Taurine deficiency, synthesis and transport in the mdx mouse model for Duchenne Muscular Dystrophy

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Abstract

The amino acid taurine is essential for the function of skeletal muscle and administration is proposed as a treatment for Duchenne Muscular Dystrophy (DMD). Taurine homeostasis is dependent on multiple processes including absorption of taurine from food, endogenous synthesis from cysteine and reabsorption in the kidney. This study investigates the cause of reported taurine deficiency in the dystrophic mdx mouse model of DMD. Levels of metabolites (taurine, cysteine, cysteine sulfinic acid and hypotaurine) and proteins (taurine transporter [TauT], cysteine dioxygenase and cysteine sulfinic acid dehydrogenase) were quantified in juvenile control C57 and dystrophic mdx mice aged 18 days, 4 and 6 weeks. In C57 mice, taurine content was much higher in both liver and plasma at 18 days, and both cysteine and cysteine dioxygenase were increased. As taurine levels decreased in maturing C57 mice, there was increased transport (reabsorption) of taurine in the kidney and muscle. In mdx mice, taurine and cysteine levels were much lower in liver and plasma at 18 days, and in muscle cysteine was low at 18 days, whereas taurine was lower at 4: these changes were associated with perturbations in taurine transport in liver, kidney and muscle and altered metabolism in liver and kidney. These data suggest that the maintenance of adequate body taurine relies on sufficient dietary intake of taurine and cysteine availability and metabolism, as well as retention of taurine by the kidney. This research indicates dystrophin deficiency not only perturbs taurine metabolism in the muscle but also affects taurine metabolism in the liver and kidney, and supports targeting cysteine and taurine deficiency as a potential therapy for DMD.

Key words

Duchenne Muscular Dystrophy; mdx mice; taurine; cysteine; taurine synthesis; taurine transport.

1. Introduction

Duchenne Muscular Dystrophy (DMD) is a lethal, X-chromosome linked muscle disease affecting about 1 in 3500-6000 boys worldwide [Reviewed in (Bushby et al., 2010, Emery, 2002)]. Mutations in the dystrophin gene result in either the absence or loss of functional dystrophin protein, with dystrophic skeletal muscles having an increased susceptibility to sarcolemma damage after muscle contraction, leading to myofibre necrosis, inflammation, regeneration and fibrosis (Grounds et al., 2008, Kharraz et al., 2014, Lapidos et al., 2004, Petrof et al., 1993). Repeated cycles of widespread myofibre necrosis and a progressive failure of regeneration lead to the loss of muscle mass and function in DMD boys with premature death often due to respiratory or cardiac failure [Reviewed in (Biggar, 2006, Bushby, 2010)].

Whilst the mechanism of necrosis in DMD and the mdx mouse model of the disease are not fully understood, inflammation, oxidative stress and disruptions in intracellular calcium homeostasis caused by sarcolemma instability are strongly implicated. Various compounds that could potentially provide a therapeutic benefit have been tested, including antioxidants, anti-inflammatory drugs and calcium channel blockers. One compound of interest is taurine (2-aminoethanesulfonic acid), a semi-essential amino acid that is found in many tissues and is especially important for the function of skeletal muscle (Bakker and Berg, 2004, Hamilton et al., 2006, Huxtable, 1992, Warskulat et al., 2004, Warskulat et al., 2007). In skeletal muscle, taurine functions in the control of ion channel function, membrane stability and calcium homeostasis (Camerino et al., 2004) and taurine treatment of mdx mice improved grip strength, prevented exercise induced muscle weakness and restored calcium homeostasis (Cozzoli et al., 2011, De Luca et al., 2003). Taurine levels are decreased in mdx muscles before and during the onset of dystropathology (between three and six weeks), and the taurine deficiency diminishes as the disease pathology stabilised in adult mice (Griffin et al., 2001, McIntosh et al., 1998). Whilst the taurine content of DMD muscle has not been reported, the urinary excretion of taurine has been

Abbreviations: 2ME, 2-mercaptoethanol; CD, cysteine deoxygenase; CS, cysteine sulfinic acid; CSD, cysteine sulfinic acid decarboxylase; DMD, Duchenne Muscular Dystrophy; EDTA, ethylenediaminetetraacetic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; HPLC, high performance liquid chromatography; HYP, hypotaurine; OPA, o-phthalaldehyde; OTC, L-2-Oxothiazolidine-4-Carboxylate; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TauT, taurine transporter protein; TCA, trichloroacetic acid.

reported to be increased in DMD boys (Engel et al., 1994), suggesting perturbations in taurine homeostasis.

