Free Radical Research, 2003 Vol. 37 (5), pp. 537–541

Taylor & Francis
healthsciences

Thiols are Main Determinants of Total Antioxidant Capacity of Cellular Homogenates

ANETA BALCERCZYK[a,*] and GRZEGORZ BARTOSZ[a,b]

[a]*Department of Molecular Biophysics, University of Łódź, 90-237 Łódź, Poland;* [b]*Department of Biochemistry and Cell Biology, University of Rzeszów, Rzeszów, Poland*

Accepted by Professor T. Grune

(Received 14 August 2002; In revised form 6 December 2002)

While the total antioxidant capacity (TAC) of blood plasma is mainly accounted for by urate, TAC of cell interior can be expected to depend more on other antioxidants, especially glutathione and protein –SH groups. We studied TAC of homogenates of several lines of cultured cells subjected to the action of thiol-modifying agents. Comparison of changes of TAC of the homogenates and of the level of total thiols (determined with a biradical spin label) demonstrates that alterations in cellular thiol content is the main determinant of changes of TAC of cell homogenates. These results show that estimation of TAC of cell extracts may be a useful parameter of assessment of oxidative stress, primarily of oxidation of thiol groups, yielding information different than TAC of body fluids.

Keywords: Total antioxidant capacity; Thiol; Antioxidants; Electron spin resonance

INTRODUCTION

Homeostasis at the cellular and organismal level includes equilibrium between the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and reactions of antioxidants maintaining appropriate levels of ROS and RNS, and minimizing their unspecific reactions with vital biomolecules. This class of compounds includes various hydrophilic and hydrophobic low-molecular weight substances such as glutathione, ascorbic acid (vitamin C), uric acid, tocopherols (vitamin E), carotenoids, coenzyme Q, bilirubin, and some amino acids (like cysteine, methionine or tyrosine) and a plethora of polyphenolic compounds in plants. Many unwanted biological oxidations proceed via a free-radical pathway, therefore these compounds are often referred to as "free-radical scavengers". However, at least some of these compounds are also effective protectors against non-radical ROS/RNS like peroxynitrite or hypochlorite.[1,2] Some amino acid residues built into proteins also exert antioxidant action. Proteins are, therefore, also antioxidants although in many cases one may ask whether we deal with protection by or rather damage to a protein. The sum of antioxidant activities of antioxidants is often referred to as total antioxidant capacity (TAC).

TAC of body fluids, especially blood plasma or serum, has been the subject of numerous studies.[3–7] They have demonstrated that TAC may be altered (generally decreased) in various diseases, and also in physiological states involving oxidative stress.[8–12] Intracellular composition of antioxidants differs greatly from that of extracellular fluids; therefore, determination of TAC of cell or tissue homogenates can be expected to provide additional information on the antioxidative status of the body, with respect to that of TAC of extracellular fluids. Extracellular practically lack the main intracellular redox buffer viz. the glutathione/glutathione disulfide couple.[13] Glutathione is present only in micromolar amounts in blood plasma and in millimolar amounts inside the cells. The oxidation state of protein thiols may be regulated differently inside and outside cells due to

*Corresponding author. Fax: +48-42-6354473. E-mail: kbm@biol.uni.lodz.pl

ISSN 1071-5762 print/ISSN 1029-2470 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1071576031000083189

the practical absence of appropriate enzymatic systems like the thioredoxin and glutaredoxin systems in the extracellular medium.[14] Antioxidants such as NADPH and NADH[15] are present only intracellularly.

The high intracellular concentration of glutathione and protein thiols can be expected to make them the main intracellular antioxidants. The aim of this paper was to verify this assumption by comparing the thiol content and TAC of homogenates of several cell lines.

MATERIALS AND METHODS

The bis-(2,2,5,5)-tetramethyl-3-imidazoline-1-oxyl-4-yl) disulfide biradical (RSSR) spin label was obtained from Alexis. All other reagents were from Sigma (Poznań) and were of analytical grade.

