Toxicology and Applied Pharmacology 267 (2013) 30–40

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

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

NRF2 activation is involved in ozonated human serum upregulation of HO-1 in endothelial cells

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ARTICLE INFO

Article history:
Received 25 August 2012
Revised 16 November 2012
Accepted 5 December 2012
Available online 16 December 2012

Keywords:
Keap1
Ozone
4-hydroxynonenal
Hydrogen peroxyde
Heme oxygenase-1

ABSTRACT

During the last decade, it has been shown that the activation of NRF2 and the binding to electrophile-responsive element (EpREs), stimulates the expression of a great number of genes responsible for the synthesis of phase I and phase II proteins, including antioxidants enzymes and heme oxygenase-1 (HO-1). This critical cell response occurs in cardiovascular, degenerative and chronic infective diseases aggravated by a chronic oxidative stress. In our previous reports we have shown that ozonated plasma is able to up-regulate HO-1 expression in endothelial cells. In the present work we investigated a candidate mechanism involved in this process. After treatment with increasing doses of ozonated serum (20, 40 and 80 μg/mL O_3 per mL of serum), a clear dose dependent activation of NRF2 and the subsequent induction of HO-1 and NAD(P)H quinone oxidoreductase 1(NQO1) was observed. This effect was also present when cells were treated with serum and hydrogen peroxide (H_2O_2) or serum and 4-hydroxynonenal (4HNE). Moreover, the treatment with ozonated serum was associated with a dose-dependent activation of extracellular-signal-regulated kinases (ERK1/2) and p38 MAP kinases (p38), not directly involved in NRF2 activation.

These data, provide a new insight on the mechanism responsible for the induction of HO-1 expression by ozonated serum in the endothelium, and have a practical importance as an expedient approach to the treatment of patients with both effective orthodox drugs and ozonated autohemotherapy, targeted to the restoration of redox homeostasis.

Introduction

During the last decade pivotal data have been published demonstrating that the activation of the transcription factor nuclear factor (erythroid-derived2)-like2 (NRF2) is associated to its translocation into the nucleus, heterodimerization with the regulatory MAF protein, and the binding to the electrophile-responsive element EpREs sequence in the promoter of several genes. This binding is therefore associated to the induction of the expression of a great number of enzymes involved in cell detoxification and antioxidant defense (Kensler et al., 2007; Kim et al., 2010a; Motohashi and Yamamoto, 2004; Taguchi et al., 2011; Zhang, 2006). *Nrf2* gene belongs to the CNC (cap'n'collar) family of human genes encoding for basic leucine zipper (bZIP) transcription factors. This family includes p45NF-E2, *Nrf1* and *Nrf3*, *Nrf2* being probably the most important within the process of cell defense. Six conserved domains are present within NRF2, the Neh2 domain allowing its binding to the cytosolic repressor Keap1 (Motohashi and Yamamoto, 2004; Yamamoto et al., 2008; Zhang, 2006).

The mechanism of activation of NFR2 is quite well known. Briefly, *Nrf2* is expressed in the kidney, muscle, lung, heart, liver and brain and normally it has a half-life of 20 min. In fact, in the absence of a specific stimulus, Keap1, which is an actin-binding protein repressing NRF2, interacts with Cullin 3 (Cul3), which is one component of the ubiquitin ligase, leading the complex into the proteasome 26S for digestion.

The following agents favor the transcription activation and translocation of NRF2 into the nucleus: a) electrophilic reagents and/or oxidative stress due to reactive oxygen species (ROS) (Petersen and Doorn, 2004); b) nitric oxide (NO) activates guanylyl cyclase, which in turn activates cGMP-dependent protein kinase (Um et al., 2011); c) protein kinase C is involved in NRF2 activation by both oxidative

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http://dx.doi.org/10.1016/j.taap.2012.12.001

stress and electrophiles through the phosphorylation of a serine residue located in the N-terminal region (Leonarduzzi et al., 2004); d) NRF2, which is a substrate of MAPK/ERK (extracellular signal-regulated kinase) or PERK (protein kinase R-like endoplasmic reticulum kinase), dissociates from Keap1 in a phosphorylation-dependent manner (Kensler et al., 2007); e) hydroperoxides are less likely to act as they are rapidly metabolized and cannot enter into cells.

On the basis of this background, the results of several experimental (Barber et al., 1999; Peralta et al., 1999) and clinical (Biedunkiewicz et al., 2004; Bocci, 2011; Bocci et al., 2007; Clavo et al., 2003; De Monte and Gori, 2011; Di Paolo et al., 2002, 2005; Rokitanski et al., 1981; Tylicki et al., 2003) studies considered the treatment of either a mixture of gas (O_2:96%–O_3:4%) or ozonated blood *ex vivo* by using small O_3 dosages (10–50 μg/ml gas per ml of blood or 0.21–1.05 μM O_3), regularly observing a constant upregulation of antioxidant proteins, phase II enzymes and HO-1. The progress of ozone therapy, revealed that these protective enzymes are able to reverse the oxidative stress induced by chronic vascular and degenerative diseases and restore redox homeostasis.

Few years ago, we have reported that O_3 immediately dissolves in the water-phase of plasma and reacts with antioxidants and albumin bound polyunsaturated fatty acids (PUFA) (Bocci et al., 1998a). Consequently, O_3 disappears generating two messengers: H_2O_2, which enters into all blood cells and activates several relevant biochemical pathways (Bocci et al., 2009). The second is represented by different alkenals (mainly 4HNE) generated by PUFA peroxidation. H_2O_2 is a ROS but it has a half-life of about 20 s in the blood, while 4HNE forms adduct with the Cys34 of albumin or with glutathione. The rapid infusion of ozonated blood into patients allows the entrance of 4HNE into many body's cells. When this electrophile binds to Cys 151 of Keap1, it suppresses the constitutive inhibition of NRF2, which then translocates into the nucleus as mentioned above. After binding to MAF, NRF2 binds to the EpREs and switches on the synthesis of a variety of highly protective enzymes (He et al., 2011; Kang et al., 2004; Kensler et al., 2007; Yang et al., 2011).

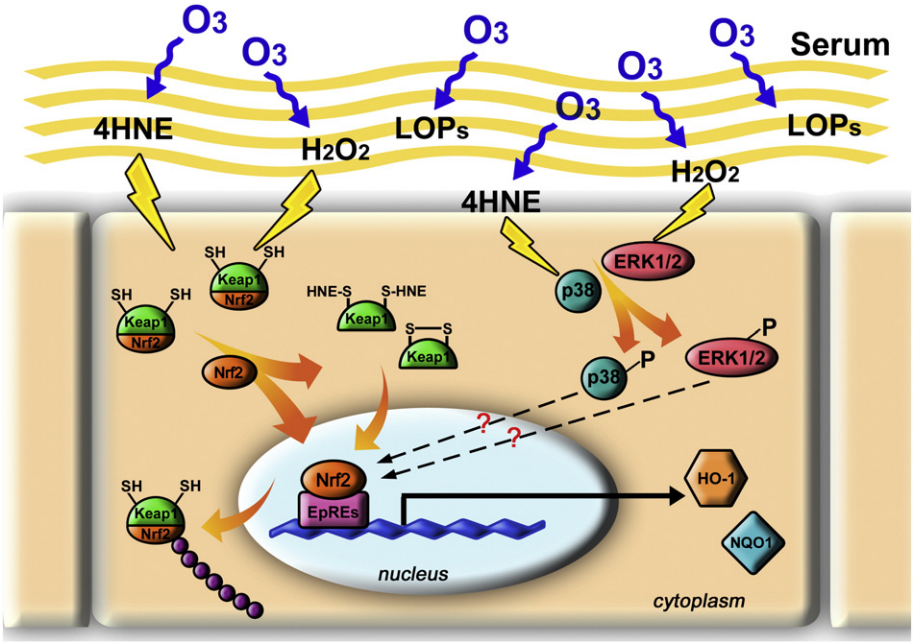
In the present study, we report that ozonated serum, acts as a calculated acute oxidative stress, triggering NRF2 nuclear translocation in a dose-dependent fashion with a subsequent induction of HO-1 and NQO1 in human endothelial cells. Owing to the fact that the NRF2 activation by ozonated serum may be due to a number of its byproducts, our work was aimed to clarify the role played by the key messengers, H_2O_2 and 4HNE (Scheme 1).

