[27] U. Kragh-Hansen, Relations between high-affinity binding sites for L-tryptophan, diazepam, salicylate and phenol red on human serum albumin, Biochem. J. 209 (1983) 135–142.
[28] U. Kragh-Hansen, Relations between high-affinity binding sites of markers for binding regions on human serum albumin, Biochem. J. 225 (1985) 629–638.
[29] U. Kragh-Hansen, Evidence for a large and flexible region of human serum albumin possessing high affinity binding sites for salicylate, warfarin, and other ligands, Mol. Pharmacol. 34 (1988) 160–171.
[30] K. Yamasaki, T. Maruyama, A. Takadate, A. Suenaga, U. Kragh-Hansen, M. Otagiri, Characterization of site I of human serum albumin using spectroscopic analyses: locational relations between regions Ib and Ic of site I, J. Pharm. Sci. 93 (2004) 3004–3012.
[31] L. Zhu, F. Yang, L. Chen, E.J. Meehan, M. Huang, A new drug binding subsite on human serum albumin and drug-drug interaction studied by X-ray crystallography, J. Struct. Biol. 162 (2008) 40–49.
[32] I. Petitpas, A.A. Bhattacharya, S. Twine, M. East, S. Curry, Crystal structure analysis of warfarin binding to human serum albumin: anatomy of drug site I, J. Biol. Chem. 276 (2001) 22804–22809.
[33] S. Wanwimolruk, D.J. Birkett, P.M. Brooks, Structural requirements for drug binding to site II on human serum albumin, Mol. Pharmacol. 24 (1983) 458–463.
[34] T. Itoh, Y. Saura, Y. Tsuda, H. Yamada, Stereoselectivity and enantiomer–enantiomer interactions in the binding of ibuprofen to human serum albumin, Chirality 9 (1997) 643–649.
[35] V.T. Chuang, M. Otagiri, Flunitrazepam, a 7-nitro-1,4-benzodiazepine that is unable to bind to the indole-benzodiazepine site of human serum albumin, Biochim. Biophys. Acta 1546 (2001) 337–345.
[36] A.J. Ryan, C.-W. Chung, S. Curry, Crystallographic analysis reveals the structural basis of the high-affinity binding of iophenoxic acid to human serum albumin, BMC Struct. Biol. 11 (2011) 18.
[37] A.A. Bhattacharya, T. Grüne, S. Curry, Crystallographic analysis reveals common modes of binding of medium and long-chain fatty acids to human serum albumin, J. Mol. Biol. 303 (2000) 721–732.
[38] I. Sjöholm, B. Ekman, A. Kober, I. Ljungstedt-Påhlman, B. Seiving, T. Sjödin, Binding of drugs to human serum albumin: XI. The specificity of three binding sites as studied with albumin immobilized in microparticles, Mol. Pharmacol. 16 (1979) 767–777.
[39] A.A. Bhattacharya, S. Curry, N.P. Franks, Binding of the general anesthetics propofol and halothane to human serum albumin. High resolution crystal structures, J. Biol. Chem. 275 (2000) 38731–38738.
[40] P.A. Zunszain, J. Ghuman, A.F. McDonagh, S. Curry, Crystallographic analysis of human serum albumin complexed with 4Z, 15E-bilirubin-IXα, J. Mol. Biol. 381 (2008) 394–406.
[41] K.L. Hein, U. Kragh-Hansen, J.P. Morth, M.D. Jeppesen, D. Otzen, J.V. Møller, P. Nissen, Crystallographic analysis reveals a unique lidocaine binding site on human serum albumin, J. Struct. Biol. 171 (2010) 353–360.
[42] K. Oettl, R.E. Stauber, Physiological and pathological changes in the redox state of human serum albumin critically influence its binding properties, Br. J. Pharmacol. 151 (2007) 580–590.
[43] R. Narazaki, M. Hamada, K. Harada, M. Otagiri, Covalent binding between bucillamine derivatives and human serum albumin, Pharm. Res. 13 (1996) 1317–1321.
[44] J. Christodoulou, P.J. Sadler, S. Tucker, Gold-induced structural switch of cys34 in albumin: comparison of auranofin with aurothiomalate, Metal-Based Drugs 1 (1994) 527.
[45] D.A. Keire, S.V. Mariappan, J. Peng, D.L. Rabenstein, Nuclear magnetic resonance studies of the binding of captopril and penicillamine by serum albumin, Biochem. Pharmacol. 46 (1993) 1059–1069.
[46] R. Narazaki, K. Harada, A. Sugii, M. Otagiri, Kinetic analysis of the covalent binding of captopril to human serum albumin, J. Pharm. Sci. 86 (1997) 215–219.
[47] C. Bertucci, B. Nanni, A. Raffaelli, P. Salvadori, Chemical modification of human albumin at cys34 by ethacrynic acid: structural characterisation and binding properties, J. Pharm. Biomed. Anal. 18 (1998) 127–136.
[48] A.I. Ivanov, J. Christodoulou, J.A. Parkinson, K.J. Barnham, A. Tucker, J. Woodrow, P.J. Sadler, Cisplatin binding sites on human albumin, J. Biol. Chem. 273 (1998) 14721–14730.
[49] D. Noort, A.G. Hulst, R. Jansen, Covalent binding of nitrogen mustards to the cysteine-34 residue in human serum albumin, Arch. Toxicol. 76 (2002) 83–88.
[50] M.C. Damsten, J.N.M. Commandeur, A. Fidder, A.G. Hulst, D. Touw, D. Noort, N.P.E. Vermeulen, Liquid chromatography/tandem mass spectrometry detection of covalent binding of acetaminophen to human serum albumin, Drug Metab. Dispos. 35 (2007) 1408–1417.
[51] D.A. Joyce, R.O. Day, B.R. Murphy, The pharmacokinetics of albumin conjugates of D-penicillamine in humans, Drug Metab. Dispos. 19 (1991) 309–311.
[52] R. Narazaki, M. Otagiri, Covalent binding of a bucillamine derivative with albumin in sera from healthy subjects and patients with various diseases, Pharm. Res. 14 (1997) 351–353.
[53] M.T. Montero, R. Pouplana, O. Valls, S. García, On the binding of cinmetacin and indomethacin to human serum albumin, J. Pharm. Pharmacol. 38 (1986) 925–927.
[54] N. Takamura, T. Maruyama, E. Chosa, K. Kawai, Y. Tsutsumi, Y. Uryu, K. Yamasaki, T. Deguchi, M. Otagiri, Bucolome, a potent binding inhibitor for furosemide, alters the pharmacokinetics and diuretic effect of furosemide: potential for use of bucolome to restore diuretic response in nephrotic syndrome, Drug Metab. Dispos. 33 (2005) 596–602.
[55] A.H. Anton, Drug-protein binding. IV. Modulation of binding Increasing activity of sulfonamides with displacing agents: a review, Ann. N. Y. Acad. Sci. 226 (1973) 273–292.
[56] R.K. Verbeeck, A. Boel, A. Buntinx, P.J. De Schepper, Plasma protein binding and interaction studies with diflunisal, a new salicylate analgesic, Biochem. Pharmacol. 29 (1980) 571–576.
[57] V.T. Chuang, A. Kuniyasu, H. Nakayama, Y. Matsushita, S. Hirono, M. Otagiri, Helix 6 of subdomain III A of human serum albumin is the region primarily photolabeled by ketoprofen, an arylpropionic acid NSAID containing a benzophenone moiety, Biochim. Biophys. Acta 1434 (1999) 18–30.
[58] B. Honoré, R. Brodersen, Albumin binding of anti-inflammatory drugs Utility of a site-oriented versus a stoichiometric analysis, Mol. Pharmacol. 25 (1984) 137–150.
[59] N. Setoguchi, N. Takamura, K. Fujita, K. Ogata, J. Tokunaga, T. Nishio, E. Chosa, K. Arimori, K. Kawai, R. Yamamoto, A diclofenac suppository-nabumetone combination therapy for arthritic pain relief and a monitoring method for the diclofenac binding capacity of HSA site II in rheumatoid arthritis, Biopharm. Drug Dispos. (2012), http://dx.doi.org/10.1002/bdd.1829.
[60] I. Mignot, N. Presle, F. Lapicque, C. Monot, R. Dropsy, P. Netter, Albumin binding sites for etodolac enantiomers, Chirality 8 (1996) 271–280.
[61] H. Meisner, K. Neet, Competitive binding of long-chain free fatty acids, octanoate, and chlorophenoxyisobutyrate to albumin, Mol. Pharmacol. 14 (1978) 337–346.
[62] P.E. Rolan, L. Court, Plasma protein binding displacement interactions-why are they still regarded as clinically important? Br. J. Clin. Pharmacol. 37 (1994) 125–128.
[63] G. Fanali, A. di Masi, V. Trezza, M. Marino, M. Fasano, P. Ascenzi, Human serum albumin: from bench to bedside, Mol. Aspects Med. 33 (2012) 209–290.
[64] S. Schmidt, D. Gonzalez, H. Derendorf, Significance of protein binding in pharmacokinetics and pharmacodynamics, J. Pharm. Sci. 99 (2010) 1107–1122.
[65] P.F. D'Arcy, J.C. McElnay, Drug interactions involving the displacement of drugs from plasma protein and tissue binding sites, Pharmacol. Ther. 17 (1982) 211–220.
[66] J. Koch-Weser, E.M. Sellers, Drug therapy. Binding of drugs to serum albumin (second of two parts), N. Engl. J. Med. 294 (1976) 526–531.
[67] S.L. Fox, Potentiation of anticoagulants caused by pyrazole compounds, JAMA 188 (1964) 320–321.
[68] P.M. Aggeler, R.A. O'Reilly, L. Leong, P.E. Kowitz, Potentiation of anticoagulant effect of warfarin by phenylbutazone, N. Engl. J. Med. 276 (1967) 496–501.
[69] S. Harder, P. Thürmann, Clinically important drug interactions with anticoagulants. An update, Clin. Pharmacokinet. 30 (1996) 416–444.
[70] Y. Hatakeyama, S. Niwano, H. Niwano, T. Kosukegawa, T. Izumi, Risks and benefits of combined use of bucolome and warfarin in anticoagulation therapy, Int. Heart J. 51 (2010) 399–403.
[71] M. Osawa, N. Hada, K. Matsumoto, T. Hasegawa, D. Kobayashi, Y. Morimoto, M. Yamaguchi, I. Kanamoto, T. Nakagawa, K. Sugibayashi, Usefulness of coadministration of bucolome in warfarin therapy: pharmacokinetic and pharmacodynamic analysis using outpatient prescriptions, Int. J. Pharm. 293 (2005) 43–49.
[72] B.B. Brodie, Displacement of one drug by another from carrier or receptor sites, Proc. R. Soc. Med. 58 (1965) 946–955.
[73] J.P. Tillement, R. Zini, C. Mattei, E. Singlas, Effect of phenylbutazone on the binding of vitamin K antagonists to albumin, Eur. J. Clin. Pharmacol. 6 (1973) 15–18.
[74] H. Abe, M. Sawada, T. Toda, N. Kurosawa, E. Owada, I. Tazawa, S. Sakuma, Evaluation of co-administration of bucolome for warfarin therapy from the view point of protein binding, Jpn. J. Ther. Drug Monit. 12 (1995) 293–298.
[75] M. Matsuoka, Thrombosis therapy, Geriatr. Med. 15 (1977) 505–512.
[76] A. Majima, T. Hosono, K. Kawabe, A new method of anticoagulant therapy used by combination warfarin and bucolome, Niigata Igaku Kai Zasshi 88 (1982) 95–119.
[77] J.O. Miners, D.J. Birkett, Cytochrome P4502C9: an enzyme of major importance in human drug metabolism, Br. J. Clin. Pharmacol. 45 (1998) 525–538.
[78] H. Takahashi, T. Kashima, S. Kimura, N. Murata, T. Takaba, K. Iwade, T. Abe, H. Tainaka, T. Yasumori, a H. Echizen, Pharmacokinetic interaction between warfarin and a uricosuric agent, bucolome: application of in vitro approaches to predicting In vivo reduction of (S)-warfarin clearance, Drug Metab. Dispos. 27 (1999) 1179–1186.
[79] S.M. Pond, D.J. Birkett, D.N. Wade, Mechanisms of inhibition of tolbutamide metabolism: phenylbutazone, oxyphenbutazone, sulfaphenazole, Clin. Pharmacol. Ther. 22 (1977) 573–579.
[80] K. Komatsu, K. Ito, Y. Nakajima, S.I. Kanamitsu, S. Imaoka, Y. Funae, C.E. Green, C.A. Tyson, N. Shimada, Y. Sugiyama, Prediction of in vivo drug-drug interactions between tolbutamide and various sulfonamides in humans based on in vitro experiments, Drug Metab. Dispos. 28 (2000) 475–481.
[81] J. Zi, D. Liu, P. Ma, H. Huang, J. Zhu, D. Wei, J. Yang, C. Chen, Effects of CYP2C9*3 and CYP2C9*13 on diclofenac metabolism and inhibition-based drug-drug interactions, Drug Metab. Pharmacokinet. 25 (2010) 343–350.
[82] K. Ogata, N. Takamura, J. Tokunaga, K. Kawai, Dosage plan of a flurbiprofen injection product using inhibition of protein binding by lipid emulsion in rats, J. Pharm. Pharmacol. 60 (2008) 15–20.
[83] N. Kuga, N. Shikano, N. Takamura, R. Nishii, K. Yamasaki, M. Kobayashi, S. Nagamachi, S. Tamura, K. Kawai, Competitive displacement of serum protein binding of radiopharmaceuticals with amino acid infusion investigated with N-isopropyl-p-^{123}I-iodoamphetamine, J. Nucl. Med. 50 (2009) 1378–1383.
[84] K. Nishi, M. Kobayashi, R. Nishii, N. Shikano, N. Takamura, N. Kuga, Pharmacokinetic alteration of (99 m)Tc-MAG3 using serum protein binding displacement method, Nucl. Med. Biol. (2013), http://dx.doi.org/10.1016/j.nucmedbio.2012.12.002.
[85] R. Gugler, D.L. Azarnoff, Drug protein binding and the nephrotic syndrome, Clin. Pharmacokinet. 1 (1976) 25–35.
[86] M.M. Reidenberg, D.E. Drayer, Alteration of drug-protein binding in renal disease, Clin. Pharmacokinet. 9 (Suppl. 1) (1984) 18–26.

