# Altered Sulfide ($H_2S$) Metabolism in Ethylmalonic Encephalopathy

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Hydrogen sulfide (sulfide, $H_2S$) is a colorless, water-soluble gas with a typical smell of rotten eggs. In the past, it has been investigated for its role as a potent toxic gas emanating from sewers and swamps or as a by-product of industrial processes. At high concentrations, $H_2S$ is a powerful inhibitor of cytochrome *c* oxidase; in trace amounts, it is an important signaling molecule, like nitric oxide (NO) and carbon monoxide (CO), together termed "gasotransmitters." This review will cover the physiological role and the pathogenic effects of $H_2S$, focusing on ethylmalonic encephalopathy, a human mitochondrial disorder caused by genetic abnormalities of sulfide metabolism. We will also discuss the options that are now conceivable for preventing genetically driven chronic $H_2S$ toxicity, taking into account that a complete understanding of the physiopathology of $H_2S$ has still to be achieved.

Because of its fame as a deadly gas, the physiological role of $H_2S$ had been long overlooked, until Abe and Kimura in 1996 (Abe and Kimura 1996) demonstrated that $H_2S$ is a neuromodulator that facilitates the induction of long-term potentiation (LTP) by enhancing the activity of *N*-methyl-D-aspartate (NMDA) receptors in hippocampal pyramidal neurons. $H_2S$ was later shown to induce relaxation of smooth muscle, to regulate the release of insulin, and to act as a mediator of inflammation.

The physiological importance of $H_2S$ has further been supported by demonstrating that $H_2S$ is endogenously produced in mammals by two pyridoxal-5′-phosphate-dependent enzymes, cystathionine β-synthase (CBS, EC 4.2.1.22) and cystathionine γ-lyase (CSE, EC 4.4.1.1), which both utilize L-cysteine as a substrate (Stipanuk and Beck 1982; Hosoki et al. 1997; Zhao et al. 2001). L-cysteine is taken up with the diet, extracted from endogenous proteins, or synthesized endogenously via *trans*-sulfuration of serine by L-methionine. In humans, the end products of cysteine sulfur catabolism are sulfate (which accounts for 77%–92% of the total sulfur excreted in the urine), sulfate esters (7%–9%), and taurine (2%–6%) (Stipanuk 2004) (Fig. 1). In some tissues, both CBS and CSE can generate $H_2S$, whereas in others only one enzyme is present (Diwakar and Ravindranath 2007). For instance, CBS is predominantly expressed in the nervous system but

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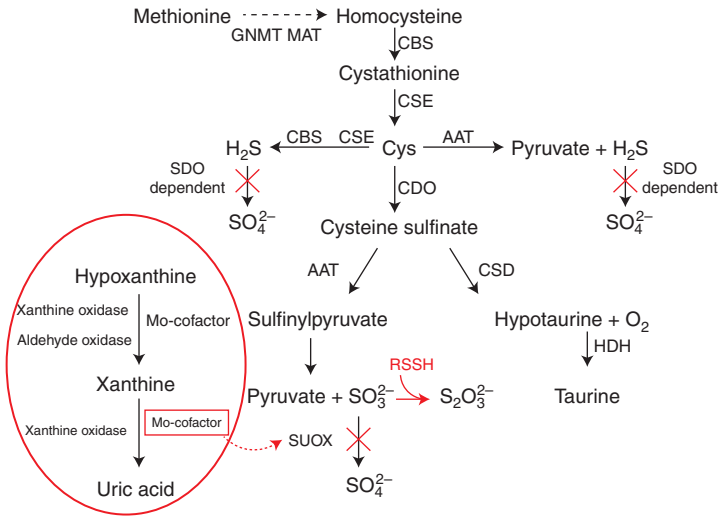


