Free Radical Research, July 2011; 45(7): 810–819

informa
healthcare

Neutrophil gelatinase-associated lipocalin: A new antioxidant that exerts its cytoprotective effect independent on Heme Oxygenase-1

MEHRYAR HABIBI ROUDKENAR[1], RAHELEH HALABIAN[1], PARISA BAHMANI[1], AMANEH MOHAMMADI ROUSHANDEH[2], YOSHIKAZU KUWAHARA[3] & MANABU FUKUMOTO[3]

[1]*Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran,* [2]*Department of Anatomy, Faculty of Medicine, Medical University of Tabriz, Tabriz, Iran, and* [3]*Department of Pathology, Institute of Development, Aging and Cancer, Tohoku University, Seiryo-cho 4-1, Aoba-ward, Sendai-city, Miyagi, prefecture, 980-8575, Japan*

(Received date: 5 January 2011; Accepted date: 11 April 2011)

Abstract

Neutrophil Gelatinase-Associated Lipocalin (NGAL/Lcn2), a member of the lipocalin family, has a variety of functions. There are extensive studies examining the expression of NGAL under harmful conditions. However, its precise function remains poorly understood. Heme Oxygenase 1 (HO-1) is an enzyme with well-established cytoprotective effects. Previous work showed that NGAL induces expression of HO-1. Interestingly, the same stimuli induced the expression of both NGAL and HO-1. The current study was designed to (1) determine whether NGAL exerts its cytoprotective effect through HO-1 and (2) compare NGAL and HO-1 with each other in terms of their protective role against oxidative stress. The current data indicate that NGAL exerts its cytoprotective effect independent of HO-1 and protects cells against oxidative stress more efficiently than HO-1. The data also strongly suggest that induction of NGAL under harmful conditions is a compensatory response to ameliorate oxidative stress-mediated toxicity. These findings may suggest new applications of NGAL, particularly when oxidative stress is a major factor.

Keywords: *NGAL, HO-1, antioxidant, oxidative stress*

Introduction

Neutrophil gelatinase-associated lipocalin (NGAL), or lipocalin-2/24p3, a member of the lipocalin family, is expressed and secreted by immune cells, hepatocytes and renal tubular cells in various pathologic states [1–3]. NGAL is a unique protein endowed with iron-carrying activity that is receiving increasing attention by researchers. Recently, NGAL has been shown to have diagnostic utility for renal failure and to be linked with a wide range of different functions, including parturition, infection, differentiation, apoptosis, inflammation and acute-phase immunity, indicating the pivotal role of this molecule in several physiological or pathological conditions [4–7]. In addition, NGAL appears to be a protein that can protect against acute ischemic renal injury [8,9]. Moreover, it has been reported that NGAL acts as a cytoprotective factor against cellular stresses such as H_2O_2 and cisplatin toxicity [10,11].

Although NGAL protects cells from oxidative stress, little is known about its biological role. In contrast, Heme Oxygenase-1 (HO-1) has been extensively studied for its cytoprotective effects. HO-1 is a rate limiting enzyme that catabolizes heme into equimolar amounts of catabolites (biliverdin, free iron, carbon monoxide), which serve regulatory and protective functions. A large number of substances, including interleukin-10 (IL-10), rapamycin, prostaglandin 15-deoxy-12, 14-prostaglandin J_2 (15-PDGJ2) and others, appear to function via HO-1 and, interestingly,

Correspondence: Mehryar Habibi Roudkenar, Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, PO Box 14665-1157, Tehran, Iran. Tel: +98 21 82052165. Fax: +98 21 88601599. E-mail: roudkenar@ibto.ir

ISSN 1071-5762 print/ISSN 1029-2470 online © 2011 Informa UK, Ltd.
DOI: 10.3109/10715762.2011.581279

HO-1 inhibitors can block their effects [12]. Furthermore, HO-1 is a stress-responsive protein induced by several agents and diseases that involve oxidative damage [13,14]. More recently, we found that NGAL induces the expression of HO-1 [15]. Interestingly, the same stimuli can induce both NGAL and HO-1, suggesting that they might collaborate together to ameliorate toxicity.

Based on these findings, we hypothesized that NGAL exerts its cytoprotective effect through HO-1. The present study was designed to test this hypothesis and to determine whether HO-1 or NGAL is the more protective factor against oxidative stress. The data indicate that NGAL exerts its cytoprotective effect independent of HO-1 and has a greater cytoprotective effect than HO-1. These findings highlight the importance of NGAL and may provide new insights for further application of NGAL, especially whenever oxidative stress is a major health problem.

Materials and methods

Cell culture

A549 (lung carcinoma), HEK293T (Human embryonic kidney) and MCF-7 (breast carcinoma) cell lines were obtained from the National Cell bank of Pasteur Institute of Iran (NCBI). The cells were cultured in 25 cm^2 cell culture flasks and were grown in RPMI 1640 medium (Gibco-BRL, Germany) with 10% heat-inactivated foetal bovine serum (Gibco-BRL) with 1% penicillin streptomycin solution (10 000 units of penicillin and 10 mg of streptomycin) in a humidified atmosphere of 5% CO_2, 95% air at 37°C.

Generation of HO-1/NGAL expression plasmids

In order to induce expression of HO-1, A549 cells were exposed to ultraviolet (UV) for 1 h. For RNA extraction, the cells were lysed by Trizol reagent (Invitrogen, Carlsbad, CA) and total RNA was extracted and purified according to the manufacturer's protocol. The yield and quality of extracted RNA were determined with a spectrophotometer (Nano Drop, Technologies Inc., USA) and electrophoresis. Full length human NGAL cDNA was synthesized by reverse transcriptase polymerase chain reaction (RT-PCR) by Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and using the random hexamer primers. Specific primers set for full-length human NGAL containing Kozak sequence, *Eco*RI (Forward)-*Not*I (Reverse) restriction enzyme site and the primers set for full-length human HO-1 containing Kozak sequence, *Bam*HI (Forward)-*Hind*III (Reverse) were designed and then the NGAL and HO-1 cDNAs were isolated by using PCR as previously described [15]. To construct plasmids containing the cDNAs, PCR products were digested with *Eco*RI, *Not* I (for NGAL) and with *Bam*HI, *Hind*III (for HO-1). These fragments were cloned into pcDNA3.1(+)vector (Invitrogen) [15].

Establishment of cell lines over-expressing NGAL, HO-1 and NGAL/HO-1

The recombinant constructs, pcDNA3.1-NGAL and pcDNA3.1-HO-1, were linearized with *Bgl*II restriction enzyme. HEK293 cells were transfected by 1 μg of the constructs using FuGENE 6 (Roche, Mannheim, Germany) according to the manufacturer's protocol. HEK293T cells were also transfected with Empty vector. Transfectants were selected in the presence of 600 μg/ml Geneticin (Roche) for at least 14 days. Single cell clones were established following serial dilution. The expression level of NGAL or HO-1 was examined by RT-PCR and western blot analysis.

Gene silencing through small interfering RNA (siRNA). The NGAL mRNA was down-regulated using Hs-Lcn2-6-HP validated siRNA (Qiagen, Hilden, Germany) as described previously [11]. The MCF-7 cell line was used for this experiment.

