Free Radical Biology and Medicine 97 (2016) 204–211

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Epidermal growth factor-induced hydrogen peroxide production is mediated by dual oxidase 1

Gábor Sirokmány [a,b], Anna Pató [a,b], Melinda Zana [a,b], Ágnes Donkó [a,b], Adrienn Bíró [c], Péter Nagy [c], Miklós Geiszt [a,b,*]

[a] Department of Physiology, Semmelweis University, Faculty of Medicine, Budapest, Hungary
[b] "Momentum" Peroxidase Enzyme Research Group of the Semmelweis University and the Hungarian Academy of Sciences, Budapest 1094, Hungary
[c] Department of Molecular Immunology and Toxicology, National Institute of Oncology, Budapest 1122, Hungary

ARTICLE INFO

Article history:
Received 9 October 2015
Received in revised form
28 April 2016
Accepted 31 May 2016
Available online 1 June 2016

Keywords:
Reactive oxygen species
Hydrogen peroxide
EGF receptor
NADPH oxidase
Dual oxidase 1

ABSTRACT

Stimulation of mammalian cells by epidermal growth factor (EGF) elicits complex signaling events, including an increase in hydrogen peroxide (H_2O_2) production. Understanding the significance of this response is limited by the fact that the source of EGF-induced H_2O_2 production is unknown. Here we show that EGF-induced H_2O_2 production in epidermal cell lines is dependent on the agonist-induced calcium signal. We analyzed the expression of NADPH oxidase isoforms and found both A431 and HaCaT cells to express the calcium-sensitive NADPH oxidase, Dual oxidase 1 (Duox1) and its protein partner Duox activator 1 (DuoxA1). Inhibition of Duox1 expression by small interfering RNAs eliminated EGF-induced H_2O_2 production in both cell lines. We also demonstrate that H_2O_2 production by Duox1 leads to the oxidation of thioredoxin-1 and the cytosolic peroxiredoxins. Our observations provide evidence for a new signaling paradigm in which changes of intracellular calcium concentration are transformed into redox signals through the calcium-dependent activation of Duox1.

1. Introduction

Reactive oxygen species (ROS) are increasingly being recognized as important signaling molecules [1,2]. Regulated production of these compounds has been described in several settings and the list of their targets is continuously growing. Among ROS, hydrogen peroxide (H_2O_2) is probably the most intensively studied molecule [3]. Oxidation of target proteins by H_2O_2 can occur on specific reactive cysteine residues and may introduce reversible changes in protein structure and function [4,5]. Prerequisite of being a signaling intermediate is the controlled production and elimination of the given molecule and H_2O_2 seems to fit in with these requirements. There are multiple stimuli that can trigger the formation of H_2O_2 in mammalian cells and growth factor-induced H_2O_2 production is thought to be a particularly important signaling event [6,7]. EGF-induced H_2O_2 production was first described in A431 cells and has been subsequently demonstrated in other cell types as well [8,9]. The ROS response elicited by EGF is thought to amplify tyrosine-kinase signaling through the inhibition of protein tyrosine phosphatases that normally antagonize this signaling route [10,7]. Although the signaling pathways triggered by EGF are mostly well-understood, the enzymatic source of EGF-induced H_2O_2 production is currently unknown. In this work we set out to study the possible involvement of Nox/Duox enzymes in EGF-stimulated H_2O_2 production. Using A431 and HaCaT cell models we demonstrate that EGF-induced H_2O_2 production originates from the calcium-dependent activation of Duox1.

