Enzymic and Non-Enzymic Antioxidants in Epidermis and Dermis of Human Skin

Yasuko Shindo, Eric Witt, Derick Han, William Epstein,* and Lester Packer
Department of Molecular and Cell Biology, University of California, Berkeley; and *Department of Dermatology, University of California, San Francisco, California, U.S.A.

We measured enzymic and non-enzymic antioxidants in human epidermis and dermis from six healthy volunteers undergoing surgical procedures. Epidermis was separated from dermis by currettage and antioxidants were measured by high-performance liquid chromatography (HPLC) or standard spectrophotometric methods. The concentration of every antioxidant (referenced to skin wet weight) was higher in the epidermis than in the dermis.

Among the enzymic antioxidants, the activities of superoxide dismutase, glutathione peroxidase, and glutathione reductase were higher in the epidermis compared to the dermis by 126, 61 and 215%, respectively. Catalase activity in particular was much higher (720%) in the epidermis. Glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase, which provide reduced nicotinamide adenine dinucleotide phosphate (NADPH), also showed higher activity in the epidermis than the dermis by 111% and 313%, respectively.

Among the lipophilic antioxidants, the concentration of α-tocopherol was higher in the epidermis than the dermis by 90%. The concentration of ubiquinol 10 was especially higher in the epidermis, by 900%. Among the hydrophilic antioxidants, concentrations of ascorbic acid and uric acid were also higher in the epidermis than in the dermis by 425 and 488%, respectively. Reduced glutathione and total glutathione were higher in the epidermis than in the dermis by 513 and 471%.

Thus the antioxidant capacity of the human epidermis is far greater than that of dermis. As the epidermis composes the outermost 10% of the skin and acts as the initial barrier to oxidant assault, it is perhaps not surprising that it has higher levels of antioxidants. *Key words: free radicals/antioxidants/human epidermis/human dermis. J Invest Dermatol 102:122–124, 1994*

Reactive oxygen species (ROS) are well known to be involved in skin cancers, inflammatory disorders, and cutaneous aging, because ROS result in harmful effects on the surrounding tissues [1]. The skin is directly and frequently exposed to ultraviolet light, ozone, and other environmental stresses generating or containing free radicals. A comprehensive and integrated antioxidant defense mechanism of the skin is thus crucial in protecting this organ from ROS.

Many researchers have studied antioxidant defense mechanisms in hairless mouse, rat, or pig [2–6]. Recently, human cultured keratinocytes have been used [7–9]. Malignant human skin tumors have been examined for certain antioxidant enzymes, especially superoxide dismutase (SOD), and lower SOD levels in malignant tumors were reported [10]. However, there are few reports on human skin because healthy human skin samples are difficult to get. In particular, to our knowledge, the levels of all of the major non-enzymic antioxidants have never been assessed in human samples.

It is important to examine both epidermis and dermis, because each has different functions. For example, the target of photoaging is mainly collagen in the dermis whereas many skin cancers due to ultraviolet light are epidermal cancers. It is also important to measure all major antioxidants, as they have different functions and interact with each other. We have previously measured activities and concentrations of the enzymic and non-enzymic antioxidants in both epidermis and dermis in murine skin [11]. In this paper, we report the levels of enzymic and non-enzymic antioxidants in human epidermis and dermis from healthy volunteers.

MATERIALS AND METHODS

Patients Six specimens of healthy human skin were obtained from patients who were undergoing surgical procedures (75-year-old woman, white, lower back; 62-year-old woman, white, groin; 64-year-old woman, white, groin; 45-year-old woman, black, groin; 72-year-old woman, Hispanic, groin; 47-year-old man, white, abdomen). The samples were placed on ice immediately, then frozen at −80°C as quickly as possible (within 15–20 min in all cases; during this time the samples remained on ice). The epidermis were then scraped from the frozen samples by curetting the samples on top of dry ice. The separated epidermis and dermis were stored at −80°C for up to 1.5 months before analysis.

Chemicals 5,5′-dithiobis-2-nitrobenzoic acid, reduced nicotinamide adenine dinucleotide phosphate, oxidized glutathione, and reduced glutathione, ferricytochrome c, hydrogen peroxide, xanthine, butylated hydroxytoluene, deferoxamine mesylate, DL-alpha tocopherol, ubiquinone 10, ascorbic acid, uric acid, buttermilk xanthine oxidase, yeast glutathione reductase, glucose-6-phosphate, and isocitrate were purchased from Sigma. 2-Vinylpyridine and 2,3-dimercapto-1-propanol were purchased from Aldrich.