Materials and methods

Chemicals. 4-hydroxynonenal (4HNE) was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany), hydrogen peroxide (H_2O_2, 30% solution) from Sigma-Aldrich (St. Louis, MO, USA), PD98059 and SB203580 from Sigma-Aldrich (St. Louis, MO, USA).

Ozone generation and measurement. Ozone was generated from medical-grade oxygen (O_2) using electrical corona arc discharge, by the O_3 generator (Model Ozonosan PM100K, Hansler, GmbH, Iffezheim, Germany), which allows the gas flow rate and O_3 concentration to be controlled in real time by photometric determination, as recommended by the Standardization Committee of the International O_3 Association. The O_3 flow rate was kept constant at 3 L/min in all experiments. Polypropylene syringes (O_3-resistant) were used throughout the reaction procedure to ensure containment of O_3 and consistency in concentrations.

Collection of human serum samples and ozonated serum preparation. Fully screened human serum samples were obtained from the blood center of our University. Each serum sample of 20 ml, contained in a 50 ml syringe, was immediately treated with an equal volume of gas mixture (95%O_2–5%O_3), with the O_3 concentration (from 20 up to 80 μg/ml of gas per ml of serum, i.e.: 0.42–1.68 μM) photometrically checked in real time. The gas withdrawn in a 20 ml syringe was introduced into the 50 ml syringe containing the serum sample via a multidirectional stopcock. We have previously determined that a



Scheme 1. Schematic model of ozonated serum activates endothelial Nrf2. Once ozone is in contact with serum it reacts with PUFA leading to the formation o the two fundamental messengers: hydrogen peroxide (H_2O_2) as a ROS and 4-hydroxynonenal (4HNE) as a lipid oxidation product (LOPs) ($-R\text{-}CH=CH\text{-}R + H_2O + O_3 \rightarrow 2\ RCHO + H_2O_2$). Under homeostatic conditions, Nrf2 is sequestered in the cytoplasm by the Keap1 complex and rapidly degraded in the ubiquitin-proteasome dependent manner. This Keap1-mediated degradation activity requires two reactive cysteine residues (Cys273 and Cys288) of Keap1. After the oxidative/electrophilic stress challenge, modification of these cysteine residues of Keap1 (S-HNE or —S—S) inhibits ubiquitin conjugation to Nrf2 by the Keap1 complex, provoking the nuclear accumulation of Nrf2. Once in the nucleus, Nrf2 is able to bind to the ARE sequences and to induce the transcription of phase II enzymes (i.e. HO-1 and NQO1). Although ozonated serum induced MAPK activation in endothelial cells, it is not fully clear whether p38 and/or ERK1/2 are directly or indirectly involved in ozonated serum induced endothelial HO-1 up-regulation.

rapid rotation of the syringe along its longitudinal axis (about 80 cycles/min) for 1 min achieved a complete mixing of the liquid–gas phases with minimal foaming and that, within this period of time, the O_3 dose reacts completely with substrates. The pO_2 reached a value of about 400 mm Hg, while the serum pCO_2 and pH values did not change. In order to obtain reproducible results, it needs to be emphasized that O_3 is a very reactive gas meaning that an extremely rapid and precise handling is required. The final gas pressure remained at normal atmospheric pressure. Control serum sample received only O_2. Immediately after either the oxygenated or the ozonated samples were promptly distributed in cell culture dishes for various treatments.

Determination of 4HNE protein adducts levels in ozonated serum. Immediately after the preparation of ozonated serum, the samples were processed for Western blotting analysis. Protein concentration was determined by the method of Bradford (Biorad Protein assay, Milan, Italy). Samples (30 μg of protein) were separated on 10% sodium dodecyl sulphate–polyacrylamide electrophoresis gels. Each gel was blotted onto nitrocellulose membrane. Blots were blocked for 1 h in Tris-buffered saline, pH 7.5, containing 0.1% Tween 20 and 3% milk and, then, incubated overnight at 4 °C with 4HNE antibody (AB5605; Millipore Corporation). After three rinses in TBST buffer, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, and the bound antibody were detected using chemiluminescence (BioRad, Milan, Italy). Images of the bands were digitized and the densitometry of the bands was performed using ImageJ software.

H_2O_2 measurement in ozonated serum. Measurement of H_2O_2 was performed as previously shown by Sticozzi et al. (Sticozzi et al., 2012) by monitoring the horseradish peroxidase (HRP)-catalyzed oxidation of the probe N-acetyl-3,7- dihydroxyphenoxazine (A6550; Molecular Probes, Eugene, OR, USA), which becomes highly fluorescent only after oxidation by H_2O_2.

Cell culture and treatments. Human endothelial EA.hy 926 cells (a cell line gift from Prof. Fabio Virgili) were grown in Dulbecco's modified Eagle's medium low Glucose (Gibco, Life Technologies, Monza, Italy), supplemented with 10% fetal bovine serum (FBS), 2% HAT supplement (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Cells were incubated at 37 °C with 5% CO_2 until 80% confluence.

Preliminary experiments were carried out to assess the toxicity of ozonated serum, H_2O_2, and 4HNE in EA.hy 926 endothelial cells, measuring the release of lactate dehydrogenase (LDH) activity in culture media, using a commercially available kit (Clontech Laboratories, Inc., Mountain View, CA, USA). Values were expressed as a percentage of the total LDH activity released by untreated cells after lysis with Triton X-100.

EA.hy 926 cells were treated with ozonated serum (20, 40 and 80 μg/ml O_3 per mL of serum) for 15, 30, 45 and 60 min to evaluate NRF2 and Keap1 nuclear translocation, 4HNE protein adducts levels, ERK1/2 and p38 activation. In an additional experiment, cells were exposed to H_2O_2 (30 and 100 μM) or 4HNE (5 and 20 μM) in medium for various time points (15, 30, 45 and 60 min) for the assessment of NRF2 nuclear migration. In parallel, we treated the cells with the higher concentrations of H_2O_2 (100 μM) or 4HNE (20 μM) in human serum. To evaluate HO-1 and NQO1 levels, cells were pretreated with ozonated serum. After 1 h, serum was replaced with medium then HO-1 and NQO1 expression were assessed after 6 and 24 h.

In some experiments, endothelial cells were pre-treated for 30 min with PD98059 (50 μM), a specific inhibitor of mitogen-activated ERK kinase (MEK) and SB203580 (10 μM) a specific inhibitor of p38 activation, before the treatment with ozonated serum (20, 40 and 80 μg/ml O_3 per mL of serum).

Preparation of total, cytoplasmic and nuclear extracts for Western blotting. For Western blotting analysis, cells were seeded (3×10^6 cells/ml) in 100 mm dishes. After treatment, they were washed twice with ice-cold PBS and then scraped with PBS. Cells were then centrifuged at 1400 rpm for 5 min. The supernatant was discarded and the pellet was lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors for total protein extracts, as previous described (Valacchi et al., 2009).

