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Mitochondrial permeabilization without caspase activation mediates the increase of basal apoptosis in cells lacking Nrf2

Julia Ariza¹, José A. González-Reyes¹, Laura Jódar¹, Alberto Díaz-Ruiz², Rafael de Cabo^{2,*}, and José Manuel Villalba^{1,*}

¹Departamento de Biología Celular, Fisiología e Inmunología, Universidad de Córdoba, Campus de Excelencia Internacional Agroalimentario, ceiA3, Spain

²Translational Gerontology Branch, National Institute on Aging, National Institutes of Health, Baltimore, MD, USA

Abstract

Nuclear factor E2-related factor-2 (Nrf2) is a cap'n'collar/basic leucine zipper (b-ZIP) transcription factor which acts as sensor of oxidative and electrophilic stress. Low levels of Nrf2 predispose cells to chemical carcinogenesis but a dark side of Nrf2 function also exists because its unrestrained activation may allow the survival of potentially dangerous damaged cells. Since Nrf2 inhibition may be of therapeutic interest in cancer, and a decrease of Nrf2 activity may be related with degenerative changes associated with aging, it is important to investigate how the lack of Nrf2 function activates molecular mechanisms mediating cell death. Murine Embryonic Fibroblasts (MEFs) bearing a Nrf2 deletion (Nrf2KO) displayed diminished cellular growth rate and shortened lifespan compared with wild-type MEFs. Basal rates of DNA fragmentation and histone H2A.X phosphorylation were higher in Nrf2KO MEFs, although steady-state levels of reactive oxygen species were not significantly increased. Enhanced rates of apoptotic DNA fragmentation were confirmed in liver and lung tissues from Nrf2KO mice. Apoptosis in Nrf2KO MEFs was associated with a decrease of Bcl-2 but not Bax levels, and with the release of the mitochondrial pro-apoptotic factors cytochrome *c* and AIF. Procaspase-9 and Apaf-1 were also increased in Nrf2KO MEFs but caspase-3 was not activated. Inhibition of XIAP increased death in Nrf2KO but not in wild-type MEFs. Mitochondrial ultrastructure was also altered in Nrf2KO MEFs. Our results support that Nrf2 deletion produces mitochondrial dysfunction associated with mitochondrial permeabilization, increasing basal apoptosis through a caspase-independent and AIF-dependent pathway.

Graphical Abstract

*Co-corresponding authors.

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1. Introduction

A range of factors which include endogenous metabolic reactions such as those related to aerobic respiration, and also exogenous agents as ionizing radiations, chemicals, xenobiotics and drugs can produce reactive oxygen/nitrogen species (ROS/RNS) and oxidative stress, an unbalanced situation between prooxidants and antioxidants leading to potential damage [1]. Although ROS/RNS can damage cellular proteins, lipids, and DNA when produced in excess [2], at physiological levels they also play a role in regulating cellular processes such as those controlling cell growth and apoptosis [3].

Nuclear factor E2-related factor-2 (Nrf2) [4] is a cap'n'collar/basic leucine zipper (b-ZIP) transcription factor [5] which acts as cellular sensor of oxidative and electrophilic stress. Kelch-like ECH-associated protein1 (Keap1), also designated as “inhibitor of Nrf2” (iNrf2), is a cytosolic inhibitor of Nrf2-mediated gene expression. Keap1 binds Nrf2 and represses nuclear activation of the antioxidant response element (ARE) signaling pathway by retaining Nrf2 in the cytosol and preventing its nuclear shuttling mechanism, which facilitates Nrf2 degradation [6].

Nrf2 and its downstream target genes are key regulators for protection against oxidative stress-related damage because Nrf2 activation under these conditions leads to the induction of phase II detoxifying enzymes and other anti-oxidative stress genes [7]. Physiological activation and termination of Nrf2-dependent pathways protects cells from harmful toxic species and has a positive impact for the organisms. In this sense, Nrf2 confers a protection against hepatotoxicity caused by a range of hepatotoxicants due to its antioxidant and cytoprotective effect [8]. Furthermore, Nrf2 induction seems to protect cells from cigarette smoke whereas its ablation in Nrf2 knockout (Nrf2KO) mice renders the cells more sensitive to oxidative stress and DNA damage [9]. In accordance, Nrf2KO mice have impaired induction of several phase II detoxifying enzymes [10] and are more sensitive to cytotoxic electrophiles when compared with wild-type mice. A physiological decline in Nrf2 function has been also observed during aging, both in organisms [11] and in cellular systems *in vitro* [12], which may be related with the functional impairment that is associated with advanced age. Increased oxidative stress and DNA damage due to the loss of Nrf2 has been also related with the initiation of cellular transformation in prostate cancer [13], and low levels of

Nrf2 activity predispose cells to chemical carcinogenesis, most likely due to poor detoxification. Of note, Nrf2KO mice are not protected by calorie restriction against carcinogenesis [14]. Moreover, a regulatory single nucleotide polymorphism residing in the NRF2 gene which results in decreased gene expression is linked to human lung cancer susceptibility [15] and conversely, upregulation of Nrf2-mediated signaling protects against different types of cancer [16–19]. Thus, Nrf2 has been recognized as a tumor suppressor gene [20] and activation of Nrf2-dependent signaling may constitute a valuable therapeutic strategy to treat oxidative stress-induced diseases, including acute lung injury, chronic obstructive pulmonary diseases, diabetic nephropathy, heart failure and cancer [21, 22].

