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L-glutamine administration reduces oxidized glutathione and MAP Kinase signaling in dystrophic muscle of mdx mice

Running title: Glutamine in dystrophic mdx mouse muscle

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ABSTRACT
To determine whether glutamine (Gln) reduces the ratio of oxidized to total glutathione (GSSG/GSH) and extracellular signal-regulated kinase (ERK1/2) activation in dystrophic muscle. 4-week old *mdx* mice, an animal model for Duchenne muscular dystrophy (DMD) and control (C57BL/10) received daily intraperitoneal injections of L-Gln (500mg/kg/d) or 0.9% NaCl for 3 days. GSH and GSSG concentrations in gastrocnemius were measured using a standard enzymatic recycling procedure. Free amino acid concentrations in gastrocnemius were determined by ion exchange chromatography. Phosphorylated protein levels of ERK1/2 in quadriceps were examined using Western Blot. L-Gln decreased GSSG and GSSG/GSH (an indicator of oxidative stress). This was associated with decreased ERK1/2 phosphorylation. Muscle free Gln, glutamate (Glu), and the sum (Gln+Glu) were higher in *mdx* versus C57BL/10, at the basal level. Exogenous Gln decreased muscle free Glu and Gln+Glu in *mdx* only, whereas Gln was not affected. In conclusion, exogenous Gln reduces GSSG/GSH and ERK1/2 activation in dystrophic skeletal muscle of young *mdx* mice, which is associated with decreased muscle free Glu and Gln+Glu. This antioxidant protective mechanism provides a molecular basis for Gln's antiproteolytic effect in DMD children.

KEYWORDS: Oxidative stress, Duchenne muscular dystrophy, Nutrition, Extracellular signal-regulated kinase (ERK1/2 p44/42), Amino acid, Nuclear factor-kappaB (NF-κB)

ABBREVIATIONS
C57, C57BL/10
DMD, Duchenne muscular dystrophy
ERK1/2, extracellular signal-regulated kinase1/2
Gln, glutamine
Glu, glutamate
GSH, total glutathione
GSSG, oxidized glutathione
IκBα, I-kappaB-alpha
MAPK, mitogen activated protein kinase
NaCl, sodium chloride
NF-κB, Nuclear factor-kappaB
TNF-α, Tumour necrosis factor-alpha

Duchenne muscular dystrophy (DMD) is a progressive muscle-wasting disease resulting from the absence of dystrophin. Dysregulation of calcium homeostasis, increased oxidative stress and proteolysis are among potential pathogenic mechanisms implicated in DMD musclewasting, downstream of the genetic defect (1-4). Oxidative stress could contribute to musclewasting by regulating specific cell signalling pathways leading to increased proteolysis (5).
We have shown that glutamine supplementation decreases whole body protein breakdown in DMD children (6, 7). However, the molecular basis for glutamine's antiproteolytic effect remains to be elucidated.
Antioxidants can improve muscle health by protecting cells from oxidative stress and reducing muscle necrosis in *mdx* mice (an animal model of DMD) (8-11). Several studies have shown that oxidative stress elicited by various stimuli is associated with activation of the extracellular signal-regulated kinase (ERK1/2) mitogen activated protein kinase (MAPK) pathway (12, 13), which could participate in pathways influencing protein breakdown (5, 14, 15). In turn, ERK1/2 activation can be inhibited by antioxidants (10). Furthermore, dystrophic muscle of DMD patients (3) and *mdx* mice (1, 2) undergo increased oxidative stress. And increased ERK1/2 activation was reported in skeletal muscle of *mdx* mice (10, 16), which was further increased in response to mechanical stress (4).
Therapeutic strategies that target downstream events in the progression of dystrophic pathology can yield worthwhile functional improvement in DMD (17). Glutamine supplementation provides a protective effect against oxidative stress in various tissues (18, 19). Thus, the primary objective of the present study is to test whether exogenous glutamine reduces the ratio of GSSG/GSH (the major regulator of the cellular redox potential and a widely used indicator of oxidative stress (20)) in skeletal muscle of *mdx* mice. And since oxidative stress mediates activation of MAPK (ERK1/2) and NF-κB transactivation (13), we determined the effect of glutamine on this pathway. Also because skeletal muscle plays a central role in glutamine metabolism (as the main producer and exporter of glutamine) and whole body glutamine exchange is decreased in DMD (21), we examined the effect of exogenous glutamine on muscle free amino acid concentrations.

