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Maternal Creatine Supplementation From Mid-Pregnancy Protects the Diaphragm of the Newborn Spiny Mouse From Intrapartum Hypoxia-Induced Damage

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ABSTRACT: We hypothesized that maternal creatine supplementation from mid-pregnancy would protect the diaphragm of the newborn spiny mouse from the effects of intrapartum hypoxia. Pregnant mice were fed a control or 5% creatine-supplemented diet from mid-gestation. On the day before term, intrapartum hypoxia was induced by isolating the pregnant uterus in a saline bath for 7.5–8 min before releasing and resuscitating the fetuses. Surviving pups were placed with a cross-foster dam, and diaphragm tissue was collected at 24 h postnatal age. Hypoxia caused a significant decrease in the cross-sectional area (~19%) and contractile function (26.6% decrease in maximum Ca^{2+}-activated force) of diaphragm fibers. The mRNA levels of the muscle mass-regulating genes *MuRF1* and *myostatin* were significantly increased (2-fold). Maternal creatine significantly attenuated hypoxia-induced fiber atrophy, contractile dysfunction, and changes in mRNA levels. This study demonstrates that creatine loading before birth significantly protects the diaphragm from hypoxia-induced damage at birth. **(*Pediatr Res* 68: 393–398, 2010)**

Hypoxia, sometimes accompanied by ischemia and metabolic acidemia, is reported to occur in 4 per 1000 live term births (1). Between 5 and 9% of these infants will not survive the neonatal period (2). In surviving infants, there is often irreversible multiorgan damage, including the brain, heart, kidney, and lung (3). Many of these infants require mechanical ventilation because of respiratory insufficiency. Despite the critical role of the diaphragm in promoting the lung liquid clearance, lung aeration, and formation of functional residual capacity at the time of birth (4), there is relatively little known of the effects of intrapartum hypoxia on this essential respiratory muscle.

In the adult, insufficient oxygen reduces the capacity for ATP production. There is an increased reliance on anaerobic pathways (5), which, when prolonged, results in an accumulation of metabolic byproducts and ionic disturbances linked with muscle fatigue (6). Excessive production of reactive oxygen species occurs and promotes myosin and actin degradation (7), leading to damage of myofibrillar proteins (8) and reduced functional capacity of skeletal muscles (9). These changes occur at the single fiber level of adult diaphragm (10). Hypoxia can also result in muscle fiber atrophy in skeletal muscle (11). Although the mechanisms are not well understood, hypoxia can increase protease activity and up-regulate the muscle-specific E3-ligases such as atrogin-1 and MuRF1 (12), which are known regulators of muscle atrophy (13).

By using a model of intrapartum hypoxia in the spiny mouse, we have previously shown that fetal creatine loading *via* maternal dietary supplementation during pregnancy reduces neonatal mortality and brain morbidity and improves postnatal growth (14,15). It is not known whether creatine can also be loaded in the fetal diaphragm and exert similar protective effects for this tissue.

There are multiple mechanisms by which creatine may protect the newborn from hypoxia. Creatine is an energy and free-radical buffer and directly scavenges free radicals (16,17). The creatine/phosphocreatine (PCr) system is also involved in coupling aerobic metabolism with ATP demand, synthesis of specific muscle proteins, acid-base balance (16), and decreasing ionic strength to improve contractile function (18). Creatine supplementation has been shown to up-regulate transcriptional regulators of myogenesis and enhance muscle growth (19,20).

In this study, we hypothesized that intrapartum hypoxia would result in structural and functional damage to the diaphragm, which would be attenuated by loading the newborn diaphragm with creatine/PCr through maternal dietary supplementation from mid-pregnancy. The precocial spiny mouse was chosen for this study as the advanced development of key organs and systems at the time of birth (*e.g.* brain, lung, liver, and kidney) is more similar to the term human fetus than other conventionally used rodent species (21).

MATERIALS AND METHODS

Ethical approval. All experiments were approved in advance by Monash University School of Biomedical Sciences Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for the Care and Use

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Abbreviations: CSA, Cross-sectional area; **PCr,** phosphocreatine; **TCr,** total creatine

of Animals for Scientific Purposes. The spiny mice were obtained from our own colony at Monash University and maintained as previously described (22).