For cytoplasmic and nuclear extracts, pellets were resuspended in 100 μl of 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 dithiothreitiol, 0.5 mmol/L phenylmethylsulphonyl fluoride, protease inhibitor cocktail, 1 mmol/L ortho-vanadate, 5 mmol/L β-glycerophosphate. The lysates were incubated for 15 min on ice and then centrifuged at 1500 g for 5 min at 4 °C for collection of the supernatants containing cytosolic proteins. Pellets containing crude nuclei were resuspended in 50 μl of extraction buffer containing 20 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$, 0.6 mol/L KCl, 0.2 mmol/L EDTA, 20% glycerol, and 0.5 mmol/L phenylmethylsulphonyl fluoride, protease inhibitor cocktail, 1 mmol/L ortho-vanadate, 5 mmol/L β-glycerophosphate and then were incubated for 30 min on ice. Samples were then centrifuged at 13000 rpm for 15 min to obtain supernatants containing nuclear fractions. Cytoplasmic and nuclear fractions were stored at −80 °C until use.

Western blotting analysis. Proteins concentration was determined by the method of Bradford (Biorad Protein assay, Milan, Italy). Samples (30 μg protein) were loaded onto 10% sodium dodecyl sulphate–polyacrylamide electrophoresis gels and then transferred onto nitrocellulose membranes. Blots were blocked for 1 h in Tris-buffered saline, pH 7.5, containing 0.1% Tween 20 and 3% milk. Membranes were incubated overnight at 4 °C with the appropriate primary antibody: NRF2 (Abcam, Cambridge, UK), Keap1 (ABS97; Millipore Corporation, Billerica, MA, USA), 4HNE (AB5605; Millipore Corporation), HO-1 and NQO1 (Abcam), phospho-p44/42 Map Kinase, p44/42 Map Kinase, phospho-p38, p38, β-actin, Lamin B (Cell Signalling; Celbio, Milan, Italy). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, and the bound antibodies were detected using chemiluminescence (BioRad, Milan, Italy). The blots were then stripped and re-probed with β-actin or Lamin B as the loading control. Images of the bands were digitized and the densitometry of the bands was performed using ImageJ software.

Immunocytochemistry. EA.hy 926 cells were grown on coverslips at a density of 4×10^4 cells/ml, and after treatment fixed in 1:1 acetone-methanol for 10 min at 4 °C. Cells were permeabilized and blocked for 30 min at room temperature with PBS-Tween 20, containing 1% BSA. Coverslips were then incubated with primary antibody in PBS-Tween 20 1% BSA at 4 °C overnight. After washing, coverslips were incubated with appropriate secondary antibody for 1 h at room temperature. Nuclei were stained with 1 μg/ml DAPI (Sigma-Aldrich) for 1 min. Coverslips were mounted onto glass slides using with anti-fade mounting medium 1,4 diazabicyclooctane in glycerine (DABCO) and examined by the Zeiss Axioplan2 light microscope equipped with epifluorescence at ×400 magnification. Negative controls for the immunostaining experiments were performed by omitting primary antibodies. Images were acquired and analyzed with Axio Vision Release 4.6.3 software.

Statistical analysis. Data are presented as means ± SD for 3 experiments in triplicate. One-way analysis of variance and Student's *t*-test were used where appropriate, and $p<0.05$ between two experimental groups was regarded as significant.

Table 1
Effects of treatments on lactate dehydrogenase release in EA.hy 926 endothelial cells.

Treatments (24 h)	LDH (% of release)
Triton 100-X	100.0 ± 2.1
Control	9.2 ± 0.9
20 μg/ml ozonated serum	11.8 ± 1.3
40 μg/ml ozonated serum	12.4 ± 1.1
80 μg/ml ozonated serum	14.8 ± 1.9
30 μM H_2O_2	9.9 ± 1.1
100 μM H_2O_2	14.8 ± 1.3
5 μM 4HNE	12.8 ± 1.2
20 μM 4HNE	14.5 ± 1.1

Endothelial cells were exposed to various treatments for 24 h. Value are the percentage ± SD of the total LDH activity released by cells treated with Triton X-100 ($n = 3$).

Results

LDH release from EA.hy 926 cells

The toxicity of the different experimental conditions (ozonated serum, H_2O_2 and 4HNE treatments) was assessed by means of LDH release in EA.hy 926 endothelial cells. As shown in Table 1, none of the treatments were associated to any significant cytotoxicity after 24 h.

Ozonated serum induces NRF2 nuclear translocation in endothelial cells

In order to examine the effects of ozonated serum on endothelial NRF2 activation, EA.hy 926 cells were exposed to increasing doses (20, 40 and 80 μg/ml) of ozonated human serum for 15, 30, 45 and 60 min. Ozonated serum increased the nuclear levels of NRF2 protein, starting at 15 min from treatment (Fig. 1A). As shown in the bottom panel of Fig. 1A, 40 μg/ml was the dose associated with a higher NRF2 translocation (2.5 fold after 30 min respect to the control). A dose response effect was detectable only when cells were treated with 20 and 40 μg/ml O_3 treated serum and no further induction of NRF2 translocation was observed when the cells were treated with 80 μg/ml (Fig. 1A). In parallel with the nuclear increase of NRF2 there was a concomitant decrease of cytoplasmic NRF2 as shown in Fig. 1B. Also in this case, the dose response was appreciable for 20 and 40 μg/ml O_3 treated serum (Fig. 1B bottom panel). These results were also confirmed by immunocytochemistry analysis (20 and 80 μg/ml are not shown). Fig. 1C shows NRF2 translocation into the nucleus when cells were treated with 40 μg/ml of ozonated human serum and NRF2 induction starts at the early time points and persists over the time points considered in this study.

Recent findings indicate that Keap1 is able to travel between the nucleus and the cytosol, chaperoning nuclear export of NRF2 and therefore repressing its transcriptional effect (Sun et al., 2007; Velichkova and Hasson, 2005). To confirm the cytoplasmic-nuclear trafficking of endogenous Keap1, we have also performed nuclear and cytoplasmic extracts immunoblotting for Keap1 of cells treated with ozonated serum (Fig. 1D–E). As expected, in untreated cells, Keap1 predominantly localizes in the cytosol (Fig. 1E) with low nuclear levels, whereas ozonated serum treatment significantly induced Keap1 translocation to the nucleus after 15 min in a dose-dependent fashion, returning to the baseline levels at later time points considered (30–45 min) (Fig. 1D). As expected the levels of Keap1 in the cytoplasm have an opposite trend of the ones in the nucleus (Fig. 1D–E).

Taken together, these results demonstrate that ozonated serum induces both NRF2 and Keap1 cytoplasmic-nuclear trafficking in endothelial cells.

Effect of ozonated serum treatment on 4HNE-NRF2 adducts

Further experiments indicate that ozonated serum treatment increased the levels of 4HNE protein adducts in EA.hy 926 cells (Fig. 2). As shown in Fig. 2A, Western blots of EA.hy 926 cells protein extracts are positive for the presence of 4HNE protein adducts starting after 30 min of ozonated serum treatment (Fig. 2A upper panel). Densitometric analysis of the bands indicates that 40 and 80 μg/ml doses significantly increased the levels of 4HNE protein adducts of 1.7 and more than 4 fold, respectively (Fig. 2A bottom panel).

Double immunofluorescence was performed to determine the co-localization of NRF2 and 4HNE immunoreactivities in ozonated serum treated endothelial cells. As shown in Fig 2B, control cells display a cytoplasmic NRF2 reactivity (left colums) and only a very low signal for 4HNE protein adducts (middle column). No merged signal was detectable (right column). Treatment with 40 μg/ml of ozonated serum induces a significant NRF2 nuclear translocation (left column), a moderate increase of 4HNE protein adducts levels (middle column) and a co-localization of 4HNE/NRF2 (right column). The levels of 4HNE and the co-localization were even more evident when cells were treated with 80 μg/ml ozonated serum.

