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**Prevention of unloading–induced atrophy by vitamin E supplementation :  
links between oxidative stress and soleus muscle proteolysis ?**

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## ABSTRACT

Exposure to reduced activity induces skeletal muscle atrophy. Oxidative stress might contribute to muscle wasting via proteolysis activation. This study aimed to test two hypotheses in rats. Firstly, supplementation of the antioxidant vitamin E, prior and during the phase of unloading, would partly counteract unloading-induced soleus muscle atrophy. Secondly, vitamin E supplementation would decrease the rate of muscle proteolysis by reducing expression of calpains, caspase-3, -9, -12 and E3 ubiquitin ligases (MuRF1 and MAFbx). Soleus muscle atrophy (- 49%) induced by fourteen days of hindlimb unloading was reduced to only 32 % under vitamin E. Vitamin E partly prevented the decrease in type I and IIa fiber size. Supplementation increased HSP72 content, suppressed the rise in muscle level of thiobarbituric acid-reactive substance caused by unloading but failed to modify the lower ratio of reduced vs. oxidized glutathione, the higher uncoupling proteins mRNA and the antioxidant enzyme activities (superoxide dismutase, catalase, glutathione peroxidase) observed after unloading. Vitamin E treatment abolished the large upregulation of caspase 9, 12 and MuRF1 transcripts in unloaded muscle and greatly decreased the upregulation of  $\mu$ -calpain, caspase 3 and MAFbx mRNA. In conclusion, the protective effect of vitamin E might be due to modulation of muscle proteolysis-related genes rather than to its antioxidant function.

Keywords: Muscle atrophy; Vitamin E; Oxidative stress; Uncoupling proteins; Heat shock protein; Caspases; Ubiquitine ligases.

## INTRODUCTION

Exposure to reduced activity induces a disuse atrophy of postural muscles which results in a decrease in muscle volume and strength and in a compromised ability to deal with physical work capacity (1, 2, 3). Multiple rodent models, including hindlimb unloading, have been developed to better understand the factors involved in muscle wasting (4). The loss in muscle mass is caused primarily by a rapid decrease in myofibril protein synthesis rate followed by a slower transient increase in myofibril protein degradation rate (5). Muscle atrophy is accompanied by a general shift in the contractile and metabolic profiles of a slow-twitch oxidative muscle toward that of a fast-twitch glycolytic muscle (6, 7). Among potential triggers and molecular signalling events underlying such skeletal muscle plasticity, oxidative stress might contribute to muscle wasting (2, 8-10). Although early studies in rats suggested that vitamin E (alpha-tocopherol), a potent antioxidative nutrient, decreased skeletal muscle atrophy induced by limb immobilization or plaster cast (11, 12), Koesterer et al. (13) demonstrated that antioxidant supplementation failed to attenuate the soleus and gastrocnemius (GS) atrophy or the decrease in Gas force generation induced by hindlimb unloading. Presumably, these discrepancies could be caused by differences in experimental design related to various disuse models and /or potential dose-dependent effects of antioxidant supplementation. More importantly, cellular functions of vitamin E that are independent of its radical-scavenging properties, have been demonstrated in the last years (14, 15). Azzi et al. (14,15) reported that alpha-tocopherol mediates cell signalling and regulates the expression of a large number of genes. A protective effect of vitamin E might be due to modulation of muscle proteolysis-related genes rather than to its antioxidant function.

Currently, unresolved questions remain concerning the specific role of reactive oxygen species (ROS) in the regulation of hindlimb unloading-induced muscle atrophy and more

specifically which protease system are controlled by ROS (9). There is evidence that both lysosomal and /or  $\text{Ca}^{2+}$  - activated (i.e. calpains) proteases contribute for a minor part to muscle wasting while the ATP-ubiquitin proteasome pathway is mainly responsible for the unloaded-soleus muscle proteolysis (16). Additionally, a cascade of cystein-dependent proteases called caspases might be involved in the loss of myonuclei by apoptosis in skeletal muscle atrophied fibers (17, 18). It therefore appeared promising to further explore the therapeutic potential of vitamin E supplementation in muscle atrophy and to advance in the understanding of signaling pathways that control muscle mass following vitamin E supplementation.

This study was designed to test two hypotheses in rats. Firstly, long-term supplementation of the lipid-soluble antioxidant vitamin E, prior (21 days) and during (14 days) the phase of unloading, would partly counteract unloading-induced soleus muscle atrophy. Secondly, vitamin E supplementation would downregulate genes involved in muscle proteolysis. To address this question, we measured biomarkers of oxidative stress (glutathione vs. glutathione disulfide ratio, level of thiobarbituric acid-reactive substance), antioxidant enzyme activities (superoxide dismutase, catalase and glutathione peroxidase) and other potent antioxidant defense systems (uncoupling proteins, UCP2, UCP3 mRNA and heat shock protein content, HSP72). We assessed muscle proteolysis by measuring the relative abundance of mRNA encoding calpains, caspase-3, -9, -12 and two atrophy-related ubiquitine ligases (Muscle Ring Finger 1, MuRF1 and Muscle Atrophy F-box, MAFbx). Here we show that prevention of muscle atrophy by vitamin E might be due to modulation of muscle proteolysis-related genes rather than to its antioxidant function.

## EXPERIMENTAL PROCEDURES

### *Animal care and protocol*

Thirty two pathogen-free male Wistar rats, weighting 350 g from Charles River, were housed in a temperature-controlled room ( $24 \pm 2^{\circ}\text{C}$ ) with a light dark cycle (12:12h). After one week of acclimatation, rats were assigned randomly to one of four experimental conditions. Two groups of eight rats received six intraperitoneal injections of vitamin E at a dose of 60 mg / kg twice a week, for 35 days according to Appell et al. (11). Vitamin E was given in the form of  $\alpha$  tocopherol acetate solubilized in soybean oil (Sigma). After 21 days of treatment, half of them were hindlimb suspended for 14 days in individual cages using Morey's tail-suspension model (4). Other rats were kept as controls. Two other groups of eight rats received soybean injections during 35 days and half of them were suspended for 14 days. This protocol generated 4 groups of rats : controls rats (C), vitamin E-supplemented control rats (C + VE), hindlimb unloaded rats (HU), vitamin E-supplemented and unloaded rats (HU+VE). The present investigation was performed following the recommendations provided by the European Convention for the protection of Vertebrate Animals used for Experimental and Scientific purposes (Council of Europe N° 123, Strasbourg, 1985). The animals were anaesthetised with halothane. Soleus muscles were excised, weighed, frozen in isopentane chilled with liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis.