Animals. Pregnant dams were fed a control diet of standard rat and mouse pellets throughout pregnancy or were fed isocaloric pellets supplemented with 5% creatine monohydrate (Specialty Feeds, Australia) from d 20 of gestation. Water was available *ad libitum*. Hypoxia was induced on d 38 of gestation (term is 38–39 d) as described by us previously (14). Briefly, the dam was killed by cervical dislocation and the isolated pregnant uterus placed in isotonic saline (0.9%) at 37°C for 7.5–8 min. Fetuses were expelled and resuscitated by gentle palpation of the chest. Control pups were delivered immediately by caesarean section. Pregnant dams were randomly allocated to either caesarean or hypoxia groups. The birth hypoxia protocol resulted in significant acidemia as shown by a fall of blood pH (control, 7.402; asphyxia, 6.906) an increase of lactate (control, 5.0 mM; hypoxia, 11.7 mM), and hypoxemia (O_2 saturation: control, 82.3%; hypoxia, 29.1%).This model produces significant mortality in hypoxic offspring, which is improved with maternal creatine supplementation (14). In this study, 45% of hypoxia pups survived compared with 60% of creatine + hypoxia pups. All caesarean-delivered controls survived. The number of neonates for each group were control, $n = 32$; creatine, $n = 24$; hypoxia, $n = 25$; creatine + hypoxia, $n = 24$, obtained from at least nine litters per group. Only surviving neonates were used in this study.

Postmortem. Twenty-four hours after delivery, neonates were killed by decapitation. The diaphragm was snap frozen in precooled isopentane, stored at −80°C or pinned onto dental wax at approximate resting length, placed into a storage solution [50% glycerol, 50% relaxing solution containing (mM): 150 propionic acid, 20 HEPES, 10 EGTA, 3 $MgCl_2$, and 2 ATP], and stored at −20°C for preparation of skinned fibers.

Measurement of total creatine. The concentration of total creatine (TCr; creatine + PCr) in the diaphragm 24 h after birth was determined in caesarean-delivered pups from control and creatine-fed mothers ($n = 6$ for both groups) as previously published (14).

Histochemistry. Frozen diaphragm samples were cut into 10-μm transverse sections. Myofibrillar ATPase staining was used to differentiate type I, type IIa and type IIb/IId fibers as previously described (23), with the following modifications: sections were preincubated in acetate buffer for 12 min (pH 4.6), then incubated for 30 min in ATPase solution at 37°C. Using this protocol, type I fibers stain dark, type IIa stain intermediate, and type IIb/IId stain pale. The distribution and cross-sectional area (CSA; μm^2) of individual fiber types were determined for ~100 fibers per transverse section (control, $n = 8$; creatine, $n = 8$; hypoxia, $n = 10$; and creatine + hypoxia, $n = 10$) using computer software (Image J).

Ca^{2+}- and Sr^{2+}-activation of single skinned fibers. Skinned diaphragm fibers were prepared as described previously (24,25). Solutions used to activate and relax the muscle fibers (26) are summarized in Table 1. Previously determined apparent affinity constants (K_{app}) were used, and the amount of free EGTA in each solution was determined by titration (26). Solutions containing Ca^{2+} (0.02–14.4 μM) or Sr^{2+} (0.03–363 μM) were obtained through combination of relaxing solution containing 50 mM EGTA (solution A) with either solution B (Ca^{2+}) or solution S (Sr^{2+}). Sr^{2+} is a similar divalent cation to Ca^{2+}, which can maximally activate mammalian skeletal muscle fibers. Sr^{2+} was used as an identification tool to determine whether changes occurred in the activation of slow and/or fast contractile and regulatory isoforms within single diaphragm fibers.

Fibers were activated using a "staircase technique" (24). Briefly, each fiber was activated in a series of increasing concentrations of Ca^{2+} or Sr^{2+} until a maximum activated force was reached. To accommodate any time-dependent decrease in force capacity during staircase activation, maximum force response was determined at the beginning and end of a series of contractions. Any decrease in force (<10%) was assumed to have declined linearly with time; thus, the force measured at each submaximal Ca^{2+} and Sr^{2+} concentration was normalized to the estimated maximum force response at that time. Maximum Ca^{2+}-activated force was obtained from the first maximum activation in the staircase protocol. Fibers were taken from 10 animals in each group (fiber number: control, $n = 24$; creatine, $n = 24$; hypoxia, $n = 28$; and creatine + hypoxia, $n = 26$). Each fiber was activated in both Ca^{2+} and Sr^{2+} solutions.