Western blot analysis

Cell culture medium from HEK293T cells was used for detection of NGAL. For detection of HO-1 total proteins were extracted by Complete Lysis-M reagent (Roche) according to the manufacturer's instruction. Total protein was boiled in loading buffer containing 4% sodium dodecyl sulphate (SDS), 20% glycerol and bromophenol blue for 5 min. Proteins were resolved on 12% SDS-PAGE and transblotted onto PVDF membrane (Roche). The membranes were incubated with monoclonal antibody at 4°C overnight and detection of proteins were carried out using specific secondary antibodies. Antibodies were obtained from the following sources: anti-human NGAL rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA), *β*-actin antibody (Sigma, USA), polyclonal anti-HO-1 antibody (Stressgen Biotechnologies, Victoria, BC, Canada), polyclonal anti-active Caspase 3 antibody (Abcam, Cambridge, UK). Following incubation with primary antibody, the membrane was washed with TBS containing 0.1% Tween 20 and the reactions were incubated with secondary antibodies (Sigma). Finally the membranes were developed by DAB solution (Sigma).

MTT assay

Exactly 2×10^4 cells/well were seeded in a 96-well plate and after 12 h the cells were exposed to H_2O_2 for different time courses. At appropriate time points, cells were incubated with 10 μl of a 5 mg/ml solution of

3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, Duesseldorf, Germany) at 37°C in a 5% CO_2 atmosphere for 4 h. Finally, the reaction was stopped by addition of 10% SDS and 0.01 M HCl. When the insoluble formazan crystals were completely dissolved, the absorbance at 570 nm was measured using a microplate reader.

Inhibition of HO-1 activity with ZPP (zinc protoporphyrin IX)

HEK293T cells were seeded in 96-well plates (2×10^4 cells/well) and incubated overnight. Zinc protoporphyrin (IX) (Sigma), a HO-1 inhibitor, was dissolved in dimethyl sulphoxide (DMSO) (Sigma, Duesseldorf, Germany) to the final stock concentration of 5 mM. Cells in presence of 12 mM H_2O_2 were treated with 50–100 μM concentrations of ZPP. At the end of treatment time, the viability of the cells was evaluated by MTT assay.

Apoptosis assay

For detection of apoptosis by Annexin-V-fluos staining kit (Roche, Germany), 6×10^5 cells/well were seeded into 6-well plates and incubated at 37°C in a 5% CO_2 atmosphere for 24 h. After incubation, the cells were treated with different concentrations of H_2O_2 (50–250 μM) at different times. Finally, cells were washed with PBS, trypsinized and centrifuged with PBS at 1500 rpm for 10 min. The pellet was re-suspended in Annexin-V-fluos labelling solution and incubated for 15 min at 20°C. Subsequently, apoptosis was analysed using a flow cytometer (PasIII, Partec, Germany).

For detection of apoptosis by TUNEL method, 2×10^5 cells were seeded on coverslips and incubated at 37°C in a 5% CO_2 atmosphere for 12 h. Then the cells were treated with different concentrations of H_2O_2 for different time lengths. Afterwards, the cells were washed twice with PBS, air dried and fixed for 60 min in freshly prepared 4% paraformaldehyde (Sigma, Germany) at room temperature. Then, they were washed again twice using PBS and incubated with 3% H_2O_2 for 10 min. Following washing with PBS, the cells were permeabilized in 0.1% Triton X-100 (Sigma, Germany) for 2 min at 4°C. The permeabilized cells were washed with PBS and the terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) assay was performed by *In Situ* Cell Death Detection Kit according to the manufacturer's protocol (Roche). To evaluate the reaction, the samples were incubated for 30 min with 100 μl DAB (Sigma, Germany) substrate in darkness. Finally, the samples were mounted and analysed under a light microscope, where the apoptotic cells are seen as brown cells. At least 300 cells were counted and the percentage of apoptotic cells was calculated.

Caspase-3 activity was measured by a colourimetric caspase-3 assay kit according to the manufacturer's protocol (PharMingen, Germany). The cells were seeded into 24-well plates at the concentrations 3×10^5 cells per well and incubated at 37°C in a 5% CO_2 atmosphere. Following treatment with different concentrations of H_2O_2, cells were washed with PBS,

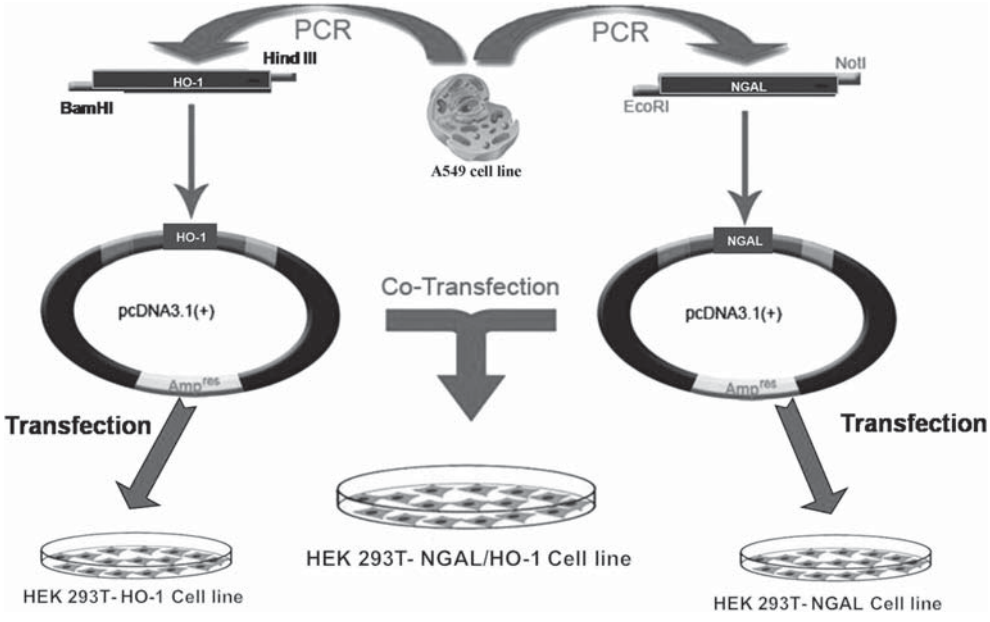

Figure 1. Summary of construction of recombinant vectors and their transfection. A549 cell line was used as a source for NGAL and HO-1 cDNAs. The isolated genes were cloned into pcDNA3.1 vector separately. To establish stable clones expressing NGAL and HO-1, the recombinant vectors were transfected to HEK293T cells separately. To establish stable clones expressing both NGAL and HO-1, the recombinant vectors were co-transfected to the cells. Stable clones expressing recombinant NGAL and HO-1 were established in the presence of geneticin. From each construct two stable clones were established.

