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Full Length Article

Methylmercury exposure causes a persistent inhibition of myogenin expression and C2C12 myoblast differentiation

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ABSTRACT

Methylmercury (MeHg) is a ubiquitous environmental toxicant, best known for its selective targeting of the developing nervous system. MeHg exposure has been shown to cause motor deficits such as impaired gait and coordination, muscle weakness, and muscle atrophy, which have been associated with disruption of motor neurons. However, recent studies have suggested that muscle may also be a target of MeHg toxicity, both in the context of developmental myogenic events and of low-level chronic exposures affecting muscle wasting in aging. We therefore investigated the effects of MeHg on myotube formation, using the C2C12 mouse myoblast model. We found that MeHg inhibits both differentiation and fusion, in a concentration-dependent manner. Furthermore, MeHg specifically and persistently inhibits myogenin (MyoG), a transcription factor involved in myocyte differentiation, within the first six hours of exposure. MeHg-induced reduction in MyoG expression is contemporaneous with a reduction of a number of factors involved in mitochondrial biogenesis and mtDNA transcription and translation, which may implicate a role for mitochondria in mediating MeHg-induced change in the differentiation program. Unexpectedly, inhibition of myoblast differentiation with MeHg parallels inhibition of Notch receptor signaling. Our research establishes muscle cell differentiation as a target for MeHg toxicity, which may contribute to the underlying etiology of motor deficits with MeHg toxicity.

1. Introduction

Methylmercury (MeHg) is a ubiquitous and pervasive environmental toxicant that has been classified as a developmental neurotoxicant. Studies of accidental poisonings in Minamata Bay, Japan and in Iraq have elucidated that the developing nervous system is a particularly sensitive target of MeHg, yielding a broad array of motor and cognitive deficits in prenatally exposed children. Various motor defects seen in these accidental poisonings and in more recent epidemiological studies include: loss of coordination, impaired gait, and delayed motor function/milestones such as sitting up and walking (Roegge and Schantz, 2006). Epidemiological studies have also associated MeHg exposure with poorer scores on tests for muscle tone and reflexes (McKeown-Eyssen et al., 1983), leg coordination (Cordier et al., 2002), and fine motor skills (Grandjean et al., 1997; Grandjean et al., 1998). Previously, researchers have attributed MeHg-associated motor deficits to the targeting of the developing nervous system. Studies on MeHg have therefore been focused on the nervous system, particularly in areas that are involved in movement such as the cerebral cortex (Eto, 1997; Harada, 1995; Kakita et al., 2002, 2003; Sakamoto et al., 1998, 2004) and the cerebellum (Choi et al., 1978; Eto, 1997; Lapham et al., 1995; Mancini et al., 2009; Pedersen et al., 1999; Sager et al., 1982, 1984). Although these brain regions are clear targets of MeHg, outcomes such as low birth weight (Grandjean et al., 2003; Karagas et al., 2012; Kim et al., 2011; Lee et al., 2010), muscle wasting (Yoo et al., 2016) and weakness seen with MeHg toxicity are not fully understood (Usuki et al., 1998). These outcomes suggest that targeting of muscle may also play a role in the motor deficits associated with MeHg exposure.

Few studies have examined a direct effect of MeHg on developing or mature muscle. In studies of MeHg in adult rats and zebrafish, muscle structure was found to be disorganized, with smaller muscle fibers and increased space between fiber bundles, and smaller mitochondria with disorganized cristae (Usuki et al., 1998; de Oliveira Ribeiro et al., 2008). Recent research in *Drosophila* has also identified muscle as a potential target of MeHg. Exposure to MeHg during development disrupts the muscle structure of *Drosophila* embryos and pupa (Engel and Rand, 2014; Montgomery et al., 2014). In a genome wide association study (GWAS), isogenic fly lines from the *Drosophila melanogaster* Genetic Reference Panel (DGRP) were screened for MeHg tolerance and susceptibility. A network analysis identified an enrichment for genes involved in muscle and neuromuscular junction development, which

