Real-Time Monitoring of Oxidative Stress in Live Mouse Skin

Alexander M. Wolf[1], Kiyomi Nishimaki[1], Naomi Kamimura[1] and Shigeo Ohta[1]

Oxidative stress is involved in many age-associated diseases, as well as in the aging process itself. The development of interventions to reduce oxidative stress is hampered by the absence of sensitive detection methods that can be used in live animals. We generated transgenic mice expressing ratiometric redox-sensitive green fluorescent protein (roGFP) in the cytosol or mitochondria of several tissues, including skin epidermal keratinocytes. Crossbreeding into hairless albino mice allowed noninvasive optical measurement of skin oxidative state. Topical application of hydrogen peroxide emulsion shifted the keratinocyte redox state toward oxidation within minutes and could be observed in real time by fluorescence ratio imaging. Exposing skin to 365 nm UVA radiation oxidized roGFP localized in keratinocyte mitochondria, but not when roGFP was localized in the cytosol. This suggests that significant amounts of the endogenous photosensitizers that mediate UVA–induced oxidative stress are located in the mitochondria. UVR is the major environmental cause of skin aging and UVA–mediated oxidative stress has been associated with the development of wrinkles in humans. Direct measurements of redox state in defined cell compartments of live animals should be a powerful and convenient tool for evaluating treatments that aim to modulate oxidative stress.

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INTRODUCTION

Oxidative stress, most often defined as an excess in the production of reactive oxygen species (ROS) over their elimination by endogenous antioxidants, is involved in the pathogenesis of cancer (Serrano and Blasco, 2007), inflammation and atherosclerosis (Lusis, 2000; Rocha and Libby, 2009), diabetes (Brownlee, 2001; Houstis *et al.*, 2006), and neurodegenerative (Esposito *et al.*, 2002; Beal, 2003; Dawson and Dawson, 2003) and other diseases (James *et al.*, 2012). During aging, depletion of endogenous antioxidants has a critical role in disease progression (Chinta and Andersen, 2006) and oxidative stress is often assumed to be the main factor driving the aging process (Balaban *et al.*, 2005; Beal, 2005; Dickinson and Chang, 2011). Even though clinical trials using antioxidants have been very disappointing (Thomson *et al.*, 2007; Bjelakovic *et al.*, 2012), treatments that can effectively modulate oxidative stress continue to be highly sought after (Finkel, 2005; Ohsawa *et al.*, 2007). The difficulty of assessing oxidative state *in vivo* remains a big hurdle in the development of effective antioxidants, and preselecting substances that can actually affect oxidative stress *in vivo* will be crucial to avoid costly failures (Vickers, 2007).

Oxidative stress is usually assessed by an increase in downstream biomarkers or fluorogenic ROS indicators, invasive single-point indicators of oxidative stress with a variety of drawbacks (Wardman, 2007; Chen *et al.*, 2010; Murphy *et al.*, 2011). Questions like *how much* of *which* ROS is produced *how*, *where*, and *when* remain mostly unanswered. Here, we report the generation of transgenic mice expressing redox-sensitive green fluorescent protein (roGFP) in the skin. RoGFP is a ratiometric redox sensor with two cysteine residues on its surface. Oxidation by hydrogen peroxide (H_2O_2) causes the formation of a disulfide bond, changing its absorption spectrum (Dooley *et al.*, 2004; Hanson *et al.*, 2004). RoGFP is reduced by the thioredoxin and glutathione systems, which also eliminate the majority of H_2O_2 (Cox *et al.*, 2010), resulting in continuous formation and release of the roGFP disulfide bridge depending on the local redox potential (Dooley *et al.*, 2004; Meyer and Dick, 2010). Although defining oxidative stress as an excess of ROS is overly simplistic, any change in roGFP redox state is a reliable indicator of oxidative stress as perturbed redox signaling (Jones, 2006, 2008). RoGFP was targeted to two subcellular locations, cytosol and mitochondria. Expressed in a defined cell type, it allowed the real-time visualization of skin redox state.