Fibroblast Cell Monolayers

V79 (Chinese hamster lung fibroblasts) cells and V79 transfected with human multidrug resistance protein (MRP1) cDNA[16] were grown in Dulbecco's modified Eagle medium (DMEM) containing glucose (4500 mg/l), sodium pyruvate and pyridoxine. Growth media were supplemented with 10% v/v heat-inactivated bovine calf serum, penicillin (100 U/l) and streptomycin (100 mg/l). MRP1-transfected cells were grown in the presence of Zeocin® (0.25 mg/ml), an antibiotic from the bleomycin family, as a selecting agent.

V79 and V79MRP1 cells were used for the experiments when they had formed confluent monolayers in culture dishes (7.06 cm^2). The medium from confluent cultures was removed and fresh medium containing different concentrations of thiol-reactive compounds (diamide or *N*-ethylmaleimide; NEM) or agents inducing oxidative stress (paraquat or *tert*-butyl hydroperoxide; *t*BOOH) was added. After 1-h incubation at 37°C, the medium was removed from cell culture dishes and the monolayers were rinsed two times with phosphate-buffered saline (PBS). Trypsin was added to the monolayers to the concentration of 0.1%. After 3 min incubation the cells were suspended in 1 ml of fresh medium and centrifuged (600*g*, 3 min). Then cells were rinsed two times with PBS and resuspended in 200 μl PBS. Then the cells were used to the evaluation of TAC and thiol content.

Suspension Cells

HL60 (human promyelocytic leukemia cells) were grown in RPMI 1640 medium with Glutamax-I, containing 25 mM HEPES, and 10% v/v heat-inactivated bovine calf serum. L5178YS and L5178YR (mouse leukemia cells) were cultured in Fischer's medium, supplemented with 10% v/v heat-inactivated calf serum. Both media contained penicillin (100 U/l) and streptomycin (100 mg/l).

Cells at the concentration of 10^6/ml were incubated for 1 h with diamide, NEM, *t*BOOH or paraquat. After incubation, cell suspensions were centrifuged (300*g*, 5 min, 4°C).The supernatant was removed and the cells, rinsed two times with PBS under the same conditions and resuspended in 200 μl of PBS, were used for estimation TAC and −SH level.

Measurement of −SH Level in the Cells

Total cellular thiol groups were estimated using biradical spin label (RSSR, bis-(2,2,5,5)-tetramethyl-imidazoline-1-oxyl-4-yl).[17] Reaction of RSSR with thiols leads to breakage of the disulfide biradical and an increase in ESR signal. RSSR was added to 100 μl of cell suspensions to the final concentration of 100 μM and the ESR spectra of the suspension were taken after 10-min incubation at room temperature. ESR measurements were made in a Bruker ESP-300E X-band spectrometer. Measurement conditions were: center field 3840 G, sweep width 80 G, modulation aplitude 1.01 G. conversion time 20.48 ms, time constant 10.24 ms, receiver gain 5×10^3. Thiol group concentration in the sample was read from calibration curve obtained with glutathione as a standard.

Measurement of Total Antioxidant Capacity of the Cells

TAC was estimated by a modified $ABTS^{*+}$ decolorization assay.[18] Briefly, preformed $ABTS^{*+}$ solution was diluted with 10 mM phosphate buffer, pH 7.4 to $A_{414\,nm} = 1$. Cell extract, obtained by lysis of the cells with 100 μM digitonin and centrifugation (600*g*, 5 min), was added to 1 ml of $ABTS^{*+}$ solution and the decrease in absorbance ($\lambda = 414$ nm) was measured after exactly 10 s. All results were expressed in Trolox equivalents.

Protein was estimated according to Lowry.[19] The results are means ± SD from at least three independent experiments.