[87] A. Dasgupta, D. Havlik, Elevated free fosphenytoin concentrations in uremic sera: uremic toxins hippuric acid and indoxyl sulfate do not account for the impaired protein binding of fosphenytoin, Ther. Drug Monit. 20 (1998) 658–662.
[88] S. Erill, R. Calvo, R. Carlos, Plasma protein carbamylation and decreased acidic drug protein binding in uremia, Clin. Pharmacol. Ther. 27 (1980) 612–618.
[89] R. Calvo, R. Carlos, S. Erill, Effects of carbamylation of plasma proteins and competitive displacers on drug binding in uremia, Pharmacology 24 (1982) 248–252.
[90] T. Sakai, T. Maruyama, H. Imamura, H. Shimada, M. Otagiri, Mechanism of stereoselective serum binding of ketoprofen after hemodialysis, J. Pharmacol. Exp. Ther. 278 (1996) 786–792.
[91] H. Watanabe, Y. Miyamoto, M. Otagiri, T. Maruyama, Update on the pharmacokinetics and redox properties of protein-bound uremic toxins, J. Pharm. Sci. 100 (2011) 3682–3695.
[92] T. Sakai, A. Takadate, M. Otagiri, Characterization of binding site of uremic toxins on human serum albumin, Biol. Pharm. Bull. 18 (1995) 1755–1761.
[93] N. Takamura, T. Maruyama, M. Otagiri, Effects of uremic toxins and fatty acids on serum protein binding of furosemide: possible mechanism of the binding defect in uremia, Clin. Chem. 43 (1997) 2274–2280.
[94] K. Mera, M. Anraku, K. Kitamura, K. Nakajou, T. Maruyama, M. Otagiri, The structure and function of oxidized albumin in hemodialysis patients: Its role in elevated oxidative stress via neutrophil burst, Biochem. Biophys. Res. Commun. 334 (2005) 1322–1328.
[95] J.P. Doweiko, D.J. Nompleggi, The role of albumin in human physiology and pathophysiology, Part III: albumin and disease states, JPEN J. Parenter. Enteral Nutr. 15 (1991) 476–483.
[96] A.H. Lau, L.E. Gustavson, R. Sperelakis, N.P. Lam, T. El-Shourbagy, J.X. Qian, T. Layden, Pharmacokinetics and safety of tiagabine in subjects with various degrees of hepatic function, Epilepsia 38 (1997) 445–451.
[97] A. Kober, A. Jenner, I. Sjöholm, Differentiated effects of liver cirrhosis on the albumin binding sites for diazepam, salicylic acid and warfarin, Biochem. Pharmacol. 27 (1978) 2729–2735.
[98] S. Klammt, S.R. Mitzner, J. Stange, J. Loock, U. Heemann, Improvement of impaired albumin binding capacity in acute-on-chronic liver failure by albumin dialysis, Liver Transpl. 14 (2008) 1333–1339.
[99] F. Ruiz-Cabello, S. Erill, Abnormal serum protein binding of acidic drugs in diabetes mellitus, Clin. Pharmacol. Ther. 36 (1984) 691–695.
[100] J. Baraka-Vidot, A. Guerin-Dubourg, E. Bourdon, P. Rondeau, Impaired drug-binding capacities of in vitro and in vivo glycated albumin, Biochimie 94 (2012) 1960–1967.
[101] G.D. Anderson, B.E. Gidal, R.J. Hendryx, A.B. Awan, N.R. Temkin, A.J. Wilensky, H.R. Winn, Decreased plasma protein binding of valproate in patients with acute head trauma, Br. J. Clin. Pharmacol. 37 (1994) 559–562.
[102] G. Arredondo, R. Calvo, F. Marcos, R. Martínez-Jordá, E. Suarez, Protein binding of itraconazole and fluconazole in patients with cancer, Int. J. Clin. Pharmacol. Ther. 33 (1995) 449–452.
[103] M.E. Winter, Clinical pharmacokinetics, in: L.Y. Young, M.A. Koda-Kimble (Eds.), Applied Therapeutics: The Clinical Use of Drugs, sixth ed., Applied Therapeutic, Inc., Washington, 1998, pp. 2-1–2-21.
[104] M.E. Winter, Basic Clinical Pharmacokinetics, fourth ed. Lippincott Williams & Wilkins, Philadelphia, 2004.
[105] S.R. Mitzner, Extracorporeal liver support-albumin dialysis with the molecular adsorbent recirculating system, Ann. Hepatol. 10 (2011) S21–S28.
[106] C.J. Karvellas, S.M. Bagshaw, R.C. McDermid, D.E. Stollery, R.T. Gibney, Acetaminophen-induced acute liver failure treated with single-pass albumin dialysis: report of a case, Int. J. Artif. Organs 31 (2008) 450–455.
[107] M. Boyle, J. Kurtovic, D. Bihari, S. Riordan, C. Steiner, Equipment review: the molecular adsorbents recirculating system (MARS), Crit. Care 8 (2004) 280–286.
[108] S.R. Mitzner, J.A.N. Stange, S. Klammt, P. Peszynski, R. Schmidt, G. Nöldge-schomburg, Extracorporeal detoxification using the molecular adsorbent recirculating system for critically ill patients with liver failure, J. Am. Soc. Nephrol. 12 (2001) S75–S82.
[109] A. Kortgen, F. Rauchfuss, M. Götz, U. Settmacher, M. Bauer, C. Sponholz, Albumin dialysis in liver failure: comparison of molecular adsorbent recirculating system and single pass albumin dialysis – a retrospective analysis, Ther. Apher. 13 (2009) 419–425.
[110] W. Laleman, A. Wilmer, P. Evenepoel, I. Elst, M. Zeegers, Z. Zaman, C. Verslype, J. Fevery, F. Nevens, Effect of the molecular adsorbent recirculating system and Prometheus devices on systemic haemodynamics and vasoactive agents in patients with acute-on-chronic alcoholic liver failure, Crit. Care 10 (2006) R108.
[111] S. Sen, N.A. Davies, R.P. Mookerjee, L.M. Cheshire, S.J. Hodges, R. Williams, R. Jalan, Pathophysiological effects of albumin dialysis in acute-on-chronic liver failure: a randomized controlled study, Liver Transpl. 10 (2004) 1109–1119.
[112] J. Stange, S.R. Mitzner, T. Risler, C.M. Erley, W. Lauchart, H. Goehl, S. Klammt, P. Peszynski, J. Freytag, H. Hickstein, M. Löhr, S. Liebe, W. Schareck, U.T. Hopt, R. Schmidt, Molecular adsorbent recycling system (MARS): clinical results of a new membrane-based blood purification system for bioartificial liver support, Artif. Organs 23 (1999) 319–330.
[113] P. Krisper, V. Stadlbauer, R.E. Stauber, Clearing of toxic substances: are there differences between the available liver support devices? Liver Int. 31 (2011) 5–8.
[114] S. Mitzner, S. Klammt, J. Stange, R. Schmidt, Albumin regeneration in liver support-comparison of different methods, Ther. Apher. Dial. 10 (2006) 108–117.
[115] J. Vienken, H. Christmann, How can liver toxins be removed? Filtration and adsorption with the Prometheus system, Ther. Apher. Dial. 10 (2006) 125–131.
[116] C.J. Karvellas, N. Gibney, D. Kutsogiannis, J. Wendon, V.G. Bain, Bench-to-bedside review: current evidence for extracorporeal albumin dialysis systems in liver failure, Crit. Care 11 (2007) 215.
[117] G. De Schoenmakere, J. De Waele, W. Terryn, M. Deweweire, A. Verstraete, E. Hoste, S. Rottey, N. Lameire, F. Colardyn, Phenytoin intoxication in critically ill patients, Am. J. Kidney Dis. 45 (2005) 189–192.
[118] S. Korsheed, N.M. Selby, R.J. Fluck, Treatment of severe theophylline poisoning with the molecular adsorbent recirculating system (MARS), Nephrol. Dial. Transplant. 22 (2007) 969–970.
[119] S. Sen, N. Ratnaraj, N.A. Davies, R.P. Mookerjee, C.E. Cooper, P.N. Patsalos, R. Williams, R. Jalan, Treatment of phenytoin toxicity by the molecular adsorbents recirculating system (MARS), Epilepsia 44 (2003) 265–267.
[120] A.C. de Pont, Extracorporeal treatment of intoxications, Curr. Opin. Crit. Care 13 (2007) 668–673.
[121] N. Pichon, B. François, C. Chevreuil, J.M. Gaulier, Albumin dialysis: a new therapeutic alternative for severe diltiazem intoxication, Clin. Toxicol. (Phila.) 44 (2006) 195–196.
[122] N. Pichon, A. Dugard, M. Clavel, J.B. Amiel, B. François, Extracorporeal albumin dialysis in three cases of acute calcium channel blocker poisoning with life-threatening refractory cardiogenic shock, Ann. Emerg. Med. 59 (2012) 540–544.
[123] C. Kawasaki, R. Nishi, S. Uekihara, S. Hayano, M. Otagiri, Charcoal hemoperfusion in the treatment of phenytoin overdose, Am. J. Kidney Dis. 35 (2000) 323–326.
[124] C.I. Kawasaki, R. Nishi, S. Uekihara, S. Hayano, U. Kragh-Hansen, M. Otagiri, How tightly can a drug be bound to a protein and still be removable by charcoal hemoperfusion in overdose cases? Clin. Toxicol. (Phila.) 43 (2005) 95–99.
[125] A. Minomo, Y. Ishima, V.T.G. Chuang, Y. Suwa, U. Kragh-Hansen, T. Narisoko, H. Morioka, T. Maruyama, M. Otagiri, Albumin domain II mutant with high bilirubin binding affinity has a great potential as serum bilirubin excretion enhancer for hyperbilirubinemia treatment, Biochim. Biophys. Acta (2013) 1–7.

Biochimica et Biophysica Acta 1830 (2013) 5465–5472

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Review

Redox properties of serum albumin ☆

Makoto Anraku [a], Victor Tuan Giam Chuang [c], Toru Maruyama [b,d], Masaki Otagiri [a,e,*]

[a] Faculty of Pharmaceutical Sciences, Sojo University, Kumamoto 860-0082, Japan
[b] Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan
[c] School of Pharmacy, Faculty of Health Sciences, Curtin Health Innovation Research Institute, Curtin University, Perth 6845,Western Australia, Australia
[d] Center for Clinical Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan
[e] DDS Research Institute, Sojo University, Kumamoto 860-0082, Japan

ARTICLE INFO

Article history:
Received 28 February 2013
Received in revised form 24 April 2013
Accepted 29 April 2013
Available online 3 May 2013

Keywords:
Human serum albumin
Oxidation
Cysteine-34
Antioxidant
Redox
S-nitrosylation

ABSTRACT

Background: Oxidative damage results in protein modification, and is observed in numerous diseases. Human serum albumin (HSA), the most abundant circulating protein in the plasma, exerts important antioxidant activities against oxidative damage.
Scope of review: The present review focuses on the characterization of chemical changes in HSA that are induced by oxidative damage, their relevance to human pathology and the most recent advances in clinical applications.
Major conclusions: The antioxidant properties of HSA are largely dependent on Cys34 and its contribution to the maintenance of intravascular homeostasis, including protecting the vascular endothelium under disease conditions related to oxidative stress. Recent studies also evaluated the susceptibility of other important amino acid residues to free radicals. The findings suggest that a redox change in HSA is related to the oxidation of several amino acid residues by different oxidants. Further, Cys34 adducts, such as S-nitrosylated and S-guanylated forms also play an important role in clinical applications. On the other hand, the ratio of the oxidized form to the normal form of albumin (HMA/HNA), which is a function of the redox states of Cys34, could serve as a useful marker for evaluating systemic redox states, which would be useful for the evaluation of disease progression and therapeutic efficacy.
General significance: This review provides new insights into our current understanding of the mechanism of HSA oxidation, based on in vitro and in vivo studies.
This article is part of a Special Issue entitled Serum Albumin.

1. Introduction

Increasing evidence indicates that reactive oxygen species (ROS) play a central role in signal transduction under physiological and pathophysiological conditions, leading to a new definition of oxidative stress in terms of the disruption of redox signaling and control [1,2]. Because proteins present in high concentrations in tissues, cells and fluids, they are the most vulnerable targets of oxidative stress [3]. Oxidative stress can cause reversible or irreversible modifications of sensitive proteins. Reversible protein modification is crucial in protecting against irreversible damage and modulating protein function. Irreversible protein modification is generally associated with a loss of function, leading to the degradation of the modified protein [4–7]. A typical example of a reversible modification is the mild oxidation of thiol groups, producing sulfenic acids and S-nitrosothiols, which is a common step in the regulatory process [8]. An irreversible modification to sulfonic acid (SO_3H) causes loss of function and protein degradation and is generally considered to be a suicide function of target proteins that are involved in antioxidation in several diseases. Other major intermediates of protein oxidation include the modification of methionine to methionine sulfoxide (MetO) or a sulfone ($MetO_2$), the oxidation of tyrosine by hypochlorous or hypobromous acids to mono and dihalo-derivatives (Cl-Tyr, di-Cl-Tyr and Br-Tyr, di-Br-Tyr) or by peroxynitrite (NO_2-Tyr) [9]. Protein carbonylation is a less specific modification that involves the oxidation of a number of amino acid side chains, i.e., proline, arginine, lysine and threonine, to form a ketone derivative. Furthermore, advanced oxidized protein products (AOPPs), which can be detected in the plasma of chronic uremic patients, frequently lead to the formation of di-tyrosine. The presence of these stable AOPP final products along the protein chain is considered to be an indicator of the extent of oxidative damage [4,10–12].

Human serum albumin (HSA), the most abundant protein in the plasma, regulates osmotic pressure while serving as a carrier for many endogenous and exogenous substances in plasma. In general, about 80% of the thiol groups in the blood can be accounted for from cysteine-34

Abbreviations: HSA, human serum albumin; HMA, human mercapto albumin; HNA, human non-mercapto albumin; ROS, reactive oxygen species; AOPP, advanced oxidized protein product; CRF, chronic renal failure; HD, hemodialysis; MCO, metal catalyzed oxidation; VEGF, vascular endothelial cell growth factor; MFI, mean fluorescence intensity

☆ This article is part of a Special Issue entitled Serum Albumin.

* Corresponding author at: Faculty of Pharmaceutical Sciences, Sojo University, Kumamoto 860-0082, Japan. Tel.: +81 96 326 3887; fax: +81 96 326 5048.
E-mail address: otagirim@ph.sojo-u.ac.jp (M. Otagiri).

0304-4165/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbagen.2013.04.036

(Cys34), which is the only Cys present in the free state in HSA [13]. Since the reversible binding of drugs to Cys34 is very important in terms of absorption, distribution, metabolism, excretion and toxicity and contributes to the analysis of early stages of the overall drug discovery process [14–18], Cys34 adducts such as S-nitrosylated and S-guanylated forms also frequently play an important role in clinical applications. In addition to these functions, the thiol-dependent antioxidant effect of HSA has recently attracted attention as a newly discovered function [19–21]. Furthermore, at physiological conditions, the Cys34 residue of HSA exists in two different forms, namely, human mercapto-albumin (HMA; reduced), the thiol group is in the free state, while in human non-mercapto-albumin (HNA; oxidized), it is present as a disulfide that is formed reversibly with Cys or glutathione, or as an oxide, such as in a sulfinic acid, sulfonic acid or a similar derivative that is formed irreversibly [22]. It has been shown that HMA and HNA are in equilibrium, depending on the redox state of Cys34, and that their ratio varies depending on age and the diseased state [23–25]. Cys34 has been shown to be a good indicator for evaluating oxidative stress in the systemic circulation, suggesting that it exerts an antioxidant effect in the blood.

In this review, the characterization of oxidized HSA, based on in vitro and in vivo studies will be discussed. Secondly, the role of Cys34 of HSA, which is the most sensitive residue to oxidative stress, as an antioxidant and its possible contribution to the maintenance of intravascular homeostasis, will be discussed. Thirdly, the usefulness of using the redox states of Cys34 of HSA as a biomarker for evaluating oxidative stress in pathological conditions will be elaborated. Finally, since Cys34 is also a binding site for NO, clinical applications of S-nitrosylation and S-guanylation of HSA will also be discussed.

2. Oxidation of human serum albumin

2.1. "In vitro" oxidation

In general, individual oxidants are known to generate different types of radicals. For example, all amino acid residues of proteins are potential targets for oxidation by $HO^{\bullet}$, which is generated upon exposure to ionizing radiation or by high concentrations of hydrogen peroxide in the presence of Cu^{2+} or Fe^{2+} [26–28]. However, these strong oxidation systems are generally associated with causing a stable loss of protein function, leading to degradation of the modified protein, which is a different process from the proteolytic attack that occurs during intracellular catabolism. Metal-catalyzed oxidation (MCO) system using very low concentrations of metal ions that will result in mild oxidation can be employed to evaluate the physiological significance and the impact of the oxidation of a protein. MCO system enables early alterations of the albumin molecule to be examined. A number of amino acid residues of HSA (Cys, Arg and Trp) were modified by the MCO system. These modifications had no detectable effect on the binding of drugs to site I in subdomain IIA. In contrast, the ligand-binding properties of the oxidized HSA for site II drugs and esterase-like activity decreased, most probably due to conformational changes that occur in subdomain IIIA as the result of oxidation [29]. In mice, the plasma half-life of oxidized HSA was substantially decreased and the liver uptake clearance of oxidized HSA was markedly increased by 11-fold [30]. In addition, the Cys and Met residues of HSA can be modified by chloramines and hydrogen peroxide, a milder oxidation system. Oxidation of these two amino acid residues had no detectable effect on the ligand-binding properties, esterase-like activity, or the plasma half-life of HSA [29]. Thus, oxidation of key amino acid residues such as Trp, Arg, and Tyr may accelerate the elimination of HSA [31]. In fact, oxidation of Arg410 has been shown to stimulate the elimination of HSA from the circulatory system [32]. Table 1 contains a summary of changes in the high affinity binding of ligands and elimination reported for oxidized albumin and the corresponding methods for achieving oxidative modification. Furthermore, protein hydroperoxides, which generate carbonyl formation and peptide cleavage, is another main form of radical damage on proteins including HSA [33–37]. In fact, protein hydroperoxides are capable of initiating further radical chain reactions both intra- and inter-molecularly, and contribute to some of the fundamental mechanisms of protein alteration and side-chain fragmentation [38–43].

On the other hand, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazol as a reaction reagent for thiol or sulfenic acid, also reacts primarily with the free thiol of Cys34 to form SO_3H, a stable adduct. The reaction of Cys34 is limited to the formation of a sulfenic (SOH) or a sulfinic acid (SO_2H), both being reversible intermediates [8], that may shift back to form the original mercapto-albumin, or react with free glutathione or homocysteine to form non-mercapto albumin (HNA), which represents 5–15% of the total amount of the protein in the serum [25]. Upon stable oxidation of the thiol group of Cys34 to SO_3H, not only are the structural and functional characteristics of HSA altered, but the protein becomes more susceptible to trypsin digestion and undergoes degradation faster than the non-oxidized counterpart, due to other major oxidation sites such as Arg, Trp and Tyr [44]. In fact, Kikugawa et al. proposed that, when Trp is oxidized, the resulting oxidized albumin becomes more susceptible to proteolytic cleavage by oxidized protein hydrolase [45]. Taking all of these factors into consideration, it appears that the preferred site of oxidation is dependent on the nature of the oxidant and the process relies on specific structural motifs in the protein to direct the oxidation reaction [46].

2.2. Relationships between "in vitro" and "in vivo" oxidation

Recent studies suggest that HSA is also oxidized in vivo. Previous reports, based on indirect techniques, indicated that only partial oxidation of HSA occurred in cases of chronic renal failure (CRF) and diabetes mellitus [47–51]. Studies of patients with CRF are particularly relevant since they have increased levels of HNA, which may represent the product of the reaction of free plasma thiols, such as glutathione and/or homocysteine, with sulfenic derivative of HSA. These studies indicated that HNA levels correlated with the degree of renal dysfunction where a marked increase of HNA level in patients undergoing hemodialysis (HD) had been reported [50]. HSA is the major plasma protein target of oxidative stress in uremia, and increased levels of carbonyl compounds are correlated with the degree of the oxidation of Cys34 in uremic patients [51]. Therefore, a variety of biological mechanisms may be responsible for these effects. In fact, oxidized HSA purified from serum of HD patients showed not only modified structural and functional properties but also acted as a mediator of the neutrophil activation state associated with chronic uremia (Fig. 1) [4]. On the other hand, the presence of elevated levels of advanced oxidation products (AOPPs), leads to the formation of di-Tyr and accounts for the excess oxidation of albumin, in several diseases, including conditions with present or developed renal lesions such as diabetes mellitus and IgA glomerulonephritis. When present, AOPPs can be used to predict the progression of renal lesions, atherosclerotic cardiovascular events and death [52,53].

3. The role of Cys34 of human serum albumin

3.1. The albumin thiol group as an antioxidant and its physiological significance

The antioxidant effect of HSA can be mainly attributed to its single thiol group, Cys34, which accounts for 80% of the reduced thiols in human plasma and was determined to be the preferred plasma scavenger of the diverse family of reactive oxygen and nitrogen species [54,55]. Studies have shown that ascorbate, urate, tocopherol and albumin are the main antioxidants in plasma. In contrast to the extrinsic nature of the former three agents, albumin is considered to be an important intravascular radical scavenger in plasma due to its intrinsic production by the liver at a consistently constant high concentration (0.5 mM) [56]. In this regard, the increase in oxidized isoforms of albumin in

Table 1
Oxidation of albumin and its effect on high affinity binding and elimination.

