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AROS-29 is involved in adaptive response to oxidative stress

NICOLA MONTESANO GESUALDI[1], GIUSEPPA CHIRICO[1], MARIA TERESA CATANESE[1], GIUSEPPE PIROZZI[2], & FRANCA ESPOSITO[1]

[1]*Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli Federico II, Via Pansini 5, Naples 80131, Italy and* [2]*Dipartimento di Oncologia Sperimentale, Istituto Nazionale Tumori, Naples, Italy*

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Abstract
Transient adaptation to mild oxidative stress was induced in human osteosarcoma cells chronically grown in sub-toxic concentrations of diethylmaleate (DEM), a glutathione (GSH) depleting agent. The adapted cells, compared to untreated cells, contain increased concentrations of GSH (4–6 fold) which, upon DEM withdrawal from the culture medium, return to normal values and are more resistant to subsequent oxidizing stress induced either by toxic concentrations of the same agent or by (H_2O_2) treatment. To investigate the molecular mechanisms involved in the adaptive response to oxidative stress, we analyzed the gene expression profiles of DEM-adapted cells by differential display. The expression of *adaptive response to oxidative stress (AROS)-29* gene, coding for a transmembrane protein of unknown function, as well as of some known genes involved in energy metabolism, protein folding and membrane traffic is up-regulated in adapted cells. The increased resistance to both DNA damage and apoptosis, in cells stably overexpressing AROS-29, demonstrated its functional role in the protection against oxidative stress.

Keywords: *Oxidative stress, gene expression, diethylmaleate, adaptive responses, reactive oxygen species*

Abbreviations: *AROS-29, adaptive response to oxidative stress; DEM, diethylmaleate; GSH, glutathione; JNK, jun kinase; ROS, reactive oxygen species*

Introduction

Reactive oxygen species (ROS) are generated as by-products of cellular metabolism, primarily in mitochondria. When their production exceeds the cell antioxidant capacity, macromolecules such as lipids, proteins and DNA can be damaged [1]. However, ROS serve as sub-cellular messengers, and play a role in gene regulation, cell cycle, apoptosis and signal transduction pathways [2,3], which may be involved in defensive mechanisms against oxidative stress as well as in adaptive response to counteract to redox perturbations [4]. Cellular redox signaling involves a variety of post-translational modifications of proteins by ROS [4]. We have demonstrated that in many cell lines exposed to the glutathione (GSH) depleting agent diethylmaleate (DEM) [5,6], different types of post-transcriptional/translational regulatory events occur; these include the p53-independent induction of $p21^{waf1}$ mRNA expression [7], and the dephosphorylation of several proteins involved in the control of cell cycle progression such as $p21^{waf1}$ [8], pRb and E2F [9]. Furthermore, we have recently reported that upon short exposure of several cell lines

Correspondence: F. Esposito, Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli Federico II, Via Pansini 5, Naples 80131, Italy. Tel: 39 817463145. Fax: 39 817464359; 39 817463650. E-mail: espositof@dbbm.unina.it

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to DEM, MAPK and AKT protein kinases are promptly activated in the absence of Tyr phosphorylation of tyrosine kinase receptors, through a mechanism involving DEM-induced activation of the Tyr kinase c-Src [10]. We also suggested that post-translational modifications of tyrosine kinase receptors different from Tyr phosphorylation, such as Tyr nitration, could be involved in the activation of mitogenic pathways [10]. One of the best characterized mechanism of adaptive response is the heat shock stress [11], but other stress as nitrosative, osmotic, hypoxic and oxidative are now being studied: the expression of heat shock proteins is involved in the adaptive response to hypoxia: in fact, hypoxia inducible factor 1 (HIF-1), a heterodimeric transcription factor [12] is a key regulator of metabolic adaptation to hypoxia, through a mechanism involving PI3K/Akt; this protein kinase contributes to HIF stabilization by inducing the expression of heat shock proteins [13].

Adaptation to oxidative stress is observed in a wide variety of cells: induced adaptive and cross-protective response to peroxides are used by bacteria to survive stressful environments. It involves the up-regulation of the peroxide (oxyR) and superoxide (soxR) gene expression, as demonstrated by the abolishment of the (H_2O_2)-induced adaptive protection in oxyR-mutants [14]. Adaptation is also observed in a wide variety of eukaryotic cells including endothelial cells exposed to nitric oxide or to oxidized lipids [15]: generally, at low concentrations, adaptation to oxidative stress appears to be mediated by induction of antioxidant defenses, mainly based on the regulation of GSH metabolism whereas at high concentrations, apoptosis frequently occurs, through mechanisms not yet completely defined [16]. Adaptation to oxidative and nitrosative stress often occurs in cells exposed to a non-toxic stress, resulting in the ability to tolerate a subsequent toxic challenge of the same or related oxidant [17]. Interestingly, after exposure to sub-lethal concentrations of different oxidizing drugs, cells become more resistant also to subsequent challenges by other stresses [18,19].

During different stresses, cells achieve phenotypic adaptation through a modulation of gene expression [20]; quite often the same family of genes can mediate more than one response, thus suggesting that common mechanisms are in adaptation to different stresses [21].

The present investigation aims at defining new genes and molecular mechanisms involved in the adaptive responses to oxidative stress in mammalian cells. We isolated a human osteosarcoma cell line transiently adapted to low doses of DEM in order to characterize the gene expression profile associated to adaptive phenotype, and identified *adaptive response to oxidative stress (AROS)-29*, a new gene whose functional role in the protection against oxidative stress has been suggested.

