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Oxidative damage in keratinocytes exposed to cigarette smoke and aldehydes

Katia Avezov [a,b], Abraham Z. Reznick [a,*], Dror Aizenbud [a,b]

[a] Department of Anatomy and Cell Biology, Rappaport Faculty of Medicine, Technion – Israel Institute of Technology, Haifa, Israel
[b] Orthodontic and Craniofacial Department, School of Graduate Dentistry Rambam Health Care Campus, Haifa, Israel

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ABSTRACT

Cigarette smoke (CS) is a significant environmental source of human exposure to chemically active saturated (acetaldehyde) and α,β-unsaturated aldehydes (acrolein) inducing protein carbonylation and dysfunction. The exposure of oral tissues to environmental hazards is immense, especially in smokers. The objectives of the current study were to examine the effect of aldehydes originating from CS on intracellular proteins of oral keratinocytes and to observe the antioxidant response in these cells.

Intracellular protein carbonyl modification under CS, acrolein and acetaldehyde exposure in the HaCaT keratinocyte cell line, representing oral keratinocytes was examined by Western blot. Possible intracellular enzymatic dysfunction under the above conditions was examined by lactate dehydrogenase (LDH) activity assay. Oxidative stress response was investigated, by DCF (2,7-dichlorodihydrofluorescein) assay and GSH (glutathione) oxidation.

Intracellular protein carbonyls increased 5.2 times after CS exposure and 2.7 times after exposure to 1 μmol of acrolein. DCF assay revealed an increase of fluorescence intensity 3.2 and 3.1 times after CS and acrolein exposure, respectively. CS caused a 72.5% decrease in intracellular GSH levels compared to controls. Activity of intracellular LDH was preserved.

α,β-Unsaturated aldehydes from CS are capable of intracellular protein carbonylation and have a role in intracellular oxidative stress elevation in keratinocytes, probably due to the reduction in GSH levels.

1. Introduction

Cigarette smoke (CS) is a multipotent mixture of numerous components associated with disorders in different organs, and involved in many pathological processes (Kuper et al., 2002). The molecular mechanisms involved in CS damage to the organism are diverse, and have not yet been fully disclosed. One of the mechanisms of CS damage is oxidative stress (OS), defined as an imbalance between the load of chemically reactive oxidants such as reactive oxygen and nitrogen species (ROS and RNS) and the ability of a biological system to detoxify them or to repair the resulting damage (Valko et al., 2007). Disturbances in the normal redox state of cells can cause toxic effects to all cellular components, including proteins, lipids, carbohydrates and DNA. OS is thought to be involved in the development of over 100 seemingly unrelated diseases including cancer, Parkinson's, Alzheimer's, atherosclerosis, myocardial infarction as well as periodontal disease (Valko et al., 2007; Lavie and Lavie, 2009; Reibel, 2003; D'Aiuto et al., 2010).

There are endogenous as well as exogenous ROS/RNS sources. Endogenously ROS are a natural byproduct of mitochondrial oxygen metabolism. ROS/RNS are also produced by leukocytes in order to destroy pathogens. Exogenously free radicals originate from sources such as ionizing radiation and environmental pollutants such as CS. The gas phase of CS contains more than 10^{15} free radicals per puff while the particulate phase contains more than 10^{17} free radicals per gram (Swan and Lessov-Schlaggar, 2007). ROS contained in the CS gas phase include hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radical ($OH^\cdot$). Additionally, cigarette smoke is a great source of RNS such as nitric oxide (NO). NO and superoxide may react and form peroxynitrite ($ONOO^-$), a potent oxidizing and nitrating compound which also has been linked to a variety of pathological conditions (Hasnis et al., 2007).

Furthermore, cigarette smoke constitutes one of the largest environmental sources of human exposure to another group of highly chemically active substances: volatile aldehydes. These include saturated (mainly acetaldehyde) and α,β-unsaturated aldehydes (mainly acrolein and crotonaldehyde) capable of protein

* Corresponding author. Address: Department of Anatomy and Cell Biology, Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, P.O. Box 9649, Haifa, Israel. Tel.: +972 48295388; fax: +972 48295403.
E-mail address: Reznick@technion.ac.il (A.Z. Reznick).

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carbonylation leading to protein dysfunction, an increase in oxidative stress and disease onset, including cancerous transformation processes (Nystrom, 2005; Dalle-Donne et al., 2003; Colombo et al., 2010; Kehrer and Biswal, 2000). Smoke from a single cigarette contains twenty times less unsaturated aldehydes (0.21 μmol crotonaldehyde and 0.8 μmol acrolein) than saturated aldehydes (19.3–22.7 μmol acetaldehyde) (Reznick et al., 1992). However, the former are a major source of reactive double bonds reacting with -SH (thiol) groups of proteins in a Michael addition reaction (Nagler et al., 2000). In this reaction aldehydic carbonyls are attached to a protein and induce structural alterations. This is particularly critical in enzymes' activity, since carbonylation can lead to their dysfunction.