Whole body taurine homeostasis is regulated by a balance between multiple processes, including absorption from food in the intestine, reabsorption from the kidney, and endogenous synthesis from cysteine (Lambert et al., 2014) as depicted in Figure 1. Maintenance of the intracellular taurine pool depends on active uptake from the extracellular space by the high affinity, low capacity, Na^+ dependent transporter, TauT (Ito et al., 2010), and taurine is released from cells via volume insensitive and volume sensitive leak pathways (Lambert, 2014). The liver is responsible for export of a substantial proportion of the taurine body pool into the plasma, and absorption of taurine from the diet in the liver by hepatocytes requires TauT. Regulation of systemic taurine levels in the plasma occurs in the kidney by reabsorption of taurine in renal tubules. This requires TauT, which is inversely regulated in response to the plasma levels of taurine (Tappaz, 2004). TauT also facilitates active transport of taurine into tissues such as muscle, along with the co-transport of sodium ions, which maintains an extraordinarily high concentration gradient (Huxtable, 1992). Muscle function is dependent on adequate TauT. Deletion of exon 1 of the TauT gene results in a striking (98%) decrease in heart and skeletal muscle taurine levels, with severely impaired skeletal muscle function and exercise capacity (Warskulat, 2004). TauT knockout mice also display severe abnormalities in muscle structure including decreased myofibre cross sectional area, myofilament fragmentation, and lipid droplets within the myofibre (Ito et al., 2008, Ito, 2010).

Another major cellular process that contributes to whole body taurine content is the synthesis of taurine from cysteine (summarised in Figure 1). Cysteine is a semi-essential amino acid, which is present in the diet and can be synthesised from the amino acid methionine. Taurine synthesis is tightly regulated by cysteine availability, and in the liver this process serves as a method to dispose of toxic excess cysteine (Huxtable, 1992). Cysteine is catabolised by the enzyme cysteine dioxygenase to cysteine sulfinic acid, which is decarboxylated to hypotaurine by the enzyme cysteine sulfinic acid decarboxylase, which is then oxidised to taurine (Huxtable, 1992). While taurine formation is proposed to occur principally in the liver, other cells contain trace amounts of these enzymes and are thus also able to form taurine from cysteine, including the brain and skeletal muscle (Stipanuk, 2004).

The cellular activity and expression of the enzymes cysteine deoxygenase and cysteine sulfinic decarboxylase are responsive to intracellular cysteine content, as well as dietary changes in protein and intake of sulfur amino acids (cysteine and methionine). Recently, we established that treatment of adult mdx mice with the cysteine precursor L-2-Oxothiazolidine-4-Carboxylate (OTC) led to an increase in the synthesis of taurine, resulting in a decreased dystropathology and reduced oxidative stress in the dystrophic muscle (Terrill et al., 2013). These data suggest that taurine deficiency in mdx muscle may be due to insufficient synthesis of taurine.

A deficiency of taurine in mdx muscle appears to be correlated with the progression of the disease, which may indicate that dystrophy is perturbing taurine metabolism. To evaluate this possibility, we investigated the multiple processes contributing to body taurine content (summarised in Figure 1). We compared mdx and wildtype C57 (C57Bl/10ScSn) mice at three ages: pre-dystrophy (18 days), during active dystropathology (4 weeks) and when **dystropathology has begun to stabilise to a lower level of active necrosis (6 weeks)**, and measured the content of taurine in liver, plasma and muscle. We examined the levels of TauT protein in liver, kidney and muscle in mdx and C57 mice at the three ages. The endogenous capacity to synthesise taurine in liver and muscle was evaluated by measuring the levels of the enzymes cysteine deoxygenase and cysteine sulfinic decarboxylase (which catalyze the synthesis of taurine from cysteine), and the intermediate metabolites cysteine sulfinic and hypotaurine. Since synthesis requires adequate cysteine availability, cysteine content was also measured in liver, plasma and muscle of all mice. The results showed perturbations in levels and metabolism of taurine in dystrophic tissue that varied with age in the growing mdx mice.

2. Methods

All reagents used were obtained from Sigma Aldrich (NSW, Australia) unless otherwise specified.