METHODS

Animals and L-glutamine administration. 4-week old dystrophin deficient (*mdx*, n=8) and control (C57BL/10, n=8) mice were housed on a 12:12h light:dark cycle and received *ad libitum* water and standard rodent chow. Mice were separated into 4 groups: 1) 0.9% NaCl-C57BL/10 (NaCl-C57), 2) L-glutamine-C57BL/10 (GLN-C57), 3) 0.9% NaCl-*mdx* (NaCl-MDX) and 4) L-glutamine-*mdx* (GLN-MDX). Mice received daily intraperitoneal (i.p.) injections for 3 days of a 3% solution (500 mg/kg body weight) of L-glutamine dissolved in 0.9% NaCl or 0.9% NaCl only. The body weight of each mouse was measured daily during the 3-day treatment period. Mice were sacrificed by cervical dislocation on the third day posttreatment.
All procedures and animal treatment followed the guidelines established by the French council on animal care.
The quadriceps and gastrocnemius muscles were rapidly dissected. Both quadriceps muscles and one gastrocnemius muscle were immediately frozen in liquid nitrogen and stored at -80°C until Western blot analyses (or ELISA) and determination of free amino acids, respectively. The other gastrocnemius muscle was rinsed in phosphate buffered saline (PBS) containing 0.16 mg/ml heparin, to remove any red blood cells and clots. Gastrocnemius muscle was homogenized in ice cold MES buffer (0.2 M 2-(N-morpholino) ethanesulphonic acid, 0.05 M phosphate, 1 mM EDTA, pH 6) centrifuged at 10 000 g for 15 minutes at 4°C. An aliquot of the supernatant was used for total protein determination with the bicinchoninic acid assay kit (Sigma-Aldrich). The remaining supernatant was deproteinated by mixing with an equal volume of metaphosphoric acid reagent (Sigma-Aldrich) and centrifuging at 2000 g for 2

minutes. The supernatant was stored at -80°C until glutathione assay.

Western blot analyses and determination of TNF-α concentrations. Frozen quadriceps muscle was homogenized in cold lysis buffer (50mM Tris HCl pH 7.4, 150mM NaCl, 5mM EDTA, 0.05% Nonidet P-40, 1% DOC, 1% Triton X-100, 0.1% SDS, 1% Protease Inhibitor Cocktail) on ice. The supernatants obtained after samples were centrifuged at 15 000 g for 20 minutes at 4°C were used for subsequent Western blot analyses or stored at -80°C prior to determination of TNF-α concentrations via ELISA according to the manufacturer's instructions (R&D Systems). Total protein was determined with DC protein assay kit (Bio-Rad Laboratories). 50 µg of protein from each sample was mixed with loading buffer (187.5mM Tris HCl pH6.8, 6% SDS, 30% glycerol, 0.03% Bromophenol blue, 5% alpha monothioglycerol), denatured for 5 minutes at 100°C and then separated by 12.5% SDSPAGE. Proteins were then transferred to a nitrocellulose membrane. The blot was blocked in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBS/T) containing 5% non-fat dry milk (w/v) washed in TBS/T (1XTBS, 0.1% Tween-20) and then incubated in primary antibody diluted in 5% bovine serum albumin (BSA) in TBS/T overnight at 4°C. Membranes were then washed in TBS/T and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. After washing in TBS/T, protein detection was performed using LumiGLO chemiluminescent substrate (Upstate cell signalling solutions) and exposure to X-ray film. Antibodies and the conditions used in these measurements were as follows: rabbit antiphospho-p44/42 MAPK (Thr202/Tyr204) (the activated form of ERK1/2), 1:1000 (Cell Signaling Technology), rabbit anti-phospho-IκBα (Ser32), 1:1000 (Cell Signaling Technology), and anti-rabbit secondary antibody conjugated to HRP, 1:2000 (Cell Signaling Technology). Troponin T (Sigma-Aldrich) (molecular weight of approximately 38 kDa) was used to control for equal loading in each lane of the gel. The molecular weights of the immunodetected proteins were verified by using the biotinylated protein ladder (Cell Signaling Technology). Protein band intensity was assessed by scanning densitometry. Arbitrary values were expressed as a percentage of values for untreated C57BL/10 muscle (NaCl-C57), the arbitrary value as 100%. TNF-α concentrations were expressed as pg/mg of protein.

Glutathione assay. Total glutathione (GSH) and oxidized glutathione (GSSG) within the gastrocnemius muscle tissue were measured with the glutathione assay kit (Cayman Chemical) as described by the manufacturer's instructions. In brief, the de-proteinated sample was used to determine GSH content via a standard enzymatic recycling procedure. To determine the GSSG content, an aliquot of the de-proteinated supernatant was mixed with 2-vinyl-pyridine (Sigma-Aldrich) and triethanolamine (Sigma-Aldrich) and then assayed.

