally, cachexia has been shown to induce heart failure, disrupt myocardial structure, and alter composition of the contractile proteins in mouse models [43, 57]. We did not identify gross systolic dysfunction by echocardiography in the present study. However, larger tumors forming over longer periods may have led to cardiac dysfunction in our model.

In the present study, we demonstrate for the first time that resveratrol directly inhibits skeletal muscle and cardiac atrophy at oral doses of 200–500 mg/kg/day in a mouse model of tumor-induced cachexia and cardiac atrophy without any apparent toxicity to the mouse. A recent study performed by Busquets, et al. found that reseveratrol did not prevent cancer-induced skeletal muscle wasting, using a rat model with Yoshida AH-130 ascites hepatoma (treated with 1 mg/kg/day resveratrol intraperitoneally) or in mice bearing the Lewis lung carcinoma (at 5 and 25 mg/kg/day resveratrol intraperitoneally)[58]. Rats were also given oral resveratrol (3 mg/kg/day) in combination with fish oil intragastrically [58]. Note that significantly lower doses were used in the intragastric model compared to our present study (100–500 mg/kg/day). The inability of resveratrol to protect against skeletal muscle mass or body weight loss in tumor-bearing rodents may therefore be due to the lower doses of resveratrol used in this earlier study. Consistent with this hypothesis, our use of 100 mg/kg/day by oral gavage did not have any effect on muscle loss, while a dose of at least 200 mg/kg/day was necessary to protect again tumor-induced cachexia. The effective doses in the current mouse study are roughly 3 times the dose tested in humans without toxicity. In s phase I clinical trial, a single dose of 5 g/day have been given to humans without obvious toxicity. [26] In an average 70 kg person, this dose would be approximately 71 mg/kg/day (5000 mg/70 kg), or approximately 2.8 times less than the minimum effective dose found in the current studies (200 mg/kg/day vs. 71 mg/kg/day).

The effects of long term resveratrol exposure in the context of treating cancer cachexia in the present model are not known. However, it should be pointed out that mice receiving 500 mg/kg/day (Figure 1B) lost weight in the presence or absence of tumor, whereas mice receiving lower doses did not. Since these small, but significant changes were seen when 500 mg/kg/day was given over a short period of time (11 days), future studies should be careful of the potential side effects with oral resveratrol therapy (i.e. weight loss) when given at doses of 500 mg/kg/day. This would be particularly important in longer term studies lasting greater than 11 days, which is likely how this drug would be used to treat cachexia in patients.

Current therapies for cachexia include single modality therapy or combinations of progestational agents, glucocorticoids, selective COX-2 inhibitors, or non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin or indomethacin. Newer treatments have been developed to inhibit cytokines or NF-κB to some degree. These include high dose progestins[59], eicosapentaenoic acid (EPA) [60], β-Hydroxy-β-methylbutyrate (HMB) [61], thalidomide [62], and NSAID's [63]. However, these agents have adverse side effects and results have been variable and marginal in reversing cachexia clinically. Resveratrol is currently being used in a variety of human diseases, including hepatocellular carcinoma[21] and neurodegenerative disease [22] with no marked toxicity [7]. It may therefore have application to cachexia (including skeletal muscle and cardiac atrophy) and more broadly to heart failure in a variety of contexts in human disease, although this has currently not been tested directly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Non-standard abbreviations

NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
IKK	IκB kinase
MAC	murine adenocarcinoma
LBM	lean body mass
FM	fat mass
MuRF1	muscle ring finger-1
UPS	ubiquitin proteasome system

