Disrupted cardiac bioenergetics linked to oxidized mitochondrial creatine kinase are rescued by the mitochondrial-targeting peptide SBT-20 in the D2.*mdx* model of Duchenne muscular dystrophy

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37 **Abbreviations**

38

39 ADP: adenosine diphosphate

40 ATP: adenosine triphosphate

41 ANT: adenine nucleotide translocase

42 cCK: cytosolic creatine kinase

43 mH_2O_2: mitochondrial H_2O_2 emission

44 mtCK: mitochondrial creatine kinase

45 PCr: phosphocreatine

46 PDC: pyruvate dehydrogenase complex

47 VDAC: voltage dependent anion carrier

Abstract

Mitochondrial creatine kinase (mtCK) regulates the "fast" export of phosphocreatine to support cytoplasmic phosphorylation of ADP to ATP which is more rapid than direct ATP export. Such "creatine-dependent" phosphate shuttling is attenuated in several muscles, including the heart, of the D2.*mdx* mouse model of Duchenne muscular dystrophy at only 4 weeks of age. Here, we determined whether such attenuations occur in later stages in D2.*mdx* (12 months of age) in relation to mtCK thiol redox state, and whether this pathway could be preserved through administration of the mitochondrial-targeting, ROS-lowering tetrapeptide, SBT-20, in the D2.*mdx* mouse. In permeabilized muscle fibres prepared from cardiac left ventricles, we found that aged male D2.*mdx* mice have reduced creatine-dependent pyruvate oxidation and elevated complex I-supported H_2O_2 emission (mH_2O_2). Surprisingly, creatine-independent ADP-stimulated respiration was increased and mH_2O_2 was lowered suggesting that impairments in the faster mtCK-mediated phosphocreatine export system resulted in compensation of the alternative slower pathway of ATP export. The apparent impairments in mtCK-dependent bioenergetics occurred independent of mtCK protein content but were related to greater thiol oxidation of mtCK and a more oxidized cellular environment (lower GSH:GSSG). We then found that 12 weeks of daily treatment with SBT-20 (from day 4 to ~12 weeks of age) increased respiration and lowered mH_2O_2 only in the presence of creatine in D2.*mdx* mice without affecting calcium-induced mitochondrial permeability transition pore activity. In summary, creatine-dependent mitochondrial bioenergetics are attenuated in older D2.*mdx* mice in relation to mtCK thiol oxidation, which can be preserved with a ROS-lowering mitochondrial-targeting peptide. These results demonstrate a specific relationships between redox stress and metabolic reprogramming during dystrophin deficiency that can be targeted with small peptide therapeutics.

Keywords: mitochondria, muscle, antioxidant, respiration, creatine, small molecule therapy

Introduction

Duchenne muscular dystrophy (DMD) is a rare neuromuscular disease affecting 1 in 5,000 boys [1]. DMD is caused by X-linked recessive mutation in the dystrophin gene, and the loss of this structural protein triggers many cellular dysfunctions including cell membrane fragility, impaired calcium homeostasis and redox and metabolic stress [2]. There remains an unmet need for new therapies [3] given that there is no cure and conventional glucocorticoid therapy targeting inflammation partially delays the progression of cardiac, respiratory and locomotor muscle dysfunction while recent exon-skipping therapies, for examples, have limited effects in skeletal muscle with no appreciable benefits being identified in the heart [4]. Identifying specific relationships between redox stress and metabolic dysfunction could provide foundational knowledge for pursuing new paradigms of therapy development.

A variety of mitochondrial stress responses have been reported in humans and mouse models, including elevated mitochondrial-induction of apoptosis through permeability transition pore activity, reduced oxidative phosphorylation, and elevated hydrogen peroxide emission (mH_2O_2) (reviewed in [5]). In young (4 weeks) D2.*mdx* dystrophin-deficient mice, we reported ADP-stimulated mitochondrial respiration and ADP-suppression of mH_2O_2 were attenuated to a greater degree when creatine was included in the experimental media compared with when creatine was absent in cardiac left ventricle and skeletal muscles [6, 7]. These comparisons were designed to test phosphate shuttling from mitochondrial to cytoplasmic compartments through two theoretical systems comprised of ATP export/ADP import (slow diffusion) and a faster phosphocreatine export/creatine import (faster diffusion) regulated in part by mitochondrial creatine kinase (mtCK) in the intermembrane space (reviewed in [8, 9]). Matrix ADP/ATP turnover is accelerated with creatine as mtCK activity reduces the diffusion distance for the slower diffusing ADP/ATP to the matrix-intermembrane space interface. This creatine-dependent enhancement of ADP-stimulated ATP synthesis also causes greater ADP-suppression of mH_2O_2 [10] by lowering membrane potential [11]. As such, the findings in D2.*mdx* mice that the faster creatine-dependent system is attenuated to a greater degree than the creatine-independent system implicates mtCK – a protein known to be inhibited by reactive oxygen species (ROS) [9] - as being particularly sensitive to the redox stress of dystrophin deficiency in the disease process.

In this study, we examined how the balance between the creatine-dependent and creatine-independent phosphate shuttling systems would respond in later stages of DMD using the D2.*mdx* mouse model. Our findings highlight considerable metabolic plasticity in these systems, particularly in the left ventricle, which is linked to greater cysteine oxidation of mtCK. Furthermore, we show proof-of-principle that creatine-dependent bioenergetics are enhanced with 12 weeks of treatment with the ROS-lowering mitochondrial-targeting tetrapeptide SBT-20 [12].