Determination of muscle free amino acid concentrations. Frozen gastrocnemius muscle was homogenized with ice-cold trichloroacetic acid (TCA 10%/EDTA 0.5 mM) containing norvaline (200µM) as an internal standard and stored on ice for 10 minutes. The supernatants obtained after samples were centrifuged at 2000 g for 10 minutes at 4°C were flash frozen in liquid nitrogen and immediately stored at -80°C for subsequent determination of free amino acid concentrations. The pellet was stored at 4°C and used for subsequent total protein determination with DC protein assay kit (Bio-Rad Laboratories). Free amino acids in the supernatant were determined by ion exchange chromatography on an AminoTac JLC/500-V analyser (JEOL) (22).

Statistical analysis. The effects of treatment (L-Gln versus 0.9% NaCl) and mouse strain (*mdx* versus C57BL/10) on variables were determined using 2-way ANOVA. Body weight data were analyzed by repeated measures ANOVA with treatment and mouse strain as between subject factors and time as the within subject factor. Post hoc comparisons were

carried out by using Fisher's PLSD test. A value for $P<0.05$ was considered statistically significant. All values are expressed as mean + SEM.

RESULTS

Body weight during L-Gln or 0.9% NaCl administration. There was a significant effect of mouse strain (C57BL/10 versus *mdx*) on body weight ($P<0.0001$) (Figure 1). Specifically, body weight was significantly lower in *mdx* mice versus age-matched wild-type C57BL/10 controls, regardless of whether they were treated with 0.9%NaCl (NaCl-MDX: 10.5+0.5 g versus NaCl-C57: 17.0+0.3 g, $P<0.0001$) or L-Gln (GLN-MDX: 10.4+0.3g versus GLN-C57: 16.0+0.7 g, $P<0.0001$). We found no significant effect of treatment (L-Gln versus 0.9% NaCl) on body weight for C57BL/10 and *mdx* mice. Over the 3-day experimental period, a significant increase in body weight was observed in all 4 groups ($P<0.0001$).

L-Gln decreases GSSG and GSSG/GSH ratio in mdx skeletal muscle. A significant effect of treatment (L-Gln versus 0.9% NaCl) was observed for GSSG ($P<0.05$) and the ratio of GSSG/GSH ($P<0.05$) (Figure 2B and 2C, respectively). After glutamine treatment, there was significant decrease in GSSG ($P<0.05$) and GSSG/GSH ratio ($P<0.05$) in *mdx* and C57BL/10 mice. There were no differences in GSH concentration between groups (Figure 2A).

Down-regulation of phospho-ERK1/2 (p44/42 MAPK) in mdx skeletal muscle following LGln. There was a significant effect of treatment on the expression of the activated form of ERK1/2 (phospho-ERK1/2) ($P<0.01$) (Figure 3). L-Gln administration significantly decreased the level of phospho-ERK1/2 (p44/42) in *mdx* and C57BL/10 mice ($P<0.01$). The magnitude of the decrease was 2 fold greater in *mdx* compared to control mice (63% versus 29%, respectively). Interestingly, the p44 band (44 kDa protein) was almost absent in the control C57BL/10, regardless of treatment (0.9% NaCl and L-Gln). Similarly, the p44 band almost completely disappeared in *mdx* mice treated with L-Gln.

TNF-α concentrations and NF-κB signaling in skeletal muscle. Figure 4 shows the effects of mouse strain (C57BL/10 versus *mdx*) and treatment (L-Gln versus 0.9% NaCl) on TNF-α concentrations and phospho-IκBα expression in quadriceps muscle. Although there were no significant effects on TNF-α concentrations, Gln treatment appeared to offset the increase in muscle TNF-α concentrations in *mdx* mice ($P=0.055$) (Figure 4A). We were able to clearly detect the phosphorylated form of IκBα in muscle of C57BL/10 and *mdx* mice (Figure 4B). However, IκBα phosporylation was not increased in *mdx* muscle. Gln treatment did not affect IκBα phosphorylation.

Skeletal muscle free amino acid concentrations. Figure 5 shows the concentration of free amino acids in gastrocnemius muscle. There was a significant effect of mouse strain (C57BL/10 versus *mdx*) on intramuscular free Gln concentrations ($P<0.05$) (Figure 5A). Muscle Gln concentrations were significantly higher in *mdx* vs C57/BL10 mice ($P<0.05$). A significant interaction (mouse strain*treatment) was observed for the concentrations of glutamate (Glu) ($P<0.05$) and the sum of Gln+Glu ($P<0.05$) (Figure 5B and 5C, respectively). Basal concentrations of Glu and Gln+Glu were significantly greater in *mdx* versus control muscle (NaCl-*mdx* vs NaCl-C57, $P<0.001$). Gln treatment significantly decreased free intramuscular Glu and Gln+Glu in *mdx* mice only ($P<0.05$). Muscle Gln concentration alone was not affected by Gln treatment. The sum of all amino acids as well as the essential amino acids did not significantly differ between groups (Figure 5D and 5E, respectively).

