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Papel da ativação do fator de transcrição NRF2 na diferenciação de células SH-SY5Y mediada por Ácido Retinóico.

Aluno: Vitor de Miranda Ramos

Orientador: Prof. Dr. José Cláudio Fonseca Moreira

Coorientador: Dr. Alfeu Zanotto Filho

Instituição sede: Centro de Estudos em Estresse Oxidativo, Departamento de Bioquímica - Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul.

Estre trabalho de conclusão será apresentado na forma de artigo científico.

Agradecimentos

Gostaria de dedicar esta parte do trabalho para expressar minha gratidão a todas pessoas que, de diferentes formas, me ajudaram a chegar até este momento.

Primeiramente, preciso agradecer as oportunidades que me foram concedidas. As mais importantes, concedidas por uma base privilegiada a qual minha família pode me proporcionar. Uma família que sempre se esforçou ao máximo para me prover as melhores experiências e que, com certeza, contribuíram essencialmente para minha formação. Sem estas oportunidades tudo teria sido mais difícil...obrigado por tudo!

Outra oportunidade chave foi-me dada pelo Prof. José Cláudio (Zé) quando, no início da faculdade, me trouxe para o seu grupo. Dentro do laboratório 32 tive, entre muitos, um fator fundamental para a realização deste trabalho...exemplos! Ali tive contato com uma diversidade de formas de pensar e agir, expressas por pessoas as quais pude me espelhar para construir meu aprendizado. Todo auxilio me foi dado dentro deste grupo, e a todos sou muito grato! Entretanto, não posso deixar de citar aqui três pessoas que têm participação primordial nesta conclusão e que se tornaram minhas principais referências dentro da prática de ciência. Primeiro agradeço imensamente ao meu orientador Zé pela liberdade e confiança que ele sempre me passou nestes últimos anos. Com certeza um exemplo de professor, pesquisador e líder. Sou fortemente grato também ao Alfeu, que teve muita paciência e dedicação, abrindo mão de muito de seu tempo para me ouvir e ajudar diretamente em todas as etapas deste trabalho. A cada experimento, aprendi muito com ele. Agradeço também ao Matheus Pasquali por ter confiado a mim muitos trabalhos (incluindo este). Mas principalmente agradeço pelo seu compromisso com o início da minha formação dentro do laboratório. Me considero de muita sorte por poder ter aprendido com estas pessoas.

Talvez uma das maiores dificuldades que tive neste processo foi manter equilíbrio entre as atividades dentro e fora do laboratório...e isto não teria sido possível sem a presença de todos amigos que me acompanharam nos últimos anos. A estes devo muito por todos os momentos que puderam me proporcionar! Devo muito por todo o carinho e lealdade que eles me passam e que talvez nem sempre eu soube retribuir da melhor forma! Também não posso deixar de fazer menção especial à minha companheira, que teve papel essencial em me ajudar a lidar com as dificuldades surgidas no caminho. Obrigado a todos vocês!

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Aqui, apenas consegui resumir a importância que todos tiveram para a construção e conclusão deste trabalho, mas acho que foi possível representar a coletividade por trás deste escrito final. Sendo assim, espero que todos possam se sentir parte dele.

Esclarecimentos do trabalho

A linha de pesquisa com Vitamina A e seus derivados sempre foi um dos carros-chefes do Laboratório 32 da Bioquímica-UFRGS. O modelo de diferenciação neuronal *in vitro* com células SH-SY5Y tratadas com Ácido Retinóico também vem sendo estudado por diferentes alunos e faz parte de um grande projeto de pesquisa do laboratório. Quando eu me juntei ao grupo em junho de 2012, comecei a aprender cultura de células utilizando-se deste modelo. Concomitantemente, aprendi diversas técnicas de análise (ex.: ensaios de viabilidade e western blot) feitas no laboratório e que me renderam alguns outros trabalhos junto com o ex-aluno de Doutorado, Dr. Matheus Pasquali. Para este trabalho de conclusão iniciei os experimentos logo em 2013, sob supervisão do Matheus que saiu do laboratório no final daquele ano. Na metade de 2014 retomei os experimentos, agora com coorientação do Dr. Alfeu Zanotto. Junto com a supervisão do Alfeu, realizei todos os experimentos os quais nós achamos necessários para poder concluir este trabalho. Em maio deste ano juntei os resultados obtidos para discuti-los com meus orientadores e assim pude começar a escreve-lo. A escrita e as figuras foram revisadas pelo Alfeu e, após as devidas correções, enviadas para a banca examinadora.

The role of Nrf2 activation in human neuroblastoma cells treated with retinoic acid.

Vitor de Miranda Ramos[1], Alfeu Zanotto-Filho[1], Matheus Augusto Bittencourt Pasquali[1], Karina Klafke[1], Juciano Gasparotto[1], Daniel Pens Gelain[1], José Cláudio Fonseca Moreira[1].

[1] Centro de Estudos em Estresse Oxidativo, Departamento de Bioquímica – Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

Abstract

Retinoic acid (RA) morphogenetic properties has been used in different kinds of therapies, from neurodegenerative disorders to some types of cancer such as promyelocytic leukaemia and neuroblastoma. However, most of the pathways responsible for RA effects remain unknown. To investigate such pathways, we used a RA-induced differentiation model in the human neuroblastoma cells, SH-SY5Y. Our data showed that n-acetyl-cysteine (NAC) reduced cells proliferation rate and increased cells sensitivity to RA toxicity. Simultaneously, NAC pre-incubation attenuated NRF2 activation by RA. None of these effects were obtained with Trolox® as antioxidant, suggesting a cysteine signalization by RA. NRF2 knockdown increased cell sensibility to RA after 96 h of treatment and diminished neuroblastoma proliferation rate. Conversely, NRF2 overexpression limited RA anti-proliferative effects and increased cell proliferation. In addition, a rapid and non-genomic activation of ERK 1/2 and PI3K/AKT pathway revealed to be equally required to promote NRF2 activation and necessary to RA-induced differentiation. Together, we provide data correlating NRF2 activity with neuroblastoma proliferation and resistance to RA treatments, thus this pathway could be a potential target to optimize neuroblastoma chemotherapeutic response as well as *in vitro* neuronal differentiation protocols.