Mode of oxidation	Indicator of oxidation	Used ligand	Change	Elimination ($T_{1/2}$)	Reference
Ascorbic acid/$FeCl_2$	Carbonyl groups	Warfarin (site I)	±	±, ↓[a]	Ref. [29]
Chloramine T		Ketoprofen (site II)	±, ↓[a]		
H_2O_2					
Ascorbic acid/$FeCl_2$	Carbonyl groups	Warfarin (site I)	±	↓	Ref. [32]
		Ketoprofen (site II)	↓		
Chloramine T	Carbonyl groups	Warfarin (site I)	±, ↓[b]	±, ↓[b]	Ref. [31]
	Fraction of HNA2	Ketoprofen (site II)			
Hemodailysis	Carbonyl groups	Warfarin (site I)	↓	±	Ref. [4]
	HNA/HMA ratio	Ketoprofen (site II)	↓		
Cystine	HNA1	L-tryptophan	↓	-	Ref. [5]
		Cefazoline	↓		
		Verapamil	↓↓		

[a] Depending on the type of oxidant.
[b] Depending on the concentration of the oxidant.

pathological conditions can be considered to constitute proof of the concept of its scavenger activity. Among the 585 amino acid residues in HSA, Bourdon et al. reported that Met and Cys accounted for 40–80% of the total antioxidant activity of HSA. They concluded that Cys functions largely as a free radical scavenger, whereas Met mainly functions as a metal chelator [19]. Carballal et al. also concluded that thiol groups of albumin may well be an important scavenger of ROS [57]. We also quantitatively evaluated the role of Cys and Mets in the antioxidative activity of HSA using recombinant mutants, in which Cys34 and/or the six Met residues had been mutated to alanine using site-directed mutagenesis [58]. Our results showed that the level of contribution of Cys34 and the Met residues to the antioxidative activity of HSA was 61% and 29% against $O_2^{\cdot-}$, 68% and 61% against H_2O_2, 38% and 6% against $HO^{\cdot}$, 36% and 13% against HOCl and 51% and 1% against NO, respectively. This further confirms the significance of Cys34 in the antioxidative activity of HSA. Moreover, we also clarified the physiological implications of the antioxidative effect of Cys34 using human umbilical venous endothelial cells (HUVEC), as a model for vascular endothelial cells model [59]. Using HSA purified from HD patients in which Cys34 had been oxidized to various degrees and Cys34 mutants [60], we found that the oxidation of Cys34 caused a decrease in the ROS scavenging activity of HSA derived from the cell system, increased vascular endothelial cell growth factor (VEGF) production, and hence a reduction in cell variability (Fig. 2). VEGF has been proposed to act as a proinflammatory and proarteriosclerotic factor in Angiotensin II (AII) induced hypertension [61]. Furthermore, AII, via its type-1 receptor, stimulates NADPH oxidase and enhances the production of reactive oxygen species, which in turn, contributes to endothelial dysfunction and vascular inflammation [62]. Thus, these findings provide evidence to support the view that HSA may have a protective effect on vascular endothelial cell function by attenuating oxidative stress to suppress VEGF production. Interestingly, these changes were well correlated with the degree of free SH content in Cys34 of HSA. A quantitative assessment indicated that Cys34 of HSA accounts for more than 40% of the antioxidant effect of HSA in vivo and under CRF conditions. In fact, Terawaki et al., using human aortic endothelial cell monolayers, also proposed that an increase in the oxidation of Cys34 induced a suppression of endothelial reduction activity that was related to the cause of vascular dysfunctions [63]. These findings point to the significance of Cys34 in HSA functions that may have possible contributions to the maintenance of intravascular homeostasis, including the protection of the vascular endothelium under oxidative stress related diseased conditions. In the future, we plan to assess the relationships between the oxidation of Cys34 and major clinical markers such as neopterin, CRP, glycoheme level etc., in order to obtain accurate information for many diseases.

3.2. The albumin thiol group as a biomarker for oxidative stress

Oxidative stress causes molecular modifications such as carbonylation of HSA, and the formation of advanced glycation end products (AGEs) and AOPP. These stable final products along with the protein chain are considered to be good indicators of the extent of oxidative damage [4,10,11]. However, since these makers are end products, sensitive markers that could be used to estimate the initial oxidative states are needed. Oxidation of the amino acyl side chains of amino acids in proteins is an attractive biomarker for oxidative reactions because of the high specificity of the end products produced via specific oxidation pathways. In particular, the detection of oxidized Cys residues currently constitutes the most sensitive and specific means available for detecting such end products of specific oxidative pathways [64].

As mentioned above, Cys34 is a major redox sensitive site of albumin and therefore it is reasonable to discuss the redox state of albumin in relation to Cys34. Era et al., developed a chromatographic analytical method for evaluating the redox state of Cys34 in HSA. The chromatographic

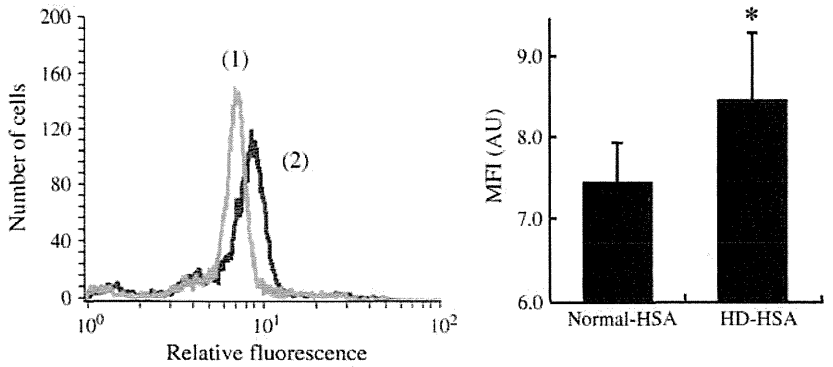

Fig. 1. ROS production by neutrophils incubated with purified HSA. (1) Normal-HSA purified from normal subjects (n = 10) (2) HD-HSA purified from HD patients (n = 20). *$P < 0.05$, compared with Normal-HSA [4].

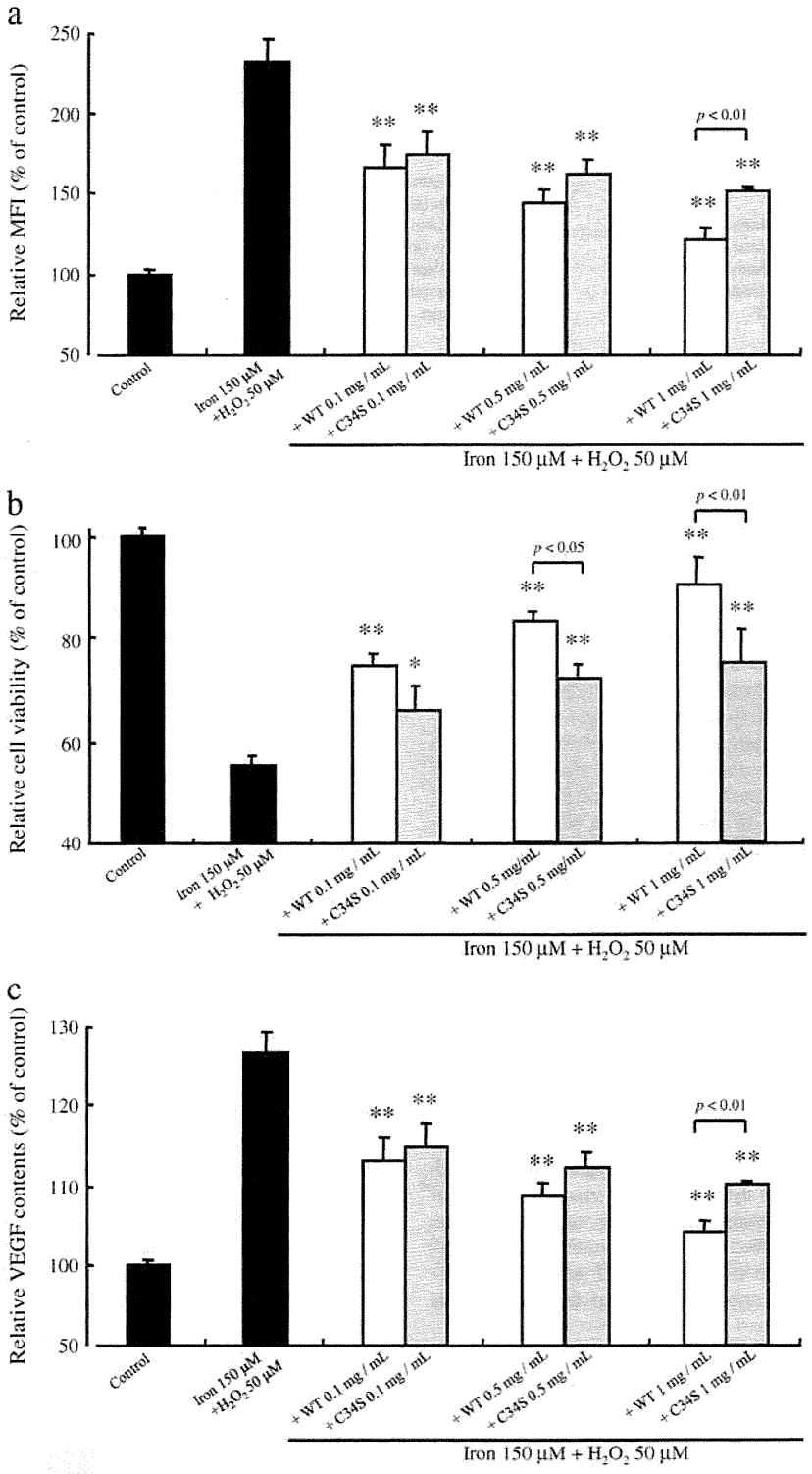

Fig. 2. Effects of Wild-type and C34S on intracellular ROS production (a), cell survival (b), and VEGF production (c) on HUVEC. Values are expressed as the mean ± S.E.; n = 3. $^{**}P < 0.01$, $^{*}P < 0.05$ as compared with iron 150 μM + H_2O_2 50 μM; $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ as compared with WT [59].

separation of albumin gives three fractions according to the state of Cys34:(i) the free thiol form, which is referred to as mercaptalbumin (HMA); (ii) the disulfide form with a small thiol compound, mainly cysteine or cysteinylglycine but also homocysteine and glutathione, referred to as human nonmercaptalbumin1 (HNA1); and (iii) a highly oxidized form in which cysteine has been oxidized to the level of a sulfinic or sulfonic acid (HNA-2) [25]. For example, in a healthy young person, HMA accounts for 70–80%, HNA-1 for 20–30%, and HNA-2 for about 2–5% of the total albumin present [65]. In the past 10 years, HMA and HNA were found to be in equilibrium, depending on the surrounding redox state of Cys34, and that their ratio is dependent on age and the specific diseased state. In fact, the HNA fraction was found to increase during the course of several pathological conditions, such as renal dysfunction, various liver diseases, coronary artery disease,

diabetes mellitus, and senile cataract, invasive surgery, and during intensive exercise and aging [22,24,25,48,49,66,67]. The HNA fraction was negatively correlated with creatinine clearance in patients with renal dysfunction and, during intrauterine growth restriction, the fraction of HNA-2 in maternal plasma was about twice as high as that in a control plasma [51,68]. Moreover, the intravenous administration of iron to anemic renal patients resulted in the development of oxidative stress and consequently an increase in the HNA-1 and HNA-2 fractions [51].

Instead of monitoring individual levels of HMA or HNA, we proposed the oxidized albumin ratio (HNA/HMA), estimated by dividing the area under the peak for the reduced form (HMA) by that for the oxidized form (HNA-1 + HNA-2), as a useful index of oxidative stress. Fig. 3 shows the oxidized albumin ratio for various pathophysiological conditions. The tendency of the changes in oxidized albumin ratio in conjunction with various pathophysiological conditions is quite consistent with previous results estimated using other oxidative stress markers. We also attempted to use the oxidized albumin ratio to evaluate therapeutic efficacy. Our previous clinical study clearly demonstrated that oxidative stress, as reflected in the oxidized albumin ratio, was significantly less evident despite equivalent anti-anemic effects, when intravenous iron was given once weekly for 3 months instead of three times weekly for 1 month. Based on these results, we proposed a change in the conventional regimen for intravenous iron treatments in Japan from the viewpoint of oxidative stress (Fig. 4) [69].

Treatment with AST-120 (Kremezin®), an oral carbonaceous adsorbent used in pre-dialysis, reduced the oxidized albumin ratio from 0.8–1.2 to 0.6–0.8 in CRF as a consequence of removing uremic toxins from the systemic circulation (Fig. 3) [70]. Similarly, olmesartan and telmisartan, inverse angiotensin II type 1 receptor antagonists, effectively reduced the extent of oxidized albumin ratio in HD patients beyond their blood lowering actions [71]. Furthermore, the intake of low-molecular weight chitosans, a natural polymer derived from chitin, may inhibit neutrophil activation and the oxidation of HSA that is commonly observed in HD patients, resulted in a reduction in uremia-related oxidative stress [72–74]. Thus, monitoring the oxidized albumin ratio would not only be a useful marker for evaluating the progression of oxidative stress related diseases, but also in predicting therapeutic efficacy (Fig. 5). In order to establish and validate the usefulness of HNA/HMA as an oxidative stress marker, examination of the correlations between HNA/HMA and other major oxidative stress markers such as AOPP or F2-isoprostane etc. will need to be conducted.

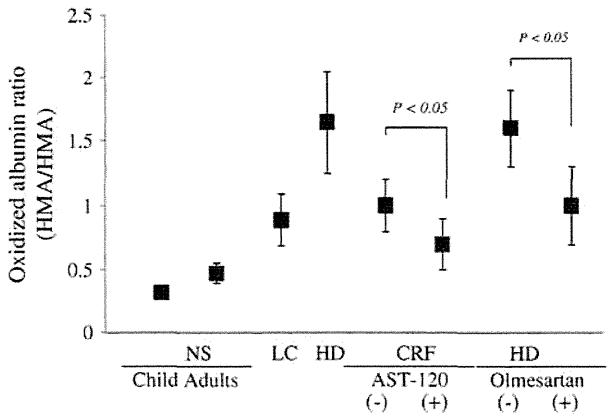

Fig. 3. Change in oxidized serum albumin ratio in healthy subjects and patients estimated by Ref. [66–71]. NS, normal subjects LC, liver cirrhosis. Values for each albumin fraction (f(HMA), f(HNA-1), and f(HNA-2)) were estimated by dividing the area for each fraction by the total area corresponding to serum albumin. AST-120 (Kremezin®), an oral carbonaceous adsorbent that has been used in pre-dialysis, reduced the oxidized albumin ratio in CRF. Olmesartan (ARB), an inverse angiotensin II type 1 receptor antagonist, effectively lowers the extent of oxidized albumin ratio in HD patients.

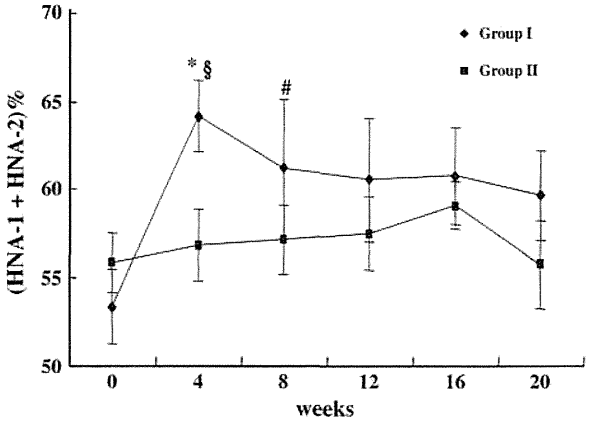

Fig. 4. Effect of the frequency of intravenous iron administration on oxidized serum albumin ratio [69]. Values for each albumin fraction (f(HMA), f(HNA-1), and f(HNA-2)) were estimated by dividing the area of each fraction by the total area corresponding to serum albumin. Trapezoid (♦) and square (■) represent patient groups I and II, respectively. Values are expressed as the mean ± SE; n = 11 for each patient group. $^{*}P < 0.01$ as compared with 0 week (group I), $^{\#}P < 0.05$ as compared with 0 week (group I), and $^{\S}P < 0.01$ as compared with 4 weeks (group II). Group I: 40 mg of iron 3 times a week for 4 weeks, group II: 40 mg of iron once a week for 3 months.

Recently, ischemia modified albumin (IMA; Ischemia Technologies, Inc.), has been shown to be a marker of myocardial ischemia. The presence of IMA was monitored by means of an albumin cobalt binding test (ACB; Ischemia Technologies). ACB measures the ability of the amino terminus (N-terminus) of human albumin to bind exogenous cobalt. Transition metals can bind tightly to the exposed N-terminus of albumin under physiological conditions [75,76]. Given the fact that a significant negative correlation has been demonstrated between IMA and serum albumin levels in pregnancy [77,78], a possible correlation between HNA/HMA and IMA might lead to a more accurate marker of HNA/HMA.

Mass spectrometry (MS) including LC-TOFMS and FTMS analyses has also recently been shown to be a powerful analytical technique capable of the rapid online analysis of HSA isoforms in diabetes [79]. In order to analyze the systemic redox states of HSA more closely, the HPLC systems that are currently in use should be combined with MS systems. Furthermore, given the fact that oxidative stress may be related to cardiovascular morbidity outcomes rather than the actual albumin levels, as reflected by hypoalbuminemia in HD patients [80], future studies involving populations with established oxidative stress may foster development of a new albumin-assay

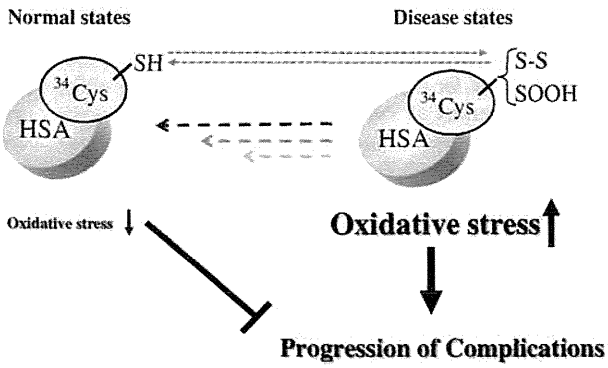

Fig. 5. Proposed mechanism for the contribution of Cys34 to the maintenance of homeostasis in blood. The treatments where the levels of Cys34 oxidation are minimized at low levels in several diseases may be beneficial for preventing the onset and progression of serious complications, which affects the prognosis for survival.

using a combination of MS and HPLC analytical methods that will allow highly sensitive and accurate albumin measurements.

3.3. Potential therapeutic applications of the albumin thiol group

Clinically, HSA is used as a plasma expander, and albumin infusion is generally considered a standard procedure for treating severe hypoalbuminemia as a result of burns, nephritic syndrome, reduction in the synthesis of HSA induced by chronic liver cirrhosis, and hemorrhagic shock. We recently found that the elimination of HSA from plasma was significantly enhanced in nephritic conditions, while dimerization of HSA via linking the Cys34 residues of two HSA led to blood retention properties similar to that observed for HSA under normal conditions. Thus, HSA dimer has good potential as a plasma expander as well as a drug carrier [81–84].

