IL-4, IL-6, IL-10, GM-CSF, INF-γ, TNF-α)。また、LPSを投与(6 mg/kg, from *E.Coli* S-type 0111:B4)した敗血症の場合と比較検討した。動物実験は学内倫理規定に従って実施した。

C. 結果および考察

LPS を投与した場合には、炎症反応の惹起により全サイトカインが顕著な増大を示し、典型的な敗血症になった。(最大値概算)IL-1α: 500 pg/mL, IL-1β□□ pg/mL, IL-2: 40 pg/mL, IL-4: 50 pg/mL, IL-6: 12000 pg/mL, IL-10: 1300 pg/mL, GM-CSF: 60 pg/mL, IFN-γ: 6000 pg/mL, TNF-α: 4000 pg/mL。他方、出血ショック後に脱血液を投与する試験では、血漿中のサイトカインレベルが出血後に上昇することを期待したが、顕著な増大は見られなかった。(最大値概算) IL-1α: 2 pg/mL, IL-1β□9 pg/mL, IL-2: 21 pg/mL, IL-4: 4 pg/mL, IL-6: 20 pg/mL, IL-10: 10 pg/mL, GM-CSF: 6 pg/mL, IFN-γ: 20 pg/mL, TNF-α: 10 pg/mL。これは、ショック状態にしておく時間が短かく、炎症惹起にまで到達しなかったこと、また、脱血液を 15 分放置した程度ではその中の白血球から炎症性サイトカインが産生されていなかったことを示す。或は、サイトカインは局所的に産生し、血漿中には遊離しない可能性も考えられる。

D. 結論

今回の実験では、出血ショック蘇生における顕著な炎症性サイトカイン産生の亢進は認められなかった。今後は、ショック状態の時間延長や、十分な蘇生をせずに放置した場合との比較を実施する予定である。

3．赤血球モデルの NO, CO 結合速度の解析

A. 研究目的

赤血球は35%濃度の Hb 溶液を細胞膜で覆った粒子 (長径 8μm)である。生命の進化の過程で赤血球は細胞核を捨て酸素運搬の役割に特化し、両凹円板の形状をした赤血球の変形能と粒子表面積が酸素運搬には有効 と考えられてはいるが、物理化学的計測では実は分子状 Hb 溶液に比べ赤血球は酸素結合/放出速度が極めて遅く非効率的であることが報告されている(Page et al., *Microvasc. Res.*, 1998;56:113-126)。

本研究では、そのような非効率な赤血球構造の生理的意義を明示することを目的とし、モデル赤血球のガス状分子メディエータ(NO, CO)との反応速度を測定し、分子状 Hb 溶液と比較検討することを目的とした。

B. 方法

赤血球は高い剪断応力によって溶血するので、モデル赤血球として力学的強度のある Hb 小胞体(HbV)を使用した。Tyrode solution (pH 7.4)に分散させ、ヘム濃度を3 μM に調節した。HbO_2溶液も同様に Tyrode solution に分散させ、2,3-DPG を 3 mM 添加して酸素親和度を調節した。ストップドフロー装置は、ユニソク社製 RSP-1000 型を使用した。NO 結合速度の測定の際には、酸素を遮断し NO/N_2 混合ガスを混合液に通気し、[NO] = 3.8 μM とした。また、CO 結合速度に際しては、CO/N_2 混合ガスを用い、混合液を[CO] = 65 μM とした。HbV または Hb 溶液側は、常に窒素ガスを通気し、deoxyHb の状態を保持した。測定は全て室温で行った。

C. 結果および考察

HbV の NO 結合速度定数は、$4.8 \times 10^6\ M^{-1}s^{-1}$ であったのに対し、Hb 溶液では、$3.0 \times 10^7 M^{-1}s^{-1}$ であった。従って、脂質膜で被覆することにより、NO 結合速度が約 1/6 に減少した。しかし、この値は、赤血球で別の方法により推定されている値($5.2 \times 10^4 M^{-1}s^{-1}$, Liu et al., 1998)に比較すると、92 倍速い値となる。

また同様に、HbV の CO 結合速度定数は、$8.6 \times 10^4\ M^{-1}s^{-1}$ であるのに対し、Hb 溶液では $4.5 \times 10^5\ M^{-1}s^{-1}$ であり、脂質膜で被覆することにより結合速度が約 1/5 に減少した。

以上のように配位子の結合速度が遅くなる理由としては、脂質界面に存在する非撹拌層における物質の拡散律速や、粒子内部の高濃度 Hb 溶液(35%)が粘稠であるため配位子が結合した Hb の拡散速度が低下し促進輸送効果が低下すること等が考えられている。

D. 結論

体内では、ガス状分子 NO, CO が様々なメディエータとして作動しており、遊離した Hb 分

子の迅速な捕捉による副作用が指摘されている。従って、赤血球のような粒子構造は、これを遅延させるために重要であることを示唆する。HbV は本物の赤血球よりも力学的強度があるため、従来出来なかっ様々な解析法を可能とする利点を有する。粒子径や、内部 Hb 濃度が大きくなるほど更に結合速度は遅くなると考えられる。今後は、結合速度を低下させる主要因子或は増大させる条件(アクアコリンなど)を整理し、本物の赤血球の様相を明らかにする予定である。

研究業績

A. 論文発表

1) H. Sakai, H. Horinouchi, Y. Masada, S. Takeoka, M. Takaori, K. Kobayashi, E. Tsuchida. Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model." *Biomaterials* **25**, 4317-4325 (2004, Aug.).

2) A. Yoshizu, Y. Izumi, S. Park, H. Sakai, S. Takeoka, H. Horinouchi, E. Ikeda, E. Tsuchida, K. Kobayashi. Hemorrhagic shock resuscitation with an artificial oxygen carrier Hemoglobin Vesicle (HbV) maintains intestinal perfusion and suppresses the increase in plasma tumor necrosis factor alpha (TNF□). *ASAIO J.* **50**, 458-463 (2004, Sept.).

3) H. Sakai, Y. Masada, H. Onuma, S. Takeoka, E. Tsuchida. Reduction of Methemoglobin via Electron Transfer from Photoreduced Flavin: Restoration of O_2-Binding of Concentrated Hemoglobin Solution Coencapsulated in Phospholipid Vesicles. *Bioconjugate Chem.* **15**, 1037-1045 (2004, Sept.).

4) H. Sakai, Y. Masada, H. Horinouchi, E. Ikeda, K Sou S. Takeoka, M. Suematsu, K. Kobayashi, E. Tsuchida "Physiologic capacity of reticuloendothelial system for degradation of hemoglobin-vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days." *J. Pharmacol. Exp. Ther.* 311:874-884 (2004, Dec.)

5) P. Cabrales, H. Sakai, A.G. Tsai, S. Takeoka, E. Tsuchida, M. Intaglietta. Oxygen transport by low and normal P_{50} Hb-vesicles in extreme hemodilution. *Am. J. Physiol. Heart Circ. Physiol.* 2005; in press.

6) C. Contaldo, J. Plock, H. Sakai, S. Takeoka, E. Tsuchida, M. Leuing, A. Banic, and D. Erni. Hemodilution with polymerized and encapsulated hemoglobins improves oxidative energy metabolism in collaterized hamster flap tissue. *Crit. Care Med.* 2005; in press.

7) H. Sakai, P. Cabrales, A.G. Tsai, M. Intaglietta, E. Tsuchida. Oxygen Releasing of Hb-vesicles with different P_{50}s from Occluded Arteriole in Hamster Skinfold Window Model. *Am. J. Physiol. Heart Circ. Physiol.* 2005; in press.

(総説、著書など)

1) 土田英俊、武岡真司、小松晃之、酒井宏水、"人工赤血球"「新訂版・表面科学の基礎と応用」第 5 章第 12 節 pp. 1333-1336、編者: 日本表面科学会、エヌ・ティー・エス社 (2004) (2004. 6. 22)

2) H. Sakai, K. Sou, S. Takeoka, K. Kobayashi, E. Tsuchida. "Hemoglobin-vesicles (HbV) as Artificial Oxygen Carriers".In: Artificial Oxygen Carrier: Its Front Line, Keio University International Symposia for Life Sciences and Medicine Vol.12, pp. 135-168, Eds. by K. Kobayashi, E. Tsuchida, and H. Horinouchi. Springer-Verlag (2005).

3) K. Kobayashi, H. Horinouchi, M. Watanabe, Y. Izumi, Y. Teramura, A. Nakagawa, Y. Huang, K. Sou, H. Sakai, T. Komatsu, S. Takeoka, E. Tsuchida. "Safety and Efficacy of Hemoglobin-Vesicles and Albumin-Hemes". In: Artificial Oxygen Carrier: Its Front Line, Keio University International Symposia for Life Sciences and Medicine Vol.12, pp. 1-21, Eds. by K. Kobayashi, E. Tsuchida, and H. Horinouchi. Springer-Verlag (2005).

4) H. Sakai, K. Sou, S. Takeoka, K. Kobayashi, and Eishun Tsuchida, "Hemoglobin-Vesicles as a Molecular Assembly: Characteristics of Preparation Process and Performances as Artificial Oxygen Carriers." In "*Blood Substitutes*" (Ed. By Robert M. Winslow, Elsevier) 2005 in press.

5) E. Tsuchida, H. Sakai, T. Komatsu, S. Takeoka, Y. Huang, K. Sou, A. Nakagawa, Y. Teramura, and K. Kobayashi. Oxygen Infusions (Hemoglobin-Vesicles and Albumin-Hemes) Based on Nano-Molecular Sciences. *Polymers Adv. Technol.* 2005; in press.