2.1. *Animal procedures*

All experiments were carried out on male dystrophic mdx (C57Bl/10ScSnmdx/mdx) and non-dystrophic control C57 (C57Bl/10ScSn) mice (the parental strain for mdx). All mice were obtained from the Animal Resource Centre, Murdoch, Western Australia. Mice were maintained at the University of Western Australia on a 12-h light/dark cycle, under standard conditions, with free access to food and drinking

water. All animal experiments were conducted in strict accordance with the guidelines of the National Health and Medical Research Council Code of practice for the care and use of animals for scientific purposes (2004), and the Animal Welfare act of Western Australia (2002), and were approved by the Animal Ethics committee at the University of Western Australia.

2.2. *Tissue collection*

Mice were sacrificed at 18 days, 4 and 6 weeks of age by cervical dislocation while under terminal anesthesia (2%v/v Attane isoflurane, Bomac, Australia). Whole blood was collected via cardiac puncture using a 27.5 gauge tuberculin syringe, into a 1.5 ml tube and spun down immediately in a refrigerated centrifuge for 5 minutes (12000 g) and plasma removed, aliquoted and frozen at -80°C until analysis. Quadriceps muscles, liver and kidney were collected and immediately snap frozen in liquid nitrogen for biochemical analyses.

2.3. *HPLC analysis of metabolites*

Taurine, cysteine, cysteine sulfinic acid and hypotaurine and in liver, plasma and quadriceps muscle was measured using reverse phase high performance liquid chromatography (HPLC) as previously described (Terrill, 2013). Plasma samples were precipitated by addition of 10 times by weight of 5% trichloroacetic acid (TCA). Frozen tissues were crushed using a mortar and pestle under liquid nitrogen and homogenized in 25 times 5% TCA. After centrifugation, supernatants were removed and stored at -80°C before analysis. Analytes were separated using HPLC with fluorescent detection, with pre-column derivitisation with o-phthalaldehyde (OPA) and 2-mercaptoethanol (2ME). OPA reacts rapidly with amino acids and sulfhydryl groups to yield intensely fluorescent derivatives, and 2ME, a reducing agent, prevents the OPA reagent from oxidising. Supernatants were mixed with iodoacetamide, dissolved in 5% TCA, to a final concentration of 25 mM. An internal standard, o-phospho-DL-serine, dissolved in 5% TCA was added to a final concentration of 5 mM. pH was adjusted to 9 with the sodium borate. Samples were placed in the autosampler, which was maintained at 4°C. Samples were mixed on sample loop with the derivitising solution, containing 40 mM OPA and 160 mM 2ME in 100 mM sodium borate, pH 12, for 30 seconds before injection onto the column. Separation was achieved with a C18 column (5 µl, 4.6 x 150 mm, Phenomenex) using a Dionex Ultimate 3000 HPLC system. Mobile phase A consisted of 50 mM potassium phosphate buffer, methanol and tetrahydrofuran (94:3:3). Mobile phase B consisted

of 90% Methanol, with a gradient increase in B from 0 to 25%. Fluorescence was set at 360 nm and 455 nm for excitation and emission respectively. Protein content of liver and muscle samples were quantified by solubilising the pellet in 0.5 M sodium hydroxide, before incubation at 80°C for 15 minutes. Once fully dissolved, protein concentrations of supernatants were quantified using a Bradford protein assay (BioRad, NSW, Australia).

2.4. Protein extraction and immunoblotting

Frozen tissues were crushed using a mortar and pestle under liquid nitrogen and homogenized in 1 ml ice-cold 1% NP40, 1 mM EDTA in phosphate buffered saline (PBS), supplemented with complete EDTA free protease inhibitor tablets and PhosSTOP phosphatase inhibitor tablets (Roche, Mannheim, Germany), and centrifuged at 13000 *g* for 10 min. Protein concentrations of supernatants were quantitated using the Detergent Compatible protein assay (Bio-Rad, NSW, Australia). Samples were resolved on 4-15% SDS-PAGE TGX gels (Bio-Rad, NSW, Australia) and transferred onto nitrocellulose membrane using a Trans Turbo Blot system (Bio-Rad, NSW, Australia). Immuno-blotting was performed with antibodies to cysteine dioxygenase type 1 (ab53436, Abcam, Cambridge, UK), cysteine sulfinic decarboxylase (ab101847, Abcam, Cambridge, UK), TauT (TAU11-A, Alpha Diagnostic, Texas USA) and GAPDH (14C10, Cell Signalling, MA, USA), all dissolved 1:1000 in 5% bovine serum albumin (BSA). HRP-conjugated secondary antibodies were from Thermo Fisher Scientific, MA, USA. The chemiluminescence signal was captured using the ChemiDoc MP Imaging System (Bio-Rad, NSW, Australia). Resultant images were quantified using ImageJ software (Schneider et al., 2012). A common sample was loaded onto each gel to normalise for detection efficiencies across membranes. GAPDH loading controls were immunoblotted on the same membrane as immunoblotted protein. All representative immunoblots in figures represent samples immunoblotted on the same membrane.