### *Myosin ATPase*

The midbelly region of each muscle was cut in serial transverse sections (10  $\mu\text{m}$ ) on a microtome at  $-25^{\circ}\text{C}$  and stained for the myosin adenosine triphosphatase (ATPase) as previously described (6). Muscle fibers were classified into two major types (I, IIa). The fiber

cross-sectional areas were calculated by means of a computerized planimetry coupled to a digitizer and the area of at least 100 fibers were measured.

#### *Glutathione levels*

Reduced (GSH) and oxidized glutathione (GSSG) were measured according to the method of Anderson (19) by monitoring the reduction by GSH of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoate (TNB) at 412 nm (25°C). Proteins were precipitated with ice-cold 5 % metaphosphoric acid and centrifuged at 5000 g for 5 min. For GSH measurements, samples were derivated with 2-vinyl-pyridin. The assay was initiated by the addition of 10 µL of 50 U/mL glutathione reductase. GSH was used as a standard and was assayed in parallel under the same condition as the tissue samples.

#### *Muscle lipid peroxidation*

The concentration of lipid peroxide was estimated by the thiobarbituric acid-reactive substances (TBARS) method described by Ohkawa et al. (20).

#### *Antioxidant enzymes activities*

A portion of soleus muscle was homogenised with a potter Elvehjem at 4°C, in buffer containing KH<sub>2</sub>PO<sub>4</sub> (100 mM), DTT (1mM) and EDTA (2 mM), pH 7.4. After centrifugation (3000 g/min, for 5 minutes), the supernatant was used for enzymatic assays. Superoxide dismutase (SOD) activity was assayed by monitoring the rate of acetylated cytochrome c reduction by superoxide radicals generated by the xanthine-xanthine oxidase system (21). One activity unit of SOD is defined as the amount of enzyme which inhibits the rate of acetylated cytochrome c reduction by 50 %. To distinguish mangano-SOD (MnSOD), exclusively located in mitochondrial matrix, from cuprozinc-SOD (CuZnSOD), which is primarily located in the cytosol, SOD activity was determined after incubation with NaCN (1 mM). At this concentration, cyanide inhibits the CuZn isoform of the enzyme, but does not affect the Mn isoform (20). The total activity of glutathione peroxidase (GPx) activity was assayed with

cumene hydroperoxide as a substrate according to Tappel (22). The activity of catalase (CAT) was determined by the method of Aebi (23). This technique used the first-order rate constant of the decomposition of H<sub>2</sub>O<sub>2</sub> by tissue CAT at 20°C. One unit of catalase activity was calculated by using  $k = (2.3/dt)(\log A_1/A_2)$ , where k is CAT activity, dt is change in time, A<sub>1</sub> is initial absorbance, and A<sub>2</sub> is final absorbance. All enzyme activities are expressed in U/ mg of proteins.

#### *HSP<sub>72</sub> content*

For HSP quantification, we performed polyacrylamide gel electrophoresis and immunoblotting as previously described (24). Briefly, 200 µL of muscle homogenate were mixed with 200 µL of buffer containing 40 mM Tris(hydroxymethyl)aminomethane pH 6.8, 1% SDS, 6% glycerol, and 1% β-mercaptoethanol. This mixture was then heated at 100°C for 10 min, and subjected to one-dimensional sodium dodecyl sulfate (SDS)-PAGE with a 5% stacking and 12.5% resolving gels for 12 hours. After electrophoretic separation, proteins were transferred at a constant voltage to nitrocellulose membranes. After protein transfer, the membranes were blocked for 2h, then incubated 2 h with a monoclonal antibody specific for HSP72 (SPA 810, StressGen, diluted 1:1000) and then exposed to the secondary antibody (goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase, Bio-Rad at a 1:10000 dilution). HSP72 were visualized by the enhanced chemiluminescence detection method (RPN 2106, Amersham). Scanning with a densitometer performed quantification of bands from blots and the data were expressed numerically as integrated optical density arbitrary units.

#### *mRNA concentration in soleus muscle*

Total RNA were extracted from muscle samples (50 mg) of rats using Trizol® (Invitrogen). Concentration and purity were verified by measuring optical density at 260 and 280 nm. Their integrity was checked by 1% agarose gel electrophoresis (Eurobio). mRNA



relative abundance was measured by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) using cyclophilin as reference. Primer sequences are shown in table 1. For each sample, a RT was performed from 1 µg of total RNA with 100 U of M-MLV Reverse Transcriptase (Promega), 5µL of M-MLV RT 5X buffer, 20 U of RNasin Ribonuclease Inhibitor, 15 nmoles of deoxynucleotide triphosphate and 1 µg of oligo dT, in a final volume of 25 µL. The reaction consisted of 5 min at 70°C (RNA and oligo dT), then 90 min at 42°C (all mix) followed by 10 min at 70°C. After chilling, 2.5 µL was used for PCR. The 2.5 µL of RT medium was added to 47.5 µL of PCR mix containing 5 µL of 10X EurobioTaq PCR buffer, 75 nmoles of MgCl<sub>2</sub>, 15 nmoles of deoxynucleoside triphosphate, 2.5 U of EurobioTaq DNA polymerase, 22.5 pmoles of corresponding antisense and sense primers. The PCR conditions were : 2 min at 94 °C followed by n cycles of PCR (1 cycle = 1 min at 94°C, 1 min at 60°C, 1 min at 72°C). The numbers of cycles is indicated in table 1 for each target. PCR was ended by 10 min at 72°C. Products were analysed on 1.5% agarose gel prestained with ethidium bromide. For quantitation of relative bands intensities, pictures were taken with a Camera DC120 (Kodak) and the ratio of each target to cyclophilin was determined for each sample with Kodak Digital Science 1D 2.0 (Kodak Scientific Imaging System).