Increased levels of 4HNE and H_2O_2 in ozonated serum

It has been shown that H_2O_2 and 4HNE act as second messenger of ozonated serum effects to cells (Fourquet et al., 2010). Therefore, we have determined whether these compounds were also present in our system. As shown in Fig. 3A serum treated with the different doses of O_3 showed a clear increased in the levels of 4HNE protein adducts in a dose dependent fashion (Fig. 3A bottom panel). The same trend was observed also for H_2O_2. As shown in Fig. 3B, serum ozonization leads to an increased levels of H_2O_2 that also in this case showed a dose-dependent relationship.

4HNE and H_2O_2 induced NRF2 translocation in endothelial cells

In order to analyze whether the induction of NRF2 translocation by ozonated serum was mediated by the detected levels of H_2O_2 and/or 4HNE (Fig. 3A–B), we treated the cells with either these compounds. As shown in Fig. 4A, when cells were treated with 30 μM of H_2O_2 there was a clear increase of nuclear NRF2 protein. This effect was also noted for Keap1, and was more evident at the highest dose of H_2O_2 (100 μM) (Fig. 4B).

As shown in Fig. 4C–D, also the treatment with 5 or 20 μM 4HNE induced the translocation of NRF2 protein to the nucleus, although no clear dose-dependence effect was observed (Fig. 4C). A similar trend was observed for Keap1 nucleus levels (Fig. 4D).

These results were also confirmed in cells treated with 4HNE or H_2O_2 added in serum to mimic the ozonated serum. While high doses of H_2O_2 (100 μM) and 4HNE (20 μM) in serum strongly activated NRF2 translocation (Fig. 5), lower doses (30 and 5 μM, respectively) did not induce any significant effect (data not shown). NRF2 protein expression peaked at 30 min after 100 μM H_2O_2 treatment (1.8-fold to control) and gradually decreased to basal levels after 60 min (Fig. 5A). In cells treated with 20 μM 4HNE, the peak of NRF2 activation was observed at 45 min, with a 1.5-fold increase with respect to the control (Fig. 5B).

Ozonated serum induces HO-1 and NQO1 protein expression

The activation of NRF2 and its binding to the EpREs site are followed by the induction of phase II enzymes, such as HO-1 and NQO1. Fig. 6A-B shows that pre-treatment with ozonated serum for 1 h significantly induced, in a dose-dependent fashion, the expression of both HO-1 (Fig. 6A) and NQO1 (Fig. 6B) protein levels in EA.hy 926 cells. This induction was already significant after 6 h of the treatment and persisted overtime (24 h).

The comparable induction of HO-1 and NQO1 levels confirms their similar transcriptional regulation (Fig. 6A-B)

Ozonated serum induces NRF2 translocation in an ERK1/2- p38- independent way

Previous studies have shown that many upstream signaling pathways such as MAPKs, PKC, and PI3K may be involved in NRF2 activation and its nuclear translocation, as well as in NRF2-mediating phase II gene expression. Thus, we have determined whether the ERK1/2 and p38 are involved in the NRF2 translocation by ozonated serum. When the cells were treated with several doses of ozonated serum (20, 40 and 80 μg/ml) at different time points (15, 30 and 60 min), there was an evident increase of phospho-ERK1/2 (p-ERK1/2) (Fig. 7A) starting at 15 min after treatment in particular in association with higher doses of ozonated serum (40 and 80 μg/ml: 3.2 and 4.7 fold increase, respectively).

The same effect was also noted for phospho-p38 (p-p38) with the maximal induction 30 min after the treatment (40 and 80 μg/ml: 4.3 and 4.7 fold increase, respectively) (Fig. 7C).

To prove the involvement of p-ERK1/2 and p-p38 in the nuclear translocation of NRF2 and in HO-1 induction by ozonated serum, the cells were pre-treated for 30 min with 50 μM of p-pERK inhibitor PD98059 or 10 μM of p-p38 inhibitor SB203580 and then incubated with ozonated serum (20, 40, 80 μg/ml). ERK1/2 and p38 inhibition did not affect NRF2 translocation and HO-1 protein levels in cells exposed to ozonated serum, as shown in Fig. 7B and D respectively.

Discussion

The conventional therapy of either cardiovascular diseases (peripheral obstructive arterial disease, chronic heart failure, stroke), degenerative-ischemic disease as the age-related macular degeneration, diabetes complicated by renal, vascular visual, neurological pathologies and chronic obstructive pulmonary disease is based upon the optimized administration of statin, antihypertensive and antiaggregant drugs, thrombolytic drugs and aspirin, insulin or oral antidiabetics, bronchodilators, antibiotics and mediator antagonists. All these drugs are effective but their utilization is limited to the correction of the metabolic derangement. There is overwhelming evidence that all the above mentioned diseases tend to aggravate because vexed by a chronic oxidative stress maintained by a markedly reduced antioxidant defenses. The use of oral antioxidants has a minimal or uncertain effect (Halliwell, 2000) as the passive intake of antioxidants is often unable to rescue the natural capacity of the organism to upregulate both the phase I reaction mediated by cytochrome P-450 mono-oxygenase system and the phase II enzymes, able to conjugate the phase I products with an increased amount of glutathione and glucuronic acid produced by phase II enzymes (Kensler et al., 2007). Although the conventional medicine has brought new and valid insights in understanding several molecular aspects of the pathogenesis of many diseases we are still far from having effective cures. It would be, therefore of interest to integrate conventional therapies with some effective and safe additional approaches able to improve the "natural" defense system.

Oxygen-ozonetherapy, in its classical form, consists in exposing for a few minutes a volume (150–200 mL) of human blood to a mixture of O_2-O_3 (96%–4% respectively). This therapeutical approach has now reached a stage where the basic biochemistry and the molecular mechanisms have been clarified and are well accepted within "orthodox medicine" (Bocci, 2012).

The present work is a conceptual follow up of our previous studies reporting the induction of HO-1 in endothelial cells (HUVEC) after exposure to ozonated plasma (Bocci et al., 2007), although at that time the molecular mechanism behind up-regulation HO-1 was not investigated. Herein, we report that ozonated serum at 0.42 μmol/mL (20 μg/mL O_3 per mL of serum), 0.84 μmol/mL (40 μg/mL O_3 per mL of serum) and 1.68 μmol/mL (80 μg/mL O_3 per mL of serum) induce HO-1 protein expression levels, confirming our previous finding and bringing new highlights on the mechanism related to HO-1 induction in endothelial cells exposed to ozonated serum.

The doses of ozone used in the present work reflect the ones used in therapy. In fact, the therapeutic dosages of ozone in patients range between 10 and 80 μg/per ml of human blood (0.21–1.68 mM). A modest toxicity in relation to the determined hemolysis (5%) has been detected only when starting with an ozone dosage over 2.5 mM (Di Paolo et al., 2004).

It has been shown (Pryor, 1994) that, once in contact with serum, O_3 instantaneously dissolves by reacting with unsaturated fatty acids and antioxidants generating mainly H_2O_2 and a variety of aldehydes such as 4HNE. Our data confirm that many of the cellular effects induced by ozonated serum are mediated by these two molecules: 4HNE and H_2O_2.

It is now well-documented that both 4HNE and H_2O_2 are a "double edged" molecules and their effect depends on the dosage. They can have protective (hermetic) or noxious effect on the cells (Leonarduzzi et al., 2004; Stone and Collins, 2002) and one way to protect the cell is via the activation of phase II enzyme in which NRF2 is involved.