B. 学会発表

1. H. Sakai, K. Sou, S. Takeoka, K. Kobayashi, E. Tsuchida. Rheological Properties of PEG-modified Hb-vesicles (HbVs) and their oxygen-transporting

capacity in vivo. / The 9th Liposome Research Days Conference / May 11-15, 2004 / Hsinchu, Taiwan

2. E. Tsuchida, H. Sakai, S. Hisamoto, I. Fukutomi, K. Sou, S. Takeoka / Detection of lipopolysaccharide in hemoglobin-vesicles by Limulus Amebocyte lysate test with kinetic-turbidimetric gel clotting analysis and pretreatment of surfactant. / The 9th Liposome Research Days Conference / May 11-15, 2004 / Hsinchu, Taiwan

3. H. Sakai, Y. Suzuki, M. Kinoshita, S. Takeoka, N. Maeda, E. Tsuchida. / O_2 release from Hb vesicles evaluated using an artificial, narrow O_2-permeable tube (comparison with RBCs and acellular Hb)/ The 9th Liposome Research Days Conference / May 11-15, 2004 / Hsinchu, Taiwan

4. E. Tsuchida, H. Sakai, H. Horinouchi, S. Takeoka, K. Kobayashi. / Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial systsem. / The 9th Liposome Research Days Conference / May 11-15, 2004 / Hsinchu, Taiwan

5. K. Sou, R. Klipper, B. Goins, H. Sakai, S. Takeoka, W.T. Phillips, E. Tsuchida. / Circulation Kinetics and biodistribution of Hemoglobin-vesicles. / The 9th Liposome Research Days Conference / May 11-15, 2004 / Hsinchu, Taiwan.

6. 小幡洋輔、武岡真司、西出宏之、酒井宏水、土田英俊／ヘモグロビン小胞体(人工赤血球)投与後のサイトカイン放出挙動／第 53 回高分子年次大会／2004.5.25-27／神戸国際会議場

7. 鈴木洋司、酒井宏水、木下めぐ美、武岡真司、土田英俊、前田信治／酸素透過性微小流路を流れるヘモグロビン小胞体からの酸素放出:赤血球浮遊液およびヘモグロビン溶液との比較／日本生理学会／2004. 6. 2-4／札幌市

8. 堀之内宏久、小林紘一、渡辺真純、泉陽太郎、山本学、藤本博行、土田英俊、武岡真司、酒井宏水、小松晃之、山本尚志／人工酸素運搬体の開発／第 52 回日本輸血学会総会／2004. 6.23-25／札幌コンベンションセンター

9. 酒井宏水、堀之内宏久、武岡真司、小林紘一、土田英俊／ヘモグロビン小胞体による 40%血液交換後の回復過程／第 11 回日本血液代替物学会／2004.7.13-14／札幌市

10. 安楽誠、浦田由紀乃、宗慶太郎、酒井宏水、土田英俊、小田切優樹／125I ラベル化ヘモグロビン小胞体の体内動態について／第 11 回日本血液代替物学会／2004.7.13-14／札幌市

11. 阿部英樹, 山口美樹, 藤原満博, 酒井宏水, 武岡真司, 土田英俊, 東 寛, 池田久實／ヘモグロビン小胞体 (HbV) 投与がラット免疫系に及ぼす影響／第 11 回日本血液代替物学会／2004.7.13-14／札幌市

12. 酒井宏水、Pedro Cabrales、Amy G. Tsai、武岡真司、Marcos Intaglietta、土田英俊／血流停止させた細動脈内のヘモグロビン小胞体からの酸素放出挙動／第 11 回日本血液代替物学会／2004.7.13-14／札幌市

13. M. Kajimura, M. Ichikawa, H. Sakai, S. Takeoka, E. Tsuchida, Makoto Suematsu. / Real-time imaging of anionic liposomes during thrombus formation and acute inflammation in rats. / Japan-United Kingdom Platelet Conference / 2004. 9

14. 酒井宏水、山本学、堀之内宏久、武岡真司、小林紘一、土田英俊／人工赤血球(ヘモグロビン小胞体)による 40%血液交換後の回復過程／第 42 回日本人工臓器学会大会／2004.10.5-7／東京

15. 武岡真司、阿閉友保、酒井宏水、土田英俊／ヘモグロビン小胞体のメトヘモグロビン消去系の構築／第 42 回日本人工臓器学会大会／2004.10.5-7／東京

16. H. Sakai, E. Tsuchida. Performances of PEG-modified Hb-vesicles as artificial oxygen carriers in Microcirculation / The 6th Asian Congress for Microcirculation (ACM'05) / 2005. 2.24-26 / Tokyo.

17. M. Kajimura, H. Sakai, T. Atoji, S. Takeoka, E. Tsuchida, M. Ichikawa, M. Suematsu. Anionic liposome protects murine brain against plasma leaking after ischemia reperfusion / The 6th Asian Congress for Microcirculation (ACM'05) / 2005. 2.24-26 / Tokyo.

(新聞報道ほか)

Medical Tribune「人工血液がもたらす未来の救急医療像を模索」(H16.8.19)

ニュートン誌「驚異の未来テクノロジー、人工赤血球で慢性的な輸血用血液の不足を補う」(H17.2 月号)

厚生労働科学研究費補助金（医薬品・医療機器等
レギュラトリーサイエンス総合研究事業）分担研究報告書

ヘモグロビンアロステリーを利用した付加価値赤血球の創剤と救急医療への応用に関する研究

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研究要旨：α-NO ヘモグロビン血の投与が、出血性ショック・再灌流後の心機能障害に与える影響をラット出血性ショック・再灌流モデルで検討した。ラットを用い、重度出血性ショックモデル（体重 100g 当たり 3.1 ml の脱血）を作成し、1 時間後、無作為に 2 群に分け、脱血相当量のα-NO ヘモグロビン血（α-NOHb group）または洗浄赤血球血(cnotrol group)を投与した。心機能の測定は、1 時間ごとに 6 時間行った。心拍出量の変化は、虚血・再灌流 1 時間後においてα-NOHb group は、control group に比較し高い値を示したが、それ以降は control group に比較し低い値を示した。動脈血中の乳酸値に関しては、2 群とも同様の変化を示した。出血性ショック・再灌流モデルに対するα-NO ヘモグロビン投与は、再灌流初期には心拍出量を保つ可能性が示唆されたが、投与 2 時間以降の効果は示されなかった。

A. 研究目的

出血性ショック・再灌流後に見られる多臓器障害は、サイトカイン、一酸化窒素（NO）、Oxygen free radical 等が関与し、さらにミトコンドリアの傷害や酸素需給バランスの障害により引き起こされる（Shock 1999 12(1):1, Am J Physiol Heart Circ Physiol 2000 278(3):H942）。また、臓器不全の一つである心機能の低下は、更なる酸素需給バランスの障害を引き起こし、悪化させる可能性がある (Lancet 2004 12;363(9425):1988)。

一方、α-NO ヘモグロビンは構造的変化から酸素放出能の増加により組織の酸素摂取率を増加させ、酸素需給バランスを改善するだけでなく、ATP 放出によるレオロジー変化により末梢循環をも改善し、出血性ショック・再灌流後の心拍出量維持に効果的である可能性がある。

そこで、α-NO ヘモグロビン血の投与が、出血性ショック・再灌流後の心機能障害に与える影響をラット出血性ショック・再灌流モデルで検討した。

B. 研究方法

α-NO hemoglobin 血および洗浄赤血球血の作成

ヒトO型血を洗浄し、ヒト O 型洗浄赤血球を作成する。希釈したヒト O 型洗浄赤血球を 90 分間のアルゴンガスにより脱酸素化後、洗浄赤血球に NO ドナーを混入後、3 時間 4℃に保存し、α-NO ヘモグロビン血を作成した。そ

の後、α-NO ヘモグロビン血は、窒素により脱酸素化した生理食塩水で再び洗浄し、ヘマトクリット 30%の洗浄血とした。

生体内での心機能評価

ラット 7 匹（Wister 系ラット　雄 360～380 g）を、pentobarbital 麻酔下に内頸動静脈にカニュレーションし、カテーテルより生理的食塩水を 10ml/kg/hr で投与を開始した。続いて、気管切開し、人工呼吸管理を行った。内頸動脈カテーテルより、圧センサー付コンダクタンスカテーテル（SPR-838　MILLAR　USA）を挿入した。圧センサー付コンダクタンスカテーテルは、容積信号ケーブル（CEC-10　MILLAR　USA）を通して、心室圧一容積測定装置（ARIA-1 MILLAR　USA）に接続し、コンピューターを用いて解析した。

その後、体重 100g 当たり 3.1 ml の脱血(Shock 2004 22(2):151)を行い、重度出血性ショックモデルを作成した。1 時間後、無作為に 2 群に分け、脱血相当量のα-NO ヘモグロビン血（α-NOHb group）または洗浄赤血球血(cnotrol group)を内頸静脈カテーテルから投与した。その後、心機能の測定は、1 時間ごとに 6 時間行った。

血液分析

1 時間ごとに 0.2 ml 採血し、動脈血中の乳酸値を測定した。

（倫理面への配慮）

ラットは、実験日まで十分な餌と水を与えて飼育した。また、実験に際しては、全例麻酔下に処置を行った。手術操作は、可能な限り無菌的に行った。

C. 研究結果

血液ガス分析装置により、投与したα-NO ヘモグロビン血中に 27%のメトヘモグロビンが検出された。また、α-NO ヘモグロビン血を投与されたラットでは、著しい血尿が観察された。