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paralleled an overt phenotype in developing flight muscles (Montgomery et al., 2014). However, parsing out effects on muscle versus effects on neurons during developmental can prove difficult. Interactions between muscle and neuron that guide the proper development and maintenance of the motor unit are mediated by an elaborate array of reciprocal signals. For example, denervation during development has been shown to reduce the size of the myoblast pool and ultimately reduce myofiber size (Fernandes and Keshishian, 1998). Likewise, myoblasts in developing systems release cues required for axon defasciculation and growth (Landgraf et al., 1999), and adult muscle satellite cells have been implicated in the maintenance of the neuromuscular junction (Liu et al., 2017).

To understand if muscle development could be directly targeted by MeHg, we turned to the C2C12 mouse myoblasts model, a premier model for studying muscle development mechanisms. C2C12 have also been utilized to study the effects of toxicants such as ethanol and arsenic on muscle development (Arya et al., 2013; Steffens et al., 2011; Yen et al., 2010). These cells are a subclone of proliferative myoblasts derived from the thigh of 2-month old C3H mice after a crush injury (Yaffe and Saxel, 1977). The differentiation profile of these cells is summarized in Fig. 1. C2C12 myoblasts can be maintained in a proliferative, undifferentiated state when cultured in serum-rich media. Upon removal of serum, the cells differentiate to non-proliferative myocytes and then fuse to develop myotubes and eventually syncytial muscle fibers (Fig. 1B). A proportion of the myoblasts also de-differentiate to a reserve cell population (Fig. 1A), which share properties of satellite cells, the adult muscle stem cells (Yoshida et al., 1998). Myoblast differentiation down each of these two paths is coordinated by expression of signature myogenic transcription factors (Buckingham and Rigby, 2014) . Whereas the myoblasts are initially elevated in expression of both MyoD and Pax7 ($MyoD^+/Pax7^+$), terminal differentiation is marked by Pax7 repression, MyoD persistence ($MyoD^+/Pax7^-$), and a sequential upregulation of myogenin (MyoG) and myosin heavy chain (MHC). The reserve cell fate is marked by persistent Pax7 expression that parallels MyoD repression ($MyoD^-/Pax7^+$) and is furthermore promoted by upregulation of Notch receptor signaling (Sun et al., 2008) (Fig. 1A).

Notch signaling has been implicated in many phases of muscle cell differentiation, particularly in satellite cell formation and maintenance of quiescence (Bjornson et al., 2012; Mourikis et al., 2012a, 2012b). Notch signaling is a conserved pathway and has been shown to be upregulated by MeHg exposure in both *Drosophila* (Bland and Rand, 2006; Engel et al., 2012; Rand et al., 2008) and rat neural stem cells (Tamm et al., 2008). In addition, MeHg susceptibility has been associated with Drosophila genes involved in myocyte adhesion and fusion, which are modulated by Notch signaling (Bour et al., 2000; Gildor et al., 2012; Montgomery et al., 2014). Therefore, we hypothesized that MeHg could inhibit myotube formation via the upregulation of Notch signaling.

We tested this hypothesis using the C2C12 mouse myoblast model. We have investigated the effects of MeHg on myoblast differentiation and fusion using immunocytochemistry. Expression levels of several myogenic gene transcripts, including core Notch pathway genes, were determined with RT-qPCR. In addition, we probed genes responsible for mitochondrial biogenesis and transcription, as mitochondria are known targets of MeHg (Caito and Aschner, 2015), and mitochondrial activity and biogenesis are thought to regulate myogenesis (Wagatsuma and Sakuma, 2013). We find that MeHg can act at distinct phases of myoblast differentiation that will substantially compromise formation of the mature myofiber.