2. Materials and methods

2.1. Materials and reagents

EGFR antibodies were from Cell Signaling Technology, the Proteome Profiler Human Phospho-Kinase Array Kit was from R&D Systems, Inc. The qPCR assays were from Applied Biosystems-Life Technologies, EGF and TGFα were from PeproTech (Rocky Hill, NJ, USA). Stealth siRNAs and Fura2-AM were from Life Technologies. Amplifu Red, thapsigargin, ATPγS, niacin, GSK1016790A were from Sigma-Aldrich. Anti-rabbit–horseradish peroxidase and anti-mouse–horseradish peroxidase were from Amersham Biosciences

Abbreviations: Duox, Dual oxidase; DuoxA1, Duox activator 1; EGF, epidermal growth factor; LPO, lactoperoxidase; Nox, NADPH oxidase; NoxA1, NADPH oxidase activator 1; Noxo1, NADPH oxidase organizer 1; PRX1, peroxiredoxin 1; PRX2, peroxiredoxin 2; PTP, protein tyrosine phosphatase; ROS, reactive oxygen species; Trx1, thioredoxin 1

* Corresponding author at: Department of Physiology, Semmelweis University, Faculty of Medicine, PO Box 259, H-1444 Budapest, Hungary.
E-mail address: geiszt.miklos@med.semmelweis-univ.hu (M. Geiszt).

http://dx.doi.org/10.1016/j.freeradbiomed.2016.05.028
0891-5849/

(Piscataway, NJ, USA). Polyclonal anti-Duox antibody was raised against the Arg618-His1044 fragment of human Duox1 [11]. EZ-Link™ Maleimide-PEG11-Biotin reagent was from Thermo Fisher Scientific, Life Technologies.

2.2. Cell culture

A431 and HaCaT cells were grown in Dulbecco's Modified Eagle's Medium with glutamin and 4.5 g/L glucose, supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin (Lonza Group Ltd., Basel, Switzerland) and 10% fetal calf serum (Biowest SAS, France, S182P-500, French origin). Cells were grown in a humidified incubator with 5% CO_2 in air, at 37°C.

2.3. siRNA transfection

Stealth siRNAs were transfected at the time of cell plating in 20 nM final concentration using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturers instructions. Measurements were carried out 48 h after transfection. Three different Duox1 specific siRNA sequences were tested and three different control siRNAs of varying GC content were used as control. All Duox1 specific sequences gave efficient knockdown of Duox1 expression. For DuoxA1 knockdown one specific stealth siRNA was used.

2.4. Quantitative PCR

RNA was isolated using Qiagen RNeasy Micro and Mini kit. During the RNA purification an on-column DNase I digestion step was also included. cDNA was synthesized from 1 μg of total RNA using oligo(dT)18 and RevertAid M-MuLV reverse transcriptase (Fermentas) in 20 μl reaction mix according to the manufacturer's recommendations. One μl of cDNA was used in a 10 μl qPCR reaction using the Taqman Gene Expression 20x assays and LightCycler 480 Probes Master in a LightCycler LC480 plate reader. For each cDNA sample the expression of the target gene was divided by the expression of the endogenous control, which was β-actin. The crossing point was determined by the second derivative method. The list of the used Taqman Gene Expression assays:

Nox1: Hs00246589_m1, Nox2: Hs00166163_m1, Nox3: Hs01098883_m1, Nox4: Hs00418356_m1, Nox5: Hs00225846_m1, Duox1: Hs00213694_m1, Duox2: Hs00204187_m1, DuoxA1: Hs00328806_m1, actinB: Hs00357333_g1, p22phox: Hs03044361_m1, NoxO1: Hs00376045_g1, NoxA1: Hs00736699_m1.

2.5. Western blot experiments

Laemmli sample buffer was added to the cell lysate samples and these were run on 8–16% SDS polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were blocked in phosphate buffered saline containing 0,1% Tween- 20 and 5% dry milk or 5% bovine serum albumin (for phospho-tyrosine specific western blots). The first antibodies were diluted in blocking buffer and used either for 2 h at room temperature or overnight at 4 °C. After several washing steps in PBS-Tween-20 membranes were incubated with HRP-linked secondary antibodies (Amersham Pharmaceuticals, Amersham, UK) diluted in blocking buffer. Antibody binding was detected using enhanced chemiluminescence and Fuji Super RX medical X-ray films. Importantly samples were never boiled when processed for western blotting with the Duox1 antibody [11].