Antioxidant Enzyme Assays Buffer A (sodium chloride 130 mM, glucose 5 mM, disodium ethylenediamine tetraacetic acid [EDTA] 1 mM, sodium phosphate 10 mM, pH 7.0), 0.75–1.5 ml, was used for homogenization. Each sample of epidermis and chopped dermis was homogenized with a

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Reprint requests to: Dr. Lester Packer, Department of Molecular and Cell Biology, 251 Life Sciences Addition, University of California, Berkeley, CA 94720.

Abbreviations: GR, glutathione reductase; G6PDH, glucose-6-phosphate dehydrogenase; ICDH, isocitrate dehydrogenase; ROS, reactive oxygen species.

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Table I. Antioxidant Enzyme Activities in Epidermis and Dermis of the Normal Human Skin[a]

	Epidermis[b]	Dermis[b]
Superoxide dismutase	816 ± 105[c]	361 ± 28
	17.8 ± 1.0	15.0 ± 1.3
Catalase	2912 ± 406[d]	355 ± 89
	62.0 ± 6.0[d]	14.6 ± 2.9
Glutathione peroxidase	0.71 ± 0.09[e]	0.44 ± 0.04
	0.013 ± 0.002	0.018 ± 0.004
Glutathione reductase	0.63 ± 0.11[c]	0.20 ± 0.01
	0.013 ± 0.001[e]	0.008 ± 0.001
Glucose-6-phosphate dehydrogenase	0.57 ± 0.09[e]	0.27 ± 0.04
	0.012 ± 0.002	0.010 ± 0.001
Isocitrate dehydrogenase	1.53 ± 0.22[d]	0.37 ± 0.04
	0.033 ± 0.045[e]	0.019 ± 0.002

[a] n = 6; the results are the mean ± SEM.
[b] Upper value, units/gm skin; lower value, units/mg protein.
[c] Epidermis different from dermis, $p < 0.05$.
[d] Epidermis different from dermis, $p < 0.01$.
[e] Epidermis different from dermis, $p < 0.001$.

Teflon homogenizer rotated by an electric drill at maximum speed for 2 min and centrifuged with a benchtop Eppendorf centrifuge Model 5415 (10,000 × *g*, 10 min). The supernatant was kept on ice and used for enzyme assays and protein determination. The activities of catalase [12], superoxide dismutase [13], glutathione peroxidase [14], glutathione reductase [15], glucose-6-phosphate dehydrogenase [16], and isocitrate dehydrogenase [17] were assayed spectrophotometrically on a Shimadzu UV 160 U Spectrophotometer according to procedures described in the cited references. One enzyme unit is equivalent to 1 μmole of product formation or 1 μmole of substrate disappearance per min under the defined conditions, except for superoxide dismutase. In the case of SOD, the amount of SOD inhibiting the cytochrome c reduction rate by 50% under the given assay conditions is defined as 1 unit. All enzyme activities were measured at 30°C. Protein concentration was determined by Bio-Rad DC protein assay.

Antioxidant Assays γ-Tocopherol, α-tocopherol, ubiquinol 10, and ubiquinone 10 contents were analyzed simultaneously by high performance liquid chromatography (HPLC) as described by Lang et al [18] using in-line electrochemical detection of tocopherols and ubiquinol 10 and UV detection of ubiquinone 10.

Glutathione was measured by the 5,5′-dithiobis-2-nitrobenzoic acid–glutathione reductase recycling assay [19]. The homogenization solution was 3.3% sulfosalycilic acid, 5 mM EDTA, and 1.5 mM butylated hydroxytoluene, ice-cold, bubbled with argon gas. Samples were homogenized with the Teflon homogenizer at maximum speed for 2 min, immediately centrifuged at 3,000 × *g* for 10 min, then 1 ml of the supernatant was added to 0.6 ml of 2 M sodium citrate (pH 5.5) and the mixture was used for total glutathione (GSH + GSSG) assay. By adjusting the pH in this manner local areas of high pH, in which GSH oxidation might occur, were avoided. For the GSSG assay, 10 μl 2-vinylpyridine was added to 500 μl of the above solution and the mixture was incubated for 1–2 h to derivatize the reduced GSH, rendering it inactive in the assay [20].

