Uniwersytet Jagielloński

Collegium Medicum

Wydział Lekarski

Patrycja Bronowicka-Adamska

Study of the activity and expression of the enzymes involved in the formation of hydrogen sulphide in various experimental systems

Badanie aktywność i ekspresja enzymów biorących udział w tworzeniu siarkowodoru w różnych układach doświadczalnych

Praca doktorska

Promotor: Prof. dr hab. Maria Wróbel

Katedra Biochemii Lekarskiej

Uniwersytet Jagielloński Collegium Medicum Wydział Lekarski

Pracę wykonano w Katedrze Biochemii Lekarskiej

Uniwersytet Jagielloński Collegium Medicum Wydział Lekarski

Kierownik jednostki: Prof. dr hab. Piotr Laidler

Kraków, 2017 rok

Składam serdeczne podziękowania wszystkim, bez których niniejsza praca nie mogłaby powstać.

Szczególne wyrazy wdzięczności kieruję pod adresem Pani Profesor Marii Wróbel, dziękując za serdeczność, zaangażowanie i ogromną pomoc merytoryczną i motywację zarówno do prowadzenia badań oraz do napisania tej pracy.

Najbliższym współpracownikom z Katedry Biochemii Lekarskiej UJCM, w której powstawała ta praca dziękuję za przyjazną atmosferę, wszelkiego rodzaju dyskusję, życzliwość oraz za nieocenioną pomoc w prowadzeniu badań doświadczalnych.

Dziękuję również swoim Bliskim, w szczególności Rodzicom, Mężowi oraz Córkom, za wyrozumiałość, cierpliwość oraz wsparcie.

SPIS TREŚCI

Wykaz zastosowanych skrótów

CAT	aminotransferaza cysteinowa
CBS	beta-syntaza cystationinowa
CSH	cysteina
CSSC	cystyna
CTH, CGL	gamma-cystationaza
CTN	cystationina
GSH	glutation zredukowany
GSSG	glutation utleniony
H_2S	siarkowodór
HSer	D,L-homoseryna
LA	mleczan
LDH	dehydrogenaza mleczanowa
MPST	transferaza siarkowa 3-merkaptopirogronianu
3MP	3-merkaptopirogronian
PA	pirogronian
PCA	kwas nadchlorowy
PPG	D,L-propargylglicyna
RP-HPLC	chromatografia w układzie odwróconych faz

Wprowadzenie

Przedstawiona rozprawa doktorska pt. *„Study of the activity and expression of the enzymes involved in the formation of hydrogen sulphide in various experimental systems”* stanowi monotematyczny cykl 4 prac oryginalnych opublikowanych w czasopismach obecnych w bazie PubMed. Doktorant jest pierwszym autorem we wszystkich pracach. Łączna wartość „Impact Factor” dla prezentowanych publikacji wynosi 10,3.

Przedstawiony cykl publikacji prezentuje wyniki prac badawczych, których tematem przewodnim było badanie tworzenia siarkowodoru (H_2S) w różnych układach doświadczalnych. Przedstawione badania miały na celu określenie udziału poszczególnych enzymów w produkcji H_2S w wybranych tkankach (ludzkich oraz ssaków) oraz w hodowlach komórkowych - prawidłowych i nowotworowych, jak również zmian zachodzących w tworzeniu siarkowodoru w stanie zapalnym śluzówki żołądka szczurzego

W cykl artykułów, o które oparta jest rozprawa doktorska, wchodzą:

1. **Bronowicka-Adamska, P**.; Zagajewski, J.; Czubak, J.; Wróbel, M. RP-HPLC method for the quantitative determination of cystathionine, cysteine and glutathione: an application for the study of the metabolism of cysteine in human brain. *Journal of Chromatography B.* **2011,** *879,* 2005-2009.
2. **Bronowicka-Adamska, P**.; Zagajewski, J.; Wróbel, M. An application of RP-HPLC for determination of the activity of cystathionine beta-synthase and gamma-cystathionase in tissue homogenates. *Nitric Oxide*. **2015**, *46*, 186-191.
3. **Bronowicka-Adamska, P**.; Bentke, A.; Wróbel, M. Hydrogen sulfide generation from L-cysteine in the human glioblastoma - astrocytoma U-87 MG and neuroblastoma SHSY5Y cell lines. *Acta Biochimica Polonica*. **2017**, *64,* 171–176. doi.org/10.18388/abp.2016_1394.
4. **Bronowicka-Adamska, P**.; Wróbel, M.; Magierowski, M.; Magierowska, K.; Kwiecień. S.; Brzozowski, T. Hydrogen sulphide production in gastric mucosa of rats exposed to stress. *Moleculs*. **2017**, *22*, 530; doi:10.3390/molecules22040530.

Wszystkie prace zostały opublikowane i są zamieszczone w rozprawie doktorskiej w wersji oryginalnej (format PDF).

Wyniki przedstawione w pracy doktorskiej były również prezentowane w formie siedmiu doniesień zjazdowych (sześciu plakatowych i jednego ustnego):

1. **Bronowicka-Adamska, P.;** Zagajewski, J.; Wróbel, M. The effect of D,L-propargylglycine on the activity of the cystathionine β-synthase/γ-cystathionase enzymatic system in mouse liver. *Acta Biochim. Pol*. **2008**, Vol. 55, Suppl. 3; 186.
2. **Bronowicka-Adamska, P**.; Wróbel, M.; Zagajewski, J. The activity of hydrogen sulfide generating enzymes cystathionine β-synthase and γ-cystathionase in mouse tissue homogenates. *Amino Acids*. **2009**, *37*, Suppl. 1; S85.
3. **Bronowicka-Adamska, P**.; Jurkowska, H.; Wróbel, M.; Zagajewski, J. Determination and quantification of cystathionine in various regions of human brain using the RP-HPLC method. *Acta. Biochim. Pol*. **2010**, Vol. *57*, Suppl. 4; 195.
4. **Bronowicka-Adamska, P**.; Jurkowska, H.; Zagajewski, J.; Wróbel, M. The activity of cystathionine β-synthase and γ-cystathionase in U-87 MG cells. *Acta. Biochim. Pol*. **2011**, Vol. *58*, Suppl. 2; 225.
5. **Bronowicka-Adamska, P.;** Wróbel, M. The activity of hydrogen sulfide generating enzymes in human cell lines. *Nitric Oxide-Biology and Chemistry*, **2014**, Vol. *39*, Suppl. 1 p. S33-S33, 3rd International Conference on H_2S Biology and Medicine, Kyoto, Japan, 4-6 Jun 2014.
6. **Bronowicka-Adamska, P.;** Wróbel, M.; Magierowski, M.; Jasnos, K.; Kwiecień, S.; Brzozowski, T. Hydrogen sulfide production in gastric mucosa of rats exposed to stress. *Nitric Oxide*, **2015**, Vol. 47, Suppl. 1, s. S38, abstr. PP63, 3rd European Conference on the Biology of Hydrogen Sulfide H_2S 2015: Athens, Greece, May 3-6.
7. **Bronowicka-Adamska, P.** Siarkowodór w stanach zapalnych żołądka. Kongres „Medycyna odkrywa nowe oblicza siarki". Kraków 30 wrzesień **2016** r. – referat.

Niniejsza rozprawa doktorska została zrealizowana ze środków pochodzących z dotacji celowej dla młodych naukowców:

1. K/ZBW/000149 „Aktywność układu enzymatycznego β-syntazy cystationinowej i γ-cystationazy związanego z tworzeniem w tkankach siarkowodoru”, kierownik projektu: mgr Patrycja Bronowicka-Adamska
2. K/ZBW/000547 „Badanie metabolizmu cystationiny w różnych regionach mózgu ludzkiego z wykorzystaniem metody wysokosprawnej chromatografii cieczowej (RP-HPLC)”, kierownik projektu: mgr Patrycja Bronowicka-Adamska
3. K/DSC/00137 „Oznaczanie siarkowodoru w układach biologicznych”, kierownik projektu: mgr Patrycja Bronowicka-Adamska
4. K/DSC/003570 „Badanie aktywności i ekspresji enzymów β-syntazy cystationinowej, γ-cystationazy oraz transferazy siarkowej 3-merkaptopirogronianu, w przebiegu procesu zapalnego związanego z uszkodzeniem śluzówki żołądka szczurów wywołanego stresem wynikającym z oziębienia i unieruchomienia (WRS ang. water immersion restraint stress)”, kierownik projektu: mgr Patrycja Bronowicka-Adamska

Wstęp

Siarkowodór (H_2S) przez wiele lat uznawany była za bezbarwny, toksyczny gaz o charakterystycznym zapachu, który blokuje oksydazę cytochromową, prowadząc do ciężkiego niedotlenienia. Przełom w dziedzinie nauki i medycyny przyniosły liczne badania prowadzone w latach dziewięćdziesiątych XX wieku wskazujące, że siarkowodór spełnia najważniejsze kryteria charakteryzujące substancje przekaźnikowe: jest syntetyzowany endogennie w regulowanych reakcjach enzymatycznych oraz wykazuje specyficzne działania biologiczne w stężeniach fizjologicznych.

H_2S jest palnym gazem, rozpuszczalny w wodzie, alkoholu oraz innych rozpuszczalnikach organicznych. Jest wysoce lipofilny – łatwo wnika do wszystkich komórek. Reaguje z nitrozotiolami (RSNO), uwalniając NO. Jest silnym czynnikiem redukującym – reaguje z anionorodnikami ponadtlenkowymi, nadtlenoazotynem, podchlorynem i nadtlenkami wodoru. W warunkach fizjologicznych, w pH = 7.4, około 1/3 H_2S występuje w postaci niezdysocjowanej, a 2/3 dysocjuje na jony H^+ i HS^-, a następnie na S^{2-}. Okres półtrwania H_2S w powietrzu wynosi od 12 do 37 h i jest uzależniony od temperatury [Wang, 2012].

H_2S w osoczu oraz w innych tkankach występuje w stężeniu około 30-100 μM. W mózgu poziom H_2S może być nawet 3-krotnie wyższy niż w osoczu [Singh i wsp., 2009]. Ze środowiska zewnętrznego wchłania się głównie przez płuca i nieznacznie przez skórę. Wydala się częściowo w stanie niezmienionym tą samą drogą, a częściowo jest przekształcany do tlenków siarki oraz kwasu siarkowego i w tych postaciach wydalany z moczem.

Endogennie utworzony H_2S służy jako neuromodulator w mózgu [Panthi i wsp., 2016, Paul i Snyder, 2015]. Poprzez aktywację receptorów NMDA (N-metylo-D-asparaginowych) i zwiększenie odpowiedzi nerwów obwodowych, H_2S może odgrywać znaczącą rolę

w procesach związanych z zapamiętywaniem i uczeniem się [Ishigami i wsp., 2009, Shibuya i wsp., 2009]. Zwiększoną syntezę H_2S zaobserwowano u pacjentów z zespołem Downa i wstrząsem septycznym, a jego zmniejszone wytwarzanie odnotowano w chorobie Alzheimera. H_2S aktywuje kanały TRPA1 w astrocytach oraz oddziałuje na homeostazę jonów Ca^{2+} w neuronach, astrocytach i mikrogleju [Kimura, 2013, Moore i Whiteman, 2005, Tan i wsp., 2010]. H_2S ma właściwości antyoksydacyjne, zwiększa produkcję glutationu w komórkach nerwowych [Kimura i Kimura, 2004, Kimura i wsp., 2010].

H_2S w układzie krążenia wykazuje między innymi działanie wazodylatacyjne, wpływa na obniżenie ciśnienia tętniczego oraz hamuje proliferację komórek mięśniówki naczyń [Olas, 2014].

H_2S jest również produkowany w przewodzie pokarmowym i odgrywa znaczącą rolę w gastroprotekcji, wpływa na mechanizmy obronne wzmacniające barierę śluzówkową żołądka, w tym na zwiększenie odporności na uszkodzenia wywołane przez niesteroidowe leki przeciwzapalne, stres oraz alkohol [Magierowski i wsp., 2013]. Eksperyment przeprowadzony przez Lou i wsp., 2008, na zwierzęcym modelu stresu indukowanego przez zanurzenie zwierząt w zimnej wodzie i ich unieruchomienie, wykazał zmniejszenie liczby uszkodzeń błony śluzowej żołądka i obniżenie peroksydacji lipidów w porównaniu z grupą zwierząt kontrolnych, co było związane z wyższym poziomem H_2S.

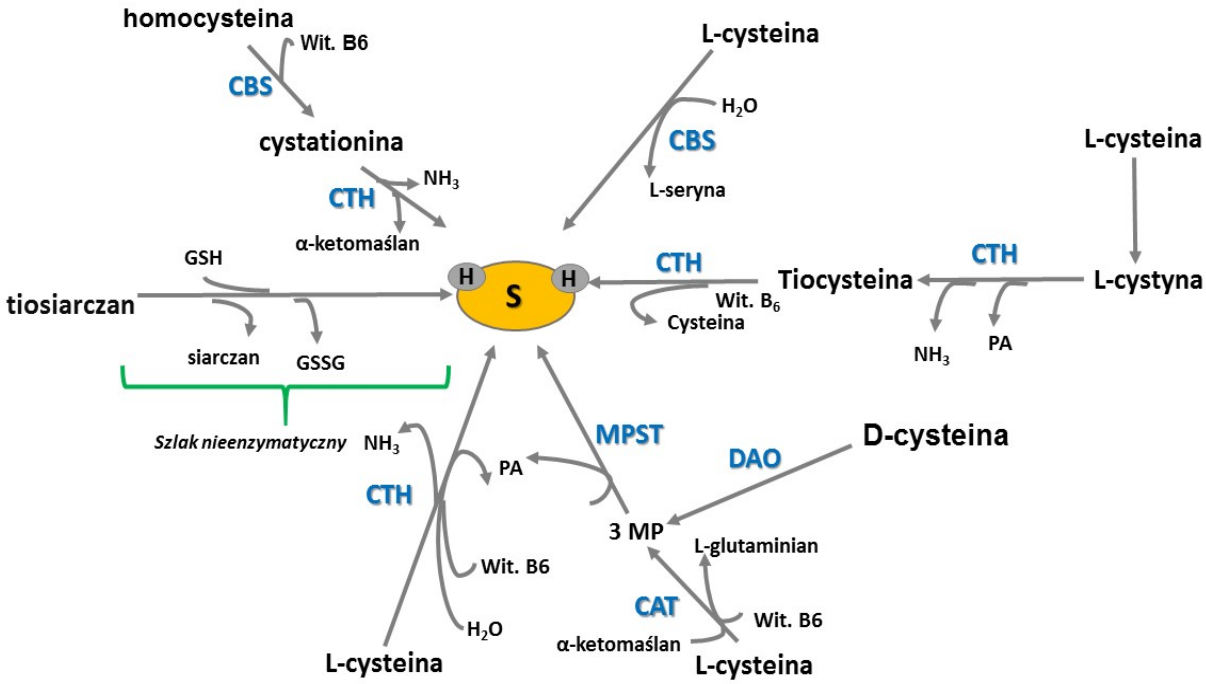
W tkankach ssaków H_2S jest syntetyzowany endogennie z L-cysteiny. W 2013 roku Shibuya i wsp. potwierdzili, że substratem do syntezy H_2S może być również D-cysteina. Szlak przemian L-cysteiny prowadzący do wytworzenia H_2S polega na nieoksydacyjnym usunięciu atomu siarki z jej struktury z udziałem enzymów, których kofaktorem jest fosforan pirydoksalu, zaangażowanych również w transulfurację homocysteiny. Są to: beta-syntaza cystationinowa (CBS, EC 4.2.1.22) oraz gamma-cystationaza (CTH, EC 4.4.1.1). L-cysteina reaguje z homocysteiną w reakcji katalizowanej przez CBS. W wyniku tej reakcji powstaje

cystationina oraz H_2S. Cystationina przy udziale CTH rozkładana jest do cysteiny, α- ketomaślanu i amoniaku. Siarkowodór powstaje w reakcji katalizowanej przez CBS, w której homocysteina reaguje z cysteiną. Cysteina utlenia się do cystyny, która w obecności CTH rozkładana jest do tiocysteiny i pirogronian (PA). Przekształcanie tiocysteiny do cysteiny związane jest z procesem uwalniania siarkowodoru. Trzecim enzymem odpowiedzialnym za produkcję H_2S jest transferaza siarkowa 3-merkaptopirogronianu (MPST, EC 2.8.1.2), która współdziała z aminotransferazą cysteinową (CAT, EC 2.6.1.3). CAT katalizuje reakcję transaminacji L-cysteiny. W wyniku tej reakcji powstaje 3-merkaptopirogronian (3MP). Transferaza siarkowa 3-merkaptopirogronianu przenosi siarkę na resztę kwasu siarkowego (IV). Produktem tej reakcji jest pirogronian i tiosiarczan, który jest następnie redukowany do siarkowodoru. Reakcja ta zachodzi w obecności zredukowanego glutationu (GSH). Synteza siarkowodoru z D-cysteiny odbywa się przy udziale dwóch enzymów: oksydazy D-aminokwasowej (DAO) oraz MPST. DAO katalizuje reakcje przekształcenia D-cysteiny do 3MP, który pod wpływem MPST jest przekształcany do PA i H_2S [Shibuya i wsp., 2013, Brodek i Olas, 2016] (Schemat 1).

Siarkowodór jest utleniany początkowo do tiosiarczanu, który ulega dalszemu przekształceniu do siarczynu, a następnie do siarczanu. Proces ten zachodzi głównie w mitochondriach. Przekształcenie tiosiarczanu do siarczynu katalizowane jest przez transferazę siarkową tiosiarczan: cyjanek (rodanaza, TST). Reakcja ta polega na przeniesieniu siarki pochodzącej z tiosiarczanu na cyjanek, w wyniku czego powstaje rodanek oraz siarczyn (Schemat 2).

Przy dostatecznym poziomie L-cysteiny, zarówno CBS jak i CTH mogą syntetyzować siarkowodór, natomiast przy braku cysteiny konieczna jest obecność obu enzymów, a substratem do wytwarzania H_2S staje się homocysteina. CBS jest głównym enzymem

odpowiedzialnym za syntezę H_2S w mózgu, CTH odgrywa podobną funkcję w układzie krążenia [Abe i Kimura, 1996].



Schemat 1. Synteza H_2S w tkankach ssaków [Wang, 2012].

CTH: gamma-cystationaza, **CBS**: beta-syntaza cystationinowa, **MPST**: transferaza siarkowa 3-merkaptopirogronianu, **3MP**: 3-merkaptopirogronian, **PA**: pirogronian, **DAO**- oksydaza D-aminokwasowa

Schemat 2. Katabolizm H_2S w mitochondrium [Kabil i Banerjee, 2010].

SQR: siarczek: ubichinon oksydoreduktaza, **TST**: rodanaza

Cel pracy

Celem pracy było:

1. Badanie możliwości różnych tkanek prawidłowych (wątroba, nerki, mózg) i nowotworowych (komórki ludzkie układu nerwowego; linia SH-SY5Y i U87-MG) w zakresie tworzenia H_2S poprzez oznaczanie aktywności i ekspresji enzymów biorących udział w jego przemianach: beta-syntazy cystationinowej (CBS), gamma-cystationazy (CTH), transferazy siarkowej 3-merkaptopirogronianu (MPST) oraz rodanazy (TST).

2. Badanie roli H_2S w przebiegu procesu zapalnego związanego z uszkodzeniem śluzówki żołądka szczurów wywołanego stresem wynikającym z oziębienia i unieruchomienia (WRS; ang. water immersion and restraint stress).

Cele szczegółowe:

1. Zastosowanie i zmodyfikowanie metody RP-HPLC (ang. Reversed phase high-performance liquid chromatography) do oznaczania poziomu zredukowanego (GSH) i utlenionego (GSSG) glutationu oraz cysteiny (CSH) i cystyny (CSSC) w próbkach o małej objętości [Publikacja nr 1].

2. Opracowanie i zastosowanie:

   - metody oznaczania aktywności CTH i CBS w próbkach o niskich aktywnościach tych enzymów oraz małych objętościach, z niską zawartością białka. W tym celu wprowadzono metodę RP-HPLC do oznaczania produktów końcowych reakcji katalizowanych przez te enzymy, α-ketomaślanu oraz cystationiny [Publikacja nr 2],
   - metody oznaczania poziomu H_2S w hodowlach komórkowych i homogenach tkankowych [Publikacja nr 3 i 4],

- badania ekspresji CTH, CBS i MPST oraz TST na poziomie mRNA oraz na poziomie białka (Western blot), ustalenia warunków reakcji odwrotnej transkrypcji i PCR [Publikacja nr 3 i 4].

3. Badanie poziomu H_2S, aktywności oraz ekspresji CTH, CBS, MPST oraz TST w prawidłowych żołądkach szczurzych, żołądkach z uszkodzeniem śluzówki w wyniku stresu wynikającego z oziębienia w zimnej wodzie i unieruchomienia przez 3,5 godziny (WRS) oraz żołądkach szczurów, które przed wywołaniem uszkodzeń śluzówki w wyniku stresu, otrzymały w iniekcji dootrzewnowej NaHS (prekursor H_2S) [Publikacja nr 4].

Materiał do badań

Tkanki ludzkie

Wycinki makroskopowo niezmienionego ludzkiego mózgu pobierano pośmiertnie w Zakładzie Patomorfologii Klinicznej i Doświadczalnej Katedry Patomorfologii CM UJ oraz w Zakładzie Medycyny Sądowej CM UJ, Kraków. Wycinki były pobierane podczas sekcji przeprowadzonych do 24 godzin od zgonu od pacjentów w wieku 40-60 lat z następujących regionów mózgu: kora czołowa (*Frontal cortex*), kora ciemieniowa (*Parietal cortex*), wzgórze (*Thalamus*), hipokamp (*Hippocampus*), móżdżek (*Cerebellum*) oraz jądra podkorowe (*Nuclei basales*). Natychmiast po pobraniu wycinki w całości zamrażano w ciekłym azocie i przechowywano w temperaturze -80ºC do momentu oznaczania aktywności CTH, poziomu białka oraz niskocząsteczkowych związków siarki (GSH, GSSG, cysteina, cystyna oraz cystationiny) metodą RP-HPLC. Przeprowadzone badania uzyskały zgody Komisji Bioetycznej UJ – opinia nr KBET/199/B/2002 z dnia 21 lutego 2002 roku.

Tkanki mysie

Do eksperymentu wykorzystano 25 myszy rasy Swiss (6 - 8 tygodniowe samice i samce), pochodzących z hodowli Zwierzętarni Katedry Immunologii Klinicznej

i Transplantologii Polsko-Amerykańskiego Instytutu Pediatrii CM UJ, Kraków. Myszy natychmiast po przywiezieniu do Katedry Biochemii Lekarskiej CM UJ były uśmiercane przez dyslokacje kręgów szyjnych przy użyciu odpowiedniej pęsety. Do doświadczenia zostały pobrane następujące tkanki: wątroba, mózg, nerki. Wypreparowane tkanki przeznaczone do doświadczenia bezpośrednio po pobraniu zamrażano w ciekłym azocie, a następnie przechowywano do czasu analizy aktywności CTH, CBS, poziomu białka oraz niskocząsteczkowych związków siarki (GSH, GSSG, cysteina, cystyna oraz cystationiny, homoseryny) metodą RP-HPLC. Wszystkie procedury wykorzystywane w eksperymencie otrzymały zgodę I Lokalnej Komisji Etycznej ds. Doświadczeń na Zwierzętach działającej przy Uniwersytecie Jagiellońskim w Krakowie - opinia nr 26/III/2009).

Tkanki szczurze

Samce szczurów rasy Wistar (220 - 300g) 24 h przed eksperymentem zostały pozbawione pożywienia, ale miały swobodny dostęp do wody. Zwierzęta zostały podzielone na 3 grupy badawcze: 1) grupa kontrolna, 2) grupa szczurów, u których wywoływano uszkodzenie śluzówki w wyniku stresu wynikającego z oziębienia w zimnej wodzie i unieruchomienia (WRS) 3) grupa szczurów, u których przed wywołaniem stresu poddano za pomocą iniekcji dootrzewnowej NaHS (prekursor H_2S) w dawce 5mg/kg. Po zakończeniu eksperymentu szczury utrzymywano w znieczuleniu pentobarbitalem (60 mg/kg, dootrzewnowo), a następnie usuwano im żołądki. Żołądek rozcinano wzdłuż krzywizny większej i pobierano błonę śluzową, natychmiast zamrażano w ciekłym azocie oraz przechowywano w temperaturze - 80°C aż do czasu analizy.

Badania prowadzone były w ramach współpracy z Katedrą i Zakładem Fizjologii Wydziału Lekarskiego Uniwersytetu Jagiellońskiego. Badania wykonano po uzyskaniu zgody dla wykonawców eksperymentu od Lokalnej Komisji Etycznej ds. Doświadczeń na

Zwierzętach (nr 68/2014 z dnia 21 maja 2014 roku) działającej przy Uniwersytecie Jagiellońskim w Krakowie, zgodnie z międzynarodową konwencją Helsińską.

Hodowle komórkowe

Nowotworowe ludzkie linie komórkowe: glioblastoma-astrocytoma (U87-MG) i neuroblastoma (SH-SY5Y) zostały zakupione w European Collection of Cell Cultures (ECACC). Komórki przechowywano w krioprobówkach w dewarach z ciekłym azotem. W celu rozpoczęcia hodowli probówkę z roztworem komórek wyciągano, ogrzewano do temperatury pokojowej, a następnie jej zawartość rozpuszczano w kilku mililitrach pożywki DMEM, uzupełnionej 10% płodową surowicą bydlęcą i antybiotykami (100 U/ml penicyliny i 100 μg/ml streptomycyny). Hodowle komórkowe prowadzono w standardowych warunkach, w inkubatorze w temperaturze 37ºC, w wilgotnej atmosferze zawierającej 5% CO_2 na płytkach do hodowli (średnica 100 mm).

Metody

Oznaczanie poziomu siarkowodoru

Oznaczanie poziomu siarkowodoru wiązanego w warstwie agarozy przeprowadzono w homogenatach komórkowych i tkankowych spektrofotometryczną metodą opisaną w pracy Kartha i wsp., 2012 z modyfikacją opisaną w Publikacji nr 4.

Oznaczanie aktywności enzymów

Gamma-cystationaza

Oznaczanie aktywności gamma-cystationazy przeprowadzono metodą Matsuo i Greenberg, 1958 z modyfikacją opisaną w pracy Czubak i wsp., 2002 oraz opartą na oznaczaniu poziomu α-ketomaślanu metodą HPLC, w obecności cystationiny (CTN) jako substratu opisaną w Publikacji nr 2. Aktywność właściwą CTH wyrażano w nmolach

α- ketomaślanu, powstałego w czasie 1 minuty inkubacji w 37ºC w przeliczeniu na mg białka.

β-syntaza cystationinowa (CBS)

Aktywność CBS badano w homogenatach tkankowych oraz komórkowych w obecności DL-homoseryny (HSer) jako substratu. Poziom cystationiny oznaczano stosując metodę HPLC opisaną w Publikacji nr 1 i 2. Kontrolne homogenaty zostały sporządzone tak samo jak homogenaty badane, ale bez dodawania HSer. Aktywność CBS wyrażona była w ilości pmol cystationiny utworzonej w ciągu 1 min inkubacji w 37°C w przeliczeniu na 1 mg białka. Aby potwierdzić całkowite zahamowanie aktywności CTH przez PPG oznaczano aktywność CTH metodą Matsuo i Greenberg., 1958 z modyfikacją opisaną w pracy Czubak i wsp., 2002.

Transferaza siarkowa 3-merkaptopirogronianu (MPST)

Oznaczanie aktywności MPST przeprowadzono metodą Valentine i Frankenfeld, 1974 z modyfikacją opisaną w Publikacji nr 3 i 4. W metodzie tej 3MP jest przekształcany w pirogronian przez MPST, a siarka przenoszona jest na siarczyn. Pirogronian jest następnie redukowany do mleczanu przy udziale dehydrogenazy mleczanowej (LDH) oraz zredukowanej formy nukleotydu nikotynoamidoadeninowego (NADH). Aktywność MPST wyrażono w nmolach PA powstałego w czasie 1 minuty inkubacji w 37°C w przeliczeniu na mg białka.

Rodanaza (TST)

Aktywność rodanazy badano metodą Sörbo, 1955 w modyfikacji opisanej w Publikacji nr 4. Aktywność rodanazy wyrażano w nmolach SCN^- wytworzonego w czasie 1 minuty inkubacji w przeliczeniu na 1 mg białka.

Oznaczanie poziomu białka i siarki sulfanowej

Poziom białka badano spektrofotometryczną metodą Lowry'ego i wsp., 1951, stosując jako standard albuminę bydlęcą. Do analizy Western blot stężenia białka oznaczano metodą Bradforda, 1976.