Materials and methods

Cell lines, culture conditions and oxidizing treatments

Saos-2 osteosarcoma cell line and SHsy5y from ATCC, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, HyClone), 100 units/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO2/95% air at 37°C. For the selection of Saos-2 cells adapted to DEM cells were grown for several months in Dulbecco's modified medium, supplemented with 10% FCS and DEM (50 and 75 μM); DEM was added to the dish every day. Cells were counted before splitting and seeded at equal number for the 3 populations (300.000/30 mm plastic dish). Saos 50 μM and 75 μM DEM, were grown under the same conditions of untreated control, plated $8*10^5$/dish and treated with DEM at the indicated concentrations every two days.

AROS-29 stable cell lines were established by transfecting Saos-2 cells with an expression vector (pRC−CMV) containing AROS-29 cDNA sequences fused to an hemoagglutinin (HA) tag at the *C*-terminus, upstream of the neomycin resistance gene; following 600 μg/ml neomycin selection, individual clones demonstrating stable AROS-29 expression, were analyzed by western blot with anti HA antibodies. All the transfection experiments were performed using lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. All the experiments were done in triplicate. Measurements of luciferase activity were performed using a Dual Luciferase reporter assay system (Promega) according to the manufacturer's instructions. For UV experiments, the monolayer of subconfluent cells was irradiated with 15 J/m^2 of UV light (254 nm) for 5 min. The cells were harvested either immediately (time 0) or after 30 min for the preparation of cell lysates and western analysis. *N*-acetylcysteine (10 mM) was added to the dishes 24 h before irradiation.

GSH assay

GSH intracellular concentration was determined according to Smith et al. [22] with minor modifications. Briefly, cells were washed twice in phosphate-buffered saline (PBS), harvested and centrifuged at 4000 rpm for 10 min at 4°C. The pellets were lysed by adding 100 μl of percloric acid (3%) for 15 s and centrifuged at 20,000 rcf for 10 min at 4°C. The supernatant was neutralized with 900 μl solution of NaH_2PO_4 0.1 M, EDTA 5 mM; glutathione reductase (1 U/ml) and NADPH (0.21 mM) were immediately added. The mix was incubated for 10 min at 37°C. GSH content was measured by adding 600 μM DTNB (5, 5′-dithio-bis(2-nitrobenzoic acid)) and quickly read at 412 nm. After the lysis pellets from percloric acid were resuspended in

NaOH 1 M and protein amount was measured by the Bradford assay (2). GSH content was expressed in nmol/mg protein.

Comet assay

DNA damages were analyzed using the Comet assay [23] with slight modifications of the manufacturer's instructions: briefly, the cells underwent the oxidizing treatments, as described in the figure legends, washed with PBS, trypsinized, re-suspended in PBS, and combined with LM-agarose (supplied in the Trevigen kit assay) at a ratio of 1:8 (cells:agarose). Electrophoretic run and qualitative and/or quantitative analyses were carried out according to the Trevigen protocol. Quantitative analyses of the results were done by using the Image software (National Institutes of Health), as suggested by the manufacturer. Data are reported as the ratio between tail and nucleus areas.

Cytoflourimetric analysis

Cell cycle distribution was analyzed by flow cytometry. Briefly, Saos-2 cells and AROS-29 stable clones were harvested in PBS containing 2 mM EDTA, washed once with PBS and lysed with hypotonic DNA staining solution (0.1% Triton X-100, 0.1% sodium citrate, 1 mg/ml RNAse A and 50 μg/ml propidium iodide) for 2 h at room temperature in the dark. DNA flow cytometry were performed using FACSVantage apparatus (Becton Dickinson, Mountain View, CA, USA) equipped with a water-cooled argon ion laser (488 nm, 150 mw), Cell Quest software (Becton Dickinson) equipped with doublet discrimination module. Data analysis was performed using a Mod-Fit (Verity, Software House, Inc.) cell cycle analysis program.

Western blot

Cells were rinsed with PBS buffer (150 mM NaCl, 0.1 M phosphate, pH 7.5) and harvested in the same buffer. Cell lysates and Western blot analysis were performed as previously described [8]. Antigen–antibody complexes were detected with a chemiluminescence reagent kit (Amersham Biosciences). Anti-Syntaxin 3 polyclonal antibody was a gift from Dr Vesa Olkkonen. Anti-Hsp 70, adaptin, HA rabbit polyclonal IgG antibodies and anti-Trap-1 and tubulin mouse monoclonal antibodies were from Santa Cruz Biotechnology; anti-phospho Jun kinase (JNK) and p38 antibodies (p-JNK, p-p38) were from New England BioLabs; all the antibodies were used in Western blot analyzes at 1 mg/ml.

Differential display technique

Differentially expressed mRNAs were detected using the Clontech "Delta Differential Display Kit" according to the manufacturer's protocol. Reamplified bands of interest were cloned into the Promega pGEM–T easy Vector and then sequenced. The identity of the bands was revealed by nucleotide blast.

Real time PCR

Total RNA was extracted using RNeasy mini kit (Qiagen). First strand of cDNA was synthesized from 2 μg of each RNA sample using an oligo dT (1 μM) and sterile water to 5 ml. The mix was incubated at 70°C for 3 min, cooled on ice for 2 min, and then dNTPs (5 mM), reverse transcriptase buffer (2X) and MMLV reverse transcriptase (200 u/ml) were added to the mix up to 10 ml and incubated at 42°C for 1 h. The reaction was terminated by the incubation at 75°C for 10 min. The reaction mix was then diluted with water to 100 μl. Real time PCR amplification mix (25 μl) contained 5 μl of first strand cDNA, 0.38 ml of forward and reverse primer mix (40 μM each), 12.5 μl of 2X Biorad iQ SYBR Green Supermix and 7.15 μl of water. Reaction was carried out on a Biorad iCycler iQ Real Time PCR detection system. The thermal conditions were: 3 min at 95°C and 40 cycles at 95°C for 15 s and 60°C for 1 min and a melting curve analysis from 55 to 95°C with 80 steps of 0.5°C for 10 s. Data analysis was carried with glucuronidase as a control gene. The following primers were used:

Trap-1 forward: 5′-GGACGCACCGCTCA-ACAT-3′
Trap-1 reverse: 5′-CACATCAAACATGGACGG-TTTC-3′
Syntaxin 3A forward: 5′-CCAACAACGTCCGG-AACAA-3′
Syntaxin 3A reverse: 5′-AGGTCTGCCGATGA-CCTGAC-3′
Glucuronidase forward: 5′-GGTTTCACCAGG-ATCCACCTCT-3′
Glucuronidase reverse: 5′-CCAACCACGTATTT-TCTGCGTT-3′
AROS-29 forward: 5′-CCTGGTCCTGCGCTT-CTTC-3′
AROS-29 reverse: 5′-ACGCCGCCCGAG-TACA -3′
$p21^{waf1}$ forward: 5′-CTGGAGACTCTCAGGGT-CGAA-3′
$p21^{waf1}$ reverse: 5′-CGGCGTTTGGAGTGGT-AGAA-3′.

Results

We previously set up an experimental procedure to manipulate intracellular redox conditions in different mammalian cells: this is based on the exposure of cells to DEM, a GSH depleting agent, and allowed us to demonstrate that the sensitivity to DEM-induced oxidative stress varies among different cells [6].

To adapt cells to mild oxidizing conditions different cell lines were cultured for several months in the presence of low concentrations (50 μM) of DEM. Higher concentrations were strongly cytotoxic for long-term treatments for any analyzed cell line. We selected for our studies the Saos-2 human osteosarcoma cell line, since it was the only one in which some cells survived after 1 month in presence of DEM. These surviving cells started to grow normally, with growth parameters (cell number and doubling time) undistinguishable from the untreated counterpart thus generating a stable cell line adapted to DEM. As a relationship between cellular resistance to H_2O_2 and changes in ploidy was previously described [19], we performed cytofluorimetric analyses to evaluate chromosomal modifications in the survived cells, but did not observe any change in ploidy (data not shown).

Since DEM induces a GSH depletion [5], we hypothesized that modifications of intracellular redox conditions (i.e. changes in GSH levels) in these cells, could be responsible for the surviving of DEM-adapted cells. As shown in Figure 1A, adapted cells contain significantly higher GSH levels than the untreated control (4 and 6 times in Saos 50 μM and 75 μM DEM, respectively). This finding suggests that the adaptation to chronic oxidizing conditions of these cells could be related to increased GSH levels. Since GSH intracellular homeostasis is maintained by a complex array of enzymatic reactions (biosynthesis, degradation, utilization as substrate of other enzymes, etc.), to characterize molecular mechanisms responsible for the increased levels observed in our cells, a quantitative analysis by real time PCR of some genes involved in GSH metabolism was performed. These experiments allowed us to demonstrate that the expression of these

