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# Deficits in Bioenergetics and Impaired Immune Response in Granulocytes From Children With Autism

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## KEY WORDS

mitochondria, autism, NFE2L2, Nrf2, oxidative stress, immune response, bioenergetics, NADPH oxidase

## ABBREVIATIONS

ASD—autism spectrum disorder  
mtDNA—mitochondrial DNA  
NADH—reduced nicotinamide-adenine dinucleotide  
NADPH—reduced nicotinamide-adenine dinucleotide phosphate  
NFE2L2—nuclear factor erythroid 2–related factor 2  
OXPHOS—oxidative phosphorylation  
PBMC—peripheral blood mononuclear cell  
PKC—protein kinase C  
PMA—phorbol 12-myristate 13-acetate  
ROS—reactive oxygen species  
TD—typically developing

Dr Giulivi conceptualized and designed the study, drafted the initial manuscript, and contributed to intellectual content; Dr Napoli carried out data analysis, contributed to biochemical data analyses and interpretation, and revised the manuscript; Ms Wong performed all experiments on molecular biology, contributed to the analysis and interpretation of data, and drafted part of the manuscript; Dr Hertz-Picciotto reviewed the manuscript; and all authors approved the final manuscript as submitted.

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## abstract



Despite the emerging role of mitochondria in immunity, a link between bioenergetics and the immune response in autism has not been explored. Mitochondrial outcomes and phorbol 12-myristate 13-acetate (PMA)–induced oxidative burst were evaluated in granulocytes from age-, race-, and gender-matched children with autism with severity scores of  $\geq 7$  ( $n = 10$ ) and in typically developing (TD) children ( $n = 10$ ). The oxidative phosphorylation capacity of granulocytes was 3-fold lower in children with autism than in TD children, with multiple deficits encompassing  $\geq 1$  Complexes. Higher oxidative stress in cells of children with autism was evidenced by higher rates of mitochondrial reactive oxygen species production (1.6-fold), higher mitochondrial DNA copy number per cell (1.5-fold), and increased deletions. Mitochondrial dysfunction in children with autism was accompanied by a lower (26% of TD children) oxidative burst by PMA-stimulated reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase and by a lower gene expression (45% of TD children's mean values) of the nuclear factor erythroid 2–related factor 2 transcription factor involved in the antioxidant response. Given that the majority of granulocytes of children with autism exhibited defects in oxidative phosphorylation, immune response, and antioxidant defense, our results support the concept that immunity and response to oxidative stress may be regulated by basic mitochondrial functions as part of an integrated metabolic network. *Pediatrics* 2014;133:e1405–e1410

A higher incidence of mitochondrial dysfunction,<sup>1</sup> altered immune response,<sup>2–4</sup> increased cellular oxidative damage<sup>1,5,6</sup> and decreased antioxidant defenses<sup>5,7–11</sup> has been reported in autism, possibly contributing to its etiology and/or morbidity. Thus, we explored the possibility that these pathways could be conceived as part of a common, integrated mechanism in which basic mitochondrial functions would play a central role.<sup>12–15</sup> To this end, oxidative phosphorylation (OXPHOS) capacity, immune response to phorbol 12-myristate 13-acetate (PMA), and markers of oxidative stress (reactive oxygen species [ROS] production, mitochondrial DNA [mtDNA] deletions) were evaluated in granulocytes from children with autism and typically developing (TD) age- and gender-matched children. Although the choice of studying peripheral blood mononuclear cells (PBMCs) as a biological material could be criticized in the context of autism research, several reports have confirmed the use of PBMC gene expression as a valid surrogate for gene expression in the brain.<sup>16–23</sup> Furthermore, the novel role for Toll-like receptor 9 (TLR9) in energy metabolism and cellular protection has reinforced the conservation of the innate immunity pathway in non-immune cells.<sup>24</sup>

The immune response was tested by following the activation of granulocytes by PMA. This soluble stimulus activates the protein kinase C (PKC)-dependent phosphorylation of cytosolic components of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase, eliciting the translocation of cytosolic components to the plasma membrane, assembly of a functional NADPH oxidase, and the ensuing “respiratory burst”<sup>25–27</sup>. In addition, gene expression of nuclear factor erythroid 2-related factor 2 (NFE2L2) was also evaluated because NFE2L2-mediated phase II antioxidant defenses seem critical for protecting memory T cells, increasing

neuronal glutathione levels,<sup>28</sup> decreasing the damage mediated by inhibitors of mitochondrial Complexes I and II,<sup>29,30</sup> and modulating the cellular response to common environmental allergens.<sup>31</sup>

## CASE REPORT

Granulocytes from 10 children with autism with severity scores of  $\geq 7$  (median [quartile1, quartile 3]: 9 [8, 9]<sup>32</sup>) and 10 age-, gender-, and race-matched TD children were sampled from individuals previously described<sup>1</sup> and enrolled in the Childhood Autism Risks from Genetics and Environment (CHARGE) Study at the University of California, Davis. All details on patient selection, diagnosis, demographic characteristics, and clinical data of the study groups used in this study were published previously<sup>1</sup> and are summarized in the Supplemental Information. Detailed descriptions of sample collection, cell isolation, and evaluation of outcomes shown in this study are included in the Supplemental Information.

