

Controlling Reactive Oxygen Species in Skin at Their Source to Reduce Skin Aging

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Abstract

Activity of an age-related, superoxide-forming, cell-surface oxidase (arNOX) comparing dermis, epidermis, serum, and saliva from female and male subjects ages 28–72 years measured spectrophotometrically using reduction of ferricytochrome *c* correlated with oxidative skin damage as estimated from autofluorescence of skin using an Advanced Glycation End products Reader (AGE-Reader; DiagnOptics B.V., Netherlands). By reducing arNOX activity in skin with arNOX-inhibitory ingredients (NuSkin's ageLOC technology), skin appearance was improved through decreased protein cross-linking and an accelerated increase in collagen.

Introduction

THE EXTERNAL NICOTINAMIDE ADENINE DINUCLEOTIDE (NADH) oxidase (ECTO-NOX), or ENOX, proteins are cell-surface located terminal oxidases involved in the plasma membrane oxidoreductase (PMOR) system.¹ Aging leads to the accumulation of mitochondrial DNA lesions² and a shift toward energy production via glycolysis resulting in a hyperactive PMOR system³. ENOX1 (CNOX) and ENOX2 (tNOX) carry out four electron transfers to molecular oxygen to form water.¹ However, ENOX3 (arNOX) is unique in that it generates superoxide at the cell surface, and its activity is elevated in individuals 50–70 years of age compared to those 20–40 years of age.^{4,5} Generated superoxide can then form hydrogen peroxide (H₂O₂) and other reactive oxygen species (ROS) capable of damaging adjacent cells, circulating lipoproteins, and components of the skin's extracellular matrix. Work was performed to demonstrate the presence of superoxide-forming, cell-surface oxidase (arNOX) in human epidermis and dermis and the possible relationship to skin damage as measured by skin autofluorescence.

Oxidative damage to proteins has been postulated as a major cause of various degenerative diseases, including deterioration of skin.⁶ A prominent target for oxidation by ROS is the tyrosine residue. We have used a highly sensitive method for the detection of tyrosyl radical formation in cells.^{6,7} The method is based on the fluorescein-labeled tyrosine analog, tyramine, which upon oxidation, may couple to proteins carrying a tyrosyl radical. Coupling of the probe to proteins such as collagen and elastin was accelerated by ROS generated by the aging-related cell-surface oxidase arNOX (results unpublished).

Methods

arNOX activity was assayed by measurement of superoxide production based on the standard method where reduction of ferricytochrome *c* by superoxide was monitored from the increase in absorbance at 550 nm with reference at 540 nm.⁸ As a further check for the specificity of the arNOX activity, 60 units of superoxide dismutase (SOD) was added near the end of the assay to ascertain that the rate returned to base line. Rates were determined using a SLM Aminco DW-2000 spectrophotometer in the dual-wavelength mode of operation.

Volunteers were recruited for studies of arNOX activity levels in biofluids. Ages ranged from 25 to 72 years. Levels of arNOX activity were determined from blinded samples. Informed consent was obtained at screening baseline visit. Both sera and saliva samples (1–3 mL) were collected at least 1 h after food consumption. For saliva, the mouth was rinsed with water and the saliva was collected 30 min later.

Punch biopsies comparing dermis and epidermis were from either sun-protected or sun-exposed skin areas plus sera from 16 female subjects, ages 28–72 years. Fitzpatrick skin type I and II were analyzed for activities based on rate of superoxide generation determined by reduction of ferricytochrome *c* measured spectrophotometrically. The 3-mm, full-thickness, punch biopsy material was hand split into epidermal and dermal portions. Each portion was exhaustively homogenized prior to assay and centrifuged briefly to remove insoluble materials. The autofluorescence, calculated as a ratio of mean intensities detected from the skin of the volar forearm between 420–600 nm and 500–420 nm, was measured noninvasively using an Advanced Glycation End

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products Reader (AGE-Reader; DiagnOptics B.V., Netherlands).

Results

arNOX is not a constitutive protein, but it appears around age 30 and increases with age thereafter in skin, serum, saliva, and urine to a maximum of about age 55 in women and age 65 in men. arNOX activity of either sun-protected or sun-exposed dermis and epidermis from subjects ages 28–72 years correlated with oxidative skin damage estimated from skin autofluorescence as measured using an AGE (Advanced Glycation End products)-Reader. arNOX is shed from the cell surface and appears in body fluids (serum, saliva, urine and perspiration). Also correlated with oxidative skin damage, estimated using the AGE reader, was arNOX activity of saliva (Fig. 1). Direct oxidative damage to collagen and elastin was demonstrated by adding arNOX from urine to the protein in solution and measuring tyrosine dimerization (cross linking) using a method based on fluorescent tyramine.^{6,7}

arNOX-catalyzed oxidation of proteins was based on oxidation of the amino acid tyrosine with focus on type I collagen and elastin. The tyrosines, once oxidized, form dimers to cross-link the proteins. Fluorescent tyramine is added to the reaction, forming fluorescent dimers with the tyrosines as they are oxidized. Their fluorescence, which is covalently linked to the protein, then serves as a measure of protein oxidation, which increases with time and is proportional to