DISCUSSION

Acute daily IP Gln administration over 3 days decreased GSSG/GSH in skeletal muscle of young *mdx* mice. Free Glu and the sum of Gln+Glu decreased in *mdx* muscle, whereas muscle free Gln was not affected by exogenous Gln. The decrease in GSSG/GSH was associated with a reduction in MAPK (ERK1/2) phosphorylation. The antioxidant mechanism may explain our previous findings in DMD children demonstrating decreased whole body protein degradation after 5 h and 10d glutamine supplementation (6, 7). This study is novel in describing a molecular basis for Gln's antiproteolytic effect in dystrophic muscle.
Here we observed that *mdx* mice (under basal conditions) showed increased muscle free Gln and Glu concentration. This could result from either an increase in proteolysis or Gln *de novo* synthesis in *mdx* muscle, both of which contribute to the muscle free intracellular Gln pool. This is consistent with increased muscle Gln production during catabolic stress, as demonstrated by enhanced Gln Synthetase expression in skeletal muscle after exogenous glucocorticoids (23). In postabsorptive humans, muscle normally releases Gln, while other tissues extract Gln, whereas Glu is taken up by muscle (24). Moreover, it has been reported in critically ill patients that Glu was the only amino acid that showed net uptake across the leg muscle and Glu net uptake correlated with Gln net release (25). Hence, the increased Gln needs by other tissue in DMD pathology may result in increased muscle protein degradation or Glu uptake by muscle to provide precursors for increased Gln *de novo* synthesis (via Gln Synthetase) for subsequent export to other tissue, all at the expense of muscle proteins. In turn after exogenous Gln, we observed a decrease in intracellular Glu and the sum of Gln+Glu in *mdx* muscle, whereas muscle free Gln was not affected. This may result from an inhibition of muscle protein degradation or a reduction in Glu uptake for Gln *de novo* synthesis for subsequent export to other tissue, since Gln availability was increased. Likewise, we showed in DMD children that exogenous Gln decreases Gln *de novo* synthesis (6). Moreover, during the dystrophic process, other tissue may preferentially make use of the provided Gln, leaving skeletal muscle Gln concentration unaltered by exogenous Gln.

The role of oxidative stress in the pathogenesis of DMD is supported by studies showing consistently higher antioxidant enzyme activities and lipid peroxidation products in *mdx* muscle (1, 2, 9, 26). Furthermore, *mdx* muscle cells and DMD skin fibroblasts show an increased susceptibility to oxidative damage (27, 28), suggesting a role of the glutathione antioxidant system in dystrophic pathology (28). Recently, increased GSSG/GSH was reported in young (pre-necrotic) *mdx* hind limb muscle, suggesting an abnormal glutathione status in dystrophic muscle under basal conditions and before muscle necrosis (2).
In the present study, Gln supplementation significantly decreased oxidized glutathione (GSSG) and the GSSG/GSH ratio in skeletal muscle. GSH was not affected, which is in keeping with the lack of increase in muscle Gln or Glu concentrations after exogenous Gln (Gln being a precursor of the Glu for GSH synthesis (20)). The effectiveness of glutathione protection in individual tissue depends on the tissue concentration of glutathione as well as the capacity of the tissue to import the reduced form of glutathione and to export GSSG (29). During catabolic conditions (e.g. critical illness) a change in the redox status occurs, indicative of an increased muscle GSSG relative to GSH concentration (29). Shifting the GSSG/GSH redox toward the oxidizing state activates specific signaling pathways (20), including those involved in proteolysis . Since the cellular redox status seems to be related to the degree of muscle protein degradation (30, 31), this may contribute to the protein catabolism observed in DMD. Exogenous Gln decreased GSSG and GSSG/GSH, and hence could protect against oxidative stress damage (i.e. decrease oxidized/damaged proteins which undergo subsequent degradation). This could then lead to a decrease in muscle necrosis in DMD, since increased oxidative stress precedes the onset of muscle necrosis, as shown by