RESULTS

Transgenic mice expressing roGFP1 under the control of the elongation factor 1a promoter were generated using nuclear injection. We chose roGFP1 over its close cousin roGFP2

[1]*Department of Biochemistry and Cell Biology, Institute of Development and Aging Sciences, Nippon Medical School, Kawasaki, Kanagawa, Japan*

Correspondence: Alexander M. Wolf, Department of Biochemistry and Cell Biology, Institute of Development and Aging Sciences, Nippon Medical School, 1-396 Kosugi, Nakahara-ku, Kawasaki, Kanagawa 211-8533 Japan. E-mail: awolf@nms.ac.jp

Abbreviations: CCD, charge coupled device; GSH, glutathione in reduced form; GSSG, glutathione in oxidized form; H_2O_2, hydrogen peroxide; LED, light-emitting diode; roGFP, redox-sensitive green fluorescent protein; ROS, reactive oxygen species

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because roGFP2 fluorescence is also sensitive to variations in pH (Hanson *et al.*, 2004), whereas roGFP1 is not (Supplementary Figure S1 online). The constructs for cytosolic and mitochondrial expression are shown in Figure 1a. For cytosolic roGFP, 17 founder transgenic mice were obtained, of which 11 yielded viable offspring containing the transgene. Of these, five lines showed appreciable roGFP fluorescence. For mitochondrially targeted roGFP, 10 of 22 founder mice passed on the transgene, with 6 lines exhibiting detectable roGFP fluorescence. For each type, two lines with the strongest roGFP expression were kept for experiments.

RoGFP fluorescence was recorded using an imaging device specially built for this purpose (Figure 1b). The object is sequentially illuminated with excitation light from three light-emitting diodes (LEDs) emitting light of around 405, 450, and 470 nm (Figure 1c). Fluorescence emission from the animal is collected synchronized to excitation with a cooled charge coupled device (CCD) camera. The fluorescence emission spectrum (Supplementary Figure S1a online) is independent of excitation wavelength, and the roGFP redox state is determined from the ratios of intensities at the different excitation wavelengths. The redox state of pure roGFP can be determined from the ratio of fluorescence at 405 versus 470 nm illumination (Supplementary Figure S1b online).

In founder mice, roGFP expression could be observed in the skin, eyes (Figure 2a and Supplementary Figure S4 online), and several internal organs including the brain, pancreas, thymus, and intestine (Supplementary Figure S2 online). For analysis of redox state, the ratio of intensities at the excitation wavelengths of 405 and 470 nm was converted to color using a scale between adjustable minimum and maximum ratios, and brightness adjusted to absolute fluorescence intensity (Figure 2b). To compare the fluorescence properties of reduced and oxidized roGFP among transgenic and wild-type animals, front paws from a single litter (11 pups, 7 roGFP positive) were either oxidized with H_2O_2 (right paw, upper row) or reduced with dithiothreitol (left paw, lower row) (Figure 2c). Although fluorescence at 405 nm excitation was much higher in transgenic animals, the contribution of mouse autofluorescence to total mouse fluorescence was not negligible. When roGFP fluorescence dominates, the ratio of fluorescence indicates the extent of oxidation and thus the roGFP redox state, while canceling out the amount of indicator and absolute fluorescence intensity (Dooley *et al.*, 2004; Hanson *et al.*, 2004). However, the amount of RoGFP expression could vary between transgenic animals. This led to an expression-dependent difference in roGFP ratio even if roGFP was fully oxidized or reduced (Figure 2c). In non-transgenic animals, the 405/470 nm fluorescence ratio was much lower and unaffected by oxidation state (Figure 2c and Supplementary Figure S3 online). We included a third illumination LED emitting around 450 nm light in the hope of using spectral unmixing to distinguish between roGFP and autofluorescence, but the 450 to 470 nm excitation fluorescence ratios of autofluorescence and roGFP were very close, making spectral unmixing impossible with the currently available wavelengths (data not shown).