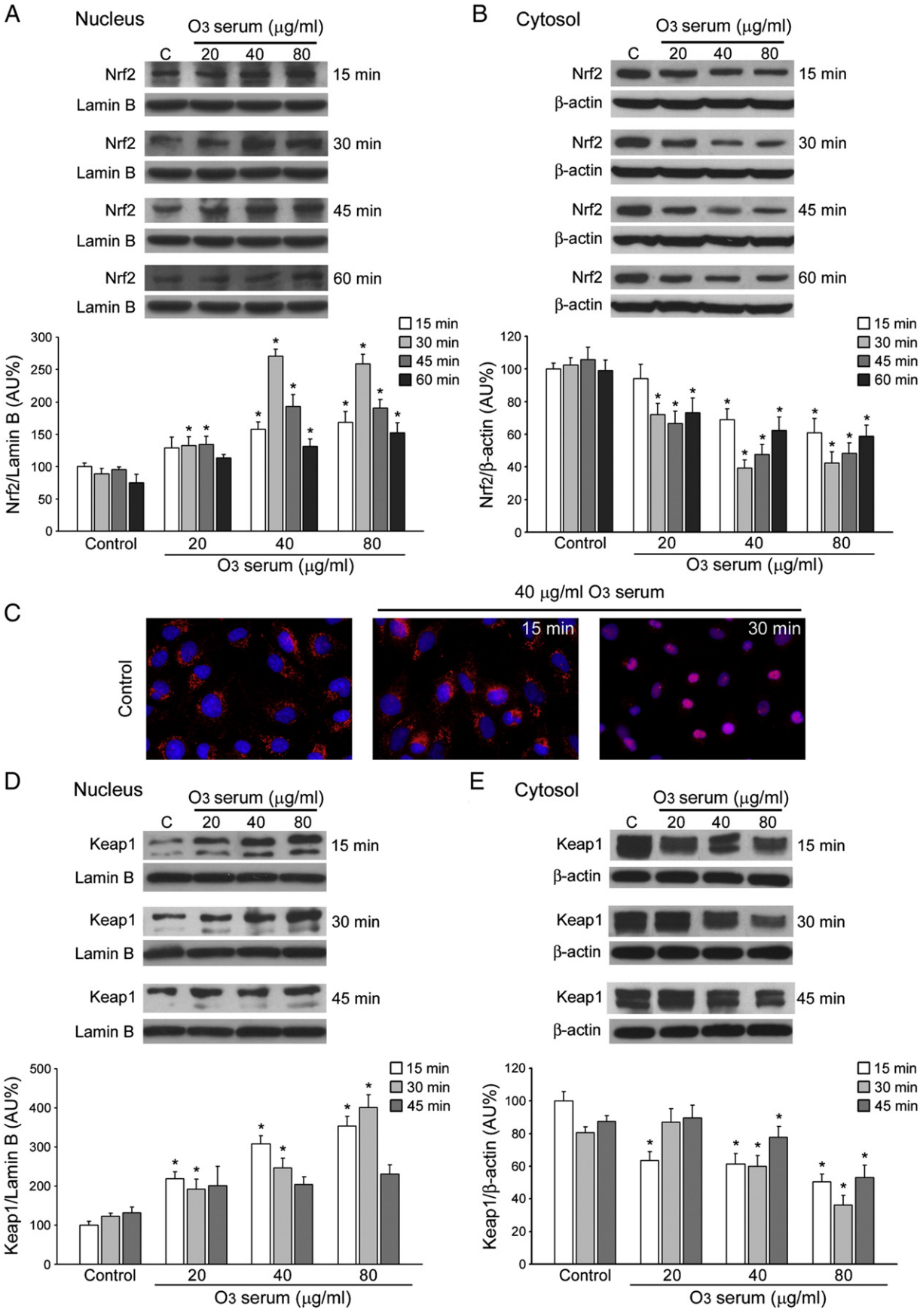
Our observations indicate that ozonated serum is able to induce NRF2 activation via the translocation of both NRF2 and Keap1 and that 4HNE and H_2O_2 might be the main molecules involved in this process. In fact, after an oxidative/electrophilic stimuli, several residues of the Keap1 protein are modified, with conformational changes and disruption of Keap1-NRF2 structure that allows in turn the translocation to the nucleus, the binding of NRF2 to the EpREs in the promoter and the subsequent expression of the enzymatic machinery involved in phase II cellular response such as HO-1 and NQO1 (Kensler et al., 2007; Kim et al., 2010a; Motohashi and Yamamoto, 2004; Taguchi et al., 2011; Zhang, 2006).

In agreement with others, we have already reported the up-regulation of a number of antioxidant enzymes by ozonated plasma, although the role of NRF2 was never investigated (Bocci et al., 1998b, 2007).

Several groups have reported that both H_2O_2 and 4HNE are also involved in the activation of cellular MAPK. Therefore we evaluated the levels of ERK and p38, as it has also been shown to be involved in the activation of NRF2 (Iles et al., 2005; Kim et al., 2010b; Leonarduzzi et al., 2004; Minelli et al., 2009).

Our data indicate that although the exposure of endothelial cells to different doses of ozonated serum activates in a dose dependent manner ERK or p38, although it seems that these MAPKs are not involved in HO-1 induction, in our experimental conditions. In fact, the pretreatment of the cells with ERK and p38 inhibitors (PD98059

Fig. 1. Both NRF2 and Keap1 redistribute to the nucleus after ozonated serum treatment in human endothelial cells. EA.hy 926 cells were exposed to 20, 40 and 80 μg/ml of ozonated serum, then nuclear (A) and cytoplasmic (B) extracts were subjected to Western blotting for NRF2 at indicated times. In the bottom panels, densitometric evaluation of Western blot of nuclear and cytoplasmic NRF2 are shown. (C) Immunofluorescence image (magnification, ×400) demonstrating NRF2 translocation after treatment with 40 μg/mL ozonated serum at 15 and 30 min. Cytosolic localization of NRF2 is indicated in red, whereas nuclear localization is given in magenta. Similar findings were observed in 3 separate experiments, also with 20 and 80 μg/mL of ozonated serum (data not shown). Nuclear (D) and cytoplasmic (E) Keap1 levels determined by Western blotting in cells exposed to ozonated serum under the same conditions described above. Quantification of proteins was expressed as a ratio to Lamin B for the nucleus extract and beta-actin for cytoplasmic exctracts. Values are mean ± SD of 3 experiments. * $P<0.05$ vs. control.


A
Nucleus
O3 serum (μg/ml)
C 20 40 80
Nrf2
Lamin B
15 min
30 min
45 min
60 min
Nrf2/Lamin B (AU%)
Control
20 40 80
O3 serum (μg/ml)
B
Cytosol
β-actin
Nrf2/β-actin (AU%)
C
40 μg/ml O3 serum
Control
15 min
30 min
D
Keap1
Keap1/Lamin B (AU%)
E
Keap1/β-actin (AU%)

