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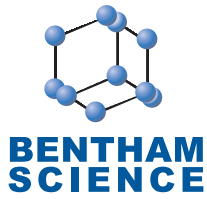
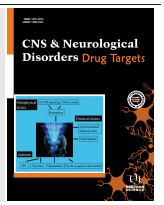


Biomarker-Guided Strategy for Treatment of Autism Spectrum Disorder (ASD)



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Abstract: Autism spectrum disorder (ASD) is a complex, life-long neurodevelopmental disorder currently affecting an estimated 1 out of 68 among children aged 8 y in the United States. ASD has complex genetic and epigenetic features that lead to the phenotype and there is no single genetic marker for the diagnosis. Therefore, the diagnosis for ASD is phenotype-based with no validated or credible laboratory tests available. Evidence-based treatments for ASD are limited. There is no FDA approved medical therapy that addresses either core ASD symptoms or pathophysiological processes associated with ASD. We outline herein, several ASD-associated basic physiological pathways that can be regulated by the small molecule phytochemical sulforaphane, as an example of a druggable small molecule target for which much *in vitro*, pre-clinical, and clinical evidence already exists: (1) redox metabolism/oxidative stress, (2) mitochondrial dysfunction, (3) immune dysregulation/neuroinflammation, (4) febrile illness and the heat shock response, and (5) synaptic dysfunction. Furthermore, we identify the biomarkers that can be used to assess the functioning of these pathways as well as suggesting how these biomarkers could guide novel treatment strategies to correct these biochemical abnormalities in order to improve core and associated symptoms of ASD.

Keywords: Heat shock response, mitochondrial dysfunction, neuroinflammation, oxidative stress, sulforaphane, synaptic dysfunction.

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INTRODUCTION

Autism spectrum disorder (ASD) is a complex, life-long neurodevelopmental disorder currently affecting an estimated 1 out of 68 among children aged 8 y in the United States, with marked male (4.5:1) preponderance [1]. ASD is characterized by a number of so-called core symptoms that reflect impaired ability to communicate and interact socially and by restricted and repetitive patterns of behavior, interests and activities [2]. In addition, many people with ASD suffer from additional mental health symptoms, cognitive deficits, epilepsy and sleep problems. Despite decades of research, our knowledge of the causes of-, and treatments for ASD remain limited [3]. ASD has complex genetic and epigenetic features that lead to the phenotype and there is no single genetic marker for the diagnosis. Therefore, the diagnosis for ASD is currently phenotype-based with no validated or credible laboratory tests available [4]. Evidence-based treatments for ASD are limited. There is no FDA approved medical therapy that addresses either core ASD symptoms or pathophysiological processes associated with ASD [5, 6]. A primary goal of much ongoing research in ASD, is thus to more precisely identify the abnormal genetic and epigenetic processes that underlie the phenotype of the disorder. Much evidence from a variety of specialties has documented that multiple non-central nervous system (CNS) abnormalities are associated with ASD. This strongly suggests that ASD may involve not just organ-specific abnormalities, but systemic abnormalities, at least in some individuals. Indeed, over the last decade, physiological systems that transcend specific organ dysfunction, such as immune dysregulation, inflammation, impaired detoxification, environmental toxicant exposures, redox regulation/oxidative stress, mitochondrial dysfunction, and gut dysbiosis have been implicated in the pathophysiology of ASD [7-11] (Fig. **1**). The importance of identifying and understanding these physiological abnormalities cannot be understated. While most genetic mutations are not directly treatable, treatments are available for metabolic disorders. Given the broad range of children with ASD that must be affected by such metabolic disturbances, targeted treatments guided by specific biomarkers have the potential to improve physiological function and health in a large number

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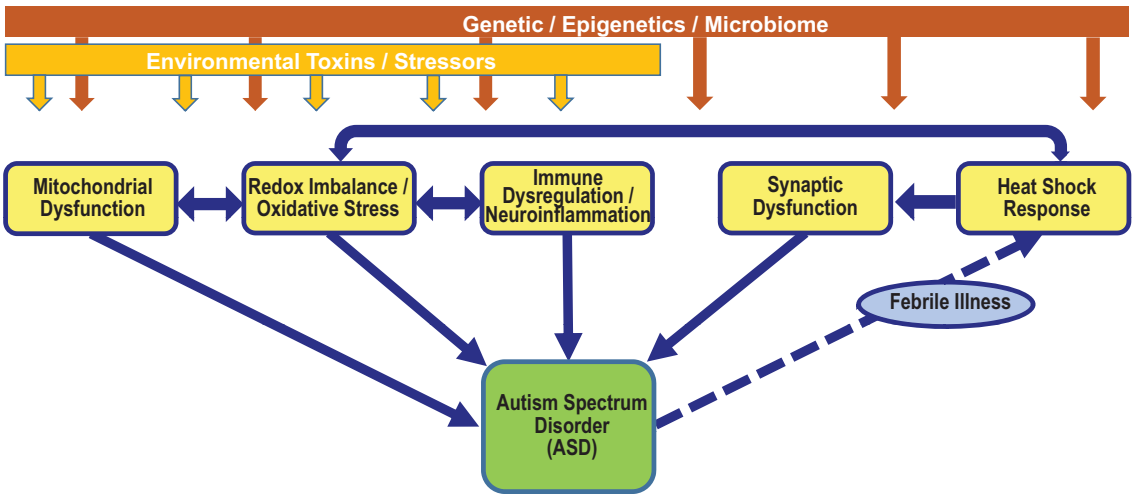

Fig. (1). Pathophysiological events associated with ASD.

of children with ASD. Although there are no autism-defining metabolic biomarkers, closer examination of the biomarkers of pathways associated with ASD would be informative regarding the pathophysiology of ASD, and might be useful in early identification, predicting risk and course. Most importantly, they might guide treatment strategy and enable clinicians to closely monitor treatment response in real time [4, 12] and without confounding by the "screen of expectation" [13] that may contribute to many neuropsychiatric evaluations.