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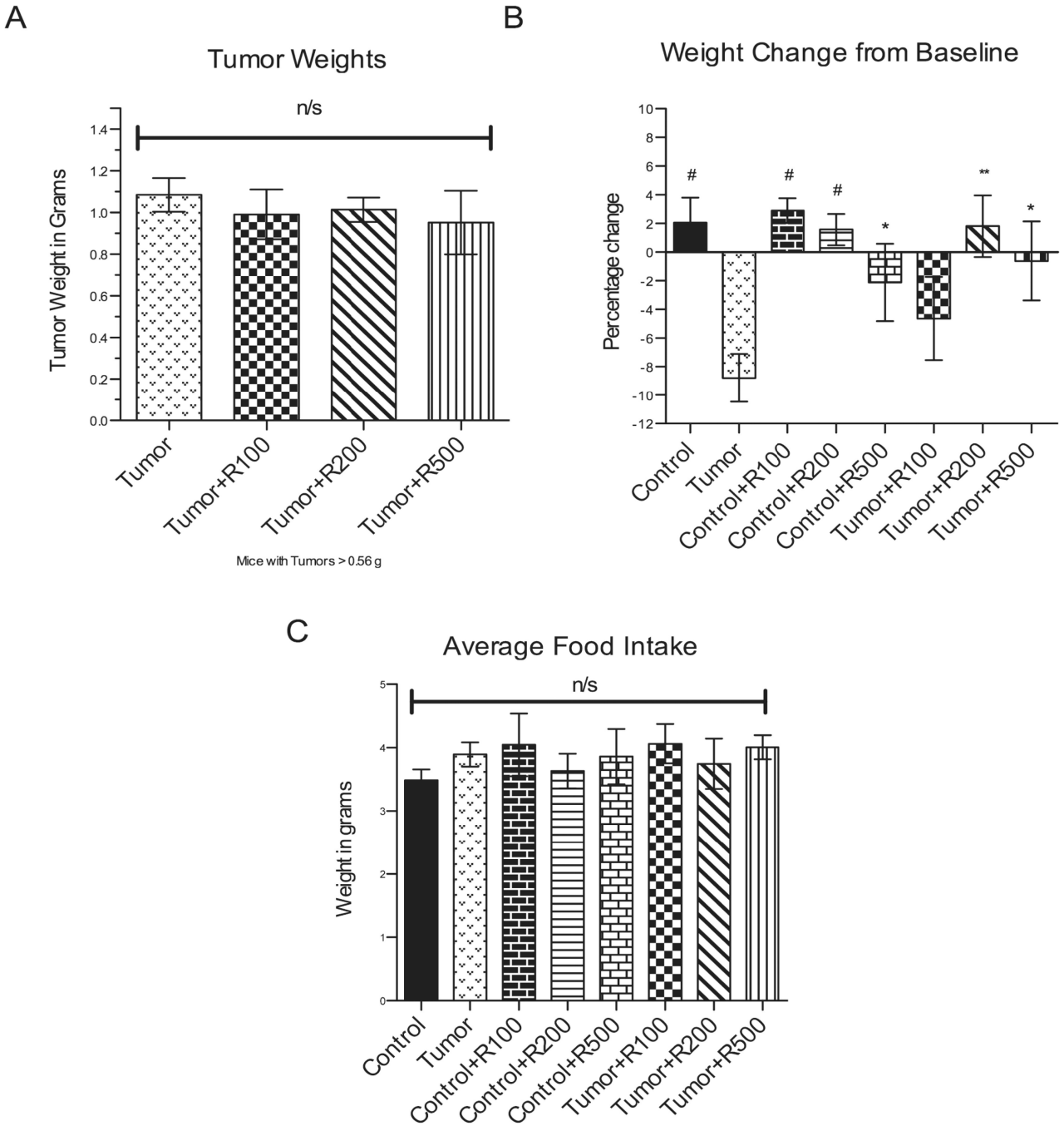

Figure 1. Final tumor weights and average food intake

(A) There were no significant differences in tumor weights amongst tumor bearing groups at all tumor weights and amongst the subset of tumors larger than 0.56 g at time of harvest using unpaired t-tests. (**B)** Significant changes in carcass weight loss was seen in the untreated tumor bearing mice compared to control mice, control mice receiving resveratrol at all doses and tumor bearing mice receiving resveratrol at the 200 mg/kg and 500 mg/kg doses. Carcass weight was determined by subtracting the weight of the tumor from the body weight. Total carcass weights were analyzed and represented by the percentage change from baseline among all groups. Also no significant differences were noted in **(C)** average food

intake per mouse per day. n/s = no significance detected ## = p <0.0001, # = p <0.001, ** = p < 0.01, * = p < 0.05. For (A) N=24 (Tumor), N=8 (Tumor +R100), N=7 (Tumor +R200), N=8 (Tumor +R500). For (B) and (C) N=24 (Tumor), N=8 (Tumor +R100), N=7 (Tumor +R200), N=8 (Tumor +R500).

