Mechanisms of Ageing and Development xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Mechanisms of Ageing and Development

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

Original Article

Circadian clock as possible protective mechanism to pollution induced keratinocytes damage

Mascia Benedusi[a,1], Elena Frigato[a,1], Mattia Beltramello[a], Cristiano Bertolucci[a,2], Giuseppe Valacchi[a,b,*,2]

[a] Department of Life Sciences and Biotechnology, Section of Medicinal and Health Products, University of Ferrara, I-44121 Ferrara, Italy
[b] NC State University, Plants for Human Health Institute, Animal Science Dept., NC Research Campus, Kannapolis, NC 28081, USA

ARTICLE INFO

Keywords:
Ozone (O_3)
Oxidative stress
Circadian clock
Antioxidant pathway
Skin homeostasis

ABSTRACT

Ozone is among the most toxic environmental stressors to which we are continuously exposed. Due to its critical location, skin is one of the most susceptible tissues to oxidative stress damaging effect of ozone. An increasing collection of data suggests a significant role of circadian system in regulation of cellular response to oxidative stress. However, the molecular mechanism linking circadian clock and antioxidant pathway it is not completely understood. Here we investigated a possible protective role of entrained circadian clock to ozone induced damage in keratinocytes, the main cellular component of human epidermis. Our results showed that, clock-synchronized keratinocytes compared to arrhythmic ones exhibited a more efficient antioxidant response, attested by a faster activation of the master antioxidant regulatory factor NRF2. Moreover, analysis of clock gene expression profiles reveals a more rapid induction of the cardinal clock gene *Bmal1* in entrained cells. Based on these findings, we suppose that an adequate coordination of circadian system and antioxidant pathway might be essential to maintain homeostasis in the skin. Alteration of metabolic pathways occurred in neurological diseases or in irregular schedule of life activity could negatively influence tissue gene expression programs and associated organ physiology *via* its effect on the circadian system.

1. Introduction

The circadian clock is an essential endogenous time-keeping mechanism evolved to synchronize (*entrain*) physiological processes to environmental changes. This fine-tuned adaptive mechanism is strictly linked to external stimuli and/or stressors.

Some of the more common environmental stress factors are chemical hazards, ionizing radiations and pollutants (Valacchi et al., 2012). Ozone (O_3) is among the most toxic stressors to which we are continuously exposed. O_3, is a small gaseous molecule characterized from an acrid and pungent smell, which concentration can exceed 0.8 ppm in highly polluted urban environment (Mustafa 1990; Valacchi et al., 2002). The toxicity of O_3 is largely due to its oxidative power and its capability to oxidize biomolecules (Khadre and Yousef, 2001). O_3, although is not a radical molecule, for its strong oxidizing power and for its instability is able to immediately reacts with other molecules found in solution, giving thus origin to the production of peroxides, aldehydes and lipid ozonation products (Laisk et al., 1989; Sarti et al., 2002). The generation of these products has been shown to be prevented by antioxidant supplementation, confirming that O_3 induces oxidative stress (Valacchi et al., 2012).

Cells have evolved an elaborate and powerful cellular defense machinery to struggle deleterious effects of oxidative stress. A key transcription factor orchestrating antioxidant defensive machinery is nuclear factor erythroid 2–related factor 2 (NRF2), a protein with a molecular weight of 95–110 kDa belonging to the cap'n'collar family of basic leucine zipper transcription factors (Kensler et al., 2007).

In particular, NRF2 is constitutively produced in the cell and in unstressed condition, it is sequestered in the cytoplasm by binding to an inhibitory protein, Kelch-like ECH associated protein-1 (KEAP1) (Kobayashi and Yamamoto, 2006). Oxidative stress leads to modification of reactive cysteines within KEAP1 that induces conformational changes resulting in NRF2 stabilization. The NRF2 protein then translocates into the nucleus where it binds to the antioxidant response elements (AREs) on the promoter of classical antioxidant enzymes (Loboda et al., 2016).

* Corresponding author at: NCSU, PHHI Building, NC Research Campus, Kannapolis, NC, USA.
E-mail address: gvalacc@ncsu.edu (G. Valacchi).
[1] Equally contributed to the manuscript.
[2] Equally supervised the work.

http://dx.doi.org/10.1016/j.mad.2017.08.017
Received 24 April 2017; Received in revised form 23 August 2017; Accepted 28 August 2017

A growing collection of data shows evidence of differences in DNA damage, lipid peroxidation and protein oxidation during the day (Kanabrocki et al., 2002; Lapenna et al., 1992; Tomás-Zapico et al., 2003). These oscillations are strongly related to the daily rhythm of antioxidant expression and protective enzyme activity levels. Interestingly, many of the key oxidative stress associated molecules are directly regulated by circadian clock molecular machinery. The basic circadian molecular clockworks consist of interacting transcriptional/translational feedback loops. Briefly, a positive loop includes CLOCK-BMAL heterodimers, transcriptional activators that bind to E-box elements located in the promoter of negative elements such as *Period* (*Pers*) and *Cryptochrome* (*Crys*). The negative limb of the feedback loop represses their own transcription by inhibiting CLOCK-BMAL activity (Reppert and Weaver, 2001).

In vertebrates the circadian clock consists of a hierarchy of multiple pacemakers. The central pacemaker is located in the suprachiasmatic nuclei of the hypothalamus and plays an essential role in coordinating the functions of the peripheral oscillators which are distributed in about all tissues, organs and cells (Mohawk et al., 2012).