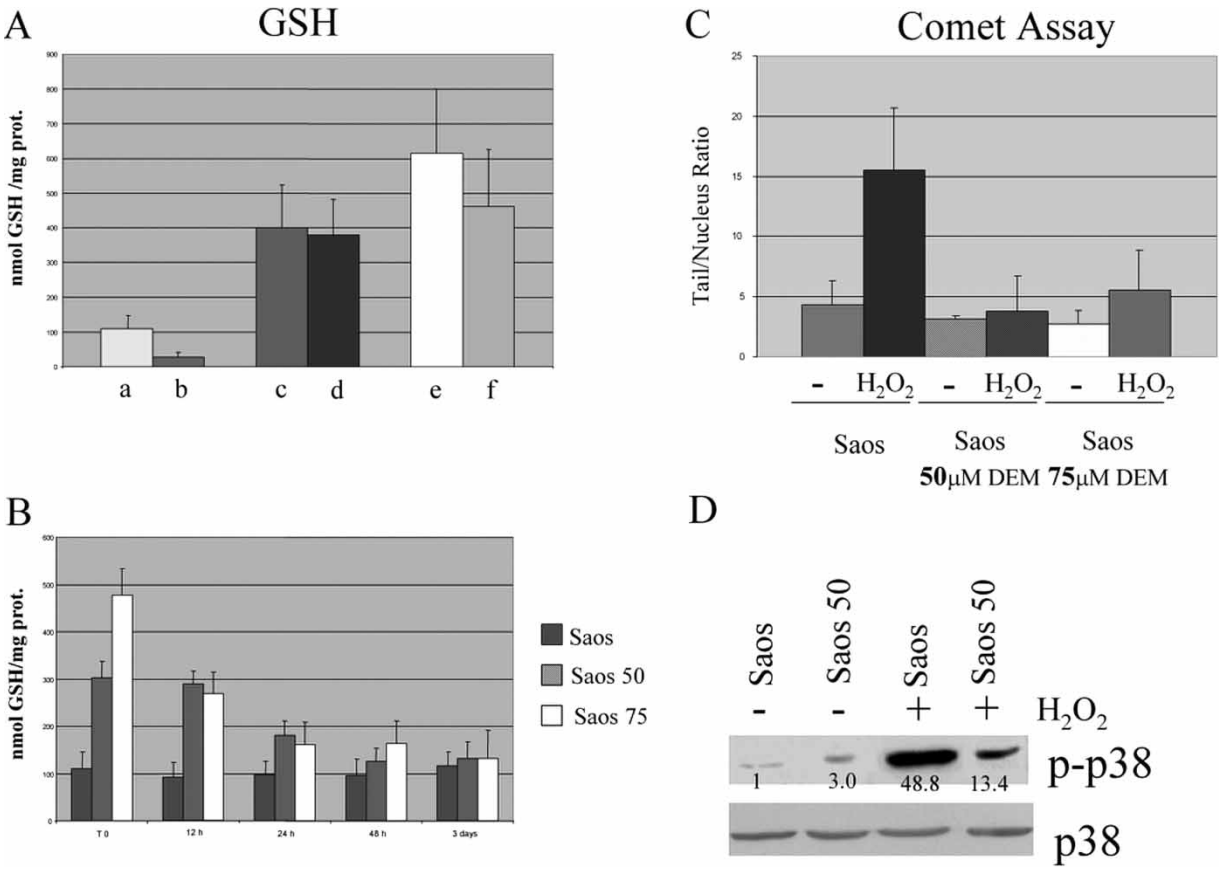

Figure 1. GSH levels and resistance to H_2O_2 of Saos-2 cells untreated and adapted to DEM. Growth conditions and measurements of intracellular GSH were as described in "Materials and methods" section. Each experiment was repeated two to three times. Panel A: GSH levels in Saos-2 cells. Lanes a, c and e, GSH basal levels in untreated, 50 and 75 μM DEM-adapted cells, respectively; lanes b, d and f, GSH depletion in the same cells upon exposure to 1 mM DEM for 30 min. All the values are expressed as nmol/mg proteins. Panel B: Measurements of GSH levels after DEM withdrawal: cells were washed with fresh medium before harvesting and GSH levels were assayed at the indicated times. All the values are expressed as nmol/mg proteins. Panel C: Effect of H_2O_2 treatment on the DNA damages: Saos-2 cells were grown as described in "Materials and methods" section and treated with H_2O_2 (1 mM for 30 min). Quantitative analysis of the results was done by the Comet assay procedure, using the NIH Image Software, as suggested by the manufacturer. Data are reported as the ratio between tail/nucleus areas and represent the mean of three independent experiments. Panel D: p38 activation upon treatment with H_2O_2: Saos-2 cells grown as described in "Materials and methods" section were treated with H_2O_2 (1 mM for 15 min), washed and harvested for the preparation of cell extract; lysates from untreated and DEM-adapted Saos cells, containing 40 μg of proteins were resolved by electrophoresis on a 10% SDS-PAGE, transferred on a nitrocellulose membrane and subjected to immunoblot analysis with anti p38 antibodies specific for the activated form (p-p38). The same filters were re-probed with anti-p38 antibodies for the normalization of cell lysates.

genes does not change significantly in the three populations, with the only exception of the increase (2.5 times) of two genes coding for the catalytic subunit of γ-glutamyl-cysteine synthetase, the enzyme involved in the first step of GSH biosynthesis (data not shown). We previously demonstrated that treatment with 1 mM DEM for 30 min yields GSH depletion (70%) in several cell lines [6]. To evaluate whether the same decrease also occurs in the adapted cells we treated them with this strong concentration of DEM. Figure 1A also shows that while in normal cells the expected GSH depletion (70%) was observed, it was significantly lower (<20%) in adapted cells. Finally, we aimed at analyzing whether the increased GSH levels in adapted cells would still be observed after washing of DEM from the culture medium. As shown in Figure 1B, within three days from DEM withdrawal, comparable GSH levels between control and adapted cells are observed. These experiments indicate that DEM-induced adaptation is a transient and reversible phenomenon.

It is reasonable to conclude, from the results obtained in these sets of experiments that DEM-induced adaptation, not only resulted in a more reduced intracellular environment, but it also yields an increased resistance to a subsequent stronger oxidative stress induced by the same agent (Figure 1A and B). To verify that DEM-adapted cells developed resistance also to other oxidizing agents, the DNA damage upon treatment of these cells with H_2O_2 was analyzed by the Comet assay. Figure 1C shows that Saos-2 cells "DEM-adapted" are more resistant to hydrogen peroxide treatment (500 μM/30 min), as demonstrated by the lower tail/nucleus ratio which, in turn, indicates a less damaged cell population. Activation of p38 stress kinase following H_2O_2 treatment, is lower in Saos-adapted cells, compared to the controls, (Figure 1D). This observation is in agreement with the results demonstrating higher resistance to stress stimuli of adapted cells.

To identify proteins involved in the resistance to oxidative stress, RNA from control and DEM-adapted Saos-2 cells was purified and used as template for the differential display technique, to analyze gene expression profiles. Among the genes found up-regulated in DEM-adapted cells, some have been identified (Table I): they include a mitochondrial acyl-CoA thioesterase, involved in the energy metabolism [24] a hsp75 protein (TRAP1), associated to the tumor necrosis factor receptor [25] with mitochondrial localization, whose involvement in the control of protein folding and the possible role in the control of apoptosis were previously suggested (see discussion); syntaxin 3A, a protein involved in intracellular membrane traffic [26], tropomyosin and PAR-1 [27], a developmental–regulatory protein.

An mRNA coding for a protein of unknown function (AROS-29) was also identified and partially characterized. The increased expression of AROS-29,

Table I. Genes up-regulated in Saos-2 adapted to DEM.