心拍出量の変化において、虚血・再灌流 1 時間後においてα-NOHb group は、control group に比較し高い値を示したが、それ以降は control group に比較し低い値を示した（図 1）。

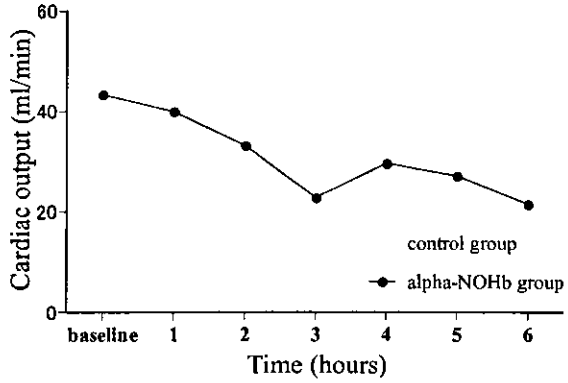

図 1.心拍出量の変化

動脈血中の乳酸値に関しては、2 群とも同様の変化を示した（図 2）。

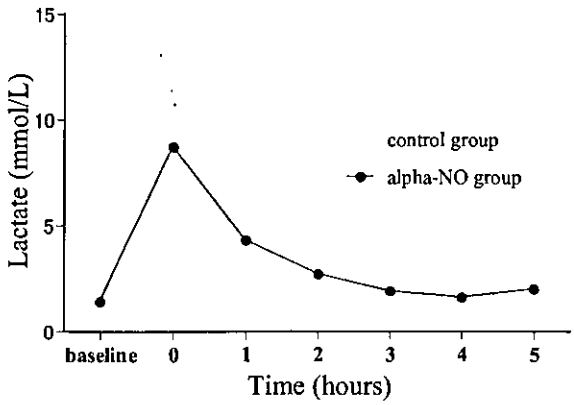

図 2.乳酸値の変化

D. 考察

この研究により、α-NO ヘモグロビン血の出血性ショックに対する投与では、再灌流初期には心拍出量を保つ可能性が示唆されたが、投与２時間後には洗浄赤血球血輸血に比較し低下した。これは、α-NO ヘモグロビン血から遊離した NO の関与が考えられた。また、抹消循環の指標である乳酸値の変化は、両群とも同様の変化を示し、α-NO ヘモグロビン血輸血による改善は示せなかった。

また、今回の問題点として、投与したα-NO ヘモグロビン血の 27%にメトヘモグロビンが検出された。これは、血ガス分析装置が NO ヘモグロビンとメトヘモグロビンとを識別できないことに起因すると予測された。今後、より精密な分析が必要であると考えられた。

さらに、重大な副作用のひとつとして、α-NO ヘモグロビン血投与による著しい血尿が上げられる。これは、溶血により引き起こされると示唆された。この原因が、ラットに対しヒトの赤血球を投与したことによるものと、脱酸素化に必要なバブリングにより赤血球の膜表面が傷害されることによることが強く疑われたが、今後の課題とした。

E. 結論

出血性ショック・再灌流モデルに対するα-NO ヘモグロビン投与は、再灌流初期には心拍出量を保つ可能性が示唆されたが、投与２時間以降の効果は示されなかった。これには、α-NO ヘモグロビン血から遊離した NO の関与が考えら、今後の検討課題とされた。

また、抹消循環の指標である乳酸値の変化は、両群とも同様の変化を示し、α-NO ヘモグロビン血輸血による改善は示せなかった。

今後の問題点として、投与したα-NO ヘモグロビン血の何%に本来のメトヘモグロビンが検出さるのか、より精密な分析が必要であると考えられた。

さらに、α-NO ヘモグロビン血投与による重大な副作用のひとつとして、著しい血尿が上げられる。これは、溶血により引き起こされると示唆された。この原因を検討することは、今後の課題と考えられた。

F. 健康危険情報

特になし

G. 研究発表

1 論文発表

なし

2 学会発表

なし

H. 知的財産権の出願･登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3.その他

なし

III 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

発表者名	論文タイトル名	発表誌名	巻名	ページ	出版年
Fujii, K., Sakuragawa, T., Kashiba, M., Sugiura, Y., Maruyama, K., Goda, N., Nimura, Y., Suematsu, M.	Hydrogen sulfide as an endogenous modulator of biliary bicarbonate excretion in the rat liver	Antioxid Redox Signal		In press	2005
Sugiura, Y., Kashiba, M., Hoshikawa, K., Sasaki, R., Saito, K., Kimura, H., Maruyama, K., Goda, N., Suematsu, M	Cadmium exposure alters metabolomics of sulfur-containing amino acids in rat testes	Antioxid. Redox Signaling		In press	2005
Katagiri, H., Ito, Y., Ishii, K., Hayashi, I., Suematsu, M., Yamashita, S., Murata, T., Narumiya, S., Kakita, A., Majima, M	Involvement of thromboxane derived from cyclooxygenase-1 and -2 in hepatic microcirculatory dysfunction during endotoxemia in mice	Hepatology	39	139-150	2004
Tomita, K., Azuma, T., Kitamura, N., Nishida, J., Inokuchi, S., Nishimura, T., Suematsu, M., Ishii, H.	Pioglitazone prevents alcohol-induced fatty liver in rats through upregulation of c-Met	Gastroenterology	126	873-885	2004
Kudo, A., Kashiwagi, S., Yoshimura, Y., Uchida, K., Arii, S., Suematsu, M.	Kupffer cells alter organic anion transport via multidrug resistance protein 2 in the post-cold ischemic rat liver	Hepatology	39	1099-1109	2004
H. Sakai, P. Cabrales, A.G. Tsai, M. Intaglietta, E. Tsuchida.	Oxygen Releasing of Hb-vesicles with different P50s from Occluded Arteriole in Hamster Skinfold Window Model	Am. J. Physiol. Heart Circ. Physiol.		In press	2005
C. Contaldo, J. Plock, H. Sakai, S. Takeoka, E. Tsuchida, M. Leuing, A. Banic, and D. Erni.	Hemodilution with polymerized and encapsulated hemoglobins improves oxidative energy metabolism in collaterized hamster flap tissue	Crit. Care Med.		In press	2005
P. Cabrales, H. Sakai, A.G. Tsai, S. Takeoka, E. Tsuchida, M.	Oxygen transport by low and normal P50 Hb-vesicles in extreme hemodilution	Am. J. Physiol. Heart Circ. Physiol.		In press	2005

Intaglietta.					
E. Tsuchida, H. Sakai, T. Komatsu, S. Takeoka, Y. Huang, K. Sou, A. Nakagawa, Y. Teramura, and K. Kobayashi	Oxygen Infusions (Hemoglobin-Vesicles and Albumin-Hemes) Based on Nano-Molecular Sciences	Polymers Adv. Technol.		In press	2005
H. Sakai, K. Sou, S. Takeoka, K. Kobayashi, and E. Tsuchida	Hemoglobin-Vesicles as a Molecular Assembly: Characteristics of Preparation Process and Performances as Artificial Oxygen Carriers	Blood Substitutes (Elsevier)		In press	2005
H. Sakai, Y. Masada, H. Horinouchi, E. Ikeda, K Sou S. Takeoka, M. Suematsu, K. Kobayashi, E. Tsuchida	Physiologic capacity of reticuloendothelial system for degradation of hemoglobin-vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days	J. Pharmacol. Exp. Ther.	311	874-884	2004
H. Sakai, Y. Masada, H. Onuma, S. Takeoka, E. Tsuchida.	Reduction of Methemoglobin via Electron Transfer from Photoreduced Flavin: Restoration of O2-Binding of Concentrated Hemoglobin Solution Coencapsulated in Phospholipid Vesicles	Bioconjugate Chem.	15	1037-1045	2004
A. Yoshizu, Y. Izumi, S. Park, H. Sakai, S. Takeoka, H. Horinouchi, E. Ikeda, E. Tsuchida, K. Kobayashi.	Hemorrhagic shock resuscitation with an artificial oxygen carrier Hemoglobin Vesicle (HbV) maintains intestinal perfusion and suppresses the increase in plasma tumor necrosis factor alpha (TNFa)	ASAIO J.	50	458-463	2004
H. Sakai, H. Horinouchi, Y. Masada, S. Takeoka, M. Takaori, K. Kobayashi, E. Tsuchida.	Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model	Biomaterials	25	4317-4325	2004

Ⅳ 研究成果の刊行物・別刷

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ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 5 & 6, 2005
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Forum Original Research Communication

Hydrogen Sulfide as an Endogenous Modulator of Biliary Bicarbonate Excretion in the Rat Liver

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AU: which affiliations?

ABSTRACT

Cystathionine γ-lyase (CSE) is an enzyme catalyzing cystathionine and cysteine to yield cysteine and hydrogen sulfide (H_2S), respectively. This study aimed to examine if H_2S generated from the enzyme could serve as an endogenous regulator of hepatobiliary function. Gas chromatographic analyses indicated that, among rat organs herein examined, liver constituted one of the greatest components of H_2S generation in the body, at 100 μmol/g of tissue, comparable to that in kidney and 1.5-fold greater than that in brain, where roles of the gas in the regulation of neurotransmission were reported previously. At least half of the gas amount in the liver appeared to be derived from CSE, because blockade of the enzyme by propargylglycine suppressed it by 50%. Immunohistochemistry revealed that CSE occurs not only in hepatocytes, but also in bile duct. In livers *in vivo*, as well as in those perfused *ex vivo*, treatment with the CSE inhibitor induced choleresis by stimulating the basal excretion of bicarbonate in bile samples. Transportal supplementation of NaHS at 30 μmol/L, but not that of *N*-acetylcysteine as a cysteine donor, abolished these changes elicited by the CSE inhibitor in the perfused liver. The changes elicited by the CSE blockade did not coincide with alterations in hepatic vascular resistance, showing little involvement of vasodilatory effects of the gas in these events, if any. These results first provided evidence that H_2S generated through CSE modulates biliary bicarbonate excretion and is thus a determinant of bile salt-independent bile formation in the rat liver. *Antioxid. Redox Signal.* 7, 788–794.