Poziom siarki sulfanowej badano metodą Wooda, 1987, w oparciu o reakcję „zimnej cyjanolizy" oraz wiązania utworzonego rodanku z jonem żelaza (III). Utworzony barwny kompleks oznaczano kolorymetrycznie. Poziom siarki wyrażano w nmolach SCN^- w przeliczeniu na 1 mg białka.

Badanie ekspresji genów dla CTH, CBS, MPST oraz TST

Badanie ekspresji genów dla CTH, CBS, MPST oraz TST było związane z izolowaniem komórkowego RNA przy użyciu odczynnika Tri-Reagent (Lab-Empire, SIGMA). Następnie wykonywano reakcję odwrotnej transkrypcji z użyciem specyficznych starterów, syntezę cDNA oraz rozdział elektroforetyczny produktów reakcji PCR w 2,5% żelu agarozowym z wizualizacją z użyciem bromku etydyny w świetle UV [Publikacja nr 3 i 4].

Western blot z immunohistochemiczną identyfikacją białek

W celu przygotowania próbek do analizy Western blot hodowle komórkowe przepłukiwano dwukrotnie zimnym PBS, a następnie zbierano do zimnego buforu lizującego RIPA z dodatkiem inhibitora proteaz, po czym lizat komórkowy odwirowywano przy 14000xg przez 15 minut w 4°C. Uzyskany supernatant przechowywano do czasu analizy w -80°C. Rozdział elektroforetyczny białek przeprowadzano w żelu poliakrylamidowym składającym się z 4,5% żelu zagęszczającego (górna warstwa) i 12-13% żelu rozdzielającego (dolna warstwa), przez 20 minut przy napięciu 60 V, a następnie 120 V przez 2,5 h. Po rozdziale żel poddawano elektrotransferowi przez noc, w temperaturze +4^{o}C, w układzie półmokrym na membranę z PVDF przy natężeniu 0,15 A. Po zakończonym transferze

membranę płukano roztworem TBS-Tween-20 (Tris, NaCl, woda, Tween) trzykrotnie przez 10 minut w temperaturze pokojowej, a następnie przez godzinę w temperaturze pokojowej blokowano membranę przez inkubację w roztworze TBS-Tween-20 z dodatkiem 5% odtłuszczonego mleka w proszku. Po skończonej inkubacji membranę ponownie płukano, po czym inkubowano z 1º-rzędowym przeciwciałem przez noc, w temperaturze +4ºC [CBS (1:1000), CTH (1: 1000), MPST (1: 1000). β-aktyny (1: 5000) i alfa-tubulinę (1:5000)].

W dalszym etapie membranę płukano przez 15 minut w roztworze TBS-Tween-20, po czym inkubowano z 2º-rzędowym przeciwciałem anty-mysim lub anty-króliczym sprzężonym z alkaliczną fosfatazą. Ostatnim etapem było 15-minutowe płukanie membrany w roztworze TBS-Tween-20, a następnie dwukrotne 5-minutowe płukanie roztworem TBS. Tak przygotowaną membranę wywoływano przez inkubację w roztworze substratu NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate) rozpuszczonym w buforze o pH=9,5 (0,1 M Tris-HCl, pH = 9,5; 0,1M NaCl, 1mM EDTA, 1% Triton X-100), aż do uzyskania wytrąconego barwnego produktu, wskazującego na obecność białka wiążącego przeciwciało 1º-rzędowe. Czas wywoływania zależał od zastosowanego przeciwciała 1º-rzędowego i wynosił od kilku sekund do kilkunastu minut [Publikacja nr 3].

Test LDH

Ocenę cytotoksyczności związków wykorzystywanych w hodowlach komórkowych, wykonywano kolorymetrycznym testem LDH (Cytotoxicity Detection Kit firmy Roche), który opiera się na przekształcaniu mleczanu w pirogronian (redukcji NAD^+ do $NADH/H^+$) w reakcji katalizowanej przez dehydrogenazę mleczanową. Dodana do mieszaniny reakcyjnej sól tetrazolowa jest redukowana do czerwonego formazanu.

Oznaczanie poziomu glutationu zredukowanego i utlenionego, cysteiny, cystyny i cystationiny zmodyfikowaną metodą RP-HPLC.

Technikę RP-HPLC zastosowano do analizy jakościowej i ilościowej związków, tj. zredukowanego (GSH) i utlenionego (GSSG) glutationu, cysteiny (CSH), cystyny (CSSC), oraz cystationiny (CTN). W tym celu związki przekształca się w N,S-dinitro- pochodne dla GSH i cysteiny oraz N,N-dinitro- pochodne dla GSSG, cystationiny i cystyny. Elucja następowała w układzie woda–acetonitryl, zgodnie ze wzrastającym stężeniem acetonitrylu.

Oznaczenia przeprowadzano na aparacie firmy Shimadzu, który wyposażony został w detektor z matrycą fotodiodową SPD–M10VP, pod kontrolą oprogramowania class VP 7,2. Rozdziały przeprowadzane były w temperaturze 20°C na termostatowej kolumnie Luna 5u C18 (Z) o wymiarach 250 mm x 4,6 mm, firmy Phenomenex. Kolumna wyposażona została w guard kolumnę o tym samym wypełnieniu. Jako eluentu w rozdziałach używano rozpuszczalników klasy grade: acetonitryl/0,1% kwas trifluorooctowy (TFA) i H_2O/0,1% TFA. Rozdział otrzymano dzięki elucji gradientowej w nieliniowym wzroście stężenia acetonitrylu od 20% do 100% w czasie 90 minut, przy przepływie 1,0 ml/min. Na kolumnę nakładano próbki o objętości 20 µl, które wcześniej filtrowano z użyciem filtrów PTFE o średnicy porów 0,2 µm firmy Supelco. Analizę związków prowadzono z detekcją UV-VIS przy 365 nm [Publikacja nr 1].

Poziom α-ketomaślanu.

Oznaczanie zawartości α-ketomaślanu metodą HPLC opisaną w Publikacji nr 2.

Publikacje składające się na dysertację

Bronowicka-Adamska, P.; Zagajewski, J.; Czubak, J.; Wróbel, M.

RP-HPLC method for the quantitative determination of cystathionine, cysteine and glutathione: an application for the study of the metabolism of cysteine in human brain

Journal of Chromatography B. 2011, 879, 2005-2009

Bronowicka-Adamska, P.; Zagajewski, J.; Wróbel, M.

An application of RP-HPLC for determination of the activity of cystathionine beta-synthase and gamma-cystathionase in tissue homogenates

Nitric Oxide. 2015, 46, 186-191

Bronowicka-Adamska, P.; Bentke, A.; Wróbel, M.

Hydrogen sulfide generation from L-cysteine in the human glioblastoma-astrocytoma U-87 MG and neuroblastoma SHSY5Y cell lines

Acta Biochimica Polonica. 2017, 64, 171–176

Bronowicka-Adamska, P.; Wróbel, M.; Magierowski, M.; Magierowska, K.; Kwiecień. S.; Brzozowski, T.

Hydrogen sulphide production in gastric mucosa of rats exposed to stress

Moleculs. 2017, 22, 530; doi:10.3390/molecules22040530

PUBLIKACJA nr 1

RP-HPLC method for the quantitative determination of cystathionine, cysteine and glutathione: an application for the study of the metabolism of cysteine in human brain

Patrycja Bronowicka-Adamska

Jacek Zagajewski

Jerzy Czubak

Maria Wróbel

Journal of Chromatography B, 879 (2011) 2005–2009

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

RP-HPLC method for quantitative determination of cystathionine, cysteine and glutathione: An application for the study of the metabolism of cysteine in human brain

Patrycja Bronowicka-Adamska, Jacek Zagajewski, Jerzy Czubak, Maria Wróbel*

Jagiellonian University, Collegium Medicum, Kopernika 7, 31-034 Cracow, Poland

ARTICLE INFO

Article history:
Received 13 April 2011
Accepted 16 May 2011
Available online 23 May 2011

Keywords:
Cystathionine
Cysteine
Glutathione
Human brain
RP-HPLC

ABSTRACT

The RP-HPLC method for a simultaneous separation and quantitation of the dinitrophenyl derivative of cystathionine (*N*,*N*′-di-DNP) in biological samples together with GSH, GSSG, cysteine and cystine, provides a very useful tool for investigation of the transsulfuration pathway in biological samples, at the same providing results which reflect the redox status (GSH/GSSG ratio) and the potential of the generation of H_2S. An application of the method for the study of the process of transsulfuration in various human brain regions shows the presence of cystathionine in all the investigated regions; it also demonstrates that cystathionine levels vary greatly between particular regions. The highest level in the thalamus and the lowest in the cerebellum were associated with respectively a low or high γ-cystathionase activity, and at the same time, a high cysteine and GSH level in the thalamus and a low value in the cerebellum. Based on the above results, one may suggest a regulatory mechanism responsible for inhibition of the CGL activity at high concentration values of cysteine and/or GSH. Simultaneous determinations of GSH and GSSG levels allow for determining the GSH/GSSG ratio, which reflects tissue redox status. The method may be also employed in determining the activity of γ-cystathionase and cystathionine-β synthase.

1. Introduction

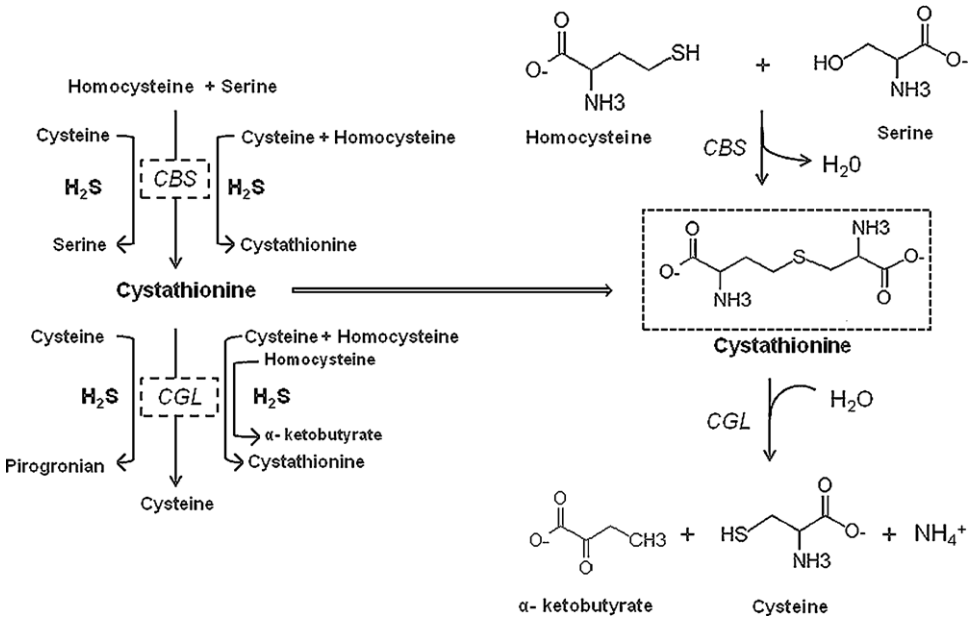
Cystathionine is an important intermediate in the L-cysteine transsulfuration pathway in mammalian tissues [1] (Scheme 1). A mean concentration of cystathionine detected in normal human serum by gas chromatographic or mass spectrometric methods equals 140 nM, with a range of 65–301 nM [2–4]. Elevated levels are found in urine of patients with neuroblastoma [5]. The level of cystathionine reflects the activity of cystathionine β-synthase ([1]CBS, EC 4.2.1.22), an enzyme responsible for cystathionine synthesis from serine and homocysteine, and cystathionine γ-lyase ([2]CGL, EC 4.4.1.1), which degrades it to cysteine, α-ketobutyrate, and ammonium ions (Scheme 1). The transsulfuration pathway is most active in such tissues as mammalian liver, kidney, pancreas, and intestine [6], but it is also present in normal brain tissue [21]. A number of reports showed the presence of cystathionine in the human brain samples collected at autopsy [7]. A relatively high level of cystathionine suggests a special relationship between the quantity and the activity of the enzymes involved in its biosynthesis and cleavage. If CGL is blocked or absent and CBS is normally active, the level of cystathionine can be elevated [6,8]. A major etiology of cystathioninuria in some neuroblastoma patients is the specific block in transsulfuration resulting from absence of CGL in the malignant tissue [5]. Cystathionine accumulates in various regions of the D,L propargylglycine-treated rat brain. The level of cystathionine in rat brain depends on the brain region. It was 10–19 times higher in the cerebellum than in the cerebral cortex and in white matter than in grey matter – this might suggests some relation of cystathionine to brain myelination [1]. In fetal brain, the concentration of cystathionine is lower than that found in the mature brain and the level increases slowly after birth until 2–3 months of age, when the value is similar to that found in the mature brain [9].

Cystathionine γ-lyase plays an important role in human brain. Diwakar and Ravindranath [10] reported that the enzyme activity was similar in most regions of the brain, except the hippocampus, where it was significantly lower as compared to cortex. The expression of CGL in all the regions of mouse and human brain as observed by *in situ* hybridization showed predominant localization in neuronal population. D,L propargylglycine inhibited the activity of CGL, what was demonstrated by the loss of [3]GSH, indicating

* Corresponding author. Tel.: +48 12 4247229; fax: +48 12 4223272.
E-mail address: mbwrobel@cyf-kr.edu.pl (M. Wróbel).

[1] Cystathionine β-synthase.
[2] Cystathionine γ-lyase.
[3] Glutathione reduced.

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.05.026



Scheme 1. Cystathionine synthesis and conversions – reactions of H_2S generation. In mammals, cysteine is made from homocysteine, which originates from methionine and supplies the sulfur atom, and serine, which supplies the carbon skeleton. The reaction is catalyzed by cystathionine-β-synthase (CBS) and yields cystathionine. In the next step, cystathionine-γ-lyase (CGL) catalyzes the removal of ammonia and cleavage of cystathionine to yield free cysteine. The two enzymes in the trans-sulfuration pathway, CBS and CGL, are believed to be chiefly responsible for H_2S biogenesis (according to Singh et al. [26]).

the importance of transsulfuration pathway in generating cysteine for GSH synthesis in [4]CNS. A significant decrease in the reducing capacity of the cellular redox couples, such as glutathione, was implicated in a number of pathologies, such as neurodegenerative disorders, epileptic seizures, demyelination (multiple sclerosis), dementia and aging [10].

The enzymatic tandem CBS/CGL is important in the production of cysteine and glutathione and also in the production of hydrogen sulfide ([5]H_2S) [11] (Scheme 1). H_2S is produced endogenously from L-cysteine in vascular smooth muscle cells and nervous system and it has a vasorelaxant property and may function as a neuromodulator. H_2S is present at a suitably high concentration in brain and CBS, which is highly expressed in the hippocampus and is involved in the production of brain H_2S [12]. In physiological concentrations, hydrogen sulfide induces the hippocampal long-term potentiation ([6]LTP), enhances [7]NMDA receptor-mediated responses and inhibits synaptic transmission in the hippocampus. The concentration of H_2S decreases in the brains of patients with Alzheimer's disease, while the overproduction of hydrogen sulfide characterizes Down syndrome patients [13].

Dominick et al. [14] developed a method for the simultaneous separation and quantitation of several thiolamines, such as glutathione reduced (GSH) and oxidized ([8]GSSG), cysteine and cystine. The procedure employs a C_{18} reversed-phase HPLC system to separate the dinitrophenyl ([9]DNP) derivatives of GSH and cysteine (*N*,*S*-di-DNP) and GSSG and cystine (*N*,*N*′-di-DNP) and relies on an internal standard, N-methyllysine, to minimize experimental error. While many methods of glutathione determination have been reported, there are a few reports concerning the simultaneous determination of GSH and GSSG [15]. We developed a modification of the method of Dominick et al. [14] allowing for the separation and quantitation of the dinitrophenyl (DNP) derivative of cystathionine (*N*,*N*′-di-DNP) in biological samples, together with GSH, GSSG, cysteine and cystine [16]. This very useful method facilitates the investigation of the transsulfuration pathway in human brain homogenates, providing results, which reflect the redox status (GSH/GSSG ratio) and the potential of the generation of H_2S.

2. Experimental conditions

2.1. Chemicals and reagents

L-Glutathione reduced, glutathione oxidized form, L-cysteine, L-cystine, cystathionine, 1-fluoro-2,4-dinitrobenzene ([10]DNFB), bathophenanthroline-disulfonic acid disodium salt ([11]BPDS), ethylenediaminetetraacetic acid ([12]EDTA), fosforan-5-pirydoksalu ([13]PLP), lactate dehydrogenase, acetonitrile were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Trifluoroacetic acid ([14]TFA) was purchased from Fluka Chemie GmbH (Buchs, Switzerland). Ethanol and 70% perchloric acid ([15]PCA) were from POCh S.A. (Gliwice). N^{ε}-methyllysine was obtained from Bachem (Bubendorf, Switzerland). All chemicals and HPLC solvents were gradient grade. Water was deionized by passing through an EASY pure RF compact ultrapure water system (MO, U.S.A.).

2.2. Methods

Various regions of human brain, collected post-mortem in the Department of Pathomorphology and Department of Forensic Medicine, Jagiellonian University Medical College, Cracow, Poland, were used in this experiment. The experimental protocol was approved by the Bioethic Commission, Jagiellonian University

[4] Central nervous system.
[5] Hydrogen sulfide.
[6] Long-term potentiation.
[7] N-methyl-D-aspartate receptor.
[8] Glutathione oxidized.
[9] Dinitrophenyl.
[10] 1-Fluoro-2,4-dinitrobenzene.
[11] Bathophenanthroline-disulfonic acid disodum salt.
[12] Ethylenediaminetetraacetic acid.
[13] Fosforan-5-pirydoksalu.
[14] Trifluoroacetic acid.
[15] Perchloric acid.

Medical College. Samples of brain (frontal cortex, parietal cortex, thalamus, hypothalamus, hippocampus, cerebellum and subcortical nuclei) were collected within 24 h of death from patients between 40 and 60 years of age if a post mortem examination performed by the attending pathologist showed that the brain was macroscopically normal. For the HPLC analysis, samples of brain were weighed and homogenized in ice-cold 10% PCA/1 mM BPDS (1 g/3 ml), during 1 min at 8000–9500 rpm using a blender homogenizer. The homogenates were centrifuged at 1400 × *g* for 10 min at 4 °C. The supernatants were used immediately or stored at −80 °C until the analysis. For CGL activity and protein determination, the brain samples were washed with cold saline and homogenized in 0.1 M phosphate buffer pH 7.5 (w/v 1:5) using a blender homogenizer (1 min, 8000–9500 rpm). The homogenates were centrifuged at 1600 × *g* for 10 min at room temperature and the obtained supernatants were used for assays.

2.2.1. HPLC instrumentation and conditions

The samples were analyzed on a 4.6 mm × 250 mm Luna C_{18} (5 μm) column with a Phenomenex Security Guard column filled with the same packing material. The chromatographic system consisted of LC-10 Atvp Shimadzu pumps, four channel degassers, column oven and a Shimadzu SIL-10 Advp autosampler. The chromatographic peaks were measured by a Shimadzu SPD-M10 Avp-diode array detector. Class VP 7.2.1 version software was used to control system operation and facilitate data collection.

A mobile phase consisting of solvent A (water/0.1% TFA) and solvent B (acetonitrile/0.1% TFA) was used for elution of samples. After injection, the column was eluted with 20% B followed with 35 min linear gradient to 55% B and 10 min isocratic period at 55% B, then 15 min linear gradient to 100% B and 10 min isocrating period. The column was then re-equilibrated to the initial conditions for 15 min. The analyses of 20 μl of each sample were performed at a flow rate of 1.0 ml/min at 20 °C temperature with diode array detection at 365 nm [16].

2.2.2. Sample preparation

The incubation mixture, total volume 456 μl, contained: 100 μl supernatant, 20 μl of 0.24 μM N^{ε}-methyllysine solution in water, 40 μl of 10% PCA/1 mM BPBS, 96 μl of 2 M KOH – 2.4 M $KHCO_3$ and 200 μl of 1% DNFB in ethanol. The samples were derivatized for about 24 h at room temperature in the dark. The reaction was stopped by adding 30μl of 70% PCA. The sample was centrifuged at 5600 × *g* for 2 min and the supernatants were filtered through a 0.20 μm PTFE Titan Syringe filter (Polygen Co., Poland).

2.2.3. Stock solutions and standard curves

Stock solutions were prepared for standard curves as follows: 2.4 μM N^{ε}-methyllysine, 1.2 μM GSH, 1.2 μM GSSG, 1.2 μM L-cysteine, 1.2 μM L-cystine, 2.2 μM cystathionine. All the stock solutions were prepared in 10% PCA/1 mM BPDS except for N^{ε}-methyllysine, which was prepared in water. A separate stock solution of the internal standard, N^{ε}-methyllysine, was prepared by dilution of 2.4 μM solution in proportion 1:10.

Standard curves were prepared in the same way as samples, by adding 20–75 μl of the stock solution of cystathionine, GSH, GSSG, cysteine and cystine instead of 40 μl 10% PCA/1 mM BPDS. Standard curves were generated in the supernatant obtained from human brain homogenate in the range from 25 to 92 nmol of each compound per ml. A linear response was shown over the concentration range investigated for all of the analytes. The linear regression analysis yielded $y = 2.28 \times 10^4 x$ ($r^2 = 0.993$) for cystathionine, where y was the peak areas (mAu) and x – the cystathionine concentration (nmol/1 ml).

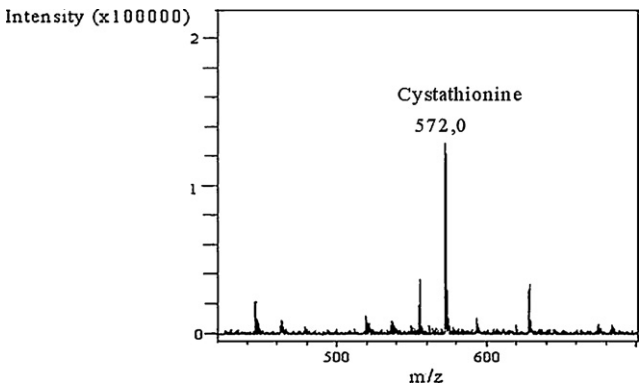


Fig. 1. The positive ionisation *m*/*z* spectrum of cystathionione.

2.2.4. Mass spectral analysis

We examined the peak areas of N,S-diDNP-cystathionine using elution gradient described in Section 2.2.1. Additionally, the fraction of cystathionine was determined by ESI/MS – the molecular ion of $m/z = 572$, $H_2O = 554$ (positive ionization M^+), corresponded to N,S-diDNP-cystathionine (Fig. 1). Mass spectrometry was performed as described elsewhere [17]. Briefly, the analyses were carried out using Esquire 3000 ESI-MS (Bruker-Daltonics, Bremen Germany) in a positive ion mode. The flow rate was set to 3 μl/min using a KD 100 Syringe pump (KD scientific, Holliston USA). Basic parameters of the ion source were as follows: heated capillary temperature: 280 °C, capillary voltage: 4.5 KV. The results were analyzed using Brukers Data Analysis software (ver 3.0).

2.2.5. Cystathionine γ-lyase assay

The CGL activity was assayed by the [18], with modifications described by Czubak et al. [19]. Each incubation mixture (650 μl) contained: 250 μl 45 mM cystathionine solution in 0.1 M phosphate buffer, pH 7.5 (10 mg of cystathionine per sample), from 275 μl to 525 μl 0.1 M phosphate buffer containing 0.05 mM 2-mercaptoethanol, pH 7.5, 25 μl 1.3 mM pyridoxal phosphate, 25 μl 13 mM EDTA–$Na_2 \cdot H_2 0$. The reaction was started by adding 75 μl of homogenate and was stopped after 30 min of incubation at 37 °C by placing 125 μl of incubation mixture in 25 μl 1.2 M perchloric acid. The samples were centrifuged at 1600 × *g* for 10 min, and 25 μl of the supernatant was transferred to 625 μl 0.194 mm NADH water solution and kept at 37 °C. The absorbance of this mixture was measured at 340 nm with a Hitachi U2000 spectrophotometer for 10 s against distilled water. After this interval, 25 μl (9.06 IU) lactate dehydrogenase from beef heart was added and the measurement was continued to 180 s [19]. The enzyme activity was expressed as pmoles α-ketobutyrate formed during 1 min incubation at 37 °C per 1 mg of protein.

2.2.6. Protein determination

Protein was determined by the method of Lowry et al. [20] using crystalline bovine serum albumin as a standard.

3. Results and discussion

The application of the modified method of Dominick et al. [14] for the study of the metabolism of low-molecular sulfur compounds in various human brain regions homogenates allows for simultaneous determinations of cystathionine, reduced and oxidized glutathione, cysteine and cystine. Examples of chromatographic distribution for homogenates of human frontal cortex, parietal cortex, thalamus, hypothalamus, hippocampus, cerebellum and subcortical nuclei are presented in Fig. 2.

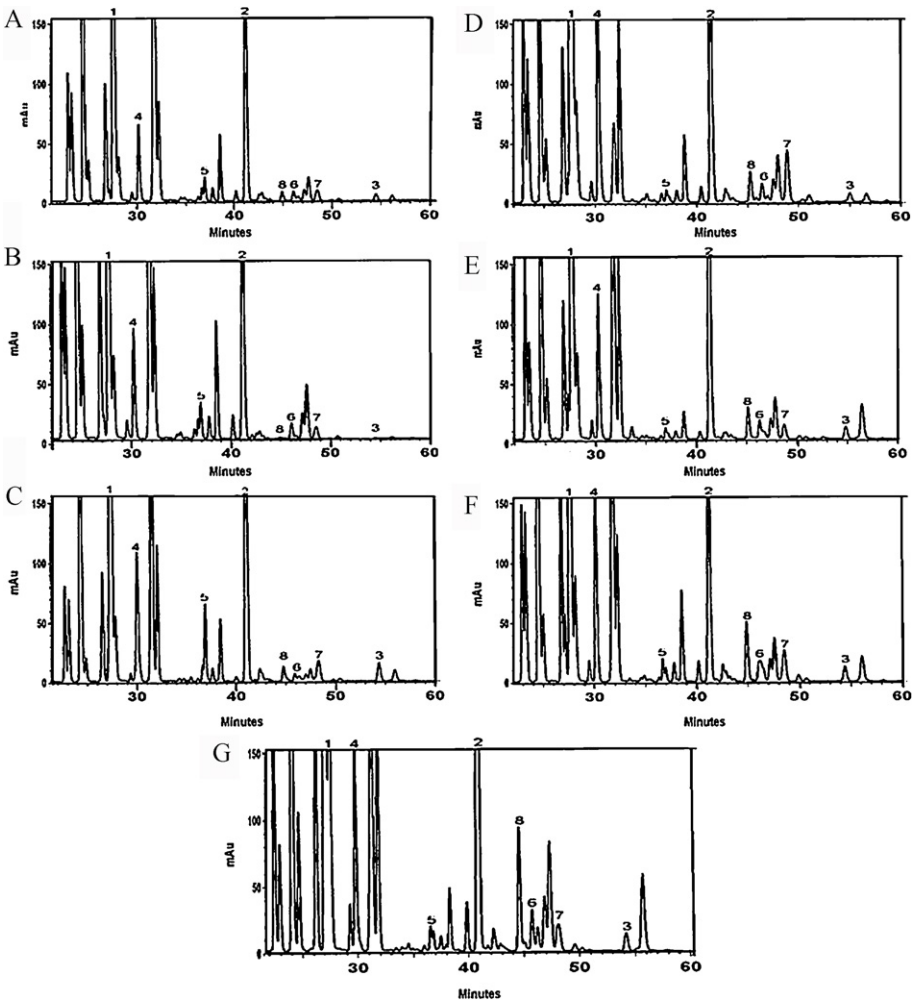


Fig. 2. Chromatograms of (A) frontal cortex, (B) cerebellum, (C) hippocampus, (D) hypothalamus, (E) parietal cortex, (F) nuclei subcortical and (G) thalamus. Peaks and retention times (min) of DNP derivatives: (1) 2,4-dinitrophenol (27.58 ± 0.24); (2) 2,4-dinitrophenyl ethyl ether (41.23 ± 0.19); (3) *N*,*N*′-di-DNP-N^{ε}-methyllysine (54.54 ± 0.27); (4) *N*,*N*′-di-DNP–GSSG (30.15 ± 0.20); (5) N,S-di-DNP–GSH (37.05 ± 0.30); (6) *N*,*N*′-di-DNP–cystine (46.08 ± 0.23); (7) N,S-di-DNP–cysteine (48.51 ± 0.28); (8) *N*,*N*′di-DNP–cystathionine (44.90 ± 0.28). Coefficient of variation (SD/mean) × 100% = 1% for all derivatives. Chromatographic conditions are described in Section 2.2.

