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認知症対策総合研究事業

認知症の新規治療法開発に向けた硫化水素の動態に関する研究

平成２３年度　総括研究報告書

独立行政法人国立精神・神経医療研究センター

神経研究所神経薬理研究部

渋谷　典広

平成２４（２０１２）年　４月

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研究代表者　渋谷　典広

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総括研究報告書

認知症の新規治療法開発に向けた硫化水素の動態に関する研究

研究代表者　渋谷　典広　独立行政法人国立精神・神経医療研究センター
神経研究所神経薬理研究部室長

研究要旨

近年、神経変性疾患の治療を目的とした生理活性物質硫化水素の応用研究が進行している。認知症に対する利用が期待される一方で、内在性硫化水素の慢性的なクリアランス異常が脳障害を引き起こすとの報告がある。硫化水素を用いた新規治療法を開発するためにはクリアランス評価が不可欠であると考え、アルツハイマー型認知症患者の死後脳を用いて動態解析を行った。硫化水素の生産・貯蔵・代謝に関する生化学的解析の結果、アルツハイマー型認知症においては健常脳と同等のクリアランス能が維持されていると判明した。硫化水素には認知症の治療及び予防の観点から効果を発揮し得る作用が *in vitro* で確認されていることから、生体への影響や投与形態の検討など臨床応用を指向した研究・開発が求められる。

A．研究目的

超高齢社会を目前に控えた現在、認知症対策の一層の推進が厚生労働行政に求められている。これまで認知症の病因候補遺伝子の導入モデル動物が多数作出されているが、モデルマウスの多くは病態の一部が再現されているのが実状であり、認知症の分子病態については不明点が多い。そのため、治療は対症療法的であり、根治困難な状況が続いている。この状況は、健康不安や介護の必要性等、患者のみならず多くの人々に漠然とした不安をもたらしている。

近年、生理活性物質としての硫化水素が注目されている。硫化水素には神経伝達の調節作用や神経細胞の保護作用が確認されており、この物質を疾患治療へ応用する研究が進行している。硫化水素には上記の作用のみならず、抗酸化ストレス作用・抗炎症作用・抗アポトーシス作用が確認されている。その多彩なはたらきは、神経変性疾患の治療に有効と考えられており、なかでも難治例の多い認知症への応用が期待されている。その一方で、脳内の硫化水素濃度がクリアランス異常によって高まり、高濃度の硫化水素に慢性的に曝露されることで脳障害が惹起されるとの報告がある。高濃度の硫化水素は、呼吸鎖系酵素複合体の阻害や神経伝達抑制などの毒性を示すことから、硫化水素を利用した治療方法を開発するためには、クリアランス評価が不可欠である。しかし、認知症脳における硫化水素の動態研究については国内・国外を問わず報告されておらず、硫化水素を利用することの妥当性は不明である。そこで本研究では、認知症の新規治療法開発を目指し、死後脳を用いた硫化水素の動態に関する検討を行った。

B．研究方法

(1) 死後脳のグループ化

Braak らのステージ分類に基づき、神経原線維変化ステージ及び老人斑ステージを組み合わせた以下の 3 群にグループ化した。

・微小変化群＝神経原繊維変化：ステージ 1-2、老人斑：ステージ 0-A

・アルツハイマー型変化群＝神経原繊維変化：

ステージ6、老人斑：ステージC
・老人斑優位型変化群＝神経原繊維変化：ステージ1-2、老人斑：ステージB-C

(2) 硫化水素クリアランスの評価

生体内で生産された硫化水素は、タンパク質のシステイン残基と結合し貯蔵される。そこで脳内における結合状態の硫化水素（結合型硫黄）を定量することで、硫化水素の曝露状況を把握した。結合型硫黄は、還元条件下で硫化水素を放出するため、脳抽出液にジチオスレイトールを添加し、37°C で 50 分間保温することで遊離する硫化水素をガスクロマトグラフィーで定量した。

硫化水素は、呼吸鎖系酵素複合体を形成する鉄硫黄クラスターの生合成に関与することが示唆されている。鉄硫黄クラスターが、内在性硫化水素の利用形態の一つである可能性を考慮し、クラスター内の硫黄含量を測定した。鉄硫黄クラスターは酸性条件下で硫化水素を放出するため、脳抽出液にトリクロロ酢酸を添加し、37°C で 30 分間保温することで放出される硫化水素を酸不安定型硫黄としてガスクロマトグラフィーで定量した。

(3) 生産酵素及び代謝酵素の定量

脳内における硫化水素の生産酵素及び代謝酵素をウエスタンブロット法で検出し、シグナル強度を定量した。

・硫化水素生産酵素
3-メルカプトピルビン酸サルファトランスフェラーゼ（3MST）及びシスタチオニンβシンターゼ（CBS）
・3MST の基質生産酵素
システインアミノトランスフェラーゼ（CAT）
・硫化水素代謝酵素
サルファジオキシゲナーゼ（ETHE1）

(4) 研究協力体制

剖検大脳皮質は、以下の研究協力者から提供を受けた。

・東京都健康長寿医療センター：
村山 繁雄　高齢者ブレインバンクリーダー
・国立国際医療研究センター国府台病院：
石田 剛　臨床検査部長
・国立精神・神経医療研究センター：
有馬邦正　第一精神診療部長
・国立精神・神経医療研究センター：
齊藤 祐子　臨床検査部医長

(5) 試料測定の流れ

盲検化を図るため、研究代表者がコード番号付き試料の測定結果を研究協力者に送付した後に、臨床情報が開示される体制に努めた。

(6) 統計解析

2 群間の比較には t 検定を行い、3 群間の比較には一元配置分散分析法を用いた。

(7) 倫理面への配慮

本研究は、当該研究機関倫理委員会において承認済みの疫学研究課題を基盤としている。

本研究では死後脳検体が対象となるため、死の尊厳への配慮と篤志ご遺族への敬意を前提に取り扱った。また、研究結果が篤志ご遺族へ不利益と危険性を及ぼす可能性について十分な配慮を払い遂行した。なお、ご遺族から本研究結果の説明を求められた場合には、医師がこれに対応できる体制とした。

C．研究結果

生体内で生産された硫化水素は、タンパク質のシステイン残基と結合し貯蔵される。そこで、硫化水素の貯蔵量を調べるため、剖検大脳皮質を用いて結合状態の硫化水素（結合型硫黄）を測定した。

はじめに比較的少数例の健常脳（control, CTL; n=5, 平均年齢 76.4, 国立国際医療研究センター）・アルツハイマー病脳（Alzheimer disease, AD; n=3, 平均年齢 69.7, 国立精神・神経医療研究センター）・筋硬直性ジストロフィー病脳（Myotonic dystrophy, DM; n=6, 平均年齢 60.2, 国立精神・神経医療研究センター）を用いて予備的な検討を行った。CTL, AD, DM における結合型硫黄含量（nmol/mg protein±S.D.）は、それぞれ 0.494±0.043, 0.858±0.042, 0.685±

0.022 であり、AD において顕著に高い結合型硫黄が検出された（AD vs CTL, $p<0.01$; AD vs DM, $p<0.05$）。硫化水素は、呼吸鎖系酵素複合体を形成する鉄硫黄クラスター（酸不安定型硫黄）の生合成に関与することが示唆されている。酸不安定型硫黄が、内在性硫化水素の利用形態の一つである可能性を考慮し、クラスター内の硫黄含量を測定した。その結果、CTL, AD, DM における酸不安定型硫黄含量（nmol/mg protein ±S.D.）は、それぞれ 0.399±0.021, 0.454±0.040, 0.313±0.019 であり、AD の硫黄含量は DM よりも高く（$p<0.05$）、CTL との差は有意ではなかった。

上記の検討は小規模であり、死後脳の提供施設が異なる。より厳密な症例対照研究を行うため、東京都健康長寿医療センターから新たに死後脳の提供を受けた。アルツハイマー型変化群（Alzheimer disease type change, ADC; 神経原繊維変化ステージ6・老人斑ステージC, n=14, 平均年齢 83.1）及び対象として微小変化群（Minimal senile change, MSC; 神経原繊維変化ステージ 1-2・老人斑ステージ 0-A, n=16, 平均年齢 82.2）の結合型硫黄（nmol/mg protein ±S.D.）を定量した結果、それぞれ 0.544±0.037 及び 0.545±0.036 であり、両群間の差は有意ではなかった。また、酸不安定型硫黄（nmol/mg protein±S.D.）を定量した結果、それぞれ 0.322 ±0.018 及び 0.304±0.012 であり、両群間の差は有意ではなかった。なお、ADC の臨床的認知症釈度（Clinical dementia rating, CDR）は 3（高度認知症）または 2（中等度認知症）であり、14 例の平均値は 2.9 であった。一方、MSC の CDR は 0（健常）または 0.5（認知症疑い）であり、未定 1 検体及び不確定 3 検体を除く 12 例の平均値は 0.1 であった。

次に、ADC とは背景病理の異なる老人斑優位型変化群（Pathological senile change, PSC; 神経原繊維変化ステージ 1-2・老人斑ステージ B-C, n=4, 平均年齢 86.0）について、東京都健康長寿医療センターから新たに提供を受けた。本症例の結合型硫黄含量（nmol/mg protein±S.D.）は、0.542±0.031 であった。一方、同センターから新たに提供を受けた MSC（n=5, 平均年齢：82.0）及び ADC（n=5, 平均年齢 84.8）における結合型硫黄含量（nmol/mg protein ± S.D.）は、それぞれ 0.718±0.028 及び 0.678± 0.074 であった。3 群間における差は有意ではなかったが、PSC において結合型硫黄含量が低下している傾向が認められた。MSC, ADC, PSC の酸不安定型硫黄含量（nmol/mg protein ± S.D.）については、それぞれ 0.399±0.021, 0.454 ±0.040, 0.313±0.019 であり、CTL との差は有意ではなかったが、PSC において比較的低濃度の酸不安定型硫黄が検出された。