INTRODUCTION

CYSTEINE METABOLISM in the liver has been shown to contribute greatly to detoxification processes through multiple mechanisms. Following reduction and decarboxylation processes, this amino acid is converted to taurine, the compound used for conjugation of bile acids. Cysteine serves as a substrate for synthesis of glutathione through reactions of glutamate ligase and glutathione synthase, and is also used to generate sulfate through aspartate transferase and sulfite oxidase; these two compounds have well been shown to play an important role in detoxification of xenobiotics such as acetaminophen. Another important substance generated upon cysteine metabolism *in vivo* is hydrogen sulfide (H_2S). This gaseous compound has recently been shown to account for a signaling molecule in neural and vascular systems. It is produced mainly by two types of pyridoxal 5′-phosphate-dependent enzymes responsible for metabolism of L-cysteine: cystathionine γ-lyase (CSE; EC 4.4.1.1) and cystathionine β-synthase (CBS; EC 4.2.1.22). In other words, although the primary role of the two enzymes is to constitute the transsulfuration pathway that provides cysteine through biotransformation of methionine derived from nutrition, both CSE and CBS are able to use cysteine as the substrate to generate H_2S. The gas synthesized by CBS in brain has been reported to execute neural transduction. On the other hand, CSE-derived H_2S was shown to relax vascular smooth muscle cells through its ability to increase the conductance of potassium channels (22); in this study, H_2S released from the enzyme blocked vasoconstriction of rat aortic rings elicited by glibenclamide, a blocker of the ATP-

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gated K^+ channel. Furthermore, the CSE activities have been reported to be altered under disease conditions; the activity in the liver is reduced in patients with liver cirrhosis and in those exposed to surgical insults or acquired immune deficiency syndrome (8, 10, 20). On the other hand, experimental models of vitamin B_6 deficiency or streptozotocin-induced diabetes revealed alterations in CSE in the liver under these disease conditions (6, 15). Until now, however, effects of such alterations in the activities on organ functions and roles of H_2S under these circumstances have not been fully investigated yet.

This study was designed to focus first on differences in contribution of CSE to tissue H_2S generation; the data indicated that the liver constitutes one of the largest organ components for the gas generation in the body. Based on this result, we further attempted to examine if H_2S derived from the enzyme could play a role in the regulation of hepatobiliary function. The current results first provided evidence that the liver utilizes this gaseous substance as a modulatory determinant of biliary bicarbonate excretion.

MATERIALS AND METHODS

In vivo *and* ex vivo *determination of bile constituents*

The experimental protocols herein described were approved by our institutional guidelines provided by the Animal Care Committee of Keio University School of Medicine. Male Wistar rats weighing 220–260 g (CLEA Japan, Tokyo, Japan) were allowed free access to laboratory chow and tap water, and were fasted for 24 h prior to experiments. As described elsewhere, rats were anesthetized with an intramuscular injection of pentobarbital sodium at 50 mg/kg, and their common bile ducts were cannulated to collect bile samples. Bile output was monitored *in vivo* according to our previous method (7). When necessary, livers of these rats were perfused *ex vivo* with the oxygenated Krebs–Henseleit buffer at a constant flow rate of 4 ml/min/g of liver in a single-pass mode (14). Bile samples collected through a cannulation were used to determine concentrations of total bile salts, phospholipids, pH values, and bicarbonate (HCO_3^-) according to previous methods described elsewhere (7, 14).

Experimental protocols

Propargylglycine (PPG) was used as a potent inhibitor of CSE. PPG was dissolved in physiological saline as a vehicle and administered intraperitoneally at a dose of 300 mmol/g of body weight at 4 h prior to the preparation for bile duct cannulation. Bile was collected every 10 min until the end of experiments according to our previous method (7). In the case of experiments using the *ex vivo* perfused preparation, livers were excised from the PPG-treated rats and perfused with the Krebs–Henseleit buffer containing 100 µmol/L PPG to avoid a possible reduction of the enzyme blockade due to elimination of the reagent from the system. To examine effects of the intraperitoneal injection of the CSE inhibitor on endogenous H_2S generation, we determined tissue contents of the gas *in vivo*. Livers were excised and snap-frozen at 4 h after the treatment with PPG or vehicle, and the samples were minced with 4% tetracholic acid to remove proteins. Amounts of H_2S in the liver tissues were determined by gas chromatography according to previous methods described elsewhere (4). In separate sets of experiments, bile output was monitored every 10 min after establishment of the bile duct cannulation, and concentrations and fluxes of bile constituents were compared between the control and PPG-treated groups. To examine if effects of PPG are attributable to a reduction of the reaction product of CSE such as H_2S, we examined effects of supplementation of NaHS, a soluble donor of the gas at desired concentrations, in the buffer for the *ex vivo* perfusion system. As a control set of the experiments, we compared effects of the same concentrations of *N*-acetylcysteine (NAC), a cysteine donor. In experiments using isolated *ex vivo* perfused livers, sodium taurocholate was added to the buffer at desired concentrations in a range between 0 and 30 µmol/L. Using data collected from these experiments, the bile acid-independent fraction of bile output was determined by plotting bile output as a function of biliary output of bile salts in the samples: the value of the output at the *y*-intercept (zero concentration of bile salts) was regarded as the bile acid-independent fraction (2).

Immunohistochemistry

Liver tissues also served as samples for immunohistochemistry. An anti-CSE antibody was prepared by immunization to chicken of the C-terminal peptide CYGGTNRYFRRVASE, the sequence of which is identical to that of the rat enzyme. The antibody was purified from the antiserum using affinity chromatography as described elsewhere (3). The specificity of the antibody was confirmed by western blot analyses. For immunohistochemistry, rat livers were removed to prepare OCT compound-embedded frozen sections (7 µm). The sections were immunostained with the anti-CSE antibody using the Vectastain ABC kit (Vector Laboratories), as previously described (5). Semiserial sections were stained with the anti-CSE antibody or with the anti-rat keratin 19 monoclonal antibody (MAB1675; Chemicon, Temecula, CA, U.S.A.) to examine colocalization of the enzyme with biliary epithelium and hepatocellular bile canaliculi, when necessary.

Statistical analyses

The statistical significance of data among different experimental groups was determined by one-way ANOVA and Fisher's multiple comparison test. $p < 0.05$ was considered significant.

RESULTS

Liver constitutes the largest organ component for CSE-derived H_2S generation

Figure 1 illustrates tissue contents of H_2S in different organs. The control liver treated with vehicle contained ~80 µmol/g of tissue of the gas (Fig. 1A). Livers from rats pretreated with 1.5 mg/kg PPG, an inhibitor of CSE, suppressed the constitutive levels of the gas by 50%. The dose of PPG used in this experiment appeared to be sufficient enough to block the enzyme, as indicated by dose responses of the H_2S contents as a function of doses of the inhibitor (Fig. 1B). When the tissue gas

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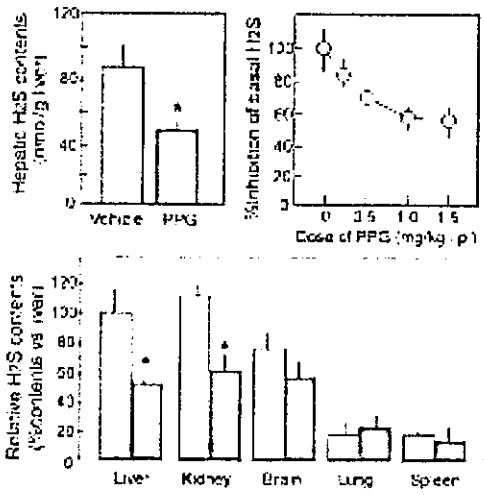

FIG. 1. Effects of administration of PPG, an inhibitor of CSE, on tissue contents of H_2S *in vivo*. (**A**) The effects of the PPG administration on hepatic H_2S contents. PPG was intraperitoneally injected at 1.5 mg/kg at 4 h prior to the experiments. Data indicate means ± SE of more than eight separate experiments. $^*p < 0.05$ as compared with the vehicle-treated control group. (**B**) Dose-dependent effects of PPG on the basal H_2S contents in rat livers. (**C**) Differences in the sensitivity to PPG administration among organs. Open and filled bars represent the tissue H_2S contents in the vehicle- and PPG-treated groups, respectively. Data indicate means ± SE of four separate experiments. $^*p < 0.05$ as compared with the vehicle-treated control group.

contents were compared among different organs (Fig. 1C), liver appeared to constitute the largest organ component for endogenous H_2S production; the level was comparable to that measured in the kidney and 1.5-fold greater than that in the brain. So far as judged by sensitivity to PPG, the gas generation in the liver and kidney depended largely on CSE, whereas that in other organs, such as brain, lung, and spleen, seemed CSE-independent; the finding is consistent with previous observations in mouse brain tissues where CBS constitutes a major source for the gas generation (1).