Table 1 presents the values of GSH, GSSG, cysteine, cystine and cystathionine levels in the investigated human brain regions. Cystathionine was detected in all the investigated human brain regions and its levels vary greatly between particular regions. The level of this amino acid was the highest in the human thalamus and it was about 11 times greater than in the cerebellum ($p < 0.05$). The concentration of cystathionine in the subcortical nuclei and hypothalamus was also significantly higher as compared to the cerebellum or frontal and parietal cortex. Similarly, our research showed that the highest concentration of cysteine was noted in the thalamus, hypothalamus and subcortical nuclei (Table 1). Cysteine is the limiting substrate in biosynthesis of GSH. The highest level of cysteine in the thalamus corresponded with the highest level of GSH and the highest ratio of GSH to GSSG. The data presented in

Table 1
The level of GSH, GSSG, cysteine, cystine and cystathionine in human brain regions.

Brain region	GSH	GSSG	Cysteine	Cystine	Cystathionine
	nmol mg of protein^{-1}				
Cerebellum	0.76 ± 0.29	1.04 ± 0.55*	0.58 ± 0.21	0.23 ± 0.09	4.71 ± 2.36
Hypothalamus	0.98 ± 0.16	0.51 ± 0.38	1.73 ± 0.34	0.22 ± 0.07	23.12 ± 10.16*
Thalamus	1.49 ± 0.31	0.58 ± 0.43*	1.92 ± 0.76*	0.28 ± 0.13	55.34 ± 15.53
Subcortical nuclei	0.74 ± 0.00	1.30 ± 0.89*	1.53 ± 0.65*	0.33 ± 0.16	32.91 ± 11.03*
Hippocampus	1.28 ± 0.43	0.56 ± 0.28	0.77 ± 0.40	0.18 ± 0.08	19.79 ± 5.97*
Frontal cortex	0.54 ± 0.26	0.97 ± 0.47	0.84 ± 0.33	0.13 ± 0.08	10.27 ± 6.16*
Parietal cortex	0.42 ± 0.11	0.17 ± 0.06	0.62 ± 0.12	0.28 ± 0.02	11.88 ± 3.23
N	2–8	2–6	3–6	2–8	3–7

The values represent the mean ± SD of eight brains regions. GSH, glutathione reduced; GSSG, glutathione oxidized; CSH, cysteine; CSSC, cystine. The Mann–Whitney's test was adopted as a criterion of statistical significance with the probability level of 0.05.

* $p < 0.05$ GSSG: cerebellum, thalamus vs. subcortical nuclei; subcortical nuclei vs. frontal cortex, CSH: Thalamus vs. subcortical nuclei; subcortical nuclei vs. hippocampus and frontal cortex, cystathionine: hypothalamus vs. hippocampus and frontal cortex; subcortical nuclei vs. hippocampus and frontal cortex; hippocampus vs. parietal cortex; frontal cortex vs. parietal cortex, GSH and CSSC: statistics could not be calculated.

Table 2
Cystathionase activity in human brain regions.

Brain region	Cystathionase
pmol mg^{-1} min^{-1}	
Cerebellum	719 ± 216*
Hypothalamus	674 ± 237*
Thalamus	342 ± 114
Subcortical nuclei	555 ± 377*
Hippocampus	454 ± 222
Frontal cortex	451 ± 291
Parietal cortex	672 ± 238

The values represent the mean ± SD of eight brains, with each determination consisting of 9–15 assays. The Mann–Whitney's test was adopted as a criterion of statistical significance with the probability level of 0.05.

* $p < 0.05$ cystathionase: cerebellum, hypothalamus, subcortical nuclei vs. frontal cortex.

Table 1 allow for determining the redox status expressed by the GSH/GSSG ratio. GSH, as a reductant plays a fundamental role in the detoxification of reactive oxygen species, which is critical to the normal function of the central nervous system and its altered levels have been reported in several pathological diseases, such as schizophrenia and cancer [22–24].

A negative correlation between the amount of cystathionine and the activity of CGL was observed. The lowest among the investigated regions activity of CGL detected in the thalamus (Table 2) reflects the high level of cystathionine (Table 1). Similarly, the highest activity of γ-cystathionase was detected in the cerebellum, with the lowest among the investigated brain regions level of cystathionine. To date, the role of cystathionine in human brain it has not been confirmed, but pharmacological studies have suggested a potential role for this amino acid as a neuromodulator [25]. High cystathionine levels in some brain regions may reflect a high CBS activity. CBS is also important in the production of H_2S [11] (Scheme 1), which may function as a neuromodulator [12]. Elevated cystathionine levels might result from a low CGL activity as it was observed in case of cystathioninuria in neuroblastoma patients [5].

The method presented in this paper may be used to investigate the activity of CBS and CGL in biological samples in the presence of homoserine, CBS substrate, and in the presence of DL-propargylglycine ([16]PAG), CST inhibitor. The difference in the cystathionine level between homogenates with and without PAG can be used for the estimation of the activity of CBS in tissue homogenates. The combined activity of the CBS and CGL tandem can be measured by a difference between cysteine levels in the control and investigated samples.

4. Conclusions

The presented RP-HPLC method allows for simultaneous determinations of cystathionine, reduced and oxidized glutathione, cysteine and cystine. Cystathionine levels depend on both the CBS and CGL activities. The method produces results, which reflect tissue redox status (GSH/GSSG ratio) and the potential of the generation of H_2S and it can be also employed in determining the activity of CGL and CBS in the presence of the CGL inhibitor.

Acknowledgments

This work was supported by a grant from Collegium Medicum, Jagiellonian University, Nos. K/ZBW/000149 and K/ZDS/001468.

References

[1] S. Yu, K. Sugahara, K. Nakayama, S. Awata, H. Kodama, Metabolism 49 (2000) 1025.
[2] A.B. Guttormsen, E. Solheim, H. Refsum, Am. J. Clin. Nutr. 79 (2004) 76.
[3] S.P. Stabler, J. Lindenbaum, D.G. Savage, R.H. Allen, Blood 81 (1993) 3404.
[4] Ø. Midttun, S. Hustad, J. Schneede, S.E. Vollset, P.M. Ueland, Am. J. Clin. Nutr. 86 (2007) 131.
[5] C.E. Klein, B. Roberts, J. Holcenberg, L.M. Glode, Cancer 62 (1987) 291.
[6] J.D. Finkelstein, J. Nutr. 136 (2006) 1750S.
[7] H.H. Tallan, S. Moore, W.H. Stein, J. Biol. Chem. 230 (1958) 707.
[8] J. Wang, R.A. Hegele, Hum. Genet. 112 (2003) 404.
[9] J.A. Sturman, E.G. Gaull, W.H. Niemann, J. Neurochem. 26 (1976) 457.
[10] L. Diwakar, V. Ravindranath, Neurochem. Int. 50 (2007) 418.
[11] G. Yang, L. Wu, B. Jiang, W. Yang, J. Qi, K. Cao, Q. Meng, A.K. Mustafa, W. Mu, S. Zhang, S.H. Snyder, R. Wang, Science 322 (2008) 587.
[12] H. Kimura, Biochem. Biophys. Res. Commun. 267 (2000) 129.
[13] P. Kamoun, Amino Acids 26 (2004) 243.
[14] P.K. Dominik, P.B. Cassidy, J.C. Roberts, J. Chromatogr. B 761 (2001) 1.
[15] D.J. Reed, J.R. Babson, P.W. Beatty, A.E. Brodie, W.W. Ellis, D.W. Potter, Anal. Biochem. 106 (1980) 55.
[16] M. Wróbel, I. Lewandowska, P. Bronowicka-Adamska, A. Paszewski, Amino Acids 37 (2009) 565.
[17] M.J. Noga, J.J. Lewandowski, P. Suder, J. Silberring, Proteomics 5 (2005) 4367.
[18] Y. Matsuo, D.M. Greenberg, J. Biol. Chem. 230 (1958) 545.
[19] J. Czubak, M. Wróbel, H. Jurkowska, Acta Biol. Cracov. Ser. Zool. 44 (2002) 113.
[20] O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.I. Randall, J. Biol. Chem. 193 (1951) 265.
[21] O. Elliot, N.A. Holtzman, V.A. McKusick, Primates 15 (1974) 227.
[22] M. Terpstra, P.G. Henry, R. Gruetter, Magn. Reson. Med. 50 (2003) 19.
[23] K. Aoyama, M. Watabe, T. Nakaki, J. Pharmacol. Sci. 108 (2008) 227.
[24] Ch. Choi, Ch. Zhao, I. Dimitrov, D. Douglas, N.J. Coupland, S. Kalra, H. Hawesa, J. Davis, J. Magn. Reson. 198 (2009) 160.
[25] V. Vitvitsky, M. Thomas, A. Ghorpade, H.E. Gendelman, R. Banerjee, J. Biol. Chem. 281 (2006) 3578.
[26] S. Singh, D. Padovani, R.A. Leslie, T. Chiku, R. Banerjee, J. Biol. Chem. 284 (2009) 22457.

[16] DL-Propargylglycine.

PUBLIKACJA nr 2

An application of RP-HPLC for determination of the activity of cystathionine beta-synthase and gamma-cystathionase in tissue homogenates

Patrycja Bronowicka-Adamska

Jacek Zagajewski

Maria Wróbel

Nitric Oxide 46 (2015) 186–191

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

An application of RP-HPLC for determination of the activity of cystathionine β-synthase and γ-cystathionase in tissue homogenates

Patrycja Bronowicka-Adamska, Jacek Zagajewski, Maria Wróbel *

Collegium Medicum, Jagiellonian University, Kopernika 7, 31-034 Kraków, Poland

ARTICLE INFO

Article history:
Available online 13 October 2014

Keywords:
Cystathionine
Gamma-cystathionase
Cystathionine-beta-synthase
Mouse tissue
H_2S

ABSTRACT

The RP-HPLC-based method of determination of the activity of cystathionine β-synthase and γ-cystathionase was undertaken in mouse liver, kidney and brain. Products of the reactions, such as cystathionine, α-ketobutyrate, cysteine and glutathione, were measured using the RP-HPLC method. A difference in the cystathionine level between homogenates with totally CTH-inhibiting concentrations of DL-propargylglycine and without the inhibitor was employed to evaluate the activity of cystathionine β-synthase. Gamma-cystathionase activity was measured using DL-homoserine as a substrate and a sensitive HPLC-based assay to measure α-ketobutyrate. The results confirmed high cystathionine β-synthase activity and no γ-cystathionase activity in brain, and high γ-cystathionase activity in mouse liver. The method presented here allows for evaluating the relative contribution of CBS and CTH to generation of H_2S in tissues. Additionally, it provides results, which reflect the redox status (GSH/GSSG) of a tissue.

1. Introduction

Hydrogen sulfide (H_2S) is produced endogenously in many different human and mammalian tissues, including the liver, kidneys, brain and blood lymphocytes (not reviewed here, but see [1,2]). Recent studies have shown that this easily diffused compound, which does not require any transporters, plays diverse biological roles determined, among others, by its solubility in aqueous vs. lipid phases, availability of H_2S detoxifying enzymes and intracellular differences in oxygen tension [1,2]. In the vascular smooth muscle cells, it manifests vasorelaxant [3–5] or vasoconstrictive properties [6], and the vasodilatory effect is O_2-dependent, similarly as its anti-inflammatory [7] and pro-inflammatory properties [8,9]. In the nervous system, H_2S functions as a neuromodulator. H_2S at physiologically relevant levels affects hippocampal long-term potentiation (LTP), enhancing NMDA receptor-mediated responses and inhibiting synaptic transmission [10]. H_2S was found to play a role of a cytoprotectant, a regulator of insulin release, inflammation and angiogenesis, and oxygen (O_2) sensor [11].

Two major sources for endogenous enzymatic production of H_2S are pyridoxal-5'-phosphate-dependent enzymes: cystathionine beta-synthase (CBS, EC 4.2.1.22) and gamma-cystathionase (CTH, EC 4.4.1.1). Hydrogen sulfide is synthesized from L-cysteine and/or homocysteine in reactions catalyzed by CTH and CBS [12–15] (Table 1).

The reaction for which CBS is the best known is the synthesis of cystathionine from homocysteine and serine. CTH splits cystathionine to cysteine, α-ketobutyrate and ammonium ions [13] (Scheme S1). CBS and CTH participate in L-cysteine desulfuration and directly influence the level of sulfane sulfur; these divalent sulfur species are easily liberated as H_2S by reduction with excess thiols. Sulfane sulfur stored as elemental sulfur can be reduced to sulfide ions (S^{2-}) [2].

CTH is predominantly expressed in the liver and in vascular and non-vascular smooth muscle with only trace amounts found in the brain [2,15,23,24]. CBS is the predominating enzyme in the brain and nervous system [25,26]. DL-propargylglycine (PPG) inhibits the activity of CTH [26] what is demonstrated by the loss of reduced glutathione (GSH), indicating that availability of a suitable amount of cysteine is important for maintenance of GSH level in the CNS [27]. There is no currently available selective CBS inhibitor [26].

This study was conducted to evaluate the RP-HPLC-based method of determination of the activity of the CBS and CTH in mouse tissues – the liver, kidneys and brain. The RP-HPLC method was used to detect and determine the amount of direct and far products of the CBS and CTH-catalyzed reactions, such as cystathionine, cysteine, α-ketobutyrate and glutathione. The level of cystathionine reflects the activity of CBS responsible for its biosynthesis, and the activity of CTH converting it to cysteine. A difference in the cystathionine level between homogenates with totally CTH-inhibiting concentrations of PPG and without the inhibitor was employed to evaluate the activity of CBS. Depending on the tissue, the activity of CBS and CTH was different and seemed to be the highest in the

Abbreviations: CBS, cystathionine β-lyase; CTH, γ-cystathionase; GSH, glutathione reduced form; GSSG, glutathione oxidized form; DNFB, 1-fluoro-2,4-dinitrobenzene; HSer, homoserine; CTN, cystathionine.

* Corresponding author. Jagiellonian University Medical College, Chair of Medical Biochemistry, Kopernika 7 St., 31-034 Kraków, Poland. Fax: +48 12 4223272.
E-mail address: mbwrobel@cyf-kr.edu.pl (Maria Wróbel).

http://dx.doi.org/10.1016/j.niox.2014.09.159
1089-8603/

Table 1
Reactions of H_2S production from L-cysteine and homocysteine, catalyzed by CBS and CTH.

Substrate	Co-substrate	Product	References
CBS			
L-cysteine	Homocysteine	L-cystathionine + H_2S	[12,16–18]
L-cysteine	H_2O	L-serine, H_2S	[19]
L-cysteine	L-cysteine	L-lanthionine + H_2S	[13]
CTH			
L-cysteine	–	Pyruvate + NH_3 + H_2S	[2,14,20–22]
L-cysteine	L-cysteine	L-lanthionine + H_2S	[12]
L-homocysteine	L-homocysteine	L-homolanthionine + H_2S	[12,13]

brain in case of CBS and in the liver in case of CTH. The method presented here allows for evaluating the relative contribution of these enzymes to generation of H_2S in tissue or cell homogenates, in particular because the technology required to measure accurately H_2S on the sub-cellular level is unavailable. It was also observed that the functional deficiency in the activity of one H_2S producing enzyme could be compensated by the activity of the other to maintain the endogenous H_2S production level [5], what could be relatively easily confirmed employing the presented method.

2. Experimental (material and methods)

2.1. Chemicals and reagents

L-Glutathione reduced (GSH), glutathione oxidized form (GSSG), L-cysteine, L-cystine, cystathionine (CTN), DL-homoserine (HSer), 1-fluoro-2,4-dinitrobenzene (DNFB), bathophenanthroline-disulfonic acid disodium salt (BPDS), acetonitrile, pyridoxal phosphate (PLP), β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH), L-lactic dehydrogenase (LDH), DL-propargylglycine (PPG), N-methyl benzothiazolone hydrazine hydrochloride (MBTH) were obtained from Sigma Chemical Co. (St. Louis, Mo, U.S.A). Trifluoroacetic acid (TFA), 2-mercaptoethanol and EDTA-Na_2·$2H_20$ were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Ethanol and 70% perchloric acid (PCA) were from Polskie Odczynniki Chemiczne S.A. (Gliwice). N^{ε}-methyllysine was obtained from Bachem (Bubendorf, Switzerland). All the chemicals and HPLC solvents were gradient grade. Water was deionized by passing through an EASY pure RF compact ultrapure water system.

2.2. Sample preparation

2.2.1. Animals

Twenty-five mice Swiss-albino mice between 6–8 weeks of age (both sexes) from Animal House, Department of Clinical Immunology, Polish-American Institute of Pediatrics, Jagiellonian University Medical College, Kraków, Poland, were used in our experiment. All the mice were sacrificed by cervical dislocation and the livers, kidneys and brains were isolated, placed in liquid nitrogen and stored at –76 °C until used in biochemical experiments. All the procedures were approved by the Ethics Committee for the Animal Research in Kraków (number 26/III/2009).

2.2.2. Tissue samples

For the experiments, the tissues were weighed and homogenized in ice-cold 0.1 M phosphate buffer pH 7.5 (1 g/5 ml) from 1 min at 8000–9500 rpm using a blender homogenizer. The homogenates were centrifuged at 1600 g for 10 min. The supernatants were used for assays immediately or stored at –80 °C until HPLC analysis.

2.3. Enzyme assays

2.3.1. Determination of CBS activity

The activity of CBS was examined in mice liver, kidneys and brain homogenates in the presence of DL-homoserine (HSer) as a substrate. The control tissue homogenates with HSer contained in a final volume of 650 μl: 25 μl 1.3 mM PLP, 25 μl 0,02 mM EDTA, 250 μl 10.8 mM homoserine and 75 μl of sample containing enzymes and 0.1 M phosphate buffer, pH 7.5 with 0.05 mM 2-mercaptoethanol. The control tissue homogenates without HSer were prepared in the same way as tissue homogenates with HSer, but without adding HSer. The tissue homogenates with HSer and PPG, in which the inhibitory effect of PPG on the activity of CTH was investigated, contained PPG in the concentration depending on the kind of tissue: 0.75 mM for the liver, 0.95 mM for the kidneys and 0.1 mM for the brain. To confirm the total CTH inhibition by PPG in the tissue homogenates with HSer and PPG, the activity of CTH was assayed by Matsuo and Greenberg's method [28] with modifications [29]. After 15 min incubation at 37 °C, the reaction was stopped by placing 125 μl incubation mixture in 25 μl 10% perchloric acid containing 1 mM BPDS. The samples were centrifuged at 1600 g for 10 min and the supernatants were stored at –80 °C until used for RP-HPLC. The level of cystathionine was determined using the RP-HPLC method described by Bronowicka-Adamska et al. [30].

2.3.2. Determination of CTH activity

The activity of CTH was examined in mice liver, kidneys and brain homogenates in the presence of cystathionine (CTN) as a substrate. The incubation mixture contained in a final volume of 650 μl: 25 μl 1.3 mM PLP, 25 μl 0,02 mM EDTA, 250 μl 10.7 mM cystathionine and 75 μl sample containing enzymes and 0.1 M phosphate buffer, pH 7.5 with 0.05 mM 2-mercaptoethanol. The control samples were prepared in the same way as the examined samples, but without CTN. The reaction was stopped after 15 min of incubation at 37 °C by placing 125 μl incubation mixture in 25 μl 10% perchloric acid containing 1 mM BPDS. The samples were centrifuged at 1600 g for 10 min and the supernatants were stored at –80 °C until used for RP-HPLC. The activity of the enzyme was expressed as nmoles of α-ketobutyrate formed during 1 min incubation at 37 °C per 1 mg of protein. The level of α-ketobutyrate was determined using the method based on the method described by Paz et al. [31–35] using the reaction of N-methyl benzothiazolone hydrazine hydrochloride (MBTH) with the carbonyl compounds. The resulting derivative was determined by RP-HPLC method with detection at 350 nm.

2.4. Protein

Protein was determined by the method of Lowry et al. [36] using crystalline bovine serum albumin as a standard.

2.5. RP-HPLC (reverse phase high performance liquid chromatography)

The level of cystathionine, cysteine, α-ketobutyrate and the reduced (GSH) and oxidized (GSSG) glutathione was determined in incubation mixtures using the RP-HPLC method of Dominick et al. [37] with modifications [30,38]. The samples were separated on a 4.6 mm × 250 mm Luna C_{18} (5u) column with a Phenomenex Security Guard column filled with the same packing material. The chromatographic system consisted of LC-10 Atvp Shimadzu pumps, four channel degassers, a column oven and a Shimadzu SIL-10 Advp autosampler. Chromatographic peaks were measured by a Shimadzu SPD-M10Avp-diode array detector. A mobile phase consisting of solvent A (water/0.1%TFA) and solvent B (acetonitrile/0.1% TFA) was used to elute the samples. The samples were eluted with 20% B after injection followed with a 35-minute linear gradient to 55% B and 10 minutes isocratic period at 55% B, then a 15-minute linear gradient to 100% B and 10 minutes isocratic period. The column was then re-equilibrated to the initial conditions for 15 minutes. All the HPLC solvents were HPLC gradient grade. The samples were filtered through a 0.20 μm PTFE filter. Analyses of 20 μl of the samples were performed at a flow rate of 1.0 ml/min at 20 °C temperature with diode array detection at 365 nm.

2.5.1. Preparation of the stock solution and standard curves

Stock solutions were prepared for standard curves as follows: 2.4 μM N^{ε}-methyllysine, 1.2 μM L-cysteine, 1.2 μM GSH, 4.2 μM homoserine and 2,2 μM cystathionine. All the stock solutions were prepared in 10%PCA/1 mM BPDS except for N^{ε}-methyllysine, which was prepared in water. A separated stock solution of internal standard, N^{ε}-methyllysine, was prepared by dilution of 2.4 μM solution in proportion 1:10 [38]. The standard curves were generated in the supernatant obtained from the homogenate in the range from 13–75 nmoles of each compound per ml. The standard curves were prepared in the same way as the samples, by adding 20–75 μl of stock solution of DL-homoserine, cystathionine, GSH, GSSG, cysteine and cystine instead of 40 μl 10% PCA/1 mM BPBS.

2.6. Statistical analysis

All data of the RP-HPLC experiments represent the average of four to ten determinations. Values in the Tables were summarized as mean ± standard deviation of the mean. The significance of the differences between the mean values was calculated using the Student's t Test.

3. Results and discussion

The RP-HPLC method for a simultaneous separation and quantification of the dinitrophenyl derivatives of cysteine and cystine, GSH, GSSG, cystathionine and homoserine (Fig. 1) provides a very useful tool for investigation of the transsulfuration pathway in biological samples, providing results which reflect the redox status (GSH/GSSG ratio). The method can be also employed in determination of CBS and CTH activity involved in the investigation of the potential of biological systems to generate H_2S.

3.1. Basal (control) levels of cysteine, cystine, GSH, GSSG, cystathionine and homoserine in mouse liver, kidney and brain

Table 2 shows basal levels of cysteine, cysteine, total free cysteine (TFC = cysteine + 2 × cystine), reduced (GSH) and oxidized (GSSG) glutathione, total free glutathione (TFG = GSH + 2 × GSSG), cystathionine and homoserine in homogenates of mouse liver, kidneys and brain. The highest level of cysteine was determined in the kidneys, glutathione in the liver and cystathionine in the brain. These observations confirm that cystathionine may be important in the function of the brain, as it has been earlier suggested for the human brain that is characterized by a higher concentration of cystathionine as compared to brains of other animals [39]. In mouse brain homogenates, the level of homoserine was also the highest, more than two times higher as compared to liver homogenates (Table 2).

3.2. Changes in levels of cysteine, cystine, GSH, GSSG and cystathionine in tissue homogenates incubated with homoserine

Rat and normal human liver homogenates were demonstrated to catalyze the formation of cystathionine from homocysteine and serine, as well as from homoserine and cysteine, although at a much lower rate than the rate for the first reaction [19,22,40–42]. A significantly elevated level of cystathionine in response to homoserine was not found in the investigated tissues, but cysteine levels were elevated in all the tissues, and the increased level of GSH was noted in liver homogenate (Table 3).

Homoserine can be converted to cystathionine by CTH [40,41] and if homoserine is available, the reaction rate depends on the availability of cysteine. In the liver, homoserine increases the level of cysteine and is further converted to glutathione. In the presence of homoserine, cysteine accumulates in brain homogenates; perhaps because of trace CTH activity in this tissue. This may also explain the highest content of cystathionine in the brain.

3.3. Effect of propargylglycine on the level of cysteine, cystine, GSH, GSSG and cystathionine in the presence of homoserine

Propargylglycine, a suicide inhibitor of CTH [43], can completely block CTH activity and H_2S formation [44]. The present authors observed a twofold increase in the level of cystathionine in the presence of PPG in the brain in comparison to the liver and kidneys (Table 4). This result suggests a higher CBS activity in the brain than in the liver, what is in accordance with the results published by Yu et al. [45] and Nishi et al. [46]. Under the present assay conditions, the fact that propargylglycine led to a significant accumulation of cystathionine in mice brain homogenate indicates that CTH exists in this tissue and takes part in the regulation of the cystathionine level. In contrast to the liver, the kidneys and brain glutathione levels were not affected in the homogenates incubated with propargylglycine. This may reflect the fact that the rate of turnover of glutathione is slow in the brain and kidneys as compared with that in the liver.

3.4. Analysis of CBS and CTH activity in mouse tissues

CBS and CTH activity was demonstrated in mouse tissues using DL-homoserine as substrate [28]. A sensitive assay, involving the use of high performance liquid chromatography, was developed to measure the small amounts of α-ketobutyrate produced by CTH [47] or cystathionine [30,31] produced by CBS. The amount of activity found was expressed in nmol of α-ketobutyrate for CTH or cystathionine for CBS formed/min mg protein.

3.4.1. CTH

α-Ketobutyrate is produced by CTH (Scheme S1). The increase in the level of α-ketobutyrate in the homogenates incubated for 15 minutes with 10.8 mM HSer reflects CTH activity (Fig. 2). In brain homogenates, α-ketobutyrate levels did not rise after the incubation with HSer; thus, this result confirms no CTH activity in the mouse brain. The liver and kidney homogenates showed the activity of CTH with a several-fold higher value for the liver.

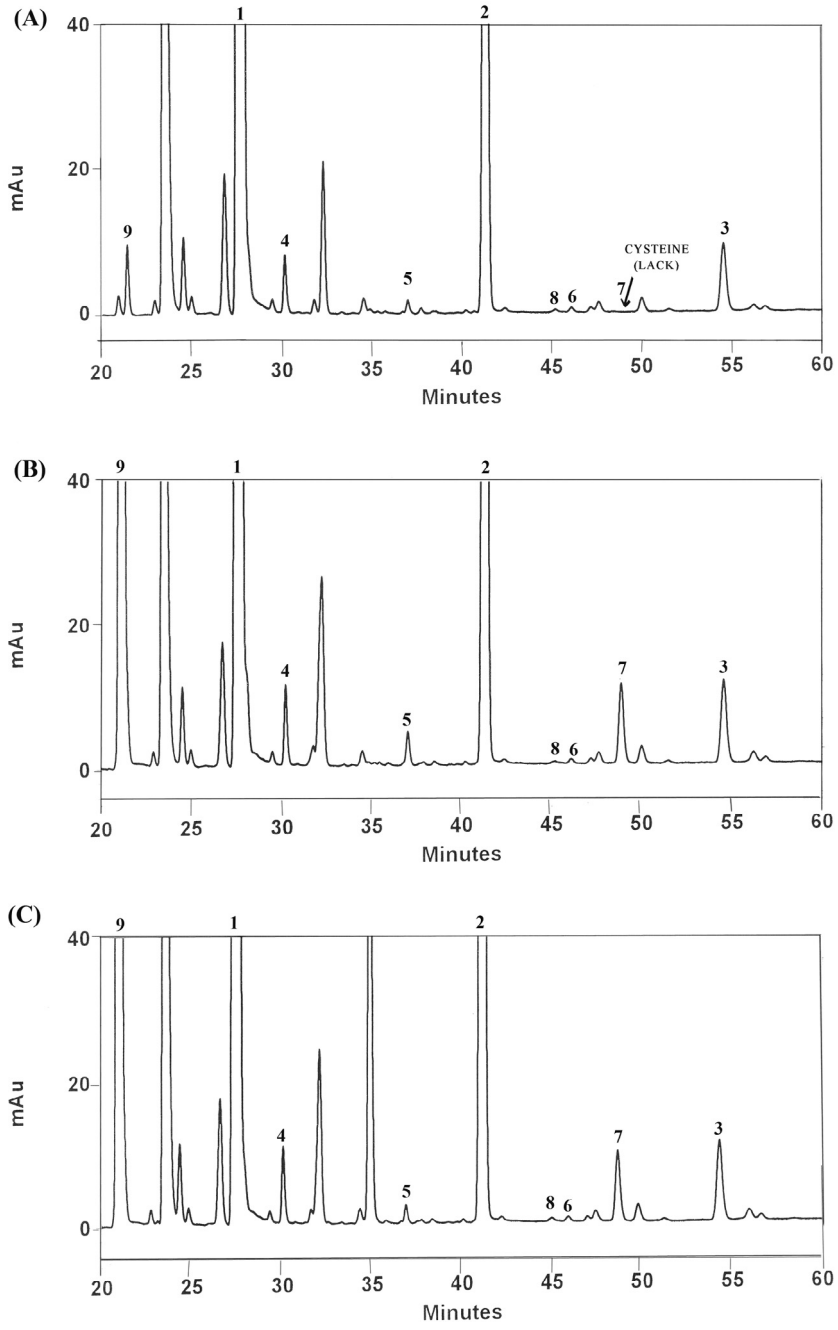


Fig. 1. Chromatograms of liver samples (A) without HSer (basal levels), (B) incubated with HSer, and (C) incubated with HSer and PPG. Peaks: 1 = 2,4-dinitrophenol; 2 = 2,4-dinitrophenyl ethyl ether; 3 = N,N′-di-DNP-N$^{\varepsilon}$-metyllysine; 4 = N,N′-di-DNP-GSSG; 5 = N,S-di-DNP-GSH; 6 = N,N′-di-DNP-cystine; 7 = N,S-di-DNP-cysteine; 8 = N,N′di-DNP-cystathionine, 9 = N-DNP-homoserine. Chromatographic conditions are described in Section 2.