ADC 及び PSC に関して、硫化水素の生産系または代謝系に変化が生じているかを調べるため、生産酵素 3-メルカプトピルビン酸サルファトランスフェラーゼ（3MST）及びシスタチオニン β シンターゼ（CBS）、3MST の基質生産酵素システインアミノトランスフェラーゼ（CAT）、代謝酵素サルファジオキシゲナーゼ（ETHE1）を定量した。まず、ADC における 3MST, CBS, ミトコンドリア型 CAT, サイトゾル型 CAT, ETHE1 の含量は、MSC のそれぞれ 1.08 倍, 0.62 倍, 0.73 倍, 1.09 倍, 1.01 倍であった。これまでの検討から、大脳皮質では錐体細胞等の比較的大型の神経細胞に 3MST が局在していることを確認している。ADC では神経細胞の変性・脱落が生じているにも関わらず、3MST 含量が MSC と同等であることから、3MST 陽性細胞が特異的に生存している可能性がある。一方、PSC における 3MST, CBS, ミトコンドリア型 CAT, サイトゾル型 CAT, ETHE1 の含量は、MSC のそれぞれ 1.17 倍, 0.96 倍, 0.99 倍, 1.16 倍, 1.25 倍であり、PSC においては 3MST の増加に加えて ETHE1 の増加が認められた。PSC における硫黄含量の低下は ETHE1 の増加に起因している可能性が示唆された。

本研究の開始当初は、硫化水素のクリアランス異常によって認知症内の貯蔵量が増加してい

た場合には、おもに毒性の観点から硫化水素の標的因子を同定する予定であった。その際、脳抽出液から標的因子を回収する方法を新規に考案したが、その後試みたところ、おもに収率の観点から同定を行うには不十分であると判明した。今後、硫化水素の動態をより詳細に解明するため、収率を向上させるための別法を確立することが新たな課題となった。

D．考察

神経変性疾患の治療に際しては、比較的長期間の投薬が予想されるため、対象疾患における硫化水素のクリアランス能を評価することはその薬理作用を効果的に発揮する上での必須条件となる。神経変性疾患に対する硫化水素の有用性については、パーキンソン病のモデル動物において組織学的及び行動学的に検討されているが、剖検脳を用いたクリアランス評価は行われていない。硫化水素による治療の可能性を模索するためにはクリアランス能の調査とその評価が前提であると考え、本研究では難治例の多いアルツハイマー型認知症の死後脳を用いて硫化水素の動態に関する検討を行った。

複数の機関から死後脳の提供を受け開始した予備検討では、健常脳よりもアルツハイマー病脳において結合型硫黄含量が有意に増加していた。一方、その後新たに東京都健康長寿医療センターから提供を受けたアルツハイマー型変化群（ADC）及び微小変化群（MSC）を用いた検討では、両群間の硫黄含量に有意な差は認められなかった。この矛盾は、今後解明するべき問題であるが、提供機関による凍結方法や保存方法の差異等が測定結果に与える可能性を現時点では排除できないことから、同一機関管理下の剖検脳を対象とした測定結果に妥当性があると判断した。

今回対象とした ADC の多くは臨床的認知症尺度（CDR）3 を示す高度認知症例であったが、硫化水素のクリアランス異常は認められなかった。老人斑優位型変化群（PSC）においては MSC よりも硫黄含量が低い傾向にあった。PSC には CDR が 0（健常）・0.5（認知症疑い）・3（高度認知症）が混在していたが、CDR と硫黄含量の間に相関関係は認められなかった。これらの結果は、アルツハイマー型認知症脳においては硫化水素のクリアランスが健常脳と同程度に維持されていることを示している。アルツハイマー型認知症患者に対して硫化水素を適用することが原理的に可能であると示唆されたことから、この物質を利用した認知症の新規治療法開発を支持するものである。

認知障害の発症原因としては、初期段階における酸化ストレス及び炎症反応が指摘されている。硫化水素には酸化ストレスや炎症反応を抑制するはたらきが確認されており、アミロイドβ タンパク質による細胞障害を抑制する作用が *in vitro* において確認されている。生体に投与した硫化水素の濃度を制御できれば、発症の予防や認知障害の軽減を目的とした利用が可能と思われる。硫化水素の実効性を *in vivo* において長期的に検証していくことが今後の課題である。

硫化水素は水溶性のガス性物質である。他のガス性因子である一酸化窒素に関しては、肺高血圧を伴った重症呼吸不全を対象とした吸入療法が新生児医療の分野で実際に行われている。硫化水素の医療応用については今後の検討課題であるが、パーキンソン病のモデル動物においては硫化水素の吸入により症状が改善されることが確認されている。硫化水素は、一酸化窒素とは異なり水に対する溶解度が高い。ガス及び水溶液の両面から効果的かつ安全な投与形態を見出すことが重要となる。その際には、クリアランスを継続的にモニターする等、より臨床応用を視野に入れた評価が必要と思われる。

本研究は、研究協力者による厳密な病理学的診断結果に基づき可能となった。本研究ではアルツハイマー型認知症が主たる対象であったが、他の神経変性疾患においても、病理診断の結果と硫化水素の動態研究を組み合わせることでクリアランスの解析及び評価が可能である。非ア

ルツハイマー型認知症を含めた他疾患の動態解析を行い、それらの結果を踏まえたうえで、認知症に対して硫化水素を利用することの妥当性を総合的に評価することが必要である。

本研究の開始当初は、認知症脳において硫化水素のクリアランス異常が認められた場合に、おもに毒性の観点から硫化水素の標的因子を同定する予定であった。その際、脳抽出液から標的因子を回収し同定する方法を考案したが、その後の検討から、同定を行うためには回収率を改善する必要があると判明した。この方法では、標的因子を捕捉する際にビオチン化マレイミドを利用する。ビオチン化マレイミドは吸湿等により加水分解する性質があり、この不安定性が回収率低下の主たる原因と思われる。また最近では、脳抽出液を調製する際の細胞破砕操作によって硫化水素が人為的にタンパク質と結合し得る可能性が指摘されている。硫化水素の標的因子を同定するためには、回収率の改善のみならず、嫌気的環境下において抽出液を調製する等、硫化水素とタンパク質の人為的結合を防ぐための措置を講じる必要がある。本研究におけるこれまでの定量解析では、結合型硫黄の総量を測定することが可能であった。一方、硫化水素の標的因子の同定方法を確立できれば、各因子を個別に定量解析できるため、より詳細な硫化水素の動態メカニズムを解明できる可能性がある。引き続き、この問題点を改善するための取り組みが必要である。

今後の検討課題としては、(i) 非アルツハイマー型認知症を含めた他疾患の動態解析、(ii) 標的因子の同定、(iii) *in vivo* における実効性の評価があげられる。これらの基礎研究の結果が医療応用を目指す際の重要な指標となる。

E．結論

死後脳を用いて硫化水素の動態を解析した結果、アルツハイマー型認知症においてはクリアランス異常に伴う硫化水素の蓄積は認められなかった。アルツハイマー型認知症患者に対して硫化水素を適用できる可能性が示唆されたことから、生体への利用を視野に入れた認知症の新規治療法開発を目指した研究が求められる。

F．健康危険情報

該当なし

G．研究発表

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H．知的財産権の出願・登録状況

該当なし

別添４

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Mikami Y, Shibuya N, Kimura Y, Nagahara N, Ogasawara Y, Kimura H.	Thioredoxin and dihydrolipoic acid are required for 3-mercaptopyruvate sulfurtransferase to produce hydrogen sulfide.	Biochemical Journal	Vol. 439, No. 3	479-485	2011
Mikami Y, Shibuya N, Kimura Y, Nagahara N, Yamada M, Kimura H.	Hydrogen sulfide protects the retina from light-induced degeneration by the modulation of Ca2+ influx.	The Journal of Biological Chemistry	Vol. 286, No. 45	39379-39386	2011
Kimura H, Shibuya N, Kimura Y.	Hydrogen sulfide is a signaling molecule and a cytoprotectant.	Antioxidant & Redox Signaling	Vol. 17, No. 1	45-57	2012

研究成果の別刷

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Thioredoxin and dihydrolipoic acid are required for 3-mercaptopyruvate sulfurtransferase to produce hydrogen sulfide

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H_2S (hydrogen sulfide) has recently been recognized as a signalling molecule as well as a cytoprotectant. We recently demonstrated that 3MST (3-mercaptopyruvate sulfurtransferase) produces H_2S from 3MP (3-mercaptopyruvate). Although a reducing substance is required for an intermediate persulfide at the active site of 3MST to release H_2S, the substance has not been identified. In the present study we show that Trx (thioredoxin) and DHLA (dihydrolipoic acid) associate with 3MST to release H_2S. Other reducing substances, such as NADPH, NADH, GSH, cysteine and CoA, did not have any effect on the reaction. We also show that 3MST produces H_2S from thiosulfate. The present study provides a new insight into a mechanism for the production of H_2S by 3MST.