Retinoic acid (RA) is a morphogen member of the retinoid family with signaling activity present since the early metazoan evolution [1]. In vertebrates, it regulates tissue growth and differentiation, including the embryo pattern of central nervous system location and later organogenesis [2;3]. Its biological properties have been explored by different cancer therapies, with successful treatments to many cases of neuroblastoma and acute promyelocytic leukemia (APL) [4;5;6]. Differently from many of the cell-cell signaling factors, classically, RA goes through the cell membrane and binds to a specific family of nuclear receptors which directly act as transcription factors to regulate target genes expression [2]. In addition to genomic mechanisms, many non-genomic actions of retinoid receptor (RAR and RXR families) have been discovered over the last years [7]. However, most of the pathways involved in RA non-genomic effects remain unknown.

A useful model to investigate RA morphogenetic and anti-proliferative effects is the human neuroblastoma lineage, SH-SY5Y. When exposed with pharmacological doses of RA, these cells undergo the initial stages of neuronal differentiation by promoting cell-cycle arrest and increasing neuronal markers gene expression [8;9]. Recently, it has been discussed the role of reactive species and oxidative stress responses during SH-SY5Y differentiation, given that an increased resistance to oxidants and xenobiotics have been observed in RA-differentiated cells [10;11;12]. These RA-induced changes in cell metabolism toward antioxidant phenotypes may have a major implication on RA therapy, and could provide a better understanding of the variable individual response and resistance to different treatments. Therefore, investigating the RA responsive pathways is a central step to elucidate the molecular mechanisms involved in both RA-induced differentiation as well as resistance to xenobiotics.

Nuclear factor erythroid 2-like factor 2 (NRF2) transcription factor is one of the most important regulator of cellular redox homeostasis by regulating the expression of a variety of genes involved in antioxidant defense, glutathione synthesis and xenobiotic detoxification [13]. At normal conditions, NRF2 protein is constantly retained in the cytoplasm and driven to proteasomal degradation by its inhibitor, Keap-1. The classical mechanism of NRF2 activation involves oxidation of specific cysteine residues with Keap-1, thus allowing NRF2 translocation to the nucleus, binding to DNA Antioxidant Response Element (ARE) and promoting gene expression [13]. However, different transcriptional, post-transcriptional [14], and post-translational (phosphorylation/acetylation) NRF2 regulations have been described as Keap-1 independent mechanism [15]. Improperly regulation of NRF2 pathway activity seems to be associated with different pathologies. For example, while NRF2 down regulation can lead to increased oxidative stress - which has a major influence on neurodegenerative diseases [16] - NRF2 up regulation is associated with chemoresistance and therapy failure [17]. It

has been demonstrated that RA induces NRF2 activation in SH-SY5Y cells, influencing cellular morphogenetic response and expression of the MAP2 differentiation marker [18]. However, the mechanisms underlying RA-induced NRF2 activation, and whether NRF2 acts as a primary differentiation factor or as a protective signal in response to RA-induced stresses remains to be depicted.

Taken the aforementioned, this study was undertaken in order to investigate the mechanisms leading to NRF2 activation by RA as well as the involvement of NRF2 and its upstream regulators on viability and morphogenesis of SH-SY5Y cells. Secondarily, we expect to promote a better understanding of the oxidative stress role in this context. In addition, we believe that this study could contribute to elucidate some mechanisms involved in RA-differentiation and cell resistance, which could be useful to overcome some issues associated with retinoid therapies

Materials and Methods

Cell culture

SH-SY5Y cells obtained from European Collection of Cell Cultures (ECACC) were grown in 75 cm^2 flasks containing DEMEM/F12 medium (1:1) supplemented with 10% fetal bovine serum (FBS) and 1× antibiotic/antimycotic solution (Sigma-Aldrich). The cells were cultured in a humidified incubator set at 37°C with 5% CO_2. When cultures reached confluence, the cells were trypsinized and seeded at a density of 15 to 30 × 10^3 cells/cm^2, and treated after 24-48 hours.

Chemicals and treatments

Before treatments, the culture medium containing 10% FBS was replaced by fresh medium containing 1% FBS. All-trans retinoic acid (RA), LY294002, UO126, SB239063 and SP600125 were dissolved in dimethylsulphoxide (DMSO) and stored protected from light at -20°C. All pharmacological inhibitors were diluted in the culture medium and added to the cells one hour before RA treatments. N-acetyl-cysteine (NAC) and Trolox® were dissolved in PBS or water and added to the cells 2-3 hours before treatments. L-buthionine-sulfoximine (BSO) was dissolved in culture medium and added to cells 12 hours before treatments to allow effective GSH depletion. All chemicals were purchased from Sigma-Aldrich®.

Nuclear extract preparation

To isolate nuclear fraction, the cells were washed with ice-cold PBS and collected by centrifugation at 500 xg for 5 min. The cells pellet were then resuspended in 200 µL of hypotonic buffer (HEPES 10 mM pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1mM phenylmethylsulphonyl fluoride and 1 mM sodium orthovanadate) and incubated on ice for 15 minutes. Next, 10 µL of 10 % Igepal was added to each sample followed by vortexing. Samples were then centrifuged at 14,000 xg for 30 seconds thus separating cell nuclei (pellet) from cytoplasmic fraction (supernatant). To isolate nuclear protein extracts, the pellets were resuspended in 80 µL of hypertonic buffer (10 mM HEPES pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 10 mM KCl, 25% glycerol, 1 mM dithiothreitol, 1mM phenylmethylsulphonyl fluoride and 1 mM sodium orthovanadate) and incubated on ice for 40 minutes. Finally, the samples were centrifuged at 14,000 xg, 4C, for 10 minutes and the supernatant containing soluble nuclear proteins was collected .

Immunoblotting

Protein lysates were prepared using RIPA buffer plus protease and phosphatase inhibitors (1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NaF, and 30 µL/mL aprotinin) and clarified by centrifugation. Protein concentrations were determined using Bio-Rad DC™ Protein Assay (#500-0111) and denatured in 2X Laemli buffer containing 10% of 2-Mercaptoethanol, and heated at 95°C for 5 minutes. The proteins (20-30 µg) were electrophoresed using 8-12% SDS-PAGE and transferred onto nitrocellulose membranes (Hybond-ECL, GE Healthcare Lifesciences). Primary antibodies were used at 1:1000 dilutions an incubated overnight at 4°C following the manufacture instructions. Afterward, membranes were washed and incubated with HRP-conjugated secondary at 1:3000 in TBS-T with 5% BSA. The bands were detected using a CCD camera (*GE* ImageQuant LAS 4000) by adding SuperSignal® West Pico chemiluminescent substrate (*Thermo Scientific*). Anti-NRF2 (#8882), anti-AKT (#9272), anti-ERK1/2 (#9102), anti-phospho-ERK1/2 (Thr202/Tyr204) (#9101) primary antibodies were from Cell Signaling Technology®. Anti-β-actin (A1978) was purchased from Sigma-Aldrich®. Anti-phospho-AKT 1/2/3 (Thr308) (sc-16646-R) was obtained from Santa Cruz Biotechnology®.