The redox state of peroxiredoxin-1, −2 and −3 was also measured by immunoblotting. HaCaT cells were treated with various stimuli as indicated in the figure legends. After the stimuli – to prevent artefactual oxidation of peroxiredoxins – cells were alkylated by incubating with 80 mM methyl-methane thiosulfonate (MMTS) in Hank's buffered salt solution. After 10 min, buffer was supplemented with 1% protease inhibitor cocktail and cells were lysed with 1% CHAPS. After 10 min incubation with CHAPS, lysates were centrifuged at 16,000x*g* for 5 min and supernatants subjected to non-reducing SDS-PAGE (12%). After transfer of proteins to PVDF membrane, membranes were blocked with 3% BSA in TBS-Tween-20 (500mM NaCl, 20 mM Tris–HCl, pH 7,4 and 0,05% Tween 20). Detection of proteins were performed using rabbit polyclonal antibodies to PRDX-1 (1:5000), PRDX-2 (1:2000), PRDX-3 (1:5000) (Sigma, St. Louis, MO, USA) and anti-Peroxiredoxin-SO3 (1:2000, AbFrontier, Seoul, Korea) in 3% BSA, probed with anti-rabbit-IgG alkaline phosphatase conjugated secondary antibody (Sigma, St. Louis, MO, USA) (1:10,000) and BCIP/NBT substrate (Merck-Millipore, Darmstadt, Germany).

The blots were analyzed on a GBox-Chemi XX6 (Syngene, Cambridge, UK) gel doc system and relative band densities of Western blots were quantified using ImageJ software.

2.6. Ca^{2+} measurements

Cell were grown on coverslips. Before the experiment cells were washed in H-medium (containing 145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.8mmol/L $CaCl_2$, 5 mmol/L glucose, and 10 mmol/L HEPES) and Fura-2-AM was loaded in a final concentration of 1 μM for 20 min at room temperature. Ratiometric fluorescence intensity measurements were performed on an inverted microscope (Axio Observer D1, Zeiss) equipped with a 40x, 1.4 oil immersion objective (Fluar, Zeiss) and a Cascade II camera (Photometrics). Excitation wavelengths were set by a random access monochromator connected to a xenon arc lamp (DeltaRAM, Photon Technology International). Images were acquired every 5 s for a period of 10–15 min with the MetaFluor software (Molecular Devices).

2.7. AmplexRed assay

Confluent cells on 24-well plates were washed in an extracellular medium “H-medium” and background fluorescence was also measured in H-medium. For the assay an H-medium-based reaction solution was used containing horse radish peroxidase and Ampliflu Red in a final concentration of 0.2 U/ml and 50 μM respectively. Agonists and inhibitors were also added to the Amplex Red containing reaction solution immediately before pipetting it on to the cells. The measurement of fluorescence started promptly after the addition of the reaction solution (0.3 ml/well) and the cells were kept at 37 °C throughout the measurement. Background fluorescence was subtracted from the fluorescence values of each well. Each experimental condition was run in 3–6 parallels on the 24-well plate.

2.8. Thioredoxin-1 labeling and detection

HaCaT cells were stimulated for 5 min at 37 °C with the indicated stimuli then washed once quickly in icecold PBS and then lysed in ice-cold RIPA buffer (containing 150 mM NaCl, 50 mM Tris pH 8.0, 0,1% SDS, 0.5% Na-deoxycholate, 1% Triton-X100 and protease and phosphatase inhibitors) in the presence of 200 μM Biotin-PEG_{11}-Maleimide. Cells were scraped on ice, collected in Eppendorf tubes and incubated at 37 °C for 25 min. Insoluble fraction was separated by centrifugation, the supernatant was mixed with non-reducing Laemmli sample buffer and heated at 80 °C for 5 min before loading onto 16% SDS-PAGE gel.