induction of expression of genes encoding antioxidant enzymes in pre-necrotic *mdx* muscle (32).
Interestingly, decreased GSSG/GSH after Gln supplementation was associated with decreased MAPK (ERK1/2) activation. Oxidative stress could contribute to muscle wasting by regulating specific cell signaling pathways. First, it is possible that oxidative stress leads to cytosolic calcium overload (33) and subsequent activation of calcium-activated proteases (calpain) in skeletal muscle (5). A second potential link between oxidative stress and muscle proteolysis is the control of caspase 3 activity, since caspase 3 activation pathways can be triggered by reactive oxygen species (ROS) (5). A third possible mechanism is the redox regulation of the ubiquitin-proteasome proteolytic system, since oxidative stress can increase muscle protein breakdown via the 26s and 20s core proteasome, which degrades oxidatively damaged proteins (34, 35). Finally, oxidative stress could increase proteolysis by control of MAPK signaling (5). ERK1/2 has been shown to be activated in *mdx* skeletal muscle at rest (10, 16) and in response to mechanical stretch (4). Importantly, ERK1/2, c-Jun N terminal kinase (JNK) and p38 MAPK have all been shown to be activated by oxidative stress in skeletal myoblasts (13). Moreover, ERK1/2 could participate in pathways influencing protein breakdown via the autophagic lysosome pathway (15). Autophagy is the major proteolytic pathway in the cell and the only pathway known to be regulated by plasma amino acids (14, 15). Gln may cause its antiproteolytic effect through osmotic swelling (36) involving the MAPK pathway (37). Likewise, amino acids can control autophagic lysosomal protein degradation by inhibiting ERK1/2 phosphorylation (15). We suggest that Gln may decrease oxidative stress and subsequent protein degradation via modulation of the ERK1/2 MAPK signaling pathway.

By inhibiting the shift toward the oxidizing state and subsequent ERK1/2 activation, exogenous Gln may also prevent the inflammatory cascade, associated with the dystrophic process. In support of this, ERK1/2 is implicated in Activator Protein-1 (AP-1) transcriptional activity and NF-κB transactivation by oxidative stress (5, 13). In turn, mechanical-stretchinduced activation of AP-1 was decreased after pre-treatment of *mdx* muscle fibres with PD98059, an ERK1/2 inhibitor (4). And recent reports in *mdx* muscle, highlight the implication of oxidative stress/lipid peroxidation via MAPK cascade and NF-κB activation, in turn triggering an inflammatory cascade that leads to muscle necrosis (9, 10). We observed that Gln treatment might offset the increase in *mdx* muscle TNF-α inflammatory cytokine concentrations (approaching significance). Interestingly, unlike classical NF-κB activation, we observed that IκBα phosphorylation was not increased in *mdx* muscle. Moreover, Gln treatment did not affect muscle IκBα phosphorylation. Thus Gln treatment might offset the increase in pro-inflammatory cytokine (TNF-α) concentrations in *mdx* muscle, without affecting IκB-α phosphorylation. Our findings are in line with a recent study showing that IκB-α phosphorylation was not associated with the dystrophic phenotype and that pharmacological inhibition of IκB kinase decreased NF-κB activity in *mdx* muscle without affecting IκB-α phosphorylation or degradation (38). Hence, NF-κB activation by IκB kinase may occur independently of classical IκB phosphorylation.

Our findings provide important and novel information that integrates the fields of nutrition, molecular biology, and muscle pathophysiology in childhood disease. The antioxidant protective mechanism of Gln has therapeutic implications for slowing disease progression in DMD children, thus improving quality of life. Moreover, therapeutic strategies (e.g. Gln nutritional therapy) to prevent oxidant production/scavenge free radicals could apply to other muscle-wasting pathologies.