Electro-mobility shift assay (EMSA)

To evaluate NRF2 DNA-binding activity, nuclear extracts were incubated with the consensus Antioxidant Response Element (ARE) double-strand sequence 5' ACTGAGGGTGACTCAGCAAAATC 3' previously labeled with biotin-11-UTP using the *Pierce™ Biotin 3' End DNA Labeling Kit*. Binding reaction was carried out according to the *LightShift™ Chemiluminescent EMSA Kit* protocol. The procedure consisted on mixing 5 µg of nuclear proteins with 1× binding buffer (10 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT), 50 ng/µL of Poly (dI-dC) and 1 µM of Biotin End-Labeled Target ARE oligonucleotides, and then incubate for

30 min at room temperature. After adding 5 µL of 5× Loading Buffer, the samples were loaded onto 6% non-denaturing polyacrylamide gels end electrophoresed in 0.5× TBE buffer at 100V. DNA complexes were then transferred to nylon membranes (100V for 30 minutes) immersed in cooled 0.5× TBE buffer using a Bio-Rad Trans-blot® cell. Immediately, the transferred DNA was cross-linked by exposure to UV light for 10 min. DNA-protein shifted complexes were blocked, then incubated with HRP-streptavidin conjugates and chemiluminescent bands were detected by in a CCD camera (GE ImageQuant LAS 4000).

Reporter gene (luciferase) assay

Cignal Antioxidant Response Reporter kit (Qiagen) was used to measure Antioxidant Response Elements (ARE) activation by active NRF2. SH-SY5Y cells plated in 96-well plates were transfected with a mixture of ARE-driven firefly luciferase and constitutive Renilla-luciferase constructs (40:1, 100 ng reporter mixture) by using Lipofectamine 3000 (*Life Technologies*) 24 h prior treatments. At the end of treatments, the cells were lysed and luciferase activity was assessed using the Dual-Luciferase® Reporter Assay (Promega). Transfection efficiency was ensured by transfecting the monster GFP and firefly luciferase constitutively expressed encoding sequence as the kit positive control (PC). Transfection of non-inducible firefly luciferase sequence was used as negative control (NC).

Intracellular reactive species production (DCF assay)

Intracellular ROS production was detected using the 2′,7′-Dichlorofluorescin (DCFH-DA, Sigma) molecule as described [19]. DCFH-DA stock solution was dissolved in DMSO at final concentration of 10 mM and stored at -20°C protected from light. Before cells were treated, DCFH-DA was diluted to100 µM into a 1% FBS supplemented medium solution, where cells remained incubated at 37°C, with 5% CO_2 and protected from light exposure for 1hour. After DCFH internalization, the medium were replaced by fresh medium and treatments were added. When internalized, RS cause DCFH oxidation and it becomes a fluorophore (DCF), which was quantified by SpectraMAX i3 (*Molecular Devices*) fluorescence plate reader (Ex/Em = 485/532 nm) or by flow cytometry (FL1-H channel, BD FACSCalibur).

Cell viability assays

Sulforhodamine B assay: The percentage of viable cells was estimated by colorimetric quantification of sulforhodamine B (SRB) stained cells [20]. The protocol consisted on fixating adhered cells with 10% trichloroacetic acid (TCA) for one hour at 4°C. Next, the precipitated proteins were stained by incubating a solution of 0.4% (w/v) SRB dissolved in 1% acetic acid for 15 minutes at room temperature. After, the exceeding unbound dye was removed by washing with 1% acetic acid (3-times), stained cells were solubilized in a 10 mM Tris base solution (pH 10.5). Absorbance was quantified using SpectraMAX i3 (*Molecular Devices*) spectrophotometer set at 515 nm.

LDH activity assay: The leakage of lactate dehydrogenase (LDH) into the culture medium was assessed as a measure of losses of cell membrane integrity typical of dead cells. LDH activity was determined by quantifying NADH decay through the conversion of pyruvate in lactate using a commercial kit (Labtest® Brazil).

Propidium Iodide (PI) uptake: After treatments, 6 µM PI was added to culture medium and incubated for 1 hour in a humidified incubator. Images were taken on a Nikon Eclipse TE 300 inverted microscope setup with a rhodamine filter.

siRNA transfection

Human NRF2 (AM16708 Silencer®) and scramble control (AM4635 Silencer®) small-interfering RNA (siRNA) were purchased from Ambion®. Reverse transfection of siRNA's were performed following the Lipofectamine® RNAiMAX (*Life Technologies)* transfection agent protocol using 40 nM of RNA final concentration. Treatments were performed 48 hours after transfection. Protein knockdown was confirmed by immunoblot.

Plasmid Construct Overexpression

The pcDNA3-EGFP-C4-NRF2 (plasmid: 21549, developed by Dr. Xiong lab (29)) and pcDNA3 empty vectors were from Addgene. Cells were transfected with Lipofectamine®-3000 (*Invitrogen*) for 24 hours, and re-plated before treatments. The pCDNA3-EGFP construct was used as a transfection efficiency control. Protein overexpression was confirmed by immunoblot.

Protein quantification

Samples total protein content was quantified using Bio-Rad DC™ Protein Assay.

Statistical analyses

Statistical tests were made using GraphPad Prism 5 software. One-way ANOVA followed by Tukey test was applied to compare more than two groups. Two-way ANOVA followed by Bonferroni post-hoc test was applied to compare multiple groups (two parameters). $P < 0.05$ was considered