Phytochemicals and small molecule analogs have long been recognized for exerting different biological effects. Since ASD is multi-factorial and no drugs are available to treat it, strategies using multi-functional phytochemicals or even the plants from which those compounds are isolated, are highly attractive. In a placebo-controlled, double-blind, randomized clinical trial, we have recently shown that the daily administration of sulforaphane for 4-18 weeks substantially ameliorated the behavioral anomalies of the majority of 26 young males with moderate to severe ASD without significant toxicity [14]. Sulforaphane is a dietary phytochemical, derived from its biologically inactive precursor (glucoraphanin) that is widely consumed in cruciferous plants (e.g. broccoli, cabbage) [15], and can be considered a food, dietary supplement, or a drug, depending on its intended use [16]. Glucoraphanin, which is present at particularly high levels in broccoli sprouts [17], is converted to sulforaphane by both the plant enzyme myrosinase and the microflora of the gastrointestinal tract [18, 19]. There is extensive evidence from unrelated *in vitro* and clinical studies, that sulforaphane counteracts many of the same biochemical and molecular (biomarker) abnormalities associated with ASD, including oxidative stress and lower antioxidant capacity, defects in reduced glutathione synthesis, mitochondrial dysfunction and low oxidative phosphorylation, increased lipid peroxidation, and neuroinflammation. Importantly, sulforaphane can cross the blood-brain-barrier and quickly reach the CNS to exert its protective effects [20-22].

We outline herein, several ASD-associated basic physiological pathways that can be regulated by sulforaphane: (1) redox metabolism/oxidative stress, (2) mitochondrial dysfunction, (3) immune dysregulation/neuroinflammation, (4) febrile illness and the heat shock response, and (5) synaptic dysfunction. We highlight sulforaphane as an example of a druggable small molecule target for which much *in vitro*, pre-clinical, and clinical evidence already exists. Furthermore, we identify the biomarkers that can be used to assess the functioning of these pathways as well as suggesting how these biomarkers could guide novel treatment strategies to correct these biochemical abnormalities in order to improve core and associated symptoms of ASD.

REDOX METABOLISM/OXIDATIVE STRESS

Oxidative Stress in ASD

Oxidative stress results from an imbalance of reactive oxygen species (ROS)/ reactive nitrogen species (RNS) and the cellular systems that cope with that oxidative stress. At high levels, ROS/RNS can react with cellular components, causing damage to lipids, protein and DNA, and modulating intracellular signaling pathways, leading to cellular degeneration and even death. ROS/RNS can also initiate proinflammatory pathways, which will further exacerbate the deleterious oxidized environment [21]. The brain is particularly vulnerable to oxidative stress because of its high oxygen consumption, high content of unsaturated fatty acids and transition metals, and low antioxidant defense capacities [23]. There is a long history of studies showing that ASD is associated with oxidative stress and diminished antioxidant capacity [8, 9, 24-27]. Lower concentrations of reduced glutathione (GSH), the major intracellular antioxidant, higher levels of oxidized glutathione (GSSG), and reduced GSH/GSSG redox ratios have been reported in plasma, peripheral blood mononuclear cells (PBMC), lymphoblastoid cell lines, brain tissue and mitochondria of autistic children in comparison to normal controls [9, 25, 28-31]. The activities of several GSH pathway enzymes have been found to be reduced in brain tissue of ASD patients [32]. In addition to alteration in glutathione homeostasis, oxidative damage to lipids, proteins and DNA in the blood, PBMC, urine and postmortem brain from ASD individuals has been reported [33-38].

Regulation of Redox Balance

Eukaryotic organisms have developed highly efficient protective mechanisms to maintain cellular redox homeostasis and reduce oxidative damage through a series of antioxidant molecules and detoxifying enzymes. Widely recognized is the Keap1/Nrf2/ARE pathway, which is controlled by a central regulator Nrf2 (nuclear factor-erythroid 2-related factor 2) (Fig. **2**). Activation of this system leads to upregulation of the phase 2 response, a large network of cytoprotective proteins (estimated to embrace as much as 4-5% of the genome), primarily enzymes, which prevent tissue injury associated with oxidative and electrophilic stress as well as other cellular insults such as radiation and carcinogen exposure [39-41]. Unstimulated, Nrf2 is sequestered in the cytoplasm by Keap1 (Kelch-like ECH-associated protein 1), promoting Nrf2 ubiquitination and degradation. When inducers, which have sulfhydryl group reactivity [42], modify specific and highly reactive cysteine residues of Keap1, Nrf2 is diverted from the proteasomal degradation and translocates to the nucleus. There it heterodimerizes with small Maf, binds to the AREs (antioxidant response elements) of phase 2 genes, and activates their transcription [43]. Nrf2 regulates the cellular redox balance by controlling the expression of genes responsible for: (i) the catalytic and the regulatory subunits of γ-glutamylcysteine ligase (GCLC and GCLM), the rate-limiting enzyme in the biosynthesis of glutathione; (ii) glutathione reductase, which regenerates oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH as the hydride donor; and (iii) all four enzymes which are responsible for the synthesis of the NADPH cofactor, namely malic enzyme 1 (ME1), isocitrate dehydrogenase 1 (IDH1), glucose-6-phosphate dehydrogenase (G6PD), and 6-phosphogluconate dehydrogenase (PGD) [41, 44-46].