Methods

Male D2.*mdx* mice originated from breeding colonies maintained at York University (Toronto, Canada) and sourced from The Jackson Laboratory (Bar Harbor, United States). DBA/2J wild type were purchased from The Jackson Laboratory at 4-5 weeks of age and aged in-house.

In the first part of this study, D2.*mdx* and wild type mice were aged to 52 weeks. Two to three days prior to tissue removal, mice were assessed for 24-hour voluntary wheel running, hang time using an inverted cage lid and forelimb grip strength. Mice also received a single micro computed tomography scan for measurement of lower limb muscle volume. In the second part of the study, beginning at 4-days of age, D2.*mdx* mice received subcutaneous injections of 5mg/kg SBT-20 (Stealth Biotherapeutics; Newton, MA, USA) 7 days/week continuously for 12 weeks. Thereafter, ultrasound assessments of cardiac function were performed, and muscles were removed.

Detailed methodology, including information on permeabilized muscle fibre bundles, high resolution respirometry, mitochondrial H_2O_2 emission, calcium retention capacity assessments, glutathione, western blots, redox assessments of mtCK, and statistical methods, are described in detail in the *Supplemental Information (**Appendix A**)*.

Results

Mitochondrial respiration and mH_2O_2: modeling metabolic demand and phosphate shuttling

At 12 months of age, D2.*mdx* mice showed reduced body weight, lower limb muscle volume and cage hang time compared with age matched DBA/2J wild type mice (**Figure 1A-E**) as expected with this model [7]

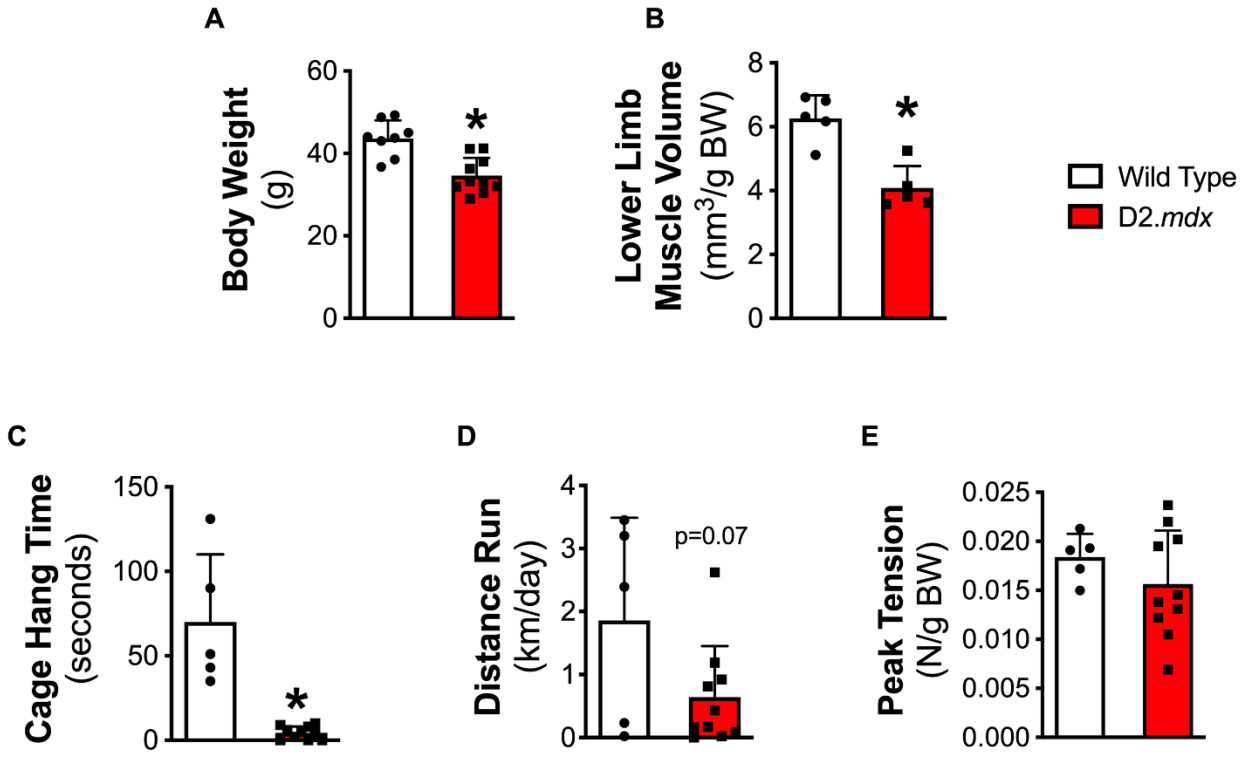
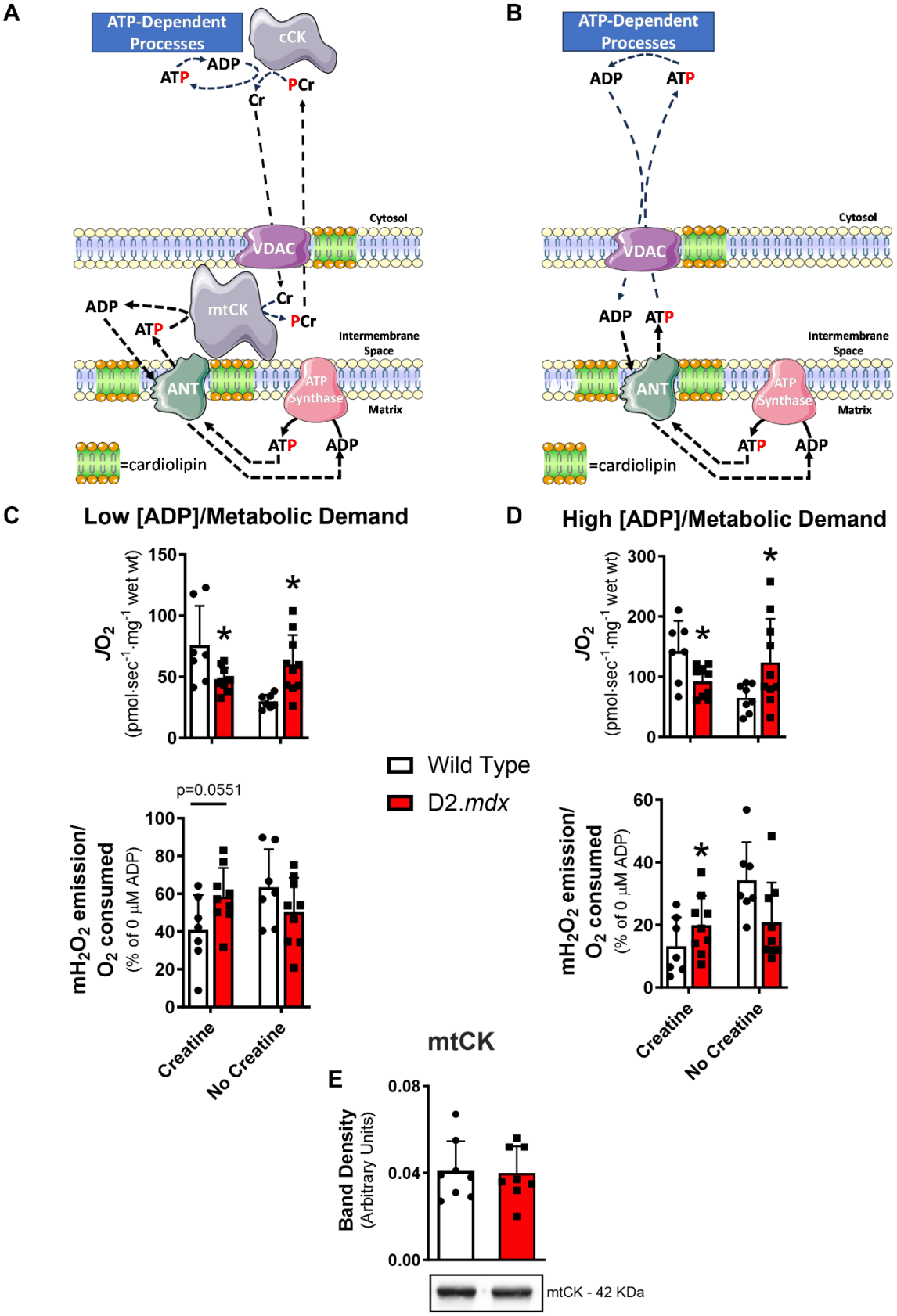