CSE-derived H_2S is a determinant of the basal bile output and biliary HCO_3^- excretion

Figure 2 demonstrates protein expression of CSE in rat liver tissues. Western blot analyses indicated that the purified polyclonal antibody used in this study specifically recognized the enzyme at 40 kDa (Fig. 2A). Immunohistochemistry using the same antibody revealed that the most intense reactivities were seen in periductal regions of portal triads, whereas walls of hepatic arterial walls and terminal portal veins displayed little reactivities, if any. In addition, a modest expression of CSE was notable in hepatocytes, indicating intralobular homogeneity in its expression (Fig. 2B), whereas nonspecific IgG did not stain the slice (Fig. 2C). Figure 2D and E illustrates semiserial sections stained with the anti-CSE and anti-keratin 19 antibodies, respectively. As seen, cytokelatin-positive ductular structures connecting to bile canalicular networks near the portal triad exhibited notable CSE expression, whereas an artery adjacent to the portal vessel did not display evident immunoreactivities. The staining disappeared when the anti-CSE antibody was absorbed by adding the antigen peptide (data not shown).

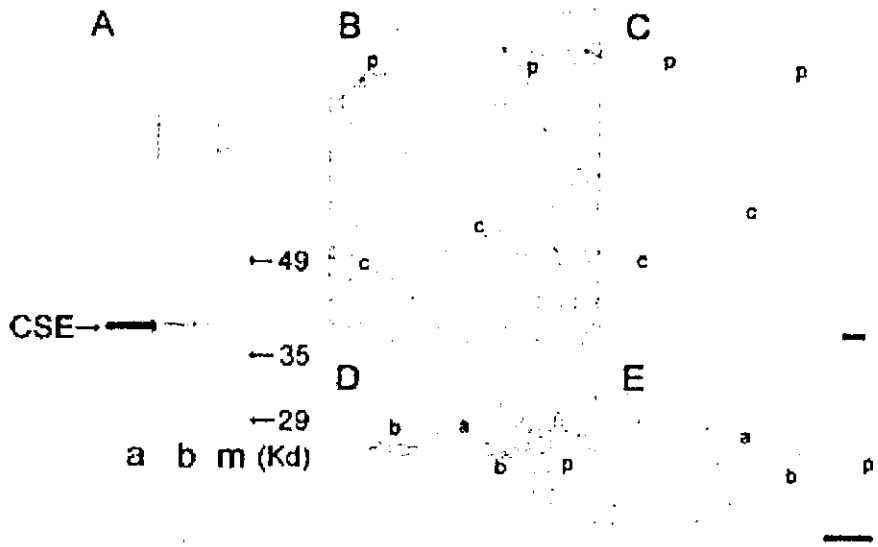

FIG. 2. Expression of CSE in the rat liver. (**A**) western blot analyses using the anti-rat CSE antiserum (lane a) and the affinity column-purified antibody (lane b). m: molecular markers. Note a single band in lane b. (**B** and **C**) Intralobular distribution of CSE in the rat liver stained with the purified anti-CSE antibody and with nonspecific chicken IgG, respectively. p and c: portal and central venules. (**D** and **E**) High magnification of a representative slice stained with the anti-CSE antibody and with the anti-keratin 19 antibody, respectively. a and b: artery and biliary duct. Bars = 50 μmm.

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TABLE 1. EFFECTS OF BLOCKADE OF CSE BY PPG ON BASAL BILE OUTPUT AND BILIARY HCO_3^- EXCRETION

Groups	*Basal bile output (μl/min/g of liver)*	*Biliary HCO_3^- concentration (mmol/L)*
Vehicle ($n = 6$)	1.73 ± 0.09	27.9 ± 1.2
PPG ($n = 6$)	2.11 ± 0.05*	33.0 ± 0.7*

*$p < 0.05$ as compared with the vehicle-treated control group.

We determined the effects of systemic administration of PPG on bile output and biliary constituents *in vivo* according to the identical protocol used in Fig. 1. As shown in Table 1, the PPG administration significantly stimulated basal bile output by 15%. The biliary concentration of HCO_3^- was also significantly elevated in the PPG-treated group. As PPG inhibits CSE and could not only reduce endogenous H_2S, but also modify cysteine metabolism, it is necessary to examine the direct effects of exogenous H_2S administration on hepatobiliary function. However, such experiments were difficult, because the administration of NaHS, the H_2S-donating reagent, is known to change systemic blood pressure *in vivo* through its vasorelaxing action (22). We thus used livers perfused *ex vivo* with the taurocholate-free Krebs solution to prove roles of CSE-derived H_2S in the basal bile excretion.

As illustrated in Fig. 3, the hepatic vascular resistance was comparable among four groups tested (*e.g.*, vehicle, PPG, PPG + NaHS, and PPG + NAC). Under these circumstances, the basal bile output was significantly elevated by 20% in perfused livers of the PPG-treated rats as compared with those treated with vehicle. This response was slightly greater than that observed in the experiments *in vivo* (Table 1), presumably because the perfusion of the organ was carried out under cholate-free conditions, as discussed later in Results. The choleretic response elicited by the PPG treatment was repressed by coperfusion of NaHS at 30 μmol/L, the concentration being comparable to the PPG-sensitive fraction of the gas generation. On the other hand, coperfusion of the same concentration of NAC, a reagent entering cells to yield cysteine, did not alter the CSE-elicited choleretic response. Like the aforementioned observations *in vivo* (Table 1), the PPG treatment significantly enhanced biliary HCO_3^- concentrations, and coperfusion of 30 μmol/L NaHS completely attenuated the changes in the perfused rat livers. On the other hand, the NAC coperfusion did not repress the PPG-induced elevation of the HCO_3^- concentration (Fig. 3B).

AU: OK? Discussion?

As HCO_3^- serves as a putative constituent yielding the driving force for bile formation, we determined if the bile acid-independent bile formation is elevated in livers of the PPG-treated groups. As seen in Fig. 4, where the output was plotted as a function of biliary fluxes of bile salts, the *y*-intercept of the line for the PPG-pretreated groups became markedly decreased and dissociated from that for the control groups. The difference between the two groups became smaller with increasing fluxes of bile salts, but the difference was still evident when the flux of bile salts reached the physiologic levels (70 nmol/min/g of liver). Such a dependency of the PPG effect on bile salts was consistent with the current data indicating differences in the choleretic responses between *in vivo*

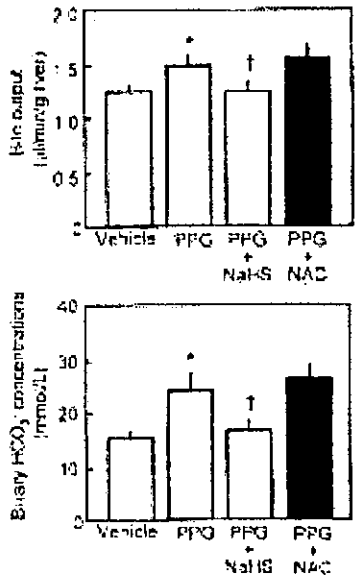

FIG. 3. Effects of the CSE blockade by PPG and supplementation of NaHS on the basal bile output and biliary HCO_3^- concentration in *ex vivo* perfused rat livers. PPG at 1.5 mg/kg was administered *in vivo* intraperitoneally at 4 h prior to the isolation of the perfused liver. Either NaHS or NAC was perfused *ex vivo* into the liver at a concentration of 30 μmol/L, when necessary. Data indicate means ± SE of seven to nine separate experiments in each group. *$p < 0.05$ as compared with the vehicle-treated group; †$p < 0.05$ versus the PPG-treated group.

(Table 1) and *ex vivo* (Fig. 3) perfused livers. We further investigated whether biliary output of glutathione, another major constituent for bile acid-independent bile formation, could also be elevated under the blockade of CSE. As seen in Fig. 5, total amounts of glutathione excreted into bile was comparable among the three groups, suggesting that this constituent plays little role in generation of the osmotic driving force, if any. Interestingly, the ratio between reduced and oxidized forms of glutathione (GSH/GSSG) was significantly elevated by the CSE blockade with PPG. Moreover, the PPG-induced elevation of GSH/GSSG in bile was further elevated with coperfusion with 30 mmol/L NaHS. As one might expect, the PPG pretreatment significantly caused a reduction of total glutathione presumably through inhibition of the transsulfuration pathway. The PPG-elicited decrease in hepatic glutathione contents was unchanged upon administration of NaHS, suggesting that the event is not mediated by endogenous H_2S. Among the three groups, >90% of glutathione was present as the reduced form (data not shown). These results suggest that suppression of CSE-derived H_2S accelerates biliary excretion of GSH, whereas its hepatic contents are reduced. Moreover, exogenous supplementation of the gas under the CSE blockade further increases its excretion into bile. Physiologic implications of this phenomenon will be mentioned later in the Discussion. Collectively, the present results suggest that H_2S endogenously generated

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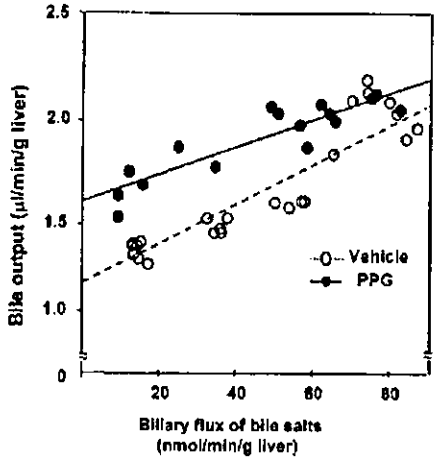

FIG. 4. Alterations in bile salt-independent fraction of bile output by the blockade of CSE by PPG. PPG at 1.5 mg/kg was administered *in vivo* intraperitoneally at 4 h prior to the isolation of the perfused liver. Note the significant elevation ($p < 0.05$) of the *y*-intercept by the PPG treatment, and the difference in the basal bile output between the two groups becomes smaller with increased excretion of bile salts in bile.