Key words: dihydrolipoic acid (DHLA), dithiol, hydrogen sulfide (H_2S), 3-mercaptopyruvate sulfurtransferase (3MST), thioredoxin.

INTRODUCTION

H_2S (hydrogen sulfide) is produced by three enzymes, CBS (cystathionine β-synthase), CSE (cystathionine γ-lyase) and 3MST (3-mercaptopyruvate sulfurtransferase) [1–6]. H_2S facilitates the induction of hippocampal long-term potentiation by enhancing the activity of NMDA (*N*-methyl-D-aspartate) receptors in neurons and induces Ca^{2+} influx in astrocytes [1,7]. Vascular smooth muscle is relaxed by H_2S, which can be released from endothelium as well as smooth muscle [2,8–10]. H_2S may also regulate insulin release, angiogenesis and sulfhydration of target molecules [11–13].

In addition to a function as a signalling molecule, H_2S has a cytoprotective effect. H_2S protects neurons from oxidative stress by reinstating the levels of GSH, a major intracellular antioxidant, by enhancing the activity of γ-glutamylcysteine synthetase and the transport of cysteine and cystine [14,15]. It also protects cardiomyocytes from ischaemia/reperfusion injury by preserving mitochondrial function [16].

3MST produces H_2S from 3MP (3-mercaptopyruvate), which is generated by CAT (cysteine aminotransferase) from cysteine and α-KG (α-oxoglutarate) [3,17–19]. 3MP provides sulfur to the active-site cysteine residue of 3MST to produce persulfide, which releases H_2S in the presence of DTT (dithiothreitol) [3,20]. However, a physiological substance corresponding to DTT has not been identified [3,20]. The 3MST orthologue of *Trichomonas vaginalis* catalyses the formation of Trx (thioredoxin) persulfide [21]. Trx, which has two redox-active cysteine residues in its active site, is abundant in cells [22]. Another physiological reducing disulfide is DHLA (dihydrolipoic acid), which is a cofactor for mitochondrial α-oxo acid dehydrogenases [23].

The present study shows that Trx and DHLA release H_2S from persulfide provided by 3MP at the active site of 3MST. We also found that 3MST produces H_2S from thiosulfate. This provides a molecular mechanism for the production of H_2S catalysed by 3MST.

EXPERIMENTAL

Chemicals

α-Lipoic acid, NADH, potassium sulfite, potassium thiosulfate hydrate, PLP (pyridoxal 5′-phosphate), sodium borohydrate and Tween 20 were purchased from Sigma–Aldrich. Citric acid monohydrate, CoA trisodium salt, L-cysteine hydrochloride monohydrate, DTT, GSH, hydroxylammonium chloride, mercaptopyruvate sodium salt, α-KG, potassium sulfate, sodium sulfide nonahydrate and sucrose were purchased from Wako Pure Chemical Industries. NADPH was purchased from Oriental Yeast. Trx was purchased from Merck Calbiochem.

Subcellular fractionation and determination of H_2S-producing activity

All animal procedures were approved by the National Institute of Neuroscience Animal Care and Use Committee. Homogenates of a whole brain were prepared from C57BL/6N mice (Clea Japan) with 9 vols of ice-cold isolation buffer A [0.1 M potassium phosphate (pH 7.4), 0.32 M sucrose and Complete™ protease inhibitor cocktail (Roche Diagnostics)] using a Potter-type glass homogenizer with a Teflon pestle (700 rev./min, eight strokes) and were centrifuged for 10 min at 4 °C at 1000 *g* (M-201 IVD centrifuge, Sakuma) to remove nuclei and intact cells. The supernatant was centrifuged for 10 min at 4 °C at 1300 *g* to obtain the post-nuclear supernatant. A discontinuous density gradient was prepared in 14×89 mm Ultra-Clear centrifuge tubes (Beckman Instruments) by applying 3 ml fractions of the supernatant on the cushion of three layers consisting of 3 ml of 10 %, 20 % and 40 % Percoll (GE Healthcare). Centrifugation was performed for 30 min at 4 °C and 20000 rev./min in an angle-headed rotor (Beckman type 41) with an ultracentrifuge (Optima XL-100K; Beckman Coulter). The broad band near the top of the gradient consisted of cytosol. Synaptosomes were localized at the interphase between 10 % and 20 % Percoll. Mitochondria

Abbreviations used: CAT, cysteine aminotransferase; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; DHLA, dihydrolipoic acid; DTT, dithiothreitol; HEK, human embryonic kidney; HRP, horseradish peroxidase; H_2S, hydrogen sulfide; α-KG, α-oxoglutarate; 3MP, 3-mercaptopyruvate; 3MST, 3-mercaptopyruvate sulfurtransferase; Nrf2, nuclear factor erythroid2-related factor 2; PLP, pyridoxal 5′-phosphate; Trx, thioredoxin.

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were at the interphase between 20 % and 40 % Percoll. The three fractions were pulled with a needle and syringe. The synaptosomes and mitochondrial fractions were diluted with 4 vols of buffer and centrifuged twice for 10 min at 4 °C and 17000 *g*, with a high-speed refrigerated microcentrifuge (MX-100; Tomy Seiko). Each pellet was resuspended in buffer containing 0.1 M potassium phosphate (pH 8.0), 0.32 M sucrose and Complete™ protease inhibitor cocktail and sonicated for 10 s (Branson Model 450 sonicator; Branson Ultrasonics). The cytosol fraction was centrifuged for 10 min at 4 °C and 17000 *g* and obtained as the supernatant. Protein concentrations were determined using the Bio-Rad DC™ Protein Assay according to the manufacturer's instructions.

HEK (human embryonic kidney)-293F cells were precipitated by centrifugation for 5 min at 4 °C and 1000 *g*. After washing with ice-cold PBS, each pellet was resuspended in ice-cold buffer A and sonicated.

Enzyme reactions were performed as described previously [3]. The substrate was added to 0.1 ml of the homogenates or cell lysates in a 15 ml centrifugation tube and incubated at 37 °C for 50 min. After adding 0.2 ml of 1 M sodium citrate buffer (pH 6.0), the mixtures were incubated at 37 °C for 10 min with shaking on a rotary shaker to facilitate a release of H_2S gas from the aqueous phase. Then, 2 ml of approximately 14.5 ml of head-space gas was applied to a gas chromatograph (GC-2014; Shimadzu) equipped with a flame photometric detector and a C-R8A Chomatopac data processor (Shimadzu). The standard curve for H_2S was prepared as described previously [3]. Briefly, 0.1 ml of 0–5 nM Na_2S in 0.01 M NaOH prepared with degassed milli-Q water, as a source of H_2S, was added in a 15 ml centrifugation tube. After adding 0.2 ml of 1 M sodium citrate buffer (pH 6.0), the mixtures were incubated at 37 °C for 10 min with shaking on a rotary shaker to facilitate a release of H_2S gas from the aqueous phase. Then, 2 ml of approximately 14.5 ml of head-space gas was applied to a gas chromatograph.

Western blot analysis

Protein samples (2.5 μg) were separated by SDS/PAGE (15 % gel) (DRC) and electroblotted on to a PVDF membrane (Millipore). After blocking with a blocking solution containing 5 % non-fat skimmed milk (Becton Dickinson) and PBST (0.1 % Tween 20 in PBS), the blots were incubated with rabbit anti-(rat 3MST) antibody (1:1500 dilution) [24], rabbit anti-(human glucose-6-phosphate dehydrogenase) antibody (1:3000 dilution; Bethyl Laboratories), rabbit anti-(human manganese superoxide dismutase) antibody (1:3000 dilution; Stressgen Bioreagents), mouse anti-(synaptophysin I) monoclonal antibody (1:150 dilution; Abcam), rabbit anti-CBS polyclonal antibody (1:3000 dilution) [25], rabbit anti-CSE polyclonal antibody (1:5000 dilution) [26] or rabbit anti-Trx2 polyclonal antibody (1:1000 dilution; Abcam) in the blocking solution for 8 h at 4 °C. The membrane was washed with PBST and incubated with a HRP (horseradish peroxidase)-conjugated anti-rabbit IgG (1:10000 dilution; GE Healthcare) or anti-mouse IgG (1:10000 dilution; GE Healthcare) in PBST for 1 h at room temperature (25 °C). HRP was visualized using Millipore Immobilon Western Chemiluminescent substrate.

Reduction of Trx and H_2S production assay

A549 cells, a human lung adenocarcinoma cell line which highly expresses Trx reductase, were maintained in Ham's F-12 medium (Sigma) containing 100 units/ml penicillin (Invitrogen) 100 μg/ml streptomycin (Invitrogen) and 10 % heat-inactivated fetal bovine serum (Japan Bio Serum) at 37 °C under an atmosphere of 90 % air and 10 % CO_2. A549 cells were harvested by scraping with a silicon rubber scraper and washed twice with ice-cold PBS. The cells were precipitated by centrifugation for 5 min at 4 °C and 1000 *g* and re-suspended with ice-cold buffer containing 0.1 M potassium phosphate (pH 8.0), 0.32 M sucrose and Complete™ protease inhibitor cocktail, and sonicated. For enzyme reactions, recombinant Trx and NADPH (final concentration of 1 mM) were added to 50 μl of A549 cell lysates in a 15 ml centrifugation tube. The tube containing the reaction mixture was incubated at 37 °C for 1 h. After incubation, 50 μl of mouse brain mitochondrial fraction and substrate were added to the reaction mixture and incubated at 37 °C for 50 min. After adding 200 μl of 1 M sodium citrate buffer (pH 6.0), the mixtures were incubated at 37 °C for 10 min with shaking, and 2 ml out of approximately 14.5 ml of headspace gas was applied to a gas chromatograph.