### *Statistical analysis*

All data reported are means ± SE. A multifactorial analysis of variance was used for intergroup comparisons. The Fisher paired least significant difference was used to identify specific means differences. Values were considered statistically different when  $P < 0.05$ .

## **RESULTS**

### *Muscle mass and fiber size.*

Soleus muscle to body mass ratio and cross-sectional areas of type I and IIa fibers are shown in Table 2. Soleus muscle atrophy (- 49%) induced by 14 days of hindlimb unloading was reduced to only 32 % under vitamin E. Unloading led to a decrease in type I (- 59 %) and IIa (- 42 %) fiber size. Vitamin E partly prevented this decrease as type I and IIa fiber cross-sectional areas were only reduced by 38 and 32 % respectively.

### *GSH/GSSG*

In control rats, there was no significant change in reduced to oxidized glutathione ratio (GSH / GSSG) after vitamin E supplementation (Fig.1a). Unloading resulted in a significant decrease (- 23 %) of GSH / GSSG in HU rats but vitamin E supplementation failed to modify the lower ratio of reduced vs. oxidized glutathione.

### *Muscle lipid peroxidation*

Thiobarbituric acid reactive substances (TBARS) content, an index of lipid peroxidation, was similar in C and C+VE ( $34.9 \pm 3.6$  and  $33.8 \pm 1.5$  nmole MDA/g tissue, respectively, Fig.1b). Vitamin E supplementation abolished the increase (+ 35 %) in TBARS content caused by unloading.

### *Antioxidant capacities.*

There was no significant difference in cytosolic (ZnCu-SOD), mitochondrial (Mn-SOD), GPx activities between C and C+VE rats (Fig. 2 abc). Hindlimb unloading similarly increased ZnCu-SOD activity (+ 47 %), GPx (+ 43%) and CAT ( + 90 %) activities in HU rats and HU+VE rats (Fig. 2 acd). CAT activity was elevated in the vitamin E supplemented control rats (+48 %) and there was a further significant increase on unloading (Fig. 2d).

### *Complementary defences : uncoupling proteins, heat shock proteins.*

Similar patterns were seen for UCP2 and UCP3 mRNA. Unloading led to higher UCP2 (+30 %) and UCP3 mRNA (+ 400%) in HU and HU+VE rats, but no obvious effect of

vitamin E was observed in C rats (Fig 3ab). No change occurred in basal HSP<sub>72</sub> level in various treatments whether supplementation or hindlimb unloading (Fig 3c). However, high levels of HSP<sub>72</sub> (+ 50 %) were found in soleus muscle of HU+VE rats (Fig 3c).

*Calpains, proapoptotic caspases, E3 ubiquitine ligases.*

In control rats, the relative abundance of caspases 3, 9, 12, MAFbx and MuRF1 mRNA were not affected by vitamin E supplementation (Fig 4, 5ab).  $\mu$ -calpain mRNA was increased to 29 % after unloading, an effect that was 93 % prevented by vitamin E supplementation (P = 0.055, Fig 5c). m-calpain mRNA was neither affected by unloading nor by vitamin E supplementation (Fig 5d).

Vitamin E supplementation abolished the unloading-induced increase in caspase -9 and -12 mRNA (+39 and +50%, respectively , Fig.4b, c) and decreased the upregulation (+ 114 %) of caspase -3 mRNA by 50 % (Fig. 4a). MAFbx and MuRF1 mRNA were increased in unloaded rats by 50 and 59 % respectively (Fig 5). This effect was significantly prevented by vitamin E supplementation for MuRF1 and at the limit of statistical significance for MAFbx (P = 0.07).

## **DISCUSSION**

Supplementation of Vitamin E prevented soleus muscle atrophy by 20 % after 14 days of hindlimb unloading. The type I and IIa fiber cross-sectional areas were only decreased by 38 and 32 % respectively after Vitamin E treatment (vs 59 and 42 % respectively for the untreated-unloaded rats). Similar results were previously observed in rats submitted to various disuse models : plaster cast (11) or immobilization of the ankle joint of one hindlimb in the extended position (10). However, using the same experimental design and the same duration of hindlimb unloading, Koesterer et al (13) reported that vitamin E supplementation was not

an effective countermeasure to soleus muscle disuse, suggesting that the mechanisms of atrophy induced by immobilization and hindlimb unloading might be fundamentally different. The dose-dependent effects, the potential composition and duration of the pretreatment (three weeks vs one) of supplementation might explain these discrepancies. In the present study, unloading, as expected (13), resulted in a lower ratio of reduced vs. oxidized glutathione and in an increased TBARS content, two biomarkers of oxidative stress and vitamin E supplementation restored the increased TBARS content caused by unloading. In fact, we have clearly demonstrated, as it was reported with other disuse models (10, 11), that vitamin E supplementation partly prevents soleus atrophy.

A complex cytoprotective system that includes antioxidant enzymes is recruited against free radical damage. Unloading significantly increased activities of ZnCu-SOD (primarily localized in the cytosol) as previously observed (8) but failed to induce a significant rise ( $P = 0.11$ ) in Mn-SOD (localized in the mitochondrial matrix) activity. Unloading induces a rise in GPx and CAT activities contrary to the decrease observed by Lawler et al. (8). Vitamin E supplementation did not confer an additional protection against oxidative damage through a further increase in antioxidant enzymes activities.

One of the main adaptations of mitochondria to oxidative stress would be a mild uncoupling of oxidative phosphorylation that might reduce the mitochondrial production of ROS by lowering mitochondrial membrane potential (25, 26). The UCPs might attenuate cellular oxidative damage in some pathophysiological states leading to increased oxidative stress such as cancer cachexia (27). Body weight loss and muscle atrophy are commonly associated with this syndrome. An activation of UCP2 and UCP3 gene expression associated with an increased catalase protein content was reported in atrophied gastrocnemius muscles of tumour-bearing rats (28, 29). Busquet et al. (29) postulate that UCPs might confers protection against muscle oxidative injury. We have previously shown a marked upregulation of UCP3

gene in unloaded soleus muscle suggesting that the rise in UCP3 expression might contribute to protect less active skeletal myocytes from oxidative damage by stimulating oxygen consumption, depleting the local concentration of oxygen and decreasing the generation of ROS (30). The current study is the first to report a similar upregulation of UCP2 and UCP3 mRNA in unloaded soleus muscle following vitamin E supplementation compared to untreated rats. These results clearly show that the effects of unloading and vitamin E treatment were found to be non-additive. Assuming a role of UCP2 as a regulator of ROS production by reducing the mitochondrial proton gradient (31-33), overexpression of UCP2 would be also beneficial for preventing unloaded cells from free-radical damage.