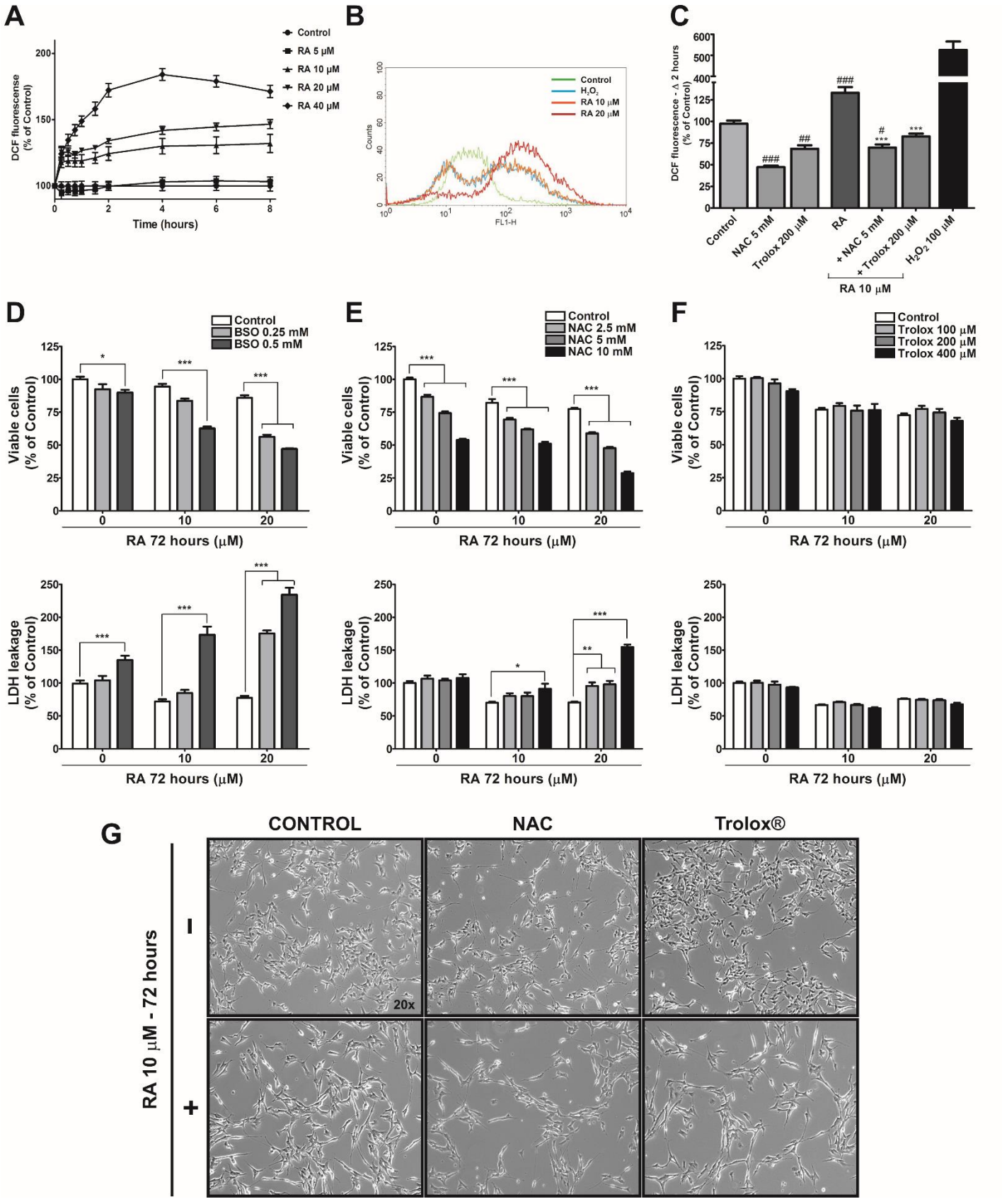

Fig. 1. *N-acetyl-cysteine pre-incubation induce RA toxicity.* (A) DCF fluorescence monitoring for 8 hours after SH-SY5Y cells treatment with increasing doses of RA. (B) Flow cytometry histogram of cells treated for 30 minutes with different doses RA and 200 µM H_2O_2 as positive control. X-axis indicates DCF fluorescence and Y-axis indicates number of cells. (C) DCF fluorescence increase in a 2 hours interval (Δ 2 hours). Cells were pre-incubated for 2 hours with NAC and Trolox® before 10 µM RA was added and fluorescence started to be monitored. 200 µM H_2O_2 fluorescence (Δ 30 min.) was used as positive control. # = different from Control and * = different from RA. (D) Percentage of viable cells and LDH leakage after 72 hours of BSO, (E) NAC and (F) Trolox® treatment combined with RA. (G) Phase-contrast picture of RA-treated cells in the presence of NAC (5 mM) or Trolox (200 µM).

statistically significant. Results are expressed by means ± SEM. * = P<0.05; ** = P<0.01; *** = P<0.001.

Results

N-acetyl-cysteine pre-incubation induce RA toxicity

Initially, we demonstrated that RA treatment was able to increase DCFH oxidation in a dose dependent manner from 15 min to 8 hours, suggesting a rapid production of reactive species (Fig 1A-B). To further experiments, we used only 10 and 20 µM doses because higher RA concentrations presented elevated toxicity (data not shown). To induce a pro-oxidant state by glutathione depletion [21], we pre-incubate low doses of BSO (0.25-0.5 mM) for 12 hours before adding RA. After 72 hours, BSO presence increased RA toxicity, reaching almost 50% less viable cells (Fig. 1D). We also pre-treated SH-SY5Y cells with two different antioxidants - the GSH precursor NAC and the GSH-unrelated vitamin E analogue Trolox® - and evaluated its effects on cell viability. Interestingly, N-acetyl-cysteine pre-treatment led to an increased sensitivity to RA instead of attenuating it, causing cell death especially when combined with 20 µM RA (Fig. 1E). In addition, NAC alone decreased the proportion of viable cells but did not increase LDH leakage (Fig. 1E – first group) meaning that it reduced cell proliferation rate without inducing cell death (see Fig. 1G). None of these effects were detected using Trolox® as antioxidant (Fig. 1F-G), even though both attenuated RS production as assessed by DCF assay (Fig. 1C). These results suggest that simple interference with general RS production is not sufficient to alter cells phenotype, yet the results with NAC and BSO suggest that a fine control of GSH/thiol homeostasis is required for cell resistance to RA.