Reduction of α-lipoic acid

Solutions of α-lipoic acid were reduced with $NaBH_4$ (sodium borohydride) [27]. α-Lipoic acid (0.4126 g) was dissolved in 10 ml of 0.2 M Tris base and 2 M $NaBH_4$ and incubated at room temperature for 15–30 min. After reduction, the solution was acidified to the point of precipitation of DHLA (approximately pH 5.0) by adding 85 % phosphate solution on ice. The acidification destroys the excess BH_4^- to borate. The DHLA solution in phosphate-borate buffer was adjusted to pH 7.3 by adding Tris base. The solution was diluted to 20 ml with 0.15 M Tris/HCl (pH 7.3). The concentrations of DHLA were determined by measuring the amount of the SH-groups using DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)].

Constructs

The construct of mouse 3MST (wild-type, R187G, R196G and C247S) expression plasmids has been described previously [3]. The coding sequence for human rhodanese was amplified by PCR from the cDNA clone MGC Collection (clone ID 4329727; Invitrogen). The amplified fragment was inserted into the pCI-neo Mammalian Expression Vector (Promega) to generate rhodanese/pCI. Human Trx2 (cDNA clone MGC Collection, clone ID 2900137) was purchased from Invitrogen as transfection-ready DNA and subcloned into the pCMV-SPORT6 vector.

Transient transfection of HEK-293F cells

Transient transfection of HEK-293F cells in suspension cultures was performed using a FreeStyle 293 Expression System (Invitrogen). HEK-293F cells efficiently express externally applied expression plasmids and grow in higher densities in suspension cultures than regular plating. For transfection, 10 μg of expression plasmids were mixed with 15 μl of the transfection reagent 293fectin™ (Invitrogen) and was then added to 1×10^7 cells in 100 ml Erlenmeyer flasks with 10 ml of FreeStyle 293 expression medium. Cells were incubated with shaking at 125 rev./min on a rotary shaker at 37 °C in a humid atmosphere with 10 % CO_2. Transfection efficiency was >90 %. The transfected cells were harvested at 48 h post-transfection.

Measurement of bound sulfane sulfur

Whole brain was homogenized with 9 vols of the ice-cold lysis buffer containing 10 mM potassium phosphate (pH 7.4), 0.5 % Triton X-100, 10 mM hydroxylamine (which was used to suppress the activity of the PLP-dependent enzymes including CAT) and

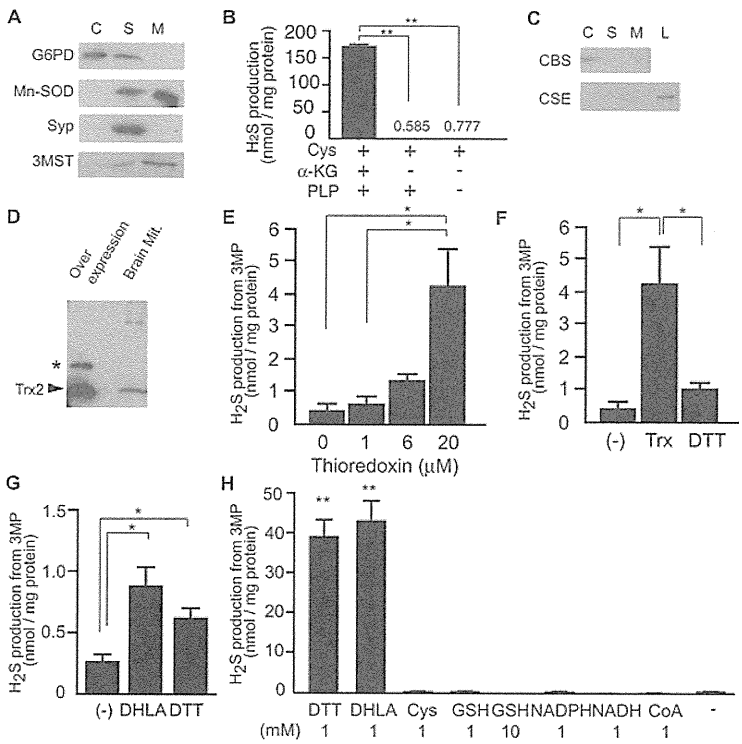

Figure 1 Endogenous reductants of 3MST for H_2S production

(**A**) Western blot analysis of 3MST and the marker proteins for mouse brain subcellular distribution. C, cytosolic fraction; S, synaptosomal fraction; M, mitochondrial fraction. Glucose-6-phosphate dehydrogenase (G6PD), manganese superoxide dismutase (Mn-SOD) and synaptophysin I (Syp) were used as markers of cytosol, mitochondria and synaptosomes respectively. (**B**) H_2S production requires cysteine and α-KG in brain mitochondria. Mouse brain mitochondrial fractions were mixed with 4 mM cysteine, 0.5 mM α-KG or 0.05 mM PLP ($n = 3$). $^{**}P < 0.01$ compared with the mixture of cysteine, α-KG and PLP. (**C**) Western blot analysis of CBS and CSE. C, brain cytosolic fraction; S, brain synaptosomal fraction; M, brain mitochondrial fraction; L, liver cytosolic fraction. (**D**) Western blot analysis of Trx2. Left-hand lane: the lysate of HEK-293F cells expressing Trx2. The arrowhead indicates Trx2, the matured form localized to mitochondria. The asterisk indicates the Trx precursor containing the mitochondrial target sequence. Right-hand lane: mouse brain mitochondrial fraction. (**E**) Concentration dependency of Trx to produce H_2S from 3MP. The mitochondrial fraction of mouse brain was mixed with 3MP in the presence of Trx and the amount of H_2S was measured. (**F**) Trx is more potent than DTT in enhancing H_2S production from 3MP. The effect of 20 μM each of Trx and DTT was compared. $^{*}P < 0.05$. (**G**) DHLA enhances H_2S production from 3MP at a similar level to DTT. The effect of 40 μM each of DHLA and DTT was compared. $^{*}P < 0.05$. (**H**) The enhancing activity of physiological reducing substances on H_2S production from 3MP. The enhancing activity of 1 mM each of DTT, DHLA, cysteine, GSH (1 mM and 10 mM), NADPH, NADH and CoA on the production of H_2S from 3MP was compared using the mitochondrial fraction of mouse brain homogenates. All data are represented as the means $\pm$ S.E.M for at least three experiments. $^{**}P < 0.01$.

Complete™ protease inhibitor cocktail using a Potter-type glass homogenizer with a Teflon pestle (1500 rev./min, ten strokes). The lysates were incubated on ice for 30 min and centrifuged for 3 min at 4 °C and 12000 *g*, and supernatants were used for the assays. For measurement of H_2S released from bound sulfane sulfur, 0.1 ml of samples and 0.1 ml of DTT or DHLA in 0.2 M Tris/HCl (pH 9.0) were placed in a 15 ml centrifugation tube, sealed and incubated at 37 °C for 50 min. After adding 0.4 ml of 1 M sodium citrate buffer (pH 6.0), the mixtures were incubated at 37 °C for 10 min by shaking on a rotary shaker to facilitate release of H_2S from the aqueous phase. Then, 4 ml of head-space gas was applied to a gas chromatograph. H_2S concentrations were determined according to the method for determination of H_2S produced by enzymes. A reaction mixture without samples was used as a control for release of H_2S from DTT or DHLA.

Statistical analysis

All statistical analyses of the data were performed using Microsoft Excel 2004 for Mac (Microsoft) with the add-in software Statcel2 (OMS). Differences between two groups were analysed with Student's *t* test. Differences between three or more groups were analysed with ANOVA. Post-hoc multiple comparisons were made using the Bonferroni-Dunn test.

RESULTS

3MST produces H_2S from 3MP in the presence of Trx and DHLA

3MST, which is localized to the mitochondrial matrix space, produced H_2S in the presence of both cysteine and α-KG [3,28] (Figures 1A and 1B). In contrast, CBS is localized to cytosol and CSE is not present in the brain (Figure 1C). In the absence of α-KG, the mitochondrial fraction produced little H_2S ($P < 0.01$; Figure 1B). These observations suggest that 3MST, along with CAT, produces H_2S from cysteine in the brain mitochondria.

3MST requires a reducing substance such as DTT to release H_2S [3]. Because Trx has two redox-active cysteine residues at its active site similar to dithiol DTT, the effect of Trx on the production of H_2S from 3MP was examined [22]. There are two forms of Trx in mammals: Trx1 localized to the cytosol and Trx2 localized to the mitochondria (Figure 1D) [22,29]. Because the structure of the CXXC motif, a sequence containing active-site cysteine residues, is highly conserved among different species, and because Trx2 is resistant to oxidative stress similar to bacterial Trx, we used *Escherichia coli* Trx [22,29]. Since Trx is readily oxidized, Trx reductase was used to reduce oxidized Trx. The mammalian Trx reductase is a selenoprotein and cannot be directly expressed in *E. coli*. [30]. Therefore we added NADPH and lysates of A549 human lung adenocarcinoma cells, which have abundant

Trx reductase, to reduce Trx [31]. The effect of reduced Trx on the H_2S-producing activity of the mitochondrial fraction from 3MP was examined. The reduced form of Trx enhanced the H_2S-producing activity in a dose-dependent manner ($P < 0.05$, 20 μM compared with 0 and 1 μM Trx; Figure 1E). Since approximately 20 μM Trx exists in cells, the effect of Trx was compared with that of DTT at 20 μM [22]. Trx had an approximately 4-fold enhanced H_2S-producing activity than DTT ($P < 0.05$; Figure 1F).