Up-regulated Genes	
Gene	ID number
Acyl-coenzyme A thioesterase 2	NM_01232
AROS-29 (FLJ20422)	NM_017814
Par-1	AF387638
Trap-1 (Hsp75)	NM_016292
Syntaxin 3A	AJ002076
Tropomyosin	X05276

Names and ID numbers were according to NCBI data bank.

syntaxin 3A and hsp75 mRNAs was confirmed by the real time PCR (Figure 2A). Moreover, to ascertain whether the increased expression observed in Saos-2 adapted-cells is a reversible process, we evaluated the expression of the three mRNAs after the removal of DEM from the culture medium. Figure 2A confirms increased expression of AROS-29, syntaxin3A and TRAP1 in DEM-adapted cells, and shows that the mRNA levels of all the proteins, within three days from DEM wash, are undistinguishable from those observed in the control cells. Since changes in gene expression correlate with modifications in GSH content we analyzed intracellular GSH levels upon AROS-29 hyperexpression and, accordingly, observed a slight increase. Finally, Western blot experiments in Saos-2 untreated and DEM-adapted cells confirm the increase of syntaxin 3A and TRAP1 at protein levels; furthermore, we aimed to analyze whether this regulatory mechanism was shared by two other proteins involved in very similar functions—i.e. adaptin, involved in vesicular transport [28] and hsp 70, another heat shock protein [29]. Figure 2B shows that the expression of these two proteins is not modified in adapted cells, thus suggesting that the observed phenomenon is specific for syntaxin 3A and TRAP-1 and not common to all the members of the class, involved in similar biological functions.

AROS-29 is annotated in data banks (GenBank accession number NM_017814) as an expressed sequences tag of unknown function; the predicted protein is highly hydrophobic with up to seven possible trans-membrane tracts. Immunostaining of cells expressing AROS-29 protein tagged either at the N- or C-terminal end, confirm the plasma-membrane topology of this protein (data not shown).

To evaluate molecular mechanisms involved in the regulation of AROS-29 expression in DEM-adapted cells, Saos-2 cells (adapted and control) were transfected with a construct containing AROS-29 promoter regions fused to the *luciferase* gene. As shown in Figure 3A, the basal level of luciferase activity in adapted cells is higher than the controls, whereas, pro-oxidant conditions induced by UV treatment in Saos-2 cells, lead to a down-regulation of AROS-29 promoter-driven luciferase activity (Figure 3B); pretreatment with the antioxidant

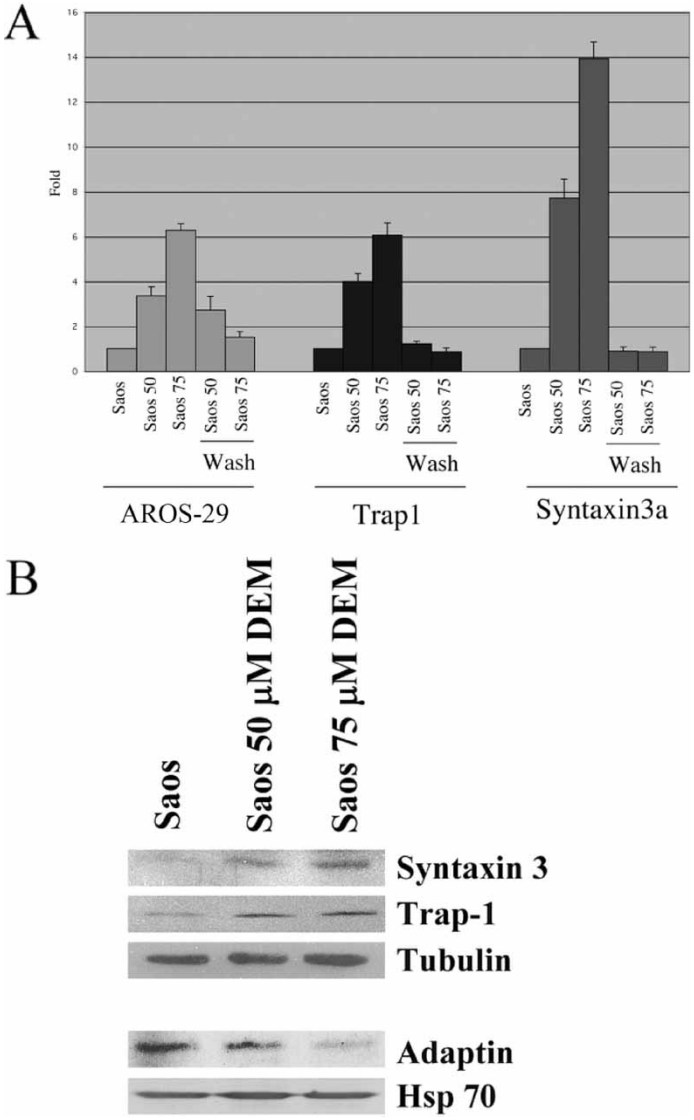

Figure 2. Adaptation to DEM induces AROS-29, syntaxin3A and Trap-1 expression in Saos-2 cells. Panel A: Induction of AROS-29, Trap-1 and syntaxin 3A mRNA expression in Saos-2 adapted cells is a reversible process. Saos-2 cells (control and DEM-adapted) were cultured as described in "Materials and methods" section. DEM was removed by washing extensively part of the dishes with fresh medium, and after one week all the cells were harvested for RNA extraction and cDNA synthesis (see "Materials and methods" section). Real time PCR condition is described in "Materials and methods" section. All the values are expressed as fold increase, by assuming mRNA levels of the untreated controls equal 1. Panel B: Induction of syntaxin 3A and Trap-1 proteins. Cell lysates from untreated and DEM-adapted Saos-2 cells, containing 40 μg of proteins were resolved by electrophoresis on a 10% SDS-PAGE, transferred on a nitrocellulose membrane and subjected to immunoblot analysis with antibodies specific for the indicated proteins (see the text). The same filters were re-probed with anti-tubulin antibodies for the normalization of cell lysates.