**Figure 1.** Sulfide production. Methionine, derived from alimentary sources, is converted into *S*-adenosylmethionine by methionine adenosyltransferase (MAT). *S*-adenosylmethionine is hydrolyzed to homocysteine by glycine *N*-methyltransferase (GNMT). Cystathionine β-synthase (CBS) transfers serine to homocysteine and produces cystathionine. Cystathionine γ-lyase (CSE) converts cystathionine to cysteine (Cys). One pathway of cysteine metabolism involves its oxidation to cysteine sulfinate by cysteine deoxygenase (CDO), which is then converted to hypotaurine by cysteine sulfinate decarboxylase (CSD); hypotaurine is eventually oxidated to taurine by hypotaurine dehydrogenase (HDH). In mitochondria, cysteine can be converted to 3-mercaptopyruvate by aspartate aminotransferase (AAT), which can then be converted to $H_2S$ by 3-mercaptopyruvate sulfur transferase. The conversion of cysteine sulfinate to sulfinyl pyruvate by AAT, followed by a nonenzymatic reaction, can also yield sulfite, which is then oxidized to sulfate by sulfite oxidase (SUOX) in a glutathione (GSH)-dependent process. The $H_2S$ produced by either AAT or CBS/CSE can be further oxidized by a sulfur dioxygenase (SDO/ETHE1)-dependent pathway (see Fig. 2). The metabolic pathway carried out by Moco-dependent enzymes is encircled in red (see text for details).

is also active in the liver and kidney (Enokido et al. 2005). CSE is mainly expressed in liver, as well as vascular and nonvascular smooth muscle (e.g., the small intestine and stomach [Awata et al. 1995; Ishii et al. 2004]).

Both CBS and CSE activities increase in rat brain after birth, reaching adult levels at week four (Awata et al. 1995).

An important additional source of $H_2S$ is the enterobacterial anaerobic flora. The intestinal epithelium expresses specialized enzyme systems that efficiently convert $H_2S$ into thiosulfate and sulfate, to both prevent the local increase of $H_2S$ to toxic levels and its entry through the portal vein system in the liver and other organs. Excessive production and absorption of $H_2S$, as well as reduced detoxification by colonic epithelial cells (colonocytes), is thought to play an important role in the mucosal damage of ulcerative colitis (Rowan et al. 2009).

A third, however marginal, source of $H_2S$ is the nonenzymatic reduction of elemental sulfur, which is present in traces in human (Westley and Westley 1991) and mouse (Buzaleh et al. 1991) blood, into $H_2S$ by electrons provided through glycolysis (Searcy and Lee 1998).

The main catabolic pathway of $H_2S$ takes place in mitochondria and consists of a series of oxidative reactions ultimately yielding sulfate ($SO_4^{2-}$), with thiosulfate ($S_2O_3^{2-}$) and sulfite ($SO_3^{2-}$) as intermediate compounds (Beauchamp et al. 1984; Szabò 2007). The existence of such a pathway has been known for more than 20 years (Powell and Somero 1986), although the enzymatic actors and detailed steps have been elucidated (Hildebrandt and Grieshaber

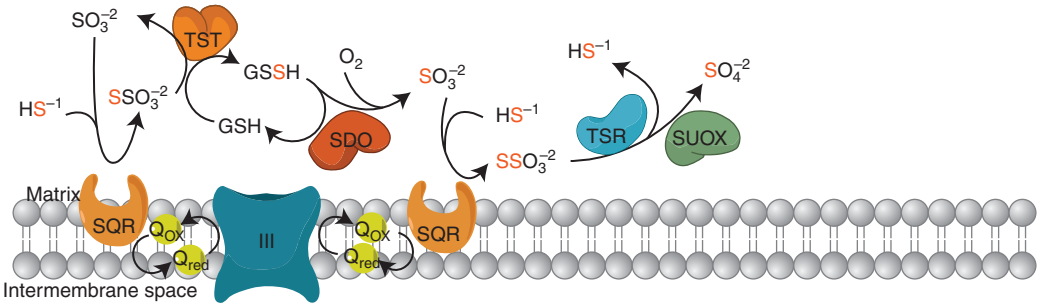


**Figure 2.** Proposed pathway for sulfide oxidation. $H_2S$ is produced from cysteine (see Fig. 1) and is initially fixed by a membrane-bound sulfide quinone oxidoreductase (SQR). The electrons extracted from $H_2S$ enter the respiratory chain at the level of the quinone pool (UQH/UQ), are then transferred to complex III (cIII), and are finally fixed by cytochrome *c* oxidase (complex IV, cIV) to molecular oxygen with the formation of water. A sulfur dioxygenase (SDO/ETHE1), present as an Fe-binding dimer in the mitochondrial matrix, oxidizes the persulfide to sulfite ($H_2SO_3$) in a reaction that includes molecular oxygen and water. See text for details. SUOX, sulfite oxidase; TST, thiosulfate sulfur transferase; TSR, thiosulfate reductase.