Figure 1. *Anthropometrics and functional testing in 12-month-old D2.mdx mice. Body weight (**A**, n=8-10), lower limb muscle volume assessed by microCT (**B**, n=5), cage hang time (**C**, n=5-10), voluntary wheel running in 24 hours (**D**, n=5-9) and grip strength (**E**, n=5-10). Data were analyzed by t-tests. Results represent mean +/- SD; *p<0.05 compared with wild type.*

Creatine-dependent versus creatine-independent regulation of ADP-stimulated respiration were assessed by placing separate permeabilized left ventricle fibre bundles in experimental media with 20mM creatine to saturate mtCK [13, 14] or in media without creatine to model both theoretical models of phosphate shuttling (**Figure 2A and B**). We found that left ventricles from 12-month-old D2.*mdx* mice have lower creatine-dependent Complex I-supported respiration when stimulated by both low and high [ADP] modeling a range of metabolic demands for mitochondrial ATP synthesis (**Figure 2C and D**). D2.*mdx* surprisingly showed increased respiration in the absence of creatine suggesting the slower but direct ADP/ATP cycling system was upregulated despite reductions in the faster creatine-dependent system. There were no changes in mtCK protein content of the left ventricles (**Figure 2E**).


A
ATP-Dependent Processes
cCK
ADP
ATP
PCr
Cr
Cytosol
VDAC
mtCK
Cr
PCr
ADP
ATP
Intermembrane Space
ANT
ATP Synthase
Matrix
ATP
ADP
=cardiolipin
B
ATP-Dependent Processes
ADP
ATP
Cytosol
VDAC
ADP
ATP
Intermembrane Space
ANT
ATP Synthase
Matrix
ATP
ADP
=cardiolipin
C
Low [ADP]/Metabolic Demand
JO2 (pmol·sec-1·mg-1 wet wt)
150
100
50
0
*
*
D
High [ADP]/Metabolic Demand
JO2 (pmol·sec-1·mg-1 wet wt)
300
200
100
0
*
*
Wild Type
D2.mdx
p=0.0551
mH2O2 emission/ O2 consumed (% of 0 μM ADP)
100
80
60
40
20
0
Creatine
No Creatine
mH2O2 emission/ O2 consumed (% of 0 μM ADP)
60
40
20
0
*
Creatine
No Creatine
E
mtCK
Band Density (Arbitrary Units)
0.08
0.04
0.00
mtCK - 42 KDa