To evaluate the OXPHOS capacity of granulocytes, resting oxygen uptake rates were evaluated with intact, not activated, cells suspended in 5 mM glucose in Hanks buffer saline solution (Table 1). For both diagnostic groups, >85% of the oxygen uptake rate was sensitive to 0.2 nmol  $\times$  (mg protein)<sup>-1</sup> oligomycin (not shown), a specific ATPase inhibitor,<sup>33</sup> suggesting that most, if not all, oxygen uptake ob-

served under these conditions is tightly coupled to OXPHOS. Granulocytes from children with autism exhibited a lower OXPHOS capacity than did those from TD children, characterized by a 2.9-fold lower oxygen uptake under resting conditions. Various segments of the electron transport chain were tested in granulocytes, namely reduced nicotinamide-adenine dinucleotide (NADH) oxidase, succinate oxidase,  $\alpha$ -glycerophosphate oxidoreductase, and cytochrome *c* oxidase activities, all normalized to citrate synthase activity, a marker of mitochondrial mass.<sup>34</sup> NADH oxidase includes the transfer of electrons from NADH (derived from malate) to oxygen through a series of 3 carriers of the electron transport chain (Complex I, III, and IV) and finally to Complex V or ATPase. The rate of oxygen consumption of permeabilized granulocytes from children with autism under phosphorylating conditions (supplemented with malate-glutamate and adenosine diphosphate) was 42% of that of TD children ( $P < .01$ ; see Table 1 for averages and Supplemental Table 3 for each individual proband). Similarly, succinate oxidase activity (which evaluates the segment encompassing Complex II, III, IV, and V) was 31% of that of TD children ( $P < .001$ ; Table 1, Supplemental Table 3). Mean Complex V or ATPase activity was also significantly decreased (by 63%;  $P < .005$ ) in children with autism (Table 1,

**TABLE 1** Mitochondrial Outcomes in Granulocytes

Outcome	Children With Autism	TD Children	<i>P</i>
Resting O <sub>2</sub> uptake	0.38 $\pm$ 0.09	1.1 $\pm$ 0.3	.05
NADH oxidase (95% CI)	4.6 $\pm$ 3.1 (2–7)	10.9 $\pm$ 4.9 (7–15)	.01
Succinate oxidase (95% CI)	1.6 $\pm$ 1.2 (1–2)	5.2 $\pm$ 2.0 (5–13)	.001
Glycerophosphate oxidoreductase (95% CI)	6.7 $\pm$ 4.0 (4–10)	13 $\pm$ 10 (3–23)	—
Cytochrome <i>c</i> oxidase (95% CI)	9.9 $\pm$ 4.3 (7–13)	16 $\pm$ 13 (5–26)	—
ATPase (95% CI)	46 $\pm$ 28 (27–66)	123 $\pm$ 54 (80–167)	.005
Citrate synthase (95% CI)	166 $\pm$ 37 (93–239)	110 $\pm$ 28 (55–166)	—
Rate of H <sub>2</sub> O <sub>2</sub> production	0.11 $\pm$ 0.02	0.07 $\pm$ 0.02	.04
mtDNA deletions at <i>CYTB</i>	0.54 $\pm$ 0.01	0.60 $\pm$ 0.02	.03
mtDNA deletions at <i>ND4</i>	0.43 $\pm$ 0.01	0.43 $\pm$ 0.02	—
NFE2L2 gene expression level	0.45 $\pm$ 0.01	1.00 $\pm$ 0.03	.01

Data are presented as means  $\pm$  SDs. All activities were expressed as nmol  $\times$  (min  $\times$  mg protein)<sup>-1</sup>, then normalized to citrate synthase also expressed as nmol  $\times$  (min  $\times$  mg protein)<sup>-1</sup>, and multiplied by 1000. CI, confidence interval; *CYTB*, cytochrome *b*; *ND4* NADH dehydrogenase subunit 4.

Supplemental Table 3), whereas mean  $\alpha$ -glycerophosphate oxidoreductase and cytochrome *c* oxidase activities were not significantly different between diagnostic groups.