However, a dark side of Nrf2 function also exists because its unrestrained activation may allow the survival of potentially dangerous damaged cells leading to oncogenesis and drug resistance. This specially applies to tumor cells containing loss-of-function mutations in the KEAP1 gene, as found in several lung cancer cells, that abolish proper interaction of the Keap1 protein with Nrf2 leading to persistent nuclear accumulation of this transcription factor, thus activating antioxidant and anti-apoptotic gene expression and favoring drug resistance [21, 23]. Therefore, Nrf2 has been also considered as a proto-oncogene and a target for pharmacological inhibition in cancer, since this inhibition would favor cell growth arrest and sensitize cancer cells to apoptosis [23].

Because a decrease of Nrf2 activity may be related with degenerative changes associated with aging, and the inhibition of Nrf2 signaling may be of therapeutic interest in cancer, it is important to investigate the relationship between Nrf2 and the mechanisms that regulate cell death. In this sense, previous investigations have demonstrated that Nrf2 exerts a direct transcriptional control of the anti-apoptotic genes Bcl-2 and Bcl-xL which contain an ARE in their promoter regions [24, 25]. In accordance, activation of Nrf2-dependent transcription results in drug resistance and cell survival while its inhibition is linked to the potentiation of etoposide and radiation-induced apoptosis in mouse and human hepatic tumor cells [24]. H₂S-mediated stabilization of nuclear Nrf2 also increased the levels of antiapoptotic proteins Bcl-2 and Bcl-xL and resulted in cardioprotective effects in mice subjected to myocardial ischemia/reperfusion [26]. In the context of aging, it has been shown that the impact of Nrf2 deletion on skeletal muscle is increased in aged animals, resulting in the increases of caspases-3 and -9, Bax, Bad, apoptosis inducing factor (AIF) and apoptosis signal-regulating kinase 1 (ASK1) levels [27]. However, whether or not the lack of Nrf2 actually increases basal rates of apoptosis has not been addressed to date. Moreover, the mechanisms involved in cell death activation in cells lacking Nrf2 are far to be fully understood.

Previous results obtained in our group showed that Murine Embryonic Fibroblasts (MEFs) isolated from Nrf2KO mice displayed diminished cellular growth rate and shortened lifespan compared with their wild-type counterparts [28]. The aim of our work was to elucidate if the lack of Nrf2 increases basal rates of apoptosis in MEFs and tissues derived from Nrf2KO mice, and to investigate how the mechanisms involved in the regulation of apoptotic signaling are altered in cells lacking Nrf2.

2. Materials and Methods

Colonies of wild-type and Nrf2 knockout mice in CD1 and C57BL6 backgrounds were maintained at the Laboratory of Experimental Gerontology (NIA, NIH, Baltimore, USA). Mice were housed four per cage in a room maintained at a constant temperature (20°–22 °C) on a light:dark 12:12h schedule. For tissue collection, male C57BL6 mice were sacrificed at 5 months of age by cervical dislocation. All procedures involving laboratory mice were carried out according to animal protocols and the guidelines issued by the Intramural Research Program of the NIH.

2.1. MEFs preparation and culture

MEFs were obtained from fetuses at day 13 post-coitum. Pregnant female CD1 mice were sacrificed by cervical dislocation. The uterus was dissected and washed in Hank's saline solution (Sigma, Spain). Each embryo was separated from its placenta and surrounding membranes. Head and dark red organs were also removed. After washing, embryos were minced and the resulting cells washed in Hank's saline solution and resuspended in 1 ml of Trypsin-EDTA per embryo for 15 minutes at 37°C. Trypsin was neutralized with Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum, 10,000 units/ml penicillin, 10 mg/ml streptomycin, 25 µg/ml amphotericin B, 2 mM L-glutamine, and 0.2% glucose (MEFs medium). Non-dispersed tissue was removed, and the cellular suspension washed with 2 volumes of MEFs medium. After centrifugation, the cell pellet was resuspended in DMEM and cells were then plated in 10 cm diameter dishes. Fibroblasts are the only cells able to adhere to the culture surface. After 24 hours the medium was changed and after few days cellular confluence was obtained (passage 0 MEFs). Cells were then frozen and maintained under liquid nitrogen. After thawing, cells were cultured in MEFs medium at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Passages of MEFs were performed according to the procedure described by Todaro and Green for obtaining the 3T3 cell line [29]. Primary fibroblasts were cultured in 75 cm² flasks at 4,000 cells/cm² and passages were performed each 3 days, avoiding confluence. After each passage, cells were detached from culture flasks using a Trypsin-EDTA detaching solution (Sigma, Spain) and the number of viable cells assessed with a haemocytometer. Unless otherwise stated, all determinations were carried out from cells grown to about 80% confluency. Genotyping of MEFs used in this study was carried out by PCR amplification using the following oligonucleotides:

Nrf2: GTGACTCGGAAATGGAGGAG (forward) and GCGGCTTGAATGTTTGTCTT (reverse); Neomycin resistance cassette: GGAGAGGCTATTCGGCTATGAC (forward), CGCATTGCATCAGCCATGATGG (reverse) (see Supplemental Methods and Supplemental Figure 1).