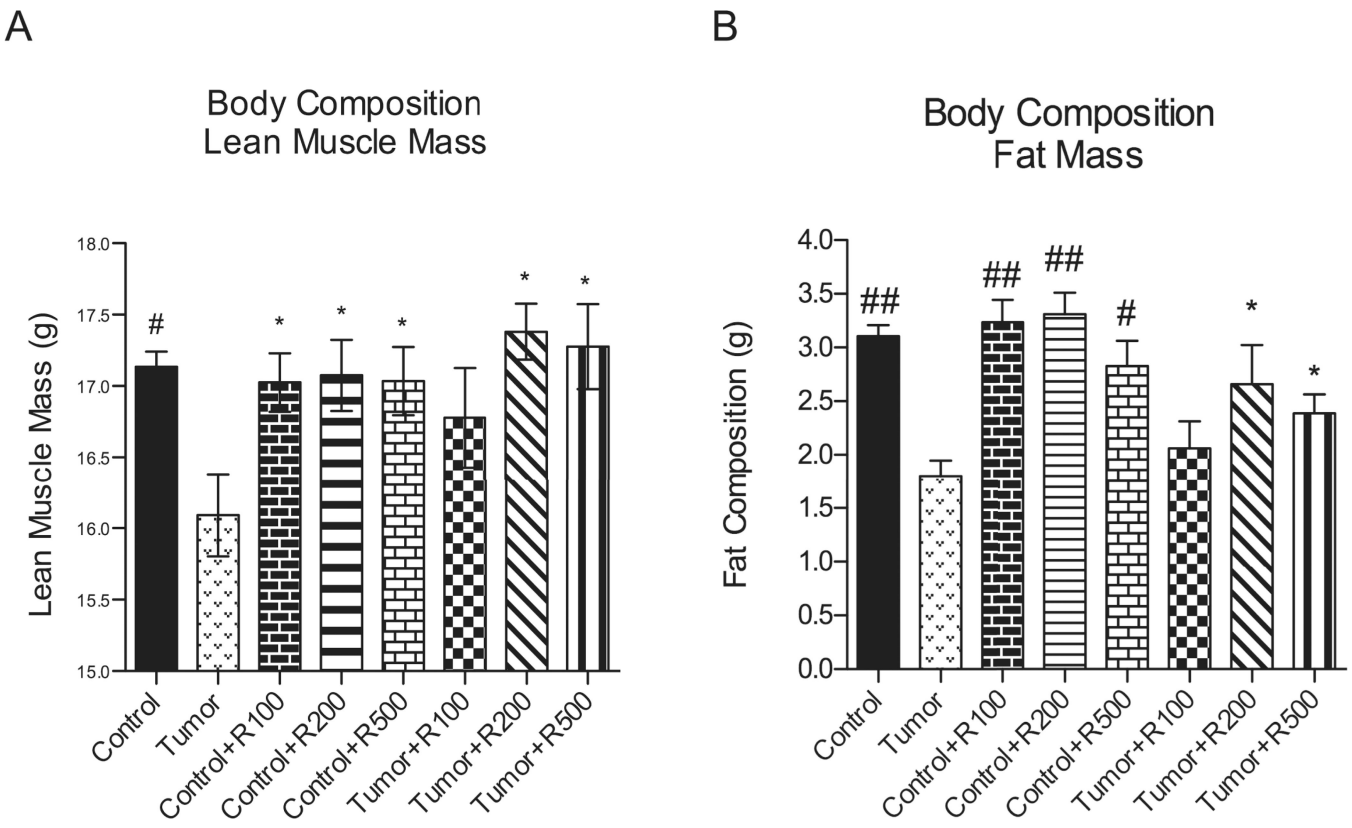

Figure 2. Final body composition among non-tumor bearing and tumor bearing mice

Whole mouse body composition was determined and graphed to show final mass in grams among all groups. **(A)** Significant lean muscle mass loss was seen in the untreated tumor bearing mice compared to control mice, control mice receiving resveratrol at all dosages and tumor bearing mice receiving resveratrol at the 200 mg/kg and 500 mg/kg levels. **(B)** Fat mass compositions were compared among the same subsets with significant fat mass loss shown in the untreated tumor bearing group. The tumor bearing groups receiving resveratrol showed significant responses by maintaining more fat mass in the 200 mg/kg and 500 mg/kg groups. The untreated and treated control groups also were significantly higher in fat mass composition compared to untreated tumor bearing. ## = $p < 0.0001$, # = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$. For (A) and (B) N=24 (Tumor), N=8 (Tumor +R100), N=8 (Tumor +R200), N=8 (Tumor +R500). For (C) N=17 (Tumor), N=6 (Tumor +R100), N=6 (Tumor +R200), N=7 (Tumor +R500).