3.4.2. CBS

A difference between the levels of cystathionine in the incubation mixtures with and without PPG can be used to estimate the activity of CBS in mouse tissue homogenates incubated with or without 10.8 mM HSer (Scheme S2). PPG concentrations causing total inhibition of CTH activity were 0.75 mM in the liver homogenate, 0.95 mM in the kidneys homogenate and 0.1 mM in the brain homogenate. The total CTH inhibition by PPG in the tissue homogenates was confirmed with the Matsuo and Greenberg's method with modifications (see chapter 2.3.1).

In the homogenates of mouse brain and kidneys, the calculated value of CBS activity was about 2–3 times higher than in the liver homogenate (Table 5B). However, in the brain, it was noted that

Table 2
Basal levels of the measured compounds in mouse tissue homogenates. Cystathionine – units [pmol/mg protein], cysteine, cystine, TFC, GSH, GSSG, TFG, homoserine – units [nmol/mg protein]. TFC = total free cysteine, TFG = total free glutathione, N.D. = not detected.

	Liver	Kidneys	Brain
Cystathionine	30 ± 8	55 ± 20	70 ± 20
Cysteine	N.D.	4.1 ± 1.8	0.34 ± 0.2
Cystine	N.D.	0.78 ± 0.10	0.11 ± 0.02
TFC	N.D.	6.26 ± 1.52	0.56 ± 0.28
GSH	0.32 ± 0.05	0.10 ± 0.04	1.4 ± 0.5
GSSG	0.86 ± 0.13	0.12 ± 0.04	10.6 ± 1.73
TFG	2.05 ± 0.3	0.28 ± 0.05	22.5 ± 3.95
Homoserine	0.32 ± 0.1	0.54 ± 0.1	0.82 ± 0.2

Table 3
Changes in levels of the measured compounds after 15 minutes of incubation with homoserine. Cystathionine – units [pmol/mg protein], cysteine, cystine, TFC, GSH, GSSG, TFG, homoserine – units [nmol/mg protein], TFC = total free cysteine, TFG = total free glutathione *P < 0.05 tissue homogenates with HSer vs. tissues homogenates without HSer, N.D. = not detected.

	Liver	Kidneys	Brain
Cystathionine	33 ± 10	78 ± 8	60 ± 10
Cysteine	9.4 ± 1.2*	19.4 ± 4.15*	38.6 ± 3.3*
Cystine	N.D.	1.24 ± 0.3*	N.D
TFC	9.4 ± 1.2*	21.6 ± 5.3*	38.6 ± 3.3*
GSH	0.86 ± 0.16*	0.09 ± 0.02	1.52 ± 0.41
GSSG	1.15 ± 0.10*	0.12 ± 0.03	10.5 ± 1.1
TFG	3.5 ± 1.2*	0.33 ± 0.06	22.5 ± 2.8

the activity of CBS without adding HSer was about one tenth only of that with HSer.

The liver and kidneys showed a relatively low CBS activity independently of homoserine (Table 5B). The brain demonstrated a relatively high CBS activity and no CTH activity (Fig. 2, Table 5B) [48], what explains high cystathionine levels (Table 2) and lack of α-ketobutyrate (Fig. 2). A huge increase in the level of cysteine (Table 3) in the brain homogenate incubated with HSer (Table 5A) suggests the biosynthesis of cystathionine from homoserine and cysteine by CBS, the activity of which was high (Table 5B). The low level of cystathionine in the liver (Table 2) together with the high level of α-ketobutyrate (Fig. 2) reflects the highest activity of CTH (Fig. 2).

Table 4
Changes in levels of the measured compounds after the incubation with HSer and PPG. Cystathionine – units [pmol/mg protein], cysteine, cystine, TFC, GSH, GSSG, TFG, homoserine – units [nmol/mg protein]. TFC = total free cysteine, TFG = total free glutathione, *P < 0.05 tissue homogenates with HSer vs. tissues homogenates without HSer, $^{\Delta}$P < 0.05 tissue homogenates with HSer and PPG vs. tissue homogenates with HSer, N.D. = not detected.

	Liver	Kidneys	Brain
Cystathionine	36 ± 5*	85 ± 25*	120 ± 37*$^{\Delta}$
Cysteine	9.7 ± 2.5*	19.25 ± 5.43*	37.1 ± 7.64*
Cystine	N.D.	1.3 ± 0.26*	0.13 ± 0.03$^{\Delta}$
TFC	9.7 ± 2.5*	21.85 ± 5.65*	37.4 ± 7.7*
GSH	0.4 ± 0.09*$^{\Delta}$	0.11 ± 0.01	1.64 ± 0.21
GSSG	1.13 ± 0.09*	0.15 ± 0.01	10.74 ± 1.3
TFG	2.7 ± 0.22*$^{\Delta}$	0.34 ± 0.12	23.12 ± 2.6

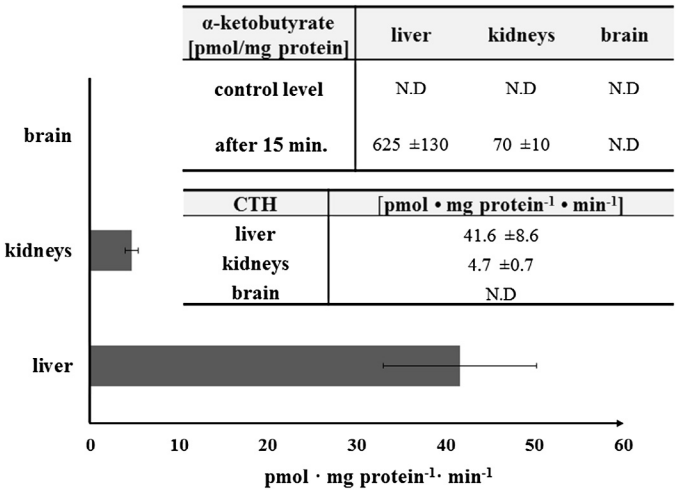


Fig. 2. The activity of CTH in mouse tissues homogenates. N.D. – not detected.

Table 5
Changes in the level of cystathionine (A) and the activity of CBS (B) in mouse tissue homogenates without and with HSer.

(A)

Cystathionine [pmol/mg protein]	Liver	Kidneys	Brain
Without HSer			
Control	27 ± 8	53 ± 15	70 ± 20
After 15 min. with PPG	30 ± 8	63 ± 31	77 ± 40
With HSer			
Control	33 ± 10	78 ± 8	60 ± 1
After 15 min. with PPG	36 ± 5	85 ± 25	120 ± 37

(B)

CBS [pmol mg protein^{-1} min^{-1}]	without HSer	with HSer
Liver	0.2	0.2
Kidneys	0.7	0.5
Brain	0.5	4.0

4. Conclusions

The RP-HPLC-based method of determination of the activity of CBS and CTH was employed in mouse tissues – the liver, kidneys and brain. The RP-HPLC method allowed for determining simultaneously, in the same sample, direct and far products of the CBS and CTH-catalyzed reactions, such as cystathionine, cysteine, and glutathione. Using the same column and elution gradient, the product of CTH, α-ketobutyrate, was measured.

A difference in the cystathionine level between the homogenates with totally CTH-inhibiting concentrations of PPG and without the inhibitor was employed to evaluate the activity of CBS. CTH activity was demonstrated using DL-homoserine as a substrate and a sensitive HPLC-based assay to measure the small amounts of α-ketobutyrate.

In the investigated tissues, the activity of CBS seemed to be the highest in the brain, while CTH demonstrated the highest activity in the mouse liver. The method presented here allows for evaluating the relative contribution of CBS and CTH to generation of H_2S in tissues. Lack of CTH activity confirms that CBS plays the main role in H_2S generation in the brain, while CTH fulfils the predominant role in the liver.

The possible explanation for the high level of cystathionine in the brain is its suggested role as a neurotransmitter and antioxidant [49]. HSer added to the brain homogenate caused an increase in the levels of cysteine and cystathionine, suggesting the possible biosynthesis of cystathionine from homoserine and cysteine catalyzed by CBS.

The low level of cystathionine in the liver together with the high level of α-ketobutyrate reflects the highest activity of CTH. The highest increase in the level of GSH in the mouse liver homogenates suggests both the important role of the CBS/CTH tandem in cysteine delivering for GSH synthesis and the high activity of the enzymes participating in glutathione synthesis.

In case of the functional deficiency in the activity of one of H_2S producing enzymes (CBS or CTH), the application of the presented method could relatively easily confirm whether the lack of this activity is compensated by the activity of the other enzyme to maintain the H_2S level, and demonstrate effects of this deficiency on the level of cysteine and glutathione, and the redox status as the changes in GSH/GSSG ratio can be investigated.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.niox.2014.09.159.

References

[1] A. Stein, S.M. Bailey, Redox biology of hydrogen sulfide: implications for physiology, pathophysiology, and pharmacology, Redox Biol. 1 (2013) 32–39.
[2] R. Wang, Two's company, three's a crowd: can H_2S be the third endogenous gaseous transmitter?, FASEB J. 16 (2002) 1792–1798.
[3] W. Zhao, J. Zhang, Y. Lu, R. Wang, The vasorelaxant effect of H_2S as a novel endogenous gaseous K(ATP) channel opener, EMBO J. 20 (2001) 6008–6016.
[4] G. Yang, L. Wu, B. Jiang, W. Yang, J. Qi, K. Cao, et al., H_2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase, Science 322 (2008) 587–590.
[5] A. Roy, A.H. Khan, M.T. Islam, M.C. Prieto, D.S.A. Majid, Interdependency of cystathione γ-lyase and cystathione β-synthase in hydrogen sulfide – induced blood pressure regulation in rats, Am. J. Hypertens. 25 (2012) 74–81.
[6] J.R. Koenitzer, T.S. Isbell, H.D. Patel, G.A. Benavides, D.A. Dickinson, R.P. Patel, et al., Hydrogen sulfide mediates vasoactivity in an O_2-dependent manner, Am. J. Physiol. Heart Circ. Physiol. 292 (2007) H1953–H1960.
[7] L.F. Hu, P.T. Wong, P.K. Moore, J.S. Bian, Hydrogen sulfide attenuates lipopolysaccharide-induced inflammation by inhibition of p38 mitogen-activated protein kinase in microglia, J. Neurochem. 100 (2007) 1121–1128.
[8] R. Tamizhselvi, P.K. Moore, M. Bhatia, Hydrogen sulfide acts as a mediator of inflammation in acute pancreatitis: in vitro studies using isolated mouse pancreatic acinar cells, J. Cell. Mol. Med. 11 (2007) 315–326.
[9] M. Whiteman, L. Li, P. Rose, C.H. Tan, D.B. Parkinson, P.K. Moore, The effect of hydrogen sulfide donors on lipopolysaccharide-induced formation of inflammatory mediators in macrophages, Antioxid. Redox Signal. 12 (2010) 1147–1154.
[10] K. Abe, H. Kimura, The possible role of hydrogen sulfide as an endogenous neuromodulator, J. Neurosci. 16 (1996) 1066–1071.
[11] H. Kimura, Metabolic turnover of hydrogen sulfide, Front. Physiol. 3 (2012) 1–3.
[12] T. Chiku, D. Padovani, W. Zhu, S. Singh, V. Vitvitsky, R. Banerjee, H_2S Biogenesis by human cystathionine γ-lyase leads to the novel sulfur metabolites lanthionine and homolanthionine and is responsive to the grade of hyperhomocysteinemia, J. Biol. Chem. 284 (2009) 11601–11612.
[13] S. Singh, D. Padovani, R.A. Leslie, T. Chiku, R. Banerjee, Relative contributions of cystathionine β-synthase and γ-cystathionase to H_2S biogenesis via alternative trans-s ulfuration reactions, J. Biol. Chem. 284 (2009) 22457–22466.
[14] O. Kabil, R. Banerjee, Redox Biochemistry of H_2S, J. Biol. Chem. 285 (2010) 21903–21907.
[15] R. Wang, Physiological implications of hydrogen sulfide: a whiff exploration that blossomed, Physiol. Rev. 92 (2012) (2012) 791–896.
[16] K.H. Jhee, W.D. Kruger, The role of cystathionine beta-synthase in homocysteine metabolism, Antioxid. Redox Signal. 7 (2005) 813–822.
[17] X. Chen, K.H. Jhee, W.D. Kruger, Production of the neuromodulator H_2S by cystathionine beta-synthase via thecondensation of cysteine and homocysteine, J. Biol. Chem. 279 (2004) 52082–52086.
[18] M.H. Stipanuk, Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine, Annu. Rev. Nutr. 24 (2004) 539–577.
[19] R. Banerjee, C.G. Zou, Redox regulation and reaction mechanism of human cystathionine-β-synthase: a PLP-dependent hemesensor protein, Arch. Biochem. Biophys. 433 (2005) 144–156.
[20] F. Binkley, Enzymatic cleavage of thioethers, J. Biol. Chem. 186 (1950) 287–296.
[21] M.H. Stipanuk, P.W. Beck, Characterization of the enzymic capacity for cysteine desulphhydration in liver and kidney of the rat, Biochem. J. 206 (1982) 267–277.
[22] E. Borcsok, R.H. Abeles, Mechanism of action of cystathionine synthase, Arch. Biochem. Biophys. 213 (1982) 695–707.
[23] C. Szabó, Hydrogen sulphide and its therapeutic potential, Nat. Rev. Drug Discov. 6 (2007) 917–935.
[24] M. Whiteman, P.G. Winyard, Hydrogen sulfide and inflamation: the good, the bad, the ugly and the promising, Expert Rev. Clin. Pharmacol. 4 (2011) 13–32.
[25] A.L. Levonen, R. Lapatto, M. Saksela, K.O. Raivio, Human cystathionine gamma-lyase: developmental and in vitro expression of two isoforms, Biochem. J. 347 (2000) 291–295.
[26] A. Asimakopoulou, P. Panopoulos, C.T. Chasapis, C. Coletta, Z. Zhou, G. Cirino, et al., Selectivity of commonly used pharmacological inhibitors for cystathionine β- synthase (CBS) and cystathionine γ-lyase (CSE), Br. J. Pharmacol. 169 (2013) 922–932.
[27] L. Diwakar, V. Ravindranath, Inhibition of cystathionine-γ-lyase leads to loss of glutathione and aggravation of mitochondrial dysfunction mediated by excitatory amino acid in the CNS, Neurochem. Int. 50 (2007) 418–426.
[28] Y. Matsuo, D.M. Greenberg, A crystalline enzyme that cleaves homoserine and cystathionine, J. Biol. Chem. 230 (1958) 545–560.
[29] J. Czubak, M. Wróbel, H. Jurkowska, Cystathionine γ-lyase (EC 4.4.1.1): an enzymatic assay of α-ketobutyrate using lactate dehydrogenase, Acta Biol. Cracov. Ser. Zool. 44 (2002) 113–117.
[30] P. Bronowicka-Adamska, M. Wróbel, J. Zagajewski, RP-HPLC method for quantitative determination of cystathionine, cysteine and glutathione: an application for the study of the metabolism of cysteine in human brain, J. Chromatogr. B 879 (2011) 2005–2009.
[31] M.A. Paz, O.O. Blumenfeld, M. Rojkind, E. Henson, C. Furfine, P.M. Gallop, Determination of carbonyl compounds with *N*-methyl benzothiazolone hydrazone, Arch. Biochem. Biophys. 109 (1965) 548–559.
[32] K. Soda, A spectrophotometric microdetermination of keto acids with 3-methyl-2-benzothiazolone hydrazone, Agric. Biol. Chem. 31 (1967) 1054–1060.
[33] K. Soda, Microdetermination of D-amino acids and D-amino acid oxidase activity with 3-methyl-2-benzothiazolone hydrazone hydrochloride, Anal. Biochem. 25 (1968) 228–235.
[34] K. Soda, T. Yorifuji, H. Misono, M. Moriguchi, Spectrophotometric determination of pyridoxal and pyridoxal 5'-phosphate with 3-methyl-2-benzothiazolone hydrazone hydrochloride, and their selective assay, Biochem. J. 114 (1969) 629–633.
[35] M. Tokoro, T. Asai, S. Kobayashi, T. Takeuchi, T. Nozaki, Identification and characterization of two isoenzymes of methionine γ-lyase from *Entamoeba histolytica*: a key enzyme of sulfur-amino acid degradation in an anareobic parasitic protist that lacks forward and reverse trans-sulfuration pathways, J. Biol. Chem. 278 (2003) 42717–42727.
[36] O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.I. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[37] P.K. Dominick, P.B. Cassidy, J.C. Roberts, A new and versatile method for determination of thiolamines of biological importance, J. Chromatogr. B 761 (2001) 1–12.
[38] M. Wróbel, I. Lewandowska, P. Bronowicka-Adamska, A. Paszewski, The level of sulfane sulfur in the fungus Aspergillus nidulans wild type and mutant strains, Amino Acids 37 (2009) 565–571.
[39] H.H. Tallan, S. Moore, W.H. Stein, L-cystathionine in human brain, J. Biol. Chem. 230 (1958) 707–716.
[40] F. Chatagner, M. Tixier, C.H. Portemer, Biosynthesis of cystathionine from homoserine and cysteine by rat liver cystathionase, FEBS Lett. 4 (1969) 231–233.
[41] P.W.K. Wong, V. Schwarz, G.M. Komrower, The biosynthesis of cystathionine in patients with homocystinuria, Pediatr. Res. 2 (1968) 149–160.
[42] P.W.K. Wong, R. Fresco, Tissue cystathionine in mice treated with cysteine and homoserine, Pediatr. Res. 6 (1972) 172–181.
[43] R.H. Abeles, C. Walsh, Acetylenic enzyme inactivators. Inactivation of γ-cystathionase, in vitro and in vivo, by propargylglycine, J. Am. Chem. Soc. 95 (1973) 6124–6125.
[44] S. Shinozijka, S. Tanase, Y. Morino, Metabolic consequences of affinity labeling of cystathionase and alanine aminotransferase by L-propargylglycine in vivo, Eur. J. Biochem. 124 (1982) 377–382.
[45] S. Yu, K. Sugahara, K. Nakayama, S. Awata, H. Kodama, Accumulation of cystathionine, cystathionine ketimine, and perhydro-1,4-thiazepine-3,5-dicarboxylic acid in whole brain and various regions of the brain of DL-propargylglycine-treated rats, Metabolism 49 (2000) 1025–1029.
[46] T. Nishi, K. Tanaka, Y. Tanaka, K. Araki, K. Furihata, M. Onodera, et al., Accumulation of L-cystathionine by an *Escherichia coli* mutant deficient in cystathionine beta-lyase, J. Biosci. Bioeng. 94 (2002) 178–181.
[47] P.L. Bergad, W.B. Rathbun, High performance liquid chromatography determination of cystathionine activity in human and rabbit lenses, Ophthalmic Res. 18 (1986) 343–348.
[48] Y. Ogasawara, S. Isoda, S. Tanabe, Tissue and subcellular distribution of bound and acid-labile sulfur, and the enzymic capacity for sulfide production in the rat, Biol. Pharm. Bull. 17 (1994) 1535–1542.
[49] V. Vitvitsky, M. Thomas, A. Ghorpade, H.E. Gendelman, R. Banerjee, A functional transsulfuration pathway in the brain links to glutathione homeostasis, J. Biol. Chem. 281 (2006) 35785–35793.
[50] A. Kato, M. Ogura, M. Suda, Control mechanism in the rat liver enzyme system converting L-methionine to L-cystine, J. Biochem. 59 (1966) 40–48.

PUBLIKACJA nr 3

Hydrogen sulfide generation from L-cysteine in the human glioblastoma-astrocytoma U-87 MG and neuroblastoma SHSY5Y cell lines

Patrycja Bronowicka-Adamska

Anna Bentke

Maria Wróbel

ACTA ABP BIOCHIMICA POLONICA

Vol. 64, No 1/2017
171–176
https://doi.org/10.18388/abp.2016_1394

Regular paper

Hydrogen sulfide generation from L-cysteine in the human glioblastoma-astrocytoma U-87 MG and neuroblastoma SHSY5Y cell lines*

Patrycja Bronowicka-Adamska, Anna Bentke and Maria Wróbel✉

Chair of Medical Biochemistry, Jagiellonian University, Collegium Medicum, Kraków, Poland

Hydrogen sulfide (H_2S) is endogenously synthesized from L-cysteine in reactions catalyzed by cystathionine beta-synthase (CBS, EC 4.2.1.22) and gamma-cystathionase (CSE, EC 4.4.1.1). The role of 3-mercaptopyruvate sulfurtransferase (MPST, EC 2.8.1.2) in H_2S generation is also considered; it could be important for tissues with low CTH activity, e.g. cells of the nervous system. The expression and activity of CBS, CTH, and MPST were detected in the human glioblastoma-astrocytoma (U-87 MG) and neuroblastoma (SHSY5Y) cell lines. In both cell lines, the expression and activity of MPST were the highest among the investigated enzymes, suggesting its possible role in the generation of H_2S. The RP-HPLC method was used to determine the concentration of cystathionine and alpha-ketobutyrate, products of the CBS- and CTH-catalyzed reactions. The difference in cystathionine levels between cell homogenates treated with totally CTH-inhibiting concentrations of DL-propargylglycine and without the inhibitor was used to evaluate the activity of CBS. The higher expression and activity of CBS, CTH and MPST in the neuroblastoma cells were associated with more intensive generation of H_2S in the presence of 2 mM cysteine. A threefold higher level of sulfane sulfur, a potential source of hydrogen sulfide, was detected in the astrocytoma cells in comparison to the neuroblastoma cells.

Key words: cystathionine, beta-synthase, gamma-cystathionase, glutathione, hydrogen sulfide, 3-mercaptopyruvate sulfurtransferase, sulfane sulfur

Received: 18 July, 2016; revised: 07 October, 2016; accepted: 05 November, 2016; available on-line: 14 March, 2017

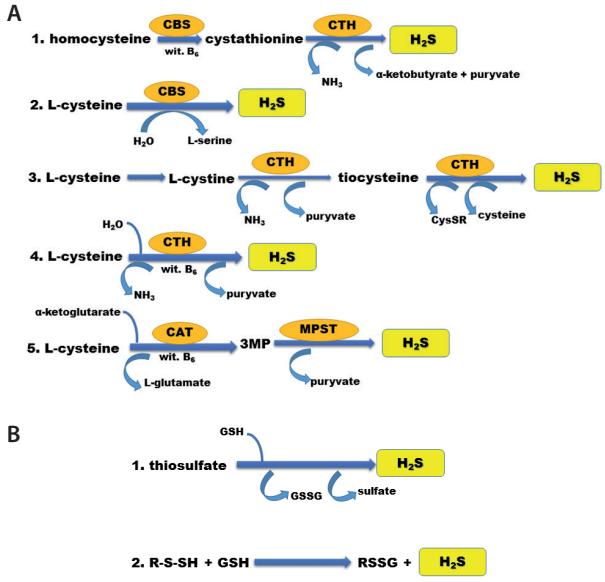
*The results were presented at the 3rd International Conference on H_2S Biology and Medicine, Kyoto, Japan, 4–6 June, 2014, as a report (The activity of hydrogen sulfide generating enzymes in human cell lines. Nitric Oxide – Biology and Chemistry, Vol. 39, Suppl. 1 p. S33-S33).

✉e-mail: mbwrobel@cyf-kr.edu.pl

Abbreviations: CAT, cysteine aminotransferase; CBS, cystathionine β-lyase; CTH, γ-cystathionase; CTN, cystathionine; GSH, glutathione reduced form; MPST, 3-mercaptopyruvate sulfurtransferase; NBT/BCIP, nitroblue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate, toluidine; PCA, perchloric acid

INTRODUCTION

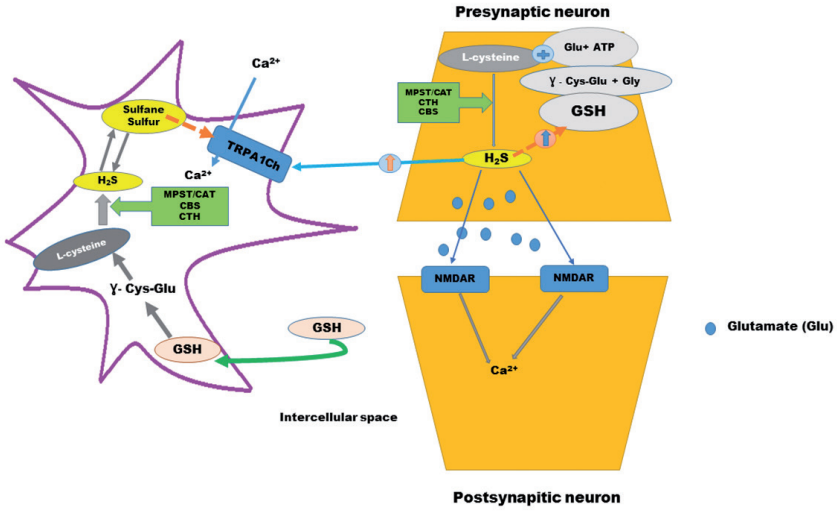
In mammalian tissues, H_2S is synthesized endogenously from L-cysteine in regulated enzymatic pathways catalyzed by pyridoxal phosphate-dependent enzymes: cystathionine beta-synthase (CBS, EC 4.2.1.22), gamma-cystathionase (CTH, EC 4.4.1.1) and cysteine aminotransferase (CAT, EC 2.6.1.3) coupled with 3-mercaptopyruvate sulfurtransferase (MPST) (Wang, 2012) (Scheme 1A).



Scheme 1. The enzymatic (A) and non-enzymatic (B) reactions generating hydrogen sulfide.

Enzymatic reactions involve L-cysteine hydrolysis by CBS to L-serine and H_2S, L-cystine transformation by CTH into thiocysteine, pyruvate (PA) and ammonia and subsequent thiocysteine transformation into H_2S and CysSR (S-thiolane). Another pathway includes the transformation of L-cysteine into 3-mercaptopyruvate (3MP) by cysteine aminotransferase (CAT) and, subsequently, 3MP desulfuration catalyzed by MPST resulting in H_2S and pyruvate formation (Scheme 1A). H_2S is formed in a redox reaction between thiosulfate or RSSH (persulfides) and biological thiols such as reduced glutathione (GSH) (Scheme 1B) (Libiad *et al.*, 2014; Predmore *et al.*, 2012). An additional pathway for the production of 3-MP and H_2S from D-cysteine by D-amino acid oxidase provides protection of cerebellar neurons from oxidative stress (Shibuya *et al.*, 2013).