2008) and further refined (Jackson et al. 2012) only recently. The pathway proposed by Hildebrandt (2008), later modified by Jackson, is shown in Figure 2. The pathway consists of (1) a mitochondrial inner-membrane-bound sulfide quinone reductase (SQR) (Theissen and Martin 2008), which fixes $H_2S$ to sulfite to produce thiosulfate; (2) mitochondrial thiosulfate sulfur transferase, also known as rhodanese, which reconstitutes sulphite from thiosulfate by fixating the sulfane sulfur of the latter onto an –SH-containing substrate, for instance glutathione, GSH, to form a persulfide (R-S-SH) species; (3) a mitochondrial matrix sulfur dioxygenase (SDO), which oxidizes the sulfur atom extracted from persulfide, converting it again into sulfite ($SO_3^{2-}$); and (4) mitochondrial sulfite oxidase, which further oxidizes sulfite into sulfate ($SO_4^{2-}$). Thiosulfate can also be converted into two molecules of sulfate by the combined action of a thiosulfate reductase (Müller et al. 2004) and sulfite oxidase (Feng et al. 2007). Additional mechanisms of detoxification rely on the fixation of $H_2S$ to methemoglobin, which is formed from hemoglobin by oxidation of the Fe center, for instance by sodium nitrate, to produce sulfo-methemoglobin, or on the administration of oxidized glutathione (GSSG) (Beauchamp et al. 1984), which is able to fixate $H_2S$ to produce glutathione persufide (GSSH), thus preventing the interaction of $H_2S$ with critical enzymatic activities. Additional sources of sulfite are known, for instance by the cysteine dioxygenase/aspartate aminotransferase (CDO/AAT) system (Cooper et al. 2002; Stipanuk and Ueki 2010), converting cysteine into β-sulfinylpyruvate, which then decomposes into pyruvate and sulfite.

## $H_2S$: PHYSICAL PROPERTIES

Sulfur, an essential element for life, occurs in different oxidation states, from –2 (as in sulfide, $S^{2-}$) to +6 (as in sulfate, $SO_4^{2-}$). Sulfur is present in living organisms as a constituent of proteins (mainly in sulfurated amino acids such as cysteine and methionine), in coenzymes (for instance CoA, biotine, and thiamine), and as a component of iron-sulfur clusters in metalloproteins. From a physical–chemical standpoint, $H_2S$ is the sulfur analog of water, but because its intermolecular bonds are weaker than $H_2O$, at room temperature and atmospheric pressure, it is a gas. In mammals, at a physiological pH of 7.4, approximately one-third of $H_2S$ exists as the undissociated form and two-thirds as the hydrosulfide anion (HS–) (Reiffenstein et al. 1992). In the undissociated form, $H_2S$ can easily penetrate the plasma membranes of cells because of its high solubility in lipids.

## PHYSIOLOGICAL ACTIONS OF $H_2S$

A preliminary question, however crucial for understanding the pathophysiology of sulfide metabolism, is how much free $H_2S$ is present in tissues and body fluids of mammalian organisms. Free $H_2S$ is difficult to measure, mainly because of its high volatility and the interference of cysteine residues contained in proteins, which hamper the results of the most common analytical methods based on spectrophotometric assays from tissue extracts. Levels as high as 30–150 μM have been reported in different tissues in normal conditions (Goodwin et al. 1989; Mitchell et al. 1993; Ogasawara et al. 1994; Wang 2002; Fiorucci et al. 2006;), a remarkable overestimation since these values are well above the olfactory threshold for $H_2S$ and are clearly associated with severe toxicity in vivo, as shown in both cells and mammalian organisms. Using a gas-chromatographic assay that avoids interference by cysteines, Furne et al. (2008) have recently shown that the concentration of free sulfide in mouse liver and brain is 15 nM, that is $10^{-3} - 10^{-4}$ less than the values obtained by spectrophotometric assays.