As the skin represents a barrier between the external milieu and the body and it is directly exposed to changes in environmental condition, many physiological processes are characterized by daily fluctuations (Le Fur et al., 2001). Also epidermal cell proliferation is associated with specific times of day and displays a circadian periodicity (Matsuo et al., 2003); cell division is regulated by circadian clockwork that minimize DNA replication during exposure to the sun's UV rays providing protection against UV-induced DNA damage (Geyfman et al., 2012). The existence of a circadian clock in skin cells has been shown in human keratinocytes, melanocytes, and fibroblasts (Spörl et al., 2011; Zanello et al., 2000; Sandu et al., 2012).

As mentioned above, the skin is chronically exposed to stress from such as O_3, which leads to antioxidant depletion as well as lipid and protein oxidation in the skin (Thiele et al., 1997) and, in particular, it has been demonstrated that O_3 induced significant skin depletion of Vitamins E and C in concert with increased lipid peroxidation (Valacchi et al., 2002, 2015).

Here we sought to identify the link between the skin circadian clock and the oxidative stress induced by ozone treatment, with the main aim to established a possible role of the circadian machinery in regulating the skin damage in response to oxidative stress and if the disruption of circadian timing mechanisms can make skin cells more vulnerable to exogenous damaging agents. To do this we performed an *in vitro* study using immortalized human keratinocyte cell line, HaCaT cells, since keratinocytes are the main cellular component of the epidermis and provides an efficient barrier against outdoor stressors, at the same time, possesses a robust peripheral molecular clock (Spörl et al., 2011).

2. Materials and methods

2.1. Cell culture

HaCaT cells line (obtained from American Type Culture Collection, ATCC), were cultured in DMEM medium (Lonza, Milan, Italy) supplemented with 10% fetal bovine serum (FBS, EuroClone, Milan, Italy), 1% of L-glutammine (Lonza, Milan, Italy) and 1% of penicillin/streptomycin antibiotics (Lonza, Milan, Italy) at 37 °C in 5% CO_2.

To synchronize the circadian clock, cells were seeded into 6 well-plates, cultured to confluence and then treated with 1 μM dexamethasone (dex, Sigma-Aldrich, Hamburg, Germany) for 1 h.

Ozone treatment was performed in previously synchronized and control cells 18 h after dex treatment.

2.2. Ozone exposure

O_3 was generated from O_2 by electrical corona arc discharge (ECO_3 model CUV-01, Torino, Italy), as previously described (Valacchi et al., 2015). The O_2–O_3 mixture (95% O_2, 5% O_3) was combined with ambient air and allowed to flow into a Teflon-lined exposure chamber, with the O_3 concentration in chamber adjusted to the ppm needed for the experiment and continuously monitored by an O_3 detector. Temperature and humidity were monitored during exposures (37 °C and 45–55%, respectively).

2.3. Cytotoxicity determination

Cytotoxicity study was performed after the different treatments by measurement of LDH (lactate dehydrogenase) release according to manufacturer's protocol (EuroClone, Milan, Italy). In order to obtain a representative maximal LDH release as the positive control with 100% toxicity, a triplicate set of samples were lysed with 2% (V/V) Triton X-100 in culture media for 30 min at 37 °C.

Cell growth and morphology were visualized directly, using a built-in camera in an inverted Nikon Eclipse microscope (20X magnification) at the same time points. A visual cytotoxicity score was attributed according to a 3-point rating scale, ranging from 1, non-cytotoxic, to 3, markedly cytotoxic, based on visible characteristics of cell distribution and lysis. A score of 1 was assigned for a confluent monolayer of well-defined cells; cell morphology and density are unchanged. No cell lysis is observed, indicating a non-cytotoxic reaction. A score of 2 was assigned when 30–50% of the cells are rounded or lysed and a score of 3 was assigned when cell viability is seriously compromised, indicating a cytotoxic effect.

2.4. RNA extraction and gene expression analysis

Total RNA was isolated from confluent cells using TRIzol Reagent (Invitrogen, Carlsbad, California, USA) according to manufacturer's protocol. The amount, quality and composition of isolated RNA were analyzed by BioSpec-nano (Shimadzu, Kyoto, Japan). One microgram of DNase-treated RNA was used to perform cDNA synthesis, using iScript cDNA Synthesis Kit (Biorad, Milan, Italy). First-strand cDNA was PCR amplified with a CFX Real-Time PCR Detection System (Bio-Rad, Milan, Italy) using SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA). After amplification, a melting curve analysis to confirm the specificity of the amplicons was performed. Gene-specific primers for human *Bmal1*, *Per3*, and *18s* have been previously described (Avitabile et al., 2014). The relative levels of each sample were calculated by the $2^{-\Delta\Delta CT}$ method (where CT is the cycle number at which the signal reaches the threshold of detection) (Livak and Schmittgen, 2001). Each CT value used for these calculations is the mean of three replicates of the same reaction.

2.5. Total protein extraction

Cells were seeded in 60 mm petri (1.5×10^6 cells). After treatments, cells were detached, washed twice with ice-cold PBS 1X and total cell lysates were extracted in ice-cold solubilization buffer containing: 20 mM Tris pH 8, 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 10 μg/ml phenylmethylsulfonyl fluoride (PMSF) and 5 mM β-glycerophosphate (Sigma, Milan, Italy). After centrifugation (15,000 × *g*, 15 min at 4 °C), the supernatants were collected. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Milan, Italy).

2.6. Nuclear-cytosolic proteins extraction

For cytoplasmic and nuclear extracts, cells were seeded in 100 mm petri (3×10^6 cells). After treatments, cells were detached, washed with ice-cold PBS 1X and cell pellets were resuspended in hypotonic buffer containing 10 mmol/l HEPES (pH 7.9), 10 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.3% Nonidet P-40, 0.5 mmol/l dithithreitiol,