2.2. Thymidine incorporation and MTT viability assays

MEFs were incubated with 0.25 µCi/ml [methyl-³H] thymidine (Amersham Pharmacia Biotech) for 24 hours and then washed with 0.9% NaCl and incubated with 5% trichloroacetic acid (Merck) for 30 minutes at 4°C. Supernatants were removed and cells were subsequently lysed with 1N NaOH (Panreac, Spain) for 90 minutes while shaken at

room temperature. One aliquot (400 μ l) of the lysates was added to 4 ml of scintillation cocktail (Cocktail Biogreen 1, Scharlau). The quantity of incorporated [methyl- 3 H] thymidine in the DNA was measured on a Beckman LS 6000TA scintillation beta-counter and then normalized to the quantity of protein using a procedure described by Stoscheck [30]. Results were expressed as cpm/ μ g protein.

In order to perform MTT viability assay, the medium was removed and the cells washed once with Hank's solution and then incubated for three hours at 37°C with 0.5 mg/ml MTT (Sigma) in DMEM supplemented with 10% bovine serum, 10,000 units/ml penicillin, 10 mg/ml streptomycin, 25 μ g/ml amphotericin B, 2 mM L-glutamine, and 0.2% glucose without phenol red. After incubation, the MTT solution was removed, the converted dye solubilized with acidic isopropanol (0.04 M HCl in absolute isopropanol), and the absorbance recorded at 570 nm in a Beckman DU-640 spectrophotometer.

2.3. Measurements of ROS levels in MEFs

The cell-permeable fluorogenic probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was used to measure the levels of intracellular peroxides. The probe (obtained from Merck) was freshly prepared in DMSO and added to the cells at a final concentration of 10 μ g/ml. MEFs were incubated with the probe for 20 minutes at 37°C. After incubation, cells were washed with Hank's saline solution to eliminate the excess of probe and then detached from culture plates using a Trypsin-EDTA solution. Cell pellets were washed twice with Hank's saline solution and resuspended in Hank's saline solution for peroxides measurements. After excitation at 488 nm, the fluorescence emission was registered at 525 nm (FL1). Specificity of the ROS signal was assessed by parallel determinations in the presence of the antioxidant N-acetylcysteine at 5 mM. The data were obtained in a logarithmic scale and were represented in histograms using the EXPO32 ADC Analysis software (Beckman-Coulter). Median data were used for quantification of the results.

2.4. Preparation of whole cell and tissue extracts

MEFs were separated from culture dishes using a trypsin-EDTA detaching solution. About $1-2 \times 10^6$ cells were recovered by centrifugation at $500 \times g$ for 5 min, washed twice with PBS (Sigma) and centrifuged under the same conditions. Subsequent extraction procedures were carried out at 4°C. Cells were disrupted by homogenization in 250 μ l of radioimmunoprecipitation assay (RIPA) buffer composed of 50 mM Tris-HCl pH 8, 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1 mM DTT, 1% Triton X-100, 1 mM phenylmethanesulfonylfluoride (PMSF), 20 μ g/ μ l of each of the following protease inhibitors: chymostatin, leupeptin, antipain and pepstatin A (CLAP), and phosphatase inhibitor cocktail 2 and 3 (Sigma) at 1:100 dilution. After gentle agitation, the suspension was centrifuged at $10,000 \times g$ for 15 minutes. Supernatants were saved for determinations of protein levels by Western blot (see below).

After euthanization of mice, the organs were collected and quickly frozen in liquid nitrogen. 50 mg of tissues were weighted and then lysed in RIPA buffer supplemented with protease and phosphatase inhibitor cocktails as indicated above for MEFs. A mechanical, motor-driven homogenizer (Ultra-Turrax T25, IKA, Staufen, Alemania) was used to aid in tissue

disruption. Lysates were centrifuged at $10,000 \times g$ for 15 minutes in a tabletop microfuge at 4°C . Supernatants were transferred to new vials and conserved at -80°C .