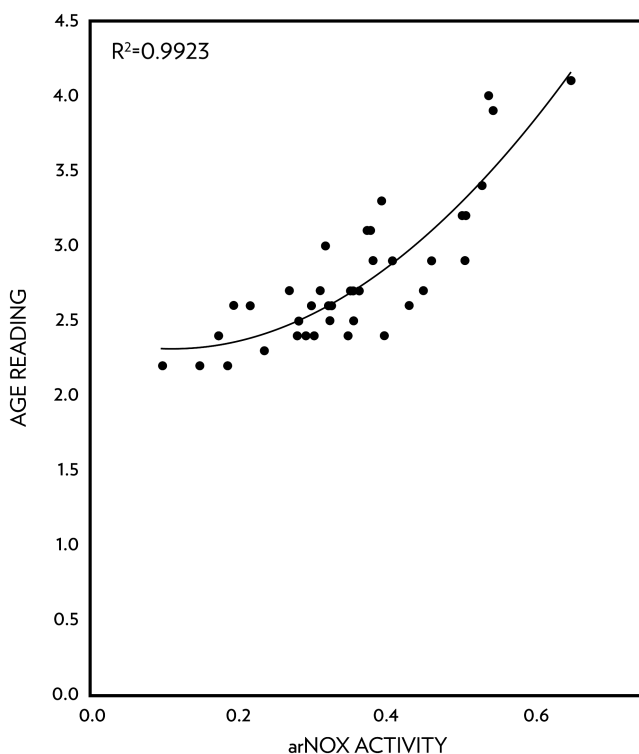


FIG. 1. Correlation between skin autofluorescence (AGE Reader, DiagnOptics B.V., Netherlands) and arNOX activity of saliva determined from superoxide dismutase inhibited reduction of ferricytochrome *c* determined spectrophotometrically. arNOX, Age-related, superoxide-forming, cell-surface oxidase.

protein amount. The arNOX source was concentrated from human urine and had a specific activity comparable to that measured in aged skin. Oxidation was blocked both by SOD and anti-arNOX antibody to demonstrate that the tyrosine oxidation was due to arNOX. Greater tyrosine oxidation was observed in aged versus young epidermal keratinocytes and proportional to the AGE score of skin.

Among the first arNOX inhibitors was coenzyme Q₁₀ (CoQ₁₀, or ubiquinone).^{9,10} Inhibitory were CoQ₈, CoQ₉, and CoQ₁₀. CoQ₀–Q₇ lacked inhibitory activity. arNOX inhibition provides a rational basis for employment of CoQ to reduce certain adverse effects of aging.

Another potent arNOX inhibitor is salicin, which in combination with other arNOX-inhibiting ingredients provides a basis for NuSkin's age-LOC technology to achieve healthier skin by controlling ROS at their source, i.e., superoxide produced by arNOX in body fluids and interstitial spaces.

Discussion

Our hypothesis is that reducing the arNOX activity in skin of older subjects with arNOX-inhibitory active ingredients will make it easier to affect antiaging changes in the skin. The result is a more youthful appearance resulting from reduced collagen cross-linking and ensuing greater collagen production.

Buffy coats and skin are included as an example of the cell-surface forms of the activity comparing old and young individuals. The activity for the older individuals without the addition of SOD shows complete loss of activity upon the addition of SOD. The correlation between patient age and arNOX of explants both epidermis and dermal punches extrapolated to zero activity at about age 30.⁹ This activity is SOD-inhibitable activity and verified to be arNOX. Activity continued to increase at least until beyond age 70 years.⁹ Findings similar to buffy coats and skin explants were found for sera, saliva, and perspiration.⁹

In cells, the electron donor for superoxide formation by arNOX is ubiquinol (CoQ₁₀H₂) or semiubiquinone of the plasma membrane. Being an external ECTO-NOX protein, the protein is shed and enters the circulation. The electron donor for the shed form of arNOX is proteins thiols. A surprising observation was that arNOX-catalyzed superoxide generation by both the plasma membrane-associated and the shed forms of the activity was inhibited by external ubiquinone (CoQ₁₀).⁹

arNOX, both at the cell surface and as a circulating shed form, affords the possibility of generating superoxide and ROS not only in skin but also in direct contact with circulating lipoproteins (i.e., low-density lipoproteins [LDLs]) in the blood to augment their oxidation as a potential major contributor to atherogenesis.¹⁰ An alternative view that continuous superoxide and H₂O₂ formation are essential for normal cell function and that they play a major role in subcellular redox state modulation has been presented as well.²

Skin health benefits of CoQ₁₀ also are indicated.⁹ In a randomized, double-blinded, placebo-controlled trial, subjects with aged skin used a skin-care preparation containing 1% CoQ₁₀ or its reduced form twice daily for 5 months. Significant reduction of wrinkle grade and an improvement of skin condition were observed as a result of the antiaging properties of CoQ₁₀.⁹

In ongoing work, an inhibitor cocktail consisting of a blend of botanical ingredients has been developed at NOX Technologies, Inc., which appears to slow production of free radicals in the skin that are associated with arNOX. The cocktail is currently under evaluation for this purpose. Inhibition of arNOX-generated free radical production may help to slow signs of aging potentially caused by accelerated free radical production.

Author Disclosure Statement

All authors are affiliated either directly or indirectly with NuSkin International, Provo, Utah, manufacturers and distributors of ageLOC® skin care products based in part on arNOX inhibitors developed by NOX Technologies, Inc., West Lafayette, IN.

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