Analysis of force-pCa and force-pSr curves. The analysis of force-pCa curves has been previously published (25). Curves were fitted to data using a Marquardt nonlinear regression algorithm. The quantitative measures obtained were pCa_x (the amount of Ca^{2+} needed to produce "x" amount of force) and n_{Ca} (maximum slope of the force-pCa curve).

The "hybrid" nature of diaphragm fibers has been described elsewhere (27). The force-pSr curves were biphasic in shape and described by two sigmoidal curves fitted by a composite Marquardt nonlinear regression algorithm and, thus, required a different analysis for force-pCa curves. The first phase (F1) of the force-pSr curve is likely to reflect activation of slow isoforms as there is minimal distance between the force-pCa and force-pSr curves, as is typical of a "pure" slow-twitch fiber (24,25). The second phase (F2, calculated as 1 − F1) shows a greater distance between force-pCa and force-pSr curves, likely reflecting activation of fast isoforms (24,25). The quantitative measures obtained were F1% (proportion of force-pSr curve described by first sigmoid), $n_{Sr}1$ and $n_{Sr}2$ (maximum slope of force-pSr curve described by F1 and F2, respectively) and pSr5.5 (%) and pSr 4.5 [%; the amount of force that is produced in 3.16 μM (pSr 5.5) and 31.62 μM (pSr 4.5) of Sr^{2+}, expressed as a percentage of the maximum Sr^{2+}-activated force]. These two measurements identify differences in Sr^{2+} sensitivity in the first [pSr 5.5 (%)] and second [pSr 4.5 (%)] phase of the force-pSr curve and will indicate whether changes occur only in fast, slow, or both types of contractile and regulatory isoforms.

Quantitative PCR (qPCR). *Atrogin-1*, *MuRF1*, and *myostatin* mRNA levels were measured in the diaphragm 24 h after birth using qPCR (control, $n = 8$; hypoxia, $n = 7$; and creatine + hypoxia, $n = 6$). RNA was extracted from 3 to 6 mg of diaphragm muscle using TRI-reagent (Applied Biosystems/Ambion, Austin, TX) combined with the Purelink RNA Extraction Kit (Invitrogen, Carlsbad, CA), as per manufacturer's instructions. Approximately 500 ng of RNA was reverse transcribed to cDNA using the Affinity Script reverse transcription kit (Stratagene, Cedar Creek, TX) followed by treatment with 1 U/mL of RNAse H (Invitrogen). The cDNA was quantified using the Quant-iT OliGreen ssDNA Reagent and Kit (Molecular Probes, Eugene, OR) with fluorescence measured at 428 nM (28). The qPCR was performed using an MX3000p thermal cycler system with Multiplex Brilliant QPCR Master Mix (Stratagene) using conditions published previously (29). The primer and probe sequences are provided in Table 2. C_t values were converted to arbitrary units (AU) using the log power calculation. All qPCR results were normalized against cDNA quantification values.

Statistical analysis. All data presented are the mean ± SE. Comparisons of CSA and contractile function were made using a two-way ANOVA and mRNA expression compared using a one-way ANOVA, with Tukey's honestly significant difference (HSD) *post hoc*. TCr content was compared using a paired t test. Statistical significance was set at $p < 0.05$.

RESULTS

Creatine in the neonatal diaphragm. At 24 h after caesarean birth, the TCr content in diaphragm of offspring from creatine-fed dams was significantly higher than controls (134.2 ± 2.3 *vs* 116.6 ± 5.9 μmol/g/dry weight; $p < 0.05$).