The sulfhydryl moiety of Cys34 can interact with nitric oxide (NO) to form S-nitrosothiols (RS-NOs). RS-NOs may function as NO reservoirs and preserve the antioxidative and other activities of NO such as cytoprotective action, antimicrobial properties and the suppression of platelet aggregation. Thus, the S-nitrosylated albumin can be considered to be a circulating endogenous reservoir of NO and may have potential as NO donors in therapeutic applications [85,86]. S-nitrosylated HSA (SNO-HSA) is believed to act primarily as a venodilator in vivo and represents a stable reservoir of NO that can release NO when the concentrations of low-molecular-weight thiols are elevated [87]. SNO-HSA has been shown to reduce ischemia or reperfusion injury in the pig heart after unprotected warm ischemia through the long-lasting release of NO. SNO-HSA prevents the uncoupling of endothelial NO synthase, which is a major contributor to the generation of vascular reactive oxygen species in ischemia and reperfusion injury, and thereby improves systolic and diastolic function, myocardial perfusion, and the energetic reserve of the heart after such insults [88]. A similar cardioprotective effect on the rabbit has also been reported [89]. We have also demonstrated that SNO-HSA is useful in preventing liver ischemia/reperfusion injury, and SNO-HSA should thus be able to prevent cell injury during liver transplantation procedures. In fact, in an attempt to improve the standard solution called the University of Wisconsin (UW) solution for hepatic transplantation, we added SNO-HSA to a UW solution and evaluated its protective effect during cold or/and warm ischemia in situ and in vitro. Although treatment with SNO-HSA or the UW solution alone is not sufficient to inhibit liver injury during both cold and warm ischemia, a combination of SNO-HSA and UW solutions have been shown to prevent these two types of ischemia via the suppression of both apoptosis and necrosis [90,91]. In addition, Ishima et al. showed, in a rat ischemia/reperfusion liver injury model, that the binding of oleate to S-nitrosylated HSA enhances its cytoprotective effect for liver cells [92]. The addition of oleate increased the accessibility of the single thiol group of albumin and improved the S-denitrosation of SNO-HSA by HepG2 cells. Oleate enhances the anti-apoptotic effect of SNO-HSA on HepG2 cells exposed to an anti-Fas antibody. These promising results promote further development of SNO-HSA into a better treatment option for ischemic conditions [93]. In view of the clinical application potential of SNO-HSA, we developed a one-step method for preparing SNO-HSA using S-nitrosoglutathione (GS-NO) and a commercial HSA formulation that contains a high concentration of caprylic acid as stabilizer that only requires incubating a commercial HSA formulation with GS-NO for only 1 min for the S-nitrosation of Cys34 in HSA.

Recently, Sawa et al. discovered that a novel nitrated cyclic nucleotide, namely, 8-nitroguanosine 3,5-cyclic monophosphate (8-nitro-cGMP), is generated in a NO-dependent manner [94]. 8-Nitro-cGMP exhibits the strongest redox activity among the nitrated guanine derivatives examined, which is different from that of activating cGMP-dependent protein kinases. Because 8-nitro-cGMP possesses an electrophilic property, this metabolite can react with sulfhydryl groups of Cys and form a protein-S-cGMP adduct via a novel posttranslational modification, namely, protein S-guanylation [94]. We found that Cys34 is a specific site for S-guanylation in HSA. S-cGMP-HSA exhibits unique biological activities that are not observed in HSA itself. Table 2 provides information on the antibacterial properties of S-cGMP-HSA as an example. Although this activity is similar to that of SNO-HSA, S-cGMP-HSA is more stable than SNO-HSA that is very sensitive to light, metals, and reducing agents [95].

Table 2
Antibacterial effects of S-cGMP-HSA against various bacteria [95].

Gram stain	IC50 (μM)	
	S-cGMP-HSA	SNO-HSA
Negative		
E. coli	1.4	0.6
S. typhimurium LT2	100	8
P. aeruginosa	14	25
K. preumoniae MGH78578	9	1.3
Positive		
B. subtilis	0.3	0.17
S. pyogenes	100	10
S. aureus OM 481	100	100
S. aureus OM 505	100	100

4. Conclusion

This review summarizes the redox properties of albumin, with a particular focus on Cys34. The antioxidant properties of HSA are largely dependent on Cys34 and its contribution to the maintenance of intravascular homeostasis, including the protection of the vascular endothelium under disease conditions related to oxidative stress. Furthermore, Cys34 adducts such as S-nitrosylated and S-guanylated HSA have shown great potential for clinical applications. On the other hand, the oxidized albumin ratio (HMA/HNA), which is a function of the redox states of Cys34, might be a useful marker for systemic redox states that is useful for the evaluation of disease progression and therapeutic efficacy.

Acknowledgement

We thank Dr Hiroshi Watanabe and Dr Yu Ishima from Kumamoto University, Japan for reviewing our article.

References

[1] H. Sies, Oxidative Stress, Academic Press, London, 1985.
[2] D.P. Jones, Redefining oxidative stress, Antioxid. Redox Signal. 8 (2006) 1865–1879.
[3] M.J. Davies, The oxidative environment and protein damage, Biochim. Biophys. Acta 1703 (2005) 93–109.
[4] K. Mera, M. Anraku, K. Kitamura, K. Nakajou, T. Maruyama, M. Otagiri, The structure and function of oxidized albumin in hemodialysis patients: its role in elevated oxidative stress via neutrophil burst, Biochem. Biophys. Res. Commun. 334 (2005) 1322–1328.
[5] A. Kawakami, K. Kubota, N. Yamada, U. Tagami, K. Takehana, I. Sonaka, E. Suzuki, K. Hirayama, Identification and characterization of oxidized human serum albumin. A slight structural change impairs its ligand-binding and antioxidant functions, FEBS J. 273 (2006) 3346–3357.
[6] G. Candiano, L. Musante, M. Bruschi, A. Petretto, L. Santucci, P. Del Boccio, B. Pavone, F. Perfumo, A. Urbani, F. Scolari, G.M. Ghiggeri, Repetitive fragmentation products of albumin and alpha1-antitrypsinin glomerular diseases associated with nephrotic syndrome, J. Am. Soc. Nephrol. 17 (2006) 3139–3148.
[7] L. Musante, G. Candiano, A. Petretto, M. Bruschi, N. Dimasi, G. Caridi, B. Pavone, P. Del Boccio, M. Galliano, A. Urbani, F. Scolari, F. Vincenti, G.M. Ghiggeri, Active focal segmental glomerulosclerosis is associated with massive oxidation of plasma albumin, J. Am. Soc. Nephrol. 18 (2007) 799–810.
[8] S. Carballal, R. Radi, M.C. Kirk, S. Barnes, B.A. Freeman, B. Alvarez, Sulfenic acid formation in human serum albumin by hydrogen peroxide and peroxynitrite, Biochemistry 42 (2003) 9906–9914.
[9] I. Dalle-Donne, A. Scaloni, D. Giustarini, E. Cavarra, G. Tell, G. Lungarella, R. Colombo, R. Rossi, A. Milzani, Proteins as biomarkers of oxidative/nitrosative stress in diseases: the contribution of redox proteomics, Mass Spectrom. Rev. 24 (2005) 55–99.

[10] A.C. Carr, C.L. Hawkins, S.R. Thomas, R. Stocker, B. Frei, Relative reactivities of N-chloramines and hypochlorous acid with human plasma constituents, Free Radic. Biol. Med. 30 (2001) 526–536.
[11] P. Salavej, H. Spalteholz, J. Arnhold, Modification of amino acid residues in human serum albumin by myeloperoxidase, Free Radic. Biol. Med. 40 (2006) 516–525.
[12] L. Selmeci, Advanced oxidation protein products (AOPP): novel uremic toxins, or components of the non-enzymatic antioxidant system of the plasma proteome? Free Radic. Res. 45 (2011) 1115–1123.
[13] T. Peters Jr., *All About Albumin, Biochemistry, Genetics, and Medical Applications*, Academic Press, San Diego, 1996.
[14] G.L. Trainor, The importance of plasma protein binding in drug discovery, Expert Opin. Drug Discov. 2 (2007) 51–64.
[15] J. Christodoulou, P.J. Sadler, A. Tucker, ^{1}H NMR of albumin in human blood plasma: drug binding and redox reactions at Cys34, FEBS Lett. 376 (1995) 1–5.
[16] N.A. Kratochwil, A.I. Ivanov, M. Patriarca, J.A. Parkinson, A. Gouldsworthy, P. del S, Murdoch, P.J. Sadler, Surprising reactions of iodo Pt(IV) and Pt(II) complexes with human albumin: detection of Cys34 sulfenic acid, J. Am. Chem. Soc. 121 (1999) 8193–8203.
[17] A.J. Stewart, C.A. Blindauer, S. Berezenko, D. Sleep, D. Tooth, P.J. Sadler, Role of Tyr84 in controlling the reactivity of Cys34 of human albumin, FEBS J. 272 (2005) 353–362.
[18] W. Hu, Q. Luo, X. Ma, K. Wu, J. Liu, Y. Chen, S. Xiong, J. Wang, P.J. Sadler, F. Wang, Arene control over thiolate to sulfinate oxidation in albumin by organometallic ruthenium anticancer complexes, Chemistry 15 (2009) 6586–6594.
[19] E. Bourdon, N. Loreau, L. Lagrost, D. Blache, Differential effects of cysteine and methionine residues in the antioxidant activity of human serum albumin, Free Radic. Res. 39 (2005) 15–20.
[20] M. Soriani, D. Pietraforte, M. Minetti, Antioxidant potential of anaerobic human plasma: role of serum albumin and thiols as scavengers of carbon radicals, Arch. Biochem. Biophys. 312 (1994) 180–188.
[21] M. Roche, P. Rondeau, N.R. Singh, E. Tarnus, E. Bourdon, The antioxidant properties of serum albumin, FEBS Lett. 582 (2008) 1783–1787.
[22] M. Sogami, S. Nagoka, S. Era, M. Honda, K. Noguchi, Resolution of human mercapt- and nonmercaptalbumin by high-performance liquid chromatography, Int. J. Pept. Protein Res. 24 (1984) 96–103.
[23] A. Soejima, F. Kaneda, S. Manno, N. Matsuzawa, H. Kouji, T. Nagasawa, S. Era, Y. Takakuwa, Useful markers for detecting decreased serum antioxidant activity in hemodialysis patients, Am. J. Kidney Dis. 39 (2002) 1040–1046.
[24] A. Hayakawa, K. Kuwata, S. Era, M. Sogami, H. Shimonaka, M. Yamamoto, S. Dohi, H. Hirose, Alteration of redox state of human serum albumin in patients under anesthesia and invasive surgery, J. Chromatogr. B: Biomed. Sci. Appl. 698 (1997) 27–33.
[25] S. Era, K. Kuwata, H. Imai, K. Nakamura, T. Hayashi, M. Sogami, Age-related change in redox state of human serum albumin, Biochim. Biophys. Acta 1247 (1985) 12–16.
[26] W.M. Garrison, Reaction mechanisms in the radiolysis of peptides, polypeptides, and proteins, Chem. Rev. 87 (1987) 381–398.
[27] H. Schuessler, K. Schilling, Oxygen effect in radiolysis of proteins. Part 2. Bovine serum albumin, Int. J. Radiat. Biol. 45 (1984) 267–281.
[28] J. Neuzil, J.M. Gebicki, R. Stocker, Radical-induced chain oxidation of proteins and its inhibition by chain-breaking antioxidants, Biochem. J. 293 (1993) 601–606.
[29] M. Anraku, K. Yamasaki, T. Maruyama, U. Kragh-Hansen, M. Otagiri, Effect of oxidative stress on the structure and function of human serum albumin, Pharm. Res. 18 (2001) 632–639.
[30] Y. Iwao, M. Anraku, M. Hiraike, K. Kawai, K. Nakajou, T. Kai, A. Suenaga, M. Otagiri, Drug Metab. Pharmacokinet. 21 (2006) 140–146.
[31] M. Anraku, U. Kragh-Hansen, K. Kawai, T. Maruyama, Y. Yamasaki, Y. Takakura, M. Otagiri, Validation of the chloramine-T induced oxidation of human serum albumin as a model for oxidative damage in vivo, Pharm. Res. 20 (2003) 684–692.
[32] Y. Iwao, M. Anraku, K. Yamasaki, U. Kragh-Hansen, K. Kawai, T. Maruyama, M. Otagiri, Biochim. Biophys. Acta 1764 (2006) 743–749.
[33] J.A. Simpson, S. Narita, S. Gieseg, S. Gebicki, J.M. Gebicki, R.T. Dean, Long-lived reactive species on free-radical-damaged proteins, Biochem. J. 282 (1992) 621–624.
[34] A.V. Babiy, S. Gebicki, J.M. Gebicki, Protein peroxides: formation by superoxide-generating systems and during oxidation of low density lipoprotein, in: G. Poli, E. Albano, M.U. Dianzani (Eds.), Free Radicals: From Basic Science to Medicine, Birkhauser Verlag, Basel/Switzerland, 1993, pp. 340–348.
[35] S. Gebicki, J.M. Gebicki, Formation of peroxides in amino acids and proteins exposed to oxygen free radicals, Biochem. J. 289 (1992) 743–749.
[36] J.M. Gebicki, Protein hydroperoxides as new reactive oxygen species, Redox Rep. 3 (1997) 99–110.
[37] H.A. Headlam, M.J. Davies, Beta-scission of side-chain alkoxyl radicals on peptides and proteins results in the loss of side-chains as aldehydes and ketones, Free Radic. Biol. Med. 32 (2002) 1171–1184.
[38] S.P. Gieseg, M.J. Davies, R.T. Dean, The protein oxidation product 3,4-dihydroxyphenylalanine (DOPA) mediates oxidative DNA damage, Biochemistry 32 (18) (1993) 4780–4786.
[39] H. Husain, S.M. Hadi, Strand scission in DNA induced by L-DOPA in the presence of Cu(II), FEBS Lett. 364 (1995) 75–78.
[40] D.I. Pattison, R.T. Dean, M.J. Davies, Oxidation of DNA, proteins and lipids by DOPA, protein-bound DOPA, and related catechol(amine)s, Toxicology 177 (2002) 23–37.
[41] F.E. Ali, K.J. Barnham, C.J. Barrow, F.J. Separovic, Metal catalyzed oxidation of tyrosine residues by different oxidation systems of copper/hydrogen peroxide, Inorg. Biochem. 98 (2004) 173–184.
[42] W.H.F. Sutherland, S.P. Gieseg, R.J. Walker, S.A. de Jong, C.A. Firth, N. Scott, Serum protein-bound 3,4-dihydroxyphenylalanine and related products of protein oxidation and chronic hemodialysis, Ren. Fail. 25 (2003) 997–1009.
[43] A.A. Platt, S.P. Gieseg, Inhibition of protein hydroperoxide formation by protein thiols, Redox Rep. 8 (2003) 81–86.
[44] E. Meucci, A. Mordente, G.E. Martorana, Metal-catalyzed oxidation of human serum albumin: conformational and functional changes. Implications in protein aging, J. Biol. Chem. 266 (1991) 4692–4699.
[45] T. Fujino, M. Kojima, M. Beppu, K. Kikugawa, H. Yasuda, K. Takahashi, Identification of the cleavage sites of oxidized protein that are susceptible to oxidized protein hydrolase (OPH) in the primary and tertiary structures of the protein, J. Biochem. 127 (2000) 1087–1093.
[46] A. Temple, T.Y. Yen, S. Gronert, Identification of specific protein carbonylation sites in model oxidations of human serum albumin, J. Am. Soc. Mass Spectrom. 17 (2006) 1172–1180.
[47] J. Himmelfarb, E. McMonagle, Albumin is the major plasma protein target of oxidant stress in uremia, Kidney Int. 60 (2001) 358–363.
[48] E. Suzuki, K. Yasuda, N. Takeda, S. Sakata, S. Era, K. Kuwata, et al., Increased oxidized form of human serum albumin in patients with diabetes mellitus, Diabetes Res. Clin. Pract. 18 (1992) 153–158.
[49] A. Soejima, N. Matsuzawa, T. Hayashi, R. Kimura, T. Ootsuka, K. Fukuoka, A. Yamada, T. Nagasawa, S. Era, Alteration of redox state of human serum albumin before and after hemodialysis, Blood Purif. 22 (2004) 525–529.
[50] H. Terawaki, K. Yoshimura, T. Hasegawa, Y. Matsuyama, T. Negawa, K. Yamada, M. Matsushima, M. Nakayama, T. Hosoya, S. Era, Oxidative stress is enhanced in correlation with renal dysfunction: examination with the redox state of albumin, Kidney Int. 66 (2004) 1988–1993.
[51] M. Anraku, K. Kitamura, A. Shinohara, M. Adachi, A. Suenaga, T. Maruyama, K. Miyanaka, T. Miyoshi, N. Shiraishi, H. Nonoguchi, M. Otagiri, K. Tomita, Intravenous iron administration induces oxidation of serum albumin in hemodialysis patients, Kidney Int. 66 (2004) 841–848.
[52] B. Descamps-Latscha, V. Witko-Sarsat, T. Nguyen-Khoa, A.T. Nguyen, V. Gausson, N. Mothu, G.M. London, P. Jungers, Advanced oxidation protein products as risk factors for atherosclerotic cardiovascular events in nondiabetic predialysis patients, Am. J. Kidney Dis. 45 (2005) 39–47.
[53] B. Descamps-Latscha, V. Witko-Sarsat, T. Nguyen-Khoa, A.T. Nguyen, V. Gausson, N. Mothu, C. Cardoso, L.H. Noel, A.P. Guerin, G.M. London, P. Jungers, Early prediction of IgA nephropathy progression: proteinuria and AOPP are strong prognostic markers, Kidney Int. 66 (2004) 1606–1612.
[54] B. Alvarez, G. Ferrer-Sueta, B.A. Freeman, R. Radi, Kinetics of peroxynitrite reaction with amino acids and human serum albumin, J. Biol. Chem. 274 (1999) 842–848.
[55] B. Halliwell, J.M. Gutteridge, The antioxidants of human extracellular fluids, Arch. Biochem. Biophys. 280 (1990) 1–8.
[56] C.C. Liu CC, J.M. Gebicki, Intracellular GSH and ascorbate inhibit radical-induced protein chain peroxidation in HL-60 cells, Free Radic. Biol. Med. 52 (2012) 420–426.
[57] S. Carballal, B. Alvarez, L. Turell, H. Botti, B.A. Freeman, R. Radi, Sulfenic acid in human serum albumin, Amino Acids 32 (2007) 543–551.
[58] Y. Iwao, Y. Ishima, J. Yamada, T. Noguchi, U. Kragh-Hansen, K. Mera, D. Honda, A. Suenaga, T. Maruyama, M. Otagiri, Quantitative evaluation of the role of cysteine and methionine residues in the antioxidant activity of human serum albumin using recombinant mutants, IUBMB Life 64 (2012) 450–454.
[59] M. Anraku, K. Takeuchi, H. Watanabe, D. Kadowaki, K. Kitamura, K. Tomita, A. Kuniyasu, A. Suenaga, T. Maruyama, M. Otagiri, Quantitative analysis of cysteine-34 on the anitioxidative properties of human serum albumin in hemodialysis patients, J. Pharm. Sci. 100 (2011) 3968–3976.
[60] H. Watanabe, K. Yamasaki, U. Kragh-Hansen, S. Tanase, K. Harada, A. Suenaga, M. Otagiri, In vitro and in vivo properties of recombinant human serum albumin from *Pichia pastoris* purified by a method of short processing time, Pharm. Res. 18 (2001) 1775–1781.
[61] Q. Zhao, M. Ishibashi, K. Hiasa, C. Tan, A. Takeshita, K. Egashira K, Essential role of vascular endothelial growth factor in angiotensin II-induced vascular inflammation and remodeling, Hypertension 44 (2004) 264–270.
[62] M. Ruiz-Ortega, O. Lorenzo, M. Rupérez, S. König, B. Wittig, J. Egido, Angiotensin II activates nuclear transcription factor κB through AT1 and AT2 in vascular smooth muscle cells: molecular mechanisms, Circ. Res. 86 (2000) 1266–1272.
[63] H. Terawaki, S. Era, M. Nakayama, T. Hosoya, Decrease in reduced-form albumin among chronic kidney disease patients: new insights in cardiovascular complications, Ther. Apher. Dial. 15 (2011) 156–160.
[64] J.W. Heinecke, F.F. Hsu, J.R. Crowley, S.L. Hazen, C. Leeuwenburgh, D.M. Mueller, J.E. Rasmussen, J. Turk, Detecting oxidative modification of biomolecules with isotope dilution mass spectrometry: sensitive and quantitative assays for oxidized amino acids in proteins and tissues, Methods Enzymol. 300 (1999) 124–144.
[65] K. Oettl, G. Marsche, Redox state of human serum albumin in terms of cysteine-34 in health and disease, Methods Enzymol. 474 (2010) 181–195.
[66] K. Kadota, Y. Yui, R. Hattori, Y. Murohara, C. Kawai, Decreased sulfhydryl groups of serum albumin in coronary artery disease, Jpn. Circ. J. 55 (1991) 937–941.
[67] H. Imai, T. Hayashi, T. Negawa, K. Nakamura, M. Tomida, K. Koda, T. Tajima, Y. Koda, K. Suda, S. Era, Strenous exercise-induced change in redox state of human serum albumin during intensive kendo training, Jpn. J. Physiol. 52 (2002) 135–140.
[68] D. Bar-Or, K.D. Heyborne, R. Bar-Or, L.T. Rael, J.V. Winkler, D. Navot, Cysteinylation of maternal plasma albumin and its association with intrauterine growth restriction, Prenat. Diagn. 25 (2005) 245–249.
[69] M. Anraku, K. Kitamura, R. Shintomo, K. Takeuchi, H. Ikeda, J. Nagano, T. Ko, K. Mera, K. Tomita, M. Otagiri, Effect of intravenous iron administration frequency on AOPP and inflammatory biomarkers in chronic hemodialysis patients: a pilot study, Clin. Biochem. 41 (2008) 1168–1174.
[70] K. Shimoishi, M. Anraku, K. Kitamura, Y. Tasaki, K. Taguchi, M. Hashimoto, E. Fukunaga, T. Maruyama, M. Otagiri, An oral adsorbent, AST-120 protects against