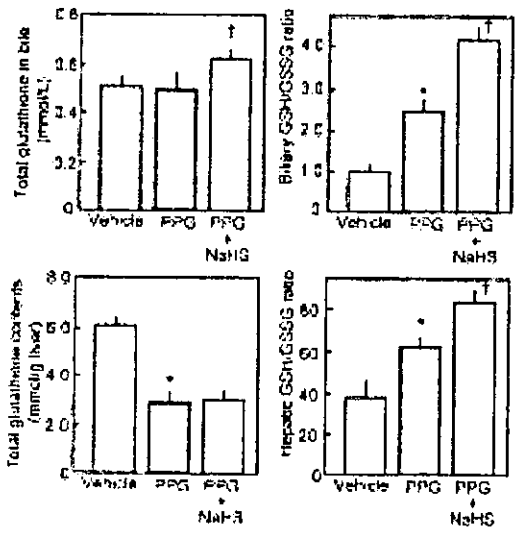

FIG. 5. Effects of the CSE blockade by PPG and supplementation of 30 mmol/L NaHS on biliary excretion and hepatic contents of glutathione in perfused rat livers. PPG at 1.5 mg/kg was administered *in vivo* intraperitoneally at 4 h prior to the isolation of the perfused liver. Data indicate means ± SE of seven to nine separate experiments in each group. $^*p <$ 0.05 as compared with the vehicle-treated group; $^\dagger p < 0.05$ versus the PPG-treated group.

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by CSE modulates the basal excretion of HCO_3^- in bile, playing a role in the regulation of the basal bile output through mechanisms dependent on bile acid-independent choleresis.

DISCUSSION

The present study first provided evidence for the presence of considerable amounts of H_2S in the liver. Furthermore, the gas appeared to serve as an endogenous modulator of the basal bile formation in the liver. Mechanisms for regulation of the basal bile formation involve the bile acid-independent process rather than bile acid-dependent one. Several lines of the current data support this concept: First, the effect of blockade of CSE, the enzyme producing ~50% of the basal H_2S generation, causes an increase in the bile acid-independent bile output *ex vivo* and *in vivo*. Second, as judged from data from *ex vivo* perfused livers, the difference in the excretion between PPG-treated and -untreated groups becomes increased as the biliary excretion of bile salts is reduced (Fig. 4), suggesting that the bile acid-independent fraction plays a major role. Thirdly and most importantly, between the two major biliary constituents for this fraction, HCO_3^-, but not glutathione, is elevated upon the CSE blockade and repressed by supplementation with H_2S, indicating that the former is attributed to generating the driving force for the bile formation. These results collectively suggest that stimulation of HCO_3^- plays an important role in the bile acid-independent choleresis elicited by suppression of CSE-derived H_2S generation.

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As seen in alterations in hepatic and biliary contents of glutathione, PPG not only suppressed CSE-derived H_2S, but also reduced the glutathione contents. As the decrease in the hepatic glutathione contents was not restored by supplementation of NaHS, this event is not mediated by the gas, but occurs as a consequence of CSE-dependent transsulfuration processes. Of interest is that biliary excretion of total glutathione [reduced (GSH) and oxidized (GSSG) forms of glutathione] was unchanged despite the reduction in their hepatic contents. Furthermore, the relative amounts of GSH in bile were increased with supplementation of NaHS. Considering biochemical properties of the gas as a potent reductant with small molecular weight, this result raised a possibility that exogenously administered H_2S is utilized to increase reducing equivalents for GSH in bile. Several possibilities should be taken into account for mechanisms by which H_2S increases the ratio of GSH/GSSG in bile: First, the blockade of CSE by PPG could inhibit the conversion of cysteine into H_2S and thereby save this amino acid for the glutathione synthesis even when the supply of the substrate from the transsulfuration pathway is inhibited. Secondly, H_2S could be used directly as a reducing equivalent to increase GSH in bile. Thus, the role of CSE-mediated conversion of cysteine into H_2S for a fail-safe mechanism to maintain the reducing equivalent deserves further studies to provide evidence that the gas serves as a novel endogenous reductant.

Among gaseous substances detected in mammalian tissues, H_2S has recently been suggested to account for a novel neurovascular transmitter, although receptor mechanisms for the gas signal transduction remain largely unknown. The current results first suggest that the liver could have the ability to ex-

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ecute remodeling of HCO_3^- excretion and increase the basal bile formation when exposed to disease conditions causing a decrease in the enzyme activity; such circumstances involve cirrhosis and surgical insults as previously reported both experimentally and clinically (8, 20). When considering effects of other gaseous mediators on the quality control of bile excretion, which were previously reported from our laboratory and other, it is not unreasonable to hypothesize that the liver could utilize multiple gases to regulate biliary function under physiologic and pathologic conditions. In the rat model of endotoxemia, nitric oxide (NO) suppresses oxidative phosphorylation via blockade of mitochondrial cytochrome *c* oxidase, and thereby down-regulates bile acid-dependent bile formation (17, 19). Although mechanisms for transcriptional regulation of the CSE expression remain largely unknown, previous studies revealed that exposure to excess NO caused up-regulation of the CSE expression in aortic tissues and increased endogenous generation of H_2S to modulate the vascular tone. As shown in the current study, the excess dose of exogenous NaHS supplementation reduced the basal bile output, suggesting that H_2S causes cholestasis with its excess amounts. In this context, quantitative determination of these two gases in the endotoxemic liver deserves further studies provided that the functional link of their overproduction to biliary function can be demonstrated.

On the other hand, the current results together with our previous data collectively suggest that a reduction of H_2S and an increase in carbon monoxide (CO) share common roles in the regulation of bile formation in that both events stimulate excretion of bile constituents besides bile salts. CO at micromolar levels not only modulates sinusoidal tone (18), but also has the ability to induce choleresis and to stimulate biliary excretion of major organic anions such as glutathione and bilirubin-IXα through mechanisms involving multidrug resistance protein 2 (13). Such effects of CO on biliary excretion occur in a concentration-specific manner, and excess concentrations of the gas repress the choleretic response and lead to cholestasis through the increase in paracellular junctional permeability and suppression of bile canalicular contractility (11, 16). In contrast to CSE, heme oxygenase-1 is up-regulated by surgical insults or by liver cirrhosis, and the parenchyma is exposed to high concentrations of CO (9, 12, 21). Thus, under disease conditions, overproduced CO and reduced H_2S could cooperatively increase the bile acid-independent fraction of bile output through increased excretion of organic anions and HCO_3^-, respectively. Although physiologic implication of the current observations remains to be fully understood, such remodeling of a quality of bile could benefit the increasing solubility of organic anions or protect against cholestasis possibly occurring under the aforementioned disease conditions. Further investigation is necessary to examine if alterations of these gases could regulate a quality of bile cooperatively with modulation of H_2S generation under a variety of hepatobiliary disease conditions.

ACKNOWLEDGMENTS

This study was supported by the 21st Century Center-of-Excellence Program and the Leading Project for Biosimulation, and partly supported by Grant-in-Aid for Creative Science Research 13GS0015 from the Ministry of Education, Sciences and Technology of Japan, as well as by Advanced Medical Technology in Health Sciences Research Grants from Ministry of Health and Welfare in Japan.

ABBREVIATIONS

CBS, cystathionine β-synthase; CO, carbon monoxide; CSE, cystathionine γ-lyase; GSH, reduced form of glutathione; GSSG, oxidized form of glutathione; HCO_3^-, bicarbonate; H_2S, hydrogen sulfide; NAC, *N*-acetylcysteine; NO, nitric oxide; PPG, propargylglycine.

REFERENCES

1. Abe K and Kimura H. The possible role of hydrogen sulfide as an endogenous neuromodulator. *J Neurosci* 16: 1066–1071, 1996.
2. Boyer JL and Klatskin G. Canalicular bile flow and bile secretory pressure. Evidence for a non-bile salt dependent fraction in the isolated perfused rat liver. *Gastroenterology* 6: 853–859, 1970.
3. Coleman PL, Walker MM, Milbrath DS, and Stauffer DS. Immobilization of protein A at high density on azlactone-functional polymeric beads and their use in affinity chromatography. *J Chromatogr* 512: 345–363, 1990.
4. Eto K, Ogasawara M, Umemura K, Nagai Y, and Kimura H. Hydrogen sulfide is produced in response to neuronal excitation. *J Neurosci* 22: 3386–3391, 2002.
5. Goda N, Suzuki K, Naito M, Takeoka S, Tsuchida E, Ishimura Y, Tamatani T, and Suematsu M. Distribution of heme oxygenase isoforms in rat liver. Topographic basis for carbon monoxide-mediated microvascular relaxation. *J Clin Invest* 101: 604–612, 1998.
6. Jacobs RL, House JD, Brosnan ME, and Brosnan JT. Effects of streptozotocin-induced diabetes and of insulin treatment on homocysteine metabolism in the rat. *Diabetes* 47: 1967–1970, 1998.
7. Kyokane T, Norimizu S, Taniai H, Yamaguchi T, Takeoka S, Tsuchida E, Naito M, Nimura Y, Ishimura Y, and Suematsu M. Carbon monoxide from heme catabolism protects against hepatobiliary dysfunction in endotoxin-treated rat liver. *Gastroenterology* 120: 1227–1240, 2001.
8. Look MP, Riezler R, Reichel C, Brensing KA, Rockstroh JK, Stabler SP, Spengler U, Berthold HK, and Sauerbruch T. Is the increase in serum cystathionine levels in patients with liver cirrhosis a consequence of impaired homocysteine transsulfuration at the level of gamma-cystathionase? *Scand J Gastroenterol* 35: 866–872, 2000.
9. Makino N, Suematsu M, Sugiura Y, Morikawa H, Shiomi S, Goda N, Sano T, Nimura Y, Sugimachi K, and Ishimura Y. Altered expression of heme oxygenase-1 in the livers of patients with portal hypertensive diseases. *Hepatology* 33: 32–42, 2001.
10. Martin JA, Sastre J, de la Asuncion JG, Pallardo FV, and Vina J. Hepatic γ-cystathionase deficiency in patients with AIDS. *JAMA* 285: 1444–1445, 2001.