2.5. Preparation of cytosolic and nuclear extracts from MEFs

All extraction procedures were carried out at 4°C . About $2-10 \times 10^6$ cells were recovered by centrifugation at $500 \times g$ for 5 min and washed twice with cold PBS. Cell pellets were resuspended in a hypotonic buffer (10mM HEPES pH 7.9 containing 1.5mM MgCl_2 , 10mM KCl, 0.5mM DTT, 1 mM PMSF, 20 $\mu\text{g}/\text{ml}$ CLAP and phosphatase inhibitor cocktail 2 and 3 at a 1:100 dilution) and incubated for 15 minutes at 4°C . NP40 was then added to a final concentration of 0.5% and the samples were gently vortexed. Samples were centrifuged at $2,800 \times g$ in a tabletop microfuge to separate nuclei-enriched pellets from crude cytosolic supernatants. These supernatants were withdrawn and centrifuged again at $135,000 \times g$ in a Beckman Optima TLX Ultracentrifuge (Beckman Coulter) to obtain clean cytosolic fractions. The nuclei-enriched pellets were resuspended in a high-salt buffer (20 mM HEPES pH 7.9 containing 1.5 mM MgCl_2 , 420 mM NaCl, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM PMSF and 20 $\mu\text{g}/\text{ml}$ CLAP and phosphatase inhibitor cocktail 2 and 3 at a 1:100 dilution). After incubating the nuclei for 30 minutes with vigorous agitation, nuclear extracts were recovered after centrifugation for 10 minutes at $10,000 \times g$ in a tabletop microfuge.

2.6. Isolation of mitochondria-enriched fractions

About $5-10 \times 10^6$ cells were detached from culture flasks by using a trypsin-EDTA solution, washed twice with PBS and centrifuged at $500 \times g$. Subsequent procedures were carried out at 4°C . Cell pellets were resuspended in a hypoosmotic buffer (10 mM pH 6.7 Tris-Acetate, 10 mM KCl and 15 mM MgCl) supplemented with PMSF 10 $\mu\text{l}/\text{ml}$ for 15 minutes. Thereafter cells were homogenized in a Potter Elvehjem at maximum speed. After the homogenization the same quantity of hyperosmotic buffer (10 mM pH 6.7 Tris-Acetate, 10 mM KCl, 15 mM MgCl and 0.5 M sucrose) was added to restore osmolarity and the tubes were filled up to 10 ml with isosmotic buffer (1:1 hypoosmotic and hyperosmotic buffer) containing PMSF. Samples were centrifuged in a Beckman J2-21 centrifuge by using a JA-20 rotor at 1,200 rpm for 5 minutes. Supernatants from previous centrifugation were subsequently centrifuged at 5,000 rpm for 15 minutes. The pellets so obtained, constituting the mitochondria-enriched fractions, were resuspended in isosmotic buffer supplemented with PMSF and CLAP. Mitochondrial fractions were stored at -80°C and used for different determinations.

2.7. Caspase activity assays

The proteolytic activity of caspases-8, -9 and -3 was assessed by fluorimetry using cytosolic fractions obtained from MEFs as previously described. The assay medium contained 25 mM HEPES-KOH buffer (pH 7.4), 10% sucrose, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 5 mM DTT and 200 μg of cytosolic protein in a final volume of 200 μl . Samples were preincubated at 37°C for 25 minutes and specific fluorogenic substrates were then added to a final concentration of 40 μM . Samples were incubated in the presence of the substrates for 1 hour at 37°C in the dark. The specific substrates (Alexis Corporation, San Diego, CA, USA) were Ac-IETD-AMC for caspase-8, Ac-LEHD-AMC for caspase-9 and Ac-DMQD-AMC for caspase-3. After incubation, the reaction was terminated with 20 μl of 1 N HCl and the

mixture was diluted by adding 1.5 ml water. Fluorescence signals were recorded with an Aminco-Bowman Series 2 Luminiscence Spectrometer by using the wavelengths of 380 nm (excitation) and 460 nm (emission). Each assay was carried out both in the absence and in the presence of the corresponding specific inhibitor for each caspase. The inhibitors (obtained from Alexis Corporation, San Diego, CA, USA) were added at a final concentration of 83 μ M. We used Ac-IETD-CHO for caspase-8, Ac-LEHD-CHO for caspase-9 and Ac-DMQD-CHO for caspase-3. Caspase activities were calculated from the difference of signal obtained in the presence and in the absence of the corresponding inhibitor. Specific activities of each caspase were expressed as arbitrary units/mg protein.

2.8. Electrophoretic separation of proteins and immunodetection

Cytochrome *c* was determined in cytosolic fractions, whereas AIF was measured both in nuclear and mitochondrial extracts. Levels of the apoptosis-related proteins Bcl-2, Bax and Bid, as well as phosphorylated and total p38, JNK-1, ERK1/2 and Akt were determined in whole extracts. Levels of procaspases and cleaved caspases (-3, -8 and -9) were also measured in whole cell extracts. The required amount of protein from the corresponding sample was mixed with concentrated SDS-dithiothreitol loading buffer (final concentration 60 mM Tris-HCl, pH 6.8, 10% sucrose, 2 mM EDTA, 1.5% (w/v) SDS, 20 mM dithiothreitol and 0.01% (w/v) bromophenol blue). Samples were boiled for 5 minutes and then separated by SDS-PAGE in gradient gels (4–20% acrylamide) and blotted onto nitrocellulose sheets. Blots were stained with Ponceau S dye for visualization of protein lanes and assesment of equal protein loading. After blocking, the blots were stained with primary antibodies at optimized dilutions (see Supplemental Table I). Secondary antibodies coupled to horseradish peroxidase were used at a 1:2000 dilution. Developing of images was performed by enhanced chemiluminescence (ECL Plus, Amersham Bioscience, Uppsala, Sweden). Photographic films obtained after ECL autoradiography were scanned in a BioRad GS800 densitometer to obtain digital images for quantification of intensity reaction using Quantity One software (BioRad). Data obtained from quantification of stained bands (in arbitrary units) were normalized to that of the corresponding lane stained with Ponceau S to correct for minor differences in protein loading between samples, as previously validated in our Group [31]. For normalization purposes, the staining density of each Ponceau S-stained lane covering from 25 to 75 kDa was quantified. Images of all Ponceau S-stained blots used for normalization in this study are depicted in Supplemental Figure 2. For measuring MAPKs and Akt activation, blots were probed first with the corresponding phospho-specific antibodies to estimate the levels of the phosphorylated proteins. After autoradiography, the blots were stripped from bound antibodies by washing membranes twice (15 minutes each) with stripping buffer (62 mM Tris pH 6.7, 2% w/v SDS, 1.4% v/v β -mercaptoethanol) at 70°C and reprobed with antibodies recognizing total levels (phosphorylated plus non-phosphorylated) of each kinase. All antibodies used in this work are listed in Supplemental Table I.