N-acetylcysteine, a GSH precursor, counteract the effect of UV irradiation on luciferase activity, thus suggesting that oxidative stress plays a role in the UV-induced down-regulation of AROS-29 promoter activity (Figure 3B). Luciferase activity correlates with AROS-29 mRNA levels assayed by RT–PCR, both in DEM-adapted cells (Figure 2A) and in Saos-2 cells upon 30 min of UV treatment (Figure 3C). The results shown in Figure 3 demonstrate that AROS-29 induction occurs at transcriptional level and is dependent on the reduced intracellular environment. This last finding is also confirmed by a similar induction of AROS-29 expression upon treatment with retinoic acid (RA), a differentiating agent provided of antioxidant properties [30]. Figure 3C shows a real time PCR analysis of AROS-29 mRNA in SHsy5y neuronal cells, containing high levels of this protein. The increased AROS-29 expression seems not related to the neuronal differentiation of these cells, being only present at early times of RA treatment (within 4 h), in absence of any differentiated phenotype: in fact, the opposite regulation of $p21^{waf1}$ mRNA, whose induction is required for the cell cycle arrest of differentiated cells [31] is demonstrated in the same experimental conditions. Furthermore, in agreement with other groups, which demonstrated an involvement of retinoids in GSH homeostasis [32],

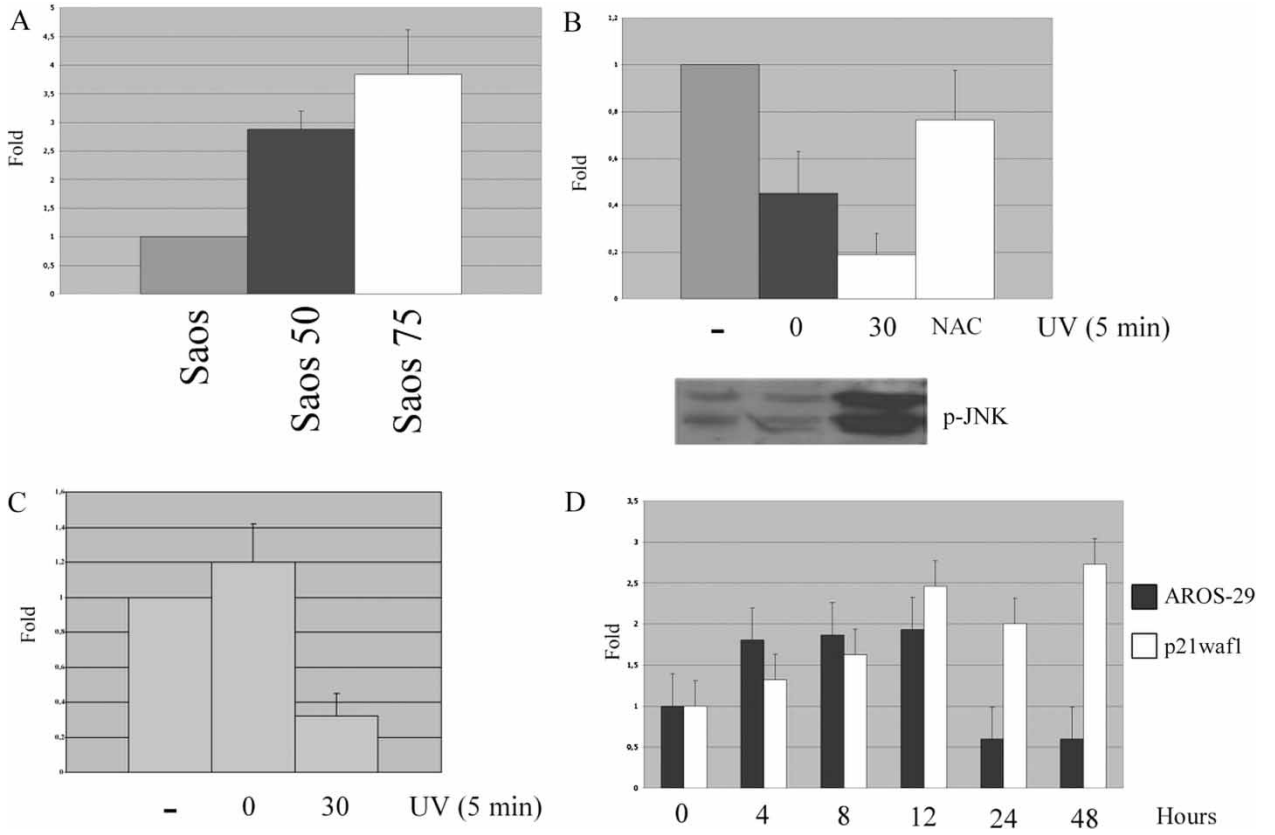

Figure 3. Redox regulation of AROS-29 expression. Panel A: Saos-2 cells (control and adapted) were transfected with a construct containing AROS-29 promoter regions (2000 bps) fused to the *luciferase* gene, as described in "Materials and methods" section. Luciferase activity represents the means of three independent transfection experiments. All the values are expressed as fold increase, by assuming luciferase activity of the untreated controls equal 1. Panel B: Upper: Saos-2 cells were transfected with a construct containing AROS-29 promoter regions fused to the *luciferase* gene and irradiated as described in "Materials and methods" section. NAC (10 mM) was added 24 h before UV irradiation. Luciferase activity represents the means of three independent experiments. All the values are expressed as fold increase, by assuming luciferase activity of the untreated controls equal 1. Lower: To control the efficacy of UV treatment, Saos-2 cells, irradiated as described in "Materials and methods" section, were harvested and cell lysates containing 40 μg of proteins were subjected to immunoblot analysis with anti-phospho JNK antibodies as described in "Materials and methods" section. Panel C: Saos-2 cells were irradiated as described in "Materials and methods" section and harvested for RNA extraction and cDNA synthesis (see "Materials and methods" section). Real time PCR conditions are described in "Materials and methods" section. All the values are expressed as fold increase, by assuming mRNA levels of the untreated controls equal 1. Panel D: SHsy5y neuronal cells were treated with RA (10 μM) for the indicated times and harvested for RNA extraction and cDNA synthesis (see "Materials and methods" section). Real time PCR conditions are described in "Materials and methods" section. All the values are expressed as fold increase, by assuming mRNA levels of the untreated controls equal 1.

we found increased levels of GSH in SHsy5y cells exposed to RA (data not shown).