Supplementation of Vitamin E failed to modify the lower ratio of reduced vs. oxidized glutathione, the increased antioxidant enzyme activities (superoxide dismutase, catalase, glutathione peroxidase) and the higher uncoupling proteins mRNA observed after unloading. Consequently, our data suggest that Vitamin E acts in a non-antioxidant way on the prevention of muscle atrophy which is consistent with the findings of previous studies (14, 15). Since 15 years, it has been well documented that Vitamin E exerts cellular functions that are independent of its radical-scavenging properties (14, 15).

To our knowledge this is the first report to demonstrate an increase in the stress-inducible form of HSP70 (HSP72) content as a result of Vitamin E supplementation in unloaded rats. In non-supplemented rats, the maintained basal HSP72 level in soleus muscle after two weeks of unloading is in accordance with our previous data (30) and those of Oishi et al. (34). The induction of HSP72 protein suggests that HSP72 acts as a complementary protective mechanism against muscle atrophy. Given the role of HSP72 as molecular chaperones preventing aggregation and facilitating the refolding of denatured proteins, increased expression of HSP72 may prevent oxidative damage and assist in repair of damaged proteins (35, 36). Moreover stress-inducible form of Hsp70 prevents cell apoptosis by

interfering with the ability of cytochrome *c* and Apaf-1 (apoptosis protease activator protein) to recruit procaspase-9 (37, 38).

In a recent review, Powers et al. (9), raised the interesting question of whether the redox control of protease activity during muscle disuse atrophy occurs by allosteric regulation (ie control of intracellular calcium levels) and /or via increased gene expression of proteases. The present study characterizes some signaling pathways that control unloading-induced muscle proteolysis following antioxidant supplementation. Vitamin E supplementation was found to prevent the upregulation of  $\mu$ -calpain mRNA while m-calpain mRNA remained unaffected during muscle wasting. Ubiquitous calpains are non-lysosomal  $\text{Ca}^{2+}$  activated cysteine proteases that cleave titin and nebulin at sites near the Z-disk, leading to a progressive disruption of the Z-disk (39, 40). They are involved in the initial breakdown of myofibrils, releasing actin and myosin from the sarcomere before they undergo ubiquitination and degradation by the proteasome. The ubiquitous  $\mu$  and m-calpains (also called 1 and 2) may have different role in the development of soleus atrophy since they have different in vitro calcium sensibility (in the range of 3-50  $\mu\text{M}$  for  $\mu$ -calpain and 400-800  $\mu\text{M}$  for m-calpain, 36). In parallel to the upregulation of  $\mu$ -calpain in our study, a 36 % increase in resting intracellular calcium (from 17 to 23 nM) was reported by Ingalls et al. (41) in mice soleus muscle after 14 days of hindlimb suspension. Goll et al. (39) postulated that the increase in intracellular calcium is not sufficiently large to activate the calpains directly but it seems probable that it alters the regulation of calpain activity by calpastatin, phosphorylation or still unknown mechanisms. Indeed, a previous study reported a sustained activation of m-calpain activity after 9 days of hindlimb suspension (16).

Vitamin E partly controls the soleus muscle proteolysis mediated by the ubiquitin proteasome pathway via two muscle-specific ubiquitin ligases (E3) : MAFbx (muscle atrophy Fbox or atrogen-1) and MuRF1, (muscle ring finger, 42, 43). MAFbx and MuRF1 are critical

regulators in the enhanced proteolysis leading to muscle atrophy in various diseases (2, 42). As Murf1 has been shown to bind titin (44), MurF1 might participate to the disruption of the Z disk in addition to the calpain system. Vitamin E supplementation prevented MurF1 induction whereas MAFbx expression was greatly reduced ( $P = 0.07$ ). It has been well documented that Vitamin E has important non-antioxidant effects and mediate cell signalling and regulation of gene expression (14, 15). For example, vitamin E downregulates a number of genes involved in acute inflammation. Given the role played by cytokines such as TNF $\alpha$  (tumor necrosis factor) which activate NF-KB in muscle cells (45) and the pronounced anti-inflammatory effects of vitamin E (15), one could possibly explain the prevention of MurF1 upregulation. However, if cytokines are involved in cachexia, Hunter et al. (46) did not find any change in TNF $\alpha$  - protein levels after a short period of muscle unloading (seven days).

Previous studies have shown that apoptosis contributes to the unloading-induced soleus muscle atrophy (17, 18). In the present study, vitamin E supplementation prevents the upregulation of caspases-9 and -12 but failed to totally abolish the increased caspase-3 mRNA. Sarcoplasmic reticulum can induce apoptosis through a calpain-mediated activation of caspase-12 and subsequent activation of caspase-9 and the key effector protease caspase-3 (47). A second intrinsic pathway of apoptosis, dependent of mitochondria, is partly initiated by reactive oxygen species (47). Release of cytochrome c into the cytosol induced the formation of an apoptosome complex with Apaf-1, ATP and procaspase-9, leading to the activation of caspase 3. Recently, Leeuwenburgh et al.(18) reported an increase in caspase-3 activity in young unloaded muscles suggesting that disuse-induced apoptosis was likely caspase-dependent whereas additional pathways such as the caspase-independent translocation of a proapoptotic endonuclease (EndoG, a mitochondrion endo-specific nuclease) might be involved in old rats. The implication of these different patterns in unloading-induced atrophy and in prevention by vitamin E should be further investigated.