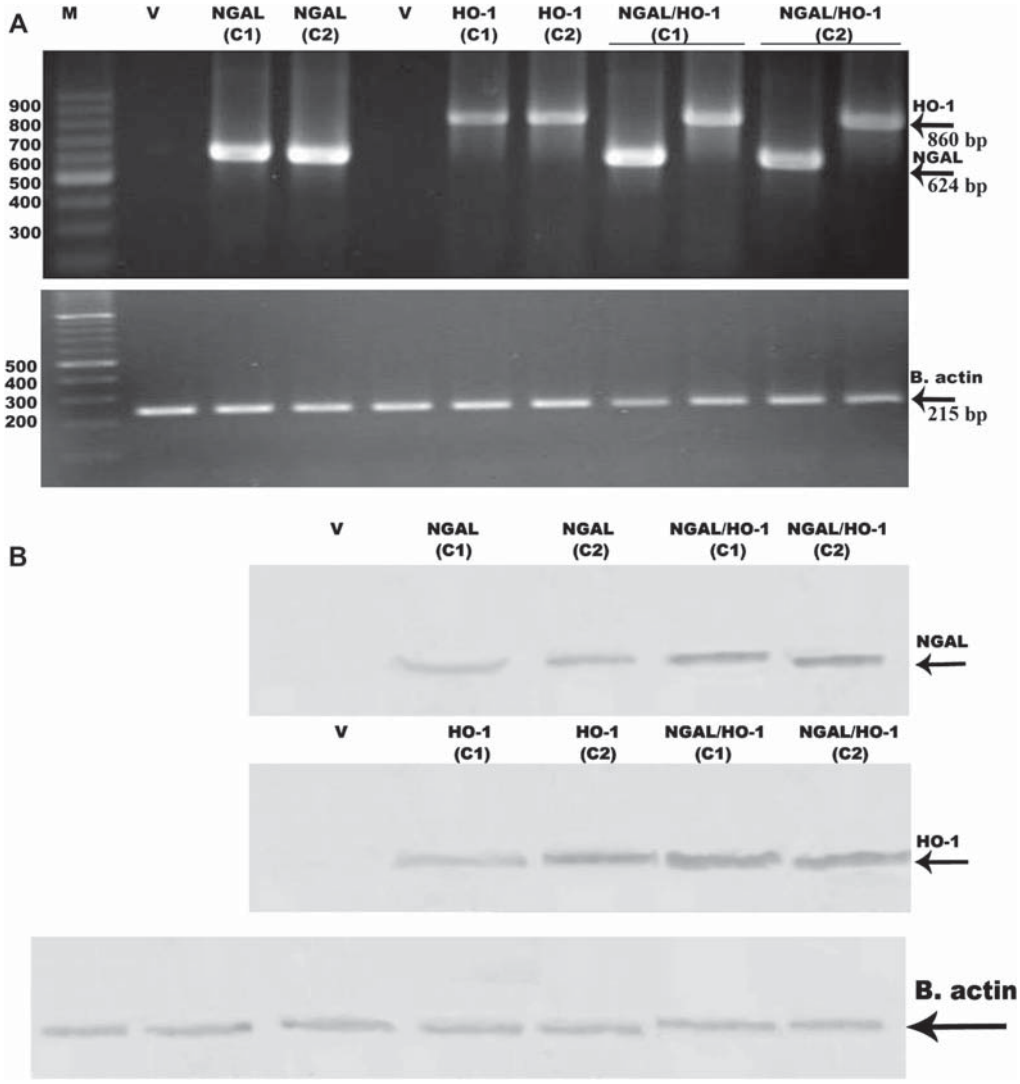

Figure 2. (A) Expression of NGAL and HO-1 in stable clones of HEK293T cells. RNA was extracted from the cells transfected with the pcDNA3.1-NGAL, pcDNA3.1-HO-1, pcDNA3.1-NGAL/HO-1 and also HEK293T cells transfected with the empty pcDNA3.1. cDNA was synthesized and reverse transcript-polymerase chain reaction (RT-PCR) was performed. HEK293T cells transfected with the recombinant constructs expressed NGAL mRNA (624 bp) and HO-1 mRNA (860 bp), while in the cells transfected with empty pcDNA3.1 vector no expression was observed. From each construct two stable clones were established (C1 and C2). Expression of beta-actin was used for normalization (pcDNA3.1 vector), C1 and C2 (clone 1 and clone 2) and M, 100 bp ladder marker. (B) Western blot analysis of the mentioned cells.

trypsinized and centrifuged at 1750 rpm for 10 min. The pellet was re-suspended in 0.5 ml of PBS and centrifuged at 3000 rpm for 10 min at 4°C and the supernatant was removed completely. Cell pellets were suspend in lysis buffer and incubated on ice for 15 min. The lysed cells were centrifuged at 19 000 rpm for 15 min at 4°C. Then, supernatants were loaded into 96-well plates in the presence of Assay Buffer and caspase 3 inhibitor, Ac-DEVD-CHO. Then, caspase 3 substrate, Ac-DEVD-pNA, was added to each well and mixed gently by shaking. After incubation of plate at 37°C for 2 h, absorbance at 405 nm was measured.

Determination of total antioxidant capacity

The antioxidant capacity of NGAL and HO-1 against oxidative stress was measured with an Antioxidant assay kit (Sigma Aldrich, Missouri, USA). The principle of this kit is formation of a ferryl myoglobin radical from metmyoglobin and hydrogen peroxidase, which oxidizes the ABTS (2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) to produce a radical cation, $ABTS^{+}$, a soluble chromogen that is green in colour and can be determined spectrophotometrically at 405 nm. Antioxidants suppress the production of the radical cation in a concentration-dependant manner. The cell culture media and/or cell lysates were harvested and antioxidant capacity was measured in 96-well plates according to the manufacturer's protocol. Trolox (water-soluble vitamin E analogue) provided by the kit was used as a positive control. To determine the antioxidant capacity of NGAL, commercially available recombinant NGAL (without iron) (R&D, Minneapolis, USA) was used. Absorbance was