145 ***Figure 2***. *Complex I-supported mitochondrial respiration and mitochondrial H_2O_2 emission*
146 *(mH_2O_2) in left ventricles from 12-month-old D2.mdx mice. Creatine-dependent (**A**) and -*
147 *independent (**B**) ADP-stimulated respiration (JO_2) and ADP-suppression of mH_2O_2 during*
148 *oxidative phosphorylation (mH_2O_2/O_2) with low [ADP] (25uM; **C**) and high [ADP] (500uM; **D**)*
149 *were assessed in cardiac left ventricle permeabilized muscle fibre bundles by stimulation with*
150 *pyruvate (5mM respiration, 10mM mH_2O_2) and malate (2mM) to stimulate Complex I with*
151 *NADH. Mitochondrial creatine kinase (mtCK) total lysate protein content were assessed in heart*
152 *samples remaining after removal of left ventricles (**E**). mH_2O_2 represents forward electron*
153 *transfer that was achieved with NADH generated by pyruvate and malate to stimulate complex I*
154 *with and without creatine in the experimental media. mH_2O_2 arising from electron slip in the*
155 *electron transport chain during oxidative phosphorylation is positively associated with*
156 *membrane potential is therefore suppressed by [ADP] [11]. For A and B, several decades of*
157 *research has contributed to this theoretical model whereby the matrix-derived ATP is transported*
158 *through the inner membrane transporter ANT (adenine nucleotide translocase) to the*
159 *intermembrane space (see [15] for review). In the presence of creatine, a phosphate is*
160 *transferred from ATP to creatine by mtCK to produce phosphocreatine (PCr). The ADP product*
161 *cycles back to the matrix while PCr is exported to the cytoplasm (theorized through VDAC;*
162 *voltage dependent anion carrier) where it is used by cytoplasmic creatine kinase (cCK) to re-*
163 *phosphorylate ADP to ATP to support ATP-dependent proteins with creatine returning to the*
164 *mitochondria. This PCr/creatine system cycles faster than ATP/ADP due to faster diffusion*
165 *kinetics of both PCr and creatine relative to ADP and ADP, and is estimated to represent up to*
166 *80% of phosphate exchange between mitochondria and cytoplasmic compartments vs 20% for*
167 *the direct ATP/ADP shuttle. mtCK, ANT and VDAC are thought to be bound to cardiolipin (see*
168 *[9] for review). Both systems are thought to be active* in vitro *in the presence of creatine.*
169 *Diffusion distances are not to scale. Data were analyzed by t-tests between wild type and*
170 *D2.mdx. Results represent means +/- SD; n=7-10. *$p<0.05$ compared to wild type within the*
171 *same "Creatine" or "No Creatine" condition.*

172

173 We also found that Complex I-supported mH_2O_2 during oxidative phosphorylation was higher in
174 the 12-month-old D2.*mdx* compared to age-matched wild type when assessed in the presence of
175 creatine during forward electron transfer (NADH) (**Figure 2C**) and reverse electron transfer to
176 Complex I from Complex II (**$FADH_2$**; **Supplemental Figure S1A and B)**. A lower emission rate
177 was observed in the absence of creatine with reverse electron transfer ($FADH_2$; Figure
178 **Supplemental Figure S1A and B**)

179 Collectively, these results demonstrate divergent remodeling of left ventricular mitochondrial-
180 cytoplasmic ADP/ATP cycling in 12-month-old D2.*mdx* mice whereby impairments in the faster
181 creatine-dependent regulation of ADP-stimulated respiration and suppression of mH_2O_2 are
182 countered by apparent compensations in the slower creatine-independent system.

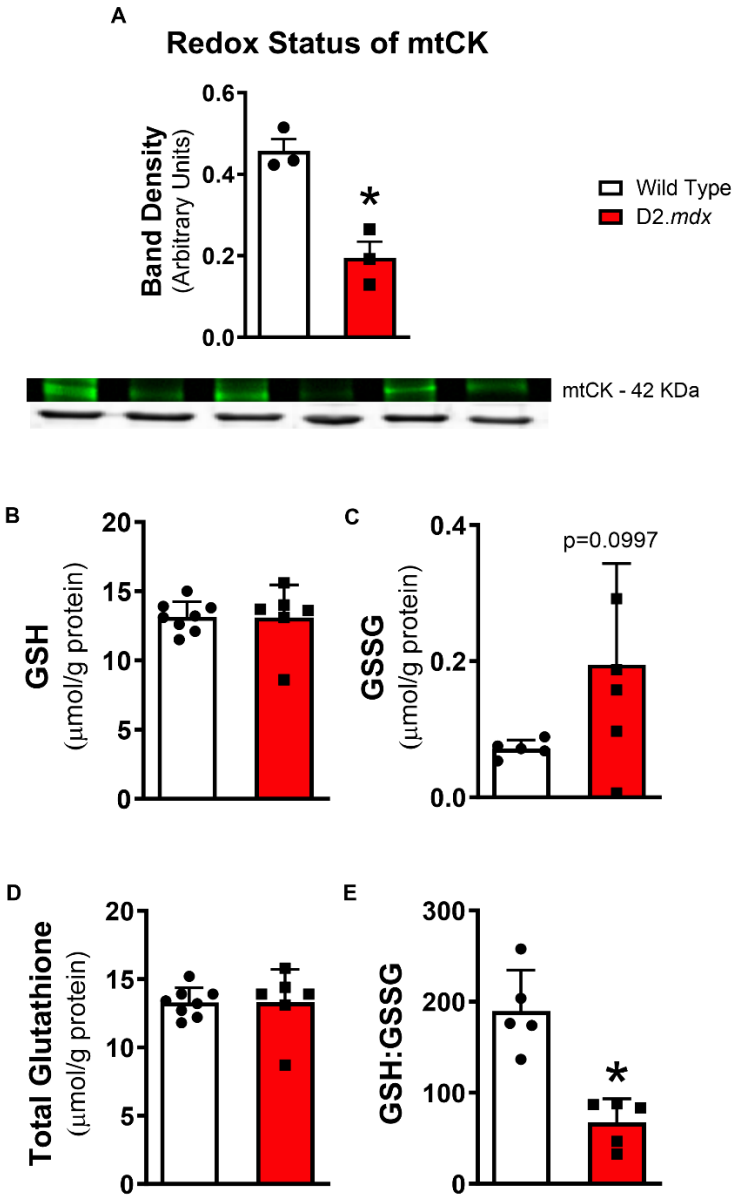
183 Similar reductions in creatine-dependent ADP governance of respiration and mH_2O_2 were
184 observed in certain skeletal muscles. However, unlike the left ventricle, there were no apparent