Protective Effects of Sulforaphane

ARE induction by chemical activators has been shown to protect neuronal cell lines against various oxidative damages induced by dopamine, hydrogen peroxide (H_2O_2) and glutamate [47-49]. In addition, a mouse study has shown that Nrf2 regulates its target genes in brain under unstressed conditions, and loss of Nrf2 affects various brain functions [50]. Sulforaphane, as the most potent naturally occurring inducer of mammalian cytoprotective enzymes regulated by the Keap1/Nrf2/ARE pathway, has been reported to have neuroprotective effects against oxidative stress mainly through Nrf2 activation and the resulting upregulation of antioxidant cytoprotective proteins and elevation of GSH [21, 41, 51-55]. Therefore, sulforaphane may be beneficial to individuals with ASD through its potent activation of the Nrf2 cytoprotective pathway.

Potential Biomarkers of ASD

Nrf2 levels are substantially depressed in granulocytes of ASD children (45% of typically developing children) [56]. Given the critical role of Nrf2 as a regulator of redox homeostasis, determination of Nrf2 levels and the expression and activities of Nrf2-dependent enzymes, such as NAD(P)H: quinone oxidoreductase-1 (NQO1), aldo-keto reductase 1 (AKR1) and aldehyde dehydrogenase (ALDH), in peripheral blood of ASD individuals before and after drug treatment will be valuable biomarkers. Oxidative stress can be moni-

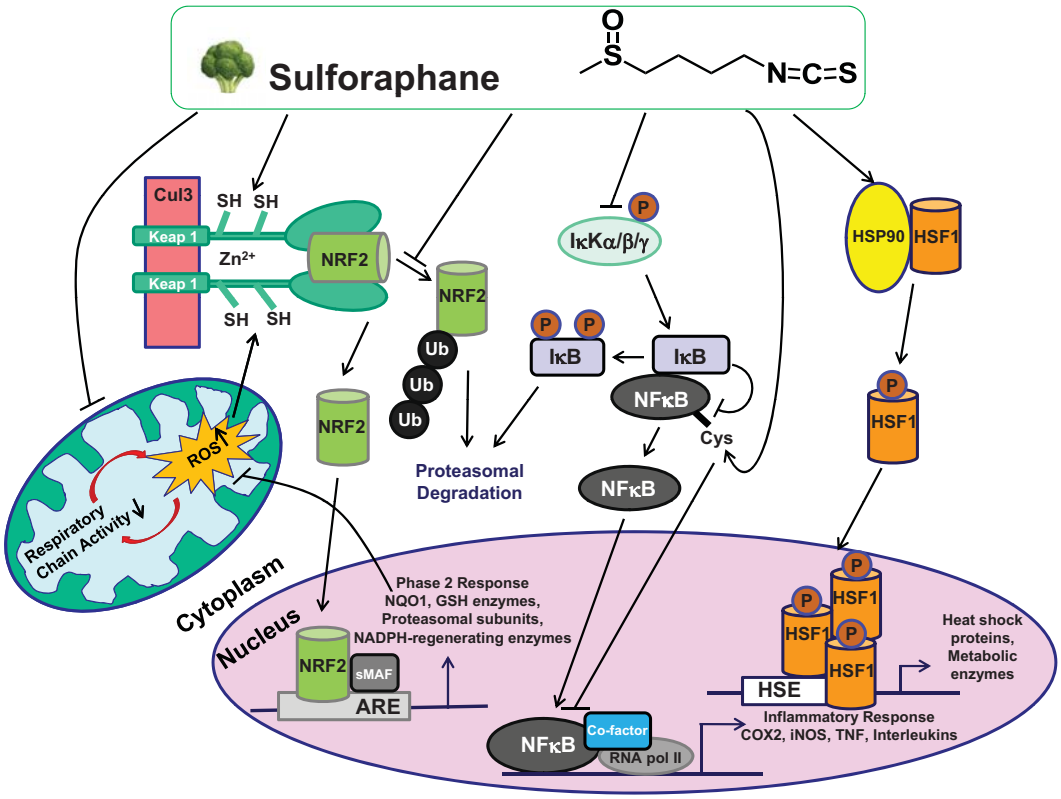

Fig. (2). Major signaling pathways for protective mechanisms against ASD by sulforaphane.

tored in plasma, PBMC, and urine by: (i) the status of direct antioxidants, e.g. levels of reduced and oxidized glutathione and their ratios; (ii) the status of antioxidant enzymes, such as glutathione peroxidase and superoxide dismutase (SOD); (iii) the generation of ROS determined by fluorescent probes; (iv) levels of F2t-isoprostanes, which are highly sensitive indicators of redox dysfunction; (v) levels of 3-chlorotyrosine (a measure of reactive nitrogen species and myeloperoxydase activity), and of 3-nitrotyrosine (a measure of chronic immune activation and oxidative protein damage); (vi) levels of 8-hydroxydeoxyguanosine (8-OHdG, a biomarker for DNA oxidation); and (vii) levels of malondialdehyde (MDA, an indicator of lipid peroxidation status) [4, 27, 37, 57, 58].