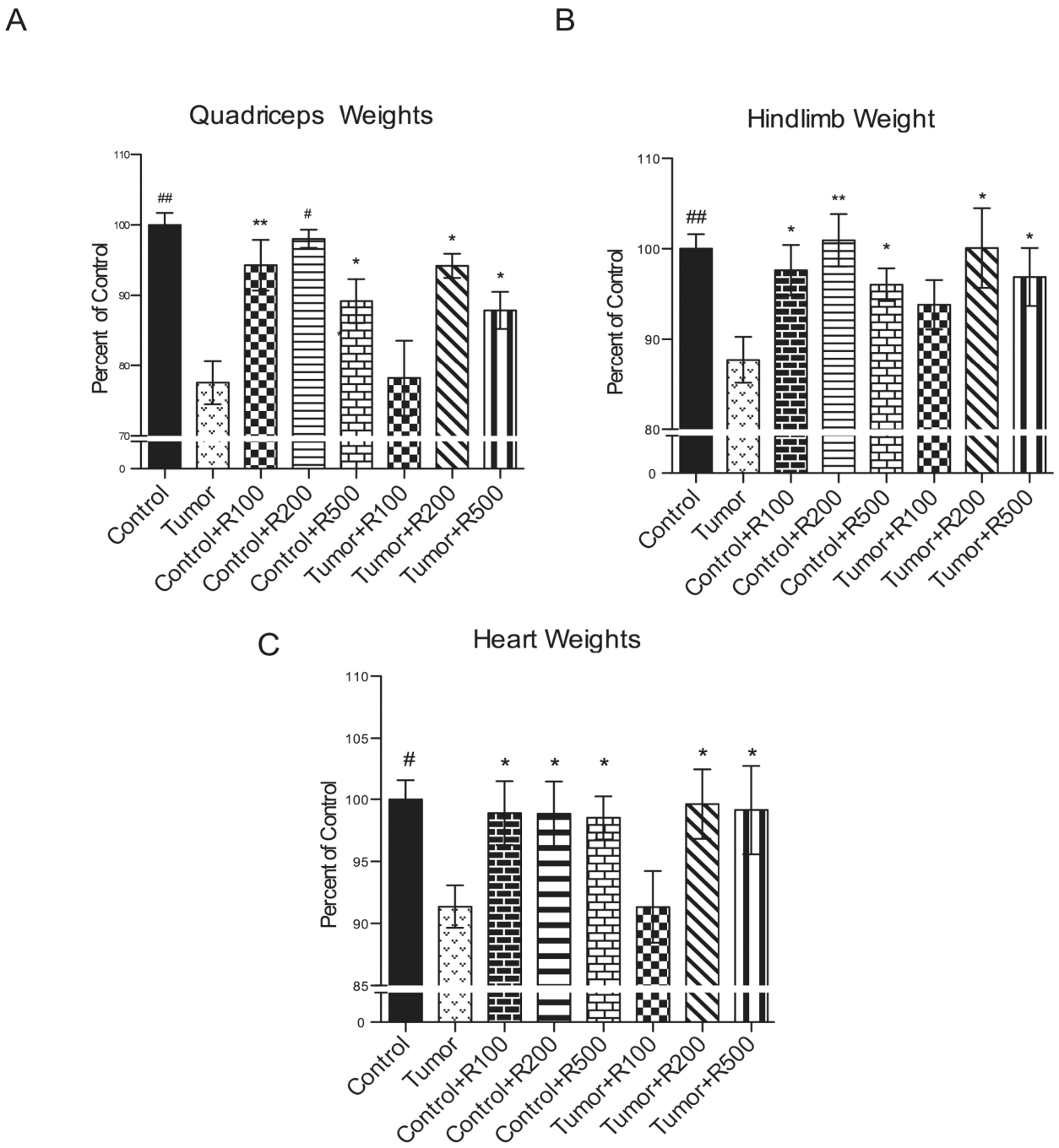

Figure 3. Final weights among non-tumor bearing and tumor bearing mice

(A) Quadriceps were individually analyzed and significant weight loss was shown in the untreated tumor bearing group compared to control, treated controls at all dosages, as well as the treated tumor bearing groups at the 200 and 500 mg/kg doses. The 100 mg/kg treated tumor bearing group was not significantly different in quadriceps weight from the non-treated tumor bearing group.**(B)** The tumor bearing groups receiving resveratrol showed significant responses by maintaining hindlimb weights comparable to control in the 200 mg/kg and 500 mg/kg groups. The untreated and treated control groups also were significantly higher in hindlimb weights compared to untreated tumor bearing mice. Hindlimb weights