the progression of oxidative stress by reducing the accumulation of indoxyl sulfate in the systemic circulation in renal failure, Pharm. Res. 24 (2007) 1283–1289.
[71] D. Kadowaki, M. Anraku, Y. Tasaki, K. Kitamura, S. Wakamatsu, K. Tomita, J.M. Gebicki, T. Maruyama, M. Otagiri, Effect of olmesartan on oxidative stress in hemodialysis patients, Hypertens. Res. 30 (2007) 395–402.
[72] M. Anraku, M. Kabashima, H. Namura, T. Maruyama, M. Otagiri, J.M. Gebicki, N. Furutani, H. Tomida, Antioxidant protection of human serum albumin by chitosan, Int. J. Biol. Macromol. 43 (2008) 159–164.
[73] M. Anraku, T. Fujii, N. Furutani, D. Kadowaki, T. Maruyama, M. Otagiri, J.M. Gebicki, H. Tomida, Antioxidant effects of a dietary supplement: reduction of indices of oxidative stress in normal subjects by water-soluble chitosan, Food Chem. Toxicol. 47 (2009) 104–109.
[74] M. Anraku, T. Fujii, Y. Kondo, E. Kojima, T. Hata, N. Tabuchi, D. Tshuchiya, T. Goromaru, H. Tsutsumi, D. Kadowaki, T. Maruyama, M. Otagiri, H. Tomida, Antioxidant properties of high molecular weight dietary chitosan in vitro and in vivo, Carbohydr. Polym. 83 (2011) 501–505.
[75] D. Bar-Or, E. Lau, J.V. Winkler, A novel assay for cobalt-albumin binding and its potential as a marker for myocardial ischemia: a preliminary report, J. Emerg. Med. 19 (2000) 311–315.
[76] P.J. Sadler, A. Tucker, J.H. Viles, Involvement of a lysine residue in the N-terminal Ni^2 and Cu^2 binding site of serum albumins: comparison with Co^2, Cd^2, Al^3, Eur. J. Biochem. 220 (1994) 193–200.
[77] D. Roy, J. Quiles, D.C. Gaze, P. Collinson, J.C. Kaski, G.F. Baxter, Role of reactive oxygen species on the formation of the novel diagnostic marker ischaemia modified albumin, Heart 92 (2006) 113–114.
[78] S. Guven, A. Alver, A. Mentese, F.C. Ilhan, M. Calapoglu, M.A. Unsal, The novel ischemia marker 'ischemia-modified albumin' is increased in normal pregnancies, Acta Obstet. Gynecol. Scand. 88 (2009) 479–482.
[79] M.R. Boisvert, K.G. Koski, C.D. Skinner, Increased oxidative modifications of amniotic fluid albumin in pregnancies associated with gestational diabetes mellitus, Anal. Chem. 82 (2010) 1133–1137.
[80] R. Michelis, B. Kristal, T. Snitkovsky, S. Sela, Oxidative modifications impair albumin quantification, Biochem. Biophys. Res. Commun. 401 (2010) 137–142.
[81] K. Taguchi, Y. Urata, M. Anraku, H. Watanabe, K. Kawai, T. Komatsu, E. Tsuchida, T. Maruyama, M. Otagiri, Superior plasma retention of a cross-linked human serum albumin dimer in nephrotic rats as a new type of plasma expander, Drug Metab. Dispos. 38 (2010) 2124–2129.
[82] S. Matsushita, V.T. Chuang, M. Kanazawa, S. Tanase, K. Kawai, T. Maruyama, A. Suenaga, M. Otagiri, Recombinant human serum albumin dimer has high blood circulation activity and low vascular permeability in comparison with native human serum albumin, Pharm. Res. 23 (2006) 882–891.
[83] T. Komatsu, Y. Oguro, Y. Teramura, S. Takeoka, J. Okai, M. Anraku, M. Otagiri, E. Tsuchida, Physicochemical characterization of cross-linked human serum albumin dimer and its synthetic heme hybrid as an oxygen carrier, Biochim. Biophys. Acta 1675 (2004) 21–31.
[84] K. Taguchi, V.T. Chuang, T. Maruyama, M. Otagiri, Pharmaceutical aspects of the recombinant human serum albumin dimer: structural characteristics, biological properties, and medical applications, J. Pharm. Sci. 101 (2012) 3033–3046.
[85] J.S. Stamler, O. Jaraki, J. Osborne, D.I. Simon, J. Keaney, J. Vita, D. Singel, C.R. Valeri, J. Loscalzo, Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 7674–7677.
[86] J.F. Keaney Jr., D.I. Simon, J.S. Stamler, O. Jaraki, J. Scharfstein, J.A. Vita, J. Loscalzo, NO forms an adduct with serum albumin that has endothelium-derived relaxing factor-like properties, J. Clin. Invest. 91 (1993) 1582–1589.
[87] N.N. Orie, P. Vallance, D.P. Jones, K.P. Moore, S-nitroso-albumin carries a thiol-labile pool of nitric oxide, which causes venodilation in the rat, Am. J. Physiol. Heart Circ. Physiol. 289 (2005) H916–H923.
[88] S. Hallstrom, M. Franz, H. Gasser, M. Vodrazka, S. Semsroth, U.M. Losert, M. Haisjackl, B.K. Podesser, T. Malinski, S-nitroso human serum albumin reduces ischaemia/reperfusion injury in the pig heart after unprotected warm ischaemia, Cardiovasc. Res. 77 (2008) 506–514.
[89] S. Semsroth, B. Fellner, K. Trescher, O.Y. Bernecker, L. Kalinowski, H. Gasser, S. Hallstrom, T. Malinski, B.K. Podesser, S-nitrosoalbumin attenuates ischemia/reperfusion injury after prolonged cardioplegic arrest in isolated rabbit hearts, J. Heart Lung Transplant. 24 (2005) 2226–2234.
[90] Y. Ishima, T. Shinagawa, S. Yoneshige, U. Kragh-Hansen, Y. Ohya, Y. Inomata, T. Kai, M. Otagiri, T. Maruyama, UW solution improved with high anti-apoptotic activity by S-nitrosated human serum albumin, Nitric Oxide (Feb 9 2013), pii: S1089-8603(13)00014-1.
[91] N. Katayama, K. Nakajou, H. Komori, K. Uchida, J. Yokoe, N. Yasui, H. Yamamoto, T. Kai, M. Sato, T. Nakagawa, M. Takeya, T. Maruyama, M. Otagiri, Design and evaluation of S-nitrosylated human serum albumin as a novel anticancer drug, J. Pharmacol. Exp. Ther. 325 (2008) 69–76.
[92] Y. Ishima, T. Akaike, U. Kragh-Hansen, S. Hiroyama, T. Sawa, A. Suenaga, T. Maruyama, T. Kai, M. Otagiri, S-nitrosylated human serum albumin-mediated cytoprotective activity is enhanced by fatty acid binding, J. Biol. Chem. 283 (2008) 34966–34975.
[93] Y. Ishima, D. Chen, J. Fang, H. Maeda, A. Minomo, U. Kragh-Hansen, T. Kai, T. Maruyama, M. Otagiri, S-nitrosated human serum albumin dimer is not only a novel anti-tumor drug but also a potentiator for anti-tumor drugs with augmented EPR effects, Bioconjug. Chem. 23 (2012) 264–271.
[94] T. Okamoto, S. Khan, K. Oyama, S. Fujii, T. Sawa, T. Akaike, A new paradigm for antimicrobial host defense mediated by a nitrated cyclic nucleotide, J. Clin. Biochem. Nutr. 46 (2010) 14–19.
[95] Y. Ishima, H. Hoshino, T. Shinagawa, K. Watanabe, T. Akaike, T. Sawa, U. Kragh-Hansen, T. Kai, H. Watanabe, T. Maruyama, M. Otagiri, S-guanylation of human serum albumin is a unique posttranslational modification and results in a novel class of antibacterial agents, J. Pharm. Sci. 101 (2012) 3222–3229.

1521-0103/345/2/271–283$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

http://dx.doi.org/10.1124/jpet.112.201814
J Pharmacol Exp Ther 345:271–283, May 2013

Long-Acting Human Serum Albumin-Thioredoxin Fusion Protein Suppresses Bleomycin-Induced Pulmonary Fibrosis Progression

Ryota Tanaka, Hiroshi Watanabe, Azusa Kodama, Victor Tuan Giam Chuang, Yu Ishima, Keisuke Hamasaki, Ken-ichiro Tanaka, Tohru Mizushima, Masaki Otagiri, and Toru Maruyama

Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences (R.T., H.W., A.K., Y.I., K.H., T.Ma.), and Center for Clinical Pharmaceutical Sciences, School of Pharmacy, Kumamoto University, Chuo-ku, Kumamoto, Japan (H.W., Y.I., T.Ma.); School of Pharmacy, Faculty of Health Sciences, Curtin Health Innovation Research Institute, Curtin University, Perth, Western Australia, Australia (V.T.G.C.); Department of Analytical Chemistry, Faculty of Pharmacy, Keio University, Minato-ku, Tokyo, Japan (K.T., T.Mi.); and Faculty of Pharmaceutical Sciences and DDS Research Institute, Sojo University, Nishi-ku, Kumamoto, Japan (M.O.)

Received November 9, 2012; accepted February 26, 2013

ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is thought to involve inflammatory cells and reactive oxygen species (ROS), such as superoxide anion radical ($O_2^{\cdot-}$). There is currently no effective treatment of IPF. We previously developed a human serum albumin (HSA)–thioredoxin 1 (Trx) fusion protein (HSA-Trx) designed to overcome the unfavorable pharmacokinetic and short pharmacological properties of Trx, an antioxidative and anti-inflammatory protein. In this study, we examined the therapeutic effect of HSA-Trx on an IPF animal model of bleomycin (BLM)-induced pulmonary fibrosis. A pharmacokinetic study of HSA-Trx or Trx in BLM mice showed that the plasma retention and lung distribution of Trxc was markedly improved by fusion with HSA. A weekly intravenous administration of HSA-Trx, but not Trx, ameliorated BLM-induced fibrosis, as evidenced by a histopathological analysis and pulmonary hydroxyproline levels. HSA-Trx suppressed active-transforming growth factor (TGF)-β levels in the lung and inhibited the increase of inflammatory cells in bronchoalveolar lavage fluid, pulmonary inflammatory cytokines, and oxidative stress markers. An in vitro EPR experiment using phosphate-buffered saline–stimulated neutrophils confirmed the $O_2^{\cdot-}$ scavenging ability of HSA-Trx. Furthermore, post-treatment of HSA-Trx had a suppressive effect against BLM-induced fibrosis. These results suggest that HSA-Trx has potential as a novel therapeutic agent for IPF, because of its long-acting antioxidative and anti-inflammatory modulation effects.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic fibrosing interstitial pneumonia with no identifiable cause. It remains a devastating disease, with a 5-year mortality rate of 50% because of its insufficient response to medical therapy. Unfortunately, with the present lack of a complete understanding of the pathogenesis of IPF, the current treatment, which involves the use of steroids and immunosuppressants, does not improve the prognosis and recovery from the acute exacerbation of the disease (American Thoracic Society and the European Respiratory Society, 2000; Luppi et al., 2004). Therefore, the development of new drugs designed to suppress the progression of the disease or to prevent the acute exacerbation of IPF is of great importance.

Recent studies have suggested that oxidative stress plays an important role in the pathogenesis and development of IPF. In fact, increased level of reactive oxygen species (ROS), such as superoxide anion radical ($O_2^{\cdot-}$), and a decrease in the levels of glutathione and superoxide dismutase (SOD) in blood and in bronchoalveolar lavage fluid (BALF) in patients with IPF have been reported (Beeh et al., 2002; Psathakis et al., 2006). In addition, the genetic knockout of NADPH-oxidase, which increases the pulmonary level of $O_2^{\cdot-}$, resulted in the suppression of bleomycin (BLM)-induced pulmonary fibrosis in an IPF animal model. An intratracheal injection of BLM into the lungs of rodents causes alveolar cell damage, an inflammatory response, fibroblast proliferation, and subsequent collagen deposition, resembling human fibrotic lung disease (Moore and Hogaboam, 2008). In contrast, the genetic knockout of extracellular SOD, which decreases pulmonary ROS levels,

This research was supported by Japan Society for the Promotion of Science [Grant-in-Aid for Scientific Research KAKENHI 21390177]; and by Japan Science and Technology Agency, A-step feasibility study program [Grant 10801043].
dx.doi.org/10.1124/jpet.112.201814.

ABBREVIATIONS: 8-OH-dG, 8-hydroxy-2′-deoxygenase; BALF, bronchoalveolar lavage fluid; BLM, bleomycin; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; HSA, human serum albumin; HSA-Trx, human serum albumin-thioredoxin 1 fusion protein; IL, interleukin; IPF, idiopathic pulmonary fibrosis; MDA, malondialdehyde; MIF, migration inhibitory factor; NO_2-Tyr, nitrotyrosine; OCT, optimal cutting temperature; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; SOD, superoxide dismutase; TCA, trichloroacetic acid; TNF, tumor necrosis factor; Trx, thioredoxin 1.

resulted in the progression of the BLM-induced pulmonary fibrosis (Fattman et al., 2003; Manoury et al., 2005). On the other hand, the role of chronic inflammation in the pathogenesis of IPF has been the focus of a number of studies, in view of the presence of interstitial and alveolar inflammatory cells and the expression of inflammatory cytokines in the lungs of patients with IPF (Keane and Strieter, 2002). The findings of a large prospective study examining the histopathologic variability of surgical lung biopsies in 109 patients with IPF suggest an evolving disease process with chronic inflammation playing a pathogenic role (Flaherty et al., 2001). In addition, it has been well established that the levels of inflammatory cytokines and chemokines, especially interleukin (IL)-6, tumor necrosis factor (TNF)-α, and migration inhibitory factor (MIF), are markedly increased in the lungs of BLM-induced disease model animals, and it has also been reported that the knockout of IL-6 or the administration of anti-TNF-α or MIF antibodies suppress BLM-induced lung disorders (Piguet et al., 1989, 1993; Tanino et al., 2002; Chaudhary et al., 2006; Saito et al., 2008). Therefore, drugs with both antioxidative and anti-inflammatory properties would be expected to be useful in the treatment of IPF.

Thioredoxin-1 (Trx) is a small redox-active protein (M_r of ~12 kDa) that is ubiquitously present in the human body and is one of the defense proteins induced in response to various oxidative stress conditions (Holmgren, 1989; Nakamura et al., 2005). In addition to its potent antioxidative effect, which is derived from dithiol-disulfide exchange in its active site, Trx also has anti-inflammatory properties, mainly because of its ability to inhibit neutrophil chemotaxis to inflammatory sites and to suppress the expression and activation of the macrophage MIF (Nakamura et al., 2001; Tamaki et al., 2006). Because of its desirable antioxidative and anti-inflammatory properties, Trx represents a new and potentially effective therapeutic agent for the treatment of IPF. However, because Trx is eliminated extensively via glomerular filtration, its plasma half-life is only approximately 1 hour in mice and 2 hours in rats, which is extremely short in terms of producing a significant therapeutic impact (Nakamura et al., 2001; Ueda et al., 2006). To obtain a satisfactory therapeutic outcome, a sustainable therapeutic concentration of Trx would be needed. To achieve this, constant infusion or frequent repeated administrations of Trx would be required (Hoshino et al., 2003; Liu et al., 2004; Ueda et al., 2006). Hoshino et al. (2003) demonstrated that exogenous recombinant Trx was effective in inhibiting BLM-induced lung damage when administered intraperitoneally or via a continuous infusion of Trx that is repeated at 2-day intervals.