2.9. Quantification of apoptotic DNA fragmentation (Apoptotic index)

The degree of apoptotic DNA fragmentation was assessed by measuring the amount of mono- and oligonucleosomes in MEFS cytosolic fractions. For this purpose the ELISA kit developed by Roche Diagnostics (Mannheim, Germany) was used according to

manufacturer's instructions. Absorbance was determined at 405 nm using a Flex Station 3 (Molecular Devices, Sunnyvale, CA, USA). Data were expressed as arbitrary OD units/mg cytosolic protein.

2.10. Determination of phosphorylated histone H2A.X

Accumulation of spontaneous DNA lesions in MEFs of the different genotypes was investigated by immunostaining for phosphorylated histone H2A.X (γ H2A.X), a marker of DNA double-strand breaks (DSBs). The intensity of γ H2A.X staining was studied by confocal microscopy. For this purpose, cells grown on coverslips were fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature. Next, cells were washed twice with PBS and subsequently incubated with blocking solution (1% BSA, 0.3% Triton X-100 in PBS) for one hour. Then, cells were incubated overnight at 4°C with the primary antibody anti- γ H2A.X (Ser-139) (Cell Signaling) diluted in blocking solution. The antibody was removed by washing three times with PBS and cells were incubated with the secondary antibody conjugated with Alexa fluor 488 (Molecular Probes, Eugene, OR, USA) at room temperature for two hours. Cells were washed three times with PBS. The coverslips were mounted on a slide with a drop of mounting medium composed of 1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI, Sigma), 0.1M Tris-HCl pH 9, 70% glycerol and 2% N-propylgallate. Preparations were observed in a LSM 5 Exciter confocal microscope (Carl Zeiss). Alexa 488 fluorochrome was excited at 488 nm and the emission fluorescence was recorded between 500 and 550 nm. For quantitative analysis of staining intensity mean grey values of the nuclei were measured using Image-J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-2014).

2.11 Electron microscopy

Cells were prefixed by adding a concentrated aqueous solution of glutaraldehyde to the cell cultures to reach a final concentration of 2.5%. After a 5 min incubation, the cells were scrapped, transferred to a vial and pelleted by centrifugation. The supernatant was discarded and replaced by 2% paraformaldehyde-2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH7.2. Fixation of cells was continued for 10 minutes at room temperature and subsequently for 2 hours at 4°C. Postfixation was in 1% osmium tetroxide in the same buffer for 1 hour at 4°C. After dehydration in an ascendant series of ethanol (50%, 70%, 90% and 100%), samples were transferred to propylene oxide and sequentially infiltrated in EMBED 812 resin using the sequence propylene oxide-resin 1:1, and 1:2 overnight. Then samples were transferred to pure resin for 24 hours and allowed to polymerize for 48 hours at 65°C. After sculpting, blocks were sectioned in an Ultracut Reicher ultramicrotome to get ultrathin (about 50nm width) sections. The sections were stained in aqueous 2% uranyl acetate for 5 minutes and Reynold's lead citrate for 5–8 minutes. The sections were viewed and photographed in a JEOL JEM 1400 transmission electron microscope. Planimetric analysis of mitochondria was performed using the Image-J software. Using the planimetric data we calculated mitochondrial area, circularity, number of mitochondria per μ m² of cytoplasm and portion of cytoplasm occupied by mitochondria.

2.12. Statistical analysis

Data shown in this work are means $\pm$ standard error of the mean (SEM) from at least three different determinations. Significant differences between means were calculated using Student's t test. Differences were considered significant with $p < 0.05$. Statistical analysis were performed using Graphpad Prism 5.03 (Graphpad Software Inc, San Diego, CA, USA).