2.5. Statistics

Significant differences between groups were determined using one way ANOVA with post hoc tests and all data are presented as mean $\pm$ standard error of the mean (SEM). Significance was set at $p < 0.05$.

3. Results

3.1. Taurine content of C57 and mdx liver, plasma and muscle

Taurine content was measured (using HPLC) in wild type C57 and dystrophic mdx liver, plasma and quadriceps muscle at three ages: pre-dystropathology (18 days), during active dystropathology (4 weeks) and **when dystropathology has begun to stabilise to a lower level of active necrosis (6 weeks)**.

In the liver of C57 mice the content of taurine declined dramatically from 18 days to 4 weeks, and remained low at 6 weeks (Fig. 2). Taurine content of 18 days mdx liver was strikingly lower (~50%) than 18 days C57 liver, but at 4 and 6 weeks, taurine levels were low and comparable in both strains (Fig. 2).

In the plasma of C57 mice, the concentration of taurine declined from 18 days to 4 weeks and then increased at 6 weeks (Fig. 2). Taurine was significantly lower (~30%) in plasma of 18 day old mdx compared with C57 mice. Taurine concentration also declined in mdx plasma from 18 days to 4 weeks, and was not significantly different from c57 mice at 4 and 6 weeks (Fig. 2).

In the quadriceps muscle of C57 mice, taurine content increased from 18 days to 6 weeks (Fig. 2). Taurine content was similar in mdx muscle at 18 days, but by 4 weeks mdx muscle had lower (~25%) taurine content than C57 muscle (Fig. 2). This deficiency was not evident at 6 weeks, with taurine content of 6 week mdx muscle also significantly higher than at 18 days (Fig. 2).

These data indicate taurine levels are perturbed in mdx mice. There is an apparent deficiency of taurine in growing mdx mice during active dystropathology at 4 weeks, with low plasma levels also evident at 18 days.

3.2. *TauT expression in C57 and mdx liver, kidney and muscle*

The maintenance of intracellular taurine pools in all tissues, including muscle, depends on uptake from the extracellular space by TauT, and we therefore measured the amount of TauT protein in liver, kidney and muscle of C57 and mdx mice, at the three ages.

In C57 liver, there were no significant changes in the amount of TauT protein from 18 days to 6 weeks. However, in mdx livers there was a striking increase (~200%) from 18 days to 4 weeks, with the mdx levels about 300% higher at 4 weeks compared

with C57 liver (Fig. 3). By 6 weeks, TauT levels in mdx liver had declined and were at levels comparable to C57 liver (Fig. 3).

In the C57 kidney, there was a striking increase (~1500%) in TauT from 18 days to 4 weeks, whereas TauT levels in mdx kidney did not change significantly with age. TauT protein levels were significantly higher (~200%) at 4 weeks in C57 compared with mdx kidney (Fig. 3).

In C57 quadriceps muscle, TauT protein levels were similar at 18 days and 4 weeks, with a significant increase by 6 weeks (Fig. 3). While levels of TauT protein in mdx muscle did not change significantly with age, levels were significantly lower for mdx compared with C57 muscles at 18 days and 6 weeks (Fig. 3).

These data indicate TauT levels were perturbed in mdx mice. In muscle it is of note that TauT levels were depressed prior to active dystrophy. The perturbation extended beyond muscle with perturbations in TauT levels in liver and kidney during active dystrophiaology.

3.3. *Cysteine content of C57 and mdx and liver, plasma and muscle*

The synthesis of taurine requires an adequate supply of cysteine, therefore the content of cysteine in liver, plasma and muscle of mdx and C57 mice was measured at the three ages.

In C57 liver, the content of cysteine declined (~50%) from 18 days to 4 weeks, and remained low at 6 weeks (Fig. 4). In contrast, cysteine did not change significantly in mdx liver with age (Fig. 4). The key difference was that cysteine content of mdx liver at 18 days was about five times lower than C57 mice (Fig. 4).

In C57 plasma, cysteine concentrations were similar at 18 days and 4 weeks, but decreased significantly by 6 weeks (Fig. 4). Cysteine levels in mdx plasma were relatively unchanged with age (Fig 4). The plasma levels of cysteine were lower (~30%) in mdx mice compared to C57 at both 18 days and 4 weeks, but levels were comparable at 6 weeks (Fig. 4).