Astrocytes secrete and store antioxidative compounds, such as glutathione or ascorbate (Bartosz, 2006; Bèlanger & Magistretti, 2009, Zablocka & Janusz, 2007). The cells play an important role in supplying precursors necessary for GSH synthesis in the neurons. GSH captured by astrocytes from the extracellular space is degraded in a reaction catalyzed by γ-glutamyl-transpeptidase (γ-GT) to free cysteine (Zablocka & Janusz, 2007) (Scheme 2). The main transport system for cysteine in the astrocytes and neurons is mediated by the Na^+ dependent X_{AG}^- and ASC (alanine-serine-cysteine) systems (Shanker *et*



Scheme 2. The possible cooperation between the astrocytes and neural cells.
The Scheme is based on the scheme presented by Kimura (2013). H_2S activates TRPA1 channels in the astrocytes in a similar way, but not as efficiently as polysulfides (Kimura, 2013; Moore & Whiteman, 2005). H_2S has antioxidative properties and it increases the production of glutathione in neural cells (Kimura *et al.*, 2010; Kimura & Kimura, 2004). TRPA1Ch, transient receptor potential cation channel; subfamily A, member 1; NMDAR, N-methyl-D-aspartate receptor; Gly, Glycine; GSH, reduced glutathione

al., 2001a; Shanker *et al.*, 2001b). The degradation product returns to neuron cells, where it is used as a substrate for glutathione synthesis. When compared to the neurons, astrocytes have the higher level of GSH, both *in vivo* and in cell cultures (Dringen *et al.*, 2000). Functional neuron-glial cell interrelations provide an important mechanism participating in brain functions control (Scheme 2).

The anaerobic conversion of cysteine can lead to the formation of hydrogen sulfide (H_2S). It is known that endogenously formed H_2S acts as a neuromodulator and neuroprotector in the brain (Panthi *et al.*, 2016; Paul & Snyder, 2015). By means of activating NMDA receptors and increasing the response of peripheral neurons, H_2S may play a significant role in processes associated with memorization and learning (Ishigami *et al.*, 2009; Shibuya *et al.*, 2009). An increased synthesis of H_2S has been observed in patients with Down's syndrome and septic shock, while its decreased generation has been noted in Alzheimer's disease. H_2S activates TRPA1 channels in the astrocytes in a similar way, but not as efficiently as polysulfides (Kimura, 2013; Moore & Whiteman, 2005). H_2S has antioxidative properties and it increases the production of glutathione in neural cells (Kimura *et al.*, 2010; Kimura & Kimura, 2004).

The study was conducted to determine the activity and expression of the enzymes: CBS, CTH and MPST involved in the production of H_2S in the human glioblastoma-astrocytoma (U-87 MG) and neuroblastoma (SHSY5Y) cell lines. The results of our previous studies (Jurkowska *et al.* 2011) showed the expression of CTH and MPST genes in the human neoplastic cell lines: astrocytoma U373 and neuroblastoma SH-SY5Y. The CTH and MPST enzymes, through an increase in sulfane sulfur levels, might increase H_2S levels. The RP-HPLC method was used to detect and determine the amount of direct and indirect products of the CBS- and CTH-catalyzed reactions, such as cystathionine, cysteine, and glutathione. The difference in the cystathionine level between the cells incubated with totally CTH-inhibiting concentrations of DL-propargylglycine (PPG) and without the inhibitor was used to evaluate the activity of CBS. Differences in the expression and activity of CBS, CTH, and MPST point to a higher intensity of H_2S generation in the neuroblastoma cells, which was confirmed by the higher level of H_2S in SHSY5Y cells determined using the H_2S trapping method of Kartha *et al.* (2012).

MATERIAL AND METHODS

Chemicals. L-Glutathione reduced (GSH), L-cysteine, cystathionine (CTN), DL-homoserine (HSer), 1-fluoro-2,4-dinitrobenzene (DNFB), bathophenanthroline-disulfonic acid disodium salt (BPDS), acetonitrile, pyridoxal phosphate (PLP), β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH), L-lactic dehydrogenase (LDH), 3-mercaptopyruvate acid sodium salt, D,L-dithiothreitol, (DTT), N-ethylmaleimide (NEM), DL-propargylglycine (PPG), sodium dihydrogen phosphate dihydrate pure, sodium sulfite, chloroform, isopropanol, agarose, sodium hydrosulfide hydrate, sodium chloride, Folin-Ciocalteu's phenol reagent, iron (III) nitrate nonahydrate, sodium thiosulfate pentahydrate, sodium carbonate and N,N-dimethyl-*p*-phenylenediamine sulfate salt, Coomassie Blue G250 were obtained from Sigma-Aldrich (Poznan, Poland). Trifluoroacetic acid (TFA), 2-mercaptoethanol were purchased from FlukaChemie GmbH (Buchs, Switzerland). Ethanol and 70% perchloric acid (PCA), 38% formaldehyde, 65% nitric acid, 38% hydrochloric acid, ammonia solution 25% pure, sodium potassium tarate, copper sulphate pentahydrate, potassium dihydrogen phosphate, ferric chloride, zinc acetate dehydrate pure, sodium hydroxide were from Polskie Odczynniki Chemiczne S.A. (Gliwice, Poland). N^{ε}-methyllysine was obtained from Bachem (Bubendorf, Switzerland). DMEM/High glucose, trypsin 0.25%, fetal bovine serum and penicillin-streptomycin solution were purchased from Thermo Scientific (Waltham, MA, USA). Trizol, ethidium bromide and EDTA-disodium salt dihydrate were obtained from Lab-Empire S.A. (Rzeszow, Poland). Potassium cyanide was from Merck Sp. z o.o. (Warszawa, Poland). Reverse Transcriptase M-MuLV was obtained from Roche Diagnostics Polska Sp. z o.o. and Promega Poland (Warszawa, Poland). Polymerase DNA Dream Taq™, Gene Ruler 100 bp DNA Ladder, Oligo(dT)18 primer and dNTP Mix were obtained from Abo Sp. z o.o. (Gdańsk, Poland). RIPA buffer was from TermoScientific (Rockford, USA). Antibodies: anti-CBS and -CTH were from Abnova (Taiwan), anti-MPST was from GeneTex (Taiwan), anti-β-actin from Sigma-Aldrich (Poznan, Poland), anti-alpha-tubuline, alkaline phosphatase-conjugated goat anti-rabbit IgG antibody and anti-mouse IgG antibody were from Proteintech (Chicago, IL, USA). NBT/BCIP (nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate), toluidine was from Roche (Warszawa, Poland). All the chemicals and HPLC solvents were

gradient grade. Water was deionized by passing through an EASY pure RFcompact ultrapure water system.

Cell lines. Cell culture. Human glioblastoma-astrocytoma (U-87 MG) and neuroblastoma (SHSY5Y) cell lines were grown in a monolayer in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), in plastic culture dishes (100 mm in diameter), at 37°C in a humidified atmosphere containing 5% CO_2. Cell lines were purchased from the European Collection of Cell Cultures (ECACC-SIGMA Aldrich, Poznań, Poland).

Cytotoxicity of L-cysteine. The cells were seeded in triplicates into 96-microwell plates at density of 20×10^3 cells/well and incubated for 24 h with or without 2 mM L-cysteine in DMEM medium supplemented with 10% FBS. Colorimetric assay was performed according to the manufacturer instructions (Cytotoxicity Detection Kit, Roche, Thermo Fisher Scientific). Absorbance of the colored product – formazane – was measured at 490 nm by microreader (EPOCH, BioTEK).

Expression of MPST, CTH, CBS in cell lines. RNA extraction. The total RNA was extracted using TRIZOL, according to the protocol provided by the manufacturer. The quality of RNA samples was determined by spectrophotometric analysis (A_{260}/A_{280}) and electrophoresis in 2.5% agarose gel followed by staining with ethidium bromide.

Reverse Transcription of RNA. Total RNA from the cell samples was reverse-transcribed using First-Stand cDNA synthesis kit according to the manufacturer instructions (Promega, Company, Warszawa, Poland). For reverse transcription (RT) 3 μg of total RNA was mixed with 1 μl Oligo $(dT)_{15}$ (0.5 μg/reaction) and nuclease-free water and heated in a 70°C heat block for 5 minutes. After preincubation the reverse transcription reaction mix containing: 4 μl of GoScript™5X reaction buffer, 3 μl of $MgCl_2$ (final concentration 1.5–5.0 mM), 1 μl of deoxyribonucleotide triphosphates (dNTPs, 10 mM), 1 μl of Recombinant RNases Ribonuclease Inhibitor (20 U/μl) and 1 μl of GoScript™ Reverse Transcriptase were prepared.

cDNA Synthesis and RT-PCR analysis. Expression of MPST, CTH, CBS and β-actin was analyzed with RT-PCR as previously described by Jurkowska *et al.* (2011) with modifications. Amplification of cDNA samples was performed in a 12.5 μl reaction volume containing: 1 μl of synthesized cDNA, 0.2 μM of each of gene-specific primer pair, 0.04 U/μl of DNA polymerase in 10 mM Tris-HCl buffer pH 8.8, 0.2 mM of each dNTPs and nuclease-free water. The temperature profile of RT-PCR amplification for the MPST consisted of activation of Taq polymerase at 94°C for 5 min, denaturation of cDNA at 94°C for 30 s, primer annealing at 56°C for 30 s, elongation at 72°C for 2 min for the following 28 cycles and was finished by the extension step for 8 min. For the CTH gene, after the initial denaturation for 5 min at 94°C, amplification was performed under the following conditions: 94°C for 30 s, 51°C for 1 min and 72°C for 8 min for 28 cycles, with the final incubation at 72°C for 10 min. For the CBS gene, after the initial denaturation for 5 min at 94°C, amplification was performed under the following conditions: 94°C for 30 s, 60°C for 30 s and 72°C for 2 min for 38 cycles, with the final incubation at 72°C for 8 min. For β-actin gene, after the initial denaturation for 5 min at 94°C, amplification was performed under the following conditions: 94°C for 30 s, 54°C for 30 s and 72°C for 2 min for 28 cycles, with the final incubation at 72°C for 8 min. The following specific primers (Oligo Company) were used:

MPST – F: 5'TCTTCGACATCGACCAGTGC' and R: 5'TGTGAAGGGGATGTTCACGG3'

CTH – F: 5'GCAAGTGGCATCTGAATTTG3' and R: 5'CCCATTACAACATCACTGTGG3'

CBS – F: 5'CGCTGCGTGGTCATTCTGCC3' and R: 5'TCCCAGGATTACCCCCGCCT3'

β-actin – F: 5'CTGTCTGTCACCACCAT3' and R: 5'GCAACTAAGTCATAGTCCGC3'

β-actin was used as the internal standard to normalize all samples for potential variations in mRNA content. PCR reaction products were separated electrophoretically in a 2.5% agarose gel, stained with ethidium bromide and directly visualized under UV light and photographed.

Western blotting analysis. The cells were suspended in RIPA buffer, containing proteinase inhibitors cocktail, sonicated 3×5 s at 4°C (BandelinSonoplus GM 70) and centrifuged at $14\,000 \times g$ for 15 min – supernatants were used for further analysis. The relative amount of CBS, CTH, MPST was determined by Western blotting using the appropriate antibody: anti-CBS (1:1 000), anti-CTH (1:1 000), anti-MPST (1:1000). Anti-β-actin (1:5 000) and anti-alpha-tubuline (1:5 000) antibodies were used to check for equal loading. Proteins of interest were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (1:2 000) or with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (1:2 000). Proteins were visualized with NBT/BCIP staining solution.

Detection of H_2S. The H_2S produced during the incubation of the cell culture with H_2S-releasing compounds was trapped as zinc sulfide in the zinc agarose layer according to Kartha *et al.* (2012). The standard curve was linear at the concentration range of 0–250 μM with correlation coefficient of 0.994.

Enzymes assay. Cell homogenization. U-87 MG and SHSY5Y cells ($3.5–5 \times 10^6$ cells) were suspended in 0.1 M phosphate buffer pH 7.5, in the proportion of 1 mln cells/0.07 ml of the buffer, sonicated 3×5 s at 4°C (BandelinSonoplus GM 70). After centrifugation at $1600 \times g$ for 10 min, the supernatant was used for the determination of protein concentration, sulfane sulfur levels and the activity of MPST and CTH. For RP-HPLC analyses cells were suspended in 0.1 ml 0.9% NaC/l 70% PCA/1 mM BPDS. The sediment was separated by centrifugation at $1400 \times g$ for 10 min, and supernatant was stored at –80°C until analysis.

MPST activity. MPST activity was assayed according to the method of Valentine and Frankelfeld, (1974) following a procedure described in our earlier paper (Wróbel *et al.* 2004). The incubation mixture contained: 250 μl of 0.12 M sodium phosphate buffer, pH 8.0, 50 μl of 0.5M sodium sulfite, 50 μl of 0.15 M dithiothreitol, 50 μl of homogenates, 50 μl of H_2O and 50 μl of 0.1 M 3-mercaptopyruvate acid sodium salt in a final volume of 500 μl. Mixture was incubated for 15 min. To stop the reaction 250 μl of 1.2 M PCA was added. Samples were centrifuged at $1600 \times g$ for 5 min, and 100 μl of supernatant was transferred to 1350 μl of mixture that contained: 1200 μl of 0.12 M sodium phosphate buffer, pH 8.0, 100 μl of 0.1 M N-ethylmaleimide, 50 μl of NADH 5 mg/ml. After equilibration at 37°C, 2.5 μl of lactate dehydrogenase (7 IU) was added, and the decrease in absorbance was measured at 340 nm. The enzyme activity was expressed as nmoles of pyruvate produced during 1 min incubation at 37°C per 1 mg of protein.

CTH activity. Cystathionase activity was determined using Matsuo and Greenberg's method (1958) with modifications described by Czubak *et al.* (2002). The

incubation mixture contained: 25 µl of 1.3 mM PLP, 25 µl of 0.02 mM EDTA, 250 µl of 45 mM cystathionine solution in 0.1 M phosphate buffer, pH 7.5 (2.5 mg of cystathionine per sample) and 75 µl of homogenate and 0.1 M phosphate buffer, pH 7.5 containing 0.05 mM 2-mercaptoethanolin the final volume of 650 µl. The reaction was stopped after 15 min of incubation at 37°C by placing 125 µl of the incubation mixture in 25 µl of 10% PCA. Samples were centrifuged at 1600 × *g* for 10 min, and 25 µl of supernatant was transferred to 625 µl of 0.194 mM NADH solution and kept at 37°C. Control samples, without 45 mM cystathionine, were prepared in the same way as the examined samples. After 10 s of the measurement (absorbance at 340 nm), 25 µl (9.06 IU) of lactate dehydrogenase (LDH) was added and measurement was continued up to 180 s. The difference between the initial value of absorbance (before adding LDH) and the lowest value (after adding LDH) corresponded to the amount of alpha–ketobutyrate formed in the course of the cystathionase reaction. Cystathionase activity was expressed as nmoles of α-ketobutyrate formed during 1min incubation at 37°C per 1 mg of protein.

CBS activity. The activity of CBS was examined in cells homogenates in the presence of DL-homoserine as substrate after 15 minutes incubation at 37°C according to the description in Bronowicka-Adamska *et al.* (2011). PPG, in the concentration of 0.7 mM, was used to completely inhibit the activity of CTH in both cell lines. The level of cystathionine was determined using the HPLC method described by Bronowicka-Adamska *et al.* (2015). The CBS activity was expressed as pmoles of cystathionine formed during 1min incubation at 37°C per 1 mg of protein.

Sulfane sulfur. Sulfane sulfur was determined by the method of Wood, (1987), based on cold cyanolysis and colorimetric detection of ferric thiocyanate complex ion. Incubation mixtures in a final volume 880 µl contained: 20 µl of 1 M ammonia solution, 20 µl of homogenate, 740 µl of H_2O and 100 µl of 0.5 M sodium cyanide. Incubation was performed for 45 min at room temperature. After incubation, thiocyanate was estimated calorimetrically at 460 nm after the addition of 20 µl of 38% formaldehyde and 40 µl of ferric nitrate reagent. Sulfane sulfur level was expressed as nmoles of SCN^-produced per 1 mg of protein.

Proteins. Protein concentration was determined with the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard. Protein concentration measurement with Bradford assay was used for the determination of protein in Western blotting analysis (Bradford, 1976).

RP-HPLC (Reverse Phase High Performance Liquid Chromatography). The level of cystathionine, alpha-ketobutyrate and the reduced glutathione (GSH) in the incubation mixtures were determined using the RP-HPLC method of Dominick *et al.* (2001) with modifications (Bronowicka-Adamska *et al.*, 2015; Bronowicka-Adamska *et al.*, 2011; Wróbel *et al.*, 2009).

Table 1. The mean value of MPST, CTH, CBS activity, and sulfane sulfur level in U-87 MG and SHSY5Y cell.

Cell line	MPST	CTH	CBS	Sulfane Sulfur
	nmol·mg^{-1} min^{-1}		pmol·mg^{-1}·min^{-1}	nmol·mg protein^{-1}
SHSY5Y	674±93	5.15±1.46	17.3±2.7	41±15
U87MG	196±23	3.29±0.83	1.34±0.2	139±47

Values are the mean of four to five measurements from three independent experiments.

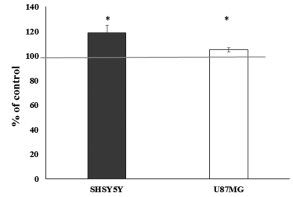


Figure 1. The level of hydrogen sulfide in homogenates of U-87 MG and SHSY5Y cells after 24 h incubation with 2 mM L-cysteine.
The experiments were carried out for control homogenates of U-87 MG, SHSY5Y cells with 2 mM L-cysteine as the main endogenous substrate for the hydrogen sulfide producing enzymes. The data represent the mean value from three independent experiments. Statistical analysis was performed using the Student's *t*-test (*P<0.05).

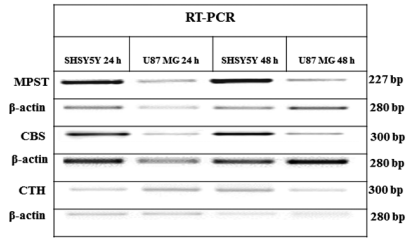


Figure 2. RT-PCR analysis of MPST, CBS and CTH expression in SHSY5Y and U-87 MG cell lines.
One set of representative results is shown. β-actin was used as the internal control. The length of the products: MPST, 227 bp; CBS, 300 bp; CTH, 300 bp; β-actin, 280 bp. Specific primers and temperature profiles for particular proteins are given in Material and Methods.

Statistical analysis. All results were expressed as means ±S.D. The significance of the differences between controls and investigated samples were calculated using Student's Test (P<0.05). Each experiment was repeated minimum three times.

RESULTS AND DISCUSSION

The studies showed U-87 MG and SHSY5Y cells capacity of hydrogen sulfide formation from L-cysteine and an increased level of hydrogen sulfide in the neuroblastoma – SHSY5Y cells (Fig. 1) by about 20%, as compared to the control cells without L-cysteine, and only by about 5% in the glioblastoma-astrocytoma (U-87 MG cells) after 24 h of incubation with 2 mM L-cysteine (Fig. 1). The cytotoxic effect on the SHSY5Y and U-87 MG cell lines after 24 hours of incubation with 2 mM L-cysteine was lower than 10% for both cell lines.

Table 1 shows the activity of CTH, MPST, CBS and the level of sulfane sulfur in the SHSY5Y and U-87 MG cells. One can summarize that all the enzymatic pathways (Scheme 1) generating H_2S can occur in the investigated cell lines. CBS is regarded as the principal enzyme responsible for H_2S synthesis in the brain, while CTH plays a similar role in the circulatory system (Abe & Kimura, 1996). More than tenfold higher CBS activity and three fold higher MPST activity were estimated in the neuroblastoma cells, as compared to the astrocytoma cells. Therefore, the neuroblastoma cells have potentially higher capacity of H_2S generation from cysteine than astrocytoma. In the neuroblastoma cells, specific activity of MPST being the highest among the investigated enzymes and the highest expression of MPST (Fig. 2 and

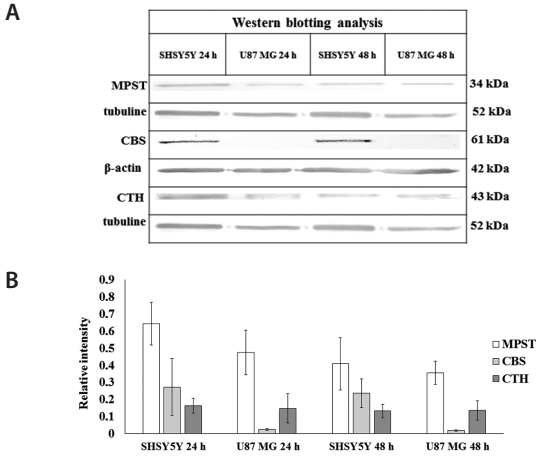


Figure 3. Western blotting analysis of MPST, CBS, CTH proteins in SHSY5Y and U-87 MG cell lines.
(**A**) Western blotting analysis. Western blot analysis of MPST, CBS and CTH was performed for the cell lines using a mixture of rabbit- (MPST) and mouse-derived (CBS, CTH, β-actin and alpha-tubuline) primary antibodies with the appropriate mixture of alkaline phosphatase-conjugated secondary antibodies. One set of representative results is shown. β-Actin and alpha-tubuline were used as the internal control of protein loading; 20 µg of protein was added to each lane. For details see Material and Methods. (**B**) The relative intensity normalized to β-actin and alpha-tubulin. Analysis of protein bands imaged with the ChemiDoc™ MP. The relative intensity value is the average from three independent experiments. The relative intensity was normalized using β-actin and alpha-tubulin signals, the average of which was taken as one.

3) can both suggest that this enzyme plays a role in the generation of H_2S.

On the other hand, the sulfane sulfur level was more than threefold higher in the astrocytoma cells, which can suggest that a nonenzymatic release of H_2S from sulfane sulfur-containing compounds (Scheme 2) is possible. It seems probable that in the neuroblastoma cells, H_2S, functioning as a neurotransmitter, is synthesized in response to the signal-to-date. The astrocytoma cells, in turn, can release H_2S from sulfane sulfur reserves to transmit a signal within the astrocyte network (Perea & Araque, 2003; Zablocka & Janusz, 2007). The conditions under which physiological signals mobilize H_2S from sulfane sulfur stores have not been elucidated so far (Paul & Snyder, 2015).

The U-87 MG astrocytoma cells had the higher level of GSH in comparison to the neuroblastoma cells, which confirms the differences in glutathione content between the astrocytes and neurons (Dringen *et al.* 2000) (Fig. 4).The high levels of glutathione in the astrocytes seem to be essential for neurons protection e.g.: against the toxicity of reactive oxygen species. In the control cultures and cultures with PPG (inhibitor of CTH) added in the concentration of 0.7 mM, both the level of α-ketobutyrate and the level of cysteine were undetectable. The inhibition of CTH with PPG in the astrocytoma cells resulted in a diminished level of GSH after 15 min of incubation, but no such effect was seen in the neuroblastoma cells (Fig. 4). This may suggest that the pathway of cysteine generation through CTH-catalyzed reaction is important in the U-87 MG cells, while the SHSY5Y cells depend to a great extent on an exogenous source of cysteine. The importance of the transsulfuration pathway in astrocytes and glioblastoma/astrocytoma cells as a reserve pathway when the demand for glutathione is high was recently discussed by McBean (2012). Changes in cystathionine levels were not

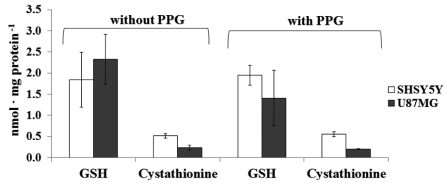


Figure 4. The level of GSH and cystathionine in U-87 MG and SHSY5Y cells.
The experiments were carried out for control homogenates of U-87 MG, SHSY5Y cells with or without PPG as the inhibitor of CTH in concentration of 0.7 mM for both cells lines. The homogenates were incubated with PPG for 15 minutes. The data represent the mean value from three independent experiments.

observed in either of the cell lines in response to PPG after 15 min of incubation (Fig. 4).

CONCLUSIONS

In the glioblastoma-astrocytoma (U-87 MG) and neuroblastoma (SHSY5Y) cells, the pathway catalyzed by enzymatic tandem CAT/MPST can play a role in the generation of hydrogen sulfide from cysteine. In the neuroblastoma cells, the pathway from methionine to cysteine through the CBS and CTH reactions seems to play a more significant role as compared to the astrocytoma cells. The higher activity and expression of enzymes involved in H_2S generation from cysteine, in the neuroblastoma cells, provide an opportunity for more rapid response in H_2S production than in the astrocytoma cells. However, in the astrocytoma cells, the elevation of H_2S seems to be possible by releasing it from the pool of sulfane sulfur. Panthi *et al.* (2016) reviewed possible physiological roles of H_2S in neurons protection from oxidative stress or in the upregulation of the GABA β-receptors at pre- and postsynaptic sites along with astrocytes roles in the regulation of neurotransmitter levels or neuronal excitability.

Acknowledgements

This work was supported by a grant from the National Committee for Scientific Research No. K/DSC/001372.

Conflicts of Interest

The authors declare no conflict of interest.

Contributions

P.B-A provided the experimental data. A.B performed and developed the Western blotting analysis. M.W. provided suggestions for the experiments. P.B-A and M.W. planned the experiments and wrote the paper.

REFERENCES

Abe K, Kimura H (1996) The possible role of hydrogen sulfide as an endogenous neuromodulator. *J Neurosci* **16**: 1066–1071. doi: 10.1017/S1740925X09000064

Araque A (2008) Astrocytes process synaptic information. *Neuron Glia Biol* **4**: 3–10.

Bartosz G (2006) Druga twarz tlenu. Wyd. PWN (in Polish).

Bélanger M, Magistretti PJ (2009) The role of astroglia in neuroprotection. *Dialogues Clin Neurosc* **11**: 281–295.

Bonansco Ch, Couve A, Perea G, Ferradas CA, Roncagliolo M, Fuenzalida M (2011) Glutamate released spontaneously from astrocytes sets the threshold for synaptic plasticity. *Eur J Neurosci* **33**: 1483–1492. doi: 10.1111/j.1460-9568.2011.07631.x

Bradford MM (1976) A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254.

Bronowicka-Adamska P, Wróbel M, Zagajewski J (2011) RP-HPLC method for quantitative determination of cystathionine cysteine and glutathione: An application for the study of the metabolism of cysteine in human brain. *J Chromatography B* **879**: 2005–2009. doi: 10.1016/j.jchromb.2011.05.026

Bronowicka-Adamska P, Wróbel M, Zagajewski J (2015) An application of RP-HPLC for determination of the activity of cystathionine beta-synthase and gamma-cystathionase in tissue homogenates. *Nitric Oxide* **46**: 186–191. doi: 10.1016/j.niox.2014.09.159

Czubak J, Wróbel M, Jurkowska H (2002) Cystathionine γ-lyase (EC 4.4.1.1): an enzymatic assay of α-ketobutyrate using lactate dehydrogenase. *Acta Biol Cracov Ser Zool* **44**: 113–117.

Deitmer JW (2001) Strategies for metabolic exchange between gila cells and neurons. *Respir Physiol* **129**: 71–81. doi: 10.1016/S0034-5687(01)00283-3

Dominik PK, Cassidy PB, Roberts JC (2001) A new and versatile method for determination of thiolamines of biological importance. *J Chromatogr B* **761**: 1–12.

Dringen R (2000) Metabolism and functions of glutathione in brain. *Prog Neurobiol* **62**: 649–671.

Dringen R, Gutterer JM, Hirrlinger J (2000) Glutathione metabolism in brain. Metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. *Eur J Biochem* **267**: 4912–4916.

Ishigami M, Hiraki K, Umemura K, Ogasawara Y, Ishii K, Kimura H (2009) A source of Hydrogen sulfide and a mechanism of its release in the brain. *Antioxid Redox Signal* **11**: 205–214. doi: 10.1089/ars.2008.2132

Jurkowska H, Placha W, Nagahara N, Wróbel M (2011) The expression and activity of cystahionine gamma-lyase and 3-mercaptoppyruvate sulfurtransferase in human neoplastic cell lines. *Amino Acids* **41**: 151–158. doi: 10.1007/s00726-010-0606-3

Kartha RV, Zhou J, Hovde LB, Belinda WY, Cheung BWY, Schröder H (2012) Enhanced detection of hydrogen sulfide generated in cell culture using an agar trap method. *Anal Biochem* **423**: 102–108. doi: 10.1016/j.ab.2012.01.001

Kimura H (2013) Physiological role of hydrogen sulfide and polysulfide in the central nervous system. *Neurochem Int* **63**: 492–497. doi: 10.1016/j.neuint.2013.09.003

Kimura Y, Goto Y, Kimura H (2010) Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria. *Antioxid Redox Signal* **12**: 1–13. doi: 10.1089/ars.2008.2282

Kimura Y, Kimura H (2004) Hydrogen sulfide protects neurons from oxidative stress. *FASEB J* **18**: 1165–1167. doi: 10.1096/fj.04-1815fje

Libiad M, Yadav PK, Vitvitsky V, Martinov M, Banerjee R (2014) Organization of the human mitochondrial Hydrogen sulfide oxidation pathway. *J Biol Chem* **289**: 30901–30910. doi: 10.1074/jbc.M114.602664

Lowry OH, Rosenbrough NJ, Farr AL, Randall RI (1951) Protein measurement with the Folinphenol reagent. *J Biol Chem* **193**: 265–275.