In an attempt to increase the blood retention time of Trx, we recently produced a genetically engineered fusion protein of human serum albumin (HSA) and Trx (HSA-Trx) with use of a *Pichia* expression system. The plasma half-life of the HSA-Trx fusion protein in normal mice was found to be similar to that of HSA, which is 10 times longer than the plasma half-life of Trx (Ikuta et al., 2010). Of interest, HSA-Trx showed a higher distribution to the lungs than did Trx. Therefore, a further attempt will be made to investigate the clinical usefulness of HSA-Trx in treating oxidative stress and inflammation-related lung disorders.

The purpose of this study was to investigate the therapeutic impact of HSA-Trx in the treatment of IPF. With use of the BLM-induced pulmonary fibrosis animal model, the results showed that HSA-Trx could prevent BLM-induced pulmonary fibrosis through its long-acting antioxidative and anti-inflammatory modulation effects.

Materials and Methods

BLM was purchased from Nippon Kayaku (Tokyo, Japan). Mayer's hematoxylin, a 1% eosin alcohol solution, mounting medium for histologic examinations (malinol), and Masson's trichrome staining reagents were from Muto Pure Chemicals (Tokyo, Japan). Optimal cutting temperature (OCT) compound was purchased from Sakura Finetek (Tokyo, Japan). Chloral hydrate, chloramine T, and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (Tokyo, Japan). Paraformaldehyde, trichloroacetic acid (TCA), perchloric acid, and 4-(dimethylamino)-benzaldehyde were from Nacalai Tesque (Kyoto, Japan). Diethylene-triamine-pentaacetic acid was purchased from Dojindo Laboratories (Kumamoto, Japan); 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Alexis Biochemicals (Lausen, Switzerland). The TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems Inc. (Minneapolis, MN). IL-6 and TNF-α ELISA kit were purchased from Biolegend (San Diego, CA). Sea-ICR mice (age, 6 weeks; male) were obtained from Kyudo Co., Ltd. (Saga, Japan). Other chemicals used were obtained from commercial suppliers.

Production of HSA-Trx Fusion Protein. Trx and HSA fusion protein was produced in accordance with the method reported previously (Ikuta et al., 2010; Furukawa et al., 2011) with a slight modification. The transformed *Pichia pastoris* was incubated in 1.25 liters of growth phase media, BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate [pH 6.0], 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 4×10^{-5} % biotin, 1% glycerol) for 2 days (OD_{600} = 2). It was then cultured in 800 ml of protein induction phase media, BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate [pH 6.0], 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 4×10^{-5} % biotin, 1% methanol) for 3 days at 30°C. Methanol was added every 24 hours so that the concentration of methanol was maintained at 1% to sustain the protein expression induction effect. The secreted fusion protein was isolated from the growth media as follows. The solution was loaded on to a column of Blue Sepharose 6 Fast Flow column (GE Healthcare, Tokyo, Japan) equilibrated with 200 mM sodium acetate buffer (pH 5.5) after the medium was dialyzed against the same buffer. The column was washed with ~5 bed volumes of 200 mM sodium acetate buffer (pH 5.5), and then the fusion protein was eluted with 20 mM sodium acetate buffer (pH 6.5) containing 3 M NaCl. Next, with use of AKTA prime, the eluate was loaded onto a column of 5-ml HiTrap Phenyl HP column (GE Healthcare) for hydrophobic chromatography with the following conditions (buffer A, 0 mM Tris-HCl/1.5 M ammonium sulfate [pH 7.0]; buffer B, 50 mM Tris-HCl [pH 7.0]; gradient, 0–100% [buffer B] 100 ml; flow rate, 3 ml/min). The desired fusion protein was obtained by delipidation with activated carbon treatment, as described by Chen (1967). The fusion protein was analyzed using SDS-PAGE and native SDS-PAGE using a 15% polyacrylamide gel, with Coomassie Blue R250 staining. The purity of the fusion protein was estimated to be more than 95% (Ikuta et al., 2010).

Production of BLM-Induced Pulmonary Fibrosis Mice Model. All animal experiments were conducted in accordance with the guidelines of Kumamoto University for the care and use of laboratory animals. BLM-induced pulmonary fibrosis model mice were produced by intratracheal injection of BLM (5 mg/kg) in phosphate-buffered saline (PBS; 1 ml/kg) under anesthesia with chloral hydrate (500 mg/kg) on day 0 (Tanaka et al., 2010). Either Trx or HSA-Trx was administered (but not both) intravenously (3.5 nmol protein in 200 μl PBS/mouse) via the mouse tail vein 30 minutes before BLM treatment (day 0). HSA-Trx was administered intravenously on days 0 and 7 (refer to every 1 week, Fig. 1A) or only on day 0 (refer to every 2 weeks, Fig. 1A). For an intervention study, the first dose of HSA-Trx was administered 1 day after the BLM treatment, and the subsequent dose was administered on day 7 after the BLM treatment. (Fig. 1C).

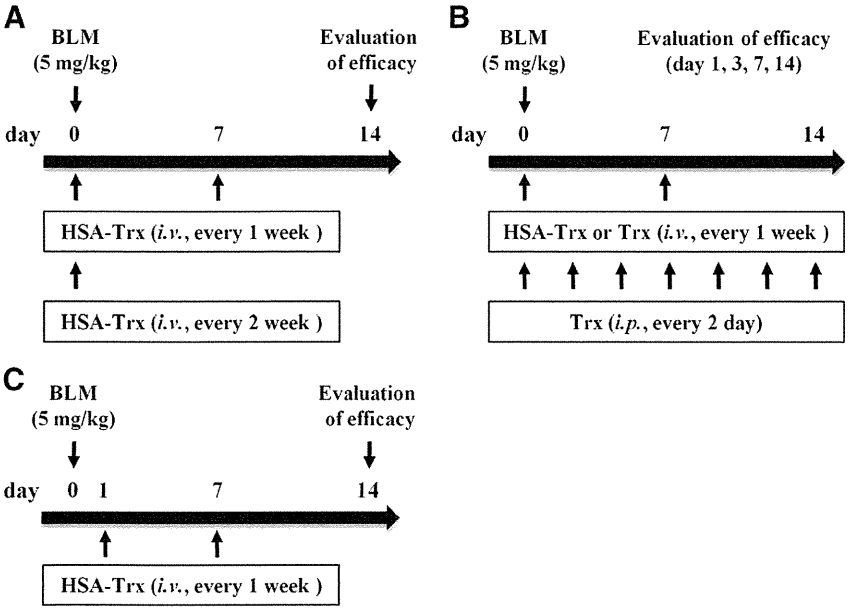

Fig. 1. Schematic summary of the experimental protocol for evaluating the effect of HSA-Trx on BLM-induced pulmonary fibrosis used in the study. HSA-Trx was administrated intravenously on days 0 and 7 (every 1 week; A) or only on day 0 (every 2 weeks; A). On day 0, HSA-Trx was administered 30 minutes before BLM treatment. For the intervention study, the first dose of HSA-Trx was administered 1 day after BLM treatment, and the subsequent dose was administered at day 7 after BLM treatment (C).

Histopathological Analysis of Lung Tissue (Hematoxylin and Eosin Staining and Masson's Trichrome Staining of Collagen). On day 14 after BLM administration, the whole lungs were flushed with 4% paraformaldehyde before being removed. The removed lungs were fixed in 4% buffered paraformaldehyde and then embedded in paraffin before being cut into 4-μm-thick sections. For hematoxylin and eosin (H&E) staining, sections were stained first with Mayer's hematoxylin and then with 1% eosin alcohol solution. For Masson's trichrome staining, sections were sequentially treated with solution A [5% (wt/vol) potassium dichromate and 5% (wt/vol) TCA], Weigert's iron hematoxylin, solution B [1.25% (wt/vol) phosphotungstic acid and 1.25% (wt/vol) phosophomolybdic acid], 0.75% (wt/vol) Orange G solution, solution C [0.12% (wt/vol) xylidine Ponceau, 0.04% (wt/vol) acid fuchsin, and 0.02% (wt/vol) azophloxin], 2.5% (wt/vol) phosphotungstic acid, and finally an aniline blue solution. H&E and Masson's trichrome staining samples were mounted with malinol and inspected using a microscope (BZ-8000; Keyence, Osaka, Japan).

Determination of Lung Fibrosis. Fibrosis score was evaluated (×100) as the quantity of the section positively stained for collagen and displaying alveolar wall thickening (1 = <25%, 2 = 25–50%, 3 = 50–75%, and 4 = 75–100%) (Gibbons et al., 2011). Only fields in which most of the field was composed of alveoli were scored. The entire lung section was analyzed. The investigator was masked to each sample.

Determination of Hydroxyproline Level in Lung Tissues. Hydroxyproline content was determined as described previously (Woessner, 1961). On day 14 after BLM administration, the right lung was removed and homogenized in 1 ml of 5% TCA. After centrifugation, the pellets were hydrolyzed in 0.5 ml of 10 N HCl for 16 hours at 110°C. Each sample was incubated for 20 minutes at room temperature after the addition of 0.5 ml of 1.4% (wt/vol) chloramine T solution and then incubated at 65°C for 10 minutes after addition of 0.5 ml of Ehrlich's reagent (1 M DMBA, 70% [vol/vol] isopropanol and 30% [vol/vol] perchloric acid). The absorbance was measured at 550 nm to determine the amount of hydroxyproline.

Quantification of Activated TGF-β1, IL-6, and TNF-α and Western Blot Analysis of Macrophage MIF in Lung Tissue. On days 3 and 7 after BLM administration, the whole lungs were removed and homogenized in 0.5 ml of buffer (PBS, 1% protease inhibitor cocktail, 10 mM EDTA, 0.05% Tween 20). After centrifugation at 21,000*g* for 10 minutes at 4°C (two times), the supernatants were recovered. The amounts of active TGF-β1 (day 7), IL-6, and TNF-α (days 3 and 7) in the supernatant were measured using ELISA, according to the manufacturer's protocol. In addition, Western blotting of MIF chemokine in the supernatant was performed using the following protocol. After measurement of the protein content with use of the BCA protein assay reagent (Pierce Biotechnology Inc., Rockford, IL), each sample was separated by 12.5% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA) by wet electroblotting. The membranes were blocked for 1 hour at room temperature with 5% skim milk in PBS. The membranes were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and incubated for 2 hours at room temperature with a 400 ng/ml primary mouse monoclonal antibody against N terminus of MIF of human origin, which has cross-reactivity with mouse MIF (sc-271631; Santa Cruz Biotechnology Inc., Santa Cruz, CA) in PBS-T. The membranes were washed 3 times with PBS-T and incubated with the secondary antibody (horseradish peroxidase-linked anti-mouse IgG [H+L]; Invitrogen, Carlsbad, CA) for 1.5 hours at room temperature. The membranes were washed 3 times with PBS-T, and immunoblots were visualized using the SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology Inc.) with LAS-4000EPUVmini (Fujifilm, Tokyo, Japan).

Counting of Cells in BALF. On days 1 and 3 after BLM administration, the mice were anesthetized with pentobarbital, the chests were opened, and blood was drained. BALF samples were collected by cannulating the trachea and lavaging the lung with 1 ml of sterile PBS containing 50 U/ml heparin (two times). Approximately 1.8 ml of BALF was routinely recovered from each animal. The BALF was centrifuged at 4100*g* for 5 minutes at 4°C to separate the cells in the BALF from the liquid. Cells were dissolved in 0.9% NaCl, and lysate was centrifuged again. From the recovered cells, the total cell number was counted using a hemocytometer. Cells were stained with Diff-Quick reagents (Kokusai Shiyaku, Kobe, Japan), and the ratios of alveolar macrophages, neutrophils, and lymphocytes to total cells were determined. More than 200 cells were counted for each sample.

Immunostaining of Lungs Tissue. On day 3 after BLM administration, the whole lungs were flushed with sterile PBS before being removed. The removed lungs were stored in 4% paraformaldehyde at 4°C for 2 hours before being immersed in a 10% sucrose solution overnight. The concentration of sucrose solution was then adjusted to 20% at room temperature, and incubation was continued for another 6 hours. The recovered lungs were covered with OCT compound and frozen at −80°C. Next, the lungs frozen with cryostat (CM3000II; Leica, Wetzlar, Germany) were sliced at a thickness of 4 μm and attached on a glass slide. The slide was then cleansed to remove OCT compound and dried. After drying the slide completely,

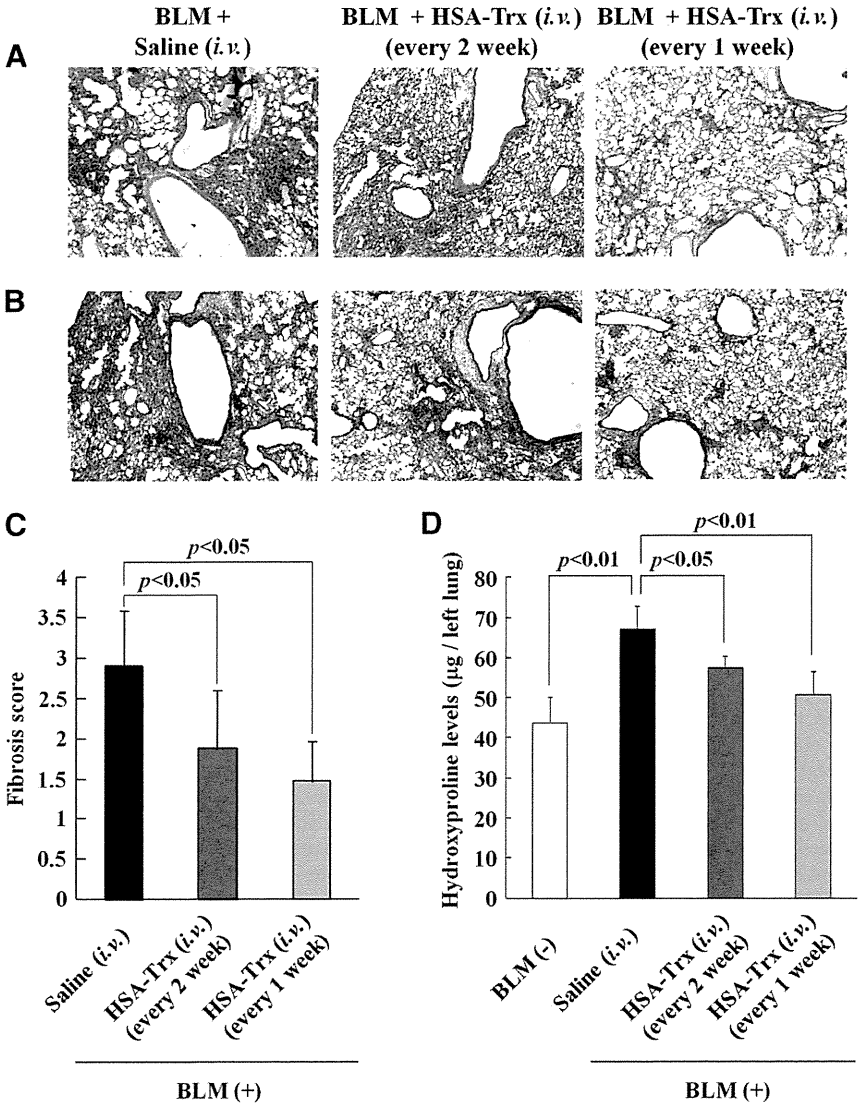

Fig. 2. Effect of HSA-Trx (intravenously; every 2 weeks or every 1 week) on the BLM-induced pulmonary fibrosis. (A and B) Sections of pulmonary tissue were prepared 14 days after BLM administration and subjected to histopathological examination [H&E (A) and Masson's trichrome staining (B)]. (C) Fibrosis score was evaluated as the quantity of the section positively stained for collagen and displaying alveolar wall thickening. (D) Hydroxyproline levels in lung were determined 14 day after BLM administration. Each bar represents the mean ± S.D. (C, n = 3–5; D, n = 5).

a solution containing 50 mM Tris/HCl + 0.1% Tween 20 (T-TB) was used to solubilize the lung slice, followed by blocking with Block Ace (Dainippon Pharmaceutics, Osaka, Japan) at room temperature for 15 minutes. Next, the primary antibody reaction was conducted below 4°C overnight. In addition, the primary mouse monoclonal antibody against 8-hydroxy-2′-deoxygenase (8-OH-dG) [15A3] (sc-66036; Santa Cruz Biotechnology Inc.), which has cross-reactivity with mouse, or the primary rabbit polyclonal antibody against nitrotyrosine (NO_2-Tyr;AB5411; Millipore, Billerica, MA), which has cross-reactivity with mouse, was diluted to 2 or 20 μg/ml before use, respectively. The lung slices were then washed with 50 mM Tris/HCl (TB) and T-TB, followed by the secondary antibody reaction at room temperature for 1.5 hours. For the secondary antibody, in relation to NO_2-Tyr and 8-OH-dG, Alexa Fluor 546 goat anti-mouse IgG (H+L; Invitrogen) and Alexa Fluor 488 goat anti-mouse IgG (H+L; Invitrogen) diluted 200 times were respectively used. After the reaction, the slide was observed using a microscope (BZ-8000; Keyence). Image analyses of the extent and intensity of 8-OH-dG and NO_2-Tyr staining were also performed using ImageJ software.

Determination of Malondialdehyde in Lung Tissue. On day 3 after BLM administration, the right lung was removed and homogenized in 0.5 ml of radioimmunoprecipitation assay/PI buffer (150 mM NaCl, 1% Nonidet P-40, 10 mM Tris-HCl, pH 7.4, including a 1% solution of a protease inhibitor; Nacalai Tesque, Tokyo, Japan). After centrifugation, the amount of malondialdehyde (MDA) in supernatant was measured using thiobarbituric acid reactive substances assay kit (Cayman Chemical, Ann Arbor, MI), which is a well-established method for measuring lipid peroxidation, according to the manufacturer's protocol.

Isolation of Polymorphonuclear Neutrophils. Whole blood was obtained from 10 mice. Heparinized blood was mixed with an equal volume of 3% dextran in 0.9% NaCl. After 30 minutes of gravity sedimentation, the upper layer, containing leukocytes, was removed and centrifuged at 620g for 10 minutes. The cell pellet was resuspended in 0.9% NaCl and underlaid with Ficoll-Paque (GE Healthcare). After centrifugation for 30 minutes at 1490g, the mononuclear cell layer was isolated, and contaminating red blood cells were removed by hypotonic lysis. After centrifugation for 10 minutes at 760g (two times), the pellet was resuspended, and the neutrophils were plated at 1.0×10^6 cells/ml in PBS.