The type of OXPHOS deficiencies and the incidence of individuals with autism with OXPHOS deficits followed the same trend as that reported for the lymphocytes from the same cohort of children (>60% for both NADH- and succinate-oxidase activities; >40% for ATPase<sup>1</sup>; Supplemental Table 3), suggesting that the observed effects are not cell-type specific. Indeed, all mitochondrial outcomes tested in lymphocytes correlated statistically with those obtained with granulocytes from the same individual (Supplemental Fig 2).

No differences in mitochondrial mass (as judged by the activity of citrate synthase<sup>34</sup>) were observed in granulocytes from children with autism and TD children. However, another putative marker for mitochondrial mass, mtDNA copy number per cell, was significantly increased in granulocytes from children with autism (1.5-fold of that from TD children<sup>1</sup>; Supplemental Table 4). Increased mtDNA copy number without increases in OXPHOS capacity and/or mitochondrial mass, as observed in this study, has been attributed to a cellular response to cope with oxidative stress<sup>35</sup> in an attempt to sustain adequate levels of mitochondrial transcripts from wild-type mtDNA.<sup>36</sup> Consistent with this view, an increased mean mitochondrial ROS production (1.6-fold; Table 1) and increased mtDNA deletions in the segment encoding for cytochrome *b* (*CYTb*) but not in that encoding for NADH dehydrogenase subunit 4 (*ND4*) were observed in granulocytes from children with autism (Table 1, Supplemental Table 4).

The respiratory burst in TD granulocytes, evaluated as the oxygen consumption to produce superoxide anion after PMA addition, was comparable to reported control values (1.1 to 7.7<sup>37</sup> vs 4

nmol O<sub>2</sub><sup>-</sup> × [10<sup>6</sup> polymorphonuclear cells (PMN) × minute]<sup>-1</sup>; Table 2, Supplemental Table 5). Upon activation, the maximum oxidative burst rate and the total amount of oxygen consumed in 5 minutes by granulocytes from children with autism was 24% and 40% of that from TD children, respectively (*P* < .05; Table 2, Supplemental Table 5). A longer interval between the addition of PMA and the start of the oxidative burst (also called latency) was observed in 7 of 9 children with autism (1.4-fold; Table 2, Supplemental Table 5), which is suggestive of a delayed or defective signal transduction pathway involving PKC alone or in combination with other pathways<sup>38</sup> (eg, NFE2L2).

In search of a common mechanism that would explain the above results, we focused on NFE2L2 because this nuclear transcription factor regulates clusters of genes that control cellular antioxidants,<sup>39-41</sup> modulate both innate and adaptive immune responses,<sup>42</sup> and has a strong association with mitochondrial function, glucose and fatty acid homeostasis, and

immune response via peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ).<sup>43-46</sup> Consistent with this hypothesis, the transcript levels of NFE2L2 (normalized to glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) evaluated by quantitative polymerase chain reaction were 45% of those of TD children (Table 1).

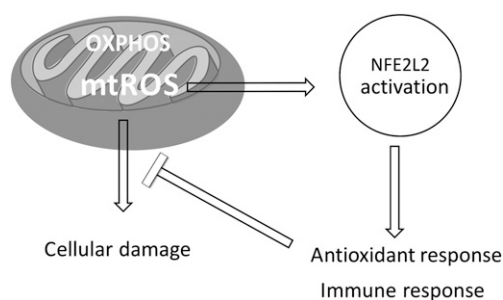
## DISCUSSION

This study performed with granulocytes from children with autism confirms and extends that previously obtained with lymphocytes from the same cohort of children.<sup>1</sup> Deficits in OXPHOS were accompanied by higher oxidative stress (increased ROS production, increased mtDNA deletions, and higher mtDNA copy number) in both cell types, suggesting that these features are not cell specific. This report broadens our knowledge because it includes studies on the immune response of granulocytes from probands. These cells presented a lower PMA-mediated oxidative

**TABLE 2** Oxygen Uptake of PMA-Stimulated Granulocytes

Outcome	Children With Autism ( <i>n</i> = 10)	TD Children ( <i>n</i> = 10)	<i>P</i>
Latency, s	42 ± 5	30 ± 6	.07
Maximum rate of O <sub>2</sub> uptake, (nmol O <sub>2</sub> ) × (min × mg protein) <sup>-1</sup>	1.9 ± 0.5	8 ± 1	.003
Total O <sub>2</sub> consumed in 5 minutes, (nmol O <sub>2</sub> ) × (mg protein) <sup>-1</sup>	28 ± 8	70.5 ± 0.4	.005

Oxygen uptake rates were evaluated in 5 mM glucose-, calcium-, and magnesium-supplemented Hanks buffer saline solution without phenol red (20°–22°C) before and after adding 20 mg PMA × mL<sup>-1</sup> and are presented as means ± SDs.