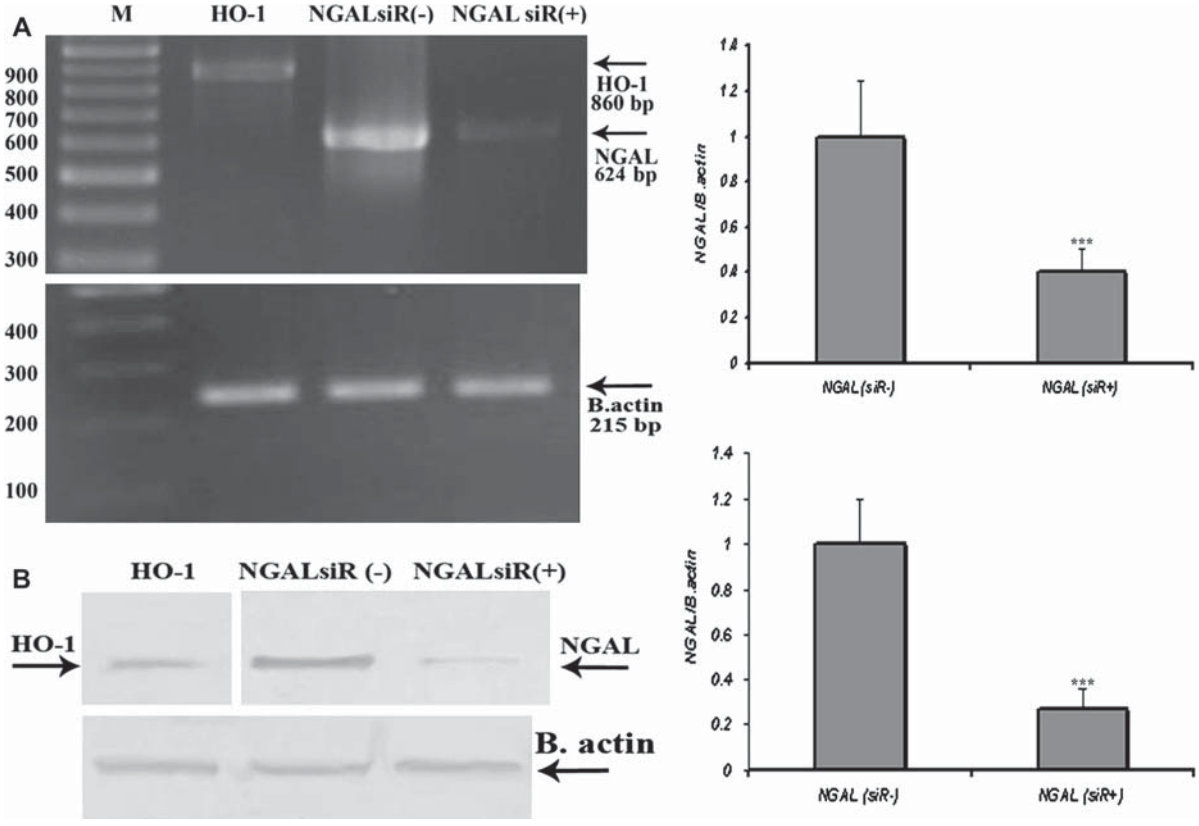

Figure 3. Expression of HO-1 and down-regulation of NGAL in the MCF-7 cell line, which is basically capable of a high level of NGAL expression (A) RT-PCR. (B) Western blot analysis. Quantification of the NGAL down-regulation at mRNA and protein levels has been determined by densitometry in three independent experiments (Mean ± SD; $^{***}p < 0.001$). M, 100-bp DNA marker; V, empty vector; siR+, transfected with NGAL siR- ; siRNA−, transfected with control siRNA.

monitored at 405 nm using an ELx800 Absorbance Microplate Reader.

Statistical analysis

Results are expressed as mean ± SD of three independent experiments. Differences between groups were compared using ANOVA with Tukey-Kramer Multiple Comparison Test as a post-test.

Results

Establishment of NGAL, HO-1 and NGAL-HO-1 over-expressing cell lines

In order to establish NGAL, HO-1 and NGAL/HO-1 over-expressing cell lines, cDNAs of NGAL and HO-1 were isolated from A549 cells and cloned into the pcDNA3.1(+) vector. Next, pcDNA3.1(+)-NGAL and pcDNA 3.1(+)-HO-1 constructs were transfected into HEK293T cells to generate HEK293T-NGAL and HEK293T-HO-1 cells. pcDNA3.1(+)-NGAL and pcDNA 3.1(+)-HO-1 constructs were co-transfected into HEK293T cells to generate HEK293T over-expressing NGAL and HO-1 cells (HEK293T-NGAL/HO-1). Stable clones were selected in the presence of Geneticin (G418). The mentioned explanations are depicted in Figure 1. The presence of NGAL and HO-1 genes in the stable clones was detected by using RT-PCR and western blot analysis (Figures 2A and B). Expression of NGAL in HEK293T-NGAL cells and HEK293T-NGAL/HO-1 cells were quantified by ELISA to assure same level expression (data not shown). Two stable clones from each of three engineered cells were established.

Down-regulation of NGAL in the MCF7 cell line

MCF-7 is one of the cell lines in which basal levels of NGAL expression are detected. In order to knock-down the NGAL expression, the MCF-7 cells were transfected with specific NGAL siRNA. As is shown in Figure 3, transfection of the NGAL siRNA resulted in a decrease of expression both in mRNA and protein levels (Figures 3A and B). Interestingly this cell line also expresses basal levels of HO-1.

The protective effect of the NGAL/HO-1 on the cells against H_2O_2 toxicity was not more than that of NGAL alone

To determine whether HEK293T cells over-expressing NGAL/HO-1 were more cytoprotective against H_2O_2 toxicity compared to HEK293T cells over-expressing only NGAL or HO-1, the cells were exposed to

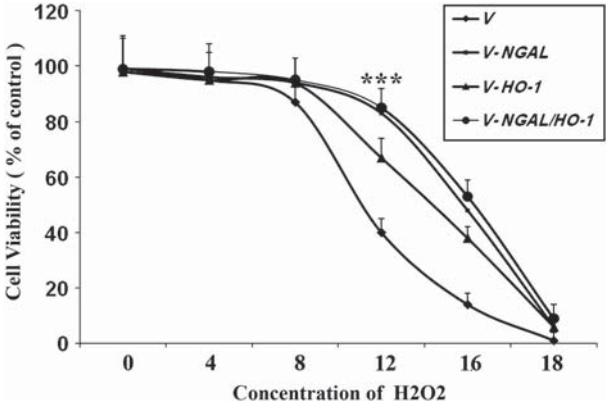

Figure 4. Cytotoxic effects of different concentrations of H_2O_2 on cells transfected with the constructs or empty vector for 2 h determined by MTT assay. Cell viability in HEK293T cells transfected with NGAL or co-transfected with NGAL/HO-1 was higher than the cells transfected with HO-1 at the concentration of 12 mM H_2O_2, indicating NGAL protects cell against H_2O_2 toxicity more than HO-1. The cells transfected with pcDNA3.1 vector were susceptible at this concentration. (Mean ± SD; $^{***}p < 0.001$; three independent experiments were carried out).

H_2O_2. As shown in Figure 4, after 2 h exposure to 12 and 16 mM H_2O_2, numbers of viable cells in HEK293T-NGAL and HEK293T-NGAL/HO-1 cells were not considerably impaired, but they were reduced in HEK293T-HO-1 and HEK293T-V (empty vector).

The number of surviving cells over-expressing HO-1 after this treatment was higher than those transfected with empty vector, but lower than those of HEK293T-NGAL and HEK293T-NGAL/HO-1 cells (Figure 4). These results suggested that, firstly, both NGAL and HO-1 protect cells against cytotoxicity induced by oxidative stress. Second, over-expression of both NGAL and HO-1 did not confer more resistance to the cells against H_2O_2 toxicity compared to NGAL alone and, finally, NGAL protects cells against H_2O_2 toxicity more than HO-1.