Because a dithiol DHLA is concentrated on mitochondria, the effect of DHLA on the H_2S-producing activity from 3MP was examined [32,33]. Because the levels of free lipoic acid are approximately 40 μM in the brain, the effect of DHLA was compared with that of DTT at 40 μM [34,35]. H_2S was produced in the presence of DHLA at a similar level as that in the presence of DTT ($P < 0.05$, DHLA and DTT compared with no-reductant control; no significant difference between DHLA and DTT; Figure 1G). We examined further the effect of other physiological reducing substances, such as cysteine, GSH, NADPH, NADH and CoA, on H_2S-producing activity from 3MP. Monothiols such as cysteine, GSH and CoA, as well as the other reducing substances, did not show any effect on H_2S production, even at 1 mM (Figure 1H). These observations suggest that 3MST depends on the dithiols Trx and DHLA for the production of H_2S from 3MP.

DHLA interacts with 3MST in a similar manner to DTT

Arg^{187} and Arg^{196} in mouse 3MST have been proposed to determine substrate specificity, and Cys^{247} is a catalytic site for 3MP [3,36]. Whether or not the H_2S-producing activity of 3MST has some difference among Trx, DHLA and DTT was examined using the mouse 3MST mutants R187G, R196G and C247S. Both C247S and R187G diminished the H_2S-producing activity in the presence of Trx, DHLA or DTT ($P < 0.05$, wild-type compared with R187G; for Figures 2A and 2C, $P < 0.05$, wild-type compared with C247S with Trx or DTT; for Figure 2B, $P < 0.01$, wild-type compared with C247S with DHLA). The production of H_2S was slightly decreased in R196G in the presence of Trx or DHLA compared with DTT (for Figure 2B, $P < 0.05$, wild-type compared with R196G with DHLA; Figures 2A–2C). These observations suggest that Trx and DHLA release H_2S by interacting with 3MST in basically the same manner as DTT.

3MST can produce H_2S from thiosulfate

It has been proposed that thiosulfate, which is known as an intermediate of sulfur metabolism from cysteine as well as a metabolite of H_2S, can produce H_2S [6,19,37]. 3MST, which is homologous with rhodanese with 60% amino acid identity conserving similar functional structure, has an affinity to thiosulfate [36,38]. We examined whether or not 3MST produces H_2S from thiosulfate. The mitochondrial fraction produced H_2S from thiosulfate in the presence of DHLA, which enhanced the production more than DTT ($P = 0.003$; Figure 3A). H_2S was not produced in the presence of 20 μM Trx or 20 μM DTT (results not shown). Monothiols, such as cysteine, GSH and CoA, and other reducing substances, such as NADPH and NADH, did not affect the production of H_2S (results not shown). These observations suggest that H_2S can be produced from thiosulfate in the presence of high concentrations of DHLA.

The mitochondrial fraction contains both 3MST and rhodanese. Because thiosulfate is known as a substrate of both rhodanese and 3MST [36], we examined the specificity of both enzymes to thiosulfate using lysates of HEK-293F cells overexpressing 3MST

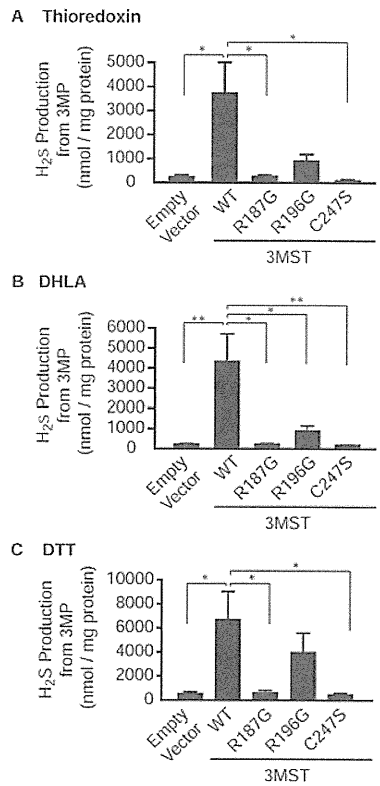

Figure 2 H_2S-producing activities of the 3MST mutants in the presence of Trx, DHLA or DTT

H_2S-producing activities of the 3MST mutants in the presence of Trx (**A**), DHLA (**B**) or DTT (**C**). Lysates of HEK-293F cells expressing 3MST mutants were mixed with 0.05 mM 3MP plus 20 μM Trx (**A**), 0.1 mM DHLA (**B**) or 0.1 mM DTT (**C**), and the amount of H_2S released was measured. All data are represented as the means $\pm$ S.E.M. for at least three experiments. $^*P < 0.05$, $^{**}P < 0.01$. WT, wild-type.

or rhodanese. 3MST produced H_2S from thiosulfate as well as from 3MP in the presence of DHLA ($P = 0.42$; Figure 3B). In contrast, rhodanese produced H_2S from thiosulfate more greatly than from 3MP ($P = 2.69 \times 10^{-7}$; Figure 3C). These observations suggest that 3MST produces H_2S from both 3MP and thiosulfate, whereas rhodanese reacts more specifically to thiosulfate.

H_2S production by 3MST and rhodanese is suppressed by sulfite

The thiosulfate cycle has been proposed to metabolize thiosulfate to produce H_2S and sulfite, which, in turn, is recycled into thiosulfate [37]. In this cycle, 3MST produces thiosulfate from sulfite and 3MP, and rhodanese metabolizes thiosulfate into H_2S and sulfite [37]. Sulfite is further oxidized into sulfate by sulfite oxidase or recycled into thiosulfate by rhodanese [6,37].

To examine whether or not the thiosulfate cycle is functioning, sulfite was added to the reaction mixture of a mitochondrial fraction with 3MP or thiosulfate, and the production of H_2S was measured. H_2S production from 3MP, as well as from thiosulfate, was suppressed in the presence of sulfite ($P < 0.01$, 0.1 or 1 mM sulfite compared with 0 mM sulfite control; Figures 4A and 4B). A similar experiment was performed to examine the effect of sulfate on this pathway. Sulfate did not have any effect on H_2S production from 3MP or thiosulfate (Figure 4C, $P = 0.99$; Figure 4D, $P = 0.98$). These observations suggest that the thiosulfate cycle may

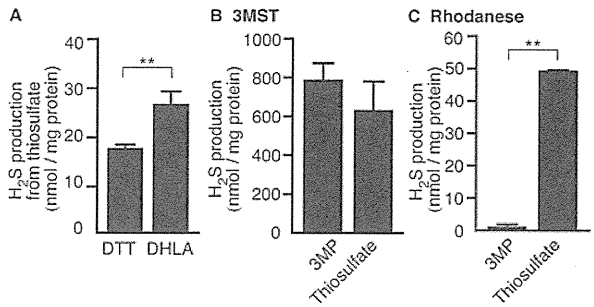

Figure 3 H_2S production from thiosulfate catalysed by 3MST and rhodanese

(**A**) The enhancing activity of DTT and DHLA on H_2S production from thiosulfate. The enhancing activity of 1 mM DTT and 1 mM DHLA on the production of H_2S from 1 mM thiosulfate was compared using a brain mitochondrial fraction. (**B** and **C**) The H_2S-producing activities of 3MST and rhodanese from 3MP and thiosulfate. Lysates of HEK-293F cells expressing 3MST (**B**) or rhodanese (**C**) were mixed with 0.1 mM DHLA and 0.01 mM 3MP or 1 mM thiosulfate, and H_2S released was measured. All data are represented as the means ± S.E.M. for three experiments. $^{**}P < 0.01$.

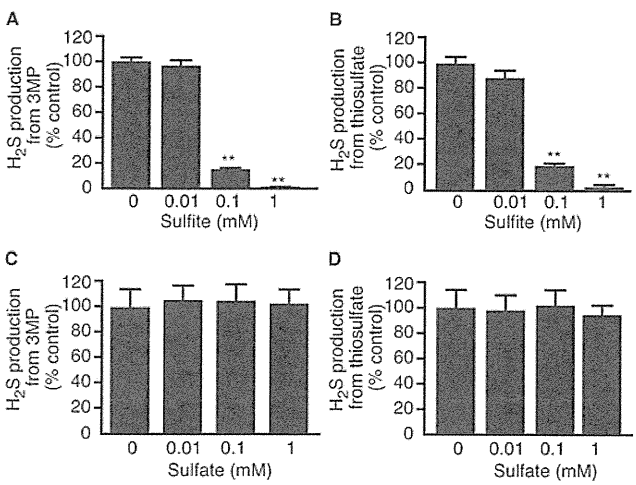

Figure 4 Sulfite suppresses H_2S production

(**A** and **B**) H_2S production from 3MP and thiosulfate is suppressed by sulfite (SO_3^{2-}). Sulfite suppresses H_2S production from 0.1 mM 3MP (**A**) and 1 mM thiosulfate (**B**) as substrates. (**C** and **D**) H_2S production from 3MP and thiosulfate is not suppressed by sulfate (SO_4^{2-}). Sulfate has no effect on H_2S production from 0.1 mM 3MP (**C**) and 1 mM thiosulfate (**D**). All data are represented as the means ± S.E.M. for three experiments. $^{**}P < 0.01$.

produce H_2S only when sulfite is immediately metabolized into non-toxic sulfate or recycled to thiosulfate.