were compared among the same subsets with significant weight loss shown in the untreated tumor bearing group. **(C)** Significant decreases in heart weight were seen in the untreated tumor bearing mice compared to control mice, control mice receiving resveratrol at all doses and tumor bearing mice receiving resveratrol at the 200 mg/kg and 500 mg/kg doses. Change in heart weights among non-tumor bearing and tumor bearing mice. Individual hearts were weighed and are graphically represented here to show heart weight as a percentage of average control heart weight among all groups. ## = $p < 0.0001$, # = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$. For (A)-(C) N=24 (Control), N=8 (Control +R100), N=8 (Control + R200), N=8 (Control +R500), N=17 (Tumor), N=6 (Tumor + R100), N=6 (Tumor + R200), N=7 (Tumor +R500).

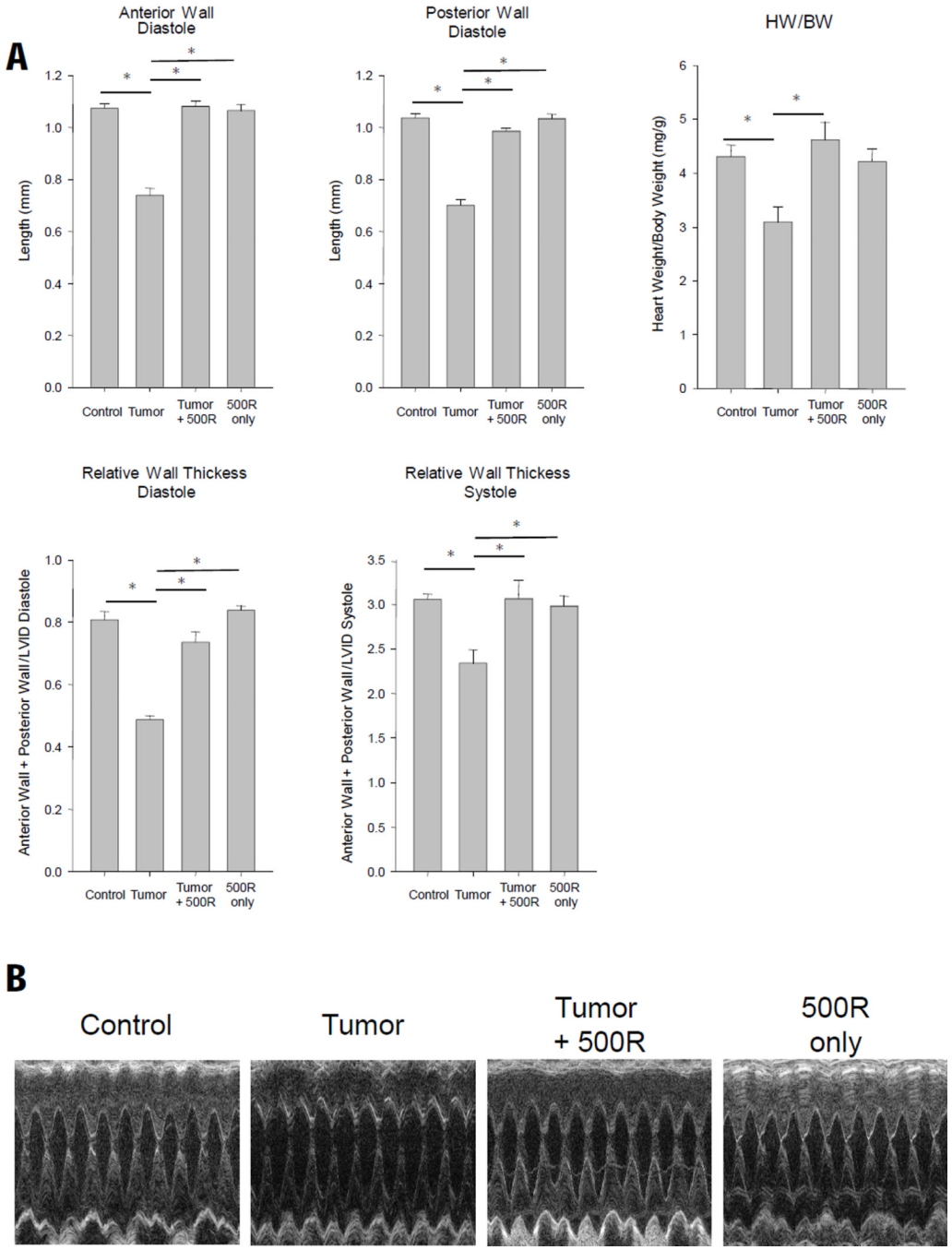

Figure 4. Resveratrol protects against tumor induced cardiac atrophy

(A) Conscious echocardiography of the left ventricle indentified significant decreases in tumor-induced anterior and posterior wall thickness in diastole, heart weight to body weight ratio (HW/BW), and relative wall thickness, that were not identified when 500 mg/kg/day resveratrol was given. **(B)** Representative M-mode recordings from images used in the analysis of (A). A one-way ANOVA was performed to determine significance, followed by a Holm-Sidak pairwise comparison to significance between groups. *p<0.05. BW, body weight, HW, heart weight; RWT=LVPW;d/s+LVAntW; d/s / LVEDD or LVEDS; LV mass (index)=[1.055* ((ExLVD;d)^3- (LVEDD;d)^3). N=4 per group.