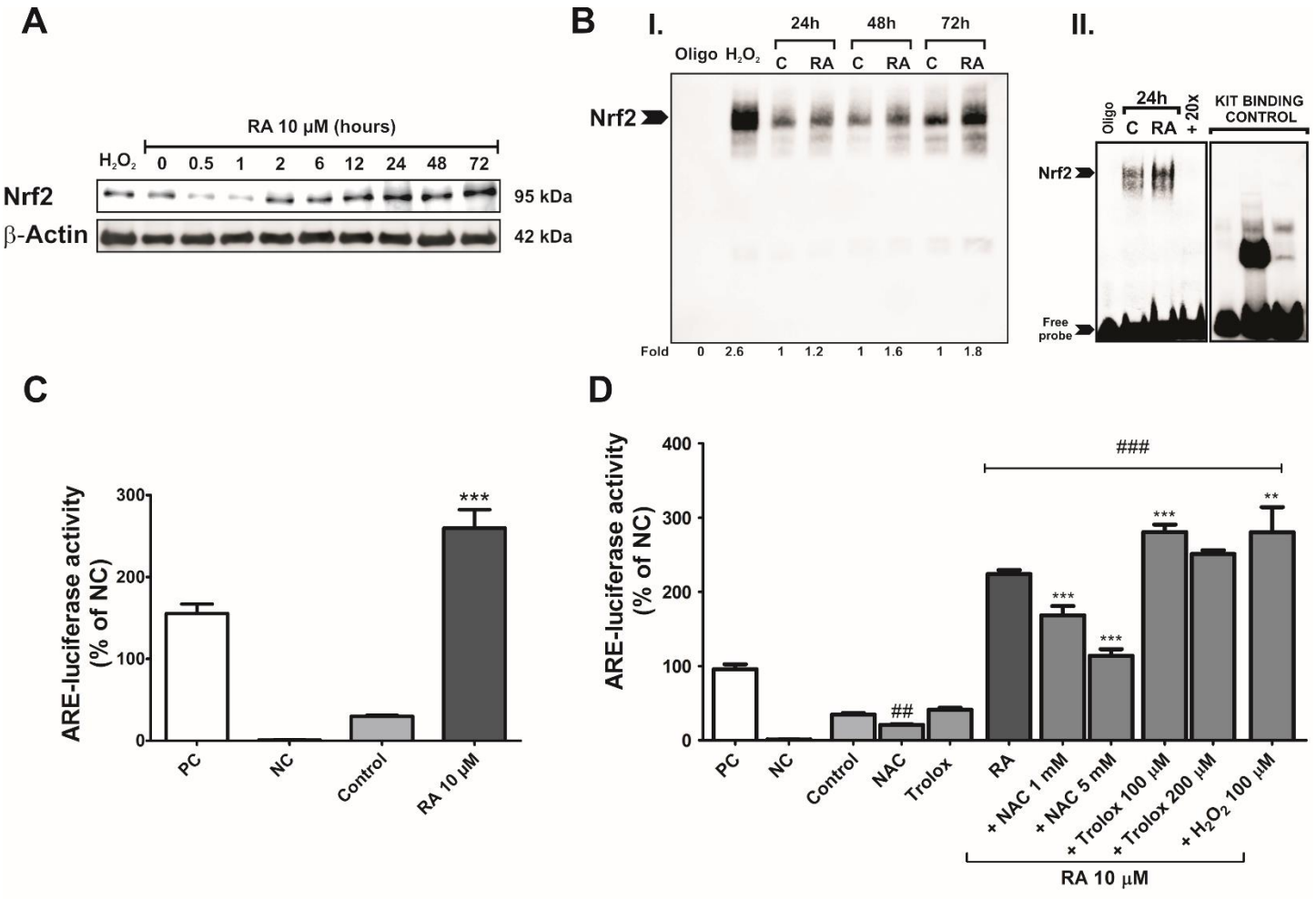

Fig. 2. *NAC, but not Trolox®, attenuates NRF2 activation by RA.* (A) Time-response curve of NRF2 immunocontent through 10 µM RA exposure. 100 µM H_2O_2 treatment was used as positive control and β-actin as loading control. (B) I. EMSA of SH-SY5Y cells nuclear extracts after 10 µM RA treatment. 200 µM H_2O_2 treatment was used as positive control. II. Binding reactions controls: *Oligo* group represents no-sample control. *20*× group represents competition control by adding 20× non-labeled ARE sequence to binding reaction, indicating no biotin interference. *Kit binding control* represents the reactions made using EBNA-DNA and nuclear extract system provided by the manufacturer. It indicates reactions and non-denaturing proper conditions. (C) ARE-luciferase activity after 24 hours of RA treatment and (D) combined with NAC and Trolox® pre-incubation. # = different from Control and * = different from RA.