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11. Mori M, Suematsu M, Kyokane T, Sano T, Suzuki H, Yamaguchi T, Ishimura Y, and Ishii H. Carbon monoxide-mediated alterations in paracellular permeability and vesicular transport in acetaminophen-treated perfused rat liver. *Hepatology* 30: 160–168, 1999.
12. Motterlini R, Gonzales A, Foresti R, Clark JE, Green CJ, and Winslow RM. Heme oxygenase-1-derived carbon monoxide contributes to the suppression of acute hypertensive responses in vivo. *Circ Res* 83: 568–577, 1998.
13. Norimizu S, Kudo A, Kajimura M, Ishikawa K, Taniai H, Yamaguchi T, Fujii K, Arii S, Nimura Y, and Suematsu M. Carbon monoxide stimulates mrp2-dependent excretion of bilirubin-IXα into bile in the perfused rat liver. *Antioxid Redox Signal* 5: 449–456, 2003.
14. Sano T, Shiomi M, Wakabayashi Y, Shinoda Y, Goda N, Yamaguchi T, Nimura Y, Ishimura Y, and Suematsu M. Endogenous carbon monoxide suppression stimulates bile acid-dependent biliary transport in perfused rat liver. *Am J Physiol* 272(5 Pt 1): G1268–G1275, 1997.
15. Sato A, Nishioka M, Awata S, Nakayama K, Okada M, Horiuchi S, Okabe N, Sassa T, Oka T, and Natori Y. Vitamin B6 deficiency accelerates metabolic turnover of cystathionase in rat liver. *Arch Biochem Biophys* 330: 409–413, 1996.
16. Shinoda Y, Suematsu M, Wakabayashi Y, Suzuki T, Goda N, Saito S, Yamaguchi T, and Ishimura Y. Carbon monoxide as a regulator of bile canalicular contractility in cultured rat hepatocytes. *Hepatology* 28: 286–295, 1998.
17. Shiomi M, Wakabayashi Y, Sano T, Shinoda Y, Nimura Y, Ishimura Y, and Suematsu M. Nitric oxide suppression reversibly attenuates mitochondrial dysfunction and cholestasis in endotoxemic rat liver. *Hepatology* 27: 108–115, 1998.
18. Suematsu M, Goda N, Sano T, Kashiwagi S, Egawa T, Shinoda Y, and Ishimura Y. Carbon monoxide: an endogenous modulator of sinusoidal tone in the perfused rat liver. *J Clin Invest* 96: 2431–2437, 1995.
19. Trauner M, Nathanson MH, Rydberg SA, Koeppel TA, Gartung C, Sessa WC, and Boyer JL. Endotoxin impairs biliary glutathione and HCO_3^- excretion and blocks the choleretic effect of nitric oxide in rat liver. *Hepatology* 25: 1184–1191, 1997.
20. Vina BJ, Gimenez A, Puertes IR, Gasco E, and Vina JR. Impairment of cysteine synthesis from methionine in rats exposed to surgical stress. *Br J Nutr* 68: 421–429, 1992.
21. Wakabayashi Y, Takamiya R, Mizuki A, Kyokane T, Goda N, Yamaguchi T, Takeoka S, Tsuchida E, Suematsu M, and Ishimura Y. Carbon monoxide overproduced by heme oxygenase-1 causes a reduction of vascular resistance in perfused rat liver. *Am J Physiol* 277(5 Pt 1): G1088–G1096, 1999.
22. Zhao W, Zhang J, Lu Y, and Wang R. The vasorelaxant effect of H_2S as a novel endogenous gaseous K_{ATP} channel opener. *EMBO J* 20: 6008–6016, 2001.

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Received for publication December 3, 2004; accepted December 10, 2004.

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ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 5 & 6, 2005
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Forum Original Research Communication

Cadmium Exposure Alters Metabolomics of Sulfur-Containing Amino Acids in Rat Testes

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ABSTRACT

This study aimed to examine distribution of cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), the hydrogen sulfide (H_2S)-generating enzymes, and metabolomic alterations in sulfur-containing amino acids in rat testes exposed to stressors. Immunohistochemistry revealed distinct distribution of the two enzymes: CBS occurred mainly in Leydig cells and was also detectable in germ cells, whereas CSE was evident in Sertoli cells and immature germ cells involving spermatogonia. The amounts of CSE and CBS in testes did not alter in response to administration of cadmium chloride, an antispermatogenic stressor leading to apoptosis. Metabolome analyses assisted by liquid chromatography equipped with mass spectrometry revealed marked alterations in sulfur-containing amino acid metabolism: amounts of methionine and cysteine were significantly elevated concurrently with a decrease in the ratio between *S*-adenosylhomocysteine and *S*-adenosylmethionine, suggesting expansion of the remethylation cycle and acceleration of methyl donation. Despite a marked increase in cysteine, amounts of H_2S were unchanged, leading to a remarkable decline of the H_2S/cysteine ratio in the cadmium-treated rats. Under such circumstances, oxidized glutathione (GSSG) was significantly reduced, whereas reduced glutathione (GSH) was well maintained, and the GSH/GSSG ratio was consequently elevated. These results collectively showed that cadmium induces metabolomic remodeling of sulfur-containing amino acids even when the protein expression of CBS or CSE is not evident. Although detailed mechanisms for such a remodeling event remain unknown, our study suggests that metabolomic analyses serve as a powerful tool to pinpoint a critical enzymatic reaction that regulates metabolic systems as a whole. *Antioxid. Redox Signal.* 7, 781–787.

INTRODUCTION

TESTIS is an organ characterized by active utilization of sulfur-containing amino acids. Metabolites derived from sulfur-containing amino acids have been shown to contribute to detoxification against noxious stressors, as well as to maturation of testicular germ cells, through multiple mechanisms. Cysteine metabolism plays a central role in such mechanisms (3, 8, 9, 14, 26). This amino acid serves as a substrate for synthesis of glutathione through reactions of glutamate ligase and glutathione synthase, and is also used to generate sulfate through aspartate transferase and sulfite oxidase. Another important substance generated upon cysteine metabolism *in vivo* is hydrogen sulfide (H_2S). This gaseous compound has recently been shown to account for a signaling molecule in neural and vascular systems (7, 10, 12, 13, 32). It is produced mainly by two types of pyridoxal 5′-phosphate-dependent enzymes responsible for metabolism of L-cysteine: cystathionine γ-lyase (CSE; EC 4.4.1.1) and cystathionine β-synthase (CBS; EC 4.2.1.22) (1, 2, 15, 30). Although the primary role of the two

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enzymes is to constitute the transsulfuration pathway that utilizes homocysteine to synthesize cysteine, both CSE and CBS are able to use cysteine as the substrate to generate H_2S (10, 13). Among the aforementioned amino acid derivatives, glutathione plays a central role in the regulation of spermatogenesis. Male mice deficient in γ-glutamyltranspeptidase exhibit testicular atrophy concurrently with oligozoospermia (16). The fluid excreted from seminiferous tubules contains ample glutathione *S*-transferase, which contributes to the transport of testosterone into the fluid. The excretion of the protein is primary supported by Sertoli cells (21). Roles of methionine metabolism in maturation of testicular germ cells have been examined extensively; *S*-adenosylmethionine (SAM) decarboxylase constitutes a major pathway for biosynthesis of polyamines, which is essential for maturation of Sertoli cells and germ cells (26).

Although these lines of information suggest active utilization of remethylation and transsulfuration pathways in testes, the whole picture of metabolic remodeling in these biochemical pathways has not been fully investigated under stress conditions. Furthermore, the distribution of the enzymes responsible for metabolism of methionine and cysteine has been largely unknown in the testis at present. Furthermore, effects of exposure to stressors causing oligozoospermia on functional outcome of metabolic remodeling in the sulfur-containing amino acids and H_2S have not fully been examined. This study was designed to investigate distribution of CBS and CSE and to examine stress-induced metabolic responses of sulfur-containing amino acids and their derivatives in this organ.

MATERIALS AND METHODS

Establishment of polyclonal antibodies against rat CBS and CSE

To generate polyclonal antibodies against rat CSE, polypeptides for the C-terminus of each enzyme were synthesized as immunoantigens and injected into rabbits. The peptide used for immunization to obtain the anti-CSE antibody was VYGGTNRYFRRVASE (1, 2). We also used a polyclonal antibody against CBS, which was prepared in previous studies (13). The antiserum for CSE was purified by affinity purification using a commercially available kit (UltraLink Immunomobilization kit, Pierce, Rockford, IL, U.S.A.). For western blot analyses, rat tissues were collected from testes and livers, homogenized in lysing buffer (50 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg of pepstatin, 5 μg/ml leupeptin), and subjected to western blot analyses as described previously (6, 22).