In C57 quadriceps muscle, the content of cysteine declined from 18 days to 6 weeks (Fig. 4). In contrast, cysteine content did not change in mdx muscle from 18 days to 4

weeks, but was lower at 6 weeks (Fig. 4). Note that at 18 days, the cysteine content of mdx muscle was about half that of C57 (Fig. 4).

These data indicate cysteine levels were perturbed in mdx mice. Notably, prior to dystropathology in 18 day old mdx mice there was an apparent deficiency in cysteine content in liver and muscle, and a lower cysteine concentration in plasma.

3.4. *Taurine synthesis in C57 and mdx and liver and muscle.*

The synthesis of taurine from cysteine begins with the catabolism of cysteine to cysteine sulfinic acid, by the enzyme cysteine deoxygenase, which is decarboxylated to hypotaurine by the enzyme cysteine sulfinic acid decarboxylase (Fig. 1). We therefore measured protein levels of the enzymes cysteine deoxygenase and cysteine sulfinic acid decarboxylase, plus the intermediate metabolites cysteine sulfinic acid and hypotaurine in C57 and mdx and C57 livers and muscles, to assess whether the ability of mdx mice to synthesize taurine is compromised.

In livers of both strains, the expression of cysteine deoxygenase dropped dramatically (to ~4%) from 18 days to 4 weeks, and remained low at 6 weeks (Fig. 5). The only significant difference in expression of cysteine deoxygenase was at 18 days where mdx liver was slightly higher than C57 (Fig. 5). The product of cysteine deoxygenase activity, cysteine sulfinic acid, declined in C57 liver from 18 days to 6 weeks but did not change significantly in mdx livers (Fig. 5). Levels of cysteine sulfinic acid decarboxylase protein increased in C57 liver from 18 days to 6 weeks (Fig. 5). In contrast, the increase in cysteine sulfinic acid decarboxylase protein was delayed in mdx liver and did not increase until 6 weeks (Fig. 5). At 4 weeks, cysteine sulfinic acid decarboxylase expression in mdx liver was 3 times lower than in C57, and remained significantly lower at 6 weeks (Fig. 5). Hypotaurine, the product of cysteine sulfinic acid decarboxylase activity, decreased in C57 liver from 18 days to 6 weeks (Fig. 5), however there was no significant change in hypotaurine content of mdx liver with age. Of note, hypotaurine content was significantly lower in 18 day mdx liver, compared to 18 day C57 liver (Fig. 5).

We also measured content of these analytes in muscle, to assess the potential for *de novo* synthesis within the muscle. In marked contrast with C57 liver (Fig. 5), cysteine deoxygenase expression in C57 quadriceps muscle did not change significantly with age (Fig. 6). While cysteine deoxygenase expression in mdx muscle decreased from

18 days to 4 weeks, it had recovered by 6 weeks (Fig. 6). At 4 weeks, cysteine deoxygenase in mdx muscle was half that of C57 (Fig. 6). The pattern of cysteine sulfinic acid change with age was similar for C57 liver and muscle with a significant decrease only at 6 weeks (Fig. 6). In mdx muscle, cysteine sulfinic acid content did not change with age (as was also seen for mdx liver), nor were there any significant differences with age matched C57 muscle.

Levels of the enzyme cysteine sulfinic acid decarboxylase declined in C57 muscle from 18 days to 4 weeks, and remained low at 6 weeks (Fig. 6). This is in direct contrast with the situation in liver where cysteine sulfinic acid decarboxylase increased with age (Fig. 5). In mdx muscle, cysteine sulfinic acid decarboxylase displayed the same pattern, with no significant difference with age matched C57 muscle (Fig. 6). The content of hypotaurine declined in C57 muscle from 18 days to 4 weeks, and remained low at 6 weeks (Fig. 6). Hypotaurine content in mdx muscle was not significantly different from C57 muscle at any age (Fig. 6).

Overall, these data indicate dystrophy may have affected taurine synthesis in the liver as indicated by the lower cysteine sulfinic acid decarboxylase levels during active dystropathology at 4 weeks and after dystropathology has stabilised at 6 weeks. Interestingly, the perturbation of cysteine sulfinic acid decarboxylase were not evident in dystrophic muscle, despite a significant decrease in cysteine deoxygenase at 4 weeks.

It should be noted, when comparing the content of these enzymes between liver and muscle, liver appears to have a much greater capacity for synthesis, with values for cysteine deoxygenase and cysteine sulfinic acid decarboxylase being 8 and 12 times higher respectively. Therefore the contribution of *de novo* synthesis of taurine in muscle to total taurine content is yet to be established.