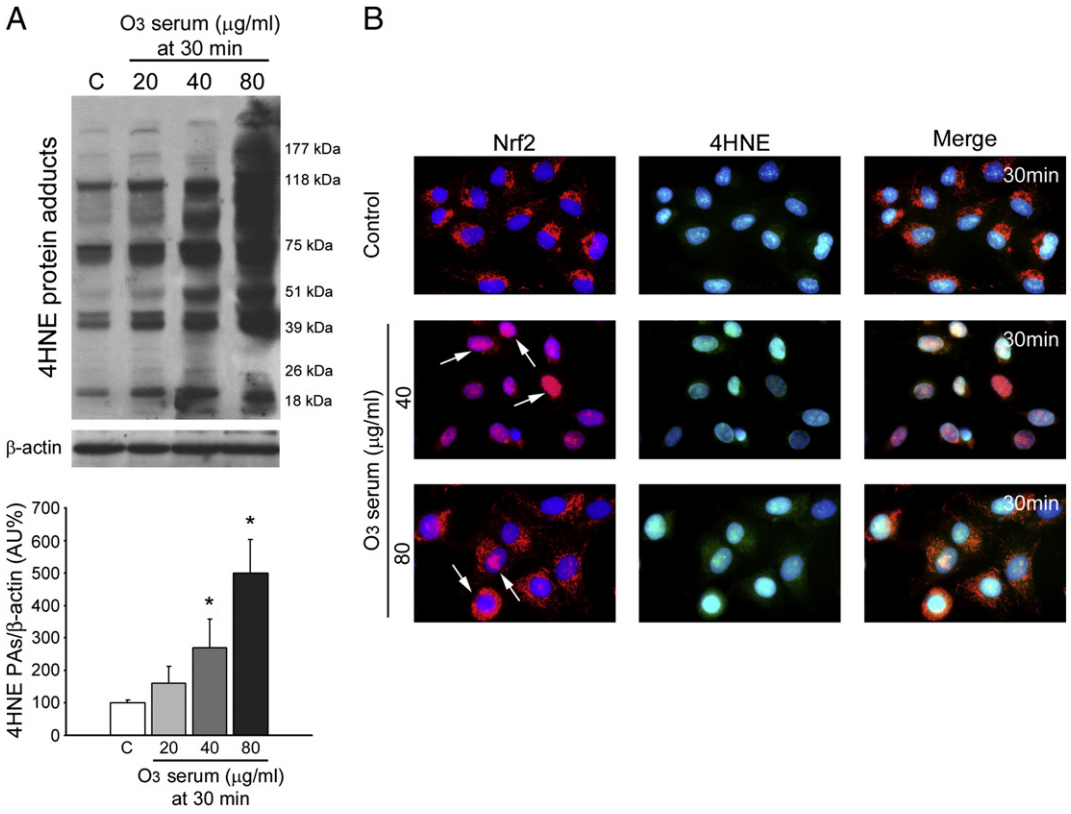


Fig. 2. Effect of ozonated serum treatment on 4HNE-NRF2 adducts. Cells were treated with ozonated serum at various time points. (A) 4HNE protein adducts were analyzed by Western blotting and quantified by densitometric analysis, normalized to β-actin. Data are means ± SD of 5 experiments. *$P<0.05$ vs. control. (B) Double immunofluorescence for NRF2 (red) and 4HNE (green) in cells exposed to 40 and 80 μg/mL ozonated serum. Arrows indicate the nuclear NRF2 traslocation.

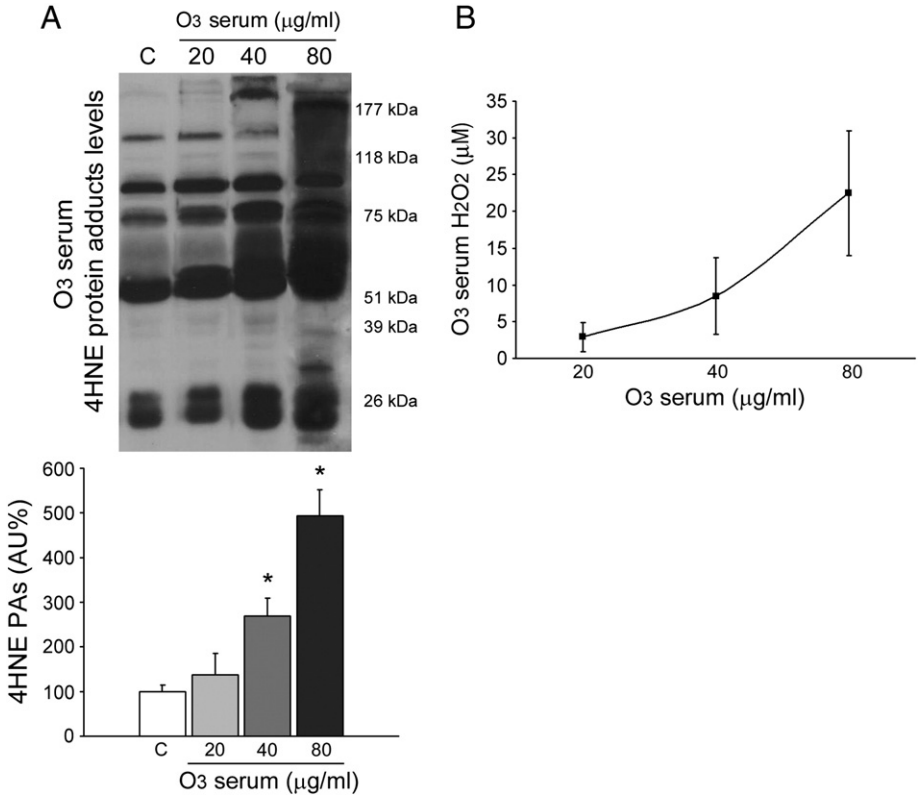


Fig. 3. 4HNE protein adducts and H_2O_2 levels in ozonated serum. Western blot analysis of 4HNE protein adducts in ozonated serum at different doses, 20, 40 and 80 μg/ml (A). *$P<0.05$ vs. control. (B) Determination of H_2O_2 concentration in ozonated serum.