To demonstrate the functional role of AROS-29 in the protection against oxidative stress, we generated Saos-2 cell lines stably transfected with AROS-29, to analyze their response to different stress. Comet assays shown in Figure 4 (Panel A) demonstrated that the levels of DNA damage upon exposure to DEM or H_2O_2 is lower in AROS-29 stable transfectants (clone 2). These results resemble the Comet analyses shown in Figure 1C. Futhermore, citofluorymetric analysis of pooled AROS-29 clones exposed for different times to cisplatin, a DNA-damaging and chemotherapeutic drug, demonstrated an increased resistance to apoptosis of these cells, compared to the controls (Panels B and C). These observations demonstrate that AROS-29 over-expression confers increased resistance to both DNA damage and apoptosis induced by different stress.

Discussion

The experiments reported in this paper were designed to investigate molecular mechanisms involved in the adaptive response to oxidative stress. The oxidizing agent used is the DEM, a GSH-depleting compound. The Saos-2 human osteosarcoma cells were selected for our studies, because it was the only cell line, among others analyzed, which survived to DEM treatment. The development of adaptive responses is achieved mainly through changes in gene expression programs; re-programming of gene expression leads to the regulation of specific genes which, depending on the kind of stress induced, contribute to restore cellular

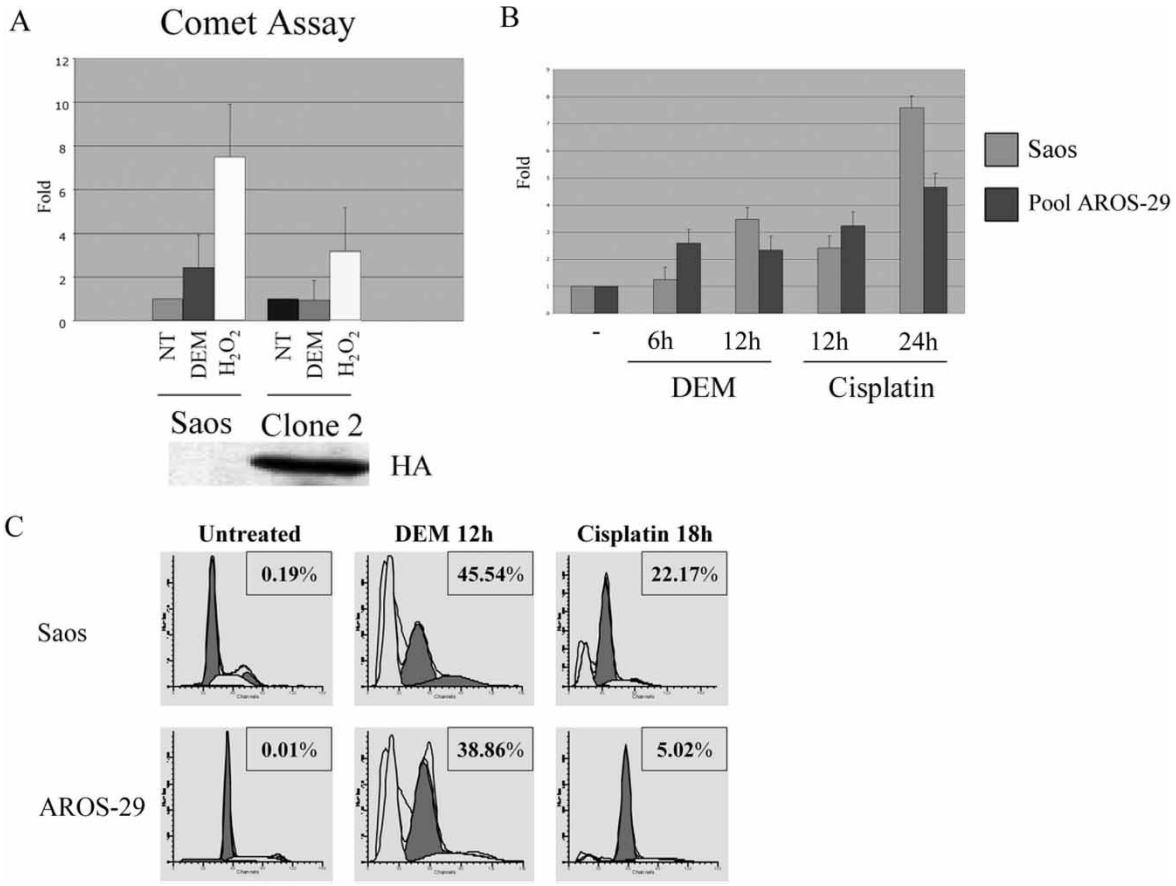

Figure 4. Stable overexpression of AROS-29 confers resistance to stress-induced DNA damage and apoptosis Panel A: Upper: Saos-2 cells and AROS-29 stable clone 2 (Cl2), generated as described in "Materials and methods" section, were treated with H_2O_2 (1 mM for 30 min). Quali/quantitative analysis of DNA damages was done by the Comet assay procedure as described in the legend of Figure 1. Lower: Immunoblot analysis of protein extracts from Saos-2 cells and AROS-29 stable clone 2 (Cl2) with anti-HA antibodies, as described in "Materials and methods" section. Panels B and C: AROS-29 stable clones, generated as described in "Materials and methods" section, were pooled and treated with DEM (1 mM) and cisplatin (50 μM). Quali/quantitative cytofluorimetric analyses of apoptosis induced by these two drugs were done as described in "Materials and methods" section. Stained nuclei were analyzed with a fluorescence-activated cell sorter (FACSVantage, Becton-Dickinson) and CellQuest software (Becton Dickinson). The data were analyzed using a Mod-Fit (Verity, Software House, Inc.) cell cycle analysis program.