4. Discussion

The key finding of this research is that in the mdx mouse, a dystrophin deficiency not only perturbs taurine metabolism in the muscle but also affects taurine metabolism in the liver and kidney. These disturbances occur both before and after the onset of dystropathology. Given that the key defect, absence of dystrophin, mainly affects muscle, perturbations of taurine metabolism in the liver and kidney presumably occur in response to metabolic disturbances in dystrophic myofibres.

In addition to establishing that taurine metabolism was perturbed in dystrophic muscle, novel data were generated on the development of taurine metabolism in normal (C57) growing juvenile mice. Our data indicate that between 18 days and 4 to 6 weeks, components of taurine metabolism undergo dramatic changes. The key findings are that at 18 days in C57 mice there are high levels of taurine in liver and plasma, elevated content of cysteine and cysteine deoxygenase in the liver, with low levels of the taurine transporter (TauT) in kidney. Most of these age-related changes can be explained by existing concepts about the regulation of taurine metabolism (table 1).

In C57 liver, taurine content is more than 10 times higher at 18 days than at either 4 or 6 weeks, suggesting that in these very young mice (around the time of weaning) there is either elevated taurine supply by the diet (milk), increased synthesis of taurine, or residual taurine derived from maternal circulation during gestation. Taurine is the most abundant amino acid in mouse milk (Rassin et al., 1978), and in newborn rats, the taurine content in milk rises dramatically from birth through to weaning (Huxtable and Lippincott, 1983). However, in 20 day old rats (at time of weaning), only 13% of body taurine has come from milk, 4% is derived from the mother and 83% is synthesised *de novo* (Huxtable and Lippincott, 1983).

The liver readily synthesises taurine when cysteine supply is abundant (Stipanuk, 2004). The key enzyme cysteine deoxygenase responds rapidly to increases in the amount of dietary protein or sulfur amino acids, with increases in cysteine deoxygenase activity and expression (Bagley and Stipanuk, 1995, Stipanuk et al., 2002). The activity and expression of cysteine sulfinatase decarboxylase is not as highly responsive to changes in sulfur amino acid intake (Jerkins et al., 1998), so cysteine deoxygenase activity is considered the major controlling factor in the synthesis of taurine from cysteine (Stipanuk, 2004). Therefore, the elevated cysteine deoxygenase levels in livers of 18 day old C57 mice, relative to 4 and 6 week old C57 mice, is likely due to high liver cysteine content. High cysteine deoxygenase in liver would enhance taurine synthesis, and this would contribute to the high taurine content in liver at 18 days. Furthermore, increased taurine synthesis by the liver, combined with taurine availability from milk, are likely to account for the high taurine concentration in plasma of 18 day old C57 mice.

One potential consequence of increased taurine in plasma is decreased expression or activity of TauT in the kidney (Tappaz, 2004). The taurine body pool is regulated

by inverse regulation of TauT in response to the plasma levels of taurine, and increasing taurine plasma levels down-regulate TauT mRNA in the kidney (Matsell et al., 1997, Tappaz, 2004). The low TauT in kidneys of 18 day old C57 mice indicates a likely down-regulation in response to increased plasma taurine levels, resulting in increased excretion of taurine in urine. For C57 mice, from 18 days to 4 weeks, there was a drop in plasma taurine levels, and a dramatic increase in TauT expression in the kidney, indicating that as the taurine availability decreases, there is increased retention of taurine.

Our data indicate that taurine content in muscle, which increased from 18 days to 6 weeks of age was not responding to plasma taurine, which decreased over the corresponding time. Additionally, there was no evidence of increased intramuscular taurine synthesis. Increased TauT is a possible cause of increased taurine content, but little is known about regulation of TauT in skeletal muscle, especially during post-natal growth. In contrast to kidney, TauT content in muscle did not respond to changes in plasma taurine levels. *In vitro* studies point to alternate mechanisms of regulating levels of TauT. In rat (L6) myoblasts, TauT activity and mRNA expression is increased in response to exposure to cortisol and IGF1 (Park et al., 2004), and TauT mRNA expression is upregulated during myogenesis in mouse (C2C12) myotubes (Uozumi et al., 2006).