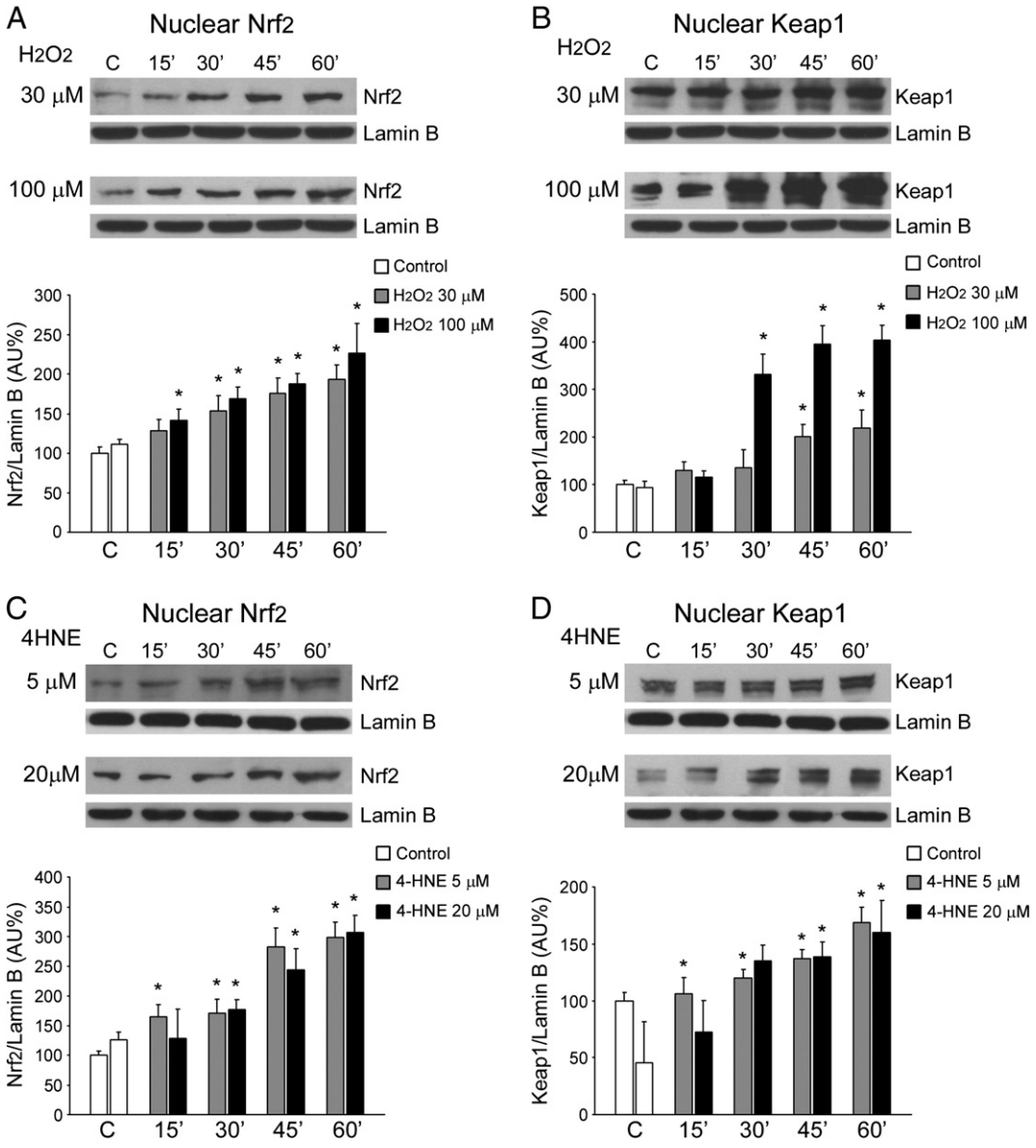


Fig. 4. NRF2 and Keap1 translocation into nucleus after treatment with H_2O_2 or 4HNE. Western blot analysis of nuclear NRF2 (A) and Keap1 (B) in endothelial cells treated with 30 or 100 μM H_2O_2. In the bottom figure are reported the protein expression of NRF2 (C) and Keap 1 (D) from cells treated with 5 μM or 20 μM 4HNE. Data are means ± SD of target protein levels normalized to Lamin B. All experiments were done in triplicate. *$P<0.05$ vs. control.

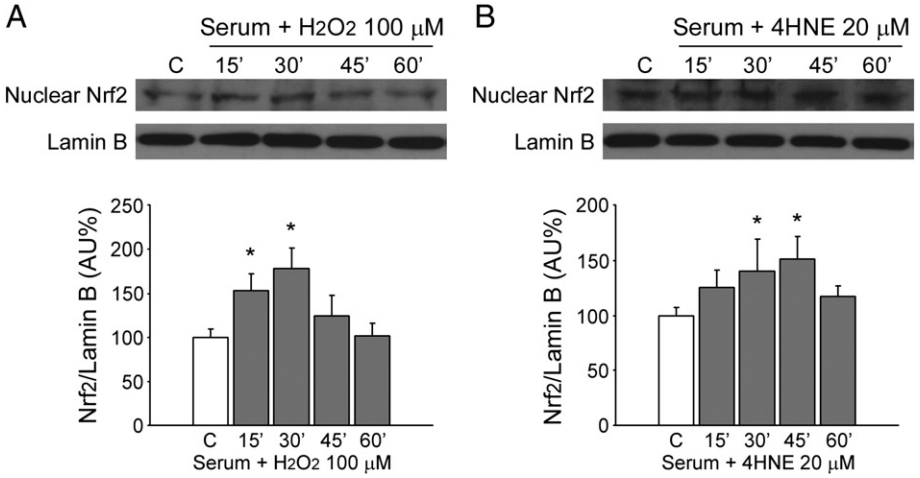


Fig. 5. NRF2 translocation following treatment of cells with 100 μM H_2O_2 and 20 μM 4HNE in serum. Cells were incubated in human serum and treated with 100 μM H_2O_2 (A) or 20 μM 4HNE (B), then nuclear extracts were subjected to Western blotting for NRF2 at indicated times. NRF2 protein was quantified by densitometric analysis, normalized to Lamin B. Data are expressed as the mean ± SD of 3 experiments. *$P<0.05$ vs. control.

and SB203580 respectively) neither affected HO-1 expression nor NRF2 translocation, after exposure to ozonated serum.

This observation is in part in contrast with the recent report by Iles et al. (2005a), where 4HNE was elegantly shown to be involved in HO-1 regulation via ERK phosphorylation in pulmonary cells. The reason of this discrepancy could be in the different experimental conditions, including the source of the cells and the peculiarity of treatments. In fact, as mentioned before, the interaction between O_3 and plasma is not only associated with the generation of 4HNE. Rather, several others lipid oxidation products (LOPs) and ROS are also generated, and it is possible that other LOPs, together with the generated ROS, could be also involved in the activation of NRF2 by inhibiting Keap1-dependent NRF2 degradation, through alkylation of specific cysteines. According to this hypothesis, Fourquet et al. (2010), observed that cells exposed to H_2O_2 promotes the formation of a disulfide bond between two Keap1 molecules via Cys(151) and activates NRF2.

In addition, also according to the report by Iles et al. (2005a), pre-treatment with ERK inhibitor did not affect HO-1 mRNA levels. This observation lead the authors to conclude that there two different mechanisms in HO-1 regulation by 4HNE are present, one ERK-independent at the transcriptional level and a second one dependent on ERK and acting at translational level. On the other hand a recent report from Sun et al. has shown the possible limited contribution of MAP kinases in NRF2 activation and this would be in line with our findings (Sun et al., 2009).

Surprisingly, only higher doses of 4HNE (30 uM) and H_2O_2 (100 μM) were able to reproduce NRF2 nuclear translocation by ozonated serum, although the normal levels of 4HNE and H_2O_2 in ozonated serum are much lower as we have shown in this study and the results are also in line with previous report (Bocci, 1996). Therefore it is possible that other molecules formed during the interaction between O_3 and serum play a role in NRF2 translocation.

In order of preference, O_3 reacts with abundant PUFA, bound to albumin, antioxidants such as ascorbic and uric acids, thiol compounds with –SH groups such as cysteine, reduced glutathione (GSH) and albumin. The interaction between PUFA and O_3 generates one mole of H_2O_2 which is a non radical *per se*, but can act as an O_3 messenger responsible for eliciting several biological effects (Bocci, 2006; Stone and Yang, 2006). A gain as noted earlier ROS are not always harmful but rather in physiological amounts, they act as regulators of signal transduction and represent important mediators of host defense and immune responses.

It has been previously shown that the interaction between O_3 and plasma causes the generation of H_2O_2 and chemiluminescence (Bocci et al., 1998a), that very rapidly decays with a half-life of less than 2 min. The transitory gradient of H_2O_2in plasma is likely to produce no more than a sub-micromolar gradient in the cytosol of endothelial cells, which can lead in turn to NRF2 translocation and also to the formation of more stable LOPs which are very heterogenous and can be classified as lipoperoxides (LOO•), alkoxyl radicals (LO•), lipohydroperoxides (LOOH), isoprostanes and alkenals. Also these compounds have been reported to be able to induce NRF2 activation (Adibhatla and Hatcher, 2010).

Small amounts of ROS and LOPs can elicit the upregulation of antioxidant enzymes on the basis of the cellular response described under the term of "hormesis" (Calabrese et al., 2011). The oxidative preconditioning or the adaptation to the chronic oxidative stress has been now demonstrated experimentally (Bocci, 1996; León et al., 1998). The increased synthesis of enzymes such as superoxide-dismutase, GSH-peroxidases, GSH-reductase and catalase has been repeatedly determined in experimental animals and in patients (Bocci et al., 2007). Interestingly, Iles and Liu (2005b) have recently demonstrated that 4HNE causes an intracellular increase of GSH, which plays a key role in antioxidant defense, by inducing the expression of glutamate cysteine ligase.