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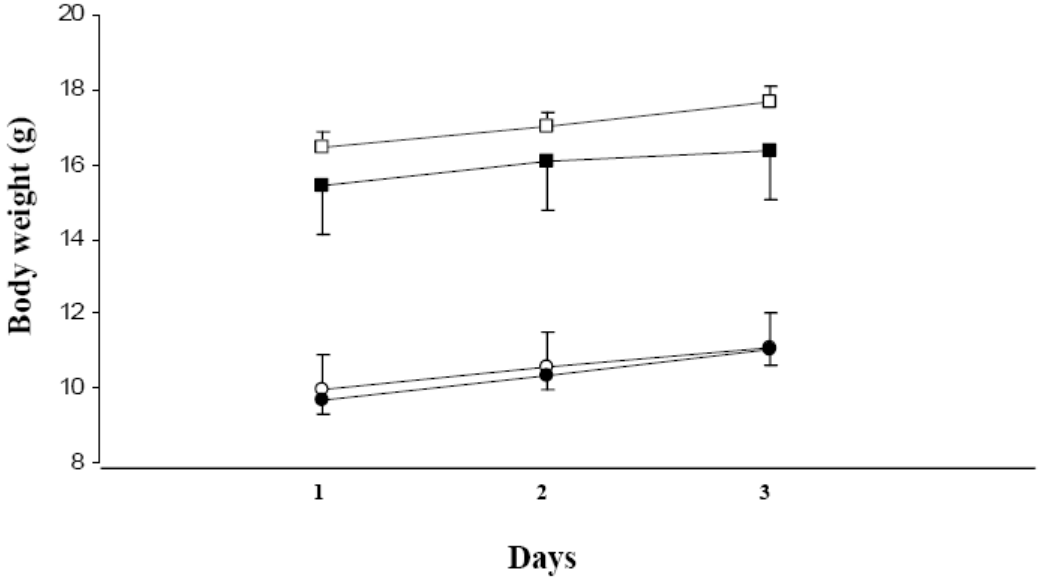
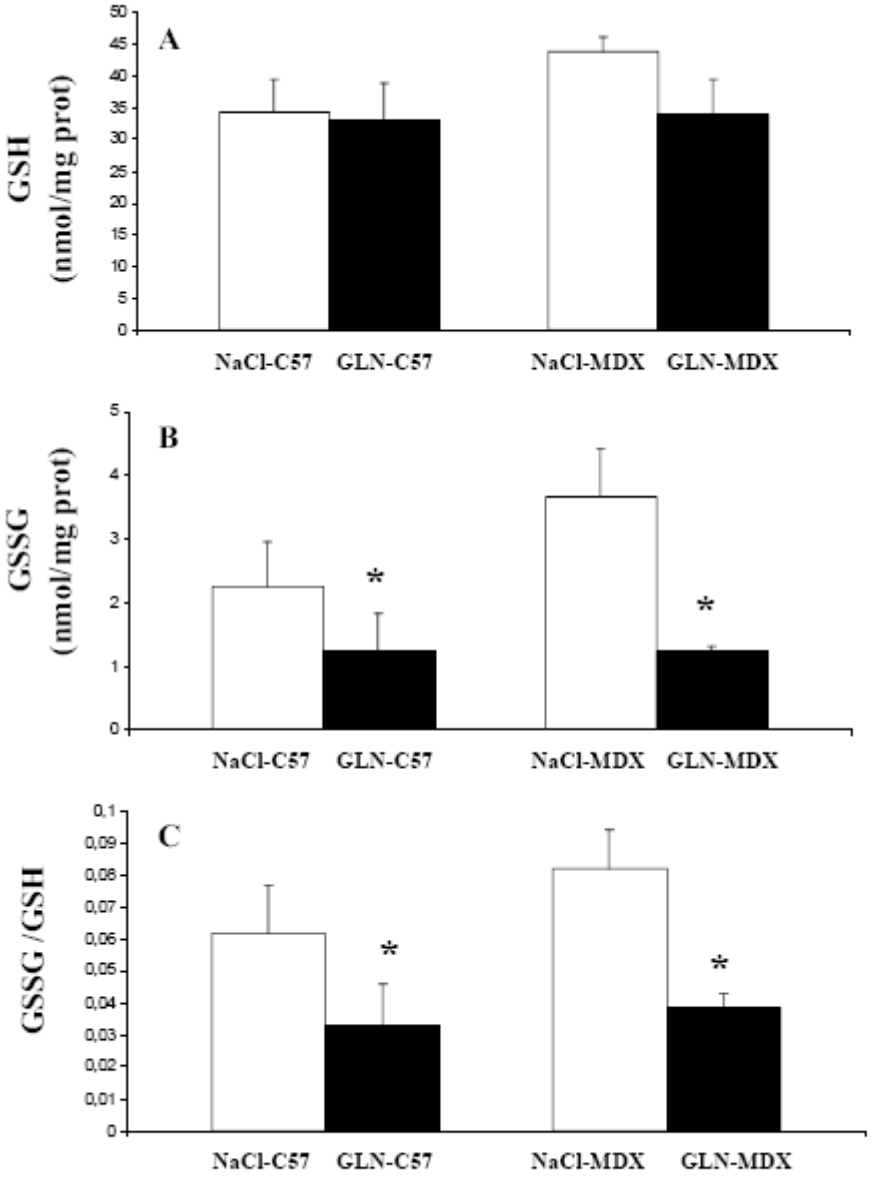