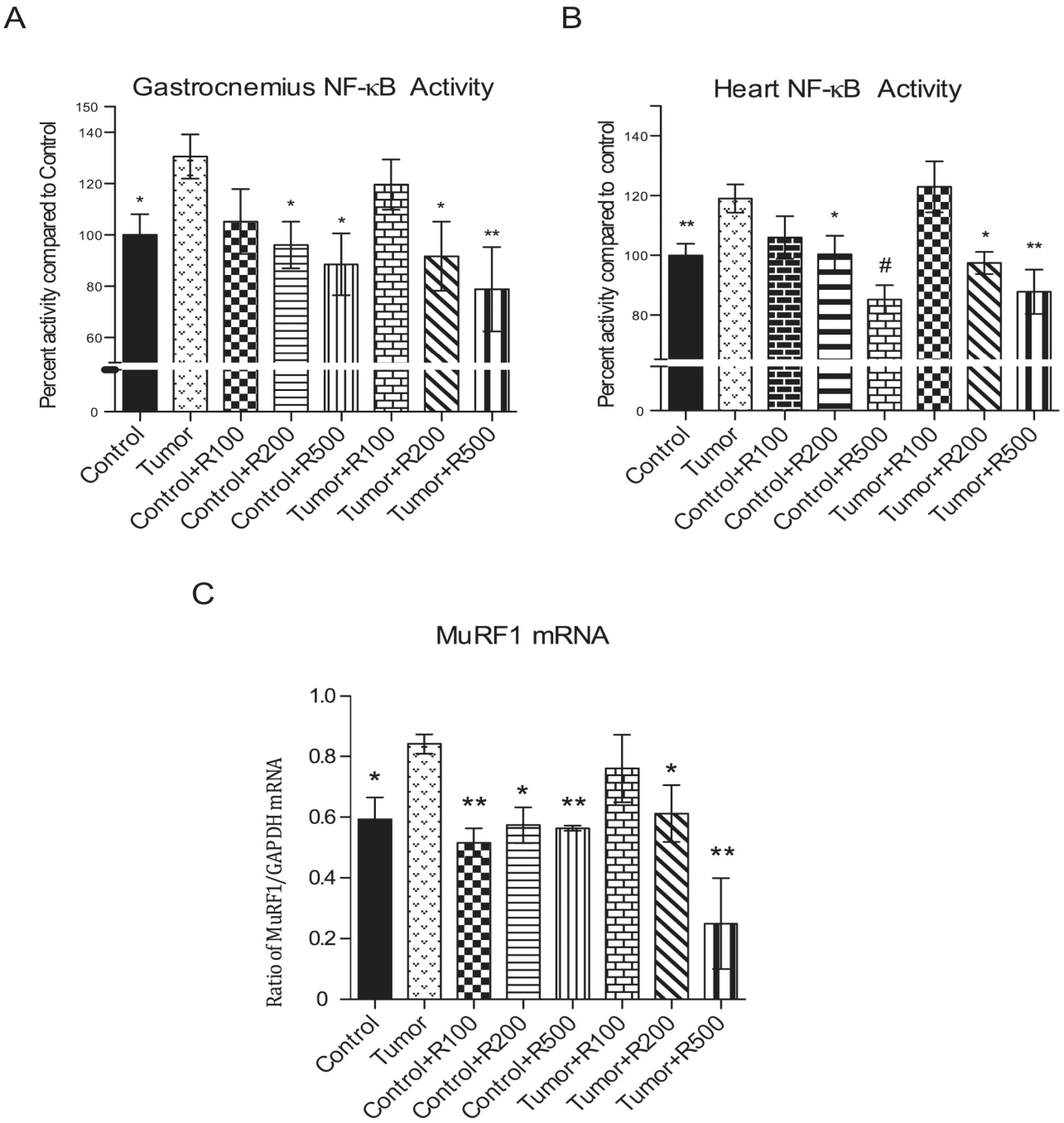

Figure 5. Nuclear factor-kappa B activity (p65)

(A) Nuclear extractions on gastrocnemius muscle were analyzed looking at p65 bound DNA activity. Significant increase in activity was noted in untreated tumor bearing mice compared to control. Tumor bearing mice treated with resveratrol showed a significant decrease in NF-κB activity at 200 mg/kg and 500 mg/kg dosages. Also of note in the treated non-tumor bearing group NF-κB activity was significantly decreased and comparable to control with administration of resveratrol at the 200 mg/kg and 500 mg/kg dosages. **(B)** The tumor bearing groups receiving 200 mg/kg and 500 mg/kg resveratrol showed significant inhibition of tumor-induced NF-κB activity. **(C)** Tumor bearing mice receiving 200 mg/kg

and 500 mg/kg resveratrol had an inhibition of tumor-induced MuRF1 expression. mRNA expression was measured and depicted here as normalized to GAPDH controls. # = p <0.001, ** = p < 0.01, * = p < 0.05. For (A) and (B): N=12 (Control); N=4 (Control + R100), N=4 (Control + R200), N=4 (Control + R500), N=4 (Tumor), N=4 (Tumor +R200), N=4 (Tumor + R500). For (C): N=4 (Control), N=3 (Control + R100), N=3 (Control + R200), N=3 (Control + R500), N=4 (Tumor), N=3 (Tumor +R100), N=3 (Control + R200), N=3 (Control +R500).