MITOCHONDRIAL DYSFUNCTION

Mitochondrial Dysfunction in ASD

Substantial evidence favors the view that impaired brain energy metabolism resulting from mitochondrial dysfunction may constitute a substantial part of the disease pathogenesis, at least in a subset of ASD individuals [7, 26, 27, 56, 59-61]. Mitochondria dysfunction has been demonstrated in animal models of ASD [62], in cell lines derived from children with ASD [63, 64], in lymphocytes and granulocytes of autistic children [56, 60], and in ASD brain samples [26, 65-67]. However, the reason for mitochondrial dysfunction in ASD is still unknown. The fact that 80% of the children with autism have lower than normal electron transport chain (ETC) activity in their lymphocytes whereas abnormalities in mitochondrial DNA are rare (less than 25%), indicates that in many ASD cases mitochondrial dysfunction may be acquired rather than inherited [7, 60].

Mitochondria are intracellular organelles that play a crucial role in oxidizing glucose and fatty acids to generate ATP, the energy carrier in most mammalian cells, through oxidative phosphorylation (OXPHOS). The OXPHOS is carried out by the ETC made up of Complexes I-V, which are located in the inner membrane of mitochondria, whereas the key energy-associated pathway, the tricarboxylic acid cycle, is located in the mitochondrial matrix. Mitochondrial dysfunction can be a downstream consequence of factors that have been proposed by Goldani and colleagues [4]. The mitochondrial ETC is both the source and target of free radicals, thus oxidative stress may impair mitochondrial function. Conversely, dysfunctional mitochondria can lead to further oxidative stress (Fig. **2**) [7, 27, 38, 56, 59, 59, 61]. Furthermore, increased levels of acyl-carnitines, the fatty acid conjugates that are imported into the mitochondria for fatty acid oxidation (FAO), were reported in autistic individuals, indicating less efficient mitochondrial FAO [68]. Deficiencies in FAO are likely to be one of the major contributors to the impaired mitochondrial phenotype. Defective mitochondrial FAO leads to accumulation of acyl-carnitines as their degradation to acetyl-CoA and subsequent entry into the tricarboxylic acid (TCA) cycle is reduced. Accumulation of these fatty acid conjugates is expected to be accompanied by depletion of free carnitine; and indeed the levels of carnitine are lower in autism [69].

Links to Nrf2

Emerging evidence suggests that Nrf2 plays a role in mitochondrial function and metabolism. In Nrf2-knockdown and Nrf2-knockout cells, mitochondrial membranes are depolarized, respiration is impaired, and oxygen consumption and ATP production are decreased, indicating compromised mitochondrial function [41]. Conversely, in cells with constitutive activation of Nrf2, the mitochondrial membrane potential, ATP levels, the rate of respiration, and the efficiency of oxidative phosphorylation are all increased [70, 71]. Glucose oxidation and substrate (oxaloacetate and acetyl-CoA) entry into TCA cycle are dramatically reduced in the absence of Nrf2 and increased upon constitutive activation of Nrf2 [72]. Nrf2 status also affects the mode of ATP production. When Nrf2 is absent, the contribution of glycolysis to ATP synthesis is profoundly enhanced, and that of oxidative phosphorylation is reduced [70]. In addition, mitochondrial FAO is accelerated under conditions of constitutive Nrf2 activation, whereas the degradation of both short- and long-chain fatty acids is less efficient in cells and mitochondria isolated from Nrf2-deficient mice [73]. Importantly, acyl-carnitine levels are reported to decrease in plasma and urine of healthy human subjects after intervention with diets rich in glucosinolates [74], suggesting that sulforaphane may improve the effiency of FAO, and thus protect mitochondrial function, most likely through activation of Nrf2.

Protective Effects of Sulforaphane

There is now an abundance of evidence for the mitochondria-protective effects of sulforaphane. Sulforaphane protected rat aortic smooth muscle cells from oxidative and eletrophilic cytotoxicity by inducing an elevation in enzymatic activity of cellular and mitochondrial SOD, catalase, glutathione S-transferase (GST), and mitochondrial GSH level [75]. In an *in vivo* approach, sulforaphane protected rat hearts against ischemic injury, partially through the activation of mitochondrial ATP-sensitive potassium channels [76]. Sulforaphane was also protective against cisplatin- and gentamicin-induced toxicity and loss of mitochondrial membrane potential in rat renal epithelial cells [77, 78]. Furthermore, sulforaphane inhibited tert-butyl hydroperoxide-mediated mitochondrial permeability transition pore opening in mitochondria isolated from rat brain and liver [79, 80]. Sulforaphane protected against 4-hydroxynonenal (4-HNE), a byproduct of lipid peroxidation, -induced mitochondrial dysfunction in mouse cerebral cortical mitochondria [81]. Brose and colleagues [82] demonstrated that sulforaphane induces mitochondrial biogenesis. Most recently sulforaphane was shown to improve mitochondrial function in a rodent hippocampal model, and to protect mice against electrically and chemically induced seizures [22].