Diaphragm muscle fiber morphology. Myofibrillar ATPase staining showed there were no differences between groups in the proportion of fiber types present in the diaphragm; the neonatal diaphragm was composed predominantly of type IIa and type IIb/IId fibers (Table 3). Figure 1 shows the CSA measurements of type I, type IIa, and type IIb/IId fibers from the diaphragm of neonates. Birth hypoxia resulted in a signif-

Table 1. *Composition of solutions for skinned fibres*

Solution	$[K^+]$	[EGTA]	[HDTA]	$[Mg^{2+}]_{total}$	$[Ca]_{total}$	$[Ca^{2+}]_{free}$	$[Sr^{2+}]_{total}$	$[Sr^{2+}]_{free}$
A	117	50	—	10.3	—	$<10^{-6}$	—	—
B	117	50	—	8.12	49.5	0.02	—	—
H	117	0.2	49.8	8.51	—	$<1.7 \times 10^{-5}$	—	—
S	114	50	—	5.95	—	—	40	0.283

All solutions contained (mM): 36 mM Na^+, 60 mM HEPES, 8 mM ATP, 10 mM creatine phosphate, 1 mM sodium azide, 1 mM Mg^{2+}; pH 7.10 ± 0.01.
A, relaxing solution; B, Ca^{2+}-activating solution; H, preactivating solution; S, Sr^{2+}-activating solution. HDTA, 1-6-diaminohexane-*N*-*N*-*N*′,*N*′-tetraacetic acid.

Table 2. *qPCR primer and probe sequences*

Gene	Forward primer	Reverse primer	Probe
Atrogin-1	ATGCCGTTCCTTGGTCAG	ACTGCTGAGGTCGCTCAC	FAM-TGCCGCTTTTCTCATCCA-BHQ1
MuRF1	AGGACTGAATTTGTGTTATATGTTG	TAGCCTCGAACTCATAGAGATC	HEX-AACTGCCTCTGCCTCCA-BHQ2
Myostatin	AGACAACTTCTGCCCAGAG	TCCGTGGTAGCGTGATAATC	ROX-CCGTCAACTGCTGTCATCC-BHQ3

Table 3. *Fibre distribution (%) in the newborn spiny mouse diaphragm*

Group	*N*	Type I	Type IIa	Type IIb/d
Control	8	17.0 ± 0.8	42.2 ± 1.3	40.8 ± 1.8
Creatine	8	17.5 ± 1.2	41.6 ± 1.3	40.9 ± 0.8
Hypoxia	10	18.2 ± 1.0	43.3 ± 1.0	38.5 ± 1.3
Creatine + hypoxia	10	17.5 ± 0.6	42.0 ± 0.5	40.5 ± 1.2

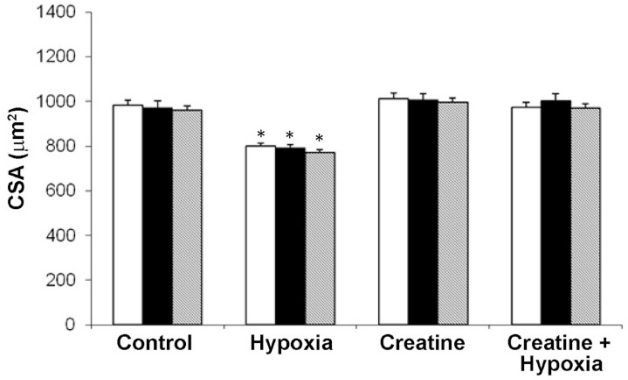

Figure 1. CSA of diaphragm fibers in spiny mouse neonates. Fiber type was determined by myofibrillar ATPase staining, and CSA was measured for type I (white columns), type IIa (black columns), and type IIb/IId (hatched columns) fibers. All fiber types from hypoxic neonates were significantly smaller than all other groups (*$p < 0.01$). Control and creatine $n = 8$, hypoxia and creatine + hypoxia $n = 10$, with 100 fibers from each diaphragm measured.