Matsuo Y, Greenberg DM (1958) A crystalline enzyme that cleaves homoserine and cystathionine. *J Biol Chem* **230**: 545–560.

McBean GJ (2012) The transsulfuration pathway: a source of cysteine for glutathione in astrocytes. *Amino Acids* **42**: 190–205. doi: 10.1007/s00726-011-0864-8

Moore PK, Whiteman M (2005) *Chemistry Biochemistry and Pharmacology of Hydrogen Sulfide*. Springer-Verlag GmbH.

Panthi S, Chung H-J, Jung J, Jeong NY (2016) Physiological importance of hydrogen sulfide: emerging potent neuroprotector and neuromodulator. *Oxid Med Cell Longev* **2016**: 1–11. doi: 10.1155/2016/9049782

Paul BD, Snyder SH (2015) Modes of physiologic H_2S signaling in the brain and peripheral tissues. *Antioxid Redox Sign* **22**: 411–423. doi: 10.1089/ars.2014.5917

Perea G, Araque A (2003) New information pathways in the nervous system: communication between astrocytes and neurons. *Rev Neurol* **36**: 137–144.

Predmore BL, Lefer DJ, Gojon G (2012) Hydrogen sulfide in biochemistry and medicine. *Antioxid Redox Signal* **17**: 119–140. doi: 10.1089/ars.2012.4612

Shanker G, Allen JW, Mutkus LA, Aschner M (2001) Methylmercury inhibits the *in vitro* uptake of the glutathione precursor cystine in astrocytes but not in neurons. *Brain Res* **894**: 131–140. doi: 10.1016/S0006-8993(01)02342-3

Shanker G, Allen JW, Mutkus LA, Aschner M (2001) The uptake of cysteine in cultured primary astrocytes and neurons. *Brain Res* **902**: 156–163.

Shibuya N, Koike S, Tanaka M, Ishigami-Yuasa M, Kimura Y, Ogasawara Y, Fukui K, Nagahara N, Kimura H (2013) A novel pathway for the production of hydrogen sulfide from D-cysteine in mammalian cells. *Nat Commun* **4**: 1366–1373. doi: 10.1038/ncomms2371

Shibuya N, Tanaka M, Yoshida M, Ogasawara Y, Togawa T, Ishii K, Kimura H (2009) 3-Mercaptopyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain. *Antioxid Redox Signal* **11**: 703–714. doi: 10.1089/ars.2008.2253

Valentine WN, Frankelfeld JK (1974) 3-Mercaptopyuruvate sulfurtransferase (EC 2.8.1.2): A simple assay adapted to human blood cells. *Clin Chim Acta* **14**: 205–210. doi: 10.1016/0009-8981(74)90031-X

Wang R (2012) Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. *Pysiol Rev* **92**: 791–896. doi: 10.1152/physrev.00017.2011

Wood L (1987) Sulfane sulfur. *Methods Enzymol* **143**: 25–29. doi: 10.1016/0076-6879(87)43009-7

Wróbel M, Jurkowska H, Śliwa L, Srebro Z (2004) Sulfurtransferases and cyanide detoxification in mouse liver kidney and brain. *Toxicol Mech Methods* **14**: 331–337. doi: 10.1080/15376520490434683

Wróbel M, Lewandowska I, Bronowicka-Adamska P, Paszewski A (2009) The level of sulfane sulfur in the fungus *Aspergillus nidulans* wild type and mutant strains. *Amino Acids* **37**: 565–571. doi: 10.1007/s00726-008-0175-x

Zablocka A, Janusz M (2007) Structure and function of the central nervous system. *Postępy Hig Med Dośw* **61**: 454–460.

PUBLIKACJA nr 4

Hydrogen sulphide production in gastric mucosa of rats exposed to stress

Patrycja Bronowicka-Adamska

Maria Wróbel

Marcin Magierowski

Katarzyna Magierowska

Sławomir Kwiecień

Tomasz Brzozowski

molecules

MDPI

Article

Hydrogen Sulphide Production in Healthy and Ulcerated Gastric Mucosa of Rats

Patrycja Bronowicka-Adamska [1], Maria Wróbel [1,*], Marcin Magierowski [2], Katarzyna Magierowska [2], Sławomir Kwiecień [2] and Tomasz Brzozowski [2]

[1] Chair of Medical Biochemistry, Jagiellonian University Medical College, Krakow, 7 Kopernika St., 31-034 Cracow, Poland; mbbronow@cyf-kr.edu.pl

[2] Department of Physiology, Jagiellonian University Medical College, 31-530 Krakow, Poland; magierowskim@yahoo.pl (M.M.); k.jasnos@interia.pl (K.M.); skwiecien@cm-uj.krakow.pl (S.K.); mpbrzozo@cyf-kr.edu.pl (T.B.)

* Correspondence: mbwrobel@cyf-kr.edu.pl; Tel.: +48-12-424-7229; Fax: +48-12-422-3272

Academic Editor: Derek J. McPhee

Received: 27 January 2017; Accepted: 22 March 2017; Published: 27 March 2017

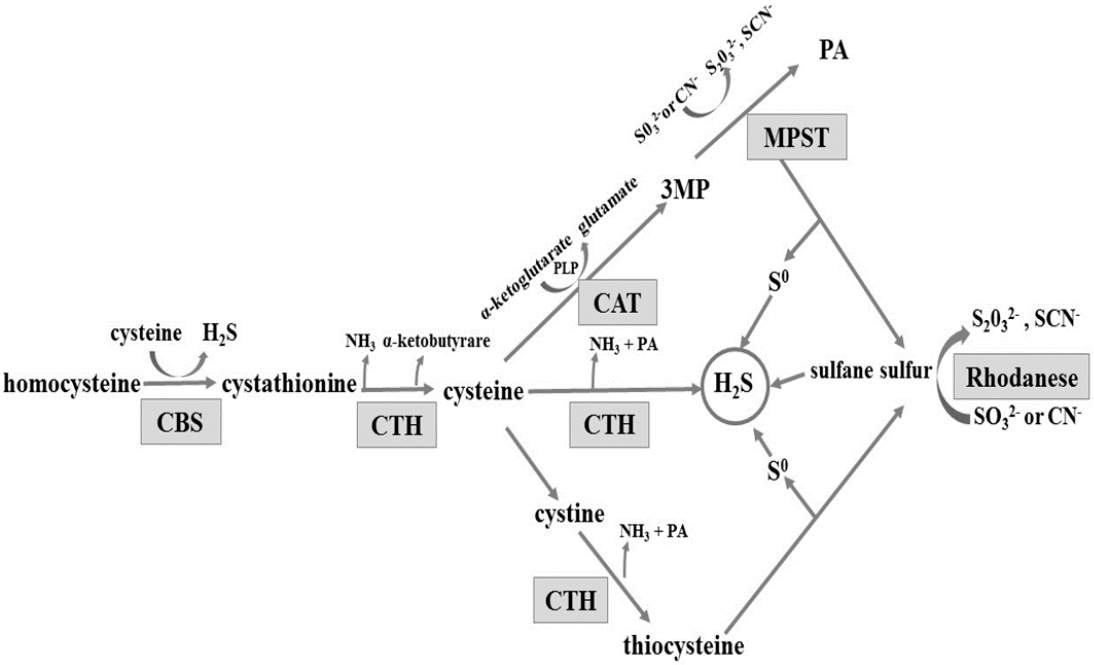
Abstract: Hydrogen sulphide (H_2S) is produced endogenously via two enzymes dependent on pyridoxal phosphate (PLP): cystathionine beta-synthase (CBS, EC 4.2.1.22), cystathionase γ-liase (CTH, EC 4.4.1.1), and a third, 3-mercaptopyruvate sulfurtransferase (MPST, EC 2.8.1.2). H_2S strengthens the defence mechanisms of the gastric mucosal barrier, and plays an important role in gastroprotection, including the increased resistance to damage caused by various irritants and non-steroidal anti-inflammatory drugs. The study was conducted to determine the role of H_2S in ulcerated gastric mucosa of rats caused by immobilization in cold water (WRS). The activity and expression of γ-cystathionase, cystathionine β-synthase, 3-mercaptopyruvate sulfurtransferase, and rhodanese was compared with healthy mucosa, together with H_2S generation, and cysteine, glutathione, and cystathionine levels. The results showed that the defence mechanism against stress is associated with stimulation of the production of H_2S in the tissue and confirmed the observed advantageous effect of H_2S on healing of gastric ulcers. In case of animals pretreated with exogenous sources of H_2S and NaHS, and some changes observed in the ulcerated gastric mucosa tend to return to values found in the healthy tissue, a finding that is in accordance with the previously determined gastroprotective properties of H_2S. The results presented in this paper point to the possible role of rhodanese in H_2S production in the gastric mucosa of rats, together with the earlier mentioned three enzymes, which are all active in this tissue.

Keywords: hydrogen sulphide; gastric mucosa; NaHS; cystathionine-β-synthase; 3-mercaptopyruvate sulfurtransferase; rhodanese; γ-cystathionase

1. Introduction

The gastrointestinal tract is exposed to various substances and factors which often cause gastric mucosal damage. Long-term exposure to these factors, or to stress, can result in pathological inflammation, such as erosions, haemorrhages, or ulcers. An important role in maintaining the integrity of the gastric mucosa is played by hydrogen sulphide (H_2S). H_2S is produced from L-cysteine enzymatically in pathways involving three enzymes: cystathionine β-synthase (CBS, EC 4.2.1.22), cystathionine γ-lyase (CTH, EC 4.4.1.1), and 3-mercaptopyruvate sulfurtransferase (MPST EC 2.8.1.2) (reviewed in [1]). CBS and CTH are enzymes dependent on pyridoxal phosphate (PLP). The MPST reaction converts 3-mercaptopyruvate produced from cysteine in a PLP-dependent transamination reaction (Scheme 1). CBS produces cystathionine and H_2S from L-cysteine or homocysteine. Cystathionine is converted by CTH to L-cysteine, alpha-ketobutyrate, and ammonia. Cysteine can be also converted

by CTH to pyruvate and H_2S. Alternatively, after oxidizing to cystine, it is converted by CTH to thiocysteine—a sulfane sulfur-containing compound. The third enzyme, 3-mercaptopyruvate sulfurtransferase, acts in combination with cysteine aminotransferase (CAT, EC 2.6.1.3) (Scheme 1). Cysteine aminotransferase catalyses L-cysteine transamination to 3-mercaptopyruvate (3MP). MPST catalyses the transfer of sulphur atom from 3MP to sulphite and the product of this reaction, thiosulfate, can be further reduced to hydrogen sulphide. Non-enzymatic reduction of sulfane sulphur to hydrogen sulphide can occur in the presence of glutathione (GSH) [2]. In the mitochondria, hydrogen sulphide is oxidized to sulphite, which is then converted to thiosulfate (a sulfane sulphur-containing compound) by thiosulfate sulfurtransferase (rhodanese; TST, EC 2.8.1.1) [1,3–5] (Scheme 1). Thus, rhodanese can be involved in H_2S generation in the mitochondria [6].



Scheme 1. Enzymatic pathways involved in hydrogen sulphide formation. CBS: cystathionine β-lyase; CTH: γ-cystathionase; MPST: 3-mercaptopyruvate sulfurtransferase; CAT: cysteine aminotransferase; 3MP: 3-mercaptopyruvate; PA: pyruvate; and PLP: pyridoxal phosphate; CN^-: cyanide; SCN^-: thiocyanate.

Stress in the gastrointestinal tract may affect the motility, secretion of glands, the mucous membrane, or the flow in small blood vessels. Water immersion restraint stress (WRS) of rats are commonly used for studying stress-induced gastrointestinal erosion and ulcers [7]. Experiments carried out by Lou et al. [8] on an animal model of stress induced by immersing the animals in water and their fixation at a low temperature showed that H_2S reduced the amount of gastric mucosal damage and statistically significantly reduced the concentration of lipid peroxidation compared to the control group exposed only to the stress from the cold and immobilization.

The study was conducted to determine the role of H_2S in the inflammatory process associated with damage to the gastric mucosa of rats caused by stress and immobilization in cold water (WRS). The investigations involved the comparison of the activity and expression of CTH, CBS, MPST, and TST in the gastric mucosa of rats not exposed and exposed to WRS. H_2S generation, and cysteine, glutathione and cystathionine levels were also compared. The results showed an increased production of H_2S as a defence against damage of the gastric mucosa caused by WRS. NaHS, a donor of H_2S, administered to animals before WRS, resulted in the reversion of some investigated parameters to values found in healthy tissue, thus confirming the gastroprotective properties of H_2S.

2. Results and Discussion

The studies were conducted in the gastric mucosa from healthy rats and rats with ulcers induced by immersion in cold water (21 °C) for 3.5 h (WRS). Differences observed in hydrogen sulphide generation (Figure 1) confirm the beneficial effect of H_2S in ulcer healing in rats [9].

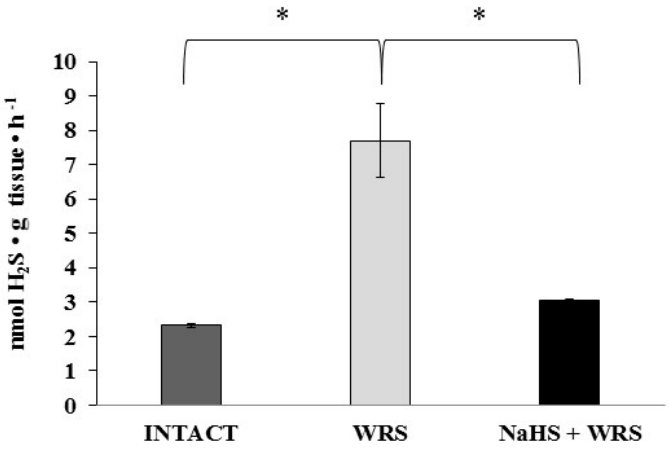


Figure 1. The level of hydrogen sulphide after 1 h of incubation with L-cysteine in the experimental group. The experiments were carried out in gastric mucosa homogenates in three experimental groups (Intact, water immersion restraint stress (WRS), WRS + NaHS), with L-cysteine as a main endogenous substrate for the hydrogen sulphide-producing enzymes. The data represent the mean value from two independent experiments. Statistical analysis was performed using the Student's *t*-test (* $p < 0.05$).

2.1. H_2S Generation in Gastric Mucosa of Healthy Rats

H_2S is produced in the gastric mucosa of healthy rats (Figure 1). In the presence of L-cysteine, 2.32 ± 0.05 nmol of H_2S is produced per 1 g of tissue during 1 h. It has also been postulated that H_2S can be generated by degradation of persulfide, i.e., sulfane sulphur may be a precursor to biological H_2S in the presence of thiols [10,11]. The determined level of sulfane sulphur in the gastric mucosa of healthy rats was 160 ± 60 nmol per 1 mg of protein (Figure 2). The GSH level equalled 17.5 ± 1.91 nmol/mg and the level of oxidized glutathione (GSSG)—3.35 ± 1.02 nmol/mg. Thus, the GSH/GSSG concentration ratio was 5.5 (Figure 3).

Table 1 shows the activity and expression of four enzymes involved in both H_2S and sulfane sulphur metabolism (Scheme 1). The results confirm the expression of MPST, rhodanese, CTH and CBS in the gastric mucosa of healthy rats. The highest specific activity expressed in nmol of product produced during 1 min per 1 mg of protein was determined for MPST. On the other hand, non-detectable activity of CBS was found.

Cysteine, cystine, and cystathionine levels were also measured in the gastric mucosa of healthy rats and are presented in Figure 4. The total cysteine level in the tissue calculated as the sum of pmol of cysteine/mg and 2× pmol of cystine/mg equalled 1.4 nmol/mg.

On the basis of these results it can be concluded that substrates for MPST, CTH, CBS, and rhodanese are available in healthy gastric mucosa and the ability to generate H_2S was confirmed in the tissue [4,12]. Mard et al. [4] and Martin et al. [12] reported the expression of CBS and CTH in the rat gastric mucosa and suggested CTH as the main enzyme responsible for H_2S production. Based on the obtained results, it seems possible that due to the low activity of CBS and CTH, the main H_2S generating pathway in the tissue involves MPST and 3-merceptopiruvate as a substrate (Scheme 1). The importance of rhodanese and sulfane sulphur can also be considered—the role of this enzyme in creation of H_2S in tissues is the least studied [6].

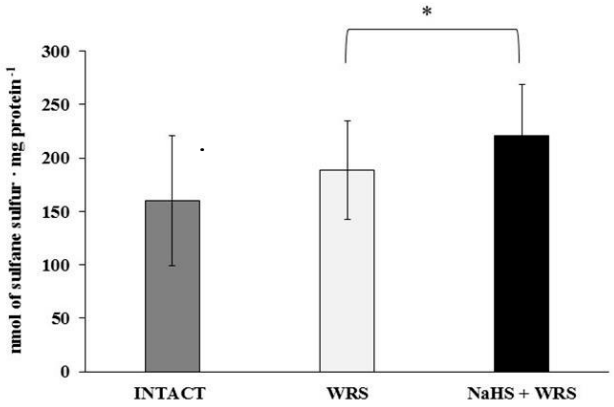


Figure 2. The level of sulfane sulphur in the experimental groups. The experiments were carried out in gastric mucosa homogenates in three experimental group (Intact, WRS, WRS + NaHS). The data represent the mean value from two independent experiments. Each value is the mean of 8–20 repeats. Statistical analysis was performed using the Student's *t*-test (* $p < 0.05$).

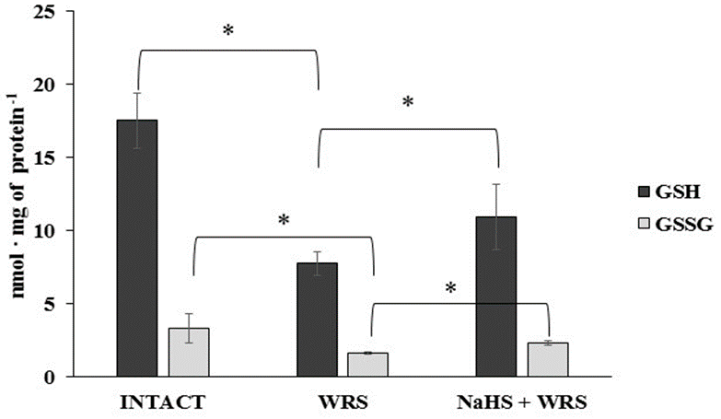


Figure 3. The level of reduced (GSH) and oxidized (GSSG) glutathione in the experimental groups. The experiments were carried out in gastric mucosa homogenates in three experimental group (Intact, WRS, WRS + NaHS) using the reversed-phase high-performance liquid chromatography (RP-HPLC) method. The data represent the mean value from two independent experiments. Each value is the mean of 8–10 repeats. Statistical analysis was performed using the Student's *t*-test (* $p < 0.05$).

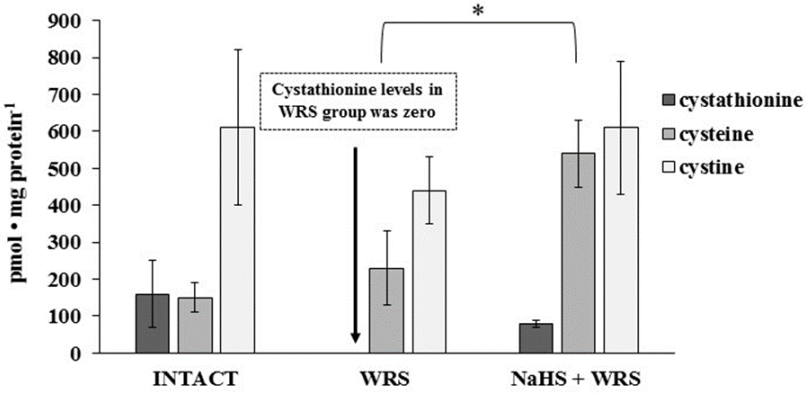
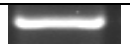


Figure 4. The level of cystathionine, cysteine, and cystine in the experimental group. The experiments were carried out in gastric mucosa homogenates in three experimental group using the RP-HPLC method. The data represent the mean value from two independent experiments. Each value is the mean of 8–10 repeats. Statistical analysis was performed using the Student's *t*-test (* $p < 0.05$).

Table 1. The activity and expression of MPST, rhodanese, CTH, and CBS in the experimental group.

Experimental Group	MPST	Rhodanese	CTH	CBS
		nmol/mg·min^{-1}		pmol/mg·min^{-1}
INTACT	2549 ± 570	1765 ± 310	0.202 ± 0.09	not detected
Expression RT-PCR				
β-Actin				
WRS	2915 ± 520 *	2567 ± 779 *	0.75 ± 0.17 *	5.5 ± 1.6
Expression RT-PCR				
β-Actin				
NaHS + WRS	4067 ± 890 *	1580 ± 520 *	0.82 ± 0.34	not detected
Expression RT-PCR				
β-Actin				

The experiments were carried out in gastric mucosa homogenates in three experimental group. The data represent the mean value from two independent experiments. Each value is the mean of 10–15 repeats. Statistical analysis was performed using the Student's *t*-test (* $p < 0.05$). MPST, TST (rhodanese), CTH, and CBS gene expression levels were evaluated by RT-PCR (reverse transcription polymerase chain reaction) analysis. One set of representative results is shown. β-Actin was used as an internal control.

2.2. Gastric Mucosa from Rats with Ulcers Induced by Water Immersion and Restraint Stress

The gastric mucosa of rats with ulcers induced by immersion in cold water (21 °C) for 3.5 h had a 2–3-fold higher ability to generate H_2S in comparison to the gastric mucosa of healthy rats (Figure 1). The level of sulfane sulphur was similar to that in the healthy mucosa (Figure 2). The GSH and GSSG levels were significantly decreased as compared to the healthy mucosa (Figure 3) and the concentration ratio of GSH/GSSG was also slightly decreased. These results may suggest that a higher capability of H_2S generation by ulcerated mucosa results from an increased specific activity of the enzymes involved in the process rather than from its increased release from sulfane sulphur stores. As it is shown in Table 1, no increased expression (messenger RNA (mRNA) levels) of the investigated enzymes was found in comparison to the healthy mucosa; however, changes in the specific activity were significant. A three-fold increased activity of CTH and a detectable CBS activity was noted in the WRS group. Similarly, the activity of MPST and rhodanese were also significantly increased. Accelerated cystathionine conversion by CTH resulted in its decreased level (Figure 4). Thus, stimulation of all enzyme-dependent pathways for H_2S generation (Scheme 1), in response to stress resulting from water immersion and restraint, was confirmed. The results show that the defence mechanism against stress is associated with stimulation of the production of hydrogen sulphide in the tissue, and confirm the observed advantageous effect of H_2S on healing of gastric ulcers [8,13,14].

2.3. NaHS Pretreatment Affects the Formation of Hydrogen Sulphide in Gastric Mucosa of Rats with WRS Induced Ulcers

The rats administered NaHS, a precursor of hydrogen sulphide, 30 min prior to WRS, demonstrated a decreased ability of endogenous H_2S generation in gastric mucosa as compared to the gastric mucosa of rats with ulcers induced by immersion in cold water, not pre-administered with NaHS (Figure 1). Figure 2 shows a significantly higher level of sulfane sulphur in the gastric mucosa of rats pre-administered NaHS. This suggests that H_2S released from NaHS results in an in-creased level of sulfane sulphur-containing compounds in the tissue [15]. The expression of all the investigated enzymes is not changed (Table 1) in the mucosa pre-treated with NaHS as compared to the gastric mucosa with ulcers induced by immersion in cold water in rats not pre-treated with NaHS. In contrast to the CTH activity, which was not affected by NaHS pre-treatment, the activity of CBS and rhodanese was found to be decreased and MPST increased.

Stress caused by immersion in cold water resulted in an increased activity of MPST and CTH in the ulcerated gastric mucosa, which remained high, independently of pre-treatment with an exogenous (NaHS) source of H_2S, whereas NaHS pre-treatment resulted in a decrease in the activity of CBS and rhodanese to the level characteristic of the healthy mucosa.

The levels of GSH and GSSG in the case of NaHS pre-treatment were significantly higher in comparison to the ulcerated mucosa (Figure 3). They were reversed to the levels found in the healthy mucosa. A similar tendency was observed in the case of the level of cystathionine, cysteine, and cystine (Figure 4).

Thus, in comparison to the healthy mucosa, the changes observed in the ulcerated gastric mucosa, such as an increased production of H_2S, an increased activity of the investigated enzymes, a decreased level of GSH, GSSG, and cystathionine, revert back to the levels/activities similar to these found in the healthy mucosa. These results confirm the beneficial effect of NaHS, as a donor of H_2S, in changing some biochemical parameters (except MPST and CTH activities and the level of cysteine), back to the values found in the healthy tissue. This is in accordance with the previously mentioned gastroprotective effect of endogenous H_2S and H_2S released from the donors (NaHS) [8,13,14]. The mechanism by which H_2S protects the gastric mucosa may involve an increase in gastric flow—the exposure of rats to 3.5 h of WRS causes gastric lesions and a significantly decreased gastric blood flow [16]. The results presented in this paper show that the defence against WRS-induced gastric mucosal lesions includes the acceleration of endogenous H_2S formation. In case of pre-administration of an exogenous source of H_2S, the changes observed in the gastric mucosa tend to maintain the

activities of some mucosal enzymes involved in H_2S generation and the levels of their substrates at levels characteristic to the healthy mucosa.

3. Material and Methods

3.1. Chemicals

L-Glutathione reduced, D,L-cystathionine (CTN), D,L-homoserine (HSer), 1-fluoro-2,4-dinitrobenzene (DNFB), bathophenanthroline-disulfonic acid disodium salt (BPDS), acetonitrile, PLP, β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH), L-lactic dehydrogenase (LDH), 3-mercaptopyruvate acid sodium salt, D,L-dithiothreitol, (DTT), *N*-ethylmaleimide (NEM), DL-propargylglycine (PPG), sodium dihydrogen phosphate dihydrate pure, sodium sulphite, chloroform, isopropanol, agarose, sodium hydrosulphide hydrate, sodium chloride, Folin-Ciocalteu's phenol reagent, iron (III) nitrate nonahydrate, sodium thiosulfate pentahydrate, sodium carbonate, *N*,*N*-dimethyl-*p*-phenylenediamine sulphate salt, and sodium thiosulfate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trifluoroacetic acid (TFA) and 2-mercaptoethanol were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Ethanol and 70% perchloric acid (PCA), 38% formaldehyde, 65% nitric acid, 38% hydrochloric acid, ammonia solution 25% pure, potassium sodium tartrate tetrahydrate, copper sulphate pentahydrate, potassium dihydrogen phosphate, ferric chloride, zinc acetate dehydrate pure, trichloroacetic acid (TCA), and sodium hydroxide were from Polskie Odczynniki Chemiczne S.A. (Gliwice, Poland). N^{ε}-methyllysine was obtained from Bachem (Bubendorf, Switzerland). Trizol, ethidium bromide and EDTA (Ethylenediaminetetraacetic acid)—disodium salt dihydrate were obtained from Lab-Empire (Rzeszow, Poland). Potassium cyanide was from Merck (Darmstadt, Germany). Reverse transcriptase M-MuLV was obtained from Promega (Madison, WI, USA). Polymerase DNA Dream Taq™, Gene Ruler 100 bp DNA Ladder, Oligo $(dT)_{18}$ primer and Deoxynucleotide (dNTP) Solution Mix were obtained from Abo (Gdańsk, Poland).

3.2. Animals

Male Wistar rats (220–300 g) were used in the experiments. They were deprived of food for 24 h with free access to tap water before the experiments. After the experiment, the rats were kept under anesthesia under pentobarbital (60 mg/kg, intraperitoneally (i.p.)) and the stomachs were removed. The stomach was slit along the curvatura major. Mucosal specimens were scraped off using a slide glass and immediately frozen in liquid nitrogen and stored at −80 °C until analysis as described in Magierowska et al. [17]. All of the experiments were conducted in cooperation with the Department of Physiology, Faculty of Medicine at the Jagiellonian University and were approved by the Institutional Animal Care and Use Committee of the Jagiellonian University Medical College in Cracow (No.: 68/2014) and performed in accordance with the Helsinki Declaration.