**FIGURE 1**

Molecular network linking NFE2L2-mediated antioxidant and immune responses and mitochondrial (mt) OXPHOS and ROS. A disruption of this network starting either with a functional disruption of NFE2L2 or OXPHOS deficits may contribute to a state of chronic inflammation, accompanied by a diminished capacity to compensate for conditions of increased oxidative stress, including exposure to environmental triggers, possibly contributing to the etiology and/or morbidity of autism.

burst with a longer latency to trigger this response. Taken together, these findings are in agreement with those reporting immune dysregulation, mitochondrial dysfunction, increased oxidative stress, and decreased antioxidant or repair capacity in some cases of autism.<sup>1–11,47–50</sup>

Several studies suggest that pre- or perinatal exposure to certain triggers might imprint a state of both dysregulated immune response and mitochondrial dysfunction in the progeny as a result of the integration of basic mitochondrial functions with the immune response and antioxidant defense mechanisms.<sup>51–55</sup> Furthermore, maternal exposure during pregnancy to various pathogens and/or the maternal immune response (fever, inflammation) have been associated with significant increased risk of autism spectrum disorder (ASD).<sup>56–59</sup> In this regard, gestational exposure to the viral mimetic poly(I:C) in rodents resulted in ASD-like behavioral abnormalities in the progeny,<sup>60</sup> and immune cells from the same animals had OXPHOS deficits still present in adulthood.<sup>61</sup> These findings are consistent with the current view of chronic inflammation in which the proinflammatory-phase response (mainly fueled by ATP generated in glycolysis<sup>13</sup>) predominates and persists unless external changes are implemented.<sup>62</sup> In our study, the frequency of children (for whom all outcomes were available) having both autism and concurrent deficits in OXPHOS, immune response, and antioxidant response (considering values outside the 95% confidence in-

terval) was 6 out of 8, supporting the concept that immunity and response to oxidative stress are interconnected and that they may be regulated by basic mitochondrial functions as part of an integrated metabolic network.<sup>14</sup>

Given the critical role of NFE2L2 as regulator of the antioxidant response, alone or in combination with PKC,<sup>38</sup> and its strong association with mitochondrial morphology,<sup>63</sup> glucose and fatty acid homeostasis, and immune response via PPAR $\gamma$ ,<sup>43–46</sup> it is likely that a dysregulation in the NFE2L2 pathway may contribute to a state of chronic inflammation with a diminished capacity to compensate for conditions of increased oxidative stress,<sup>64</sup> including exposure to environmental triggers,<sup>65,66</sup> thereby limiting the mitochondrial switch to a phase with increased OXPHOS and more reparative features and lower inflammatory/cytotoxic responses<sup>14,15</sup> (Fig 1). Our findings should be interpreted with caution because this is a case-control study, in which blood samples were collected postdiagnosis in a small number of probands.

Although we cannot exclude other mechanisms that could account for our findings, comparisons between our study and others reporting a downregulation in the expression of genes encoding for Complexes I, III, IV, and V<sup>67,68</sup> or the occurrence of pathogenic mtDNA mutations<sup>69–72</sup> are limited because of the following issues: (1) the age of the individuals used spanned from 2 to 60 years old,<sup>67,68</sup> above the age range of our subjects (2–5 years)<sup>1</sup>; (2) levels of

transcripts from postmortem (frozen and with varied post-mortem intervals) brain samples<sup>67,68</sup> are likely not comparable to OXPHOS activities obtained with freshly obtained PBMCs; (3) messenger RNA expression does not necessarily predict protein expression and/or activity<sup>73–75</sup>; and (4) sequencing of mtDNA segments of our samples did not reveal a high incidence of any pathogenic mutation<sup>6</sup> as observed by others.<sup>76</sup> Although some of these observations could be reconciled considering differences between tissues or proportions of mutant mtDNA, no study has evaluated the gene expression for NFE2L2 in children with autism. Thus, we cannot exclude the possibility that the reported downregulation could be downstream from NFE2L2.

In conclusion, this is the first report to our knowledge suggesting a molecular network linking mitochondrial OXPHOS and the inflammation/immune response, opening new doors for future studies and pharmacologic targets. In this regard, activation of the NFE2L2 pathway has been reported as being beneficial at decreasing the behavioral abnormalities and brain pathology in a murine model of Huntington disease.<sup>77</sup>

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