In dystrophic mice, our data show that taurine content was lower in mdx muscle than C57 muscle at 4 weeks, during active dystropathology. Since taurine treatment improves mdx muscle pathology (Cozzoli, 2011, De Luca, 2003), this decline in taurine content may exacerbate the breakdown of dystrophic muscle. The cause of this low muscle taurine is unclear. A decline in taurine uptake does not appear to be causal as plasma levels of taurine and Taut content in muscle were similar to C57 levels at 4 weeks. One possibility is deficient taurine synthesis by mdx muscle, as cysteine deoxygenase was significantly lower than C57 muscle at 4 weeks, however, the overall contribution of *de novo* synthesis in muscle to taurine content is yet to be established.

A key finding is the evidence of perturbations in taurine metabolism that extended beyond the muscle of dystrophic mice. **Future experimental research to assess the extent of this perturbation may be useful, for example to examine the cysteine and taurine content of tissues such as brain and heart, and expression of TauT in tissues such as the gut.** The pathogenic pathway(s)

causing the perturbation in taurine metabolism remain to be elucidated. One possibility is that in 4 week mdx mice, the presence of dystropathology could be a contributing factor. Inflammatory responses associated with dystropathology result in the release of cytokines which have the potential to affect tissues distal to the site of injury (Tidball, 2005). However, taurine levels in liver and plasma are lower in 18 day old mdx mice relative to C57 mice. These perturbations in taurine metabolism are occurring when the mdx limb muscles are still intact, prior to the acute onset of dystropathology around 21 days. Another possibility is that these perturbations are a consequence of changes in metabolic demands of dystrophic muscles. Energy expenditure, muscle protein synthesis, and whole body protein turnover rates are considerably higher in mdx mice relative to age-matched controls (Radley-Crabb et al., 2014). It is plausible that the increase in metabolic demand is responsible for the lower cysteine content in liver, plasma and muscle of 18 day old mdx mice relative to C57 mice. The liver readily synthesises taurine when cysteine supply is adequate, but when cysteine is lacking it is conserved for other functions (Stipanuk, 2004). As a consequence, the decreased availability of cysteine would lead to decreased synthesis of taurine and a decrease in taurine content of liver and plasma.

In summary, we have established that dystrophy perturbs taurine metabolism in muscle. Whether the perturbation in juvenile mice is a cause or consequence of dystropathology requires further investigation. Our finding that perturbations of taurine metabolism are also occurring in liver and kidney raises the larger question as to whether other aspects of liver and kidney function are affected by dystrophy and whether there are any adverse effects on the progression of pathology in dystrophic muscle. **Investigating perturbations in taurine homeostasis in older mdx mice (once muscle pathology has fully stabilised) may therefore be useful.** Furthermore, this research supports targeting cysteine and taurine metabolisms as a potential therapy for DMD.

Conflict of interest statement

All authors have no financial or personal conflict with other people or organisations that could inappropriately influence our work.

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Figure captions

Fig. 1. Schematic of taurine metabolism. The schematic illustrates the complexity of regulating muscle taurine content: it is dependent on cysteine availability, expression of cysteine deoxygenase (CD) and cysteine sulfinate decarboxylase (CSD) in the liver, and expression of taurine transporter (TauT) in multiple tissues including liver, kidney and muscle. Methionine, cysteine and taurine (dotted arrows) are absorbed in the gut into blood plasma, with cysteine and methionine metabolised mainly in the liver. Taurine is taken up by tissues via TauT (only shown for liver, kidney and muscle). Primarily in the liver, cysteine is synthesised from methionine via transsulfuration. When cysteine is abundant, the synthesis of taurine occurs via actions of the enzymes CD and CSD. Hypotaurine is chemically unstable, and is thus readily oxidised to taurine. Taurine (solid arrows) is transported from the liver in the blood plasma. The kidney is the primary site of regulating taurine abundance, where taurine is filtered from plasma and reabsorbed via the actions of TauT: taurine that is not reabsorbed is excreted in urine.

Fig. 2. Taurine content of liver, plasma and muscle from 18 day (18d), 4 week (4w) and 6 week (6w) C57 and dystrophic mdx mice. Values are nmol per mg protein for liver and muscle, and μM for plasma. Bars represent mean $\pm$ SEM and $n=7-8$. Symbols for significant differences ($p<0.05$) are: * - between C57 and age matched mdx; \$ - compared with 18d mice of same strain; # - compared to 4w mice of same strain.

Fig. 3. Levels of the taurine transporter (TauT) protein in liver (L), kidney (K) and muscle (M) from 18d, 4w and 6w C57 and mdx mice. Representative immunoblots show data for 2 mice per group. Values represent arbitrary units, and are not comparable between tissues. Bars represent mean $\pm$ SEM and $n=7-8$. Symbols for significant differences ($p<0.05$) are: * - between C57 and age matched mdx; \$ - compared with 18d mice of same strain; # - compared to 4w mice of same strain.