3. Results

We previously showed that cultures of wild-type MEFs grew steadily until passage 62 although they could not surpass passage 64. In the case of Nrf2KO MEFs, lifespan was considerably shortened. These cells displayed a stabilized doubling rate between passages 20 and 52 but then underwent a sharp decline in growth rate and could not divide beyond passage 54 [28]. For the determinations described in this paper we used wild-type and Nrf2KO MEFs obtained from passages 25 to 35. We previously demonstrated that Nrf2KO MEFs exhibited a slight, although statistically significant, decrease in doubling rate in comparison with the wild-type controls [28]. Thus, we first wanted to confirm that growth rate was indeed lower in MEFs lacking Nrf2 by using a specific proliferation marker as the incorporation of [^3H]-thymidine, and results were fully consistent with our previous observations since the level of incorporated [^3H]-thymidine was significantly lower in Nrf2KO MEFs compared with the wild-types (Supplemental Figure 3A).

Basal apoptosis is increased in MEFs and in tissues from Nrf2KO mice

Nrf2 has been recently shown to control the expression of the antiapoptotic proteins Bcl-2 and Bcl-xL [24, 25]. Thus, we hypothesized that decreased growth of Nrf2KO MEFs could be related with enhanced rates of basal apoptosis in the absence of defined proapoptotic stimuli. Thus, using ELISA we determined the abundance of oligonucleosomal chromatin fragments released to the cytosol as a quantitative estimation of apoptosis levels. This method was chosen because of its high sensitivity and also because it allows to easily distinguish apoptosis from necrotic cell death, since during necrosis oligonucleosomal fragments are not retained in cytosol but released to the culture medium. As depicted in Figure 1A, the basal rate of apoptotic DNA fragmentation was dramatically increased in Nrf2KO MEFs in comparison with wild-type MEFs. Since the level of basal apoptosis shows a high dependence on cell density in MEFs [32], we also measured apoptotic DNA fragmentation at varying cellular densities from sparse (about 4,000 cells/cm²) to sub-confluency (about 50,000 cells/cm²). We found that DNA fragmentation was always higher in Nrf2KO than in wild-type MEFs at all culture densities, although the increase of DNA fragmentation in Nrf2KO MEFs was much more pronounced in dense cultures (Supplemental Figure 3B). In addition, we aimed to measure by immunocytochemistry the levels of histone H2A.X phosphorylated at serine-139 ($\gamma\text{H2A.X}$), as a specific indicator of cell response to the existence of DNA double strand breaks [33]. As depicted in Figures 2B and C, the intensity of $\gamma\text{H2A.X}$ staining was significantly increased in Nrf2KO MEFs indicating the activation of a DNA damage response in cells lacking Nrf2. We also studied whether or not deletion of this factor led to alterations in ROS levels in the MEFs. However, despite the well-characterized role of Nrf2 as a master regulator of cellular response to pro-

oxidants [7], steady state levels of ROS did not show a significant increase in immortalized Nrf2KO MEFs under basal culture conditions (Supplemental Figure 3C).

Nrf2 signaling and pathophysiology in liver have been widely linked, with Nrf2 playing a role in hepato-carcinogenesis and other hepatic chronic diseases [33]. In addition, oncogenesis in lung is also particularly sensitive to alterations in Nrf2 status [15]. We thus measured apoptotic DNA fragmentation in liver and lung samples obtained from wild-type and Nrf2KO mice and demonstrated that apoptotic DNA fragmentation was also increased in tissues isolated from Nrf2KO mice (Figure 1D and E).

Apoptotic signaling alterations in Nrf2KO MEFs

Bcl-2 and Bax—Permeabilization of the outer mitochondrial membrane (OMM) is one of the most important events to trigger apoptosis. To gain insights about the cellular mechanisms that could account for the increase of basal apoptosis in MEFs lacking Nrf2, we first evaluated the levels of anti-apoptotic Bcl-2 and pro-apoptotic Bax. The abundance of Bcl-2 relative to Ponceau S staining was greatly diminished in Nrf2KO compared with wild-type MEFs (Figure 2A) with no significant change being observed for Bax (Figure 2B), which resulted in a substantial decrease of Bcl-2/Bax ratio in MEFs lacking Nrf2 (Figure 2C).

Cytosolic cytochrome c and nuclear AIF—The balance between pro- and anti-apoptotic members of the Bcl-2 family defines the permeabilization of the OMM and the release of pro-apoptotic mitochondrial factors that trigger an apoptotic signaling cascade. Thus, we next determined the levels of cytosolic cytochrome *c* as an estimate of its release/accumulation from the mitochondrial intermembrane space into the cytosol, and the levels of nuclear AIF as an estimate of its release from the mitochondria and its further accumulation into the nucleus. In accordance with their decreased Bcl-2 levels and Bcl-2/Bax ratio, both cytosolic cytochrome *c* (Figure 2D) and nuclear AIF (Figure 2E) were dramatically increased in Nrf2KO MEFs. However, levels of AIF in isolated mitochondria were not decreased in Nrf2KO MEFs when compared with the wild types (Supplemental Figure 3D).

Apaf-1 levels—We also wanted to elucidate whether or not genetic deletion of Nrf2 affected levels of Apaf-1 which, together with dATP and cytochrome *c*, recruits and activates caspase-9 in the apoptosome [34]. Interestingly, we found that this pro-apoptotic adaptor protein was also substantially increased in Nrf2KO MEFs (Figure 2F).