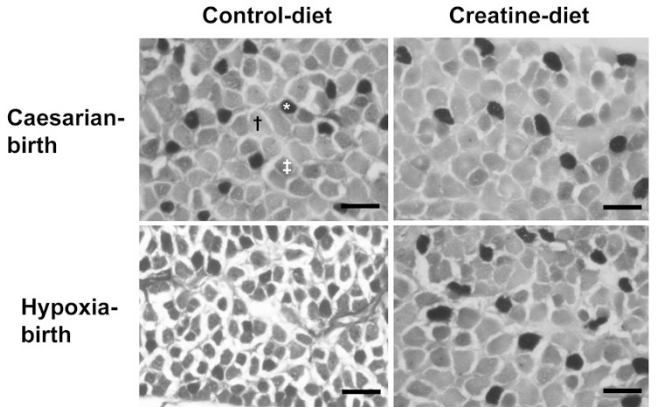

Figure 2. Transverse sections of the diaphragm from spiny mouse neonates. CSA measurements were taken from sections stained for myofibrillar ATPase. Fibers were classified based on staining intensity: type I fibers stain dark (*), type IIa fibers stain intermediate (‡), and type IIb/IId fibers remain pale (†). In comparison with controls and creatine + hypoxia neonates, all fiber types of hypoxic neonates are significantly smaller ($p < 0.01$), with a larger space between fibers. Black bar is 100 μM.

icant reduction in the CSA of all fiber types, which was completely prevented with maternal creatine supplementation ($p < 0.01$; Figs. 1 and 2). Figure 2 shows an example of reduced fiber size in the diaphragm of hypoxia neonates. A large increase in the space between fibers can also be seen. The reduction in CSA occurred in all fibers; thus, no particular fiber type was especially vulnerable to the effects of hypoxia.

Ca^{2+} and Sr^{2+} sensitivity in single skinned muscle fibers. The contractile function of individual diaphragm fibers was examined by inducing contraction with solutions containing Ca^{2+} and Sr^{2+}. The activation parameters obtained for neonates from all groups are shown in Table 4. Birth hypoxia significantly reduced fiber sensitivity to both Ca^{2+} and Sr^{2+} ($p < 0.05$). In comparison, offspring from mothers fed the creatine diet showed significantly greater sensitivity than Ca^{2+} and Sr^{2+}, so that creatine + hypoxia pups were not different from controls; in addition, caesarean-delivered creatine pups were more sensitive to Ca^{2+} and Sr^{2+} than both control and creatine + hypoxia pups ($p < 0.05$; Table 4). The changes in sensitivity to Ca^{2+} and Sr^{2+} between groups could not be attributed to a change of sarcomere length ($p > 0.05$, Table 4).

Maximum Ca^{2+}- and Sr^{2+}- activated force. Individual fibers were submerged in a solution containing sufficient Ca^{2+} (pCa 4.84 or 14.4 μM) to completely activate the contractile apparatus, identifying the maximum Ca^{2+}-activated force response that was then normalized to the CSA of the individual fibers. Hypoxia caused a significant reduction in Ca^{2+}-activated maximum force, which was completely prevented by maternal creatine (5.13 ± 0.15 *vs* 6.46 ± 0.43 N/cm^2; $p < 0.05$; Fig. 3*A*). Creatine supplementation alone did not alter the force produced.

The force-pCa and force-pSr curves were normalized and expressed as a percentage of the maximum Ca^{2+}- or Sr^{2+}-activated force obtained for control fibers (Fig. 3*B–E*). Hypoxia significantly decreased overall contractile function (sensitivity and force), whereas offspring from creatine-fed dams showed no such decrease in contractile function in response to hypoxia. The effect of hypoxia was observed in both the first and second phase of the force-pSr curve. As single diaphragm fibers contain both fast and slow contractile and regulatory isoforms, this indicates that hypoxia induced a similar effect on both the isoforms.

Gene expression. The mRNA levels of *atrogin-1*, *MuRF1*, and *myostatin*—genes known to negatively regulate muscle mass—were measured in diaphragm tissue 24 h after birth. Hypoxia significantly increased mRNA expression of *MuRF1* and *myostatin* (~2- and 2.4-fold increase, respectively; $p < 0.05$; Fig. 4). *Atrogin-1* mRNA levels also increased by 36%, although this was not significant ($p = 0.17$; Fig. 4). Expres-

Table 4. *Ca^{2+}– and Sr^{2+}– activation parameters of diaphragm fibres from spiny mouse neonates*