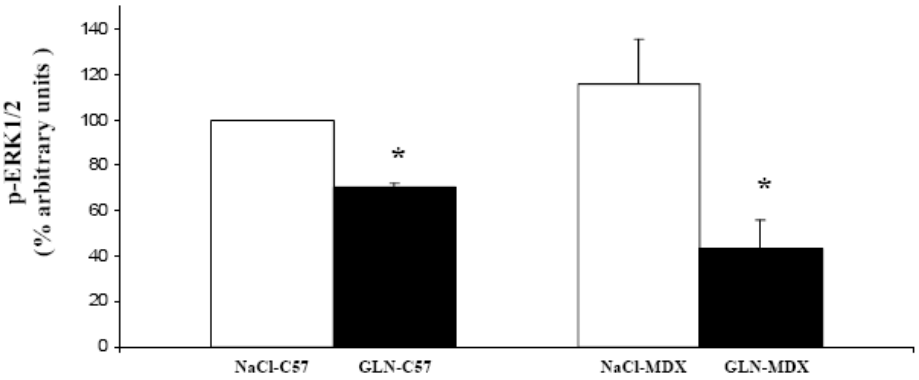
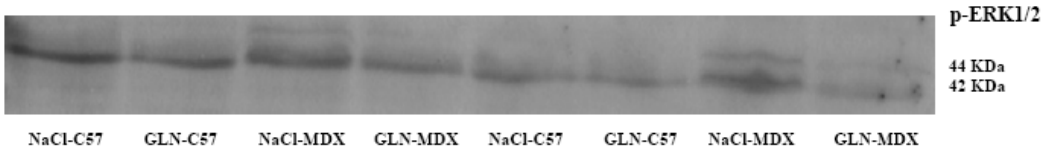
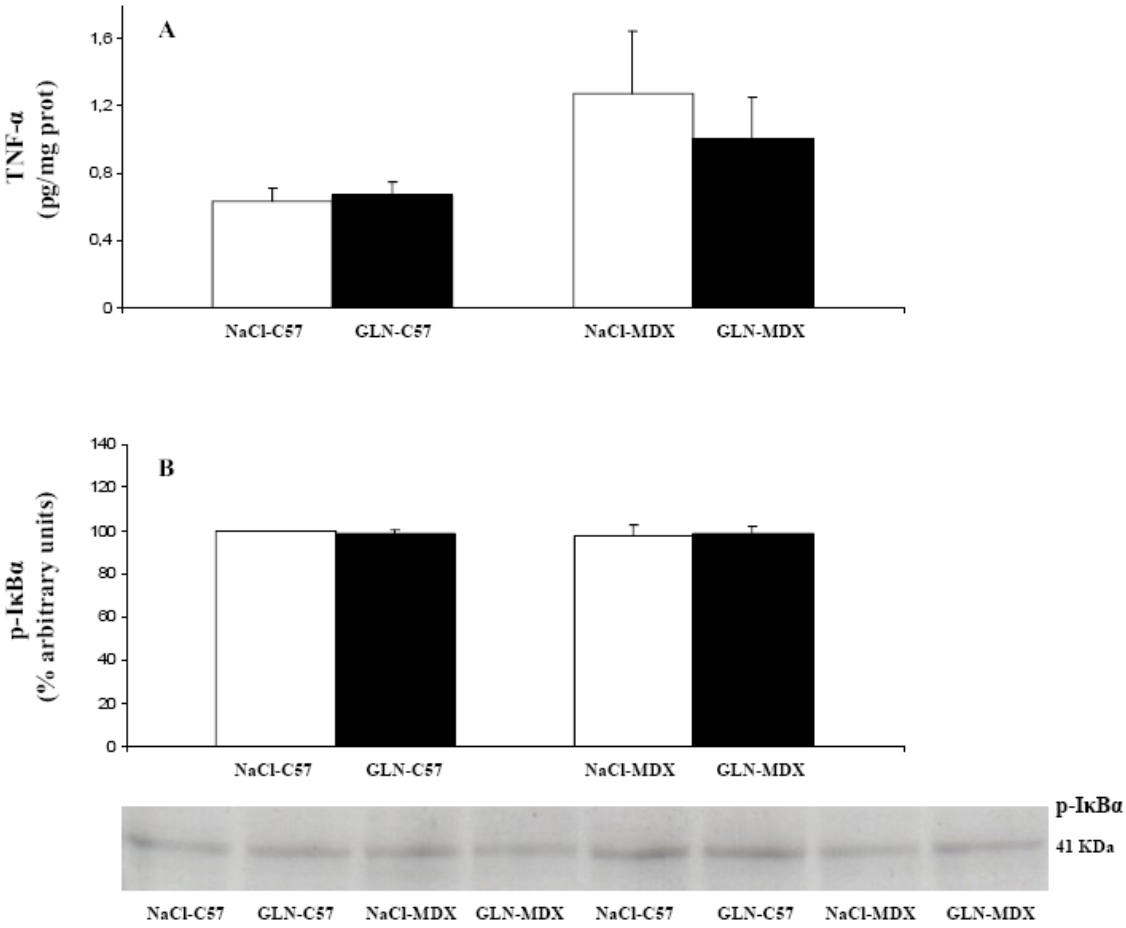
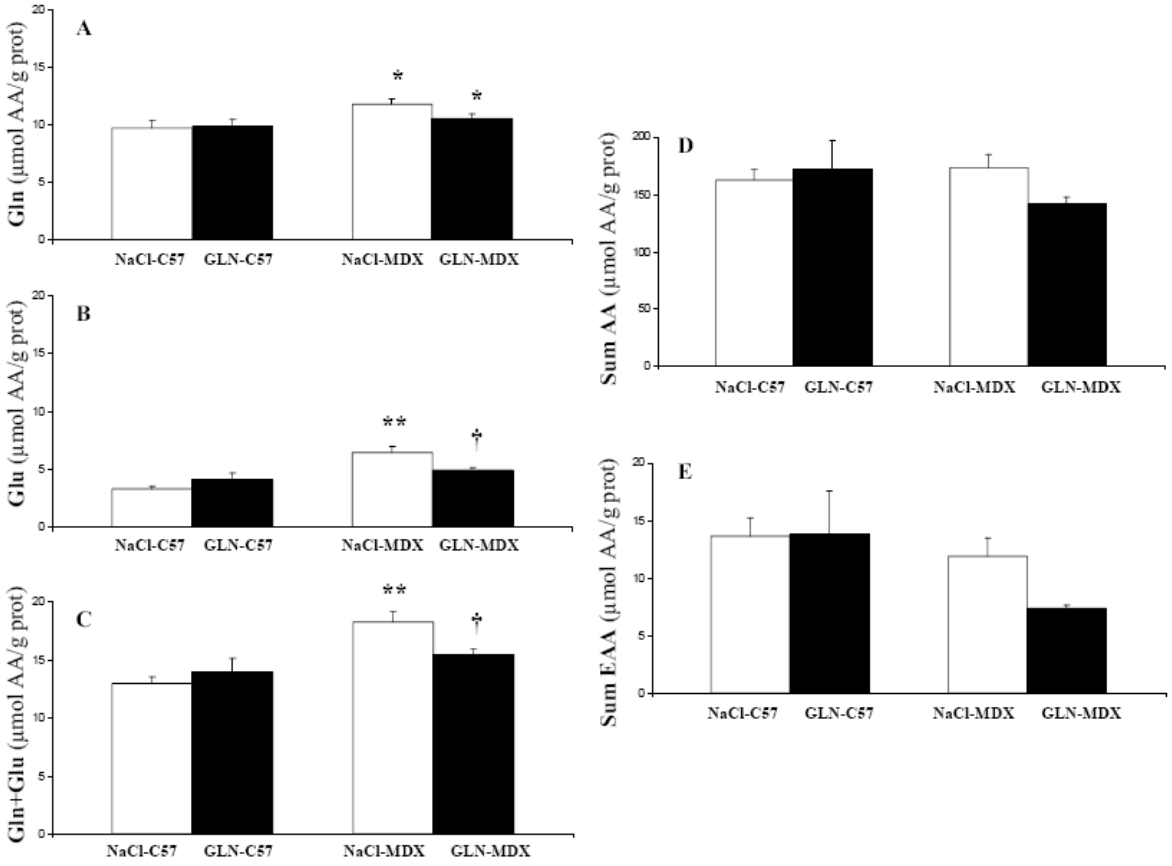