Recent studies have demonstrated for $H_2S$ a multiplicity of physiological functions, including up-regulation of antioxidant systems and amplification of the effects produced by known antioxidants such as *N*-acetylcysteine (NAC) and glutathione (Whiteman 2004; Yan 2006); up-regulation of anti-inflammatory and cytoprotective genes including heme oxygenase (HO1, also known as HMOX1) in pulmonary smooth-muscle cells (Qingyou et al. 2004) and macrophages (Oh et al. 2006). In the CNS, low concentrations of $H_2S$ are sufficient to increase the production of cAMP, enhance NMDA-receptor-mediated responses, and facilitate the induction of long-term potentiation in hippocampal neurons (Kimura and Kimura 2004).

$H_2S$ displays a number of different effects on myocardial cells and vascular smooth muscle. By activating $K_{ATP}$ channels, $H_2S$ increases the membrane potential of vascular smooth-muscle cells that become hyperpolarized, therefore causing vasorelaxation (Zhao et al. 2001; Elsey et al. 2010). Accordingly, hypertension is a consistent feature of CSE knockout mice, confirming that $H_2S$ plays a role as a smooth-muscle relaxant and suggesting that it may physiologically regulate blood pressure (Yang et al. 2008).

This effect could, however, be mediated by a quenching action of $H_2S$ on nitric oxide (NO), possibly via the formation of a nitrothiol intermediate (Whiteman et al. 2006). A counteracting action by $H_2S$ on vasodilation induced by NO could explain why low concentrations of $H_2S$ (10–100 μM) induce vasoconstriction, whereas concentrations over 100 μM induce vasorelaxation (Ali et al. 2006). A second mechanism of interaction between $H_2S$ and NO may involve transcriptional regulation of CSE, which is induced by NO (Zhao et al. 2001). It is thus possible that the vasorelaxant effect of $H_2S$ may be mediated, at least in part, by a local regulatory effect on the NO concentration.

$H_2S$ induces the proliferation of smooth-muscle cells by activating the PI3 Kinase/Akt pathway, thus stimulating angiogenesis (Cai et al. 2007) and apoptosis by activating the mitogen-activated protein kinase (MAPK) pathway (Yang et al. 2004).

Finally, $H_2S$ can directly modulate the function of the heart, with negative inotropic and chronotropic effects (Ferdinandy et al. 2007), which are mediated by activation of $K_{ATP}$ channels. This mechanism results in the hyperpolarization of the membrane potential, closure of L-type calcium channels, and consequent reduction of contraction strength dependent on calcium-activated calcium release. In addition, the activation of $K_{ATP}$ channels depresses the activity of the sinoatrial pacemaker, thus reducing the heart rate (Xu et al. 2008).

The several effects that $H_2S$ exerts on the cardiovascular system suggest that it may have a protective role during ischemia/reperfusion injury. During ischemia, cells are in hypoxic conditions, and subsequent reperfusion and reoxygenation lead to inflammation and cell death by increasing reactive oxygen species (ROS) production, increasing mitochondrial $Ca^{2+}$ overload, and opening the mitochondrial permeability transition pore (mPTP) (Ferdinandy et al. 2007). In experimental ischemia/

reperfusion tests, the treatment with $H_2S$ is protective, either before the induction of ischemia (preconditioning) or before the reperfusion phase (postconditioning). These effects are mediated by activation of the cardioprotective PI3K/Akt pathway, activation of $K_{ATP}$ channels, and increase of the free thiol pool, which acts as ROS scavenger, thus inhibiting the opening of mPTP. The protective effects of $H_2S$ in ischemia/reperfusion experiments have been further confirmed in a mouse model characterized by a twofold increase of $H_2S$ in the myocardium due to transgenic overexpression of CSE (Elrod et al. 2007).

## TOXIC EFFECTS OF $H_2S$

Whereas at physiological (nanomolar) concentrations, Although $H_2S$ acts as a cytoprotective agent, at micromolar concentrations, it can interfere with a variety of cellular functions, including mitochondrial respiration, via inhibition of cytochrome *c* oxidase (COX, EC 1.9.3.1) (Hill et al. 1984). COX activity is inhibited by the formation of a covalent bond between $H_2S$ and the Fe atom coordinated by heme a, which is exquisitely sensitive to the toxic action of $H_2S$ (and cyanide as well). The chronic exposure and binding of $H_2S$ to COX causes accelerated degradation of its protein subunits (Di Meo et al. 2011), thus reducing the amount of fully assembled and functionally active enzyme.