	Control	Hypoxia	Creatine	Creatine + hypoxia
pCa_{10}	6.38 ± 0.02	6.27 ± 0.02*	6.42 ± 0.02†	6.34 ± 0.01†‡
pCa_{50}	6.07 ± 0.03	5.97 ± 0.02*	6.14 ± 0.03†	6.09 ± 0.00†‡
pCa_{90}	5.68 ± 0.02	5.66 ± 0.00	5.78 ± 0.04*†	5.75 ± 0.04†
n_{Ca}	2.73 ± 0.29	2.89 ± 0.21	3.27 ± 0.27	3.73 ± 0.35*†
pSr 5.5 (%)	8.25 ± 1.08	0.98 ± 0.27*	12.55 ± 1.33*†	1.87 ± 0.52*†‡
pSr 4.5 (%)	75.70 ± 0.05	42.35 ± 3.17*	86.02 ± 1.88*†	56.74 ± 1.93*†‡
$n_{Sr}1$	1.71 ± 0.50	2.31 ± 0.33	1.63 ± 0.45	2.45 ± 0.36
$n_{Sr}2$	4.92 ± 0.23	4.61 ± 0.38	4.85 ± 0.27	4.83 ± 0.22
F1%	26.31 ± 5.51	24.28 ± 6.45	30.19 ± 6.60	26.78 ± 8.10
SL (μm)	2.38 ± 0.05	2.40 ± 0.06	2.42 ± 0.04	2.38 ± 0.04

Control and creatine $N = 24$ fibres, hypoxia $N = 28$ fibres, creatine + hypoxia $N = 26$ fibres. Fibres were taken from 10 animals in each group. Mean ± SE.

* Different from control, where $p < 0.05$.

† Different from hypoxia, where $p < 0.05$.

‡ Different from creatine, where $p < 0.05$.

SL, sarcomere length.

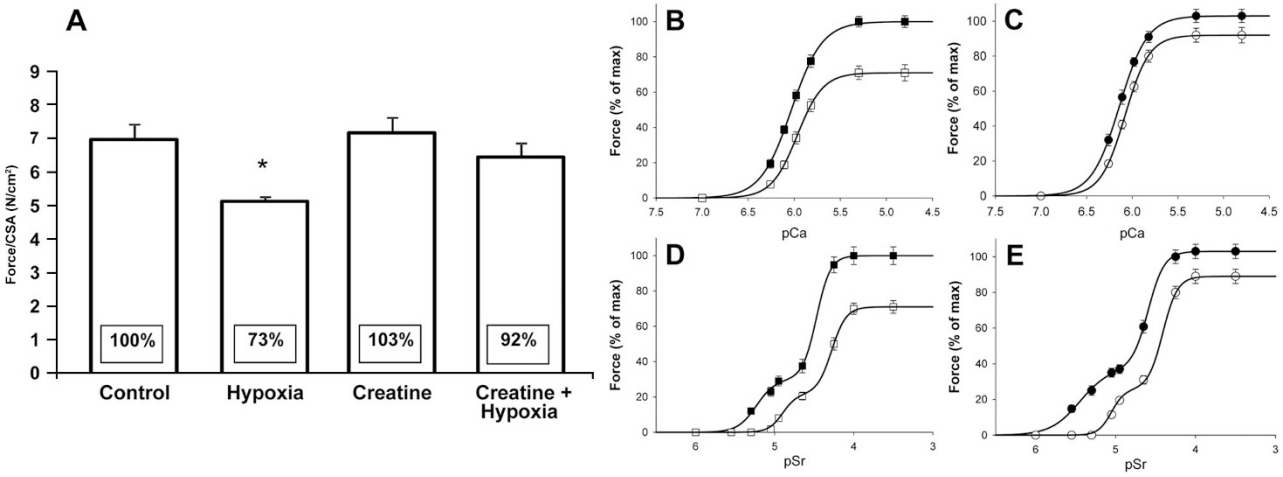

Figure 3. Contractile function of diaphragm fibers in spiny mouse neonates. *A*, Maximum Ca^{2+}-activated force from single diaphragm fibers. The value in box shows the amount of force produced expressed as a percentage of the maximum Ca^{2+}-activated force of fibers from control neonates. Maximum force was significantly smaller for hypoxia neonates compared with all other groups (*$p < 0.01$). *B–E*, The sensitivity to Ca^{2+} (*B*, *C*) and Sr^{2+} (*D*, *E*) plotted against force (normalized to CSA) for control (closed squares) and hypoxia (open squares) neonates, and creatine (closed circles) and creatine + hypoxia (open circles) neonates. Control and creatine, $n = 24$ fibers; hypoxia, $n = 28$ fibers; and creatine + hypoxia, $n = 26$ fibers. Fibers were taken from 10 animals in each group.