The present study demonstrates that acute dosing of vitamin E, prior and during unloading, partly prevents soleus muscle atrophy. Vitamin E failed to modify markers of oxidative stress (GSH/GSSG, SOD, GPx, CAT, UCPs), suggesting a non-antioxidant mechanism for Vitamin E action. A number of genes involved in muscle proteolysis such as  $\mu$ calpain, caspases-3,-9,-12 and two atrophy-related ubiquitine ligases (MuRF1 and MAFbx), were downregulated by Vitamin E while HSP72 was upregulated. These results provide an alternative signaling mechanism for understanding the muscle protective effects of Vitamin E during unloading, via muscle proteolysis-related genes regulation. Vitamin E may be regarded as an adjuvant to protect muscle cells from atrophy.

## **LISTE OF ABBREVIATIONS**

CAT: catalase

GPx: glutathione peroxidase

GSH : glutathione

GSSG : glutathione disulfide

HSP<sub>72</sub>: heat shock protein of 72 KDa

MAFbx: muscle atrophy F- box/ atrogen-1

MuRF1 : muscle ring finger 1

ROS: reactive oxygen species

SOD: superoxide dismutase

TBARS = thiobarbituric acid-reactive substance

UCP : uncoupling protein



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**Table 1 : Primer sequences used for RT-PCR**

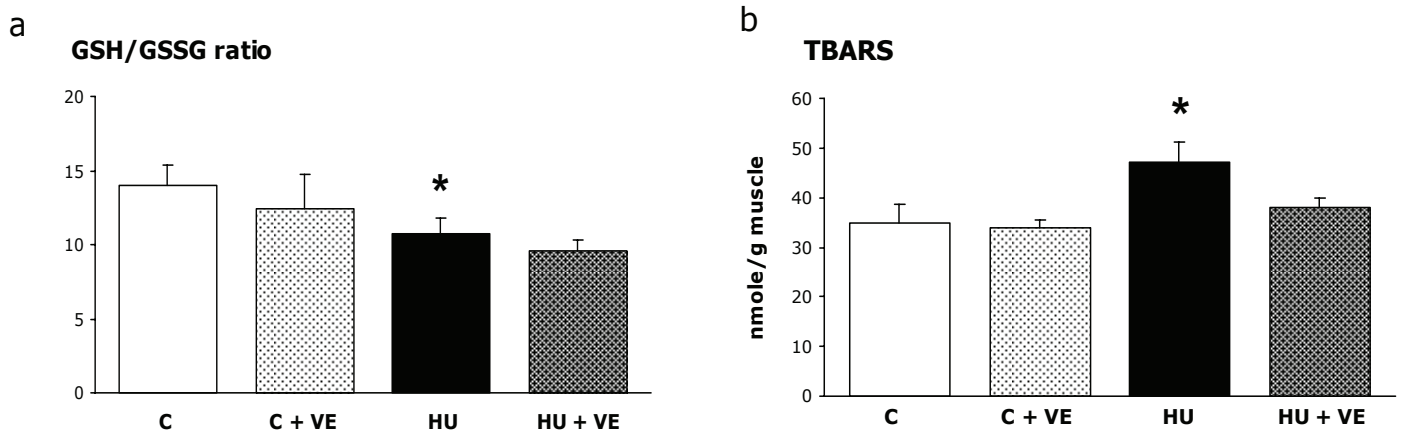
	Sense (5' → 3')	Antisense (5' → 3')	Size (bp)	n cycles
<b>cyclophilin</b> (NM_017101)	<i>GTG GCA AGT CCA TCT ACG GAG</i>	<i>CCA CAG TCG GAG ATG GTG ATC</i>	<b>265</b>	<b>25</b>
<b>MAFbx</b> (AY059628)	<i>GCC TGA ACT ACG ATG TTG CAG</i>	<i>GCT GGT CTT CAA GAA CTT TC</i>	<b>323</b>	<b>29</b>
<b>MuRF1</b> (AY059627)	<i>AGG TGC CTA CTT GCT CCT TGT</i>	<i>GCT GTT TCC ACA AGC TTG GTC</i>	<b>378</b>	<b>27</b>
<b>caspase 3</b> (NM_012922)	<i>CAC TGG AAT GTC AGC TCG CAA</i>	<i>CCA CTG TCT GTC TCA ATA CCG</i>	<b>359</b>	<b>32</b>
<b>caspase 9</b> (NM_031632)	<i>GCA GGA TCC AGA AGC TGT TAC</i>	<i>GCC ATA TCT GCA TGT CTC TCG</i>	<b>281</b>	<b>36</b>
<b>caspase 12</b> (NM_130422)	<i>CCG ACA AAC AGC TGA GTT TAC</i>	<i>GTC AGT CTC AGC ATC ATC TCT</i>	<b>361</b>	<b>32</b>
<b>μ-calpain</b> (NM_019152)	<i>CCG ATA TCC GTG ATT TGG AGG</i>	<i>TCG ACT GTC ATA GTC GTC TGC</i>	<b>478</b>	<b>36</b>
<b>m-calpain</b> (NM_017116)	<i>CAT CGC GAT GAA ACT GGC CAA</i>	<i>GGA AGC TCT GGT CTA GAG GCA CA</i>	<b>388</b>	<b>36</b>
<b>UCP2</b> (NM_019354)	<i>AGC AGT TCT ACA CCA AGG GC</i>	<i>AGC ATG GTC AGG GCA CAG TG</i>	<b>471</b>	<b>28</b>
<b>UCP3</b> (NM_013167)	<i>ATG CAT GCC TAC AGA ACC AT</i>	<i>CTG GGC CAC CAT CCT CAG CA</i>	<b>314</b>	<b>28</b>

**Table 2 : Soleus muscle to body mass ratio and cross-sectional areas of type I and IIa fibers in soleus muscle**

Parameters	Soleus (mg/100 g)	Fiber Areas ( $\mu\text{m}^2$ )	
		Type I	Type IIa
Control + saline (C)	44.7 $\pm$ 1.7	4078 $\pm$ 224	3866 $\pm$ 227
Control + vitamin E (C+VE)	40.7 $\pm$ 1.2	3763 $\pm$ 158	4299 $\pm$ 178
Hindlimb unloading + saline (HU)	22.9 $\pm$ 1.4 *	1661 $\pm$ 65 *	2251 $\pm$ 105 *
Hindlimb unloading + vitamin E (HU+VE)	27.8 $\pm$ 1.1 † #	2348 $\pm$ 139 † #	2937 $\pm$ 230 † #