Ascorbic acid and dehydroascorbic acid were measured by HPLC using electrochemical detection [21]. In brief, samples were homogenized in ice-cold 90% methanol, 1 mM EDTA, 50 μM deferoxamine mesylate, and 1.5 mM butylated hydroxytoluene solution bubbled with argon gas, with the Teflon homogenizer at maximum speed for 2 min. After centrifugation (3,000 × *g*, 3 min), a 20-μl sample of supernatant was immediately analyzed by HPLC for ascorbic acid. Simultaneously, uric acid was detected in the same column. For dehydroascorbic acid, a sample of supernatant was incubated in the dark at room temperature for 10 min with an equal volume of 10 mM 2,3-dimercapto-1-propanol. After incubation the solution was extracted three times with three volumes of water-saturated ethyl ether. After extraction, samples were purged with nitrogen for 2 min and immediately analyzed by HPLC. Dehydroascorbic acid was calculated as total ascorbic acid minus reduced ascorbic acid. Ascorbic acid (2 μM) and uric acid standard (2 μM) were freshly prepared for each day's assay. The concentration of ascorbic acid was determined spectrophotometrically using an extinction coefficient at 265 nm of 14,500 $m^{-1}cm^{-1}$.

We have previously shown that these procedures do not affect the concentration or redox status of the antioxidants [11].

Statistics Statistical significance was determined by Student pooled t test.

RESULTS

Reference Base We have referenced the data to gm skin, not mg protein. The usual method of extraction can not extract the collagen protein in the dermis, hence data shown in terms of mg/protein in the skin will not include the contents of collagen. Collagen should be contained in the data because it is a major target of photoaging. We have discussed the advantages of reference to wet weight versus reference to protein in a previous paper [11].

Antioxidant Enzymes (Table I) We measured four antioxidant enzymes and two other enzymes that produce NADPH. These are reported as units/gm skin. The activity of every enzyme was higher in the epidermis than in the dermis. The activities of superoxide dismutase, glutathione peroxidase, and glutathione reductase (GR) were higher in the epidermis than the dermis by 126%, 61%, and 215%, respectively. Catalase activity in particular was higher, by 720%. Glucose-6-phosphate dehydrogenase (G6PDH) and isocitrate dehydrogenase (ICDH) produce NADPH, which is a substrate of glutathione reductase. G6PDH and ICDH activities were higher in the epidermis than in the dermis by 111 and 313%, respectively. When values are expressed as units/mg protein, catalase, GR, and ICDH activities were higher in the epidermis.

Non-enzymic Antioxidants (Table II) Every non-enzymic antioxidant was also higher in concentration in the epidermis than in the dermis. Of the lipophilic antioxidants, the concentration of α-tocopherol was higher in the epidermis by 90%, and the concentration of ubiquinol 10 was especially higher, by 900%.

Among the hydrophilic antioxidants, ascorbic acid and uric acid were higher in the epidermis than in the dermis by 425% and 488%, respectively. Reduced glutathione and total glutathione were higher in the epidermis than in the dermis by 513% and 471%, respectively.

DISCUSSION

The skin is the largest organ and constitutes the surface layer of the body. Its antioxidant defense capacity would thus be expected to be greater than that of internal organs. Studying antioxidant defense mechanisms of the skin can be of benefit in discovering protective

Table II. Antioxidants of Epidermis and Dermis of Human Skin[a]