Collectively, sulforaphane may protect mitochondrial function by regulation of mitochondrial redox through Nrf2 pathway, preservation of mitochondrial respiratory complex activity, oxygen consumption and bioenergetics, regulation of mitochondrial permeability transition (MPT) pore opening, and induction of mitochondrial biogenesis [83], and could thus benefit individuals with ASD.

Potential ASD Biomarkers

Among the biomarkers of mitochondrial function that may be related to ASD and that can be monitored are lactate, pyruvate and their ratios (evidence for increased glycosis) in plasma, free and total carnitine, mitochondrial membrane potential, ROS generation, activities of each of the respiratory chain complexes, and oxidative phosphorylation [4, 38, 56, 64].

IMMUNE DYSREGULATION/ NEUROINFLAMMATION

Immune Dysregulation and NO Accumulation in ASD

In individuals with ASD, inflammation and immune dysregulation have been observed both within the brain [26, 84-87] and in the periphery [10, 26, 88-96]. Evidence for disruption in immune regulation in ASD patients is supported by altered microglial cell activation, atypical proinflammatory cytokine production, increased expression of immune-related genes, and other biomarkers of inflammation [26, 97], all of them leading to chronic state of inflammation in the CNS and in the peripheral immune system. Nitric oxide (NO) levels in plasma are indicative of enhanced inflammatory processes, and are believed to be etiologically associated with a variety of neuropsychiatric disorders. NO levels have been found to be substantially (40-70%) elevated both in the plasma and in the red blood cells of autistic children [34, 98-100], and there was a positive correlation between NO and interferon-γ (IFN-γ) levels [100]. This suggests that high NO may be related to IFN-γ -mediated upregulation of inducible nitric oxide synthase (iNOS), which may contribute to the pathophysiology of autism because of the vulnerability of the developing brain to oxidative injury. The cholinergic receptors known to be sensitive to NO toxicity were decreased in the cortex of autistic patients [101]. Additionally, treatment with cholinergic agonists improved behavioral abnormalities in autism [102].

Protective Effects of Sulforaphane

Sulforaphane inhibits pro-inflammatory responses in various settings, including animal models of neuroinflammation [20, 103, 104]. The anti-inflammatory effects of sulforaphane are mediated at least in part through inhibition of the nuclear factor-κB (NF-κB) pathway, resulting in decreased expression of many proinflammatory factors, such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), and various interleukin cytokines (e.g., IL-1β and IL-6) [105-107]. The NF-κB proinflammatory pathway can be disrupted by sulforaphane through inhibition of IκB phosphorylation, thus blocking NF-κB translocation to the nucleus, or by directly interacting with cysteine residues in the DNA-binding region of the NF-κB transcription factor [104, 108, 109] (Fig. **2**). Emerging evidence suggests that Nrf2 may play an important role in regulating inflammation in the brain [110]. Although the relation between Nrf2 and NF-κB is not well characterized, the identification of NF-κB binding sites in the promoter region of the *nrf2* gene suggests cross-talk between these two regulators [111]. The NF-κB subunit p65 has been shown to act as a negative regulator of Nrf2 activation, and Keap1 is a negative regulator of NF-κB signaling [110]. In Nrf2-deficient mice, the inflammatory response in the brain and microglia activation is much more pronounced in response to lipopolysaccharide (LPS) when compared to normal animals. Critically, systemic administration of sulforaphane has been shown to attenuate microglia-induced inflammation in the hippocampus of mice that had been challenged with LPS, as evidenced by reduction of iNOS levels and production of the pro-inflammatory cytokines IL-6 and TNF-α [112]. Sulforaphane also inactivates the tautomerase activity of the proinflammatory cytokine macrophage inhibitory factor (MIF), which may reduce the proinflammatory effects of MIF by blocking its interaction with the CD74 receptor of macrophages [113, 114]. We recently demonstrated that systemic administration of sulforaphane in a rat model of traumatic spinal cord injury led to reduced levels of inflammatory cytokine mRNA in the injured spinal cord, and to inactivation of urinary MIF tautomerase activity, which supported the neuroprotective effects of sulforaphane in the spinal cord after injury [20]. It is thus possible that sulforaphane may be beneficial to ASD patients through its anti-inflammatory activity.

Potential ASD Biomarkers

Pro-inflammatory cytokines in plasma are major biomarkers for immune dysfunction; they include TNF-α, MIF, IL-6, and IL-1β. In PBMCs, the expression levels of COX-2 and iNOS can be readily measured. Concerted attention to a coordinate regulation of these markers may be highly instructive.

FEBRILE ILLNESS AND HEAT SHOCK RESPONSE (HSR)

Febrile Illness and HSR in ASD

Widespread anecdotal reports have suggested that fever can dramatically but temporarily ameliorate the disturbed behavior of many autistic patients. Notably, the degree of improvement (mostly in stereotypic behavior and inappropriate speech) was unrelated to the severity of fever or of autism [115]. Elucidation of the fever response might provide insight into the mechanisms of ASD and point to new therapeutic approaches [115, 116]. Although the mechanisms of the fever effects in ASD patients are unclear, they may be both direct and indirect. Fever activates heat shock response (HSR), and thus up-regulates heat shock proteins (HSPs), which are central to multiple cellular processes in the CNS, including synaptic transmission [117, 118], and may improve long-range cerebral cortical connectivity that is depressed in ASD [119].