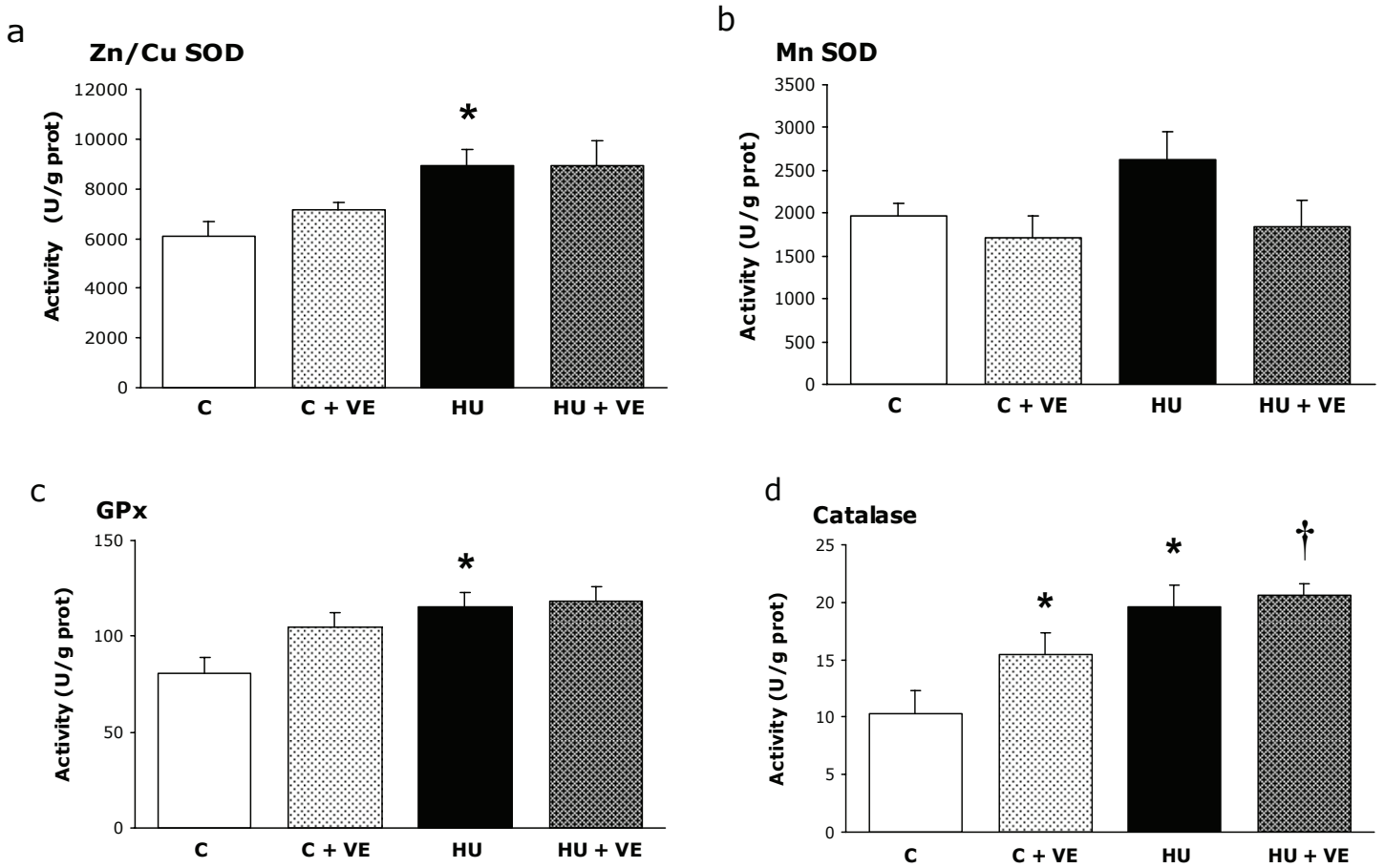
Values are means  $\pm$  SE for 8 animals. \*  $P < 0.05$ , significantly different from control + saline rats. †  $P < 0.05$ , significantly different from vitamin E-supplemented control rats. #  $P < 0.05$ , significantly different from hindlimb unloaded rats