compensations in the absence of creatine except for a similar pattern for mH_2O_2 in the diaphragm (**Supplemental Figure S2**).

As there were no changes in mtCK protein content of the left ventricles (*see* **Figure 2E**), we next questioned whether altered post-translational modifications of mtCK were related to the attenuations in creatine-dependent mitochondrial bioenergetics.

Cellular redox state and mtCK thiol oxidation

The increased creatine-dependent mH_2O_2 during oxidative phosphorylation in aged D2.*mdx* mice guided us to hypothesize that mtCK thiols would be more oxidized than in wild type given mtCK is a redox-sensitive protein (reviewed in [9]). We next immunoprecipitated mtCK from left ventricles and incubated the extract in a maleimide-tagged fluorophore that binds irreversibly to reduced cysteine thiols, with no affinity to oxidized thiols, as previously described ([16-18], **Supplemental Figure S3**). This experiment demonstrated that mtCK is more oxidized in aged D2.*mdx* mice (**Figure 3A**). This was related to a more oxidized cellular environment as reflected by a lower GSH:GSSG (reduced to oxidized glutathione redox buffer) in lysate due apparently to high variability in GSSG (**Figure 3B-E**). This oxidized environment in the left ventricle was more pronounced than other specific muscles, although the diaphragm also showed a lower GSH:GSSG despite increases in both GSH and GSSG (**Supplemental Figure S3A)**. No changes were observed in quadriceps and white gastrocnemius (**Supplemental Figure 3A**).



203

204

205 ***Figure 3***. *Mitochondrial creatine kinase (mtCK) cysteine redox state and cellular glutathione*
206 *equilibrium in the heart from 12-month-old D2.mdx mice. Measurements were made in frozen*
207 *heart following the removal of left ventricles. Greater cysteine oxidation on immunoprecipitated*
208 *mtCK from D2.mdx left ventricles is demonstrated by lower binding of the maleimide-tagged*
209 *fluorescent IR-dye 800 CW probe compared to wild type (**A**). Glutathione was measured in left*
210 *ventricle lysates using HPLC-UV for the detection of GSH (**B**) and HPLC-fluorescence for GSSG*
211 *(**C**). The GSH:GSSG ratio (**D**) and total glutathione (GSH + 2x GSSG; **E**) were calculated from*
212 *GSH and GSSG. Data were analyzed by t-tests between wild type and D2.mdx Results represent*
213 *mean +/- SD; n=4-8. *p<0.05 compared to wild type.*

214

There were no differences in ANT1, VDAC2 protein contents or subunits of the electron transport chain (**Supplemental Figure 3B and C**). While we did not assess their post-translational modifications, these collective observations guided us to examine creatine-dependent bioenergetics in more detail.

In vivo treatment with the mitochondrial ROS-lowering peptide SBT-20

Given we observed attenuated creatine-sensitive control of bioenergetics by ADP in the left ventricle, as the second part of the study we performed a pilot study with *in vivo* injections of the mitochondrial targeting ROS-lowering peptide SBT-20 in D2.*mdx* mice from 4 days of age to ~12.5 weeks of age. Treatment at an earlier age was chosen given a 12-month protocol was not possible. This 12-week treatment protocol increased pyruvate-supported ADP-stimulated respiration in the presence of creatine compared to saline-treated D2.*mdx* mice and had no effect on creatine-independent respiration at low or high [ADP] (**Figure 4A and C**). Likewise, mH_2O_2 suppression by ADP was enhanced by SBT-20 treatment but only in the presence of creatine (**Figure 4B and D**). Similar responses to SBT-20 were noted when we assessed separate permeabilized fibre bundles in media containing high PCr and low creatine (**Supplemental Figure 4A and B**) to reflect an equilibrium of the creatine kinase system closer to what might exist *in vivo* [13, 19].