$H_2S$ is also an inhibitor of carbonic anhydrase (EC 4.2.1.1) (Coleman 1967), an effect that could explain the alterations of ventilatory dynamics in response to inhaled $H_2S$ due to changes in the reactivity and distribution of intrapulmonary $CO_2$ receptors (Klentz and Fedde 1978).

A further effect of $H_2S$ is inhibition of enzymatic activity of short-chain acylCoA dehydrogenase (SCAD), which can cause dicarboxylic aciduria (Pedersen et al. 2003).

Acute exposure to gaseous $H_2S$ causes anoxic brain injury, pulmonary edema, and death (Yalamanchili and Smith 2008). Neurological impairment is consistent with prolonged brain hypoxia, whereas respiratory insufficiency and pulmonary edema are consistent with acute respiratory syndrome. These two injuries are the most likely cause of death after acute exposure to $H_2S$, whereas chronic exposure to low levels determines irritant effects including upper airway inflammation, conjunctivitis, and wheezing. A direct effect of $H_2S$ on the respiratory center in the brainstem, resulting in apnea, is likely to be caused by inhibition of COX activity (Yalamanchili and Smith 2008).

## $H_2S$ IN ETHYLMALONIC ENCEPHALOPATHY (EE)

Ethylmalonic encephalopathy (EE) (Online Mendelian Inheritance in Man [OMIM] #602473) (Burlina et al. 1991) is a severe mitochondrial disease of early infancy, clinically characterized by a combination of progressive encephalopathy, vascular lesions determining bouts of petechial purpura and orthostatic acrocyanosis (Fig. 3A), and chronic hemorrhagic diarrhea. Neurological symptoms include psychomotor regression, spastic tetraparesis, axial hypotonia, dystonia, and seizures, reflecting the presence of patchy, bilateral lesions in the basal nuclei and brainstem gray matter (Fig. 3B). The onset is in the first months after birth; children usually die within the first decade.

Biochemically, EE is characterized by the unusual combination of severe deficiency of COX, the terminal component of the mitochondrial respiratory chain, in muscle (Fig. 3C) and brain, leading to high levels of lactate in blood, and accumulation of ethylmalonate (ethylmalonic acid, EMA) in urines (Fig. 3D). EMA, a dicarboxylic organic acid produced by the carboxylation of butyrate, is a hallmark of enzymatic defects of β-oxidation of fatty acids and branched-chain amino acids, for instance defects of the short-chain and branched-chain acylCoA dehydrogenase (SCAD, BCAD) (Burlina et al. 1991; Gregersen et al. 2000; Korman 2006). Like in SCAD and BCAD deficiency, accumulation of C4- and C5-acylcarnitines has been documented in blood of EE patients. However, SCAD and BCAD activities are normal in EE fibroblasts (Nowaczyk et al. 1998) and muscle, therefore excluding them from being the primary abnormality in this disease. For still