Table 1

Transthoracic echocardiography on unanesthetized sham, tumor, or tumor plus resveratrol treatment in age-matched mice 16 days after tumor implantation (±SE)

A one-way ANOVA was performed to determine significance.

	Control N=8	Tumor N=4	Tumor + 500R N=4	Control + 500R N=4
Body Weight (g)	21.8 ± 0.4	20.4 ± 1.1	21.6 ± 0.8	20.6 ± 0.4
HR (bpm)	651 ± 9	617 ± 27	640 ± 13	659 ± 13
LVEDD (mm)	2.63 ± 0.09	2.95 ± 0.08 *	2.82 ± 0.13	2.50 ± 0.07
LVESD (mm)	1.11 ± 0.05	1.35 ± 0.07	1.19 ± 0.10	1.08 ± 0.06
LV %EF	88.97 ± 0.46	85.78 ± 2.78	88.64 ± 1.86	88.20 ± 0.94
LV Vol; d (ml)	25.9 ± 2.2	33.8 ± 2.2	30.6 ± 3.5	22.8 ± 1.5
LV Vol; s (ml)	2.9 ± 0.3	4.7 ± 0.7	3.5 ± 0.8	1.7 ± 1.5

BW, body weight; LV, left ventricle; HR, heart rate; LVEDD, LV end diastolic dimension; LVESD, LV end systolic dimension; LV EF%=[(LV Vol;d-LV Vol;s/LV Vol;d)*100];LV Vol; d, LV volume in diastole; LV Vol;s, LV volume in systole; LV Post W.

* $p<0.05$ vs Control, Tumor + 500R, and Control + 500R.