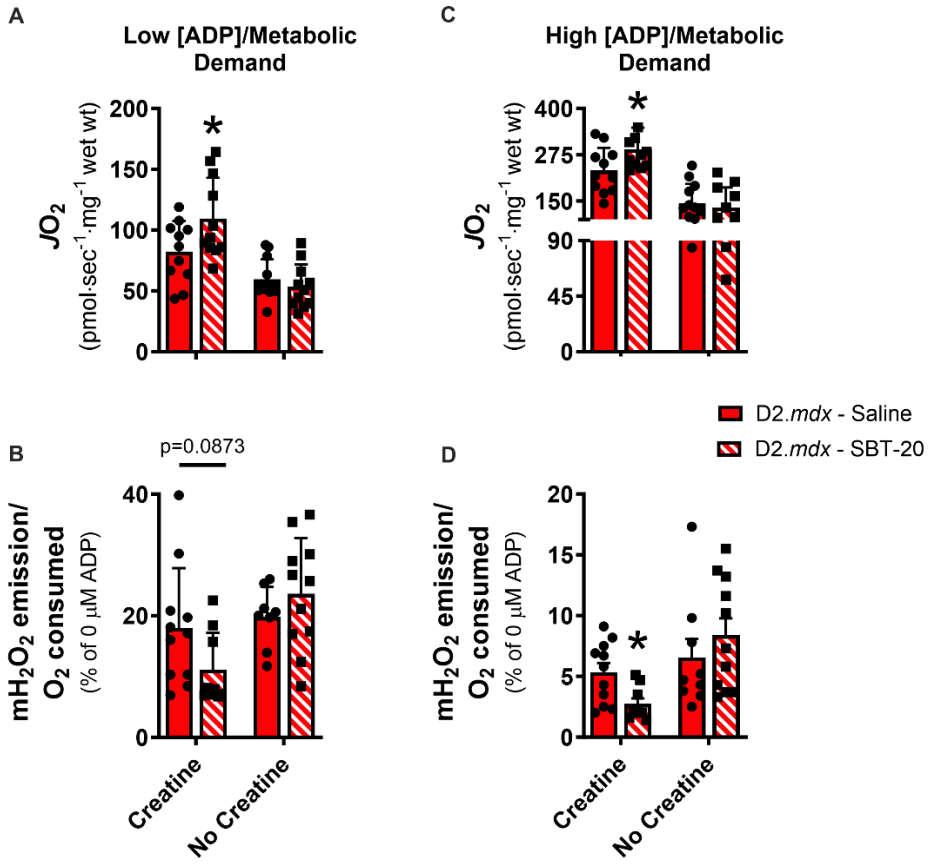


***Figure 4**. The effects of SBT-20 on complex I-supported mitochondrial respiration and mitochondrial H_2O_2 emission (mH_2O_2) in left ventricles from D2.mdx mice. Mice received daily*

subcutaneous injections of SBT-20 from day 4 to ~12.5 weeks of age. Creatine-dependent and -independent ADP-stimulated respiration (JO_2) and ADP-suppression of mH_2O_2 during oxidative phosphorylation (mH_2O_2/O_2) with low [ADP] (25uM, ***A, B****) and high [ADP] (500uM;* ***C, D****) were assessed in cardiac left ventricle permeabilized muscle fibre bundles by stimulation with pyruvate (5mM respiration, 10mM mH_2O_2) and malate (2mM) to stimulate Complex I with NADH. Data were analyzed by t-tests between wild type and D2.mdx Results represent mean +/- SD; n=8-12. *p<0.05 compared to D2.mdx-saline treated mice in the same "Creatine" or "No Creatine" condition.*

Increased susceptibility to calcium-induced mitochondrial permeability transition pore activity was observed given calcium retention capacity was reduced in 12-month-old D2.*mdx* mouse left ventricles (**Supplemental Figure S1C**). There was no effect of SBT-20 on this measure compared to saline-treated D2.*mdx* mice at ~12.5 weeks of age (**Supplemental Figure S1D**).

Select cardiac functional parameters were not altered by SBT-20 compared to saline-treated D2.*mdx* mice (**Supplemental Figure S4C-F**).

Lastly, to determine if higher mH_2O_2 in the presence of creatine was unique to the ADP-sensitive regulation of ROS production, we also assessed Complex III- and pyruvate dehydrogenase complex (PDC)-supported mH_2O_2 in the absence of ADP. In 12-month-old D2.*mdx*, Complex III-supported mH_2O_2 was lower in white gastrocnemius whereas PDC-supported mH_2O_2 was lower in the left ventricle, diaphragm, and quadriceps (**Supplemental Figure S1E)**. SBT-20 had no effect on these pathways. While this lower mH_2O_2 in aged D2.*mdx* mice warrant further investigation into how the contents or post-translational regulation of these pathways are altered during dystrophin deficiency, these data suggest the loss of creatine-dependent bioenergetics during pyruvate oxidation (**Figure 2**) is a unique mechanism contributing to higher mH_2O_2 during attenuated oxidative phosphorylation in DMD.

Discussion

Creatine enhances the ability of ADP to stimulate oxidative phosphorylation and attenuate H_2O_2 emission in mitochondria. Here, we show aged D2.*mdx* mice have lower creatine-dependent bioenergetics that are related to greater cysteine oxidation of mtCK. Moreover, 12 weeks of treatment with the ROS-lowering mitochondrial-targeting peptide SBT-20 increased creatine-dependent respiration and lowered mH_2O_2 emission, particularly in the left ventricle. These results demonstrate a specific mechanism linking redox and metabolic stress in mitochondria arising from dystrophin mutations and serve as a direction for continued development of mitochondrial-targeted therapies designed to restore metabolic and redox balance in DMD.