Involvement of DHLA in release of H_2S from bound sulfane sulfur

Bound sulfane sulfur can release H_2S under physiological stimulations [3,39,40]. Since DHLA and the reduced form of Trx have a similar effect to DTT on persulfide, it is possible that both substances release H_2S from bound sulfane sulfur. This possibility was examined using brain post-nuclear supernatant, which abundantly contains bound sulfane sulfur. DHLA released H_2S from bound sulfane sulfur more efficiently than DTT ($P < 0.01$, 10 mM DHLA compared with 10 mM DTT; Figure 5), whereas 20 μM Trx did not release H_2S (results not shown). This observation suggests that DHLA may be a physiological releaser of H_2S from bound sulfane sulfur.

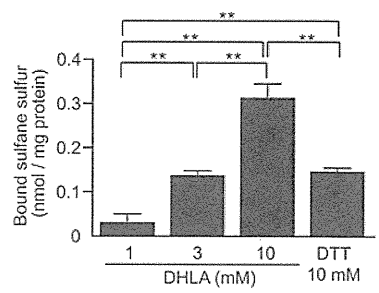

Figure 5 Release of H_2S from bound sulfane sulfur by DHLA

The post-nuclear supernatant was incubated at pH 8.4 with DHLA or DTT, and H_2S released was measured. All data are represented as the means ± S.E.M. for at least three experiments. $^{**}P < 0.01$.

DISCUSSION

The present study shows that the reduced form of Trx and DHLA are physiological disulfides associated with 3MST to produce H_2S from 3MP. 3MST can also produce H_2S from thiosulfate.

Roles of Trx and DHLA

3MST of *T. vaginalis* catalyses the formation of Trx persulfide, suggesting that 3MST can interact with Trx [21,41]. The present study shows that the reduced form of Trx is a potent physiological substance, which releases H_2S from persulfide produced at the active site of 3MST (Figures 1E and 1F). α-Lipoic acid/DHLA, which is synthesized in the mitochondria by lipoic acid synthase and also absorbed from food or supplement, is a cofactor for mitochondrial α-oxo acid dehydrogenases [23,32,33]. There has been little quantitative data on the lipoic acid concentration in biological samples. α-Lipoic acid is readily reduced to DHLA in cells [42]. Most lipoic acid is considered to be present as a protein-bound form in physiological systems [34]. The present study shows that DHLA is as potent as DTT for 3MST to produce H_2S (Figure 1G).

α-Lipoic acid induces Nrf2 (nuclear factor erythroid2-related factor 2), which increases the expression of Trx and Trx reductase [43–45]. α-Lipoic acid/DHLA may regulate the levels of Trx. Since H_2S induces Nrf2 [46], the production of H_2S may also regulate the levels of Trx and Trx reductase. In conjunction with the present finding that Trx enhances the H_2S-producing activity of 3MST (Figure 1E), Trx may induce positive feedback that promotes H_2S production through Nrf2 induction.

Dithiols are required to release H_2S from 3MST

Dithiols such as DTT and DHLA have negative redox potentials in the range from −0.29 to −0.33 V, and the redox potential of the active site dithiol of Trx is −0.26 V [47,48]. The redox potentials of monothiols such as GSH, cysteine and CoA are from −0.22 to −0.35 V [49], and those of NADH and NADPH are −0.320 V and −0.324 V [50]. Trx has two cysteine residues at its active site and DHLA is a dimercapto derivative of octanoic acid [33,47]. Both Trx and DHLA are effective at producing H_2S, whereas monothiols are not effective, even with greater redox potentials (Figure 1). Disulfide is required for a release of H_2S from 3MST.

T. vaginalis 3MST can produce Trx persulfide [21]. The present study suggests that 3MST can produce H_2S in the presence of dithiols such as Trx and DHLA. A possible mechanism is that

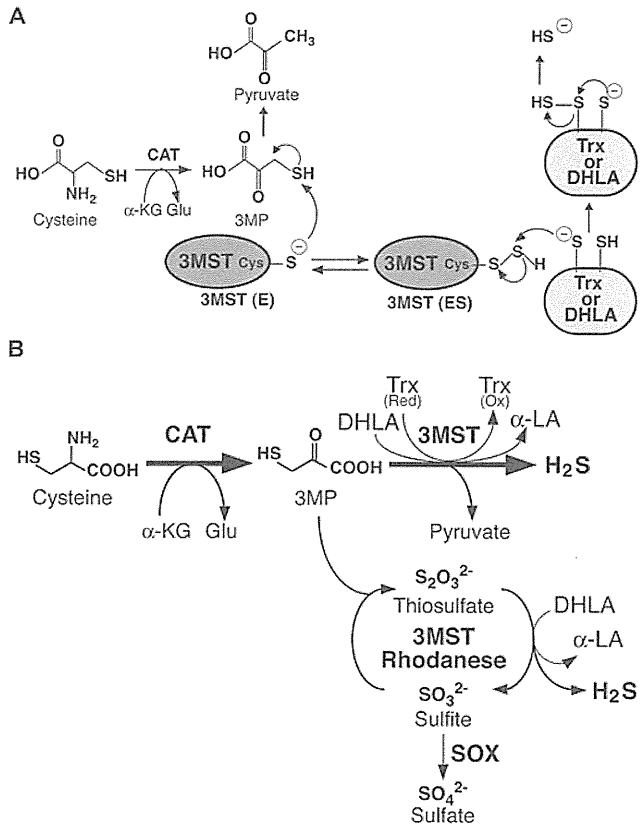

Figure 6 Production of H_2S by 3MST

(**A**) The production of H_2S by 3MST in the presence of Trx or DHLA. 3MST reacts with 3MP to produce H_2S via a persulfide intermediate. Trx or DHLA accepts a sulfur atom from a persulfide intermediate which is attacked by another thiol and releases H_2S. (**B**) The production of H_2S from 3MP and thiosulfate. Glu, glutamate; α-LA, α-lipoic acid; SOX, sulfite oxidase; Trx (Ox), oxidized form of Trx; Trx (Red), reduced form of Trx.

persulfide, which is produced at the active site Cys^{247} of 3MST by receiving thiol from 3MP, is attacked by one of the thiols in Trx or DHLA and transferred to them (Figure 6A). The transferred persulfide at one thiol is attacked by another thiol to release H_2S (Figure 6A).

H_2S production from thiosulfate

H_2S was produced in the presence of 1 mM DHLA or 1 mM DTT, whereas it was not produced in the presence of 20 μM Trx or 20 μM DTT (Figure 3A and results not shown). Thiosulfate forms sulfenyl thiosulfate with the cysteine residue of the 3MST catalytic site (Cys^{247}) and inhibits 3MST activity [36]. High concentrations of small molecules such as DHLA and DTT reduce sulfenyl thiosulfate to release H_2S, whereas a large molecule such as Trx has difficulty to access to sulfenyl thiosulfate at the catalytic site and does not produce H_2S at low concentrations [36].

Both 3MST and rhodanese can produce H_2S from thiosulfate (Figures 3B and 3C). It has been proposed that H_2S is produced from thiosulfate by rhodanese [6,37]. Although the affinity is weak compared with rhodanese, 3MST has an affinity to thiosulfate [36]. 3MST produces H_2S from both thiosulfate and 3MP, whereas rhodanese is more specific to thiosulfate than to 3MP (Figures 3B and 3C).

The thiosulfate cycle was initially studied in the presence of a high concentration (10 mM) of 3MP and thiosulfate [6]. Although the endogenous levels of sulfite have not been measured, the K_m value of mitochondrial sulfite oxidase, which oxidizes sulfite to sulfate, is 17 μM [51]. This observation suggests that the physiological levels of sulfite are less than 100 μM and may not suppress the production of H_2S from 3MP or thiosulfate. However, in the mitochondrial sulfite oxidase-deficient patients, the K_m value for sulfite of the sulfite oxidase mutant R160Q was 1.7 mM [51]. This observation suggests that the levels of sulfite are much greater than in the normal individuals and that sulfite oxidase deficiency may suppress H_2S production.

In conclusion, Trx and DHLA are physiological substances required for 3MST to produce H_2S. 3MST may also catalyse the reaction to produce H_2S from thiosulfate. The present study provides a new insight into a molecular mechanism for the production of H_2S catalysed by 3MST.

AUTHOR CONTRIBUTIONS

Yoshinori Mikami, Norihiro Shibuya and Yuka Kimura provided the experimental data. Noriyuki Nagahara provided an antibody against 3MST and contributed to the discussion. Yuki Ogasawara provided suggestions for experiments and contributed to the discussion. Hideo Kimura planned the experiments.

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Hydrogen Sulfide Protects the Retina from Light-induced Degeneration by the Modulation of Ca^{2+} Influx*

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Background: Hydrogen sulfide (H_2S) has been recognized as a signaling molecule as well as a cytoprotectant.
Results: Ca^{2+} regulates the 3-mercaptopyruvate sulfurtransferase/cysteine aminotransferase pathway to produce H_2S production. H_2S, in turn, regulates Ca^{2+} influx and protects retinal neurons from light-induced degeneration.
Conclusion: H_2S regulates Ca^{2+} levels and protects retinal neurons.
Significance: It provides a possible role of H_2S and its therapeutic application in the retina.