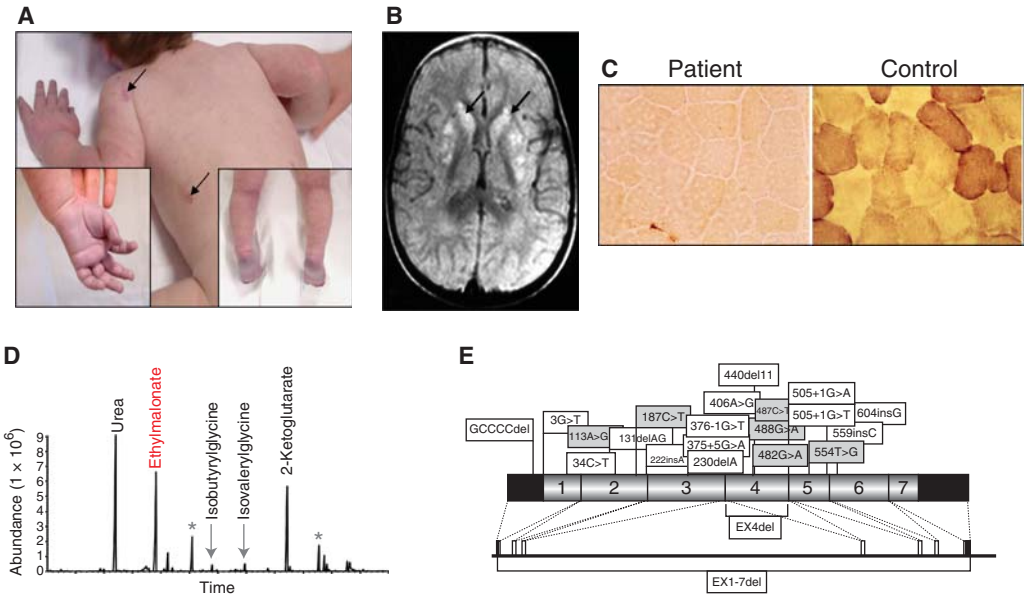


**Figure 3.** Ethylmalonic encephalopathy: clinical and molecular features. (*A*) Skin areas with petechial hemorrhages are indicated by arrows. The *insets* show the acrocyanosis. (*B*) T2-weighted MRI images of a transverse section of the brain: symmetrical, patchy, high-intensity signals are present in the head of nucleus caudatus and putamen (arrows). (*C*) COX activity is virtually absent in skeletal muscle of an EE patient as compared to normal reactivity present in control. (*D*) Gas-chromatographic profile of urinary organic acids in EE. The abnormal peak of ethylmalonic acid (EMA) is indicated in red. Asterisks indicate internal standards. (*E*) ETHE1 mutations identified in our patients. Missense (gray) and nonsense (white) mutations are indicated along the schematic representation of the ETHE1 cDNA subdivided in the corresponding exons (indicated by numbers). The black areas represent the 5′ and 3′ UTRs. The genomic organization is shown below the cDNA.

unexplained reasons, in virtually all cases, the origin of these EE families is from or could be traced to different regions of the Mediterranean basin or the Arabic peninsula.

In 2004, we demonstrated the presence of several pathogenic mutations in a gene dubbed *ETHE1* for EE 1 (Tiranti et al. 2004). We have reported a series of 29 patients, presenting a fairly homogeneous clinical and biochemical presentation, in spite of a wide spectrum of *ETHE1* mutations including missense, nonsense, frame-shift and deletion of single exons or of the entire gene (Tiranti et al. 2004, 2006) (Fig. 3E, shows only part of the mutations identified). Mutations in the *ETHE1* gene have been found in 32 patients affected by EE worldwide (Di Rocco et al. 2006; Merinero et al. 2006; Zafeiriou et al. 2007; Mineri et al. 2008). A total of 18 nonsense (Tiranti et al. 2004, 2006; Merinero et al. 2006) and nine missense (Tiranti et al. 2004, 2006; Di Rocco et al. 2006; Zafeiriou et al. 2007) mutations has so far been described. To date, a total of 70 mutant patients have been identified in our laboratory (V Tiranti and M Zeviani, unpubl.). Western-blot analysis of the Ethe1 protein (Ethe1p) indicated that some of the missense mutations are associated with the presence of the protein, suggesting that the corresponding wild-type amino acid residues have a catalytic function (Tiranti et al. 2006). In fact, a 3D model of the Ethe1p makes it possible to analyze the spatial location of the amino acid changes predicted by missense mutations and to classify the mutation as catalytic versus structural (Tiranti et al. 2006). More recently, the availability of the crystal structure of the Ethe1p from *Arabidopsis thaliana* (Holdorf et al. 2008) allowed us to demonstrate that the predicted 3D

model of human Ethe1p was a faithful in silico reconstruction of the presumed real structure of the native protein (Mineri et al. 2008).

The Ethe1p, a 30 kDa polypeptide located in the mitochondrial matrix, functions in vivo as a homodimeric, Fe-containing sulfur dioxygenase (SDO) activity (Tiranti et al. 2009), which we have already mentioned as one of the reactions involved in the catabolic oxidation of sulfide to sulfate.