At 12 months of age, the left ventricle of D2.*mdx* mice also demonstrated a surprising increase in creatine-independent respiration, which contrasts the decreases we previously reported in this pathway in 4-week-old mice [6, 7]. This observation suggests the slower mitochondrial ADP-ATP system may compensate for impairments in the faster creatine-dependent phosphate shuttling mechanism [9]. The greater abundance of cysteine oxidation on mtCK was consistent with higher rates of creatine-sensitive mH_2O_2 during oxidative phosphorylation and the more oxidized cellular environment (lower GSH:GSSG). mtCK oxidation may be unique to advanced stages of this disease, given we previously reported no differences in 4-week D2.*mdx* mice, at least in skeletal muscle [16]. Further studies could examine the precise form of thiol modification that occurs in aged D2.*mdx* mice, such as glutathionylation, considering its emerging role in linking redox signaling to metabolic control [20, 21] and considering the shift in GSH:GSSG noted in the present study. Likewise, the degree to which mtCK thiol redox state was preserved by SBT-20 could be considered given the positive effects noted in this part of the study that was otherwise limited by tissue availability. Further studies could assess the specific cystines that were oxidized given cysteine 278, which regulates mtCK activity, and C358, which may regulate mtCK tethering to the inner mitochondrial membrane, were previously shown to be redox sensitive [22]. ANT and VDAC thiol oxidation could also be assessed to explain the apparent compensatory increases in creatine-independent bioenergetics at late stages of disease given their protein contents did not change, although reconciling these measures with the divergent response of creatine-dependent and -independent systems may be challenging given both proteins are thought to be primary regulators in either system.

We were unable to assess cardiac function in aged D2.*mdx* mice due to their qualitatively frail nature. Furthermore, while we did not see robust differences in grip strength compared to age-matched wild type mice, we did note that the absolute values of grip strength in the 12 month old wild type mice are ~50% of the values we have reported previously in this strain at 4 weeks of age [7, 16] suggesting an aging effect occurred in the control group. Nonetheless, the other parameters demonstrate a severe myopathy in the aged D2.*mdx* mice. Also, the effect of age on mitochondrial reprogramming in locomotor and respiratory muscle pathology compared to muscle dysfunction could also be considered, particularly in relation to the earlier remodeling seen in 4-week-old D2.*mdx* [6, 7, 16, 23].

SBT-20 is a small tetrapeptide with high cell-penetrating potential that accumulates on cardiolipin in the inner mitochondrial membrane similar to the mitochondrial-targeting peptide elamipretide

(formerly SS-31) [24-26] that prevents cytochrome *c* peroxidase activity, preserves oxidative phosphorylation, and prevents increases in superoxide production in response to stressors [27, 28]. SBT-20 also preserved mitochondrial respiration in H_2O_2-treated cells and partially prevents cardiac infarct size in response to ischaemia reperfusion injury [12]. To our knowledge, this is the first study to report a unique creatine-specific preservation of bioenergetics by cardiolipin-targeting peptides. As mtCK is thought to be bound to cardiolipin [9], future studies could consider whether the age-related impairment in creatine-dependent reductions in respiration seen in the present study was due to altered cardiolipin tethering to mtCK, and whether the preserved creatine-dependent bioenergetics by SBT-20 preserved such interactions. As ANT and VDAC are also thought to be bound to cardiolipin, the potential for SBT-20 to regulate the system as a whole could be considered.

While an aged-match wild type control group (~12.5 weeks of age) was not included for the SBT_20 experiments, we have previously demonstrated that creatine-dependent respiration is lower in 4-week-old D2.*mdx* mouse left ventricles [6]. Therefore, as creatine-dependent respiration is lower at both 4 weeks and 12 months as seen in **Figure 2**, it is likely that similar reductions would exist at ~12.5 weeks as well. Likewise, although select cardiac functional parameters were not altered by SBT-20 compared to saline-treated D2.*mdx* mice, we cannot determine if a dysfunction existed at this age in comparison to wild type. In fact, prior work at younger ages in the D2.*mdx* mouse have reported no overt cardiomyopathy [6] while much older ages are known to have a moderate left ventricular cardiomyopathy in this model [15], at least as assessed with non-invasive approaches.

SBT-20 did not alter calcium retention capacity suggesting that it did not have an effect on mitochondrial permeability transition – an event that links mitochondrial calcium overload to apoptosis [29]. This is in contrast to previous reports showing SBT-20 prevents cytochrome *c* release, a key trigger of mitochondrial-induced apoptosis, in response to ischaemic stress [28] that is known to trigger mitochondrial permeability transition [29]. As our pilot study did not include a wild type control group, we are unable to determine whether calcium retention capacity was reduced in the D2.*mdx* saline control group at this age in contrast to the clear reductions seen at 12 months of age (**Supplemental Figure S1**).

Collectively, these findings suggest that a time-course design could be employed to determine whether SBT-20 prevents the unique time-dependent signatures of mitochondrial stress and delays the eventual onset of cardiomyopathy. To this end, we did not see an effect of SBT-20 on cardiac function assessed with echocardiography on ~12.5-week-old mice. However, previous reports have shown an absence of overt cardiac dysfunction in D2.*mdx* mice at 4 weeks [6] or 7 weeks [15] of age but is apparent at 28 weeks and 52 weeks [15], with the latter age corresponding to our observation of oxidized mtCK.