Hydrogen sulfide (H_2S) has recently been recognized as a signaling molecule as well as a cytoprotectant. Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) are well-known as H_2S-producing enzymes. We recently demonstrated that 3-mercaptopyruvate sulfurtransferase (3MST) along with cysteine aminotransferase (CAT) produces H_2S in the brain and in vascular endothelium. However, the cellular distribution and regulation of these enzymes are not well understood. Here we show that 3MST and CAT are localized to retinal neurons and that the production of H_2S is regulated by Ca^{2+}; H_2S, in turn, regulates Ca^{2+} influx into photoreceptor cells by activating vacuolar type H^+-ATPase (V-ATPase). We also show that H_2S protects retinal neurons from light-induced degeneration. The excessive levels of light exposure deteriorated photoreceptor cells and increased the number of TUNEL- and 8-hydroxy-2′-deoxyguanosine (8-OHdG)-positive cells. Degeneration was greatly suppressed in the retina of mice administered with NaHS, a donor of H_2S. The present study provides a new insight into the regulation of H_2S production and the modulation of the retinal transmission by H_2S. It also shows a cytoprotective effect of H_2S on retinal neurons and provides a basis for the therapeutic target for retinal degeneration.

Hydrogen sulfide (H_2S) is synthesized by three enzymes: cystathionine β-synthase (CBS),[2] cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3MST) along with cysteine aminotransferase (CAT) (1–6). 3MST produces H_2S from 3-mercaptopyruvate (3MP), which is produced from cysteine and α-ketoglutarate (α-KG) by CAT that is identical with aspartate aminotransferase (4, 7, 8). In the central nervous system, CBS is mainly localized to astrocytes, and 3MST to neurons (4, 9, 10). H_2S facilitates the induction of hippocampal long-term potentiation by enhancing the activity of NMDA receptors and induces Ca^{2+} waves in astrocytes (2, 11, 12). It also relaxes smooth muscle by activating K_{ATP} channels, regulates insulin release and induces angiogenesis (3, 13–16).

In addition to its role as a signaling molecule, H_2S has a cytoprotective effect. H_2S protects neurons from oxidative stress by enhancing the activity of γ-glutamylcysteine synthetase (γ-GCS) as well as the transport of cysteine and cystine, leading to reinstating the levels of GSH decreased by oxidative insults (17, 18). H_2S also protects cardiac muscle from ischemia-reperfusion injury (19). H_2S facilitates the nuclear localization of a transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2), which increases the expression of antioxidants such as thioredoxin and heme-oxygenase1 (20). H_2S produced by 3MST along with CAT can directly react with several cytotoxic oxidant species in mitochondria to protect cells (18, 21).

Calcium ion (Ca^{2+}) acts as a second messenger involved in a broad spectrum of intracellular signaling pathways. A number of enzymes are regulated by Ca^{2+}. NOS and heme oxygenase-2 are regulated by Ca^{2+}/calmodulin (22, 23). There are several enzymes that are directly regulated by Ca^{2+} without being mediated by Ca^{2+}-binding proteins. For examples, three intramitochondrial citrate cycle dehydrogenases; pyruvate dehydrogenase, NAD-isocitrate dehydrogenase, and oxoglutarate dehydrogenase were activated by Ca^{2+} (24). Recently, we demonstrated H_2S production by 3MST depends on thioredoxin and dihydrolipoic acid (DHLA) (25). However, the regulation of the activity of 3MST is not well understood.

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[2] The abbreviations used are: CBS, cystathionine β-synthase; 3MP, 3-mercaptopyruvate; 3MST, 3-mercaptopyruvate sulfurtransferase; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; Baf A1, bafilomycin A1; BBS, bicarbonate buffer saline; CAT, cysteine aminotransferase; CSE, cystathionine γ-lyase; DHLA, dihydrolipoic acid; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; α-KG, α-ketoglutarate; NEM, *N*-ethylmaleimide; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment; PLP, pyridoxal 5′-phosphate; ROS, reactive oxygen species; Trx, thioredoxin; V-ATPase, vacuolar type H^+-ATPase.

The present study shows that the H_2S production by 3MST and CAT is regulated by intracellular Ca^{2+}. H_2S, in turn, suppresses Ca^{2+} channels by activating vacuolar type H^+-ATPase (V-ATPase). Under physiological conditions, H_2S may maintain intracellular Ca^{2+} in low levels. The regulation of Ca^{2+} by H_2S may be failed by the excessive levels of light, and the photoreceptor cell degeneration occurs. Even under such conditions the administration of sodium hydrosulfide (NaHS), a donor of H_2S, suppresses photoreceptor degeneration. These observations suggest that H_2S protects photoreceptor cells from the insult caused by excessive levels of light.

EXPERIMENTAL PROCEDURES

Chemicals—Bafilomycin A1, calcium chloride, L-cysteine hydrochloride monohydrate, DTT, eosin Y, *N*-ethylmaleimide, magnesium sulfate, Mayer's hematoxylin solution, mercaptopyruvate sodium salt, α-ketoglutarate, potassium chloride, potassium dihydrogen phosphate, sodium chloride, sodium hydrogen carbonate, sodium sulfide, and sucrose were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Bicuculline methiodide, CremophorEL, diltiazem hydrochloride, EGTA, glucose, nifedipine, picrotoxin, pyridoxal 5′-phosphate monohydrate, sodium hydrosulfide, and Tween 20 were purchased from Sigma-Aldrich (St. Louis, MO). Calmodulin was purchased from Merck Calbiochem (Darmstadt, Germany) and W-7 from BIOMOL International (Plymouth Meeting, PA).

Determination of H_2S Producing Activity—All animal procedures were approved by the National Institute of Neuroscience Animal Care and Use Committee. Homogenates of the mouse retina were prepared in the ice-cold isolation buffer containing 50 mM potassium phosphate (pH7.4), 1 mM DTT, the protease inhibitor mixture complete EDTA-free (Roche Diagnostics, Mannheim, Germany) and 10 mM EGTA, and sonicated for 10 s using a sonicator (Branson Model 450; Branson Ultrasonics, Danbury, CT). Protein concentrations were determined by Bio-Rad D_c Protein Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

The enzyme reaction was performed as described (4) with modifications. The substrate was added to 0.1 ml of homogenates or cell lysates in a 15 ml centrifuge tube and incubated at 37 °C for 30 min. After adding 0.2 ml of 1 M sodium citrate buffer, pH 6.0, the mixtures were incubated at 37 °C for 10 min with shaking on a rotary shaker to facilitate a release of H_2S gas from the aqueous phase. Two ml of approximate 14.5 ml of head-space gas was applied to a gas chromatograph (GC-2014; Shimadzu, Kyoto, Japan) equipped with a flame photometric detector and a data processor C-R8A Chomatopac (Shimadzu). The concentrations of H_2S were calculated using a standard curve of 0 to 5 nM of Na_2S, as a source of H_2S.

Constructs and Transient Transfection of HEK 293-F Cells—The constructs of 3MST and CAT expression plasmids were described previously (4). Transient transfection of HEK 293-F cells in suspension cultures was performed using a FreeStyle 293 Expression System (Invitrogen Life Technologies Corp., Carlsbad, CA). HEK 293-F cells efficiently express externally applied expression plasmids and grow in higher densities in suspension cultures than regular plating. For transfection, 10 μg of expression plasmids were mixed with 15 μl of transfection reagent 293 fectin (Invitrogen) and then added to 1×10^7 cells in 100 ml Erlenmeyer flasks with 10 ml of FreeStyle 293 expression medium. Cells were incubated with shaking at 125 rpm on a rotary shaker at 37 °C in a humid atmosphere with 10% (v/v) CO_2. Transfection efficiency was >90%. Cells were harvested at 48 h post-transfection. The HEK 293-F cells were precipitated by centrifugation at 1000 × *g* for 5 min. After washing with ice-cold PBS, cell pellets were resuspended in ice-cold buffer A and sonicated.

Immunohistochemistry—Mouse eyecups were fixed overnight at 4 °C with 4% (w/v) paraformaldehyde in PBS. After rinsing in PBS, tissues were cryoprotected in 5, 15, and 30% (w/v) sucrose/PBS in order and embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical, Tokyo, Japan) in an aluminum boat and frozen in liquid nitrogen. Retinal sections with 12-μm thickness were cut by a cryostat (CM1900, Leica Instruments, Nussloch, Germany) at −20 °C and mounted on MAS-coated slides (Matsunami, Osaka, Japan) and dried. The sections were incubated with 10% (v/v) normal goat serum (Nichirei, Tokyo, Japan) or blocking solution (Roche Diagnostics) at room temperature for 1 h. For double immunofluorescence staining, sections were incubated overnight at 4 °C with antibodies against rabbit anti-3MST polyclonal antibody (1:3000) (26), sheep anti-aspartate aminotransferase (AST; cytosolic CAT) antibody (1:3000; Rockland, Gilbertsville, PA), sheep anti-glutamic-oxaloacetic transaminase 2 (GOT2; mitochondrial CAT) antibody (1:1000; Lifespan biosciences, Seattle, WA), mouse anti-calbindin-D antibody (1:1000; clone CB-955, Sigma), rabbit anti-CSE antibody (1:3000) (27), rabbit anti-CBS antibody (1:3000) (9), or mouse anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) antibody (5 μg/ml, Japan Institute for the Control of Aging, Nikken SEIL, Shizuoka, Japan). The immunoreaction was visualized with secondary subclass-specific Alexa Fluor 488-, Alexa Fluor 555-conjugated (Invitrogen) or Cy3-conjugated (Jackson ImmunoResearch laboratories, West Grove, PA) antiserum at a dilution of 1:200. For nuclear staining, sections were incubated with 2 μg/ml Hoechst 33342 (Invitrogen) in PBS for 2 min at room temperature. The specimens were observed under a laser confocal microscope (TCS SP2; Leica Microsystems, Wetzlar, Germany) mounted on a Leica Microsystems inverted microscope (DMIRE2) using the oil-immersion objective (63×; 1.32 NA; Leica Microsystems).