Inhibition of HO-1 activity with ZPP does not affect cytoprotective properties of NGAL

Next, to determine whether inhibition of HO-1 could affect the cytoprotective property of NGAL, HO-1 activity was suppressed by using ZPP and the transfected cells were treated with H_2O_2 followed by an analysis of cell viability by MTT assay. Inhibition of HO-1 activity after administration of 12 mM H_2O_2 resulted in cell death in HEK293T-HO-1 (Figure 5A). Interestingly, this treatment induced cell death in HEK293T-NGAL/HO-1 cells but had no effect on HEK293T-NGAL cells. Overall, the results again indicated that the cytoprotective effect of NGAL was higher than HO-1 and independent of this enzyme. In order to support the mentioned conclusion and to extend our findings on non-transfected cells, we

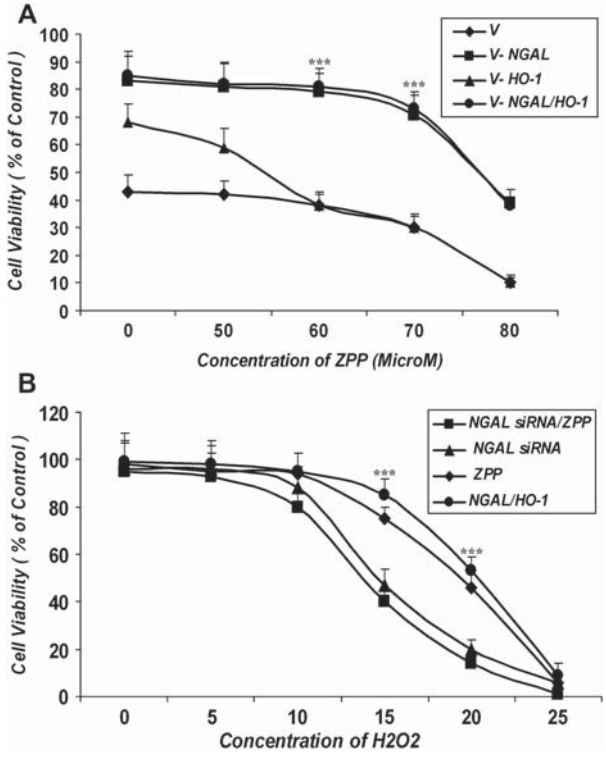

Figure 5. (A) Cytotoxic effects of 12 mM H_2O_2 on cells transfected with the constructs or empty vector in the presence of different concentrations of ZPP, an inhibitor of HO-1, for 2 h determined by the MTT assay. This treatment in the concentration of 60 and 70 μM of ZPP resulted in cell death in HEK293T-HO-1 but slightly induced cell death in HEK293T-NGAL/HO-1 and had no effect on HEK293T-NGAL. (B) Cytotoxic effects of different concentrations of H_2O_2 on MCF-7cells. The expression of NGAL was down-regulated by siRNA and HO-1 activity was suppressed by using 70 μM ZPP. Following treatment of the cells with H_2O_2, analysis of the cell viability was performed by MTT assay. Treatment of the cells with 15 and 20 mM H_2O_2 for 3 h resulted in slight cell death in the MCF-7 cells treated with ZPP, but a considerable cell death induced in the cells transfected with the NGAL siRNA. There was no significant difference in terms of cytotoxicity between MCF-7 cells in which both NGAL and HO-1 were suppressed (NGAL siRNA/ ZPP) and those only transfected with NGAL siRNA (NGAL siRNA). (Mean ± SD of three independent experiments; $^{***}p < 0.001$).

performed some experiments on MCF-7 cells. This cell line expresses basal levels of NGAL and HO-1 (Figure 3). Here, the expression of NGAL was down-regulated by siRNA technology (Figure 3) and HO-1 activity was suppressed by using 70 μM ZPP. Then, the cells were treated with H_2O_2 followed by an analysis of cell viability using MTT assay. While the administration of 15 and 20 mM H_2O_2 for 3 h to the MCF-7 cells in which the HO-1 activity had been suppressed resulted in slight cell death, a considerable cell death was observed in the cells transfected with NGAL siRNA (Figure 5B). Interestingly, the inhibition of both NGAL and HO-1 (NGAL siRNA/ ZPP) did not sensitize cells against H_2O_2 toxicity compared to the NGAL (NGAL siRNA) (Figure 5B). In other words, an additive effect was not produced.