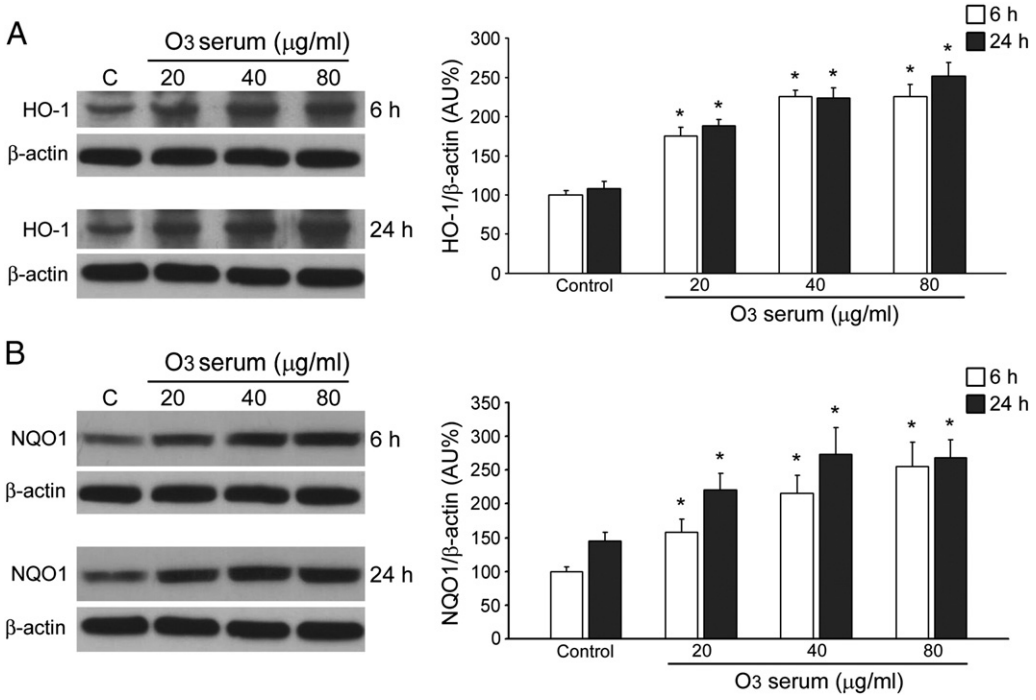


Fig. 6. Ozonated human serum increases HO-1 and NQO1 protein expression in human endothelial cells. The cells were treated with ozonated serum for 1 h. Protein expression of HO-1 (A) and NQO1 (B) was analyzed by Western blotting after 6 and 24 h. Quantification of proteins was expressed as a ratio to β-actin. Data are mean ± SD of 3 experiments. *$P<0.05$ vs. control.

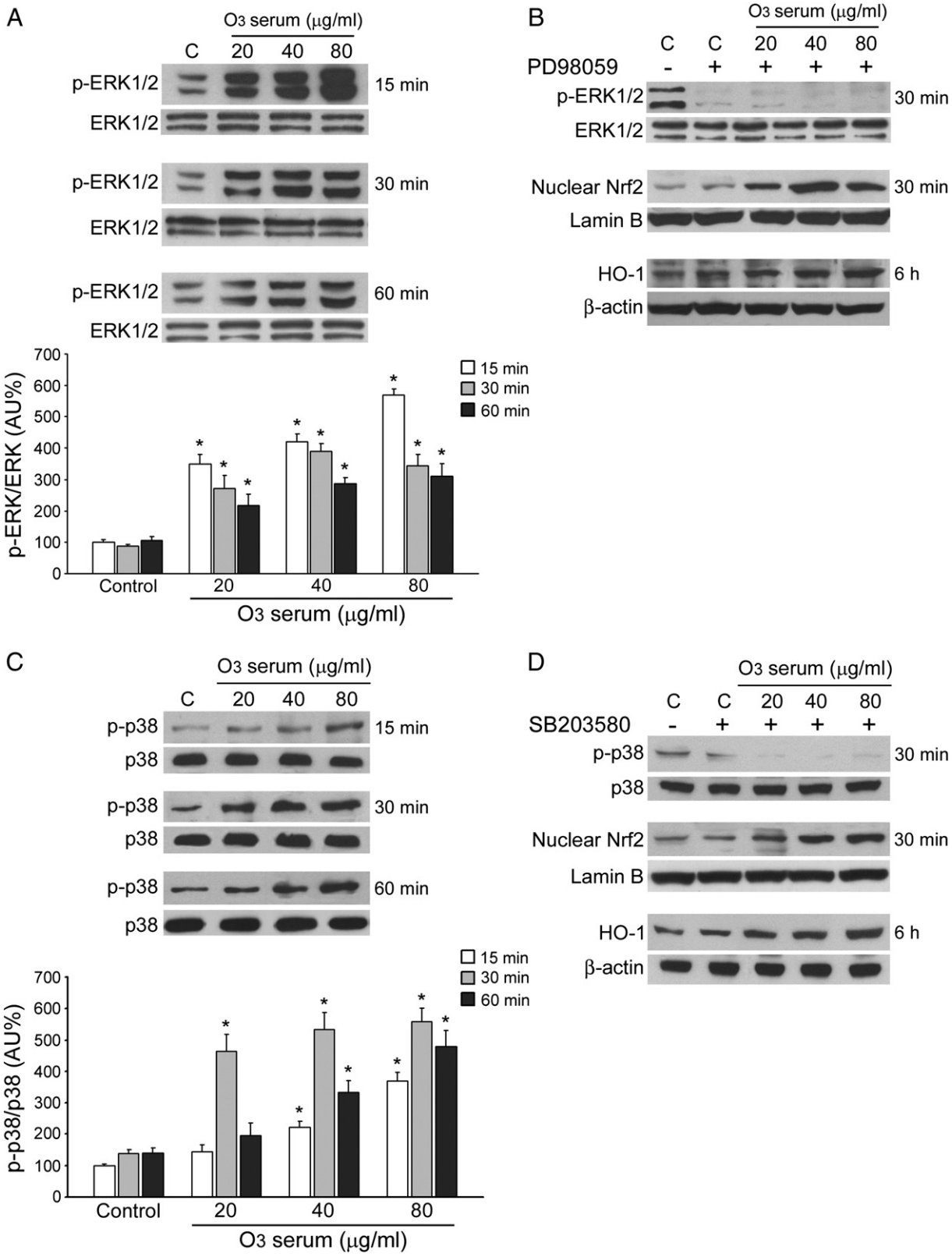


Fig. 7. Ozonated human serum induces NRF2 translocation in an ERK1/2- p38- independent way. Western blot analysis of p-ERK1/2 (A) and p-p38 (C) in endothelial cells treated with ozonated serum at various doses and times. p-ERK1/2 and p-p38 levels were estimated by densitometry, and data are expressed as mean ± SD of 3 experiments. Cells were pre-incubated for 30 min with PD98059 (B) or SB203580 (D) and then treated with ozonated serum. Nuclear NRF2 translocation and HO-1 levels were analyzed by Western blotting. Data are mean ± SD of 3 experiments. *$P<0.05$ vs. control.

In conclusion, the optimized daily drug administration, associated with ozonetherapy can represent a good integrative approach to a wide spectrum of degenerative diseases on the basis of a complementary activity: while drugs help to correct the dysmetabolism, ozonetherapy will reverse the chronic oxidative stress, probably restoring a redox homeostasis.

A final useful comment is that the induction of HO-1 has been surprisingly obtained by administering simvastatin (Lee et al., 2004), rosuvastatin (Grosser et al., 2004), aspirin (Grosser et al., 2003), and curcumin (Maheshwari et al., 2006), suggesting the protective activity of this enzyme with other drugs.

Conflict of interest statement

On behalf of all the authors I disclose any actual of potential conflict of interest.

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