Preparation of Mouse Retinal Slices and Ca^{2+} Imaging—The mice were kept in a room maintained under a 12 h/12 h light cycle. Mice were dark-adapted for 2 h before experiments. Subsequent manipulations were performed under dim red light. The mice were anesthetized and killed by cervical dislocation. The eyes were enucleated and immediately put in ice-cold bicarbonate-buffered saline (BBS), composed of: 120 mM NaCl, 22.6 mM $NaHCO_3$, 3 mM KCl, 0.5 mM KH_2PO_4, 2 mM $CaCl_2$, 0.5 mM $MgSO_4$, and 6 mM glucose. The BBS solution (pH 7.4) was continuously bubbled with 95% (v/v) O_2 and 5% (v/v) CO_2. After enucleation, the anterior segment of the eye including the lens was removed. The resulting eyecup was detached from the pigmented epithelium, and a section of retina was placed vitreal side down on a piece of filter paper (13 mm in diameter, Type HAWP, 0.45 μm pores, Millipore, Billerica, MA). After adher-

ing to the filter paper, retinal slices with 200–250 μm thickness were manually cut with a razor blade.

For Ca^{2+} imaging, retinal slices were loaded with a cell-permeant Ca^{2+} indicator, Calcium Green-1 AM (Invitrogen), by incubating slices for 50 min in the dark with 1 μM Calcium Green-1 AM and 0.01% (v/v) CremophorEL in BBS solution. The slices were positioned in the glass-bottom dish (Matsunami) with high-pure white vaseline (Wako) for viewing the retinal layers. The dish was mounted on an upright fixed stage microscope (Leica DMLFS, Leica Microsystems). The images were acquired using a xenon lamp (Osram, Augsburg, Germany), a water immersion objective (40X; 0.80 NA; Leica Microsystems) and a CCD camera (C4742–95-12ER; Hamamatsu Photonics, Shizuoka, Japan). Excitation and emission were controlled by LEP MAC5000 filter wheel and a shutter controller (Ludl electronic products, Hawthorne, NY). Frame duration ranged from 20–35 ms and each image was acquired at 5-s intervals. Images were acquired and analyzed using Aquacosmos software Ver. 2.0 (Hamamatsu Photonics).

Light Damage—Male ICR mice (Japan Clea), aged 8–10 weeks were kept under controlled lighting conditions (12h-light/12h-dark). After dark adaptation for 24 h, NaHS (0.4375 μmol/kg) or vehicle (PBS) was administered intraperitoneally to mice 15 min before exposure to light. Mice were dilated with 5% (w/v) phenylephrine eye drops (Kowa, Tokyo, Japan) 5 min before exposure to light. Nonanesthetized mice were exposed to while fluorescent light (1220 lm) for 2 h in cage (375 cm^2) with reflective interior. After light exposure, animals remained in darkness until they are analyzed.

For analysis with a light microscopy, the eyes were enucleated under dim red illumination, fixed with 4% (w/v) paraformaldehyde in PBS at 4 °C overnight and immersed in 5, 15, and 30% (w/v) sucrose in PBS in order at 4 °C. The eyes were then submerged into an O.C.T. compound and frozen. The retinal sections with 12-μm thickness were cut by a cryostat at −20 °C and stored at −80 °C until staining. The sections were stained with Mayer's hematoxylin and eosin for histological analysis. To detect the retinal cell death, TUNEL staining was performed. The sections were washed three times in PBS and incubated in PBS containing 2 μg/ml proteinase K (Merck) at 37 °C for 10 min. The sections were washed three times in PBS and incubated in 0.3% (w/v) hydrogen peroxide with methanol at room temperature for 5 min to block endogenous peroxidase activity. The sections were washed and incubated with terminal deoxyribonucleotidyl transferase (TdT; Invitrogen) with biotin-labeled dCTP (Invitrogen) at 37 °C for 1 h. The biotin-labeled dCTP was detected colorimetrically using peroxidase-conjugated streptavidin (Nichirei) and its chromogenic substrate 3, 3′-diaminobenzidine (Dojindo, Kumamoto, Japan). Sections were imaged by an epifluorescence microscopy (Axiophot, Carl Zeiss, Germany) using a Plan-NEOFLUAR 40× objective (Carl Zeiss). Three light-microscope images were photographed from each eye sample, and the number of TUNEL-positive cells in the outer nuclear layer was averaged for five eye samples.

Statistical Analysis—All statistical analyses of the data were performed using Microsoft Excel 2004 for Mac (Microsoft, Redmond, WA) with the add-in software Statcel2 (OMS, Saitama, Japan). Differences between two groups were analyzed with Student's *t* test. Differences between three or more groups were analyzed with one-way analysis of variance (ANOVA).

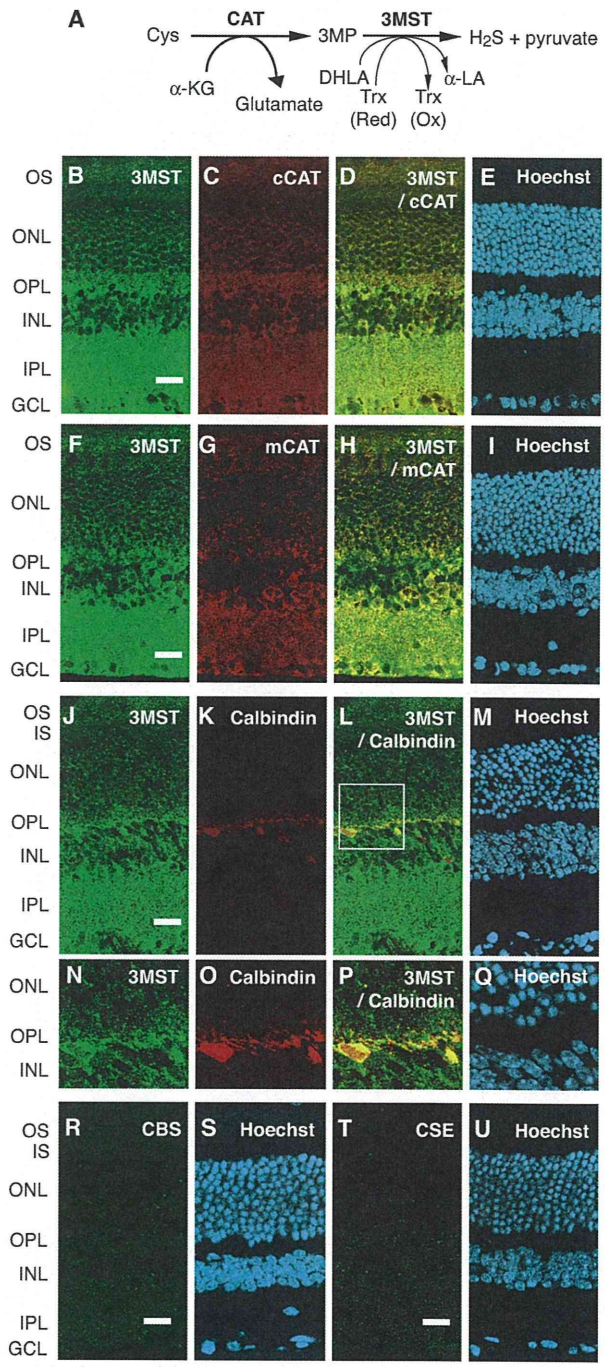

FIGURE 1. **Localization of 3MST and CAT in the retina.** *A*, schematic representation of the production of H_2S by 3MST and CAT. *Cys*, cysteine; *α-KG*, α-ketoglutarate; *3MP*, 3-mercaptopyruvate; *Trx* (ox), oxidized form of thioredoxin; *Trx* (*red*), reduced form of thioredoxin; *DHLA*, dihydrolipoic acid; *α-LA*, α-lipoic acid. *B–I*, 3MST (*B*, *F*) co-localizes with cytosolic CAT (cCAT, *C*) and mitochondrial CAT (mCAT, *G*). 3MST and cCAT (*D*) or mCAT (*H*) are merged. *J–Q*, 3MST is localized to horizontal cells. 3MST (*J*, *N*) colocalizes with calbindin, a specific marker of horizontal cells (*K*, *O*). 3MST and calbindin were merged (*L*, *P*). The square area in *L* is magnified and shown in *N–Q*. *R–U*, neither CBS (*R*) nor CSE (*T*) is found in the retina. Nuclei were stained by Hoechst 33342 (*E*, *I*, *M*, *Q*, *S*, *U*, respectively). *B–U*, scale bars, 20 μm. *OS*, outer segment; *IS*, inner segment; *ONL*, outer nuclear layer; *OPL*, outer plexiform layer; *INL*, inner nuclear layer; *IPL*, inner plexiform layer; *GCL*, ganglion cell layer.