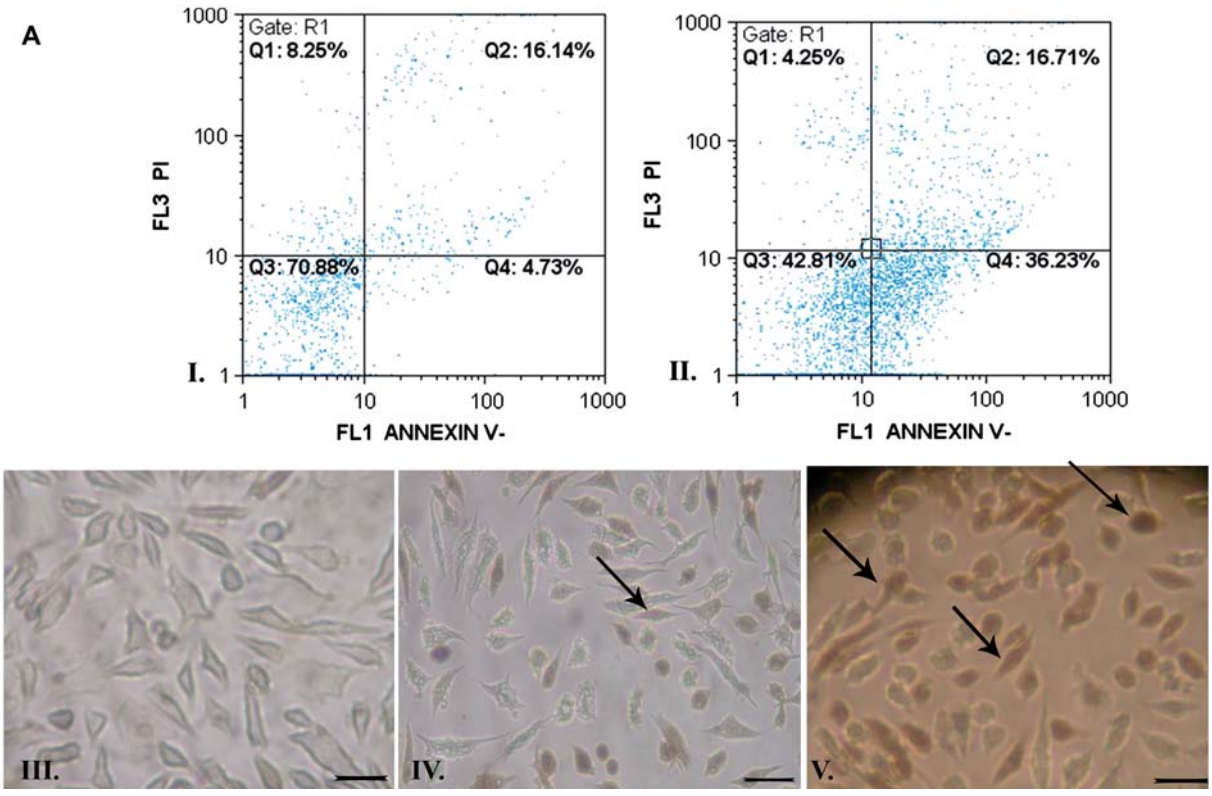

Figure 6. Apoptotic effects of 200 mM H_2O_2 on the stable cells for 12 h. (A) Detection of apoptosis using Annexin-V. Analysis of the experiments outcomes resulted in four types of cells. Those viable cells which were both Annexin V-FITC and PI negative (A-I and A-II, lower and left), the cells in early apoptosis, which were Annexin V-FITC positive and PI negative (A-I and A-II, lower and right), the cells in late apoptosis, which were both FITC Annexin V and PI positive (A-I and A-II, upper and right) and the dead cells which were Annexin V-FITC negative and PI positive (A-I and A-II, upper and left). A-III, A-IV and A-V show light microscopic images of apoptosis, detected by TUNEL method. Apoptotic cells can be observed as brown. While there is no apoptotic cell in the control sample (without H_2O_2 treatment, A-III), a number of apoptotic cells can be observed in the H_2O_2 treated cells (A-IV and A-V). (B) Early and late apoptosis obtained by Annexin-V assay and those by TUNEL (three independent experiments) have been shown by diagram. The number of apoptotic cells transfected with empty vector (V) was higher than those transfected with only NGAL or HO-1. There was no significant difference between the cells co-transfected with NGAL and HO-1 and the cells transfected with only NGAL. The number of apoptotic cells in the cells transfected with NGAL was lower than the cells transfected with HO-1, suggesting NGAL more protects cells against apoptosis induced by oxidative stress. B-II shows apoptosis detection in MCF-7 cells by using Annexin V and TUNEL methods. B-III shows detection of apoptotic activity using caspase-3 assay kit. The lowest level of the activated caspase 3 was observed in HEK293T-NGAL and HEK293T-NGAL/HO-1 cells. The highest level of the activated form of caspase 3 was observed in HEK293T cells transfected with empty vector (V). There was no significant difference between HEK293T-NGAL and HEK293T-NGAL/HO-1 in terms of caspase 3 activity. B-IV shows apoptosis detection in MCF-7 cells by caspase-3 assay kit. (Mean ± SD of three independent experiments). (C) western blot analysis of active caspase-3. Quantification of active caspase-3 expression has been determined by densitometry in three independent experiments (Mean ± SD of three independent experiments; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

NGAL protects cells more against oxidative stress induced apoptosis than HO-1

The inhibition of apoptosis is one of the well-known functions of NGAL and HO-1 [16–19]. Therefore, we determined whether there was any difference between NGAL and HO-1 in terms of protection against apoptosis induced by oxidative stress. We also determined whether HEK293T cells over-expressing both NGAL and HO-1 were more resistant to apoptosis induced by oxidative stress compared to cells over-expressing NGAL or HO-1 alone. Cells were exposed to sub-lethal doses of H_2O_2 (50–250 μM) followed by detection of apoptosis using Annexin V, TUNEL and caspase-3 assays kits. After incubation of the cells with 200 μM H_2O_2 for 12 h, the number of apoptotic cells transfected with empty vector was higher than those transfected with only NGAL or HO-1, indicating HO-1 and NGAL protect cells against apoptosis induced by oxidative stress (Figures 6A and B). However, similar to the results from the cell survival assay, there was no significant difference between the cells co-transfected with NGAL and HO-1 and the cells transfected with NGAL alone in terms of the number of apoptotic cells. This suggested that in the cells co-transfected with NGAL and HO-1, HO-1 does not confer more anti-apoptitic property than NGAL. Furthermore, the number of apoptotic cells in the cells transfected with NGAL was lower than that transfected with HO-1, suggesting NGAL provides more protection against apoptosis induced by oxidative stress than HO-1 (Figures 6A and B).

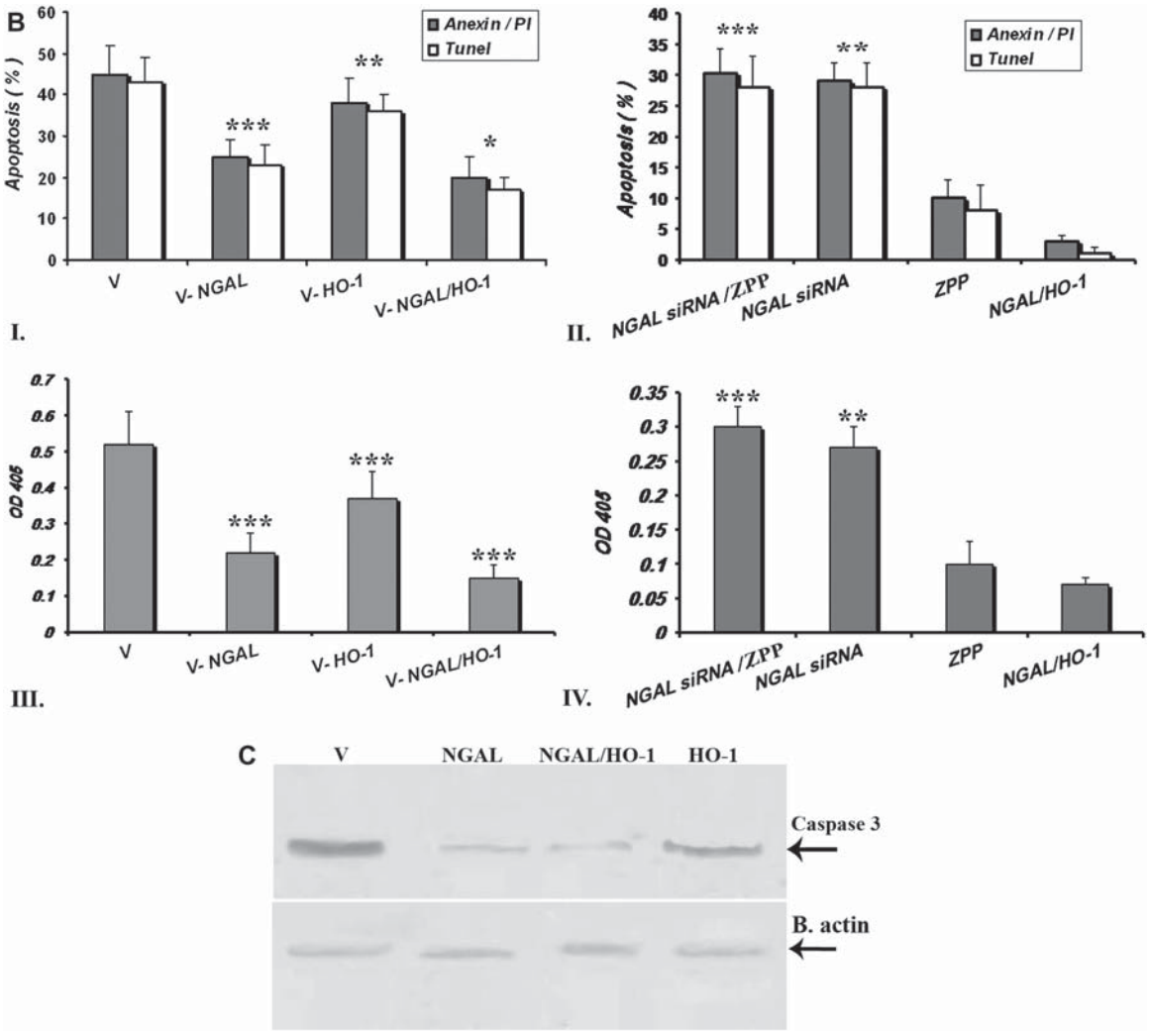

Figure 6. *(Continued)*.