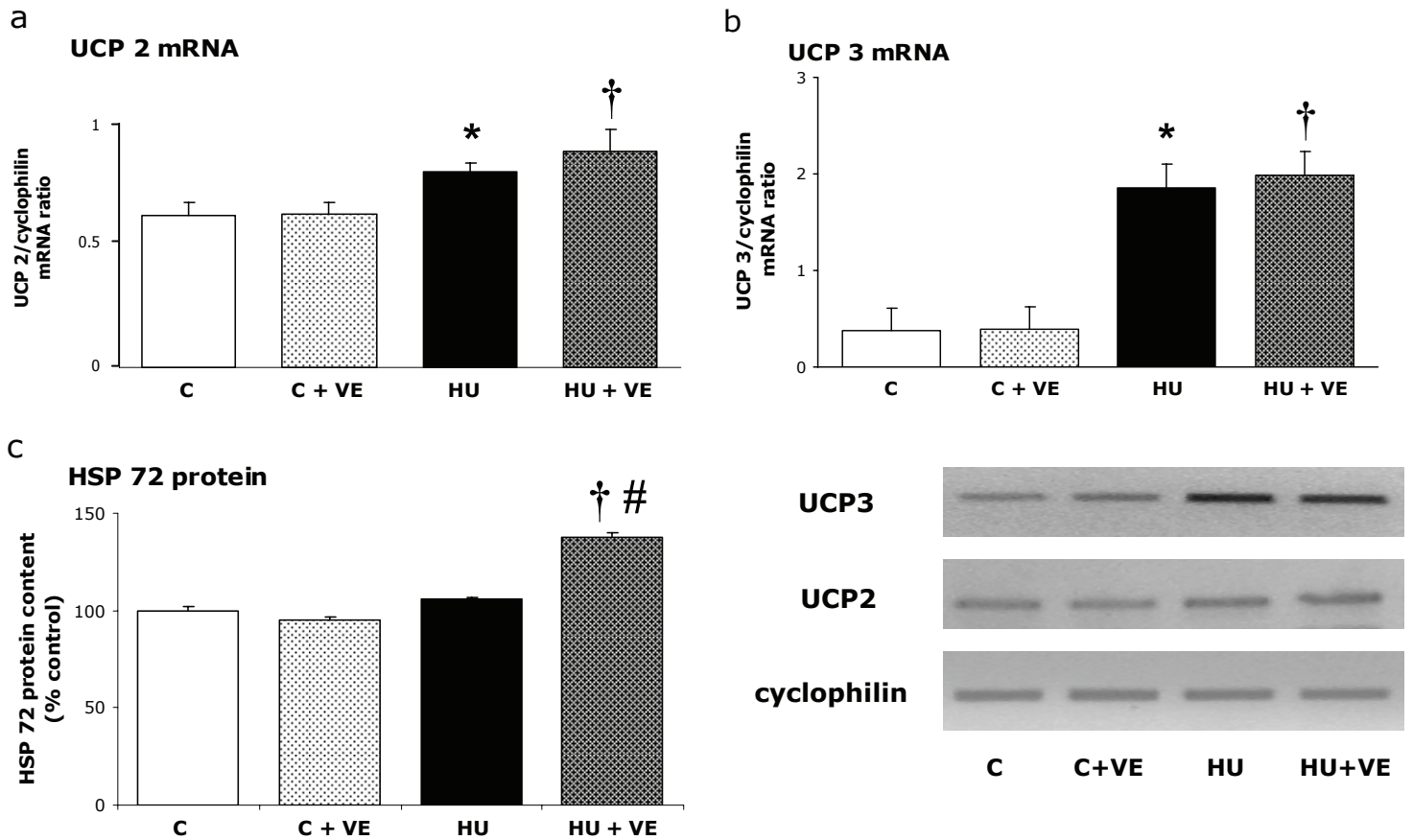
**Fig. 1.** Effect of unloading and vitamin E supplementation on reduced to oxidized glutathione ratio (GSH / GSSG) and thiobarbituric acid reactive substances (TBARS) content.

C: control rats, C+VE : vitamin E-supplemented control rats, HU : hindlimb unloaded rats, HU+VE : vitamin E-supplemented and unloaded rats. Values are means  $\pm$  SE for eight animals. \* Significantly different from control rats.



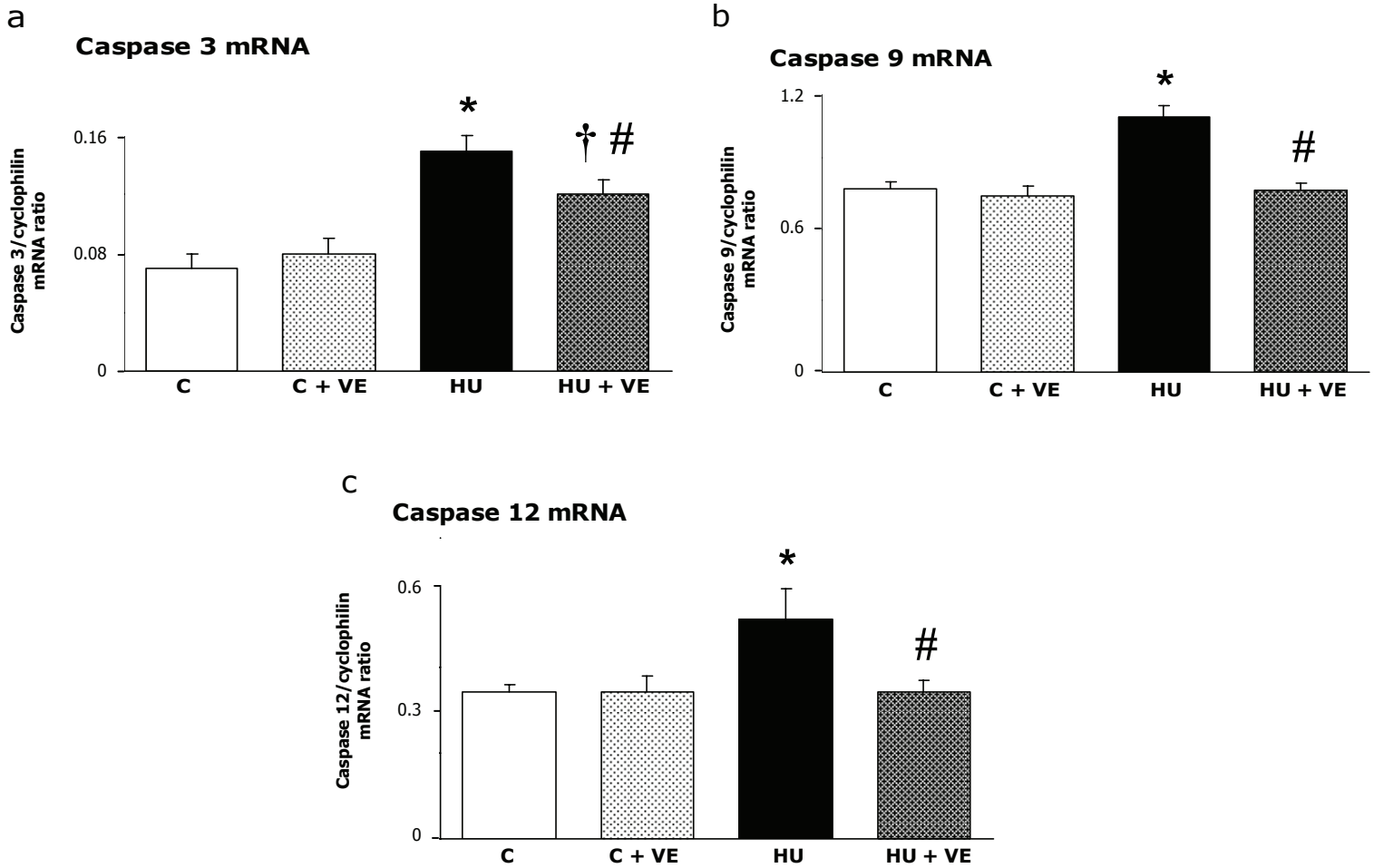
**Fig. 2.** Effect of unloading and vitamin E supplementation on activity of a) cytosolic (SOD  $Zn^{2+}$ ,  $Cu^{2+}$ ) and b) mitochondrial ( $Mn^{2+}$ ) superoxide dismutase, c) glutathione peroxidase (GPx) and d) catalase (CAT).