	Concentration (nmol/gm skin)	
Antioxidant	Epidermis	Dermis
γ-Tocopherol	3.26 ± 1.00	1.78 ± 0.15
α-Tocopherol	31.0 ± 3.8[b]	16.2 ± 1.10
Total vitamin E	34.2 ± 4.6	18.0 ± 1.06
Ubiquinol 10	3.53 ± 0.79[b]	0.35 ± 0.08
Ubiquinone 10	4.12 ± 0.59	2.86 ± 0.84
Total (ubiquinol + ubiquinone)	7.66 ± 0.45	3.15 ± 0.87
% ubiquinone 10	54.6 ± 8.25	90.4 ± 3.02
Ascorbic acid	3,798 ± 1016[b]	723 ± 320
Dehydroascorbic acid	3,802 ± 1552	588 ± 240
Total (ascorbic acid + dehydroascorbic acid)	7,600 ± 2498[b]	1,311 ± 559
% dehydroascorbic acid	46.6 ± 4.0	44.9 ± 1.9
Uric acid	1071 ± 242[c]	182 ± 24
Reduced glutathione	460.9 ± 77.4[d]	75.1 ± 9.0
Oxidized glutathione	23.3 ± 6.41	9.6 ± 3.8
Total (reduced glutathione + oxidized glutathione	484.3 ± 81.4[d]	84.8 ± 11.5
% oxidized glutathione	5.0 ± 0.86	10.5 ± 2.7

[a] n = 6; the results are the mean ± SEM.
[b] Epidermis different from dermis, $p < 0.05$.
[c] Epidermis different from dermis, $p < 0.01$.
[d] Epidermis different from dermis, $p < 0.001$.

procedures against skin cancers, cutaneous aging, and skin inflammatory disorders.

The present study represents an effort to establish baseline values for the concentration and redox status of all major antioxidants in the human epidermis and dermis. Previous studies of human skin have focused on one or at most a few antioxidants, usually enzymes. But skin, like other organs and tissues, contains a large variety of antioxidants that serve different functions and interact with each other; it is essential to measure as many of these antioxidants as possible. In addition, different pathologies involve different skin layers; hence, the antioxidant capacities of the epidermis and dermis should be evaluated and compared.

Until now, reports concerning antioxidants of human skin have chiefly dealt with enzyme activities in pathologic skin. Oberley and Buettner reported decreased SOD activity, especially the mitochondrial Mn-SOD, in various cancer tissues [22]. Hamanaka [10] reported that all the Cu,Zn-SOD activities in the 15 malignant skin tumors (four malignant melanomas, three squamous cell carcinomas, four Bowen's diseases, two basal cell epitheliomas, one sebaceous epithelioma, and one extramammary Paget's disease) were significantly lower than those in adjacent normal tissues of the same samples. Schallreuter [23] reported low catalase levels in the epidermis of patients with vitiligo and suggested that hydroxyl radicals are capable of bleaching constitutional melanin and causing membrane lysis through lipid peroxidation reactions.

Recently, the antioxidant enzyme capacity of cultured human keratinocytes has been examined. Destruction of catalase and SOD has been observed in UVA- and UVB-irradiated keratinocytes [7,8]. Others have compared antioxidant enzymes in cultured cell types that represent the major cell types in the epidermis and the dermis [9]. Antioxidant enzymes were present in descending order from fibroblasts to keratinocytes to melanocytes.

However, there is scant information about non-enzymic antioxidants in tumors, keratinocytes, or normal skin. Because a change in antioxidant enzyme activity can be due to many non-oxidative factors, whereas changes in concentration or oxidation status of non-enzymic antioxidants provide indirect indicators of oxidative stress, it is important to measure all major antioxidants before drawing conclusions. In this study, only six samples from six people of various ages, races, and both genders were measured. These variations in subjects and in the location of the samples may explain the large variability seen in some of the antioxidants measured. Further investigation is needed to establish any variations in antioxidant concentrations depending on race, age, and region of the body.

The function and capacity of antioxidants in the epidermis and dermis are probably different. The target of photoaging is mainly collagen in the dermis, whereas UV-induced cancers such as melanoma, basal cell carcinoma, and squamous cell carcinoma are derived from epidermis. We previously reported that the activities of many antioxidants were higher in the epidermis than in the dermis in hairless mouse skin [11]. In this study, we found that the capacity of antioxidants in the human skin was also higher in the epidermis than the dermis, and the difference was much greater in human skin than in murine skin.