Induction of apoptosis was also studied by assessing caspase-3 activity. As shown in Figure 6C, the highest level of activated caspase-3 was observed in HEK293T-V. In HEK293T-HO-1 the activated caspase 3 was lower than HEK293T-V. The lowest level of the activated caspase-3 was detected in HEK293T-NGAL and HEK293T-NGAL/HO-1 cells. There was no significant difference between HEK293T-NGAL and HEK293T-NGAL/HO-1 in terms of caspase-3 activity. These observations were further confirmed by western blot analysis for activated caspase- 3 (Figure 6C).

Apoptosis was also investigated in MCF-7 cells in which the expression of NGAL was down-regulated and/or HO-1 activity was suppressed by ZPP. After incubation of these cells with 250 μM H_2O_2 for 12 h, the number of apoptotic cells transfected by NGAL siRNA was higher than those treated with ZPP (Figures 6B-II and IV). Interestingly, suppression of HO-1 activity by ZPP did not induce any considerable apoptotic death. However, similar to the results of the cell survival assay, there was no significant difference in term of the number of apoptotic cells between the cells in which both NGAL and HO-1 were suppressed and the cells in which only NGAL was knocked-down. Taken all together, these results indicated that, following exposure to oxidative stresses, the NGAL capability on decreasing the apoptosis was higher than HO-1.

NGAL exhibits antioxidant properties

The current results, as well as our previous studies [11,15], revealed that NGAL can protect cells against H_2O_2 toxicity but its mechanism was obscure. Because NGAL and HO-1 are induced by similar stresses, we assumed that they might exert their cytoprotective effect through similar mechanisms, i.e. scavenging free radicals. To examine the hypothesis, the antioxidant capacity of the engineered cells was evaluated. For HEK293T cells expressing HO-1, there was no increased antioxidant activity in cell culture medium. The antioxidant assay of lysed cells over-expressing NGAL showed a low level of antioxidant activity in accordance with the secretion property of NGAL

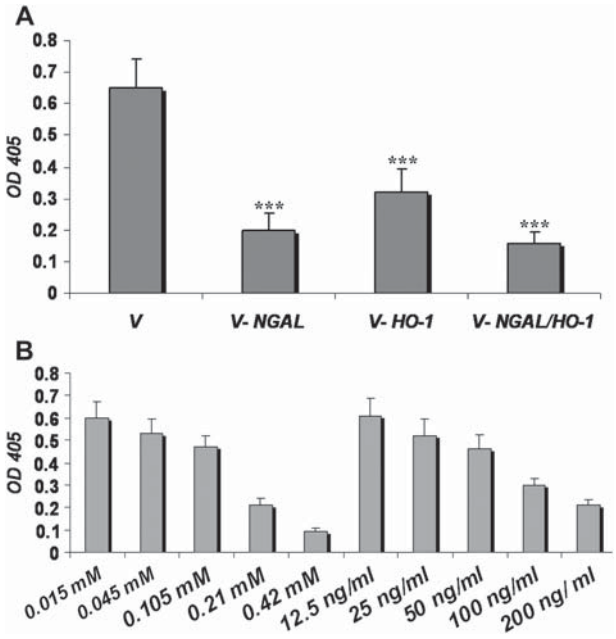

Figure 7. Determination of antioxidant capacity of cells expressing NGAL and HO-1. (A) For NGAL cell culture medium and for HO-1 cell lysate were used. For the cells transfected with both NGAL and HO-1, a mixture of cell culture medium and cell lysate were used. The antioxidant capacity of the cell expressing NGAL was higher than HO-1 and there was no significant difference between NGAL and NGAL/HO-1. Purified recombinant NGAL in a concentration-dependent manner was also able to scavenge the free radicals. (B) Torlox was used as a positive control. Note that when a molecule contains antioxidant property, the number of free radicals is lower so there is less dye and absorbance.

(data not shown). Therefore, in the case of analysing NGAL effects cell culture medium was used, while in the case of analysing HO-1, cell lysate was applied. Also, for the cells transfected with both NGAL and HO-1, a mixture of cell culture medium and cell lysate were used. As shown in Figure 7A, the antioxidant capacity of NGAL was higher than HO-1 and there was no significant difference between NGAL and NGAL/HO-1. Together, these results suggested that the higher cytoprotective effect of NGAL compared to HO-1 could be attributed to higher antioxidant capacity of NGAL. Next we analysed the antioxidant capacity of purified recombinant NGAL. NGAL was able to prevent or scavenge the free radicals in a concentration-dependent manner (Figure 7B).

Discussion

In medicine, there is increasing evidence showing that the ability of cells to produce appropriate compensatory stress responses defines their capability of surviving and adapting to severe systemic physiological stresses. In a previous study, we found that oxidative stress induced the expression of NGAL [20]. In another study, we showed that NGAL acts as a cytoprotective factor against H_2O_2 toxicity [11]. We also showed that the induction of NGAL was associated with the expression of HO-1, a well-known antioxidant molecule (15). Hence, we proposed that NGAL might exert its cytoprotective effect through HO-1. Based on the studies presented here, we hypothesized that the cells expressing both NGAL and HO-1 are more refractory to oxidative stress compared to cells over-expressing either NGAL or HO-1 alone.

Unexpectedly, the data showed that cell survival in HEK293T NGAL/HO-1 cells was the same as in HEK293T-NGAL cells. This indicated that the cytoprotective property of NGAL was independent of HO-1. To study the effect of inhibiting HO-1 on the cytotoxicity of H_2O_2, HO-1 activity was suppressed by using ZPP. These results showed that ZPP does not effect cytoprotective ability of HEK293T-NGAL/HO-1 cells but it diminished the cytoprotective ability of HEK293T-HO-1 cells. This again supported the finding that the cytoprotective effects of NGAL are independent of HO-1.

HO-1 expression is highly inducible after exposure to a wide variety of pro-oxidant stimuli and the antioxidant properties of this protein are established [21,22]. To assess the antioxidant capability of NGAL we determined its total antioxidant capacity. NGAL demonstrated antioxidant activity in a concentration-dependent manner.

Our results also showed that the antioxidant capacity of NGAL was higher than HO-1. HO-1 localizes to the endoplasmic reticulum, caveoli and mitochondria to provide cellular protection against oxidative stress [23], whereas NGAL first is produced in endoplasmic reticulum then secreted to the medium that could prevent intracellular and extracellular free radicals. Thus, the higher antioxidant ability of NGAL could be reasonable. It has been reported that Lipocalin α 1-Microglobulin, one of the lipocalin protein family, is also a free radical scavenger [3]. Antioxidant properties have also been reported for tear lipocalin (Lcn 1) [24]. Together, these data indicate that the lipocalin family of proteins has similarity in terms of function as well as amino acid sequences. It is noteworthy that homology sequences in the lipocalins family are very low but they share tertiary structure.

Protective molecules are often induced in response to harmful conditions such as intoxication, infection, inflammation or other forms of cellular stresses. Indeed, increased levels of NGAL and HO-1 are detected in these conditions [4,19,25–27]. Therefore, it seems that NGAL and HO-1 may work together for protection against oxidative stress. To support this concept, Mori et al. [28] found that treatment with NGAL resulted in low levels of HO-1 expression in normal proximal tubules of the kidney but markedly enhanced expression of HO-1 in ischemic tissues.