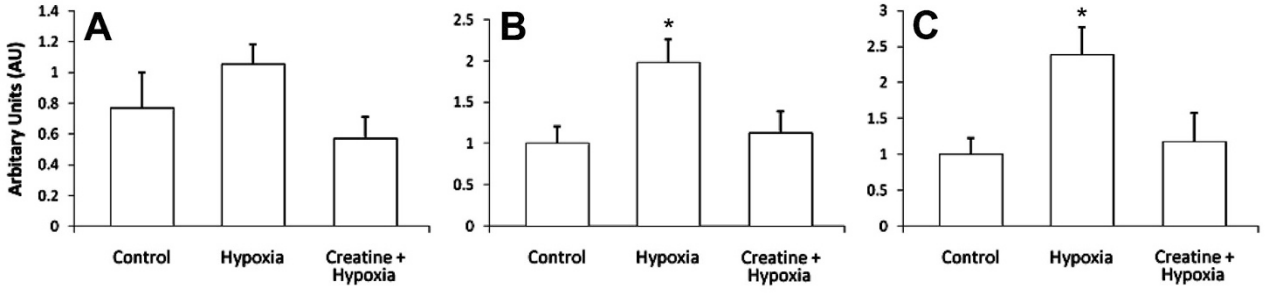

Figure 4. Expression of atrophy and muscle growth-regulating genes in the diaphragm in response to hypoxia. The mRNA expression of atrogin-1 (*A*), MuRF1 (*B*), and myostatin (*C*) in the diaphragm in response to hypoxia. *$p < 0.05$.

sion of MuRF1, myostatin, and atrogin-1 mRNA in creatine + hypoxia pups was not different from controls (Fig. 4).

DISCUSSION

This study has shown that intrapartum hypoxia causes significant structural and functional damage to the newborn diaphragm. This was attenuated by loading the fetal diaphragm with creatine through maternal dietary supplementation from mid-pregnancy. Birth hypoxia caused a decrease in CSA of all fiber types, reduced force-generating capacity, and decreased sensitivity to Ca^{2+} and Sr^{2+}, reflecting contractile dysfunction. Hypoxia also increased *MuRF1* and *myostatin*

mRNA, genes known to negatively regulate muscle mass (30). These changes were not observed in hypoxic offspring whose mothers received creatine in their diet for the second half of pregnancy.

Creatine loading in the fetal diaphragm. We have previously shown in this model that creatine accumulates in the placenta and fetal brain, heart, liver, and kidney in offspring from mothers fed the supplemented diet (14). This study shows this occurs also for the fetal diaphragm. The concentration of TCr was measured only in caesarean-delivered pups from control and creatine-fed dams. It is reasonable to assume that TCr levels in the birth hypoxia groups would be similar to their respective control and creatine-fed groups, at least immediately before the hypoxic insult. To our knowledge, this study is the first to measure TCr concentration in neonatal diaphragm. The values obtained for control animals are similar to that reported for adult rat diaphragm (31).

Creatine protects against hypoxia-induced muscle fiber atrophy. Intrapartum hypoxia caused structural changes in the neonatal diaphragm, significantly decreasing CSA of all fiber types. Hypoxia has been shown to increase protein degradation and attenuate protein synthesis (12) and myogenesis by reducing both muscle cell proliferation and differentiation (32). Our observations that *MuRF1* and *atrogin-1* mRNA were increased, products of two genes involved in the ubiquitin proteasome pathway (UPP), supports *in vitro* observations made in L6 muscle cells subjected to hypoxia (12). Similarly, in patients with chronic obstructive pulmonary disease, a condition associated with prolonged tissue hypoxia, an increase in atrogin-1 and proteasome activity in the diaphragm has been observed (33). *Myostatin*, a known inhibitor of myogenesis, was also increased after hypoxia in this study. Of clinical relevance is the observation that *myostatin* is upregulated in children with cerebral palsy who exhibit muscle atrophy; a common consequence of severe birth hypoxia (34). Further studies are required to determine whether hypoxia-induced atrophy of the diaphragm is caused directly through pathways involving MuRF1/atrogin-1 and myostatin or indirectly through effects on protein degradation, protein synthesis, and myogenesis.