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REFERENCES

1. Cross CC, Halliwell B, Borish ET, Pryor WA, Ames BN, Saul RL, McCord JM, Harman D: Davis conference: oxygen radicals and human disease. *Ann Intern Med* 107:526-545, 1987
2. Fuchs J, Huflejt ME, Rothfuss LM, Wilson DS, Carcamo G, Packer L: Acute effects of near ultraviolet and visible light on the cutaneous antioxidant defense system. *Photochem Photobiol* 50:739-744, 1989
3. Fuchs J, Huflejt ME, Rothfuss LM, Wilson DS, Carcamo G, Packer L: Impairment of enzymic and nonenzymic antioxidants in skin by UVB irradiation. *J Invest Dermatol* 93:769-773, 1989
4. Miyachi Y, Imamura S, Niwa Y: Decreased skin superoxide dismutase activity by a single exposure of ultraviolet radiation is reduced by liposomal superoxide dismutase pretreatment. *J Invest Dermatol* 89:111-112, 1987
5. Hashimoto Y, Ohkuma N, Iiauka H: Reduced superoxide dismutase activity in UVB-induced hyperproliferative pig epidermis. *Arch Dermatol Res* 283:317-320, 1991
6. Darr D, Combs S, Dunston S, Manning T, Pinnell S: Topical vitamin C protects porcine skin from ultraviolet radiation-induced damage. *Br J Dermatol* 127:247-253, 1992
7. Punnonen K, Puntala A, Jansen CT, Ahotupa M: UVB irradiation induces lipid peroxidation and reduces antioxidant enzyme activities in human keratinocytes in vitro. *Acta Dermatol Venerol* 71:239-273, 1991
8. Punnonen K, Jansen CT, Puntala A, Ahotup M: Effects of in vitro UVA irradiation and PUVA treatment on membrane fatty acids and activities of antioxidant enzymes in human keratinocytes. *J Invest Dermatol* 96:255-259, 1991
9. Yohn JJ, Norris DA, Trastorza DG, Buno IJ, Leff JA, Hake SS, Repine JE: Disparate antioxidant enzyme activities in cultured human cutaneous fibroblasts, keratinocytes, and melanocytes. *J Invest Dermatol* 97:405-409, 1991
10. Hamanaka H, Miyachi Y, Tzchibana T, Imamura S: Lowered Cu,Zn-superoxide dismutase activity in human malignant skin tumors. *J Dermatol* 18:258-261, 1991
11. Shindo Y, Witt E, Packer L: Antioxidant defense mechanisms in murine epidermis and dermis and their responses to ultraviolet light. *J Invest Dermatol* 100(3):260-265, 1993
12. Aebi H, Catalase: In: Bergmeyer HU (ed.). *Methods of Enzymatic Analysis.* Academic Press, New York, 1974, pp 673-684
13. Floche L, Otting F: Superoxide dismutase assays. In: Packer L (ed.). *Methods Enzymol, Vol. 105.* Academic Press, 1984, pp 93-105
14. Floche L, Gunzler W: Assays of glutathione peroxidase. In: Packer L (ed.). *Methods Enzymol, Vol. 105.* Academic Press, 1984, pp 114-121
15. Bergmeyer HU, Gawehn K, Grassl M: Glutathione reductase. In: Bergmeyer HU (ed.). *Methods of Enzymatic Analysis.* Academic Press, New York, 1974, pp 465-466
16. Bergmeyer HU, Gawehn K, Grassl M: Glucose-6-phosphate dehydrogenase. In: Bergmeyer HU (ed.). *Methods of Enzymatic Analysis.* Academic Press, New York, 1974, pp 458-459
17. Bernt E, Bergmeyer HU: Isocitrate dehydrogenase. In: Bergmeyer HU (ed.). *Methods of Enzymatic Analysis.* Academic Press, New York, 1974, pp 624-631
18. Lang JK, Gohil K, Packer L: Simultaneous determination of tocopherols, ubiquinols, and ubiquinones in blood, plasma, tissue homogenates, and subcellular fractions. *Anal Biochem* 157:106-116, 1986
19. Anderson ME: Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol* 113:548-552, 1985
20. Griffith OW: Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Bioch* 106:207-212, 1980
21. Dhariwal KR, Hartzell WO, Levine M: Ascorbic acid and dehydroascorbic acid measurements in human plasma and serum. *Am J Clin Nutr* 54:712-716, 1991
22. Oberley LW, Buettner GR: Role of superoxide dismutase in cancer: a review. *Cancer Res* 39:1141-1149, 1979
23. Schallreuter KU, Wood JK, Berger J: Low catalase levels in the epidermis of patients with vitiligo. *J Invest Dermatol* 97:1081-1085, 1991