2. Methods

2.1. C2C12 cell culture

C2C12 cells were ordered from ATCC (#CRL-1772, Lot # 61633507). Cells were cultured in growth media (GM), DMEM with high glucose, L-glutamine, and sodium pyruvate (Gibco, #11995) supplemented with 20% fetal bovine serum (Atlanta Biologicals, #s11150) and Penicillin/Streptomycin at 50 µg/mL (Gibco, #15140).

C2C12 were plated in GM until reaching 90% confluency after 2 days. GM was then replaced with differentiation media (DM), DMEM supplemented with 10% horse serum (ATCC, # 30–2040) and 50 µg/mL Penicillin/Streptomycin. DM was replaced every 24 h.

2.2. Cell treatment

MeHgCl (Sigma-Aldrich, #215465–5 g) was kept as a 50 mM stock in DMSO (Thermo-Fisher Scientific, #BP231-100) and diluted in DM to create a 10 µM stock. For 0 µM control stocks, equivalent amounts of DMSO (vehicle) were added to DM. The 10 µM MeHg stock was diluted further in the DMSO stock to create appropriate concentrations, all containing 0.02% DMSO. MeHg was added to the cells for 24 h in DM, then MeHg-DM was replaced with MeHg-free DM for either 3 or 6 days. DM was replaced every 24 h.

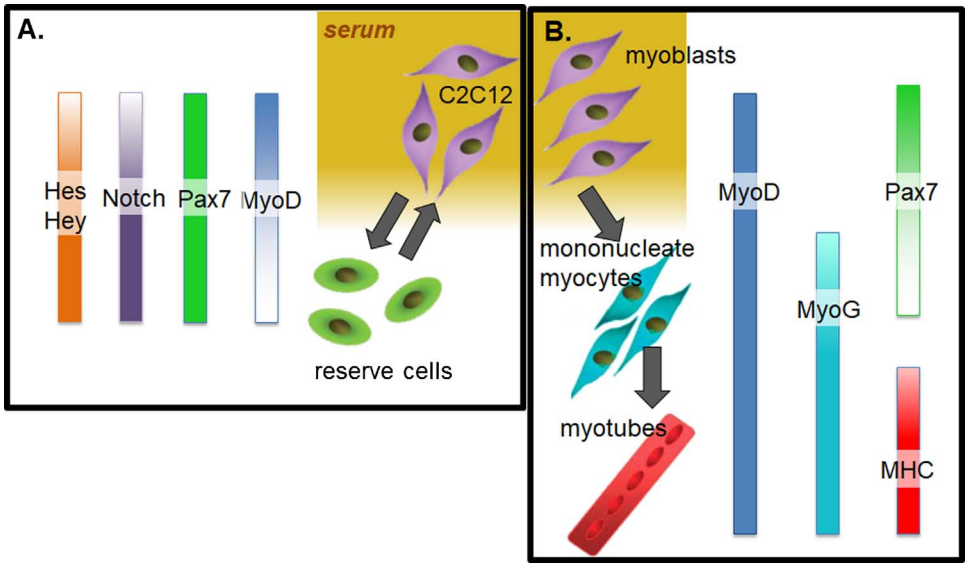

Fig. 1. Schematic of C2C12 Differentiation. (A) In the C2C12 cell culture system, upon removal of serum, proliferating myoblasts can de-differentiate into quiescent reserve cells. Reserve cells are capable of differentiating into myoblasts with the addition of serum. Myoblasts are $Pax7^+/MyoD^+$; whereas, reserve cells are $Pax7^+/MyoD^-$. Cells are guided to the reserve cell fate by expression and activation of the Notch receptor, which gives elevated Hes and Hey gene expression and maintains the self-renewing state. (B) Upon serum removal, C2C12 cells also differentiate into myocytes, which are $Pax7^-/MyoD^+$. Myocytes lose their proliferative capabilities, express myogenin (MyoG), followed by myosin heavy chain (MHC), and ultimately fuse to form syncytial myotubes.