The heat shock response is complex and evolutionarily conserved. It results in the transcriptional activation of genes encoding cytosolic molecular chaperones, proteases, and other proteins essential for protection and recovery from cellular damage. Under stress conditions, many heat shock proteins (Hsp) (e.g., Hsp70, Hsp27) bind and refold denatured proteins, preventing them from aggregating, and/or regulating their degradation. Some Hsps (e.g., Hsp90) play integral roles in signal transduction, immunity, and apoptosis. Thus, modulation of the HSR may be directly beneficial

for a variety of human diseases [120, 121]. Normally Hsp90 is bound to the transcription factor heat shock factor 1 (HSF1), and is biologically "dormant". Upon activation of the heat shock response by thermal stress, denatured proteins or small-molecule inducers, HSF1s dissociate from the Hsp90 complex and the resultant free HSF1s are phosphorylated and form trimers, which translocate into the nucleus and bind to heat shock elements (HSEs) of the target genes to induce a number of HSP genes that amplify defense capacity (Fig. **2**). Since inducible Hsp70 is usually not detectable under normal conditions and its expression is the most robust of the HSPs, it often is regarded as a diagnostic marker for stress [122]. Much evidence points to the neuroprotective role of HSPs, and the enhanced susceptibility of cells to damage when HSPs are depressed [123]. In the nervous system, Hsp70 overexpression has been reported to protect against insults such as heat shock and metabolic stresses through multiple mechanisms, such as its chaperone functions, anti-inflammation and anti-apoptosis [122, 124, 125].

Protective Effects of Sulforaphane

It is therefore of interest and probable importance that sulforaphane has been recently shown to have extensive effects on HSPs [82, 126] (Fig. **2**). It has been known for a long time that sulforaphane is a very efficient inducer of a classic HSP heme oxygenase 1 (HO-1 or Hsp32) [127]. More recently sulforaphane was shown to powerfully activate heat-shock transcription factor 1 (HSF1)-mediated heat-shock response in HeLa and COS1 cell lines, and to elevate proteasomal activity through upregulation of Hsp27 [126]. This is an additional, new and potentially important mechanism for the protective action of sulforaphane since proteasomal function plays a role in many types of cellular signal transduction and transcriptional regulation, and proteasomal dysfunction has been associated with many types of chronic diseases, especially those associated with aging [126]. Sulforaphane has also been reported to enhance the expression of Hsp70, Hsp90 and Hsp40 in normal human fibroblasts [82]. The upregulation of HSPs is considered a major cellular protective mechanism, and may therefore play a role in the observed clinical effects of sulforaphane on ASD [14].

There are a number of small molecules such as 4-phenylbutyrate, trichostatin, hydroxyurea, that, as well as sulforaphane, can provide benefits in a spectrum of genetic disorders including ASD [14, 82]. It was concluded that despite the belief that these agents had rather different primary modes of action, they were all concerned with activation of generalized stress responses, also known as adaptive cell survival responses, which have been evolutionarily conserved from archaea to eukaryotes [128]. Evidence is therefore mounting that activation of what has been called "the stress proteome" may involve at least two widely-studied cellular signaling pathways: regulation of the Keap1/Nrf2/ARE cytoprotective genes, and the heat shock protein/proteasomal pathway which protects against a wide variety of disturbances of cellular function. Furthermore, these two major cytoprotective mechanisms are interrelated. Similar to sulforaphane, many cysteine-reactive Nrf2 activators, including sulfoxythiocarbamate analogs of sulforaphane, also induce the HSR [129-131]. An interaction between Hsp90 and Keap1, the major negative regulator of Nrf2, has been reported to occur during heat shock, causing activation of Nrf2 [132]. Several cytoprotective proteins can be upregulated by both the Nrf2 pathway and the HSR, such as HO-1 [127, 133], Hsp70 [134, 135], proteasomal subunits [126, 136], and the autophagy cargo protein p62 [137-139]. Induction of p62 by the HSR may activate Nrf2, since p62 can displace Nrf2 from Keap1 [137, 140]. In addition, both Nrf2 pathway and the HSR affect the redox balance of the cell, and promote a more reduced environment [141]. The levels of glucose-6-phosphate dehydrogenase, which regenerates NADPH that is used by glutathione reductase to reduce GSSG to GSH, are increased by both activation of Nrf2 [45, 72] and upregulation of Hsp25/27 [142].

Potential ASD Biomarkers

Expression levels of HSF1 and HSPs, such as HSP70, HSP90, HSP40 and HSP27 can all be measured directly in PBMCs.

SYNAPTIC DYSFUNCTION

Synaptic Dysfunction in ASD

Synapses are critical points of information transfer between neurons. It is critical to the organism that their functionality be preserved during stressful conditions in order to prevent communication breakdown in the nervous system. Synaptic connections are recognized as being particularly vulnerable regions of neurons that are involved in the physiological process of neurotransmission that links neurons to functional networks [143]. Synaptic dysfunction caused by aberrant protein synthesis is believed to be a key pathogenic mechanism for ASD [144]. Many ASD brains exhibit both disrupted mTOR (mammalian target of rapamycin) signaling and synaptic defects during childhood and adolescence [145], suggesting that mTOR signaling may provide a common mechanism involved in ASD synaptic pathology [146].