Enzymatic activities of caspases—We next analyzed how deletion of Nrf2 affected activities of different caspases. This analysis was first carried out by measuring their enzymatic activities by a fluorimetry assay using fluorogenic peptides as substrates. We measured enzyme activities of caspase-8, which is activated *via* the extrinsic pathway; caspase-9, a key factor of the intrinsic (mitochondrial) pathway; and caspase-3 activity, the most representative executioner caspase. Despite the substantial decrease of Bcl-2/Bax ratio and the elevation of cytosolic cytochrome *c* and Apaf-1 levels in Nrf2KO MEFs, proteolytic activities of caspases-9 and -3 were not increased significantly in Nrf2KO MEFs in

comparison with the controls (Figures 3A and B). On the other hand, a significant increase of caspase-8 activity was observed for Nrf2KO MEFs (Figure 3C).

Levels of pro-caspases, cleaved caspases and truncated Bid—Caspase activation was also studied by western blot determination of pro-caspases and their corresponding cleaved caspases in order to confirm the results obtained with the enzymatic assay. When focusing on procaspases, we found that the level of procaspase-9 was substantially increased in Nrf2KO MEFs (Figure 4A), procaspase-3 was maintained (Figure 4B), and procaspase-8 was slightly decreased (Figure 4C). Cleaved activated caspases could be only revealed in these western blots after prolonged developing of the enhanced chemiluminescence reaction, which suggests the presence of low rates of caspase activation in these cells. We observed a trend towards an increase in the 37/39 kDa activated fragments of caspase-9 in Nrf2KO MEFs (Supplemental Figure 4A) but no activation of caspase-3 was found, neither for the 19 kDa (Supplemental Figure 4B) nor the 17kDa (Supplemental Figure 4C) fragments. In agreement with the results obtained with the enzymatic assay, levels of cleaved caspase-8 were significantly elevated in Nrf2KO MEFs, both for the 25 kDa (Figure 5A) and the 18 kDa (Figure 5A) fragments.

MEFs are well-characterized type II cells, which transduce apoptotic signaling to the mitochondria *via* the caspase-8-dependent cleavage of Bid [35]. Thus, we wanted to test if caspase-8 activation played a significant role in the increase of basal apoptosis in MEFs lacking Nrf2KO. To this purpose we followed two different approaches: (i) to measure levels of Bid and tBid, its truncated form, and (ii) to test the effect of Z-IETD-FMK (a cell permeable non-toxic and irreversible caspase-8 inhibitor) on apoptotic DNA fragmentation. Levels of full-length Bid did not change among wild-type and Nrf2KO MEFs (Figure 6A) and, strikingly, levels of tBid were even decreased in Nrf2KO MEFs (Figure 6B) which is in contrast with their enhanced activation of caspase-8. For the experiments with the caspase-8 inhibitor, cells were cultured for 24 hours in the presence Z-IETD-FMK at concentrations ranging from 5 to 25 μ M before they were lysed to obtain cytosolic fractions which were used for the ELISA quantification of apoptotic DNA fragmentation. We observed no effect of the caspase-8 inhibitor on DNA fragmentation at any of the concentrations tested (Figure 6C).

XIAP levels and effect of XIAP inhibition—Inhibitors of apoptotic proteases (IAPs), such as XIAP, can avoid the engagement of mitochondrial apoptosis by inhibiting both caspase-9 and -3, and this anti-apoptotic activity is antagonized by pro-apoptotic factors which are also released from the mitochondria, such as Smac/DIABLO [36, 37]. Since XIAP levels were found unchanged among wild-type and Nrf2KO MEFs (Figure 7A), we hypothesized that, at these endogenous levels, XIAP could be sufficient for inhibiting caspases under basal culture conditions. We thus decided to investigate how BV-6, a Smac/DIABLO mimetic and XIAP inhibitor [38], affected viability of wild-type and Nrf2KO MEFs. For these experiments, cells were treated with BV-6 for 48 hours and a MTT assay was then performed to assess for cell viability. Interestingly, treatment with BV-6 decreased viability in a concentration-dependent manner only in Nrf2KO MEFs, with no effect of this inhibitor being observed in the wild-types, and differences between the two genotypes were

observed from the lowest concentration of BV-6 tested (Figure 7B). We also aimed to investigate if BV-6 treatment led to a variation of procaspase-3 processing. Interestingly, a significant decrease of procaspase-3 levels was observed in Nrf2KO MEFs treated with 12.5 μ M BV-6, with no effect being observed on the wild-types (Figure 7C).

MAPKs and Akt phosphorylation

Mitogen-activated protein kinases (MAPKs) regulate many cellular processes including proliferation, differentiation, survival, and death [39]. We thus measured phosphorylated and total levels of the main MAPKs: p38, JNK1 and ERK1/2 (see Figure 8A for representative results of western blots). Nrf2 deletion resulted in a significant increase of phosphorylated p38 (Figure 8A and B) although no significant change was observed in JNK1 activation (Figure 8A and C). On the other hand, phosphorylated forms of ERK1/2 were also increased significantly in Nrf2KO MEFs (Figure 8A and D). We also measured the total levels and phosphorylation state of Akt, which protects cells against apoptosis [40], and found a trend toward decreased levels of phosphorylated Akt in Nrf2KO MEFs, although differences did not reach statistical significance (Figure 8A and E).

