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Original Contribution

Enzyme-independent nitric oxide formation during UVA challenge of human skin: characterization, molecular sources, and mechanisms

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Abstract

Many of the local UV-induced responses including erythema and edema formation, inflammation, premature aging, and immune suppression can be influenced by nitric oxide synthase (NOS)-produced NO which is known to play a pivotal role in cutaneous physiology. Besides NOS-mediated NO production, UV radiation might trigger an enzyme-independent NO formation in human skin by a mechanism comprising the decomposition of photo-reactive nitrogen oxides. Therefore, we have examined the chemical-storage forms of potential NO-generating agents, the mechanisms and kinetics of their decomposition, and their biological relevance. In normal human skin specimens we find nitrite and S-nitrosothiols (RSNO) at concentrations 25- or 360-fold higher than those found in plasma of healthy volunteers. UVA irradiation of human skin leads to high-output formation of bioactive NO due to photo-decomposition of RSNO and nitrite which represents the primary basis for NO formation during UVA exposure. Interestingly, reduced thiols strongly augment photo-decomposition of nitrite and are essential for maximal NO release. The enzyme-independent NO formation found in human skin opens a completely new field in cutaneous physiology and will extend our understanding of mechanisms contributing to skin aging, inflammation, and cancerogenesis.

Keywords: Nitric oxide; Human skin; UVA; Nitrite; Photolysis; *S*-Nitrosothiols

Introduction

Nitric oxide is formed endogenously by NO synthases (NOS),[1] an enzyme family comprising three members, the two constitutively expressed neuronal nNOS and endothelial eNOS, and the cytokine-inducible isoform (iNOS) [1]. In skin several lines of evidence indicate that NO is involved in the control of wound healing processes, in allergic skin manifestations, in microbicidal activity, in antigen presentation, in hair growth, in proliferation and differentiation of epidermal cells, and in the regulation of innate immune reactions and inflammatory responses. In addition, UV-induced processes such as erythema and edema formation as well as melanogenesis are also affected by NO [2]. Furthermore, NO is an effective inhibitor of lipid peroxidation [3], and the coordinated action of NO on gene expression and preservation of membrane function plays an

Abbreviations: NOS, nitric oxide synthase; nNOS, neuronal NOS; eNOS, endothelial NOS; iNOS, inducible NOS; ROS, reactive oxygen species; NEM, *N*-ethylmaleimide; DTNB, 5,5′ -dithiobis(2-nitrobenzoic acid); FAMOS, Faraday modulation spectroscopy; QCL, quantum cascade laser; AEC, aorta endothelial cell; DMEM, Dulbecco's modified Eagle's medium; TCEP, tris-(2-carboxyethyl)phosphine.

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important role in protection against either UVA- or reactive oxygen species (ROS)-induced apoptotic as well as necrotic cell death [4,5].

The action of NO is largely determined by its rapid diffusion and its ability to penetrate cell membranes. The diffusion coefficient of NO at 37°C has been found to be 1.4-fold higher than that of oxygen or carbon monoxide and thus a diffusion distance of 500 μm was calculated for NO in tissue [6]. Within tissues, some of the NO may react with SH groups leading to the formation of comparatively more stable nitrosothiols (RSNO) [7]. RSNOs like *S*-nitrosoalbumin, *S*-nitrosoglutathione, or *S*-nitroso-L-cysteine have been detected and quantified in vivo [8,9] and are thought to be responsible for some of the well-documented physiological cardiovascular processes that had been previously attributed to NO itself [10–12].

Both nitrite and RSNO compounds potentially represent enzyme-independent sources for NO either in acidic milieu or during UVA exposure via photolysis, respectively [13–15]. With respect to its biological impact on human cutaneous physiology [16] and the protective role of NO as an important modulator of UV-induced cytotoxicity [17], we have determined the local concentrations of NO-derived products in human skin and examined the mechanisms and conditions of their decomposition or ability to form NO upon UVA irradiation, respectively.

Materials and methods

Materials

If not indicated, chemicals or media noted were from Sigma (Deisenhofen, Germany) or PAA (Linz, Austria), respectively.

UVA sources

The UVA sources are as follows: (1) Philips 400 W mercury arc lamp (HPA 400 W Cleo lamp; Phillips, Hamburg, Germany) emitting a UVA spectrum (320–420 nm) with a maximum of intensity between 340 and 380 nm (34 mW/cm^2 in a distance of 25 cm) [18]; (2) Sellas 4000 W mercury arc lamp unit (Sellas Medizinische Geräte, Gevelsberg, Germany) emitting a UVA1 spectrum (340–410 nm) with a maximum of intensity at 366 nm (84 mW/cm^2 in a distance of 25 cm); and (3) Sellas 2000 W mercury arc lamp unit emitting a UVA spectrum (320–400 nm) with a maximum of intensity between 350 and 370 nm (70 mW/cm^2 in a distance of 25 cm).

Human skin samples and preparation of skin homogenates

Human skin specimens were derived from mammoplastic surgery (Department of Plastic Surgery, Florence Nightingale Hospital, Düsseldorf, Germany), within 15 min at 4°C cut into 10-mm squares, immediately embedded in Tissue-Tek (Reichert-Jung, Vienna, Austria), and snap-frozen in liquid N_2 for immunohistochemical characterization.

Skin homogenates were prepared by cutting embedded skin specimens parallel to the epidermis into 20-μm thin Sections 2 mm deep into the dermis. The material was weighed, diluted in 3 w/vol of NEM buffer (PBS containing 5 mM *N*-ethylmaleimide (NEM), 2.5 mM EDTA, protease inhibitor cocktail), and homogenized. After a short centrifugation supernatants were collected, diluted to a protein content of 10 mg/ml, and immediately used or frozen at −20°C for maximally 2 weeks. Additionally, skin homogenates were prepared in the absence of the alkylating agent NEM. All solutions generating NO upon UVA challenge were adjusted to pH 7.0. This pH has been kept constant during the entire experiment.

Quantification of nitrite, nitrate, S- or N-nitroso compounds and sulfhydryls in human skin

Dermal nitroso species were quantified by reductive denitrosation of skin homogenate samples prepared in the presence or absence of NEM using a mixture of iodine/iodide in glacial acetic acid and subsequent detection of the released NO by the chemiluminescence detector (CLD 88 AM, from Eco Physics, Munich, Germany) exactly as described [8,19]. Nitrate concentrations of skin homogenates were obtained by the difference of nitrite values obtained after reduction with VCl_3 [20].

Determination of total sulfhydryl (SH) groups, protein-bound SH groups, and free SH groups in homogenates of human skin was performed by the Ellman assay using DTNB [21] and glutathione as a standard. Additionally, glutathione concentrations were determined by using a glutathione assay kit (Biozol Diagnostica, Munich, Germany) by which total, reduced, and oxidized glutathione (GSSG also) concentrations can be quantified.

Detection of UVA-induced NO liberation from human skin or skin homogenates

UVA-induced (400 W lamp) NO release from human skin was measured using a quartz glass-covered (16 cm^2) stainless-steel chamber, permanently exhausting the gases for detection. Using a UV filter (opening above 420 nm), we examined UV-independent effects, e.g., temperature, on NO release. The NO amounts detected were calculated as micromolar NO/min x square centimeter of skin.

Skin homogenates (15 ml; 2 mg protein/ml) were UV-irradiated (400 W lamp) in a quartz glass cylinder, permanently exhausting the gas phase for detection. Differential reduction or deprivation of nitrogen oxide species from samples allows examination of the input of those agents on UVA-induced NO formation via photo-decomposition. Nitrate was removed by reaction with nitrate