homeostasis. It has been previously described that among the genes most frequently involved in the adaptation to oxidative stress, there is the family of genes related to GSH biosynthesis and degradation, as well as antioxidant enzymes such as catalase, superoxide dismutase and other proteins that decrease the extent of oxidative stress [33]. However, some results demonstrate that H_2O_2-resistant cell lines have been isolated without a significant increase in transcription and/or translation of scavenging enzymes [33]. In Saos-2 DEM-adapted the expression of several genes of GSH metabolism does not show significant modifications compared to the controls. Increased gene expression could be related to chromosomal aberrations (i.e. gene duplication): in fact, a relationship between stable cellular resistance to H_2O_2 and changes in the ploidy that was related to increased catalase activity, was previously described in CHO variants cells [19]: we performed cytofluorimetric studies to analyze chromosomal modifications of Saos-2 cells adapted to DEM, but did not find any modification in ploidy (data not shown). Our results demonstrate that DEM-induced adaptation is a reversible process, since withdrawal of the GSH depleting agent from the culture medium restores molecular phenotypes of control cells (i.e. GSH concentrations); furthermore, mRNA/protein levels of DEM-induced genes return to normal values within short times; our results suggest an involvement of both transcriptional and post-transcriptional/translational mechanisms in the development of stress resistance in our experimental models.

Increased resistance to oxidant-induced DNA damages in adapted cells (see Comet assays) is confirmed by a lower p38 phosphorylation upon treatment of these cells with H_2O_2, and by a decreased activation of NF-κB, a stress-responsive transcription factor, whose DNA binding is decreased in presence

of high levels of GSH ([34] and our unpublished observations).

Several other experimental approaches, including cDNA arrays and proteomic analyses, have been used to identify the genes induced upon adaptive mechanisms to oxidative stress [35–37]: they include genes and/or proteins involved in mitochondrial energy metabolism, in RNA processing and translation, chaperoning, protein folding, cellular signaling and redox regulation. In agreement with these observations, all the genes identified in this paper by the mRNA differential display belong to the above-described classes, thus confirming the involvement of specific families of genes in adaptive molecular programs. Modification of protein folding seems to be one of the first mechanisms involved in the adaptation [38]: reasonably, it can be considered a defense mechanism against oxidant insults that cells set up to avoid incorrect shape of the proteins and to prevent their degradation. Molecular chaperones play a fundamental role in this process: they are required for the folding and the stabilization of many cellular proteins; upon several kinds of stress, form molecular complexes with different proteins to protect them from possible damaging and degradative mechanisms.

TRAP-1/hsp75, one of the products identified in the present study, shares striking homology with hsp90, one of the best known molecular chaperones [25]. It will be of interest to verify whether these two proteins share similar functions in the control of protein folding, thus confirming that up-regulation of TRAP-1 expression can be considered as an adaptative response of Saos-2 cells to DEM-induced oxidative stress.

Other proteins, whose expression is found consistently regulated by oxidative insults, are the mitochondrial ones: we found that an acylCoA thioesterase is several fold induced in our experimental conditions. Thioesterases control the acylation rate of some proteins and their intracellular localization. It was recently demonstrated that the acyl protein thioesterase I was involved in the deacylation of Ha-*ras in vivo* [24], thereby regulating its membrane association. It will be interesting to investigate whether oxidants play roles in the activation of small G proteins, as *ras*, through post-translational modifications, as the acylation and subsequent membrane localization.

Another observation that arises from our studies concerns with the identification of syntaxin3A that was found to be up-regulated in DEM-adapted cells: syntaxins include a family of compartment-specific membrane-anchored proteins, known as SNARE, highly involved in the control of vesicle transport [26]. It will be worthwhile to investigate on possible relationships between intracellular traffic and oxidant adaptation. Again, the up-regulation of syntaxin3A expression upon DEM treatment could be interpreted as a modification in the normal transport of proteins to the apical plasma membrane that is probably one of the sub-cellular compartment more sensitive to oxidant insults. Lastly, the identification and partial characterization of AROS-29 as a new trans-membrane protein, and the finding that stable over-expression of this protein in Saos-2 cells confers increased resistance to both oxidants-induced DNA damage and apoptosis, confirms important functional roles of the identified genes in the protection against oxidative stress.

Oxidative stress is involved in several diseases, including Alzheimer's disease and other neurological, lung and blood disorders; it is also implicated in aging. AROS-29 modifications could be investigated in diseases involving oxidative stress. In addition, a "reductive" induction of AROS-29 expression may occur in response to antioxidant therapy, thereby providing a reliable marker of its efficacy.

Finally, since several chemotherapeutic drugs are known to function as anti-tumor agents by producing ROS, it has been suggested that the resistance of cancer cells to pro-oxidant conditions and apoptosis may represent one of the mechanisms involved in the resistance to chemotherapy. For example, it was demonstrated that the pretreatment with hydrogen peroxide is responsible for the adaptation to Adriamycin in some melanoma cell lines [39]. Preliminary data suggest that the expression of some genes, identified in this study, is likewise increased in cells resistant to anticancer drugs (unpublished results). Future experiments may, ultimately, contribute to the identification of new molecular mechanisms involved in chemoresistance.

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