Regulation of Synaptic Function

The phosphoinositide-3 kinase/protein kinase-B/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway plays central roles in synaptic protein synthesis [147], and its dysregulation results in many behavioral abnormalities related to ASD [148] (Fig. **3**). PI3K can be activated by multiple extracellular signals whose effects are transduced via receptors on the cell membrane. Activated PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) and to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which recruits 3-phosphoinositide dependent protein kinase 1 (PDK1) and Akt, and subsequently activates Akt. This process is negatively regulated by phosphatase and tensin homolog (PTEN), a lipid phosphatase which convert PIP3 back to PIP2. In the cytosol, activated Akt phosphorylates and inhibits tuberous sclerosis complex (TSC1/2), a negative regulatory complex of mTOR, which in turn promotes mTOR-mediated protein synthesis. Mutations in genes upstream of mTOR, such as those detected in TSC1/2, and PTEN, cause hyperactivity of the mTOR pathway, which can result in excess synaptic protein synthesis, thereby giving rise to abnormal synaptic function, and lead to ASD phenotypes

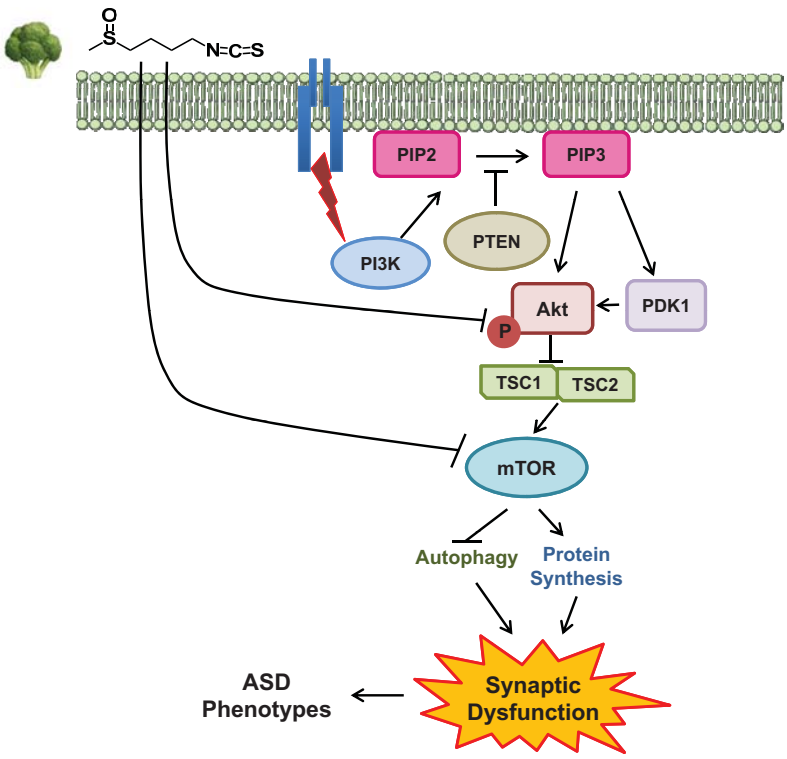

Fig. (3). Proposed role of mTOR signaling in ASD.

[149-151]. Signaling molecules in the downstream of the mTOR pathway have also been demonstrated to play crucial roles in ASD pathogenesis through stimulating cap-dependent translation initiation and elongation, thereby increasing protein synthesis [152, 153]. Synapses, however, must balance protein synthesis and degradation to maintain homeostasis and support plasticity [145, 154]. One of the two major protein degradation pathways is autophagy, a process downstream of mTOR signaling, which is directly linked to synaptic plasticity [155]. Autophagy deficiency in the postmortem brain, which is a consequence of mTOR overactivation, has been reported to strongly correlate with ASD dendritic spine pathology [145].

Protective Effects of Sulforaphane

Although there is no direct evidence that sulforaphane could protect synaptic function, sulforaphane has been reported to regulate PI3K/Akt/mTOR signaling pathway and autophagy in multiple cultured cell lines and in mice. The levels of phosphorylated Akt, mTOR and its downstream effectors have been commonly used as biomarkers of the PI3K/Akt/mTOR pathway, and levels of LC3-II, a biomarker that indicates the abundance of autophagosomes, is commonly considered an excellent indication for the activation of the autophagic pathway. In several breast cancer cell lines, sulforaphane decreased phosphorylation of Akt and an mTOR downstream effector, inhibited protein synthesis, and induced autophagy [156]. Sulforaphane also triggered the down-regulation of the Akt/mTOR pathway, and thus decreased excess protein synthesis in cardiomyocytes and prostate cancer cells [157, 158]. In neuronal cells, sulforaphane has been shown to induce autophagy through extracellular signal-regulated kinase [159]. More importantly, sulforaphane treatment upregulated LC3-II in mouse brain, which suggests enhanced autophagy [160]. These results suggest that sulforaphane might protect synaptic function by regulating mTOR signaling and autophagy, and thus benefit ASD patients. In addition, induction of HSR has been reported to protect the nervous system at the functional level and permits neurotransmission events to proceed at synaptic connections between neurons under stressful conditions [123, 161, 162]. Therefore, another mechanism for sulforaphane to protect synaptic function, may be through its upregulation of HSPs.

Potential ASD Biomarkers

The levels of Akt, mTOR, and their phosphorylated forms for mTOR signaling, and the levels of LC3-II for autophagy, are particularly promising biomarkers for monitoring synaptic function.