RESULTS AND DISCUSSION

In this study, we were interested in the contribution of total, both protein and non-protein −SH groups to the TAC of homogenates of various cell types. We included in the comparison the MRP1-transfected cell line since it differs significantly in the glutathione level from the parental cell line.[20]

The paucity of the material prompted us to employ the spin-label method of estimation of thiol group content of the cells and the $ABTS^{*+}$ decolorization assay for the assay of TAC. The RSSR spin label used penetrates the cell membrane and, therefore, thiol groups may be measured in whole cells which simplifies the procedure and avoids artifacts of thiol oxidation after cell lysis. The level of thiols estimated by this method may be slightly underestimated as nitroxides may be reduced by such cell components as ascorbate and NAD(P)H but their content is much lower with respect to –SH groups (especially in cells cultured in the absence of ascorbate) and the error does not excide several per cent.[17]

The thiol group content of the cell lines studied estimated by this method ranged from 8.3 ± 2.0 nmol/mg protein in L5178YS cells to 16.3 ± 3.2 nmol/mg protein in V79 cells (Fig. 1). *t*BOOH in the concentration range employed induced a loss of thiols only in L5178YS and L5178YR cells while the other stressors used brought about a concentration-dependent diminution of the thiol content of all cell types studied.

From among numerous method of TAC determination we choose the $ABTS^{*+}$ decolorization method due to its simplicity, speed, relatively high accuracy and sensitivity. The $ABTS^{*+}$ cation is promiscuous and reacts with many compounds if present at high concentrations; i. a., such detergents as Triton X-100, SDS and Tween 20 which, therefore, could not be used for cell lysis. We lysed the cells with digitonin which did not react with $ABTS^{*+}$, and employed a very short reaction time (10 s). Typical antioxidants react with $ABTS^{*+}$ within this time while "slow antioxidants" such as tyrosyl and tryptophanyl residues of proteins (whose biological significance as antioxidants seems doubtful) show only very limited reactivity. In this respect, this method is superior to the commercial test of Total Antioxidant Status which employs 3-min reaction time allowing for a higher contribution of slow-reacting antioxidants.[21]

The TAC of cell extracts ranged from 15.3 ± 2.4 (L5178YS cells) to 28.7 ± 3.1 nmol Trolox equivalents/mg protein (V79MRP1 cells). Although in some cases (e.g. after treatment with paraquat and with the lowest concentration of NEM) the decrease in the thiol level of thiols was not accompanied by alterations in TAC, the pattern of changes of TAC of cell extracts under the influence of thiol-reactive and oxidative stress-inducing compounds generally reflected that of changes of thiol group content (Fig. 2).

Comparison of all values of thiol group content and TAC of cell extracts (Fig. 3) indicates that changes in the thiol group content determine the TAC of cell extracts in about 70%. It demonstrates that estimation of TAC of cell extracts may be a valid

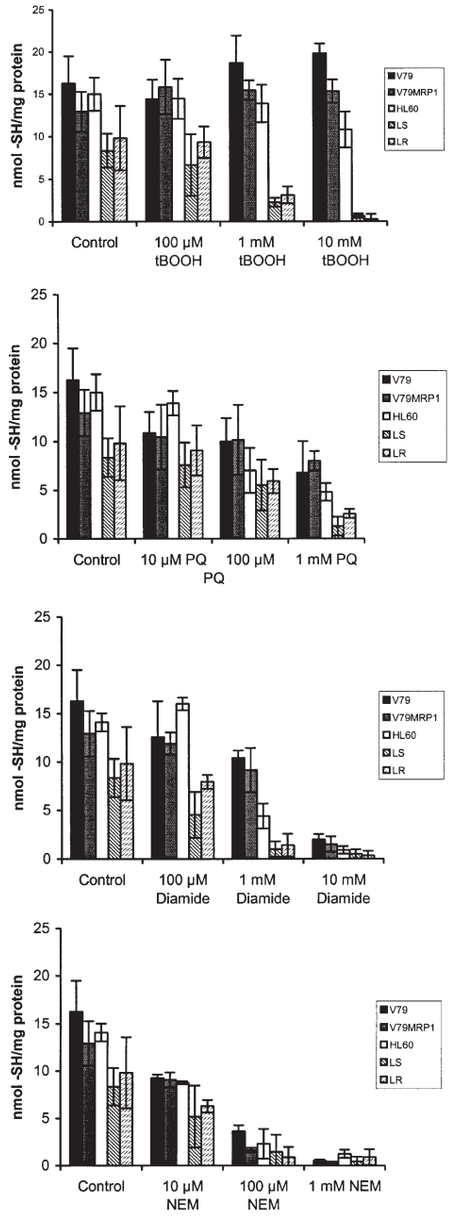

FIGURE 1 Effect of *tert*-butyl hydroperoxide (*t*BOOH), paraquat (PQ), diamide and *N*-ethyl maleimide (NEM) on the thiol group content in various cell lines.