C: control rats, C+VE : vitamin E-supplemented control rats, HU: hindlimb unloaded rats, HU+VE : vitamin E-supplemented and unloaded rats. Values are means  $\pm$  SE for eight animals. \* Significantly different from control rats. † Significantly different from vitamin E-supplemented control rats.



**Fig. 3.** Influence of unloading and vitamin E supplementation on UCP 2 (a) and UCP3 (b) mRNA and HSP 72 protein content (c). Note that HSP72 contents are expressed as a percentage of HSP72 levels in control group.

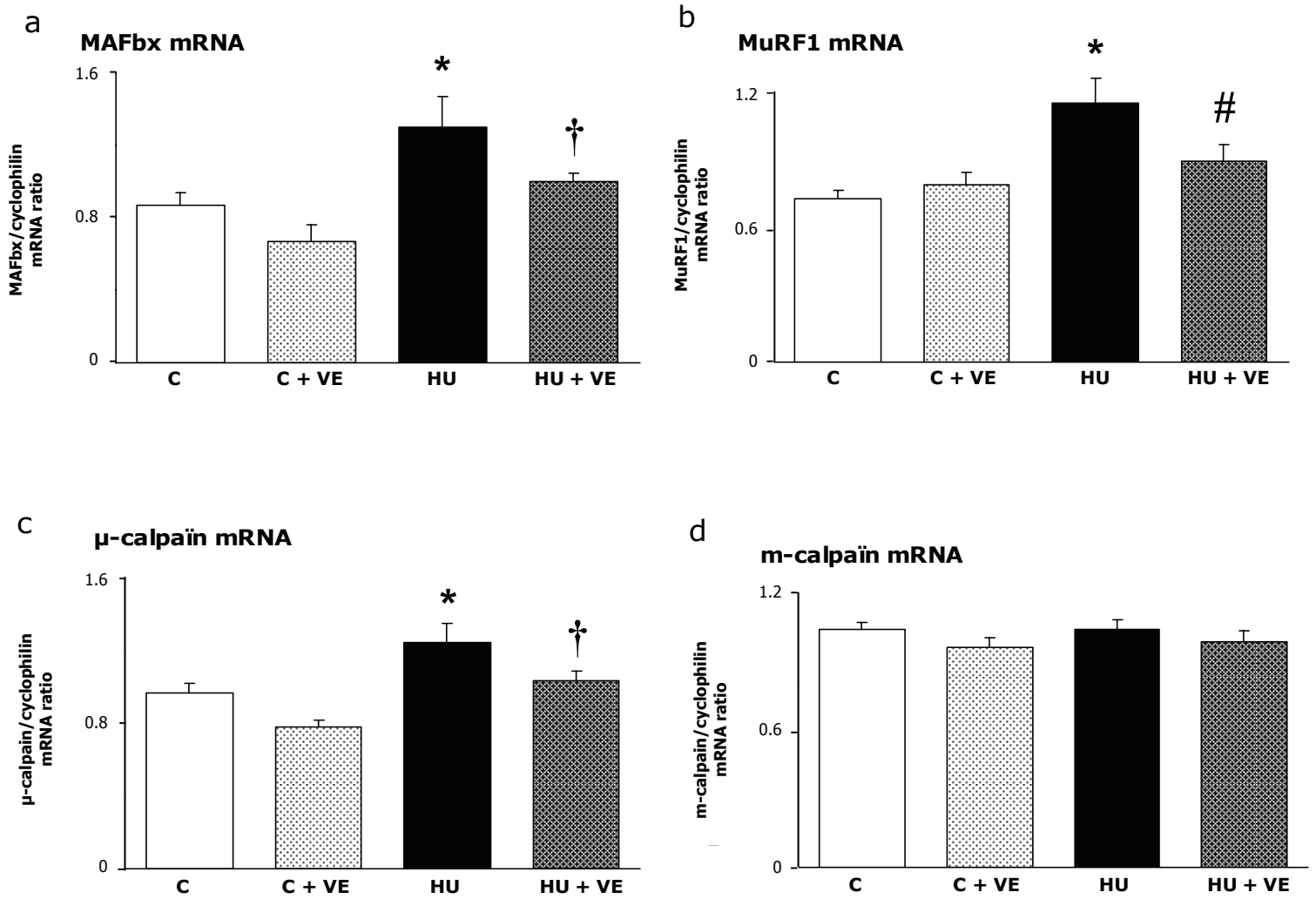
C : control rats, C+VE : vitamin E-supplemented control rats, HU : hindlimb unloaded rats, HU+VE : vitamin E-supplemented and unloaded rats. Values are means  $\pm$  SE for eight animals.\* Significantly different from control rats. † Significantly different from vitamin E-supplemented control rats.



**Fig. 4.** Effect of vitamin E on Caspase 3, 9, 12 mRNA in control and unloaded soleus muscles.

C : control rats, C+VE : vitamin E-supplemented control rats, HU : hindlimb unloaded rats, HU+VE : vitamin E-supplemented and unloaded rats. Values are means  $\pm$  SE .

\* Significantly different from control rats. † Significantly different from vitamin E-supplemented control rats. # Significantly different from hindlimb unloaded rats.



**Fig. 5.** Effect of vitamin E on expression of two atrophy-related ubiquitin ligases, MAFbx and MuRF1.

C : control rats, C+VE : vitamin E-supplemented control rats, HU : hindlimb unloaded rats, HU+VE : vitamin E-supplemented and unloaded rats. Values are means  $\pm$  SE .

\* Significantly different from control rats. † Significantly different from vitamin E-supplemented control rats. # Significantly different from hindlimb unloaded rats.