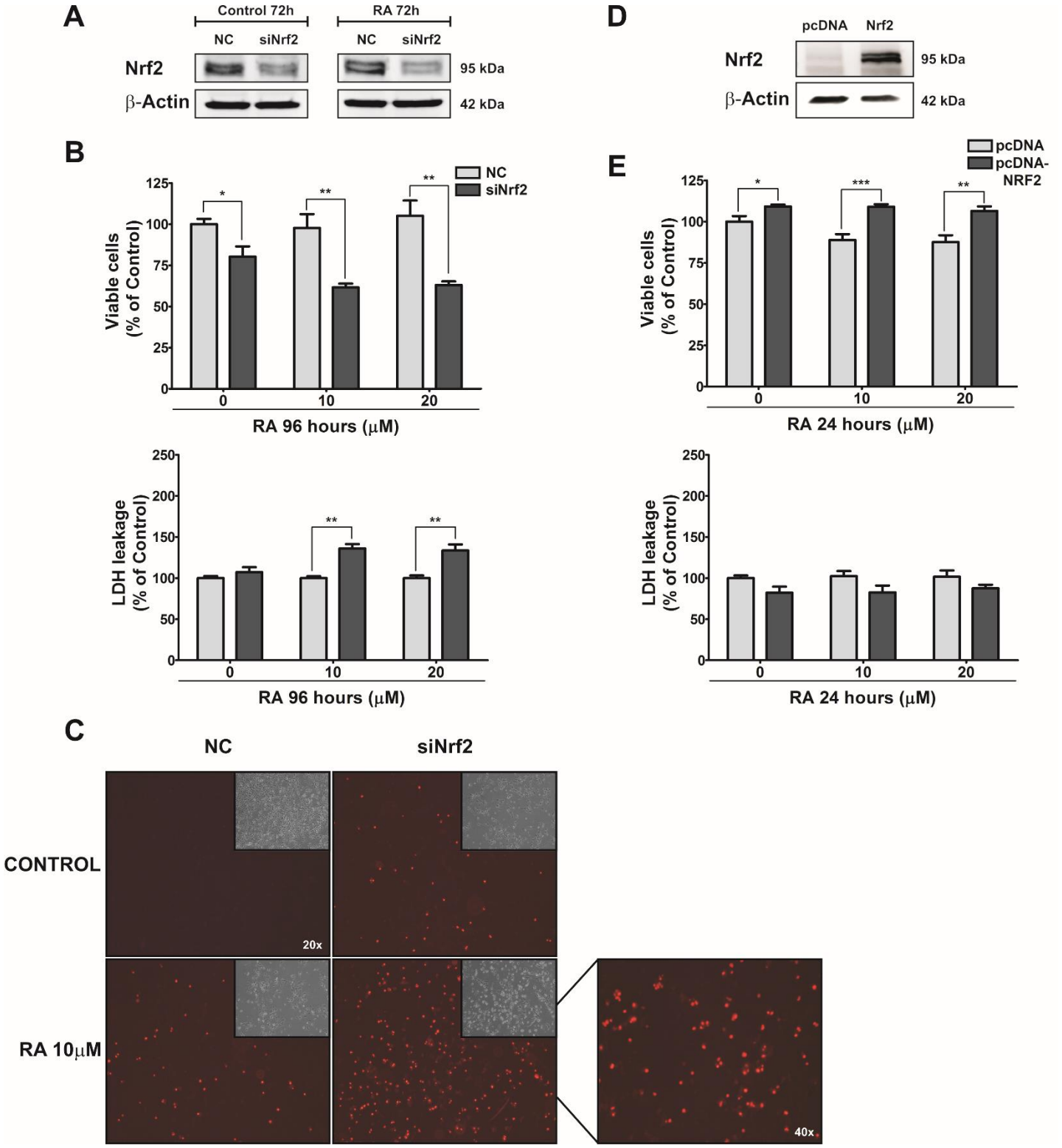

Fig. 3. *NRF2 activation importance on SH-SY5Y cells proliferation and survival.* (A) Western blot indicating NRF2 immunocontent after siNRF2 and scramble (NC) transfection in SH-SY5Y cells.10 μM RA was added 48 hours after transfection. (B) Percentage of viable cells and LDH leakage 96 hours after RA treatment in silenced cells. (C) PI uptake images of NRF2 knockdown cells combined with 10 μM RA. (D) NRF2 immunocontent 24 hours after pcDNA3-EGFP-C4-NRF2 and pcDNA3 empty vectors transfection. (E) Percentage of viable cells and LDH leakage after 24 hours RA treatment in NRF2 overexpressed cells.

NAC, but not Trolox®, attenuates NRF2 activation by RA

Previous studies demonstrated in SH-SY5Y cells that diamide, a sulfhydryl oxidant, induces NRF2 activation preventing its toxicity [22]. In addition, it has been already reported that a 10 μM dose of RA can activate this pathway to induce NQO1 and NF-M genes [18]. Considering these observations, we focus to further investigate NRF2 activation mechanism. We first validated that RA was able to increase NRF2 protein content after 2 hour, which lasted

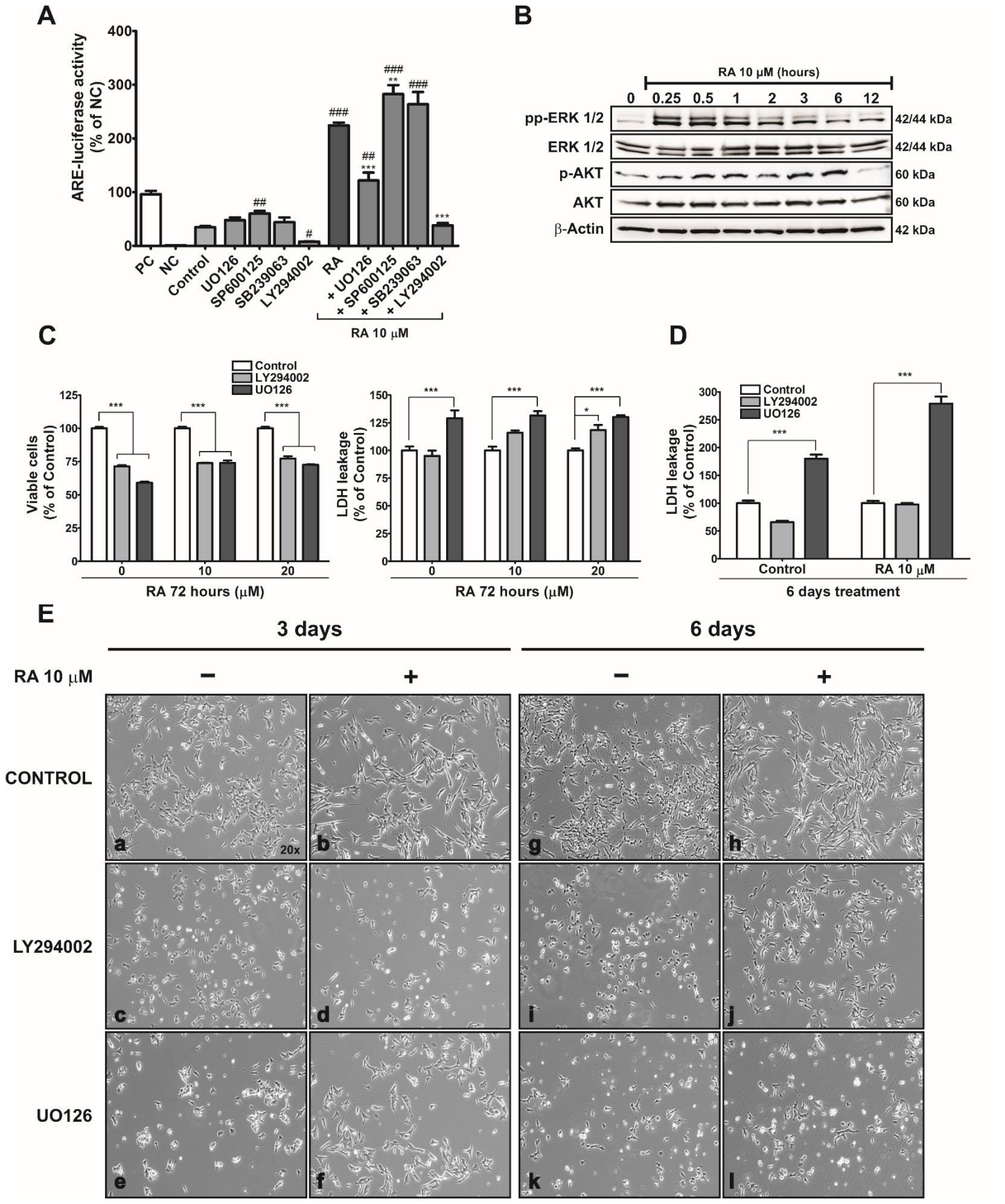

Fig. 4. *RA actions upon ERK1/2 and PI3K regulate Nrf2 activation, cell survival and neurite formation in SH-SY5Y cells.* (A) ARE-luciferase activity 24 hours after RA treatment combined with pharmacological inhibitors to MEK/ERK1/2 (UO126), p38^{MAPK} (SB203580), JNK1/2 (SP600125) and PI3K/Akt (LY294002) pathways. # = different from Control and * = different from RA. (B) Western blot representing time-response curve of ERK1/2 and AKT phosphorylation mediated by 10 μM RA treatment. (C) Percentage of viable cells and LDH leakage after 72 hours of RA treatment combined with LY294002 and UO126. (D) LDH leakage after 6 days of RA-induced differentiation in the presence of LY294002 and UO126. (E) Phase-contrast pictures of RA treated SH-SY5Y cells, co-treated with LY294002 and UO126 for 3 and 6 days.