Impaired activity of ETHE1-SDO leads to the accumulation of $H_2S$ in critical tissues, including colonic mucosa, liver, muscle, and brain, up to concentrations that inhibit SCAD and COX activities, therefore accounting for EMA aciduria and high levels of C4 and C5 acylcarnitines, EMA, and lactate in plasma (Tiranti et al. 2009). Chronic COX inhibition by accumulated $H_2S$ leads to accelerated degradation of the COX protein backbone (Di Meo et al. 2011). Although ETHE1 activity is measurable in all organs, muscle and brain of *Ethe1*-less (*Ethe1*$^{-/-}$) mice show both defective COX activity and reduced COX amount, whereas liver does not. This may reflect the presence of alternative and highly effective metabolic pathways protecting hepatocytes from $H_2S$ toxicity.

In addition to $H_2S$, which is a highly unstable, difficult-to-measure gas, its stable derivative thiosulfate is also present at very high concentrations in tissues and body fluids of *Ethe1*$^{-/-}$ mice and EE patients, and can well be considered a specific biomarker of the disease. The pathway outlined in Figure 1 explains these findings: $H_2S$ is converted into thiosulfate by the SQR activity. In normal conditions, rhodanese-TST catalyzes the transfer of the sulfane sulfur from thiosulfate to GSH (or other R-SH substrate) to form persulfide, which is the substrate of the Ethe1-SDO activity, but since the latter is missing, the disposal of the sulfane sulfur is also blocked and consequently both thiosulfate and sulfide accumulate.

We have demonstrated that the ablation of *Ethe1* restricted to muscle or brain is clearly associated with an isolated COX deficiency in the targeted tissue but not in other, *Ethe1*-competent, tissues (Di Meo et al. 2011). This data unequivocally demonstrates that failure to neutralize the endogenous production of $H_2S$ is sufficient for COX activity to decrease, but not for the animals to become sick or for thiosulfate to increase. This observation suggests that multiorgan accumulation of $H_2S$ and diffusion of exogenously released $H_2S$ from the bacterial flora are both needed to determine the severe metabolic impairment and the fatal clinical course of *Ethe1*-less mice and humans.

Besides COX and SCAD deficiency, other symptoms of EE are explained by accumulation of $H_2S$, including damage of endothelial cells and vasodilation, which account for the petechiae and the acrocyanosis. Since a substantial amount of $H_2S$ derives from the anaerobic bacterial flora residing in the intestinal lumen, COX activity is markedly reduced in the luminal colonocytes of *Ethe1*$^{-/-}$ mice, whereas it is normal in cryptal colonocytes that are relatively secluded from the luminal surface. This difference is likely to reflect different exposure of the two cell populations to the inhibitory action of exogenous $H_2S$. Excessive production and absorption of $H_2S$, as well as reduced detoxification by colonocytes, is regarded to play an important role in the mucosal damage of ulcerative colitis. A similar mechanism can well account for the severe chronic diarrhea afflicting EE patients.

## THERAPEUTIC APPROACHES IN EE

In principle, $H_2S$ production from the anaerobes of the large intestine can be attenuated by reducing the bacterial contingent, and the excess of free $H_2S$ can be buffered by suitable compounds in critical tissues. For instance, metronidazole is a drug widely used to combat anaerobic infections by reducing the bacterial load in the large intestine (Kang et al. 2008). Metronidazole is a nitroimidazole anti-infective compound that is in fact a pro-drug, being activated in anaerobic organisms by the redox enzyme pyruvate-ferredoxin oxidoreductase. Metronidazole is selectively taken up by anaerobic bacteria and sensitive protozoal organisms (Samuelson 1999) because of the unique ability of these organisms to reduce uptaken metronidazole to its active form within their cell bodies. The nitro group of metronidazole is chemically

reduced by ferredoxin (or a ferredoxin-linked metabolic process), and the products are responsible for disrupting the DNA helical structure, thus inhibiting nucleic-acid synthesis (Eisenstein and Schaechter 2007). NAC is a cell-permeable precursor of glutathione (GSH), an abundant compound in mitochondria (Marí et al. 2009), where it acts as one of the physiological acceptors of the sulfur atom of $H_2S$ operated by SQR. This reaction leads to the formation of GSSH persulfide, which acts in turn as a substrate for the Ethe1-SDO. If Ethe1-SDO activity is missing, GSH can buffer, at least in part, accumulating $H_2S$ by forming GSSH, which unlike free $H_2S$, is a nontoxic compound (Atkuri et al. 2007).