Immunohistochemistry

Male Wistar rats were fasted overnight and anesthetized with an intramuscular injection of 50 mg/kg pentobarbital sodium. The testes of rats treated with an intraperitoneal injection of $CdCl_2$ at a dose of 20 μg/kg or with vehicle were removed and fixed for 4 h at 4°C in periodate–lysine–paraformaldehyde solution as described previously (6, 22). The samples were washed sequentially for 4 h with phosphate-buffered saline (PBS) containing 10, 15, and 20% sucrose, embedded in OCT compound and processed for preparing 4-mm slices to apply anti-CBS or -CSE antibody at a final concentration of 1 μg/ml at 4°C. After several washes with PBS, the sections were stained with a biotinylated anti-rabbit IgG for 1 h (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA, U.S.A.). To inhibit endogenous peroxidase reactions, the samples were pretreated with 0.3% hydrogen peroxide in cold methanol for 30 min and subsequently incubated with avidin and horseradish peroxidase-conjugated biotin for 30 min. Finally, 0.1 mg/ml diaminobenzidine tetrahydrochloride was applied to sections for 3 min. The sections were counterstained with methyl green after fixation with 20% formaldehyde for 20 min. In some experiments, the antibodies preabsorbed with an excess of antigens in advance were applied for immunohistochemistry as negative controls.

In separate sets of experiments, sections of testes were double-stained by a method using diaminobenzidine and nickel chloride according to our previous method (6, 22) to examine cell types expressing CBS or CSE. To this end, we applied the antibody against adrenal 4 binding protein (Ad4BP), an intranuclear DNA-binding protein expressed in steroidogenic cells (a gift from Professor Kenichiro Morohashi, National Institute of Molecular Biology, Okazaki, Japan). As described previously (21), this antibody allowed us to stain nuclei of Leydig cells and Sertoli cells. Leydig cells are located in the interstitial space of seminiferous tubules, whereas Sertoli cells stand in the distal basement region of the tubules. Because of such anatomical topography of these cells, the Ad4BP staining led us to distinguish easily Sertoli cells from Leydig cells and also from testicular germ cells in the tubules (22). By this protocol, cells reacting only with the initial primary antibody were stained light brown, whereas those reacting with the second primary antibody were stained bright purple. When reacting with both primary antibodies simultaneously, cells were identified as those stained dark brown.

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Sulfur-containing amino acid metabolome analyses in testes of $CdCl_2$-treated rats

Amounts of H_2S in tissues were determined by gas chromatography according to previous methods described elsewhere (12, 13). To determine amounts of metabolites in remethylation and transsulfuration pathways, HPLC was used with three different detection systems. Approximately 1 g of the testes was homogenized in 10 ml of 10% trichloroacetic acid (TCA; Sigma, Inc., St. Louis, MO, U.S.A.) containing 1 m*M* EDTA. The homogenate was then centrifuged at 15,000 rpm for 15 min at 4°C, and that supernatant was collected and stored at −80°C. Amounts of cysteine were determined by HPLC with fluorimetric detection and isocratic elution. The last step was derivatization with 7-fluorobenzene-2-oxy-1,3-diazolic-4-ammonium sulfate (SBD-F; Wako, Inc., Tokyo, Japan) (0.3 g/L 500 m*M* potassium borate, pH 11.5) at 60°C for 60 min. The HPLC system (Shimazu, Kyoto, Japan) was used with a SIL-10Advp automatic sample injector and an RF-10AXL fluorescence detector. Chromatographic separation was performed on an ODS column (C18, 250 × 4.6 mm) using 0.1 *M* potassium dihydrogen phosphate as mobile phase at a flow

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rate of 1 ml/min and a column temperature of 30°C. The fluorescence of the separated compounds was detected with a detector adjusted for excitation at 385 nm and emission at 515 nm. Amounts of the compounds were calculated with a calibration curve established by measurements of known concentrations of the standard compounds (4, 20). Contents of methionine, SAM, *S*-adenosylhomocysteine (SAH), cystathionine, serine, and taurine were determined by a liquid chromatography assisted by double mass spectrometry (API 3000 LC-MS/MS) system. TCA-treated hepatic samples were pre-column-derivalizated with a Waters AccQ-Fluor Reagent Kit (Waters, Milford, MA, U.S.A.) to determine these compounds. To determine hepatic SAM levels, hepatic samples were added to 100-fold 100 m*M* ammonium acetate. Chromatographic separation was performed on an Atlantis column (dc18, 2.1 × 150 mm) using 5 m*M* ammonium acetate, acetonitrile as mobile phase at a flow rate of 1 ml/min, and a column temperature of 30°C.

Statistical analyses

Differences in mean values among groups were examined by Fisher's multiple comparison analyses combined with ANOVA. $p < 0.05$ was considered statistically significant.

RESULTS

Immunohistochemical detection of CBS and CSE in rat testes

The specificity of the polyclonal antibody against CBS used in the current study was characterized using rat liver lysates by western blot analysis. As seen in Fig. 1A, a major band was observed at 63 kDa in the liver, suggesting the specificity to use this antibody for immunodetection. The CBS expression was unchanged in the liver of the cadmium-treated rats. When using the lysate derived from testes, CBS was undetectable irrespective of the presence or absence of the cadmium exposure, suggesting a paucity of the protein expression in the whole organ. The anti-CSE antibody used in the current study was identical to that used in our other study (5), and western blot analyses for the liver revealed that the expression of CSE, as recognized at 40 kDa, in the intact liver was unchanged irrespective of the stimulus, such as cadmium. We thus hypothesized that these enzymes could be expressed locally in particular cell types, and attempted to examine the cellular localization immunohistochemically. Figure 1B–D illustrates representative pictures showing localization of CBS in the control and $CdCl_2$-treated rat testes. As seen in low-power images (Fig. 1B), CBS occurred mainly in cells in the interstitial space and in the basement membrane of seminiferous tubules. We then examined if CBS is localized in Leydig cells and Sertoli cells. To distinguish these cells from others, immunostaining with Ad4BP, a nuclear transcriptional factor for steroidogenic cells, was conducted with CBS staining using double immunohistochemistry. As seen in Fig. 1C, Leydig cells in the interstitial space (arrows) expressed this enzyme abundantly. The cells expressing CBS notably in the basement membrane of the tubules were Sertoli cells as characterized by

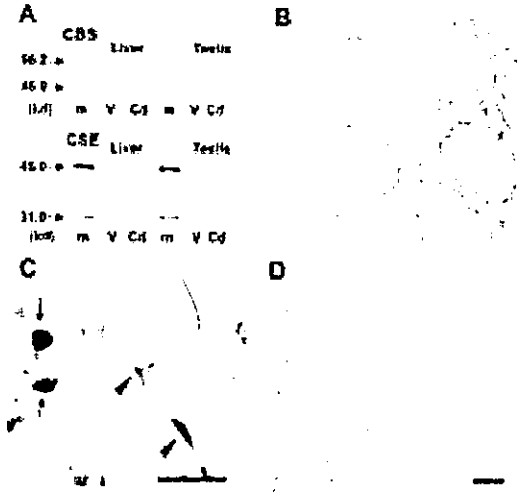

FIG. 1. Characterization of antibodies used in the study and immunohistochemistry illustrating the expression of CBS in rat testes. (**A**) Western blot analysis showing specificity of the anti-CBS and anti-CSE antibodies. CBS and CSE were evident in the liver samples at 63 kDa and 40 kDa, respectively. V, vehicle-treated controls; Cd, cadmium-treated groups; m, molecular markers. (**B**) Immunohistochemical analysis of the topographic distribution of CBS in the rat testis. (**C**) Double immunostaining with Ad4BP (purple) and CBS (brown). Note marked expression of the enzyme in Ad4BP-positive cells in the interstitial space, suggesting the presence of CBS in Leydig cells. (**D**) Effects of administration of $CdCl_2$ on the expression of CBS in rat testes. Note the absence of alterations. Bars = 100 μm.

their shape with cell processes protruding toward the central region of tubules (Fig. 1C). At the same time, immature germ cells adjacent to Sertoli cells express the enzyme modestly. On the other hand, mature germ cells observed in the central region of tubules express the enzyme little, if any (Fig. 1B). Such distribution patterns and immunoreactivities were unchanged in response to exposure to $CdCl_2$, so far as judged at 12 h after the administration (Fig. 1D). These results suggest that testicular CBS is expressed in a cell type-specific manner, resulting in the failure of detection by western blot analyses.

As seen in Fig. 1A and also in our other article in this *Forum* (5), the anti-rat CSE antisera were purified through an affinity column and turned out to be usable for specific detection of the antigen at 40 kDa. Using the same antibody, distribution of CSE was examined in testes. As indicated in Fig. 2A, CSE was abundantly expressed in vascular walls in the interstitial space of testes, as well as in Sertoli cells, which constitute the basement membrane of the tubules. The localization of CSE was also notable in immature germ cells occurring in the marginal regions of the tubules (Fig. 2B). Furthermore, the enzyme expression in the individual cells appeared to be condensed in nuclei, whereas the expression in cytoplasm of these cells was relatively modest. Like that of the CBS expression, the response of the CSE expression was not altered by exposure to $CdCl_2$ (data not shown). Collectively, these results

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