parameter of assessment of oxidative stress, primarily of oxidation of thiol groups. In this sense, it may be a better indicator of oxidative stress than TAC of blood plasma which is determined mainly by urate and responds only weakly to oxidation of thiol groups.[22] Sensitivity of the test may allow estimation of TAC of small samples obtained by tissue biopsy which may be of value especially when the oxidative stress is localized primarily in one organ, e.g. liver (xenobiotic exposure)[23] or muscle (physical exercise).[24,25] Paradoxical increases in blood plasma TAC were noted in cases

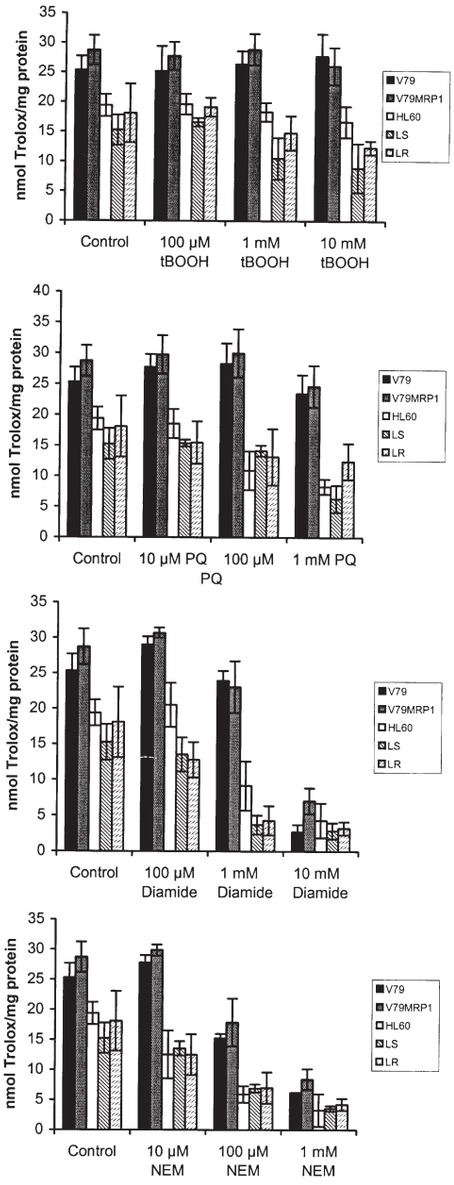

FIGURE 2 Effect of *tert*-butyl hydroperoxide, paraquat, diamide and *N*-ethyl maleimide on the total antioxidant capacity of extracts of various cell lines.

(including HIV and strenuous physical exercise) when oxidative stress lead to cell injury and leakage of intracellular antioxidants;[22] in such cases estimation of TAC of cell homogenates would give more straightforward estimate of the redox balance. Cyclic voltammetry studies pointed to considerable differences in antioxidant profile between different tissues which may underlie their sensitivity to oxidative stress;[26] such differences should be evident in the TAC assay. Moreover, TAC assay of cellular homogenates can be useful in cellular toxicology for the assessment of effect of oxidants on cultured cells, including microorganisms.

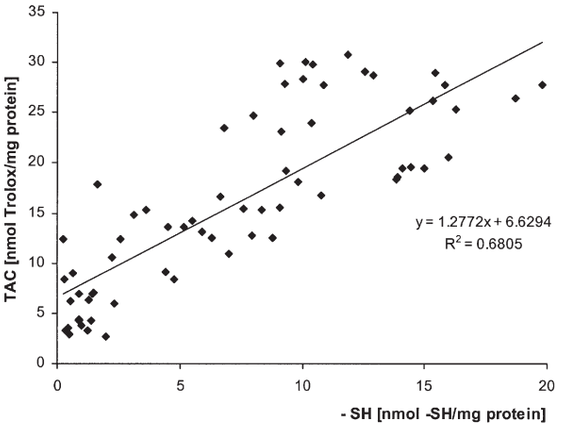

FIGURE 3 Correlation between the thiol content and TAC of extracts of various cell lines (pooled data for control and treated cells).