Prior studies have not examined whether NGAL is implicated as a first line of defence during oxidative stresses. It is noteworthy that kidney epithelia express massive quantities of NGAL within 30 min in the

urine when damaged by ischemia-reperfusion [8], but HO-1 levels are not increased until 3–6 h after reperfusion [29]. Considering this notion with our findings as well as the secretion property of NGAL to scavenge both intra- and extra-cellular radicals, it could be suggested that NGAL is the first defence line compared to HO-1 during oxidative stress. While we propose a tentative reaction scenario for radical scavenging function of NGAL, the mechanism by which it exerts this role is still unknown. Further studies are needed to characterize the relationship between HO-1 and NGAL and to determine the interactions between these proteins. Investigating the down-stream mediators of NGAL functions would be one of the future studies.

Acknowledgements

We thank M. Ebrahimi, M. Mohamadipoor and P. Hamedi for their technical help. We also thank Dr J. Kahrer and Dr A. J. Najafabadi for their help in preparation of the manuscript.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Kjeldsen L, Bainton DF, Sengelov H, Borregaard N. Identification of neutrophil gelatinase-associated lipocalin as a novel matrix protein of specific granules in human neutrophils. Blood 1994;83:799–807.

[2] Schmidt-Ott KM, Mori K, Li JY, Kalandadze A, Cohen DJ, Devarajan P, Barasch J. Dual action of neutrophil gelatinase-associated lipocalin. J Am Soc Nephrol 2007:18:407–413.

[3] Triebel S, Blaser J, Reinke H, Tschesche H. A 25 kDa alpha 2-microglobulin-related protein is a component of the 125 kDa form of human gelatinase. FEBS Lett 1992;314:386–388.

[4] Cowland JB, Borregaard N. Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans. Genomics 1997;45:17–23.

[5] Flower DR. The lipocalin protein family: structure and function. Biochem J 1996;318:1–14.

[6] Friedl A, Stoesz SP, Buckley P, Gould MN. Neutrophil gelatinase-associated lipocalin in normal and neoplastic human tissues. Cell type-specific pattern of expression. Histochem J 1999;31:433–441.

[7] Owen HC, Roberts SJ, Ahmed SF, Farquharson C. Dexamethasone-induced expression of the glucocorticoid response gene lipocalin 2 in chondrocytes. Am J Physiol Endocrinol Metab 2008;294:1023–1034.

[8] Mishra J, Ma Q, Prada A, Mitsnefes M, Zahedi K, Yang J, et al. Identification of neutrophil gelatinase associated lipocalin as a novel early urinary biomarker for ischemic renal injury. J Am Soc Nephrol 2003;14:2534–2543.

[9] Mishra J, Morib K, Maa Q, Kellya C, Baraschb J, Devarajana P. Neutrophil gelatinase-associated lipocalin: a novel early urinary biomarker for cisplatin nephrotoxicity. Am J Nephrol 2004;24:307–315.

[10] Roudkenar MH, Ghasemipour Z, Halabian R, Roushandeh AM, Yaghmai P, Gharehbaghian A, et al. Lipocalin 2 acts as a cytoprotective factor against cisplatin toxicity, an *in vitro* study. Daru 2008;16:106–111.

[11] Roudkenar MH, Halabian R, Ghasemipour Z, Roushandeh AM, Rouhbakhsh M, Nekogoftar M, et al. Neutrophil gelatinase-associated lipocalin acts as a protective factor against H(2)O(2) toxicity. Arch Med Res 2008;39:560–566.

[12] Bach FH. Heme oxygenase-1 and transplantation tolerance. Hum Immunol 2006;67:430–432.

[13] Takahashi T, Morita K, Akagi R, Sassa S. Protective role of heme oxygenase-1 in renal ischemia. Antioxid Redox Signal 2004;6:867–877.

[14] Takahashi T, Morita K, Akagi R, Sassa S. Heme oxygenase-1: a novel therapeutic target in oxidative tissue injuries. Curr Med Chem 2004;11:1545–1561.

[15] Bahmani P, Halabian R, Rouhbakhsh M, Roushandeh A, Masroori N, Ebrahimi M, et al. Neutrophil gelatinase-associated lipocalin induces the expression of heme oxygenase-1 and superoxide dismutase 1, 2. Cell Stress Chaperon 2010;15:395–403.

[16] Dulak J. Changing faces of heme oxygenases. Antioxid Redox Signal 2007;9:2043–2047.

[17] Jozkowicz A, Was H, Dulak J. Heme oxygenase-1 in tumors: is it a false friend? Antioxid Redox Signal 2007;9:2099–2117.

[18] Kehrer JP. Lipocalin-2: pro- or anti-apoptotic? Cell Biol Toxicol 2009;26:83–89.

[19] Tong Z, Wu X, Ovcharenko D, Zhu J, Chen C-S, Kehrer JP. Neutrophil gelatinase-associated lipocalin as a survival factor. Biochem J 2005;391:441–448.

[20] Roudkenar MH, Kuwahara Y, Baba T, Roushandeh AM, Ebishima S, Abe S, et al. Oxidative stress induced lipocalin 2 gene expression: addressing its expression under the harmful conditions. J Radiat Res (Tokyo) 2007;48:39–44.

[21] Applegate LA, Luscher P, Tyrrell RM. Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells. Cancer Res 1991;51:974–978.

[22] Deshane J, Chen S, Caballero S, Grochot-Przeczek A, Was H, Li Calzi S, et al. Stromal cell-derived factor 1 promotes angiogenesis via a heme oxygenase 1-dependent mechanism. J Exp Med 2007;204:605–618.

[23] Majewska M, Zajac K, Dulak J, Szczepanik M. Heme oxygenase (HO-1) is involved in the negative regulation of contact sensitivity reaction. Pharmacol Rep 2008;60:933-9-40.

[24] Akerström B, Maghzal GJ, Winterbourn CC, Kettle AJ. The lipocalin alpha1-microglobulin has radical scavenging activity. J Biol Chem 2007;282:31493–31503.

[25] Flo TH, Smith KD, Sato S, Rodriguez DJ, Holmes MA, Strong RK, et al. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. Nature 2004;432:917–921.

[26] Hemdahl AL, Gabrielsen A, Zhu C, Eriksson P, Hedin U, Kastrup J, et al. Expression of neutrophil gelatinase-associated lipocalin in atherosclerosis and myocardial infarction. Arterioscler Thromb Vasc Biol 2006;26:136–142.

[27] Zhang J, Wu Y, Zhang Y, LeRoith D, Bernlohr DA, Chen X. The role of lipocalin 2 in the regulation of inflammation in adipocytes and macrophages. Mol Endocrinol 2008;22:1416–1426.

[28] Mori K, Lee HT, Rapoport D, Drexler IR, Foster K, Yang J, et al. Endocytic delivery of lipocalin-siderophoreiron complex rescues the kidney from ischemia-reperfusion injury. J Clin Invest 2005;115:610–621.

[29] Tanaka Y, Maher JM, Chen C, Klaassen CD. Hepatic ischemia-reperfusion induces renal heme oxygenase-1 via NF-E2-related factor 2 in rats and mice. Mol Pharmacol 2007;71:817–825.

This paper was first published online on Early Online on 12 May 2011.