Fig. 4. Cysteine content of liver, plasma and muscle from 18d, 4w and 6w C57 and mdx mice. Representative immunoblots show data for 2 mice per group. Values represent nmol per mg protein for liver and muscle, and μM for plasma. Bars represent SEM and $n=7-8$. Symbols for significant differences ($p<0.05$) are: * - between C57 and age matched mdx; \$ - compared with 18d mice of same strain; # - compared to 4w mice of same strain.

Fig. 5. Quantification of levels of the enzymes and their metabolites involved in taurine synthesis in liver. Cysteine deoxygenase (CD, western blot), cysteine sulfinic acid (CS, HPLC), cysteine sulfinic acid decarboxylase (CSD, western blot) and hypotaurine (HYP, HPLC) from 18d, 4w and 6w C57 and mdx mice. Representative immunoblots show data for 2 mice per group. Values represent arbitrary units for CD and CSD, and nmol per mg protein for cysteine sulfinic acid and hypotaurine. Bars represent SEM and n=7-8. Symbols for significant differences (p<0.05) are: * - between C57 and age matched mdx; \$ - compared with 18d mice of same strain; # - compared to 4w mice of same strain.

Fig. 6. Quantification of levels of the enzymes and their metabolites involved in taurine synthesis in muscle. Cysteine deoxygenase (CD, western blot), cysteine sulfinic acid (CS, HPLC), cysteine sulfinic acid decarboxylase (CSD, western blot) and hypotaurine (HYP, HPLC) from 18d, 4w and 6w C57 and mdx mice. Values represent arbitrary units for CD and CSD, and nmol per mg protein for cysteine sulfinic acid and hypotaurine. Bars represent SEM and n=7-8. Symbols for significant differences (p<0.05) are: * - between C57 and age matched mdx; \$ - compared with 18d mice of same strain; # - compared to 4w mice of same strain.

Table 1. Model describing developmental changes in taurine metabolism in C57 mice. Changes in content or concentration of key analytes that occur after 18 days are summarised in the table. We propose taurine metabolism in C57 mice is driven by the availability of both cysteine and taurine. Prior to weaning (at 18 days), abundant cysteine upregulates taurine synthesis in the liver and this plus taurine from milk causes high plasma taurine resulting in the down regulation of TauT in the kidney to facilitate excretion of taurine into the urine. In older mice, either decreased availability of cysteine in the diet or decreased synthesis of cysteine causes a down regulation of taurine synthesis in the liver. This, combined with limited taurine in the diet, lead to decreased plasma taurine and upregulation of TauT in the kidney to decrease taurine excretion into urine. While taurine content of C57 muscle remains quite constant across the three ages, there is an upregulation of TauT in the muscle at 6 weeks, resulting in a slight increase in taurine content. The mechanism driving this upregulation is not understood. Arrows represent changes from 18 day to 4 week C57: —, no change; ↗, increasing; and ↘, decreasing levels of analytes. n/a indicates not applicable.

Table 2. Summary of perturbations of taurine metabolism in mdx mice before (18 days) and during (4 weeks) active dystropathology.

Data presented in this research indicate that the disturbances in taurine metabolism observed in mdx mice occur before and after onset of pathology, and occur in tissues such as liver, plasma, kidney and muscle. Arrows represent differences between mdx relative to age matched C57 tissue: —, no change; ↑, increased; and ↓, decreased levels of analytes. n/a indicates not applicable.

Fig. 2.

Fig. 4.

Fig. 5.

Fig. 6.

—	Muscle	Liver	Kidney	Plasma
Taurine	—	↘	n/a	↘
TauT	—	—	↗	n/a
Cysteine	↘	↘	n/a	—
CD	—	↘	n/a	n/a
CSD	↘	↗	n/a	n/a

Table 1.

	Muscle		Liver		Kidney		Plasma	
	Pre-dyst.	Active	Pre-dyst.	Active	Pre-dyst.	Active	Pre-dyst.	Active
Taurine	—	↓	↓	—	n/a	n/a	↓	—
TauT	↓	—	—	↑	—	↓	n/a	n/a
Cysteine	↓	—	↓	—	n/a	n/a	↓	↓
CD	—	↓	↑	—	n/a	n/a	n/a	n/a
CSD	—	—	—	↓	n/a	n/a	n/a	n/a

Table 2.