Combined exposure to metronidazole and NAC has been effective in prolonging the survival of *Ethe1*$^{-/-}$ mice and in improving the main symptoms in a pilot study on EE patients (Viscomi et al. 2010), including marked attenuation or disappearance of the vascular lesions and diarrhea, as well as amelioration of some neurological abnormalities. These encouraging results suggest that the invariably fatal clinical course of EE could be modified by a pharmacological protocol based on the off-label use of low-cost, relatively safe drugs.

More recently, Hildebrandt (2011) demonstrated that sulfide toxicity can be modified by the addition of dehydroascorbic acid (DHA) in rat mitochondria. DHA significantly reduced the inhibitory effect of sulfide on COX, resulting in higher rates of respiration and sulfide oxidation. For this purpose, DHA is more suitable than ascorbic acid, the main functionally active form of vitamin C, because its concentration is lower and more flexible. The fraction of oxidized vitamin C reflects the redox state of the cell or organelle and can therefore induce a specific modification of sulfide metabolism. This observation has important clinical implications because DHA could be used during the treatment of sulfide poisoning either after accidental environmental exposure or in patients with EE.

Additional useful information, potentially relevant in the treatment of EE, was recently obtained by Palmfeldt and colleagues (2011), who performed a proteomics study on mitochondria derived from cultured patient fibroblast cells and compared it with healthy controls. Sulfide:quinone oxidoreductase (SQRDL), which takes part in the same sulfide pathway as ETHE1, was also underrepresented in EE patients. Two proteins, the mitochondrial superoxide dismutase (SOD2) and aldehyde dehydrogenase X (ALDH1B) involved in pathways of detoxification and oxidative/reductive stress, were down-regulated in EE. These authors propose that redox perturbation could be an additional factor in the molecular mechanism of EE and an additional therapeutic target.

Another approach to scavenge circulating sulfide coming from the gut could be restoration of the ETHE1-SDO activity in liver of constitutive *Ethe1*$^{-/-}$ individuals by using adeno-associated virus (AAV)-based gene replacement. Because liver is the main checkpoint organ against toxic compounds from the gut, AAV-mediated liver-specific ETHE1 expression may be beneficial to *Ethe1*$^{-/-}$ organisms. This very strategy has recently been successful in markedly prolonging the survival of constitutive *Ethe1*$^{-/-}$ mice (Di Meo et al. 2012).

A complementary strategy, based on the same rationale, is bone marrow transplantation (BMT). BMT has emerged as a major therapeutic option in the treatment of malignant diseases and has also become the treatment of choice for a number of nonmalignant disorders. As for liver-targeted, AAV-based gene replacement, the rationale of BMT-based therapy in the context of EE is to provide a substantial contingent of Ethe1-SDO proficient cells able to clear the excess of $H_2S$ from plasma and other body fluids. A similar strategy has recently been proposed for mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), due to loss of activity of thymidine phosphorylase (TP), resulting in high plasma and tissue levels of thymidine (Thd) and deoxyuridine (dUrd), which become mutagenic on mitochondrial DNA (Halter et al. 2011).

## CONCLUDING REMARKS

Because of the multiple functions of mitochondria, not only does OXPHOS impairment

determine various degrees of energy failure, but so also do a host of additional effects that account for the intricacy and complexity of the pathophysiology of mitochondrial disorders.

The recent elucidation of the pathogenetic basis of EE has provided evidence for the existence of yet another mechanism of mitochondrial energy impairment, namely the genetically determined failure to detoxify OXPHOS poisons, such as $H_2S$. In principle, accumulation of a harmful compound can be contrasted more effectively than intrinsic failure of OXPHOS energy metabolism. Therefore, we believe that the specific toxic mechanism underpinning EE makes effective therapy a realistic goal. The results of our effort will constitute a fundamental step toward the development of a cure for this fatal mitochondrial disease in humans.

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