Conclusions

These findings demonstrate that left ventricle mitochondrial creatine metabolism is attenuated in relation to oxidized mitochondrial creatine kinase in late stages of disease in 1-year-old D2.*md*x mice. The ability of the ROS-lowering mitochondrial peptide SBT-20 to increase creatine-

342 dependent pyruvate oxidation and lower creatine-dependent mitochondrial H_2O_2 emission
343 demonstrates that redox and metabolic stress are intertwined. This finding supports continued
344 development of a new paradigm of redox and metabolic enhancing therapeutics that do not exist
345 in the current standard of care of anti-inflammatory and exon-skipping approaches.

Declaration of competing interests.

Stealth Biotherapeutics provided SBT-20 through a material transfer agreement but did not provide funding for this study.

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Supplemental Information.

Supplemental methods (Appendix A) and data (Appendix B) can be found in the separate file *Supplemental Information*.

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Author Contributions

M.H., S.V.R., P.B., J.S. and C.G.R.P. contributed to the conception or design of the work. M.H., S.V.R., A.B., P.C.T., N.P., U.S., T.H., P.B., J.S. and C.G.R.P. contributed to acquisition, analysis and/or interpretation of data. All authors contributed to drafting the manuscript and approved the final version of the manuscript.

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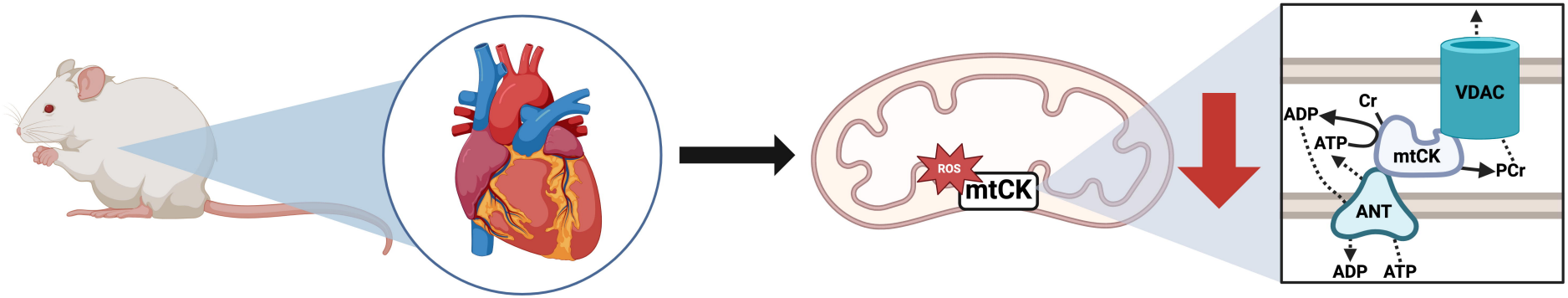
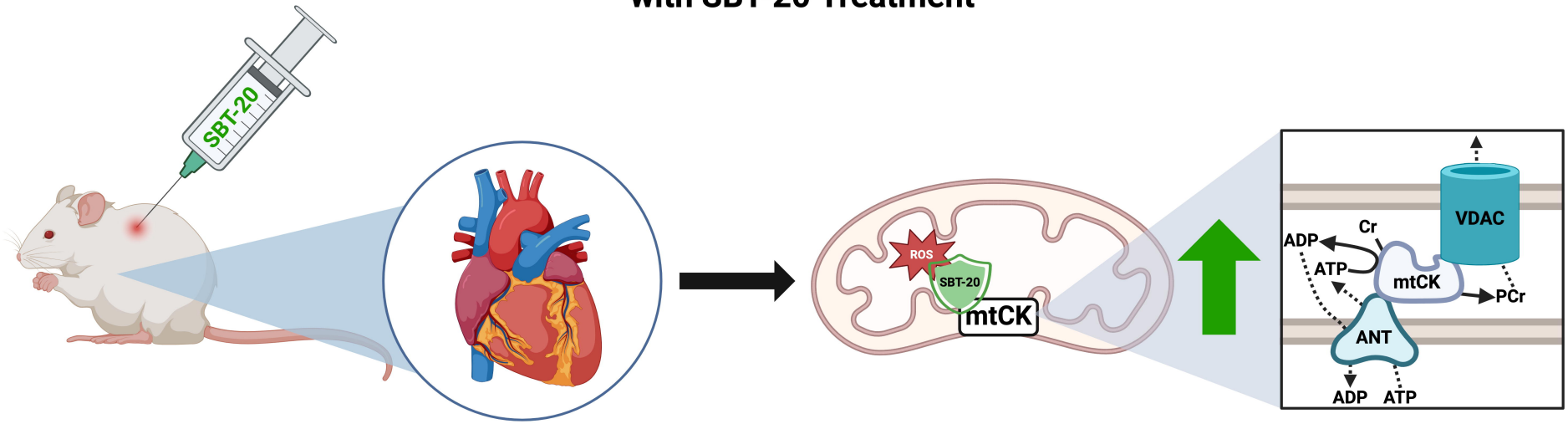
Aged D2.*mdx* mice
(Duchenne Muscular Dystrophy)



D2.*mdx* mice
with SBT-20 Treatment