Measurement of Neutrophil-Derived ROS. The scavenging activity of HSA-Trx against $O_2^{\cdot-}$ released from neutrophils was determined using EPR spin trapping with DMPO. The neutrophils (1.0×10^6 cells/ml) were pretreated with PMA (1 μg/ml) for 7 minutes at 37°C to activate the cells and generate ROS. Aliquots of this cell suspension were combined with 100 μM DTPA in Hanks' balanced salt solution in the absence or presence of either HSA-Trx (10, 30, 50 μM), HSA (30 μM), or Trx (30 μM). After activation of neutrophils,

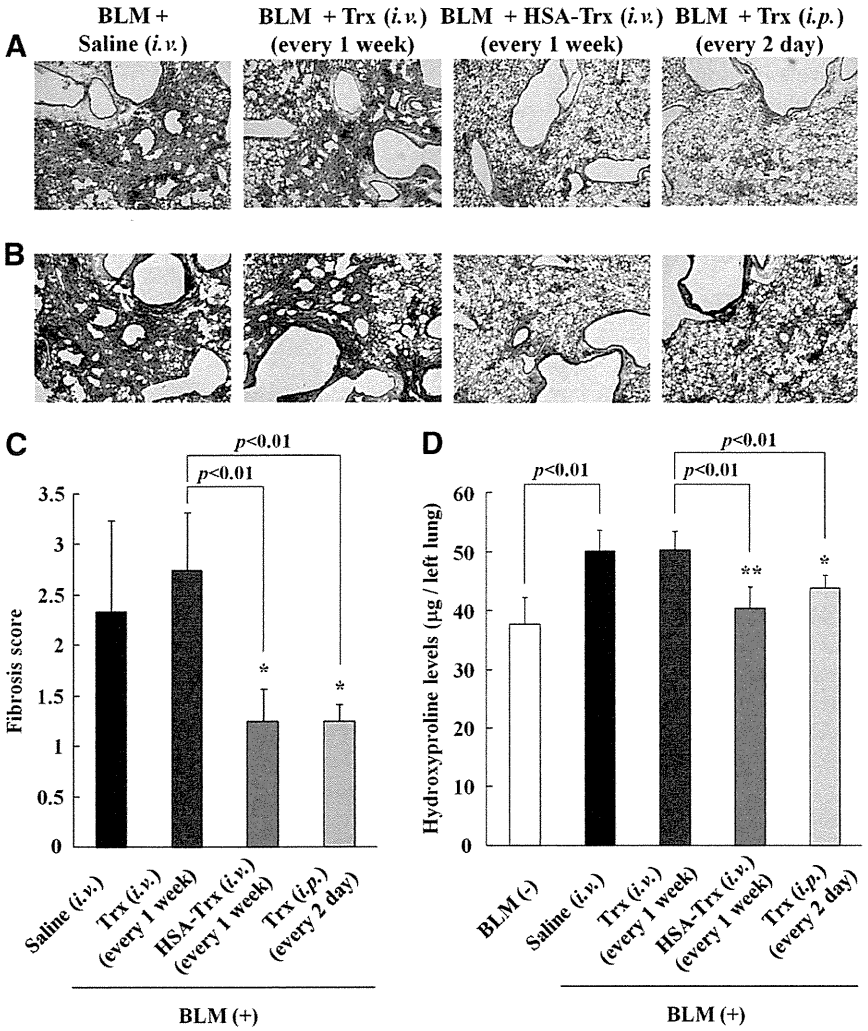

Fig. 3. Effects of HSA-Trx (intravenously; every 1 week), Trx (intravenously; every 1 week), or Trx (intraperitoneally; every 2 days) on the BLM-induced pulmonary fibrosis. (A and B) Sections of pulmonary tissue were prepared 14 days after BLM administration and subjected to histopathological examination ([A] H&E and [B] Masson's trichrome staining). (C) Fibrosis score was evaluated as the quantity of the section positively stained for collagen and displaying alveolar wall thickening. (D) Hydroxyproline levels in lung were determined 14 days after BLM administration. Each bar represents the mean ± S.D. (C, $n = 4$; D, $n = 5$). $^{**}P < 0.01$; or $^{*}P < 0.05$ versus BLM(+), saline (i.v.).

this reaction mixture was added with DMPO (27 mM). After 6 minutes of addition of DMPO, EPR spectra were recorded at room temperature in a JES-TE-200 spectrometer (JEOL, Tokyo, Japan) under the following conditions: modulation frequency, 100 kHz; microwave frequency, 9.43 GHz; microwave power, 40 mW; scanning field, 335.2 ± 5 mT; sweep time, 2 minutes; field modulation width, 0.25 mT; receiver gain, 630; and time count, 0.3 seconds.

Radiolabeling of Proteins with ^{125}I. HSA-Trx and Trx were radiolabeled with ^{125}I according to the procedures reported previously (Watanabe et al., 2001; Furukawa et al., 2011) and purified using a Pharmacia Bio-Gel PD-10 column. The radiolabeled proteins were diluted with nonlabeled protein before conducting the pharmacokinetic experiments to adjust the dose (mg/kg) of protein in each group.

Pharmacokinetics of Trx and HSA-Trx in BLM Mice. On day 14 after BLM administration, the ^{125}I proteins (0.1 mg/kg) were injected into the tail vein of mice (~10^5 cpm/mouse). Approximately 500 μl of blood was collected from the vena cava at 0.05, 0.167, 0.5, 1, 2, 4, 6, and 12 hours after the injection of these radiolabeled proteins with the mice under ether anesthesia, and plasma was obtained. Degraded proteins and free ^{125}I were removed from plasma by centrifugation in 1% bovine serum albumin and 40% TCA. At each of these time points, the mice were sacrificed. The organs were rinsed with saline and weighed, and the ^{125}I radioactivity contained in each tissue was determined using a gamma-counter (ARC-5000; Hitachi Aloka Medical, Tokyo, Japan).

Data Analysis. Noncompartment model was used in the pharmacokinetic analyses after ^{125}I proteins administration. Each parameter was calculated using the program MOMENT (BS) (Tabata et al., 1996). Data are means ± S.D. for the indicated number of animals. Significant differences among each group were examined using a one-way of analysis of variance followed by Tukey's multiple comparison. A probability value of $P < 0.05$ was considered to indicate statistical significance.

Results

Evaluation of the Administration Schedule of HSA-Trx in BLM-Induced Pulmonary Fibrosis. Pulmonary fibrosis was induced in mice by a single intratracheal administration of BLM (day 0). Because Hoshino et al. (2003) found that the multiple administration of Trx (3.5 nmol/body) prevented BLM-induced pulmonary fibrosis, we adopted a dose of HSA-Trx equivalent to one Trx treatment (3.5 nmol/body). Figure 1 shows a schematic summary of the experimental protocols used in the study. The effect of HSA-Trx on BLM-induced pulmonary fibrosis was evaluated using H&E staining (Fig. 2A), Masson's trichrome staining (Fig. 2B), the quantity analysis of the section positively stained for collagen (Fig. 2C), and the measurement of hydroxyproline levels in lung tissue (Fig. 2D) on day 14.

H&E staining showed that BLM administration induced severe lung damage (thickened and edematous alveolar walls

and interstitia) and the infiltration of inflammatory cells into these regions (Fig. 2A). Masson's trichrome staining of collagen and the measurement of hydroxyproline levels in lung tissue indicated that BLM induced the deposition of collagen in lung (Fig. 2, B–D). These phenomena induced by BLM were all significantly suppressed by the HSA-Trx treatment. In addition, the administration of HSA-Trx at weekly intervals had a more potent effect than its use at 2-week intervals (Fig. 2, A–D). Therefore, HSA-Trx was administered once per week in the subsequent experiments.

Effect of HSA-Trx on Histologic Alterations and Hydroxyproline Levels in Lung Tissue. Hoshino et al. (2003) demonstrated that, when Trx is administered every 2 days, it prevents BLM-induced pulmonary fibrosis. Thus, we compared the effect of the intravenous administration of HSA-Trx at weekly intervals with that for the intravenous administration of Trx at weekly intervals or the intraperitoneal administration of Trx at 2-day intervals (Fig. 1B).

H&E staining (Fig. 3A), Masson's trichrome staining (Fig. 3B), the quantity analysis of the section positively stained for collagen (Fig. 3C), and hydroxyproline levels in lung tissue (Fig. 3D) clearly indicated that HSA-Trx, when administered at 1-week intervals, significantly suppressed the lung injury and fibrosis in diseased model mice. Intraperitoneal administration of Trx at 2-day intervals resulted in similar therapeutic effects as those for the HSA-Trx treatment at weekly intervals, but intravenous administration of Trx at weekly intervals showed no suppressive effect.

Effect of HSA-Trx on Active TGF-β1 Levels in Lung Tissue. TGF-β1 plays an important role in BLM-induced pulmonary fibrosis (Kinnula et al., 2005; Chaudhary et al., 2006). To reveal the mechanism underlying the suppressive effect of HSA-Trx on BLM-induced pulmonary fibrosis, the levels of active TGF-β1 in lung tissue on day 7 were determined. As shown in Fig. 4, the level of active TGF-β1 was increased in BLM mice [BLM(+), saline (i.v.)]. In contrast, HSA-Trx decreased the level of active TGF-β1 to the same level as the normal group [BLM(−)].

Effect of HSA-Trx on BALF Cells. To evaluate the effects of HSA-Trx on the inflammatory response induced by BLM, the cells in BALF were analyzed. As shown in Fig. 5, the administration of BLM resulted in an increase in the number of inflammatory cells (Fig. 5A), including alveolar macrophages (Fig. 5B), neutrophils (Fig. 5C), and lymphocytes (Fig. 5D) on days 1 and 3 after BLM administration. The HSA-Trx treatment significantly reduced the number of total cells and neutrophils on both days 1 and 3 and alveolar macrophages and lymphocytes on day 3. These results suggest that HSA-Trx ameliorates the BLM-induced pulmonary inflammatory response.

Effect of HSA-Trx on Inflammatory Cytokines and Chemokine Levels in Lung Tissue. We also examined the effect of HSA-Trx on IL-6, TNF-α, and MIF levels in the lung tissue of BLM-induced pulmonary fibrosis on days 3 and 7. As shown in Fig. 6, the levels of IL-6 (Fig. 6A), TNF-α (Fig. 6B), and MIF (Fig. 6, C and D) in lung tissue that were increased by BLM were significantly decreased as the result of the HSA-Trx treatment. These data suggest that HSA-Trx exerts an anti-inflammatory action against BLM-induced pulmonary damage.

Effect of HSA-Trx on Oxidative Stress in Lung Tissue. Recently reported findings suggest that ROS released from activated leukocytes, especially alveolar macrophages and neutrophils,

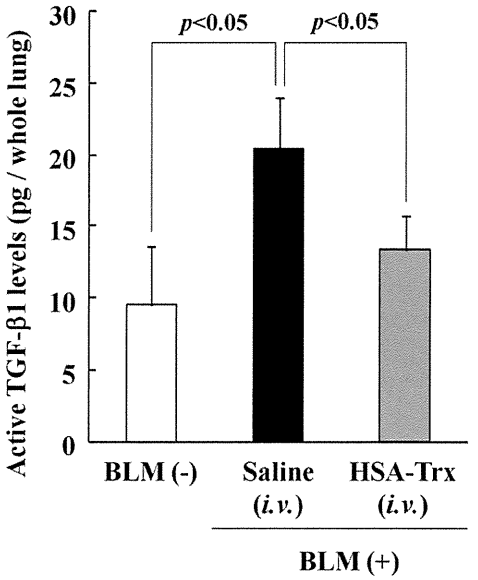

Fig. 4. Effect of HSA-Trx on active TGF-β1 levels in BLM mouse lung. Active TGF-β1 levels in lung were determined 7 days after BLM administration. Each bar represents the mean ± S.D. (n = 5).

are associated with the development of BLM-induced lung injury (Manoury et al., 2005). To evaluate the effect of HSA-Trx on the oxidative stress induced by BLM in the lung, immunostaining of 8-OH-dG and NO_2-Tyr, oxidized product of nucleic acids and proteins, respectively, and also quantification of lipoperoxidation final reaction substances, MDA, in lung sections were performed on day 3 after BLM administration. As shown in Fig. 7, the accumulation of 8-OH-dG, NO_2-Tyr, and MDA in lung tissue increased in BLM-mice [BLM(+), saline (i.v.)], compared with normal mice [BLM(−)], whereas HSA-Trx clearly suppressed the levels of these oxidative stress markers in the lungs.

To confirm whether HSA-Trx shows scavenging activity against $O_2^{\cdot -}$ generated from neutrophils, we conducted ex vivo EPR spectroscopy with use of a DMPO spin-trapping technique. As shown in Fig. 8, although PMA-stimulated neutrophils generated $O_2^{\cdot -}$ and increased EPR signaling, HSA-Trx significantly decreased the EPR signaling in a concentration-dependent manner (Fig. 8, A and B). We also compared the $O_2^{\cdot -}$ scavenging activity among HSA-Trx, Trx, and HSA. At a 30 μM concentration of each protein, HSA-Trx and Trx significantly decreased the EPR signal by 40 and 85%, respectively, whereas HSA alone did not significantly change the intensity of the signal (Fig. 8, C and D).

Effect of Post-Treatment of HSA-Trx on BLM-Induced Pulmonary Fibrosis. For future clinical applications, the postadministration effect of HSA-Trx against BLM-induced fibrosis was examined (Fig. 1C). Because HSA-Trx suppressed ROS production by neutrophils (Fig. 8), the effect of the postadministration of HSA-Trx was examined at 1 and 7 days after BLM treatment, when a marked increase in neutrophils in BALF was observed (Fig. 5C). The results of H&E staining (Fig. 9A), Masson's trichrome staining (Fig. 9B), the quantity analysis of the section positively stained for collagen (Fig. 9C), and the determination of hydroxyproline levels (Fig. 9D) on day 14 showed that the post-treatment of HSA-Trx suppressed the progression of BLM-induced fibrosis.

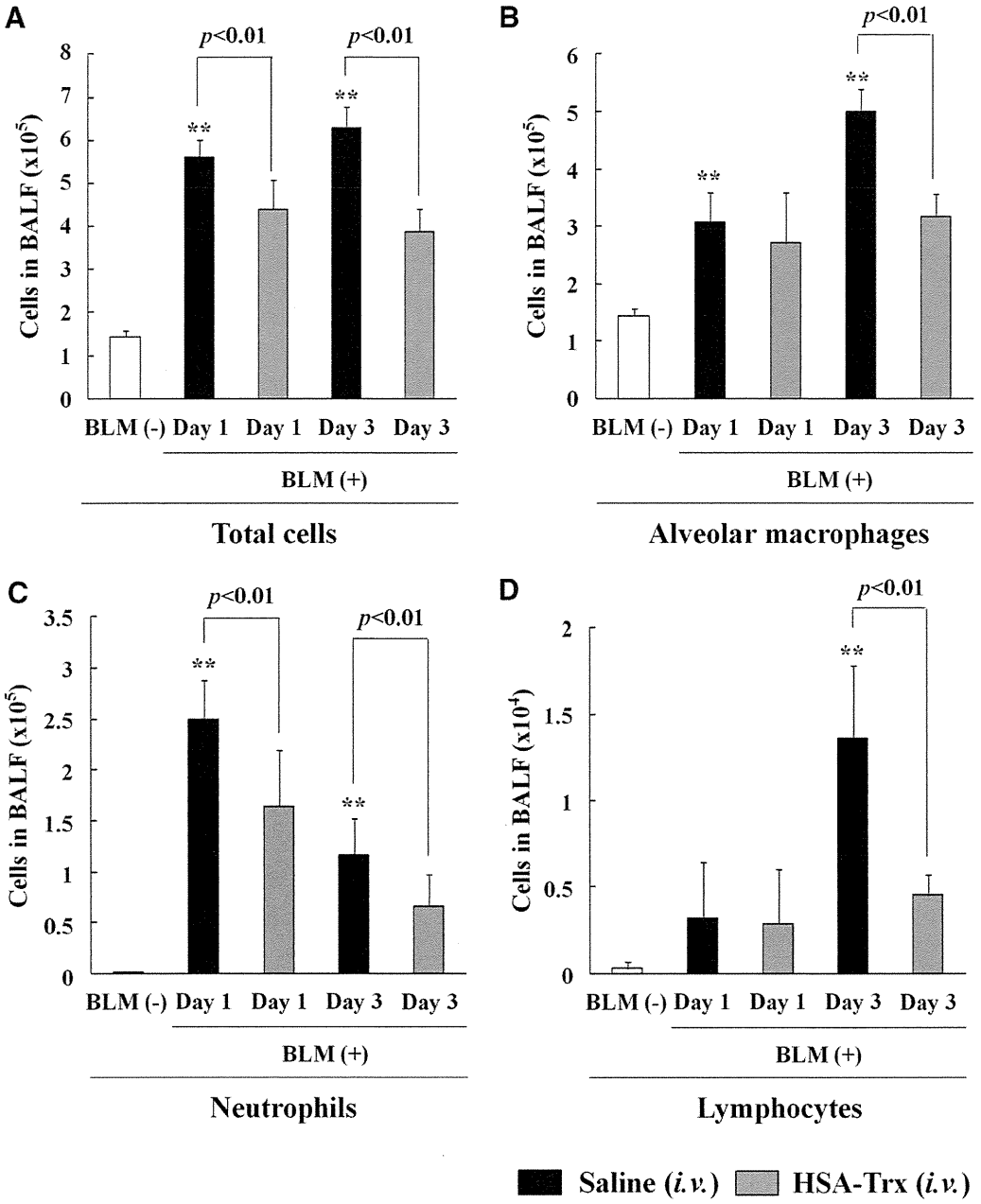

Fig. 5. Effect of HSA-Trx on BALF cells in BLM mice. Inflammatory cells number, including total cells (A), alveolar macrophages (B), neutrophils (C), and lymphocytes (D), was determined 1 and 3 days after BLM administration. Each bar represents the mean ± S.D. ($n = 6$). $^{**}P < 0.01$ versus BLM(−).

Pharmacokinetics of HSA-Trx in BLM Mice. A pharmacokinetics study of ^{125}I-labeled HSA-Trx or Trx was performed using BLM mice. As shown in Fig. 10, no significant difference between the plasma concentration-time profiles for HSA-Trx in BLM(+) mice and that in BLM(−) mice were found. The half-life of HSA-Trx was approximately 8 hours in both BLM (8.45 ± 0.85 hours) and normal (7.89 ± 0.23 hours) mice, which is more than 10 times longer than that of Trx (0.50 ± 0.02 hours) in the model mice (Fig. 10A). In addition, no significant difference was observed in the tissue distribution patterns of the HSA-Trx in BLM and normal mice, in which the highest concentration was found in the plasma, followed by the kidney, lung, spleen, and liver (Fig. 10B). On the other hand, the tissue distribution of Trx in the kidneys was the highest in BLM mice (Fig. 10B). These pharmacokinetic properties of HSA-Trx or Trx alone were similar to previously reported results using normal and ovalbumin-induced lung injury mice (Ikuta et al., 2010; Furukawa et al., 2011).