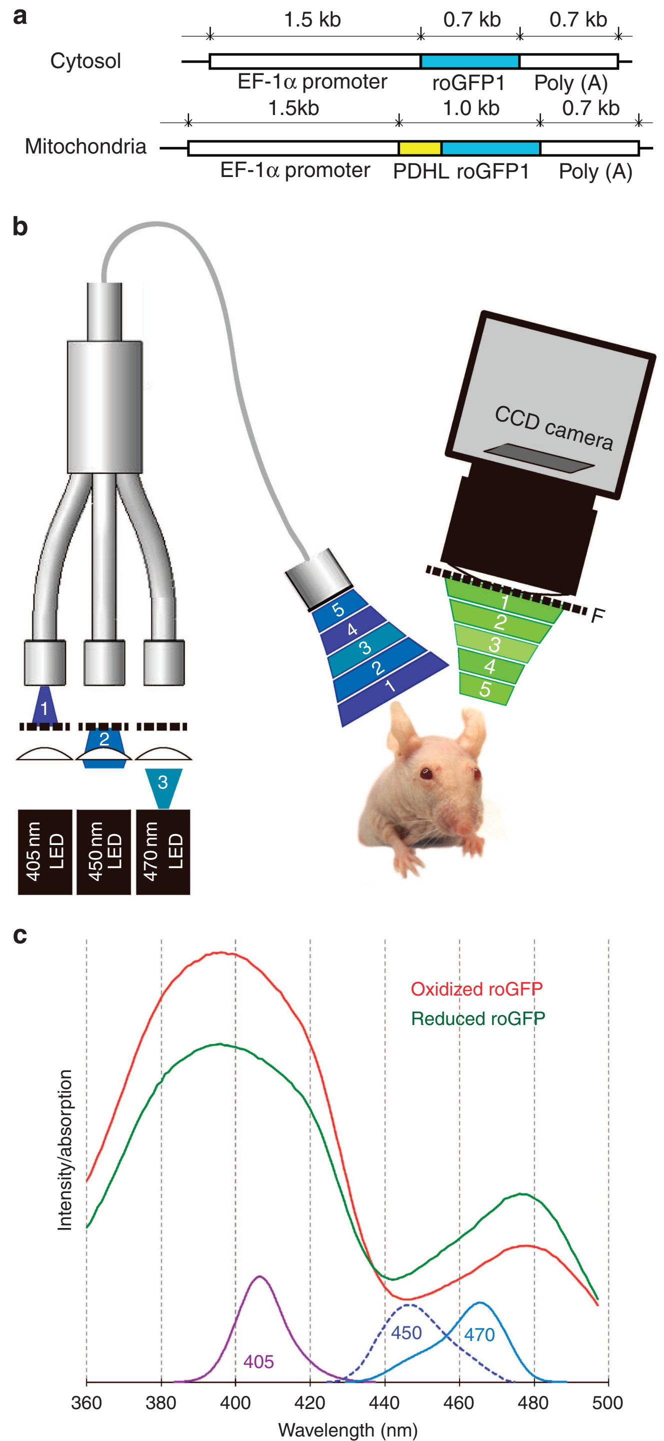

Figure 1. *In vivo* redox state recording system. (**a**) Representation of the construct used to express redox-sensitive green fluorescent protein (roGFP1) in the cytosol and mitochondria of transgenic mice. (**b**) Scheme of the setup used to record roGFP fluorescence. Light from three light-emitting diodes (LEDs) turned on sequentially synchronized to the charge coupled device (CCD) camera is combined with a randomized fiber bundle. (**c**) Absorption spectra of the oxidized and reduced forms of roGFP and emission spectra of the LEDs used for excitation ratio imaging. High 405/470 nm fluorescence ratios indicate oxidation, whereas low ratios indicate reduction of roGFP.

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