Body weight (g)
20
18
16
14
12
10
8
1
2
3
Days


A
GSH
(nmol/mg prot)
50
45
40
35
30
25
20
15
10
5
0
NaCl-C57
GLN-C57
NaCl-MDX
GLN-MDX
B
GSSG
(nmol/mg prot)
5
4
3
2
1
0
*
*
NaCl-C57
GLN-C57
NaCl-MDX
GLN-MDX
C
GSSG /GSH
0,1
0,09
0,08
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0
*
*
NaCl-C57
GLN-C57
NaCl-MDX
GLN-MDX


p-ERK1/2
(% arbitrary units)
140
120
100
80
60
40
20
0
*
*
NaCl-C57
GLN-C57
NaCl-MDX
GLN-MDX


p-ERK1/2
44 KDa
42 KDa
NaCl-C57
GLN-C57
NaCl-MDX
GLN-MDX
NaCl-C57
GLN-C57
NaCl-MDX
GLN-MDX


A
TNF-α
(pg/mg prot)
1,6
1,2
0,8
0,4
0
NaCl-C57
GLN-C57
NaCl-MDX
GLN-MDX
B
p-IκBα
(% arbitrary units)
140
120
100
80
60
40
20
0
NaCl-C57
GLN-C57
NaCl-MDX
GLN-MDX
p-IκBα
41 KDa
NaCl-C57
GLN-C57
NaCl-MDX
GLN-MDX
NaCl-C57
GLN-C57
NaCl-MDX
GLN-MDX


A
Gln (µmol AA/g prot)
20
15
10
5
0
*
*
NaCl-C57
GLN-C57
NaCl-MDX
GLN-MDX
B
Glu (µmol AA/g prot)
20
15
10
5
0
**
†
NaCl-C57
GLN-C57
NaCl-MDX
GLN-MDX
C
Gln+Glu (µmol AA/g prot)
20
15
10
5
0
**
†
NaCl-C57
GLN-C57
NaCl-MDX
GLN-MDX
D
Sum AA (µmol AA/g prot)
200
150
100
50
0
NaCl-C57
GLN-C57
NaCl-MDX
GLN-MDX
E
Sum EAA (µmol AA/g prot)
20
15
10
5
0
NaCl-C57
GLN-C57
NaCl-MDX
GLN-MDX