Oral cavity tissues are the first to encounter cigarette smoke and its toxic constituents entering the body. Moreover, oral cells are uniquely susceptible to free radical damage because the oral mucus membranes allow rapid absorption of substances across their surface. All oral tissues are affected by CS: teeth, mucosa, salivary glands and the saliva. The effects of cigarette smoke on the oral cavity range from simple tooth staining, inflammatory conditions to oral cancer (Reibel, 2003). In addition, macromolecules such as enzymes found in the saliva may lose their activity (Zappacosta et al., 2002). For instance, lactate dehydrogenase (LDH) activity was shown to decrease after exposure to CS (Nagler et al., 2001). Although the effect of CS on saliva is transient as it is continuously secreted, CS components such as aldehydes, which are readily dissolved in saliva, can potentially penetrate into oral keratinocytes and inactivate intracellular as well as extracellular proteins (Lambert et al., 2005). This specifically applies to heavy smokers suffering from an additive effect. Furthermore, the increase in oral oxidative stress from free radical formation through periodontal infection, alcohol, dental procedures and substances leads to further breakdown of cell walls of oral tissues and exacerbates inflammation (Ismahil et al., 2011). Recent studies have shown that this is a major contributing factor to systemic inflammatory diseases, including rheumatoid arthritis, vascular and cardiovascular diseases (D'Aiuto et al., 2010; Lee and Park, 2013).

Biological systems have developed the ability to detoxify both endogenously generated ROS and environmental oxidative and chemically active agents (Tomitori et al., 2012). One of the immediate antioxidant agents is glutathione (GSH), a tripeptide containing a thiol group acting as a reducing agent. GSH reduces disulfide bonds in cytoplasmatic proteins by serving as an electron donor. In this process, GSH is converted to its oxidized form, glutathione disulfide (GSSG). Once oxidized, GSSG can be reduced to GSH by the glutathione reductase enzyme (GSH-RD), using NADPH as an electron donor. The ratio between GSH and GSSG is used as a measure of the cellular oxidative status.

Despite constant exposure of oral cavity tissue in smokers to CS and its chemically active constituents such as aldehydes, the effect of aldehydes on oral cells and antioxidant defense systems was not extensively studied. The objectives of the current study were to examine the effect of aldehydes originating from CS on intracellular proteins of keratinocytes and to observe the antioxidant response in these cells.

2. Materials and methods

2.1. Cell culture and cell viability assay

HaCaT keratinocyte cell line acquired from CLS Cell Lines Service (Eppelheim, Germany) was used in the experiments. HaCaT are in vitro spontaneously transformed keratinocytes from histologically normal human skin. The line is referred to as immortal (>140 passages), maintains full differentiation capacity and is non-tumorogenic (Boukamp et al., 1988). These cells are widely used (Ge et al., 2012) as a model for epithelial tissue studies, including oral epithelium investigations, due to their high proliferation rate. The cells were cultured in 100 mm Nunclon cell culturing dishes and incubated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine, 10% fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37 °C humidified atmosphere containing 5% CO_2. Results of tests for mycoplasma, bacteria and fungi were negative. Before each set of experiments, cells from a frozen stock were thawed and passaged for 6–10 times. After each passage, the cells were grown for 4 days until 90% confluency was reached, then the experiments were performed. All the experiments were executed in 100 mm Nunclon cell culturing dishes submerged in 10 ml of cell culturing medium. For the DCF assay, the cells were cultured on 0.17 mm coverslips placed in 100 mm petri dishes. Viability was assessed by the trypan blue exclusion method. Immediately after the experiments, the cells were lysed, centrifuged to remove cell debris and preserved in an ultra-low temperature freezer (−80 °c) for carbonylation and GSH/GSSG assays for up to 15 days. Fresh cell lysates were used for the LDH enzyme activity assay.

2.2. Exposure of cell cultures to CS and aldehydes

The study was carried out using cigarettes (filtered "Time" cigarettes, Dubek, Israel, containing 14 mg of tar and 0.9 mg of nicotine per cigarette) combined with a source of lowered pressure system as previously described (Rom et al., 2013). The CS exposure system is schematically shown in Fig. 1I. In short: an open 100 mm Petri dishes with cell cultures submerged in 10 ml of culture medium (as mentioned before) were placed in a sealed reservoir with a sidearm to which a cigarette was attached. A reproducible low pressure was created in the reservoir by a vacuum pump and valve A was closed. When the attached cigarette was lit, valve B was opened for 10 s and the smoke from the lit cigarette was drawn in. This was considered a single "puff". According to previous works in our laboratory, a single cigarette smoked in the above apparatus statistically contains nine "puffs" (Weiner et al., 2008).

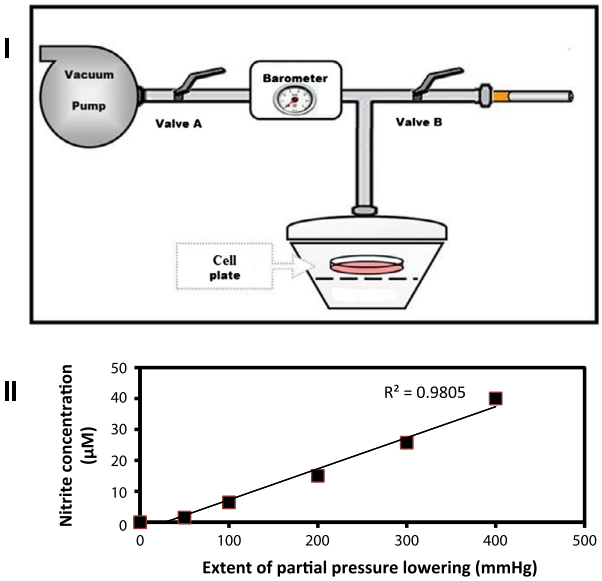

Fig. 1. Exposure of cell cultures to CS. 1 I: CS exposure system. Reproduced with permission of the copyright owner. From: Rom O, Mech Ageing Dev (2012). 1 II The correlation between the extent of lowering the pressure in the reservoir and the detected nitrite concentrations in PBS samples.