OTHER BIOMARKERS

In addition to the above reviewed physiological abnormalities, prenatal or postnatal environmental exposure to pro-oxidant factors such as mercury, lead, viruses, air pollutants, pesticides and other toxins has been associated with ASD risk [163] (Fig. **1**). Biomarkers for heavy metals, solvents, pesticides, polychlorinated biphenyls (PCBs), phthalates and polybrominated diphenyl ethers have been studied in the blood, urine, hair, brain or teeth of ASD patients. In several studies, biomarkers of environmental toxicants were associated with physiological abnormalities, which include depleted GSH levels, increased oxidative stress, impaired cellular signaling, dysregulated immune system and impaired

mitochondrial function, in some individuals with ASD [24, 163]. Activation of the cellular stress response is known to protect cells from environmental toxins. Recently, we reported that intervention with sulforaphane-rich broccoli sprouts enhances the detoxication of some airborne pollutants in a clinical trial in China [164]. Although the underlying mechanisms are unclear, the actions of sulforaphane on air pollutants are most likely through activation of the Nrf2 cytoprotective signaling pathway.

CONCLUSION

We have outlined several physiological abnormalities in ASD, and the associated signaling pathways, and we have highlighted the regulatory roles that sulforaphane (and likely other phytochemicals) may play. An interrelationship between oxidative stress, mitochondrial dysfunction, and/or immune dysregulation/inflammation has been reported in some individuals with ASD [8, 26], and there is clearly cross-talk between the related signaling pathways. The Keap1/Nrf2/ARE system appears to play a central role in connecting the anti-oxidative stress, anti-inflammatory, preserving mitochondrial function, and heat shock response systems. Notably, the Nrf2-mediated defense system should be of particular importance for post-mitotic cells, such as neurons, because of their limited regenerative ability. Although it is unclear whether those physiological abnormalities are etiological or secondary manifestations, they are likely to contribute significantly to the behavioral symptoms intrinsic in ASD, and some studies have suggested that treatments that address these anomalies, such as oxidative stress and mitochondrial dysfunction, may improve core and associated symptoms of ASD [3]. Unfortunately, many of these studies did not use related biomarkers to monitor the status of the abnormalities or the effects of the treatments.

A consensus panel at the National Institutes of Health defines biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [165]. Therefore, the use of potential biomarkers that indicate specific mechanisms would lead to a better understanding of ASD pathophysiology, help to identify meaningful subtypes of autism, and finally tailor treatment or prevention strategies. It is important to keep in mind the characteristics of an ideal biomarker. A biomarker should be quantifiable, and the assay method should be accurate and reproducible; samples should be readily obtainable in quantities needed for measurement, and sampling should be minimally invasive. In addition, a biomarker should be responsive to changes in disease activity, and sensitive to effective intervention. The search for biomarkers for ASD has been intense, and a wide range of autism biomarkers has been proposed, but none has yet demonstrated enough sensitivity and specificity to be translated into clinic [4, 166]. Although brain biopsy is highly desirable, biomarkers in peripheral blood or urine, which are discussed in this article, appear to have practical advantages because they are better standardized, less- or non-invasive, cost effective, and readily available in clinical settings. A vast variety of peripheral blood biomarkers based on pathophysiologic processes have been evaluated in ASD patients. For instance, Napoli and colleagues showed the statistically significant difference of several markers for mitochondrial function and oxidative stress in granulocytes of children with autism comparing to that of typically developing children, such as the oxidative phosphorylation capacity of granulocytes was 3-fold lower, and the gene expression level of Nrf2 was 55% lower in autistic children [56]. However, singly, none of these markers is specific or sensitive enough for diagnosis or for tracking response to therapy. Given the clinical complexity of ASD, therefore, approaches combining panels of existing biomarkers related to the physiological abnormalities show promise for discovering biomarker profiles that are characteristic of ASD, and further providing guidance for the selection and efficacy of biomedical interventions.

Multiple metabolic systems associated with ASD are influenced by sulforaphane and a variety of small molecules with similar activities. The capacity of these molecules to activate the Keap1/Nrf2/ARE cytoprotective signaling pathway is especially strong, suggesting that they may therefore protect against both environmental and endogenous risk factors that affect brain development in ASD [14, 167], and provide potential benefits for the prenatal prevention of ASD as well as for the early treatment of young children with this disorder. With the guidance from combination of biomarkers discussed in this article, translation of these potential interventions to improve the health outcomes of ASD patients will be promising.

LIST OF ABBREVIATIONS

AKT = Protein Kinase B

ARE = Antioxidant Response Element

ASD = Autism Spectrum Disorder

CNS = Central Nervous System

ETC = Electron Transport Chain

FAO = Fatty Acid Oxidation

GSH = Reduced Glutathione

GSSG = Oxidized Glutathione

HSF1 = Heat Shock Factor 1

HSP = Heat Shock Protein

HSR = Heat Shock Response

IL-6 = Interlukin-6

iNOS = Inducible Nitric Oxide Synthase

Keap1 = Kelch-like ECH-Associated Protein 1

LC3 = Microtubule-Associated Protein 1 Light Chain 3

MIF = Macrophage Inhibitory Factor

mTOR = Mammalian Target of Rapamycin

NF-κB = Nuclear Factor Kappa B

NO = Nitric Oxide

Nrf2 = Nuclear Factor-Erythroid 2-Related Factor 2

PBMC = Peripheral Blood Mononuclear Cell

PI3K = Phosphoinositide-3 Kinase

RNS = Reactive Nitrogen Species

ROS = Reactive Oxygen Species

TNF-α = Tumor Necrosis Factor-α

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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