An obvious drawback of the TAC assay is the necessity of homogenization of the cells which is relatively troublesome.

TAC of cell or tissue homogenates have been measured by various Authors[27–31] but the contribution of individual antioxidants to TAC of this material has rarely been analyzed. Systematic study of TAC of homogenates of several rat tissues demonstrated that low-molecular weight antioxidants account for nearly half of TAC of homogenates of liver, brain and kidney homogenates while the other half can be ascribed to cellular proteins.[32] In the protein molecules, thiols are the main determinants of antioxidant activity although other residues may contribute as slow-reacting antioxidants, depending on the method of TAC measurement.[21] The present study, including both low-molecular and protein-bound thiols, provides proof that, contrary to what is observed in extracellular fluids, the thiol level is the decisive factor determining TAC of cellular homogenates.

Acknowledgements

We are indebted to Prof. B. Burchell (University of Dundee) for giving us V79 and V79MRP1 cells, to Dr M. Kruszewski (Institute of Radiation Chemistry and Technology, Warsaw) for L5178YS and L5178YR cells and to Dr M. Center (University of Kansas, Manhattan) for HL60 cells.

References

[1] Regoli, F. and Winston, G.W. (1999) "Quantification of total oxidant scavenging capacity of antioxidants for peroxynitrite, peroxyl radicals, and hydroxyl radicals", *Toxicol. Appl. Pharmacol.* **156**, 96–105.

[2] Santos, C.X.C., Anjos, E.I. and Augusto, O. (1999) "Uric acid oxidation by peroxynitrite: multiple reactions, free radical

formation, and amplification of lipid oxidation", *Arch. Biochem. Biophys.* **372**, 285–294.

[3] Ghiselli, A., Serafini, M., Natella, F. and Scaccini, C. (2000) "Total antioxidant capacity as a tool to assess redox status: critical view and experimental data", *Free Radic. Biol. Med.* **29**, 1106–1114.

[4] Prior, R.L. and Cao, G. (1999) "*In vivo* total antioxidant capacity: comparison of different analytical methods", *Free Radic. Biol. Med.* **27**, 1173–1181.

[5] Rice-Evans, C. and Miller, N.J. (1994) "Total antioxidant status in plasma and body fluids", *Methods Enzymol.* **234**, 279–293.

[6] Rice-Evans, C.A. (2000) "Measurement of total antioxidant activity as a marker of antioxidant status *in vivo*: procedures and limitations", *Free Radic. Res.* **33**, S59–S66.

[7] Schlesier, K., Harwat, M., Bohm, V. and Bitsch, R. (2002) "Assessment of antioxidant activity by using different *in vitro* methods", *Free Radic. Res.* **36**, 177–187.

[8] Severin, E., Nave, B., Stander, M., Ott, R. and Traupe, H. (1999) "Total antioxidative capacity is normal in sera from psoriasis patients despite elevated bilirubin, tocopherol and urate levels", *Dermatology* **198**, 336–339.

[9] Meucci, E., Littarru, C., Deli, G., Luciani, G., Tazza, L. and Littarru, G.P. (1998) "Antioxidant status and dialysis: plasma and saliva antioxidant activity in patients with fluctuating urate levels", *Free Radic. Res.* **29**, 367–376.

[10] Miller, N.J., Johnston, J.D., Collis, C.S. and Rice-Evans, C. (1997) "Serum total antioxidant activity after myocardial infarction", *Ann. Clin. Biochem.* **34**, 85–90.

[11] MacKinnon, K.L., Molnar, Z., Lowe, D., Watson, I.D. and Shearer, E. (1999) "Measures of total free radical activity in critically ill patients", *Clin. Biochem.* **32**, 263–268.

[12] Leinonen, J.S., Ahonen, J.P., Lonnrot, K., Jehkonen, M., Dastidar, P., Molnar, G. and Alho, H. (2000) "Low plasma antioxidant activity is associated with high lesion volume and neurological impairment in stroke", *Stroke* **31**, 33–39.