up to 72 hours (Fig. 2A). EMSA experiments confirmed NRF2 translocation to the nucleus in its active conformation and able to bind ARE consensus sequence after RA treatment (Fig. 2B). We also determined the transcriptional activity of RA-induced NRF2 by reporter gene assay. This approach revealed to be more sensitive to measure NRF2 activation, showing a 10-fold increase on RA group compared to control group after 24 hours (Fig 2C). To evaluate the effects of RS depletion on NRF2 activation, cells were pre-incubated with NAC or Trolox® two hours before adding 10 µM RA. After 24 hours treatment, only NAC attenuated NRF2 binding, reducing luciferase luminescence up to 50% when compared to RA alone (Fig. 2D). Surprisingly, Trolox® treatment combined with RA had higher luminescence than RA only (Fig. 2D). It is worth noticing that SH-SY5Y cells presented a basal NRF2 activation (see basal ARE-luciferase, EMSA and immunoblot bands in untreated samples) that was also modulated by NAC (34.67 ± 1.98 vs. 20.77 ± 0.75, p = 0.0022) suggesting that this mechanism is present in proliferative cells and not only induced by RA.

NRF2 activation is important to SH-SY5Y cells proliferation and survival.

Considering that NAC increased cell sensitivity to RA treatment and concomitantly attenuated NRF2 activation, we analyzed the effects of NRF2 knockdown and overexpression on SH-SY5Y cells viability. Fig. 3A western blot shows that NRF2 immunocontent remained reduced even after 72 hours RA treatment in NRF2 silenced cells. NRF2 knockdown by siRNAs led to a higher sensibility to RA exposure as determined after 96 hours of treatment (fig.3B). PI uptake clearly shows the increased cell death and number of apoptotic bodies (see 40X magnification images), especially in the RA+NRF2 siRNA combinations (Fig. 3C). Interestingly, NRF2 knockdown alone diminished the proliferation rate when compared to scrambled siRNA negative controls (Fig. 3B – first group), similar to the effect observed in NAC treated cells.

In a second approach, the consequences of overexpressing NRF2 (using the pcDNA3-EGFP-C4-NRF2 construct) upon the toxicity of RA in SH-SY5Y cells were evaluated. Fig. 3D shows NRF2 overexpression validation in SH-SY5Y 24 h post transfection. Opposite to the effect of NRF2 knockdown, cells transfected with pcDNA-NRF2 alone had a higher proliferation rate compared to empty-pcDNA transfected cells (Fig. 3E – first group). In the presence of RA, NRF2 overexpression seems to attenuate RA-induced cell number decreases (Fig. 3E). No significant difference on LDH leakage was detected on this experiment as expected (Fig. 3E).

Rapid non-genomic actions of RA upon ERK1/2 and PI3K regulate NRF2 activation, cell survival and neurite formation in SH-SY5Y cells.

It has been amply demonstrated in literature that some protein kinases such as MAPKs, PI3K, PKC and GSK3β pathways can regulate NRF2 activation in different cellular contexts [15;22]. While RA genomic actions take several hours or even days to alter cell proteome and phenotype, rapid and non-genomic actions of retinoids upon protein kinases phosphorylation have been described by our group and others [23;24]. Given the rapid induction of NRF2 by RA observed in Fig. 2A immunoblots, we sought to investigate some candidate protein kinases modulating NRF2 pathway. To this end, the cells were pre-incubated with specific pharmacological inhibitors of MEK/ERK1/2 (UO126), p38^{MAPK} (SB203580), JNK1/2 (SP600125) and PI3K/Akt (LY294002) before adding RA. Inhibition of MEK/ERK pathway by UO126 led to a 50% decrease in ARE reporter gene activity when compared to RA alone (Fig. 4A). The p38 and JNK1/2 inhibitors exerted no effects upon NRF2 reporter gene activity (fig.4A). In addition, the PI3K inhibitor also reduced NRF2 activation by RA to control levels. In the absence of RA, the inhibition of PI3K/AKT pathway, but not MEK-ERK1/2, caused a 5-fold decrease in basal NRF2 activity (Fig.4A). Fig. 4B immunoblots confirmed that RA treatment rapidly activated ERK1/2 and AKT pathways, agreeing with previous works [25].

Long-term blocking of MEK-ERK1/2 with UO126 (5 to 20 µM) caused cytoxicity irrespective of the presence of RA. After 72 hours, cells that received UO126 had higher mortality in all groups (Fig 4C). It is worth noticing that surviving cells showed no neurite projections even in presence of RA (Fig 4E – compare pic. b to f and). On the other hand, LY294002 (20 µM) showed no significant toxicity

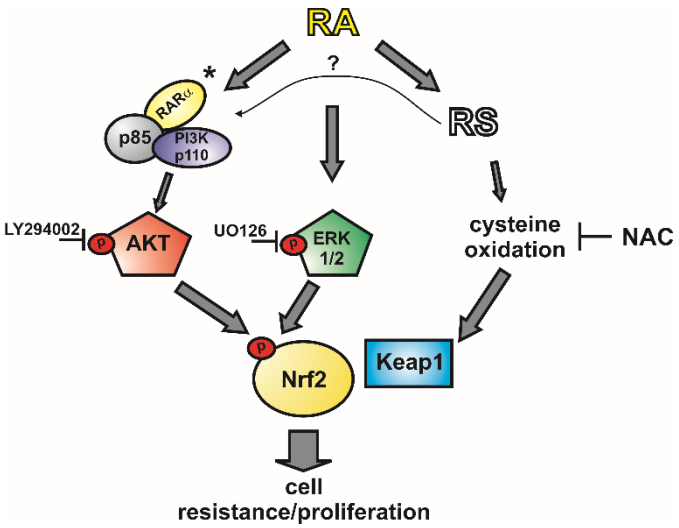

Fig. 5. Schematic representation of RA-induced NRF2 activation in SH-SY5Y cells. * Masiá, S. *et al.*, 2007

alone or with 10 µM RA given that LY294002- treated cells showed about 30% of reduction on the percentage of viable cells without detectable LDH leakage. When combined with 20 µM RA, a significant increase in LDH leakage was observed (Fig. 4C). These results indicate that PI3K inhibition caused a reduction on cell proliferation and diminished their resistance to RA. Furthermore, LY inhibited any visible morphogenetic effect caused by RA (Fig 4E – compare pic. b to d and h to j neuritis formation). Similar results were obtained when taking treatment up to 6 days. Neuritogenesis could not be evaluated in UO126 treatments due to increased death (Fig. 4D).