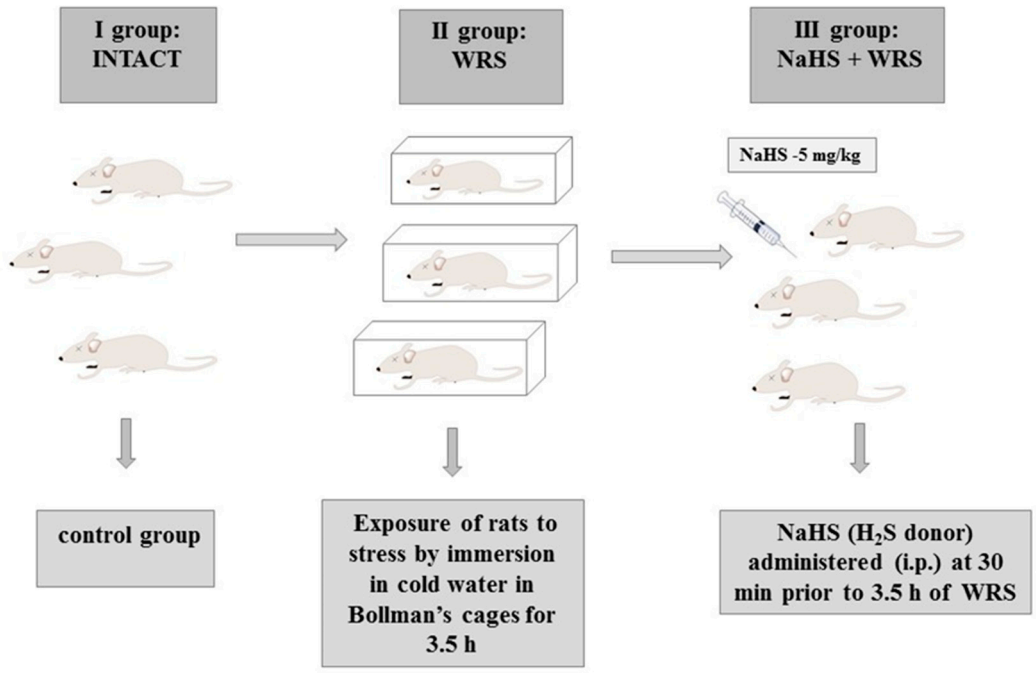
3.3. Experimental Group

Stress lesions were caused by immobilizing the rats in individual Bollman's cages and immersing the animals in cold water (21 °C) for 3.5 h as described in previous studies [18]. The experiment was carried out in three experimental groups: (1) the control group (intact); (2) vehicle (saline)-pre-treated 30 min prior to 3.5 h of water immersion and restraint stress (WRS); or (3) NaHS (H_2S donor) administered i.p. at a dose of 5 mg/kg (Scheme 2).

3.4. Tissue Homogenates

For determinations of the enzyme activities (CTH, MPST, CBS, rhodanese) and the level of sulfane sulphur, the gastric mucosa samples were weighed and homogenized in ice-cold 0.1 M phosphate buffer pH 7.5 (1 g/4 mL) for 1 min at 8000–9500 rpm using a blender homogenizer. The homogenates were centrifuged at 1600 g for 10 min. After centrifugation, the supernatants were used

for the determination of the enzyme activities (CTH, MPST, CBS rhodanese) and the level of sulfane sulphur and protein content.



Scheme 2. Experimental group. i.p.: intraperitoneally.

For the reversed-phase high-performance liquid chromatography (RP-HPLC) the tissues were weighed and homogenized at 8000–9500 rpm in ice-cold 10% PCA/1 mM BPDS (1 g/3 mL) (1 g tissue/3 mL solution). The homogenates were centrifuged for 10 min at 4 °C at 1400× *g*. The supernatants were used for assays immediately or stored at −80 °C until HPLC analysis. The tissues were homogenized using an Ultra-Turrax T 25 (Janke and Kunkel IKA-Labortechnik Company, Staufen, Germany). The homogenates were centrifuged using a MPW 375 centrifuge (MPW MED Instruments, Warszawa, Poland) or a Hettich Universal 16 centrifuge (Hettich AG, Kloten, Switzerland).

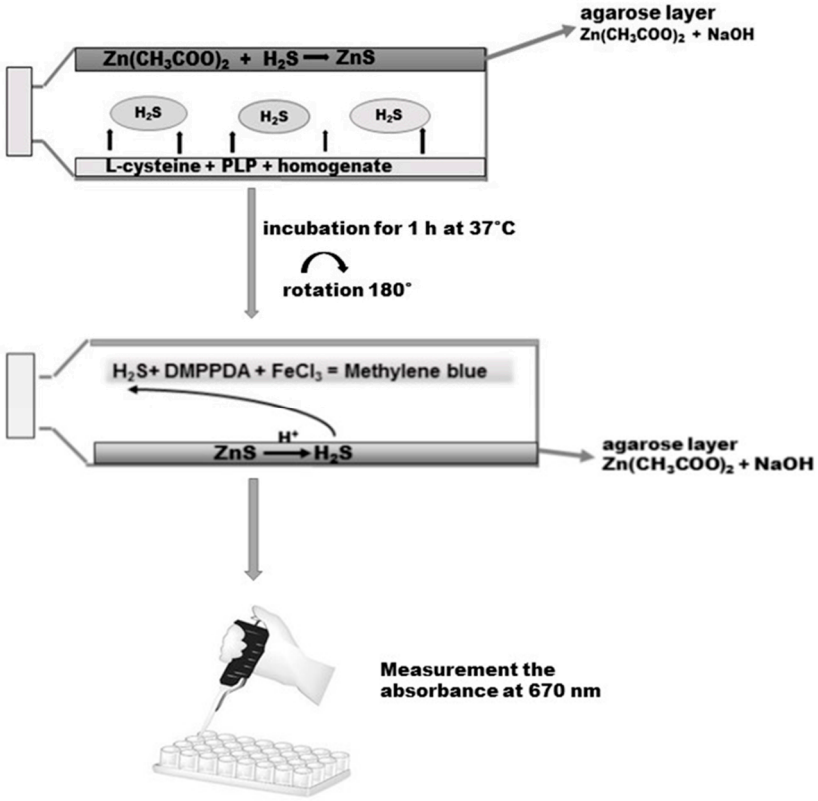
3.5. RP-HPLC

The RP-HPLC method of Dominick et al. [19] with modifications [20–22] was used for the detection and quantitation of the levels of direct and non-related products of the CBS- and CTH-catalysed reactions, such as cystathionine, reduced (GSH) and oxidized (GSSG) glutathione, cysteine, and cystine.

3.6. Determination of H_2S in the Homogenate of the Gastric Mucosa

The gastric mucosa tissue samples were homogenized at a ratio of 1/8 with 50 mM phosphate buffer, pH 8.0. Then, the homogenates were incubated for 5 min at 37 °C on ice. Before the experiment, 50 mL tissue culture flasks with unventilated caps were covered with a layer of agarose mixed with 1% of zinc acetate and 3 M sodium hydroxide as described by Karth et al. [23]. On the opposite side of the layer of agarose, 5000 µL of a reaction mixture containing 4500 µL homogenate, 250 µL 2 mM of pyridoxal phosphate, and 250 µL of 10 mM L-cysteine (final concentration) was added. The caps of the flasks were secured with parafilm. The incubation was initiated by the transfer of the bottles with ice to 37 °C. After the 90-min incubation, 2500 µL 50% TCA was added to the reaction mixture. Another 60 min was allowed for the trapping of evolved H_2S by the layer of agarose. After incubation the reaction mixture was removed from the flasks, the flasks were rotated through 180 °C and the reaction was conducted on the layer of agarose by adding 2 mL of 40 mM *N*,*N*-dimethyl-*p*-phenylenediamine sulphate salt (DMPPDA) and subsequently incubating for 10 min at room temperature, followed by addition of 400 µL 1% ferric chloride and re-incubation for 20 min at room temperature (Scheme 3). As a result of the reaction, methylene blue was formed, which was

spectrophotometrically determined at 670 nm. The standard curve was linear over the concentration range of 0–250 μM with a correlation coefficient of 0.994.



Scheme 3. Determination of H_2S in the homogenate of the gastric mucosa according to Kartha et al. [23]. Details are described in Materials and Methods. DMPPDA: *N*,*N*-dimethyl-*p*-phenylenediamine sulphate salt.

3.7. Enzymes Assay in the Gastric Mucosa Homogenates

The MPST activity was assayed according to the method of Valentine and Frankelfeld [24], following a procedure described in our earlier paper [25]. The incubation mixture contained in a final volume of 500 μL: 250 μL, 0.12 M sodium phosphate buffer, pH 8.0, 50 μL, 0.5 M sodium sulfite, 50 μL 0.15 M dithiothreitol, 50 μL homogenates, 50 μL H_2O, and 50 μL 0.1 M 3-mercaptopyruvate acid sodium salt. The mixture was incubated for 15 min. To stop the reaction, 250 μL of 1.2 M PCA was added. The samples were centrifuged at 1600× *g* for 5 min, and 100 μL of supernatant was transferred to 1350 μL mixture that contained: 1200 μL, 0.12 M sodium phosphate buffer, pH 8.0, 100 μL 0.1 M *N*-ethylmaleimide, and 50 μL NADH 5 mg/mL. After equilibration at 37 °C, 2.5 μL of L-lactic dehydrogenase (7 IU) were added, and the decrease in absorbance at 340 nm was measured. The enzyme activity was expressed as nmol of pyruvate produced during 1 min incubation at 37 °C per 1 mg of protein.

The γ-cystathionase activity was determined by the Matsuo and Greenberg's method [26] with modifications described by Czubak et al. [27]. The incubation mixture contained: 25 μL 1.3 mM PLP, 25 μL 13 mM EDTA, 250 μL 45 mM cystathionine solution in 0.1 M phosphate buffer, pH 7.5 (2.5 mg cystathionine per sample), 75 μL homogenates, and 0.1 M phosphate buffer, pH 7.5 containing 0.05 mM 2-mercaptoethanol, in a final volume of 650 μL. The reaction was stopped after 15 min of incubation at 37 °C by placing 125 μL incubation mixture in 25 μL 10% PCA. The samples were centrifuged at 1600× *g* for 10 min, and 25 μL of supernatant was transferred to 625 μL 0.194 mM NADH solution and kept at 37 °C. The control samples, without 45 mM cystathionine, were prepared in the same way as the examined samples. After 10 s of the measurement (absorbance at 340 nm), 25 μL (9.06 IU) L-lactic dehydrogenase were added and the measurement was continued to 180 s. The

difference between the initial value of absorbance (before addition of LDH) and the lowest value (after adding LDH) corresponded to the amount of α-ketobutyrate formed in the course of the γ-cystathionase reaction. The γ-cystathionase activity is expressed as nmol of α-ketobutyrate formed.

Sulfane sulphur was determined by the method of Wood [28], based on cold cyanolysis and colorimetric detection of the ferric thiocyanate complex ion. Incubation mixtures in a final volume 880 µL contained: 20 µL 1 M ammonia solution, 20 µL homogenate, 740 µL H_2O, and 100 µL 0.5 M sodium cyanide. The incubation was performed for 45 min at room temperature. After incubation, thiocyanate was estimated calorimetrically at 460 nm after the addition of 20 µL 38% formaldehyde and 40 µL ferric nitrate reagent. The sulfane sulphur level is expressed as nmol of SCN^- (thiocyanate) produced per 1 mg of protein during 1 min incubation at 37 °C per 1 mg of protein.

The activity of CBS was examined in homogenates in the presence of D,L-homoserine (HSer) as a substrate. After 15 min of the incubation at 37 °C, the methods described in Bronowicka-Adamska et al. [21] were used. The level of cystathionine was determined using the HPLC method described by Bronowicka-Adamska et al. [20]. The CBS activity is expressed as pmol of cystathionine formed during 1 min incubation at 37 °C per 1 mg of protein.

Rhodanese in the gastric mucosa homogenates was assayed according to Sörbo [29] with modifications. Incubation mixtures in a final volume 500 µL contained: 200 µL, 0.125 M sodium thiosulfate, 100 µL, 0.2 M potassium phosphate (KH_2PO_4), 100 µL, 0.25 M sodium cyanide, and 100 µL homogenate. The incubation was performed during 5 min at 20 °C, after which thiocyanate was estimated colorimetrically at 460 nm after the addition of 20 µL of 38% formaldehyde and 40 µL ferric nitrate reagent. The enzyme units are defined as µmol of SCN^- generated per minute per 1 mg of protein at 20 °C under the prescribed assay conditions.

The protein concentration was determined by the method of Lowry et al. [30] using crystalline bovine serum albumin as a standard.

3.8. Expression of MPST, CTH, and CBS in the Gastric Mucosa Homogenates

3.8.1. RNA Extraction

Total RNA was extracted using TRIzol (Lab-Empire S.A (Rzeszow, Poland)), according to the protocol provided by the manufacturer. The quality of the RNA samples was determined by spectrophotometric analysis (A_{260}/A_{280}) and electrophoresis in 2.5% agarose gel followed by staining with ethidium bromide.

3.8.2. Reverse Transcription of RNA

Total RNA from the cell samples was reverse-transcribed using a First-Stand complementary DNA (cDNA) synthesis kit according to the manufacturer instructions (Promega, Madison, WI, USA). For reverse transcription (RT), 3 µg of the total RNA was mixed with 1 µL of Oligo $(dT)_{15}$ (0.5 µg/reaction) and nuclease-free water and heated in a 70 °C heat block for five minutes. After pre-incubation, the reverse transcription reaction mix containing: 4 µL GoScript 5× reaction buffer (Promega, Madison, WI, USA), 3µL $MgCl_2$ (final concentration 1.5–5.0 mM), 1 µL dNTPs (10 mM), 1 µL Recombinant RNases Ribonuclease Inhibitor (20 U/µL), and 1 µL GoScript Reverse Transcriptase was prepared.

3.8.3. cDNA Synthesis and RT-PCR Analysis

Expressions of MPST, CTH, CBS, rhodanese, and β-actin were analysed by RT-PCR. Amplification of cDNA samples was run in a 12.5 µL reaction volume containing 1 µL of synthesized cDNA, 0.2 µM of each of the gene-specific primer pair, 0.04 U/µL DNA polymerase in 10 mM buffer Tris–HCl pH 8.8, 0.2 mM each of dNTPs and nuclease-free water. The temperature profile of RT-PCR amplification for MPST consisted of activation of Taq DNA polymerase (Abo, Gdansk, Poland) at 94 °C for 5 min, denaturation of cDNA at 95 °C for 30 s, primer annealing at 54 °C for 30 s, elongation at 72 °C for 1 min for the following 28 cycles, and finishing by the extension step for 8 min. For the *CTH* gene,

after an initial 5 min at 94 °C denaturation, amplification was performed under the following conditions: 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min for 36 cycles, with a final incubation at 72 °C for 8 min. For the *CBS* gene [4], after an initial 10 min of denaturation at 95 °C, amplification was performed under the following conditions: 94 °C for 20 s, 60 °C for 1 min, and 72 °C for 1 min for 40 cycles, with a final incubation at 72 °C for 5 min. For the *β-actin* gene, after an initial 5 min denaturation at 94 °C, amplification was performed under the following conditions: 94 °C for 30 s, 53.6 °C for 30 s, and 72 °C for 2 min for 28 cycles, with a final incubation at 72 °C for 8 min. For the *rhodanese* gene, after an initial 5 min denaturation at 94 °C, amplification was performed under the following conditions: 94 °C for 30 s, 55.6 °C for 30 s, and 72 °C for 2 min for 38 cycles, with a final incubation at 72 °C for 8 min. The specific primers (Oligo.pl, Warszawa, Poland) were used (Figure 5). The PCR reaction products were separated electrophoretically in a 2.5% agarose gel, visualized with ethidium bromide, directly visualized under ultraviolet (UV) light, and photographed.

GENE	PRIMER	PCR product size	Annealing temperature
MPST	F: 5' TCCTGGGTGGAGTGGTACAT 3' R: 5' GTGAAACAAGCTAGGTGGGC 3'	339 bp	54 °C
CBS	F: 5' CTGTGAAGGGCTATCGCTGC 3 R: 5' CTGGCATTGCGGTACTGGTC3'	205 bp	60 °C
CTH	F: 5' TTTGTATACAGCCGCTCTGGA 3 R: 5' ACAAGCTTGGTCTGTGGTGT 3'	290 bp	54 °C
TST	F: 5' CTCTATCGAGCCGCTGGTCTC 3 R: 5' TCGTAAGGCGAAGTCGTGTC 3'	200 bp	55.6 °C
β-ACTIN	F: 5' ACCCGCGAGTACAACCTTCTT 3 R: 5' GCCGTGTTCAATGGGGTACT 3'	285 bp	53.6 °C

Figure 5. Forward and reverse primers used in the RT-PCR reaction to assess messenger RNA (mRNA) expression for MPST, CBS, CTH, TST, and β-actin.

3.9. Statistical Analysis

All results are expressed as means ± SEM (standard error of the mean). The significance of the differences between the controls and the investigated samples was calculated using the Student's *t*-test ($p < 0.05$) (MS Excel 2013). Each experiment was repeated a minimum of three times.

4. Conclusions

Endogenous synthesis of H_2S is stimulated in the gastric mucosa as a compensatory mechanism to damage induced by WRS. Hydrogen sulphide is produced in the gastric mucosa in response to injury and acts to promote healing when its precursor, NaHS, is administered prior to WRS. The results suggest that H_2S-releasing drugs could be employed to accelerate healing of gastric ulcers.

Acknowledgments: This work was supported by a grant (P.B.-A) from the National Committee for Scientific Research No. K/DSC/003570.

Author Contributions: P.B.-A., M.M., S.K. and K.M. performed the experiments and collected the data. M.W. and T.B. provided suggestions for experiments. P.B.-A. and M.W. planned the experiments and wrote the paper. We would like to thank Dominika Szlęzak for her help with the graphs.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Stipanuk, M.H.; Ueki, I. Dealing with methionine/homocysteine sulfur: Cysteine metabolism to taurine and inorganic sulfur. *J. Inherit. Metab. Dis.* **2011**, *34*, 17–32.
2. Kabil, O.; Vitvitsky, V.; Banerjee, R. Sulfur as a Signaling Nutrient through Hydrogen Sulfide. *Annu. Rev. Nutr.* **2014**, *34*, 171–205.

3. Stein, A.; Bailey, S.M. Redox biology of hydrogen sulfide: Implications for physiology, pathophysiology, and pharmacology. *Redox Biol.* **2013**, *1*, 32–39.
4. Mard, S.A.; Veisi, A.; Ahangarpour, A, Gharib-Naseri, M.K. Mucosal acidification increases hydrogen sulfide release through up-regulating gene and protein expressions of cystathionine gamma-lyase in the rat gastric mucosa. *Iran. J. Basic Med. Sci.* **2016**, *19*, 172–177.
5. Tadeusiewicz, J.; Olas, B. Siarkowodór—Gaz nie tylko o właściwościach toksycznych. *Kosmos* **2014**, *63*, 125–135.
6. Zaichko, N.V.; Melnik, A.V.; Yoltukhivsky, M.M.; Olhovskiy, A.S.; Palamarchuk, I.V. Hydrogen sulfide: Metabolism, biological and medical role. *Ukr. Biochem. J.* **2014**, *86*, 5–25.
7. Takagi, K.; Okabe, S. The effects of drugs on the production and recovery processes of the stress ulcer. *Jpn. J. Pharmacol*. **1968**, *19*, 9–19.
8. Lou, L.X.; Geng, B.; Du, J.B.; Tang, C.S. Hydrogen sulphide-induced hypothermia attenuates stress-related ulceration in rats. *Clin. Exp. Pharmacol. Physiol*. **2008**, *35*, 223–228.
9. Wallace, J.L.; Dicay, M.; McKnight, W.; Martin, G.R. Hydrogen sulfide enhances ulcer healing in rats. *FASEB J.* **2007**, *21*, 4070–4076.
10. Ida, T.; Sawa, T.; Ihara, H.; Tsuchiya, Y.; Watanabe, Y.; Kumagai, Y.; Suematsu, M.; Motohashi, H.; Fujii, S.; Matsunaga, T.; et al. Reactive cysteine persulfides and *S*-polythiolation regulate oxidative stress and redox signaling. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 7606–7611.
11. Toohey, J.I.; Cooper, A.J.L. Thiosulfoxide (Sulfane) Sulfur: New Chemistry and New Regulatory Roles in Biology. *Molecules* **2014**, *19*, 12789–12813.
12. Martin, G.R.; McKnight, G.W.; Dicay, M.S.; Coffin, C.S.; Ferraz.; J.G.; Wallace, J.L. Hydrogen sulphide synthesis in the rat and mouse gastrointestinal tract. *Dig. Liver Dis.* **2010**, *42*, 103–109.
13. Magierowski, M.; Jasnos, K.; Kwiecień, S.; Brzozowski, T. Rola siarkowodoru w fizjologii przewodu pokarmowego i w mechanizmie gastroprotekcji. *Postępy Hig. Med. Dośw.* **2013**, *67*, 150–156.
14. Wallace, J.L. Physiological and Pathophysiological Roles of Hydrogen Sulfide in the Gastrointestinal Tract. *Antioxid. Redox Signal*. **2010**, *9*, 1125–1133.
15. Toohey, J.L. Sulfur signaling: Is the agent sulfide or sulfane? *Anal. Biochem*. **2011**, *413*, 1–7.
16. Czekaj, R.; Majka, J.; Ptak-Belowska, A.; Szlachcic, A.; Targosz, A.; Magierowska, K.; Strzalka, M.; Magierowski, M.; Brzozowski, T. Role of curcumin in protection of gastric mucosa against stress-induced gastric mucosal damage. Involvement of hypoacidity, vasoactive mediators and sensory neuropeptides. *J. Physiol. Pharmacol.* **2016**, *67*, 261–275.
17. Magierowska, K.; Magierowski, M.; Surmiak, M.; Adamski, J.; Mazur-Biały, A.I.; Pajdo, R.; Sliwowski, Z.; Kwiecien, S.; Brzozowski, T. The protective role of carbon monoxide (CO) produced by heme oxygenases and derived from the CO-releasing molecule CORM-2 in the pathogenesis of stress-induced gastric lesions: Evidence for non-involvement of nitric oxide (NO). *Int. J. Mol. Sci.* **2016**, *17*, 442.
18. Konturek, P.C.; Brzozowski, T.; Meixner, H.; Ptak, A.; Hahn, E.G.; Konturek, S.J. Central and peripheral neural aspects of gastroprotective and ulcer healing effects of lipopolysaccharides. *J. Physiol. Pharmacol*. **2001**, *52*, 611–623.
19. Dominik, P.K.; Cassidy, P.B.; Roberts, J.C. A new and versatile method for determination of thiolamines of biological importance. *J. Chromatogr. B* **2001**, *761*, 1–12.
20. Bronowicka-Adamska, P.; Wróbel, M.; Zagajewski, J. RP-HPLC method for quantitative determination of cystathionine, cysteine and glutathione: An application for the study of the metabolism of cysteine in human brain. *J. Chromatogr. B* **2011**, *879*, 2005–2009.
21. Bronowicka-Adamska, P.; Wróbel, M.; Zagajewski, J. An application of RP-HPLC for determination of the activity of cystathionine β-synthase and γ-cystathionase in tissue homogenates. *Nitric Oxide* **2015**, *46*, 186–191.
22. Wróbel, M.; Lewandowska, I.; Bronowicka-Adamska, P.; Paszewski, A. The level of sulfane sulfur in the fungus *Aspergillus nidulans* wild type and mutant strains. *Amino Acids*. **2009**, *37*, 565–571.
23. Kartha, R.V.; Zhou, J.; Hovde, L.B.; Belinda, W.Y.; Cheung, B.W.Y.; Schröder, H. Enhanced detection of hydrogen sulfide generated in cell culture using an agar trap method. *Anal. Biochem.* **2012**, *423*, 102–108.
24. Valentine, W.N.; Frankelfeld, J.K. 3-Mercaptopyuruvate sulfurtransferase (EC 2.8.1.2): A simple assay adapted to human blood cells. *Clin. Chim. Acta* **1974**, *14*, 205–210.
25. Wróbel, M.; Jurkowska, H.; Sliwa, L.; Srebro, Z. Sulfurtransferases and cyanide detoxification in mouse liver, kidney, and brain. *Toxicol. Mech. Methods* **2004**, *14*, 331–337.
26. Matsuo, Y.; Greenberg, D.M. A crystalline enzyme that cleaves homoserine and cystathionine. *J. Biol. Chem*. **1958**, *230*, 545–560.

27. Czubak, J.; Wróbel, M.; Jurkowska, H. Cystathionine γ-lyase (EC 4.4.1.1): An enzymatic assay of α-ketobutyrate using lactate dehydrogenase. *Acta Biol. Crac. Zool.* **2002**, *44*, 113–117.
28. Wood, L. Sulfane sulfur. *Methods Enzymol*. **1987**, *43*, 25–29.
29. Sörbo, B. Rhodanese. In *Methods in Enzymology*; Colowick, S.P., Kaplan, N.O., Eds.; Academic Press: New York, NY, USA, 1955; Volume 2, pp. 334–337.
30. Lowry, O.H.; Rosenbrough, N.J.; Farr A.L.; Randall, R.I. Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. **1951**, *193*, 265–275.

Sample Availability: Not available.

Najważniejsze wyniki i wnioski

1. Zmodyfikowaną metodę RP-HPLC (Dominick i wsp., 2011) opisano w Publikacji nr 1 i zastosowano ją do równoczesnego oznaczania, w czasie jednego rozdziału, w 20 µl próbce, poziomu zredukowanego (GSH) i utlenionego (GSSG) glutationu, cysteiny (CSH), cystyny (CSSC) oraz cystationiny w mózgu ludzkim.

   Wnioski:

   - poziom cystationiny jest bardzo zróżnicowany w zależności od regionu mózgu ludzkiego i skorelowany z aktywnością CBS i CTH,
   - wyniki uzyskane dzięki opisanej metodzie pozwalają na określenie statusu redoks tkanek poprzez podanie stosunku GSH/GSSG.

2. Opracowaną metodę badania aktywności układu enzymatycznego CBS/CTH w homogenatach tkankowych opisano w Publikacji nr 2.

   - aktywność CTH badano na podstawie przyrostu stężenia α-ketomaślanu (produktu reakcji katalizowanej przez CTH),
   - aktywność CBS oznaczano na postawie zmian poziomu cystationiny w homogenacie zawierającym inhibitor CTH - D,L-propargylglicynę (PPG) w stężeniu całkowicie hamującym aktywność CTH.

   Wnioski:

   - aktywności CBS oznaczono we wszystkich badanych tkankach mysich (mózg, wątroba, nerki), a CTH w wątrobie i nerce – w tworzeniu siarkowodoru w wątrobie główną rolę odgrywa CTH, a w mózgu CBS.

3. Metodę oznaczania siarkowodoru tworzonego w obecności L-cysteiny i wiązanego w warstwie agarozy w hodowlach komórkowych oraz homogenatach tkankowych opisano w Publikacji nr 3 i 4. Porównano dwie linie komórkowe: neuroblastomy ludzkiej

(linia SH-SY5Y) oraz glioblastomy - astrocytomy ludzkiej (linia U87-MG) oraz śluzówkę żołądka szczura nieuszkodzoną i uszkodzoną w wyniku narażenia na stres, w zakresie ich potencjalnych możliwości w tworzeniu siarkowodoru.

Wnioski:

- komórki neuroblastomy mają potencjalnie większą zdolność do tworzenia H_2S z cysteiny niż komórki astrocytomy – duże znaczenie w wytwarzaniu siarkowodoru z cysteiny w tych komórkach może mieć MPST,
- w stresie, który prowadzi do uszkodzenia śluzówki żołądka wzrasta produkcja siarkowodoru – H_2S ma korzystny wpływ na proces gojenia uszkodzonej śluzówki.

4. Opracowano warunki badania ekspresji na poziomie mRNA (RT-PCR) oraz białka (Western-blot) dla enzymów biorących udział w tworzeniu i metabolizmie siarkowodoru (CBS, CTH, MPST, TST) - opisane w Publikacji 3 i 4.

Wnioski:

- w komórkach neuroblastomy potwierdzono największą ekspresję MPST (mRNA oraz białko),
- w prawidłowej błonie śluzowej żołądków szczurów potwierdzono ekspresję (mRNA) wszystkich czterech enzymów: MPST, CTH, CBS, TST,
- w owrzodzonej błonie śluzowej żołądków szczurów nie obserwowano zmian w ekspresji (mRNA) badanych enzymów (MPST, CTH, CBS, TST) w porównaniu do prawidłowej błony śluzowej żołądka.