[13] Schafer, F.Q. and Buettner, G.R. (2001) "Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple", *Free Radic. Biol. Med.* **30**, 1191–1212.

[14] Halliwell, B. and Gutteridge, J.M.C. (1999) *Free Radicals in Biology and Medicine* (Oxford University Press, Oxford).

[15] Kirsch, M. and De Groot, H. (2001) "NAD(P)H, a directly operating antioxidant?", *FASEB J.* **15**, 1569–1574.

[16] Cuff, R.L., Wade, L.T., Rychlik, B., Jedlitschky, G.A. and Burchell, B. (2001) "Characterisation of glucuronidation and transport in V79 cells co-expressing UGT1A1 and MRP1", *Toxicol. Lett.* **120**, 43–49.

[17] Weiner, L.M. (1995) "Quantitative determination of thiol groups in low and high molecular weight compounds by electron paramagnetic resonance", *Methods Enzymol.* **251**, 87–105.

[18] Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. (1999) "Antioxidant activity applying an improved ABTS radical cation decolorization assay", *Free Radic. Biol. Med.* **26**, 1231–1237.

[19] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) "Protein measurement with the Folin phenol reagent", *J. Biol. Chem.* **193**, 265–275.

[20] Balcerczyk, A., Rychlik, B., Kruszewski, M., Burchell, B. and Bartosz, G. (2002) "MRP1-Transfected cells do not show increased resistance against oxidative stress", *Free Radic. Res.*, In press.

[21] Bartosz, G. and Bartosz, M. (1999) "Antioxidant activity: what do we measure?", *Acta Biochim. Pol.* **46**, 23–29.

[22] Bartosz, G. (2002) "Total antioxidant capacity", *Adv. Clin. Chem.*, In press.

[23] Jaeschke, H., Gores, G.J., Cederbaum, A.I., Hinson, J.A., Pessayre, D. and Lemasters, J.J. (2002) "Mechanisms of hepatotoxicity", *Toxicol. Sci.* **65**, 166–176.

[24] McArdle, A., Vasilaki, A. and Jackson, M. (2002) "Exercise and skeletal muscle ageing: cellular and molecular mechanisms", *Ageing Res. Rev.* **1**, 79–93.

[25] Ji, L.L. (2002) "Exercise-induced modulation of antioxidant defense", *Ann. N. Y. Acad. Sci.* **959**, 82–92.

[26] Kohen, R., Vellaichamy, E., Hrbac, J., Gati, I. and Tirosh, O. (2000) "Quantification of the overall reactive oxygen species scavenging capacity of biological fluids and tissues", *Free Radic. Biol. Med.* **28**, 871–879.

[27] Popov, I.N. and Lewin, G. (1999) "Antioxidative homeostasis: characterization by means of a chemiluminescent technique", *Methods Enzymol.* **300**, 437–456.

[28] Jaruga, E., Lapshina, E.A., Biliński, T., Płonka, A. and Bartosz, G. (1995) "Resistance to ionizing radiation and antioxidative defence in yeasts. Are antioxidant-deficient cells permanently stressed?", *Biochem. Mol. Biol. Int.* **37**, 467–472.

[29] Skrzydlewska, E. and Farbiszewski, R. (1997) "Trolox-derivative antioxidant protects against methanol-induced damage", *Fundam. Clin. Pharmacol.* **11**, 460–465.

[30] Tsakiris, S., Angelogianni, P., Schulpis, K.H. and Behrakis, P. (2000) "Protective effect of L-cysteine and glutathione on rat brain Na^+, K^+-ATPase inhibition induced by free radicals", *Z. Naturforsch.* **55c**, 271–277.

[31] Cao, G. and Prior, R.L. (1999) "Measurement of oxygen radical absorbance capacity in biological samples", *Methods Enzymol.* **299**, 50–62.

[32] Evelson, P., Travacio, M., Repetto, M., Escobar, J., Llesuy, S. and Lissi, E.A. (2001) "Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols", *Arch. Biochem. Biophys.* **388**, 261–266.