Discussion

In our study, we demonstrated that a non-genomic effect of RA upon ERK1/2 and AKT pathways promotes NRF2 pathway activation and a survival strategy to counteract RA toxicity in the model of RA-induced differentiation in SH-SY5Y neuroblastoma cells. The protective role of NRF2 against xenobiotic toxicity has been broadly explored in literature both in the context of chemoresistance as well as chemical toxicology [13;16;17]. In fact, it has been demonstrated, *in vivo* and *in vitro*, that RA toxicity in hepatocytes is counteracted by glutathione up regulation mediated by NRF2 [26]. In our model, BSO experiments showed that GSH content was pivotal to cell survival in RA treatment. On the other hand, even knowing that the glutathione synthesis genes GCLC and GCLM are typically regulated by NRF2-ARE binding [13], the lack of protection observed with NAC (a GSH precursor that impeded NRF2 activation by RA) supports that likely other NRF2 targets are contributing to NRF2-mediated RA resistance in SH-SY5Y. As potential candidates, our recent work showed that other NRF2 targets such as *TXN* (thioredoxin-1) and *TXNRD1* (thioredoxin reductase-1) have their expression up regulated by RA [10]. In addition, Trolox® - which we herein found to stimulate NRF2 - potentiated RA-induced expression of these targets [10]. Thus, the individual contribution of these various NRF2 targets on RA resistance requires further investigation.

RA therapy has been extensively used as a tumor/growth suppressor treatment for different types of cancer, especially for its anti-proliferative effect [27]. Recently, some authors have discussed that NRF2 activation can promote anabolic metabolism in cancer cells accelerating its proliferation [28;29]. In agreement, our data showed that NRF2 overexpression limited anti-proliferative effects of RA in human neuroblastoma cells (Fig. 3F) and favored cell proliferation instead. Corroborating, the opposite effect was observed through NRF2 knockdown and NAC treatment, which contributed to RA anti-proliferative effects. Although a previous study argued that increased NRF2 activation benefits RA induction of neurite outgrowth [18], our results points that NRF2 ensures a better survival to cellular stresses caused by RA exposure. This could indirectly improve cell survival along the differentiation protocol, instead of acting as a direct promoter of neuronal differentiation genes. Taking the SH-SY5Y model in cancer perspective, one of major concerns about retinoid therapies is that they frequently fail to inhibit cell growth and, instead, promote cancer cells survival [30]. In this context, neuroblastoma patients can rapidly become resistant to RA effects, thus limiting its therapeutic efficacy [4]. In agreement, our previous data [10] together with other authors [11] show that RA-differentiated SH-SY5Y cells exhibit expression of different antioxidant enzymes (ex.: Catalase and SOD2) which confer resistance to oxidative treatments when compared to parental undifferentiated cells [12]. Additionally, our data exposed a significant correlation between NRF2 activity and neuroblastoma proliferation. Thus, the NRF2 pathway could be considered as a potential target to circumvent the cases of neuroblastoma low response to RA differentiation chemotherapy.

With regard to antioxidants biology, our results demonstrated that NRF2 activation was not generally sensible to RS depletion by Trolox®, but specific to NAC treatment. NAC acts directly as a thiol antioxidant, either by reducing cysteine groups with proteins and small peptides or by acting as a GSH precursor, which can directly or enzymatically lead to RS detoxification, especially hydrogen peroxide. NAC probably inhibits NRF2 responses both by keeping specific cysteine residues with Keap1 in the reduced form or/and by providing an overall thiol reducing cellular environment which impedes activation of thiol oxidation sensitive proteins such as the NRF2-Keap1 complex [15]. On the other hand, other classes of antioxidants like tocopherols can actually induce NRF2 activation by antagonizing NRF2 interaction with its negative regulators or because they can increase oxidative stress during their metabolism [31]; which could explain the higher NRF2 activation in the presence of Trolox® (Fig. 2D).

It has been demonstrated that RA activates ERK1/2 and PI3K/AKT in neuroblastoma cells [25][32]. In addition, it has been reported that ERK phosphorylation is required to activate NRF2 in SH-SY5Y cells treated with other types of oxidants [22]. Here we demonstrated that both pathways have a role to induce NRF2 protein activation in response to RA treatment; p38 and JNK1/2 were irrelevant to this phenomenon. PI3K has an oxidative interface that can lead to its activation [33], but it is also modulated by RA binding-mediated RAR receptor interaction with PI3K subunits through a non-genomic action [34]. Noteworthy, this RA effect occurred at doses (1 µM RA) not enough to induce a significant increase in RS production, suggesting that, in fact, PI3K/Akt activation is redox independent. Either way, this pathway is extremely necessary to induce RA morphogenetic responses [25] and confers to RA treated

cells better resistance to oxidative toxins [12]. In line with previous studies, even though the PI3K/Akt pathway has a myriad of cellular downstream targets, the PI3K-NRF2 axis herein elucidated seems to be committed to warrant cell survival and morphogenesis during RA-induced differentiation. On the other hand, the ERK1/2-pathway showed to be key for basal rates of SH-SY5Y survival, while the RA-induced ERK1/2-NRF2 activation probably sustains cell adaptation RA-induced xenobiotic stress. Although we believe that further studies are requested to better understand the mechanisms elucidated thus far, our current hypothesis is represented in Fig 5.

Conclusions

Despite the growing number of literature pointing to NRF2 roles in a variety of physiological and pathological responses to xenobiotics and oxidants in different tissues [16;35;36], we could only find one study that discuss NRF2 functions in the context of RA treated neuroblastoma [18] besides our previous publication [10]. Even though retinoid differentiation therapy approach has been used for more than 30 years to treat neuroblastomas [37]. We herein depicted three major factors that are related to NRF2 activation under this context: (1) a rapid non-genomic action of RA upon PI3K/Akt and ERK1/2 pathways leading to NRF2 activation; (2) a role of NRF2 in the control of cell proliferation and resistance to cytotoxic levels of RA; (3) the secondary relevance of RA-induced RS in activating NRF2, but the differential roles of thiol and tocopherol related antioxidants in sustaining or blocking it. In the light of these observations, we understand that more in-depth investigation is needed to elucidate the real contribution of these mechanisms in the context of chemotherapeutic responses as well as neuronal differentiation protocols in vitro and in vivo.

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