Streszczenie

Siarkowodór (H_2S) jest obok tlenku azotu oraz tlenku węgla kolejnym nieorganicznym gazowym mediatorem. H_2S jest lipofilny - jego wnikanie do komórek nie wymaga transporterów. Jest produkowany endogennie w wątrobie, nerkach, mózgu oraz wielu innych tkankach ludzkich i ssaków. Jego synteza odbywa się przy udziale enzymów, których kofaktorem jest fosforan pirydoksalu: beta-syntazy cystationinowej (*CBS*, EC 4.2.1.22), gamma-cystationazy (*CTH*, EC 4.4.1.1) oraz zależnej od jonów cynku transferazy siarkowej 3-merkaptopirogronianu (*MPST*, EC 2.8.1.2). H_2S może być wytwarzany enzymatycznie z L-cysteiny, L-cystyny, 3-merkaptopirogronianu, L-homocysteiny oraz D-cysteiny. Synteza H_2S z D-cysteiny odbywa się przy udziale MPST i oksydazy D-aminokwasowej (DAO). H_2S może być również uwalniany nieenzymatycznie z nadsiarczków w warunkach redukujących.

Celem podjętych badań było oznaczanie tworzenia H_2S w różnych tkankach oraz hodowlach komórkowych poprzez badanie aktywności enzymów mających znaczenie dla jego powstawania i metabolizmu (CBS, CTH, MPST, TST) oraz oznaczanie poziomu H_2S w tkankach prawidłowych i objętych stanem zapalnym. Opracowana została metoda oznaczania aktywności układu enzymatycznego CBS/CTH w obecności homoseryny (substrat dla CBS) oraz cystationiny (substrat dla CTH). W celu analizowania zmian stężenia cystationiny (produktu przekształcenia homoseryny przez CBS) zmodyfikowana została metoda RP-HPLC (Dominik i wsp., 2011). Modyfikacja pozwoliła również na oznaczanie, w czasie jednego rozdziału, poziomu zredukowanego (GSH) i utlenionego (GSSG) glutationu, cysteiny (CSH), cystyny (CSSC) oraz cystationiny.

Doświadczenia związane z modyfikacją metody RP-HPLC zostały przeprowadzone w homogenatach tkankowych pochodzących z różnych regionów mózgów ludzkich. Efektem tych badań było zarówno opracowanie modyfikacji, jak również oznaczenie poziomu cystationiny w badanych tkankach. Najwyższy poziom cystationiny oznaczono we wzgórzu

i był on około 11 razy większy w porównaniu do móżdżku. Wysoki poziom cystationiny we wzgórzu był skorelowany z niską aktywnością CTH. Najwyższy poziom cysteiny oznaczono we wzgórzu, podwzgórzu i jądrach podkorowych. Wysoki poziom cysteiny we wzgórzu był związany z wysokim poziomem GSH. Sugerowana jest rola cystationiny w mózgu ludzkim jako neuromodulatora, oprócz jej udziału w metabolizmie cysteiny i tworzeniu H_2S. Wysoki poziom cystationiny w niektórych regionach mózgu ludzkiego może być związany z wysoką aktywnością CBS. CBS podobnie jak CTH katalizuje reakcje, w których powstaje H_2S [Publikacja nr 1].

Kolejnym etapem badań było opracowanie metody oznaczania aktywności układu enzymatycznego CBS/CTH, ważnego zarówno w tworzeniu H_2S w tkankach, jak i w dostarczaniu cysteiny do syntezy glutationu. Prace doświadczalne zostały przeprowadzone w homogenatach wątroby, nerek oraz mózgu myszy przy użyciu dwóch substratów: homoseryny oraz cystationiny. Aktywność CTH badano na podstawie przyrostu stężenia α-ketomaślanu (produktu reakcji katalizowanej przez CTH). Metoda po raz pierwszy została opisana w Publikacji nr 2. Aktywność CBS oznaczano na postawie zmian poziomu cystationiny w homogenacie zawierającym inhibitor CTH - D,L-propargylglicynę (PPG) w stężeniu całkowicie hamującym aktywność CTH. W tkankach myszy aktywność CBS najwyższa była w mózgu. W mózgu myszy nie stwierdzono aktywności CTH, co potwierdziło główną rolę CBS w wytwarzaniu H_2S. W obecności homoseryny oznaczano w mózgu zwiększony poziom cystationiny, co potwierdza wysoką aktywność CBS. W wątrobie niski, w porównaniu do mózgu, poziom cystationiny oraz wysoki poziom α-ketomaślanu, są związane z wysoką aktywnością CTH. Równocześnie wysoki poziom GSH w homogenatach wątroby myszy, świadczy o roli CBS i CTH w dostarczaniu cysteiny do syntezy GSH [Publikacja nr 2].

Opracowaną metodę oznaczania aktywności układu enzymatycznego CBS/CTH zastosowano do porównania dwóch linii komórkowych: neuroblastomy ludzkiej (linia SH-SY5Y) oraz glioblastomy - astrocytomy ludzkiej (linia U-87 MG) w zakresie ich potencjalnych możliwości w tworzeniu siarkowodoru. Kolejną metodą wdrożoną w czasie realizacji projektu była metoda oznaczania poziomu H_2S wiązanego w warstwie agarozy i dostosowanie jej do oznaczeń w hodowlach komórkowych oraz homogenatach tkankowych. Badania wykazały, że linie: U-87 MG oraz SH-SY5Y są zdolne do tworzenia siarkowodoru w obecności L-cysteiny, odpowiednio o 20% dla linii SH-SY5Y oraz 5% dla linii U-87 MG w porównaniu do komórek kontrolnych bez L-cysteiny. W obu liniach komórkowych potwierdzono również aktywność MPST, CBS oraz CTH. Stwierdzono, że aktywność badanych enzymów w neuroblastomie ludzkiej jest większa w porównaniu do komórek glioblastomy ludzkiej i największą aktywność właściwą oznaczono dla MPST. Sugeruje to, że komórki neuroblastomy mają potencjalnie większą zdolność do tworzenia H_2S z cysteiny niż komórki astrocytomy i głównym enzymem odpowiedzialnym za wytwarzanie siarkowodoru z cysteiny w tych komórkach jest MPST. Aktywność enzymów zależy od ekspresji genów, co zasugerowało konieczność opracowania warunków badania ekspresji na poziomie mRNA oraz białka dla badanych enzymów. Przeprowadzone badania potwierdziły największą ekspresję MPST w komórkach neuroblastomy. W komórkach glioblastomy ludzkiej (U-87 MG) oznaczono wysoki poziom glutationu, co wydaje się mieć zasadnicze znaczenie dla ochrony neuronów, np. przed toksycznym działaniem reaktywnych form tlenu. Ponad trzykrotnie większy poziom siarki sulfanowej w komórkach glioblastomy ludzkiej może sugerować możliwość nieenzymatycznego uwalniania H_2S z tej puli w warunkach redukujących [Publikacja nr 3].

Metody przedstawione w publikacjach 1, 2, 3 pozwoliły na badanie w rożnych układach doświadczalnych udziału poszczególnych enzymów - CBS, CTH i MPST w tworzeniu H_2S oraz na ilościowe oznaczanie tworzenia H_2S.

Ostatnim etapem badań było zastosowanie wcześniej opracowanych metod do określenia tworzenia H_2S w żołądkach szczurzych ze śluzówką uszkodzoną, z owrzodzeniami, będącymi wynikiem stresu wynikającego z oziębienia i unieruchomienia w wodzie (WRS) w porównaniu do żołądków z nieuszkodzoną śluzówką. Badano również wpływ prekursora H_2S – NaHS, podanego przed wywołaniem uszkodzeń śluzówki, na powstawanie i gojenie się uszkodzeń wywołanych stresem. W pierwszym etapie badań potwierdzono, że H_2S jest wytwarzany w błonie śluzowej żołądka zdrowych szczurów. Potwierdzono ekspresję czterech enzymów: MPST, CTH, CBS, TST, biorących udział w tworzeniu H_2S oraz w metabolizmie siarki sulfanowej. Oznaczona aktywność MPST i CTH oraz nieoznaczalna aktywność CBS sugerują, że MPST i CTH są głównymi enzymami odpowiedzialnym za wytwarzanie H_2S w prawidłowej błonie śluzowej żołądka. W homogenatach błony śluzowej żołądków szczurów z regionów z owrzodzeniami zaobserwowano 2-3-krotnie większą zdolność do tworzenia H_2S w porównaniu do błony śluzowej żołądka zdrowych szczurów, co sugeruje, że może to być wynikiem zwiększonej aktywności enzymów w stanie zapalnym. Potwierdzono trzykrotnie zwiększoną aktywność CTH, podwyższoną aktywność MPST i TST oraz wzrost aktywności CBS do wartości oznaczalnych w grupie WRS. Nie stwierdzono natomiast zmian w ekspresji badanych enzymów w porównaniu do zdrowej błony śluzowej żołądka. Podobnie, poziom siarki sulfanowej był porównywalny z poziomem siarki sulfanowej w zdrowej śluzówce. Poziom GSH i GSSG był natomiast znacznie obniżony w porównaniu do zdrowej błony śluzowej żołądka. Uzyskane wyniki wskazują na to, że w stresie, który prowadzi do uszkodzenia śluzówki żołądka wzrasta produkcja siarkowodoru, co ma korzystny wpływ na proces gojenia uszkodzonej śluzówki. Szczury, którym podawano NaHS, wykazywały

zmniejszoną zdolność do tworzenia endogennego H_2S w błonie śluzowej z owrzodzeniami i zmiany niektórych parametrów biochemicznych (z wyjątkiem aktywności MPST i CTH oraz poziomu cysteiny) w kierunku wartości oznaczonych w zdrowej błonie śluzowej. Przeprowadzone badania potwierdziły, że endogenny H_2S oraz H_2S uwolniony z donorów (NaHS) chroni błonę śluzową żołądka, co jak można przypuszczać związane jest ze zwiększeniem przepływu w naczyniach [Publikacja nr 4].

Wyniki przedstawione w zaprezentowanych publikacjach przyczyniły się do opracowania metod badania aktywności układu enzymatycznego CBS/CTH, metod badania ekspresji enzymów odpowiedzialnych za wytwarzanie siarkowodoru w tkankach ludzkich, mysich, szczurzych i hodowlach komórkowych oraz metod oznaczania poziomu H_2S. Badania z wykorzystaniem opracowanych metod pozwoliły na określenie możliwości różnych tkanek (wątroba, nerki, mózg) oraz komórek nowotworowych (komórki ludzkie układu nerwowego: linia SH-SY5Y i U-87 MG) w zakresie tworzenia H_2S oraz na wyjaśnienie roli H_2S w wywołanym stresem procesie zapalnym, związanym z uszkodzeniem śluzówki żołądka szczurów. Uzyskane wyniki dają podstawę do dalszych badań w kierunku wyjaśnienia roli H_2S w schorzeniach związanych ze zmianą jego poziomu oraz w kierunku modulowania poziomu H_2S zarówno poprzez stosowanie różnych szybko lub wolno uwalniających prekursorów H_2S, jak i poprzez zmiany aktywności oraz ekspresji (wyciszanie genów) enzymów odpowiedzialnych za tworzenie siarkowodoru.

Summary

Apart from nitric oxide and carbon monoxide, hydrogen sulphide (H_2S) is another inorganic gaseous mediator. H_2S is lipophilic - its penetration into cells does not require transporters. It is produced endogenously in the liver, kidneys, brain and many other human and mammalian tissues. Its synthesis is carried out with the participation of enzymes, where the co-factor is pyridoxal phosphate: cystathionine beta-synthase (CBS, EC 4.2.1.22), gamma-cystathionase (CTH, EC 4.4.1.1) and zinc ions-dependent 3-mercaptopyruvate sulfurtransferase (MPST, EC 2.8.1.2). H_2S can be produced enzymatically from L-cysteine, L-cystine, 3-mercaptopyruvate, L-homocysteine and D-cysteine. The synthesis of H_2S from D-cysteine takes place with the participation of MPST and D-amino acids oxidase (DAO). H_2S can be also released non-enzymatically from the persulfates under reducing conditions.

The aim of the study were to determine the production of H_2S in various tissues and cell cultures by examining the activity of the enzymes responsible for its formation and metabolism (CBS, CTH, MPST, TST) and to determine H_2S levels in healthy and inflammatory tissues. A method of determination the activity of the CBS/CTH tandem in the presence of homoserine (CBS substrate) and cystathionine (CTH substrate) was developed. For the analysis of changes in cystathionine concentration (a product of homoserine transformation by CBS), the RP-HPLC method was modified (Dominick et al., 2011). The modification also allowed for determination, during one separation, of the level of reduced (GSH) and oxidized (GSSG) glutathione, cysteine (CSH), cystine (CSSC) and cystathionine.

The experiments related to RP-HPLC modification were carried out in homogenates of tissue from different regions of the human brains. The effects of these studies were developing a modification and also determining the level of cystathionine in the examined tissues. The highest level of cystathionine was detected in the thalamus and it was about 11 times higher as

compared to the cerebellum. A high level of cystathionine in the thalamus was correlated with a low CTH activity. The highest level of cysteine was detected in the thalamus, hypothalamus and subcortical nucleus. A high level of cysteine in the thalamus was associated with a high level of GSH. The role of cystathionine as a neuromodulator has been suggested in the human brain, in addition to its contribution to cysteine metabolism and production of H_2S. A high level of cystathionine in some regions of the human brain might be associated with a high CBS activity. CBS, similarly as CTH, catalyzes reactions, in which H_2S is produced [Publication No. 1].

The next step in the study, important for the production of H_2S in tissues and for delivery of cysteine to GSH synthesis, was developing a method for determination of the activity of the CBS/CTH tandem. The research was carried out in homogenates of the mouse liver, kidneys and brain in the presence of two substrates: homoserine and cystathionine. The CTH activity was examined through the increase of the amount of α-ketobutyrate (a product of catalyzed reaction through CTH). The method was described for the first time in Publication No. 2. A difference in the cystathionine levels between the homogenates with totally CTH-inhibiting PPG concentrations and without the inhibitor was employed to evaluate the activity of CBS. The CBS activity was the highest in the mice brain. The CTH activity was undetectable in the mice brain, which was confirmed by the major role of CBS in H_2S production. In the presence of homoserine, increased levels of cystathionine were determined in the brain, which confirmed the high CBS activity. In the liver, the low – as compared to the brain - level of cystathionine and high levels of α-ketobutyrate were associated with a high CTH activity. Simultaneously, a high level of GSH indicated the main role of CBS and CTH in delivering cysteine for GSH synthesis in homogenates of the mouse liver [Publication No. 2].

A method was developed for determination of the activity of the CBS/CTH tandem and used to compare potential possibilities of H_2S generation in two cell lines: the human

neuroblastoma (SH-SY5Y cells) and the human glioblastoma (U87-MG cells). Another method employed during the project was determination of the amount of H_2S bound in the agarose layer and its adaptation to analysis in the cell cultures and homogenates of tissues. The studies showed the U-87 MG and SHSY5Y cells capacity of hydrogen sulfide formation from L-cysteine, 20% for SH-SY5Y cells and 5% for U-87 MG cells, respectively, as compared to the control cells without L-cysteine. The activities of MPST, CBS and CTH were also confirmed in both the cell lines. A higher activity of the investigated enzymes was found in the neuroblastoma cells as compared to the astrocytoma cells, and the highest appropriate activity of MPST was examined. The findings suggested that the neuroblastoma cells had a potentially higher capacity of H_2S generation from cysteine than the astrocytoma cells, and MPST was the main enzyme responsible for H_2S production from cysteine in either of the cell lines. The activity of the enzymes depended on gene expression, which suggested a necessity of determining the conditions necessary for analyzing the expression (mRNA and protein) for the investigated enzymes. The results confirmed the highest expression of MPST in the neuroblastoma cells. The human glioblastoma cells (U-87 MG cells) demonstrated a higher level of GSH in comparison to the neuroblastoma cells, what seemed to be essential for neuron protection, e.g. against the toxicity of reactive oxygen species. The sulfane sulfur level was more than three-fold higher in the human glioblastoma cells, which might suggest a nonenzymatic release of H_2S from sulfane sulfur-containing compounds under reducing conditions [Publication No. 3].

The methods presented in the publications No. 1, 2, 3 allowed for studying the participation of several enzymes - CBS, CTH and MPST - in the production of H_2S in various experimental systems, as well as for quantitative determination of H_2S.

The final step in the research was employing the previously developed methods to determine the production of H_2S in rat stomachs with damaged mucosa, with ulcers, caused by stress and immobilization in cold water (WRS) in comparison to the healthy mucosa.

The effect of NaHS – a precursor of H_2S, administered prior to WRS, on the production and healing of stress-induced damage was also investigated. In the first stage of the research, it was confirmed that H_2S was produced in the gastric mucosa of healthy rats. The results confirmed the expression of MPST, CTH, CBS and TST involved in the production of H_2S and the metabolism of sulfane sulfur. In the gastric mucosa of healthy rats, the activity of MPST and CTH were determined, the activity of CBS was undetectable, what suggested the main role of MPST and CTH in H_2S production. The gastric mucosa of rats with ulcers had a 2–3-fold higher ability to generate H_2S in comparison to the gastric mucosa of healthy rats, what suggested increased activities of the enzymes in the inflammatory process. A three-fold increase of the CTH activity, the elevated activity of MPST and TST and the increased activity of CBS were confirmed as compared to the healthy mucosa. No increased expression of the investigated enzymes was found when compared to the healthy mucosa. Similarly, the level of sulfane sulfur was comparable to that in the healthy mucosa. The GSH and GSSG levels were significantly decreased as compared to the healthy mucosa. These results may suggest that a higher capability of H_2S generation by ulcerated mucosa resulted from an increased specific activity of the enzymes involved in the process, which had a beneficial effect on the healing process of the damaged mucosa. The rats administered NaHS demonstrated a decreased ability of endogenous H_2S generation in the gastric mucosa and changes of some biochemical parameters (except the MPST and CTH activities and the level of cysteine) back to the values found in the healthy tissue.

The research confirmed that endogenous H_2S and H_2S released from the donors (NaHS) protected the gastric mucosa, which may be associated with an increase in gastric flow [Publication No. 4].

The results shown in the presented publications contributed to the development of methods of determining the activity of the enzyme CBS/CTH tandem, expression of the enzymes responsible for the production of hydrogen sulfide in the human, mouse and rat tissues and cell cultures, and methods of determining the amount of H_2S. The research using the developed methods allowed for defining the capabilities of different tissues (the liver, kidneys, brain) and cancer cells (human cells of the nervous system: the SH-SY5Y cells and U87-MG cells) in the production of H_2S and explained the role of H_2S in the inflammation caused by stress, associated with damage to the gastric mucosa.

The results provide the foundation for further research aiming at explanation of the role of H_2S in conditions associated with the changes of its level and at modulating the amount of H_2S through using a variety of fast or slow-release H_2S precursors, as well as through changes of the activity and expression (gene-silencing) of enzymes participating in the production of hydrogen sulfide.

Literatura

Abe, K.; Kimura, H. The possible role of hydrogen sulfide as an endogenous neuromodulator. J. Neurosci. **1996,** 16: 1066-1071.

Bradford, M. M.. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Analytical. Biochemistry*. **1976**, *72,* 248-254.

Brodek, P.; Olas, B. Biochemistry and therapeutic potential of hydrogen sulfide - reality or fantasy? *Postepy Hig Med Dosw*. **2016**, *11*, 820-829.

Czubak, J.; Wróbel, M.; Jurkowska, H. Cystathionine γ-lyase (EC 4.4.1.1): An enzymatic assay of α-ketobutyrate using lactate dehydrogenase. *Acta Biol. Crac. Zool.* **2002**, *44*, 113–117.

Dominick, P.K.; Cassidy, P.B.; Roberts, J.C. A new and versatile method for determination of thiolamines of biological importance. *J. Chromatogr. B.* **2001**, *761*, 1-12.

Ishigami, M.; Hiraki, K.; Umemura, K.; Ogasawara, Y.; Ishii, K.; and Kimura, H. A source of Hydrogen sulfide and a mechanism of its release in the brain. *Antioxid. Redox Signal*. **2009**, *11*, 205–214

Kabil, O.; Banerjee, R. Redox biochemistry of hydrogen sulfide *J Biol Chem*. **2010**, 16, 285 (29): 21903-21907.

Kartha, R. V.; Zhou, J.; Hovde, L.B.; Belinda, W. Y.; Cheung, B.W. Y.; Schröder, H. Enhanced detection of hydrogen sulfide generated in cell culture using an agar trap method. *Analytical Biochemistry*. **2012,** 423, 102-108.

Kimura, H. Physiological role of hydrogen sulfide and polysulfide in the central nervous system. Neurochemistry International. **2013.** *63*, 492-497.

Kimura, Y.; Goto, Y.; Kimura, H. Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria. Antioxid Redox Signal. **2010**, *12*: 1-13.

Lou, L. X.; Geng, B.; Du, J. B.; Tang, C.S. Hydrogen sulphide-induced hypothermia attenuates stress-related ulceration in rats. *Clin. Exp. Pharmacol. Physiol*. **2008,** *35,* 223-228.

Lowry, O.H.; Rosenbrough, N.J.; Farr, A.L.; Randall, R.I. Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. **1951**, *193,* 265-275.

Magierowski, M.; Jasnos, K.; Kwiecień, S.; Brzozowski, T. Rola siarkowodoru w fizjologii przewodu pokarmowego i w mechanizmie gastroprotekcji. *Postepy Hig Med Dośw.* **2013**, *67*, 150-156.

Matsuo, Y.; Greenberg, D.M. A crystalline enzyme that cleaves homoserine and cystathionine. J. Biol. Chem. **1958**, *230*, 545-560.

Olas, B. Hydrogen sulfide in hemostasis; friends or foe. *Chem. Biol. Interact*. **2014**. *217*, 49-56.

Panthi, S.; Chung, H-J.; Jung, J.; Jeong, N. Y. Physiological Importance of Hydrogen Sulfide: Emerging Potent Neuroprotector and Neuromodulator. *Oxid. Med. Cell. Longev*. **2016**, 1-11.

Paul, B. D.; Snyder, S. H. Modes of physiologic H_2S signaling in the brain and peripheral tissues. *Antioxid. Redox. Sign*. **2015**, *22*, 411-423.

Shibuya N.; Tanaka M.; Yoshida M.; Ogasawara Y.; Togawa T.; Ishii K.; Kimura H. 3-Mercaptopyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain. *Antioxid. Redox Signal.* **2009**, *11*, 703-714.

Shibuya, N.; Koike, S.; Tanaka, M.; Ishigami-Yuasa, M.; Kimura, Y.; Ogasawara, Y.; Fukui, K.; Nagahara, N.; Kimura, H. A novel pathway for the production of hydrogen sulfide from D-cysteine in mammalian cells. *Nat. Commun*. **2013**, *4*, 1366-1373.

Sörbo, B. Rhodanese. In *Methods in Enzymology*; Colowick, S.P., Kaplan, N.O., Eds.; Academic Press: New York, NY, USA, **1955**, 2, 334–337.

Valentine, W. N.; Frankelfeld, J. K. 3-Mercaptopyuruvate sulfurtransferase (EC 2.8.1.2): A simple assay adapted to human blood cells. *Clin. Chim. Acta.* **1974**, *14*, 205-210.

Wang, R. Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. *Pysiol. Rev.* **2012,** *92*, 791-896.

Wood, L. Sulfane sulfur. *Methods Enzymol*. **1987**, *143*, 25-29.

Oświadczenia współautorów

Kraków, dnia. 09.05.2017

Mgr Jacek Zagajewski
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „*RP-HPLC method for the quantitative determination of cystathionine, cysteine and glutathione: an application for the study of the metabolism of cysteine in human brain* " oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to: współudział w wykonywaniu oznaczeń metodą RP-HPLC.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Patrycję Bronowicką - Adamską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Patrycji Bronowickiej - Adamskiej przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

.....

(podpis współautora)

Kraków, dnia. 05.05.2017

Dr Jerzy Czubak
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „ *RP-HPLC method for the quantitative determination of cystathionine, cysteine and glutathione: an application for the study of the metabolism of cysteine in human brain* " oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

współudział w przygotowaniu materiału biologicznego do badań oraz oznaczaniu aktywności gamma-cystationazy (CTH).

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Patrycję Bronowicką - Adamską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Patrycji Bronowickiej - Adamskiej przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

.....

(podpis współautora)

Kraków, dnia. 09.05.2017

Prof. Dr hab. Maria Wróbel
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „*RP-HPLC method for the quantitative determination of cystathionine, cysteine and glutathione: an application for the study of the metabolism of cysteine in human brain* " oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

...współudział w przy planowaniu zakresu, metodologii badań, formowaniu wniosków oraz edycji tekstu publikacji.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Patrycję Bronowicką - Adamską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Patrycji Bronowickiej - Adamskiej przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

.....Maria Wróbel.....

(podpis współautora)

Kraków, dnia. 09.05.2017

Mgr Jacek Zagajewski
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „... An application of RP-HPLC for determination of the activity of cystathionine beta-synthase and gamma-cystathionase in tissue homogenates. ..." oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

współudział w wykonywaniu oznaczeń metodą RP-HPLC oraz oznaczania poziomu α-ketomaślanu.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Patrycję Bronowicką - Adamską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Patrycji Bronowickiej - Adamskiej przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

.....
(podpis współautora)

Kraków, dnia. 09.05.2017

Prof. Dr hab. Maria Wróbel
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „. An application of RP-HPLC for determination of the activity of cystathionine beta-synthase and gamma-cystathionase in tissue homogenates. " oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

współudział w przy planowaniu zakresu, metodologii badań, formowaniu wniosków oraz edycji tekstu publikacji

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Patrycję Bronowicką - Adamską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Patrycji Bronowickiej - Adamskiej przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

.....

(podpis współautora)

Kraków, dnia. 09.05.2017

Dr Anna Bentke
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „. Hydrogen sulfide generation from L-cysteine in the human glioblastoma-astrocytoma U-87 MG and neuroblastoma SHSY5Y cell lines. " oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

.współudział w wykonywaniu badań metodą Western blot

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Patrycję Bronowicką - Adamską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Patrycji Bronowickiej - Adamskiej przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

.....

(podpis współautora)

Kraków, dnia. 09.05.2017

Prof. Dr hab. Maria Wróbel
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „. Hydrogen sulfide generation from L-cysteine in the human glioblastoma-astrocytoma U-87 MG and neuroblastoma SHSY5Y cell lines.. “ oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

...współudział w przy planowaniu zakresu, metodologii badań, formowaniu wniosków oraz edycji tekstu publikacji

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Patrycję Bronowicką - Adamską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Patrycji Bronowickiej - Adamskiej przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

.....Marek Wróbel.....

(podpis współautora)

Kraków, dnia. 09.05.2017

Prof. Dr hab. Maria Wróbel
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „...Hydrogen sulphide production in gastric mucosa of rats exposed to stress" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

...współudział w przy planowaniu zakresu, metodologii badań, formowaniu wniosków oraz edycji tekstu publikacji

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Patrycję Bronowicką - Adamską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Patrycji Bronowickiej - Adamskiej przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

Maria Wróbel
.....

(podpis współautora)

Kraków, dnia. 08.05.2017

Dr Marcin Magierowski
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „...Hydrogen sulphide production in gastric mucosa of rats exposed to stress. " oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

współudział w przygotowaniu modelu badawczego oraz w pobieraniu materiału do badań

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Patrycję Bronowicką - Adamską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Patrycji Bronowickiej - Adamskiej przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

Magierowski Marcin

(podpis współautora)

Kraków, dnia. 08.05.2017

Mgr Katarzyna Magierowska
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „...Hydrogen sulphide production in gastric mucosa of rats exposed to stress. ..." oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

współudział w przygotowaniu modelu badawczego oraz w pobieraniu materiału do badań

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Patrycję Bronowicką - Adamską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Patrycji Bronowickiej - Adamskiej przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

Katarzyna Magierowska
(podpis współautora)

Kraków, dnia. 08.05.2017

Dr hab. Sławomir Kwiecień
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „...Hydrogen sulphide production in gastric mucosa of rats exposed to stress. ..." oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

współudział w przygotowaniu modelu badawczego oraz w pobieraniu materiału do badań

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Patrycję Bronowicką - Adamską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Patrycji Bronowickiej - Adamskiej przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

Sławomir Kwiecień

(podpis współautora)

Kraków, dnia. 08.05.2017

Prof. dr hab. Tomasz Brzozowski
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „...Hydrogen sulphide production in gastric mucosa of rats exposed to stress. ..." oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

współudział w przy planowaniu zakresu, metodologii oraz interpretacji wyników badań

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr Patrycję Bronowicką - Adamską jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład mgr Patrycji Bronowickiej - Adamskiej przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

.....

(podpis współautora)