Pups from creatine-treated mothers showed resistance to hypoxia-induced muscle fiber atrophy. Unlike hypoxia pups from control-fed dams, they showed no reduction in fiber size 24 h after birth. This supports the finding that short periods of creatine supplementation can prevent muscle atrophy and wasting (19). The mechanisms by which creatine protected against hypoxia-induced muscle atrophy are unknown, and their identification were not within the scope of this study. However, creatine loading has been shown to increase growth factor signaling such as IGF-1 (35), which can suppress atrogin-1 and MuRF1 levels (36), a creatine-associated response observed in this study.

A known side-effect of creatine administration is accumulation of water and increased intracellular volume to maintain osmotic balance and muscle weight (37). As there were no differences in the CSA of fibers from caesarean-delivered control and creatine neonates, it is unlikely that this accounts for the observed differences in CSA between hypoxia and creatine + hypoxia pups.

Creatine protects against hypoxia-induced contractile dysfunction. Intrapartum hypoxia caused contractile dysfunction in the neonatal diaphragm, significantly reducing the force-generating capacity and sensitivity to Ca^{2+}. This finding is in agreement with studies in the adult diaphragm (9,10). Supplementation of the maternal diet with creatine completely prevented contractile dysfunction in offspring from hypoxic births.

Oxidative stress is known to reduce contractile function by altering the intracellular environment, leading to a rise in inorganic phosphate and a decline in pH, PCr, and adenine nucleotide content (38). In this study, the effects of hypoxia on contractile function were still evident when fibers were activated under conditions where the intracellular composition was controlled (*i.e.* by using "skinned" fibers). This suggests that although the decrease in function may have been initially triggered *in vivo* where the muscle fiber is intact, lasting damage occurred to individual fibers at the level of the contractile apparatus.

The significant reduction in the maximum Ca^{2+}-activated force response in birth-hypoxia neonates was confirmed when this was normalized to fiber size (CSA). As significant fiber atrophy was observed in these neonates, the absolute change in force production would likely be even more pronounced. A significant decrease in Sr^{2+} sensitivity was also observed in fibers from birth-hypoxia pups. This was observed in the F1 and F2 portions of the force-pSr curve. This suggests that hypoxia-induced changes to the intracellular environment affect the activation of both slow and fast contractile and regulatory isoforms in diaphragm fibers.

The most important response to severe hypoxia is gasping, which is a very forceful diaphragmatic contraction. Although it was not directly measured in this study, it is possible that creatine loading improved the neonates' ability to gasp after being expelled from the uterus. This, in conjunction with increased cerebral creatine that may prevent hypoxic ventilatory depression, may contribute to the improved survival rate observed in this model (14).

It is possible that creatine acted as an energy and free-radical buffer during the acute hypoxic episode and reoxygenation period, providing additional energy for the diaphragm and reducing the accumulation of reactive oxygen species whereas attenuating the increase in proteasome activity (39). Creatine itself has been shown to have direct antioxidant properties (17), and consistent with this is the finding that antioxidant therapy improves contractile function in the adult rat diaphragm under hypoxic conditions (9).

SUMMARY

This study demonstrated that a short period of intrapartum hypoxia caused significant structural and functional damage to the neonatal diaphragm. This was effectively prevented by loading the fetal diaphragm with creatine through maternal dietary supplementation during pregnancy. These results provide evidence that creatine supplementation during pregnancy

may protect respiratory function in the neonate during the critical period immediately after birth. Although the many benefits of creatine therapy have been reviewed recently (40), its use in pregnancy has not been fully considered. This study also confirmed that dietary intervention during pregnancy provides a simple and effective means to prevent many of the deleterious effects arising from intrapartum hypoxia. This is particularly relevant for developing countries where maternal nutrition is often suboptimal, and intrapartum hypoxia remains a leading cause of neonatal mortality.

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