Discussion

IPF is a refractory lung disorder for which no effective treatment is available. Trx was recently shown to be present at higher concentrations in the serum and lungs of patients with IPF, compared with healthy subjects (Iwata et al., 2010). In addition, in vivo and in vitro investigations have shown that BLM treatment caused Trx to be expressed at high levels

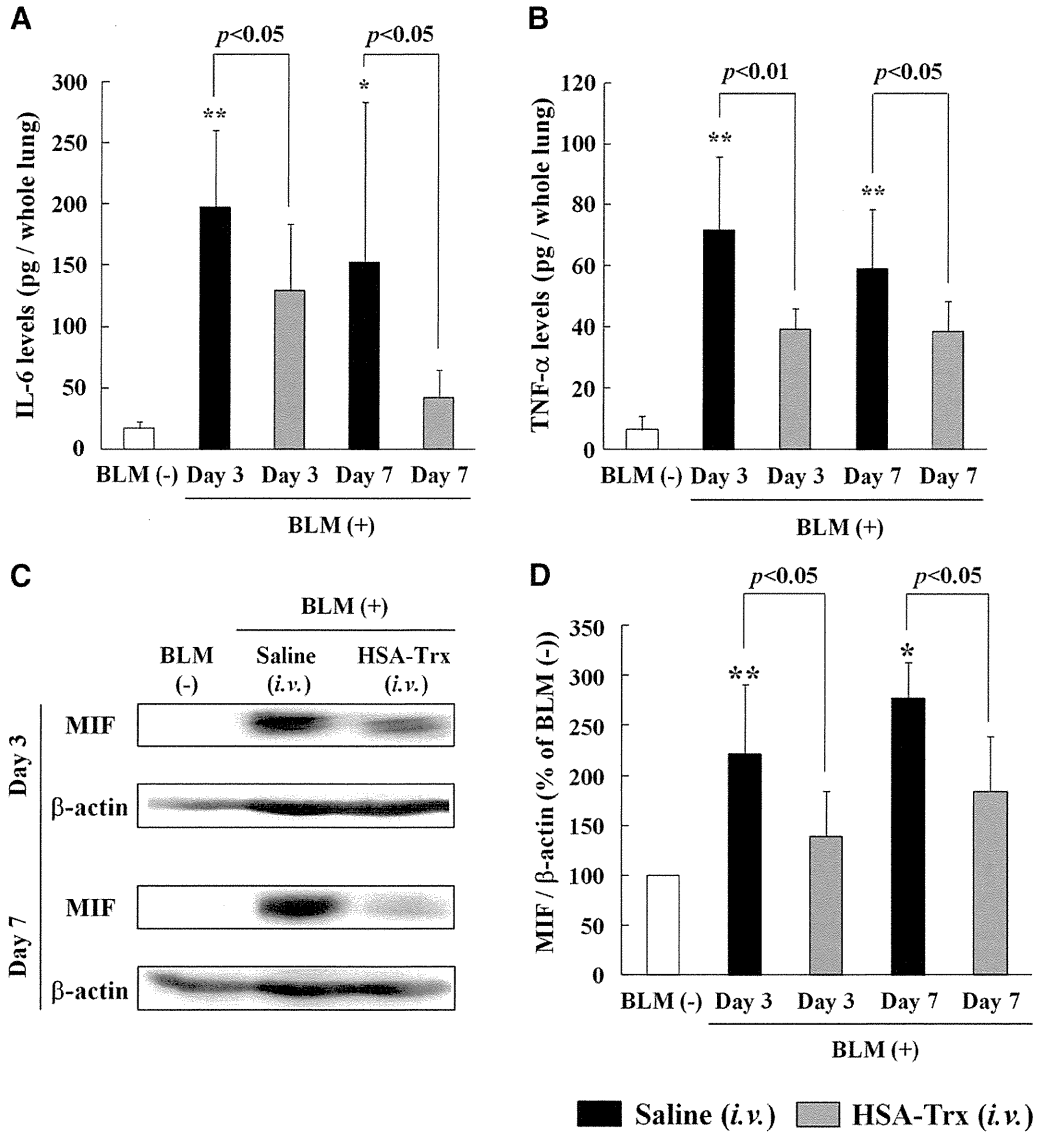

Fig. 6. Effect of HSA-Trx on pulmonary inflammatory cytokines and chemokine in BLM mice. IL-6 levels (A) and TNF-α levels (C) in lung were determined, and (C and D) MIF expression in lung were confirmed and analyzed 3 and 7 days after BLM administration. Each bar represents the mean ± S.D. (A and B, n = 5–6; D, n = 3–4). $^{**}P < 0.01$; or $^{*}P < 0.05$ versus BLM(−).

in bronchial epithelial cells, although no other endogenous antioxidative proteins (i.e., Cu/Zn-SOD, Mn-SOD, catalase, and glutathione peroxidase) were induced (Gon et al., 2001). These collective findings suggest that Trx may play an important role in the protection of lungs in patients with IPF. In this study, we evaluated the therapeutic effects of HSA-Trx on mice with BLM-induced pulmonary fibrosis and obtained four important findings. First, HSA-Trx suppressed the progression of pulmonary fibrosis when given once weekly. Second, the mechanism by which HSA-Trx inhibits lung damage induced by BLM can be attributed to the long-acting antioxidative and anti-inflammatory modulation effects of Trx. Third, the postadministration of HSA-Trx was effective in suppressing lung damage. Fourth, in the BLM-induced pulmonary fibrosis animal model, HSA-Trx exhibited similar pharmacokinetic properties to that in healthy animals (e.g., increased blood retention and enhanced distribution to the lungs).

Because the BLM-induced lung disorder animal model used in this study manifested lung disorders with a greater severity than did other conventional pathologic models, it is possible that the pathologic conditions contributed to the changes in the pharmacokinetics of HSA-Trx. However, the administration of BLM and the subsequent pulmonary fibrosis did not change the pharmacokinetic behavior of HSA-Trx (Fig. 10). This observation led to assumption that HSA-Trx could be used as a long-acting Trx that will persistently exhibit a lung-protective action in the BLM-induced lung disorders animal model. In fact, consistent with the results reported by Hoshino et al. (2003), we found that the intravenous administration of Trx at weekly intervals did not prevent pulmonary fibrosis because of its short half-life, whereas the once weekly intravenous administration of HSA-Trx suppressed the progression of fibrosis to the same extent as the intraperitoneal administration of Trx at 2-day intervals (Fig. 3). Such a reduction in the dose-frequency of Trx by fusing the protein

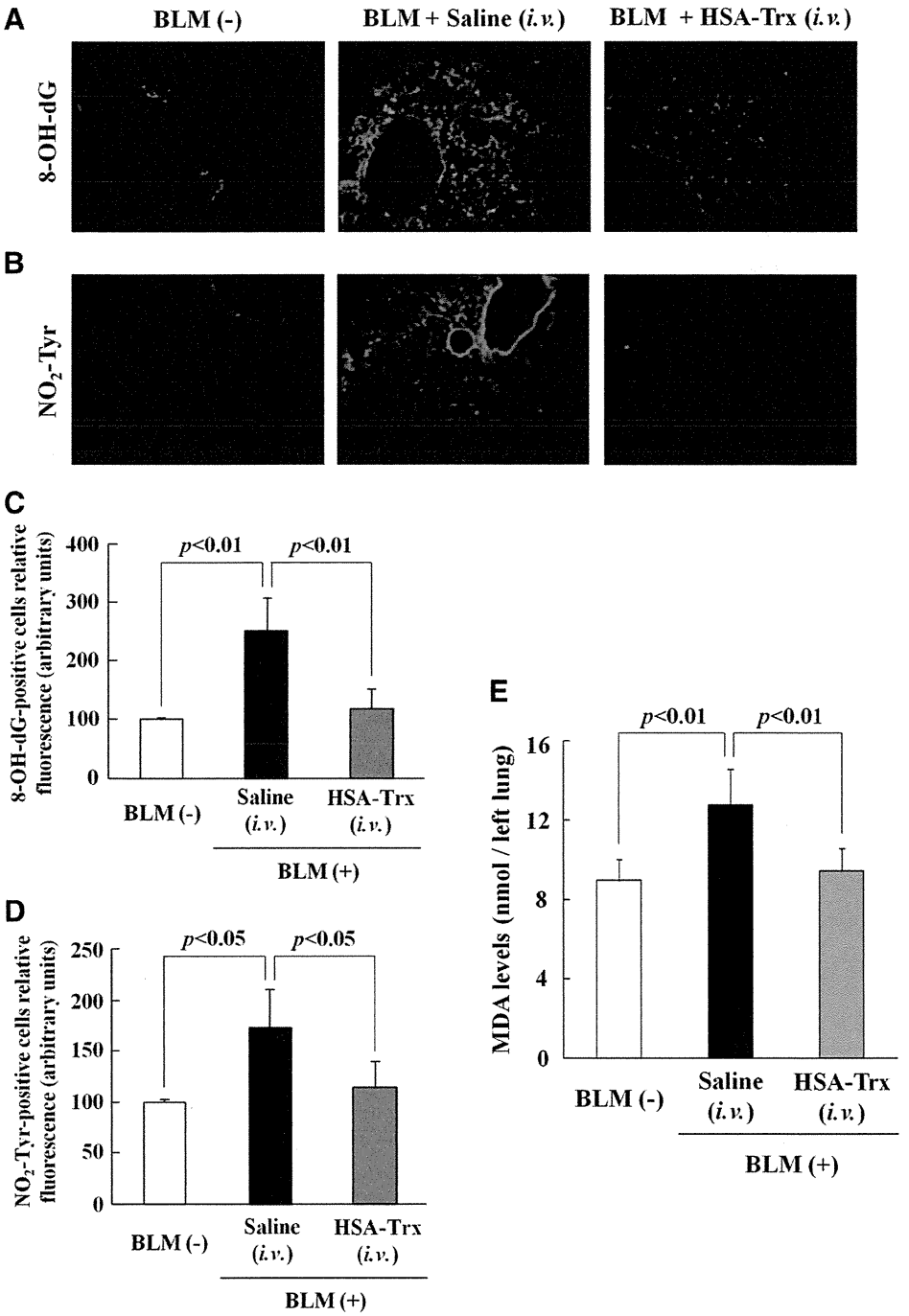

Fig. 7. Effect of HSA-Trx on pulmonary oxidative stress in BLM mice. The immunostaining of the frozen lungs slice for the oxidative stress markers of nucleic acid 8-OH-dG and amino acid (A) NO_2-Tyr (B) were performed 3 days after BLM administration. Image analysis of the extent and intensity of 8-OH-dG (C) and NO_2-Tyr (D) staining was performed. (E) The MDA levels in lung were determined 3 days after BLM administration. Each bar represents the mean ± S.D. (C and D, n = 4; E, n = 6).

to HSA is a favorable outcome from the view point of clinical applications. To the best of our knowledge, this is the first demonstration of HSA-Trx being used as an effective long-acting therapeutic agent against BLM-induced lung disordered models.

BLM-induced pulmonary fibrosis is a pathologic condition in which ROS is produced at an early stage of BLM treatment, serves as a key trigger to cause the further production of ROS that, in turn, promotes inflammatory cell infiltration and the associated secretion of inflammatory cytokines, resulting in the progression of lung injury and inflammation and, eventually, fibrosis. The suppressive effect of HSA-Trx on BLM-induced pulmonary fibrosis could be attributable to the antioxidative activity of Trx, because Trx exhibits superior antioxidative characteristics via a redox reaction of SH and S-S at its active site (Holmgren, 1989). In fact, we found that HSA-Trx significantly suppressed the accumulation of oxidative stress markers in the lungs (8-OH-dG, NO_2-Tyr and MDA) at day 3 of a BLM treatment (Fig. 7). Another investigation involving EPR experiments using a DMPO spin trapping agent examined the direct scavenging capacity of HSA-Trx for neutrophil-derived ROS, revealing the concentration-dependent suppression of $O_2^{\cdot -}$ production by HSA-Trx (Fig. 8).

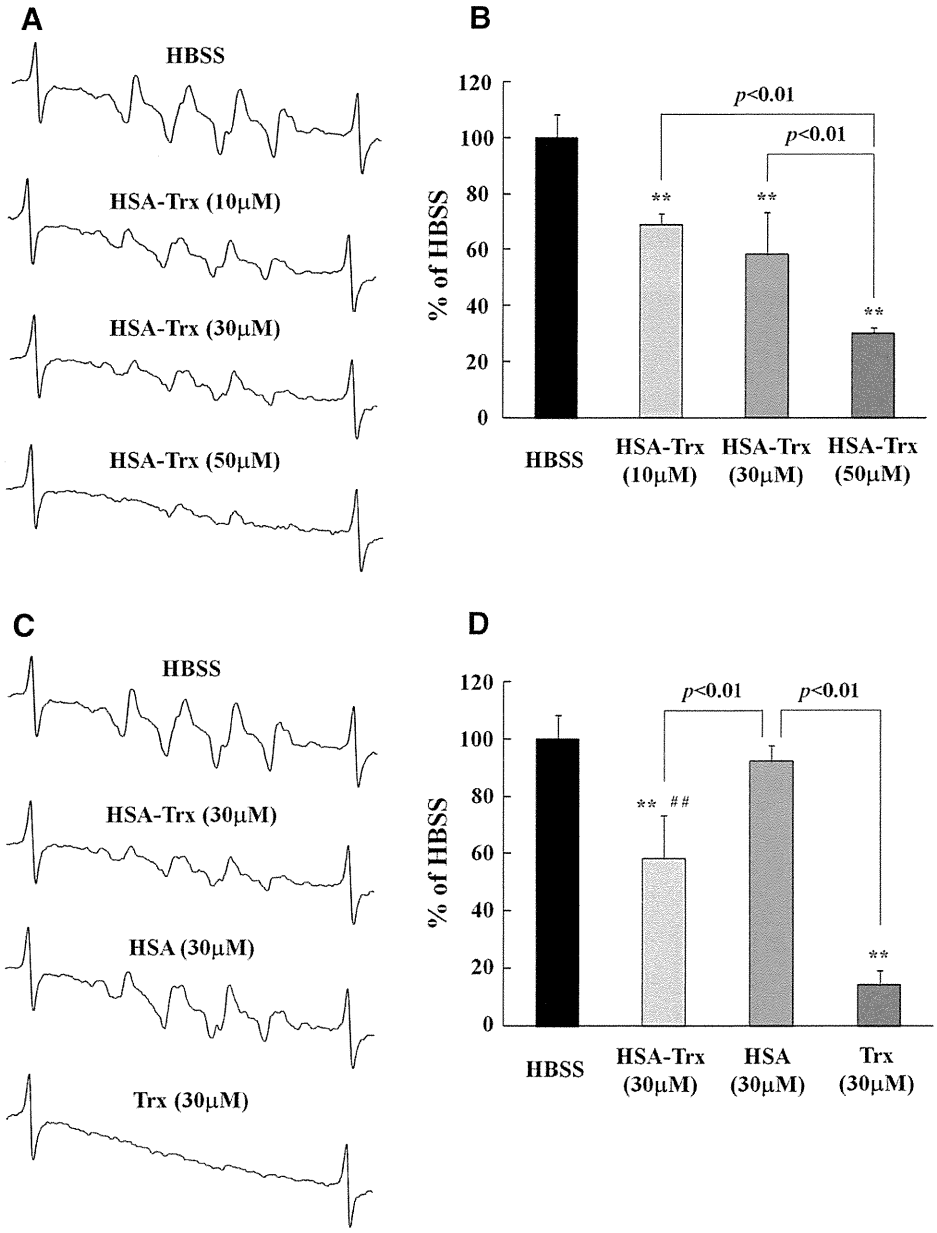

Fig. 8. Effect of HSA-Trx on neutrophil-derived ROS using EPR spectroscopy. The concentration-dependent scavenging activity of HSA-Trx against ROS released from neutrophils was determined using EPR spin trapping with DMPO. EPR spectrum of DMPO spin adducts of HO (A), and the quantitation of the HO concentration (B) were shown. The scavenging activity of HSA-Trx, HSA, and Trx (30 μM) against ROS released from neutrophils was determined by EPR spin trapping with DMPO. EPR spectrum of DMPO spin adducts of HO (C), and the quantitation of the HO concentration (D) were shown. Each bar represents the mean ± S.D. (n = 3). **P < 0.01 versus Hanks' balanced salt solution (HBSS); or ##P < 0.01 versus Trx.

Inflammatory cells, such as activated leukocytes, especially neutrophils, are considered to be major contributors to the onset and progression of IPF and BLM-induced pulmonary fibrosis because of their dramatic increases at the beginning of inflammation (Fig. 5C) and subsequent release of a large amount of $O_2^{\cdot-}$ (Manoury et al., 2005; Kinder et al., 2008). A comparison of the neutrophil-produced $O_2^{\cdot-}$ scavenging capacities of HSA-Trx and Trx showed that HSA-Trx retained ~ 50% of the specific activity of Trx (Fig. 8). This reduced activity of HSA-Trx is likely to be attributable to microenvironmental changes in the active center of Trx or steric hindrance as the result of the formation of a fusion complex (Muller et al., 2007).

TGF-β1 promotes interstitial collagen production via fibroblast activation and epithelial cell transition to mesenchymal cells (epithelial-mesenchymal transition) (Willis and Borok, 2007). A recent in vitro study showed that ROS and inflammatory cytokines promoted TGF-β1 production by pulmonary epithelial cells and its activation (Barcellos-Hoff and Dix, 1996; Bellocq et al., 1999). Because HSA-Trx scavenges ROS, it should be able to suppress the production of active TGF-β1 in lung tissue. The findings reported here indicate that HSA-Trx significantly reduced the active TGF-β1 content in lung tissue induced by the BLM treatment (Fig. 4). Therefore, the results suggest that the suppression of ROS production and inflammatory cell infiltration at an early-stage of BLM treatment by HSA-Trx eventually led to the suppression of active TGF-β1 production.

Active TGF-β1, in turn, activates NADPH oxidase in fibroblasts, thereby enhancing ROS production and downregulating the expression of glutamate cysteine ligase (Thannickal and Fanburg, 1995; Arsalane et al., 1997), resulting in aggravation of the redox balance in the body. It is therefore necessary to block the vicious circle of oxidative stress caused by active TGF-β1 to suppress BLM-induced pulmonary fibrosis.