Since phosphorylated forms of both ERK1/2 and p38 were significantly increased in Nrf2KO MEFs, we next studied if these pathways were involved in enhancing basal apoptosis of Nrf2KO MEFs by testing the effect of well-established pharmacological inhibitors on apoptotic DNA fragmentation. The MEK inhibitor PD98059 was used to test the putative participation of ERK1/2, whereas both SB203580 and PD169316 were used to test the involvement of p38 MAPK. Despite the observed changes in ERK1/2 no effect on DNA fragmentation was observed when MEFs (either wild-type or Nrf2KO) were cultured in the presence of PD98059 at a concentration range from 1 to 25 μ M (Supplemental Figure 5A), indicating that activation of ERK1/2 is not related with the regulation of basal apoptosis in the MEFs. When we studied the effects of the two p38 inhibitors we found that, at some of the concentrations, PD169316 decreased basal DNA fragmentation in wild-type MEFs (Supplemental Figure 5B), although a similar effect was not observed for SB203580 (Supplemental Figure 5C). In either case, neither SB203580 nor PD169316 altered the abundance of cytosolic oligonucleosomal fragments in Nrf2KO MEFs at any of the tested concentrations (Supplemental Figures 5B and C). The lack of effect of the two p38 inhibitors on basal apoptosis in Nrf2KO MEFs was striking, provided the well-established proapoptotic role of this stress-activated kinase in MEFs [32]. Thus we also studied if increased phosphorylation state of p38 was also present in lung and liver tissues from Nrf2KO mice, which had exhibited increased basal apoptosis levels (see Figure 1D and E). Although p38 phosphorylation was increased in lung from Nrf2KO mice (Supplemental Figure 5D), no changes were observed in liver (Supplemental Figure 5E), which suggests that the effect of Nrf2 deletion on the activation of p38 pathway is tissue specific, and probably unrelated to the alterations in basal apoptosis observed in cells lacking Nrf2.

Nrf2KO MEFs exhibit mitochondrial structural alterations

Our work focused on apoptotic signaling is consistent with the idea that deletion of Nrf2 produces mitochondrial alterations that lead to enhanced mitochondrial permeabilization and the release of pro-apoptotic factor. Using electron microscopy techniques we studied directly

whether or not genetic deletion of Nrf2 produced alterations of mitochondrial ultrastructure. The observation of micrographs showed an apparent decrease of mitochondrial size in Nrf2KO MEFs when compared to their wild-type counterparts (Figure 9A and B), which was confirmed by planimetric analysis of these micrographs (Figure 9C). However, circularity did not change (Figure 9D). In addition, we evidenced the existence of a higher number of mitochondria per μm^2 of cytoplasm in Nrf2KO MEFs (Figure 9E), and the portion of cytoplasm occupied by mitochondria was also higher (Figure 9F).

4. Discussion

Basal apoptosis is increased in MEFs bearing a genetic deletion of Nrf2 gene and in tissues from Nrf2KO mice

Nrf2 has been recently shown to control the expression of several antiapoptotic proteins, as Bcl-2 and Bcl-xL [24, 25]. Thus we hypothesized that decreased growth of Nrf2KO MEFs could be due to enhanced rates of basal apoptosis. We evaluated the rates of apoptosis-related DNA damage by measuring the abundance of oligonucleosomal fragmentation and found out that levels of basal apoptosis were increased not only in Nrf2KO MEFs but also in lung and liver tissues isolated from Nrf2KO mice. Previous work in the field has reported that the inhibition of Nrf2-dependent transcription by gene silencing is linked to the enhancement of etoposide and radiation-induced apoptosis in mouse and human hepatic tumor cells [24]. In addition, Nrf2KO mice show higher levels of several pro-apoptotic factors in skeletal muscle from aged mice [27, 41], although a putative increase of apoptosis of the skeletal muscle fibers was not tested directly in those previous studies. Of note, it has been recently reported that the expression of a constitutively active form of Nrf2 (caNrf2) delayed proliferation and induced apoptosis of hepatocytes in the regenerating liver of caNrf2-transgenic mice [42], suggesting that a homeostatic balance of Nrf2 function is needed to protect mitotic tissues from apoptosis. In accordance, oncogenesis has been reported to increase in lung not only by decreased expression of Nrf2 gene [15] but also by unrestrained Nrf2 activation due to inactivating mutations in the Keap1 gene [43].

Staining for $\gamma\text{H2A.X}$ histone in nuclei is a way to quantify DNA double strand breaks (DSBs) since $\gamma\text{H2A.X}$ is needed for the collection of several DNA damage response (DDR) proteins at DSBs to allow for DNA repair [44]. The increase in $\gamma\text{H2A.X}$ signal intensity in the Nrf2KO MEFs is consistent with higher DNA fragmentation. However, levels of ROS were not elevated in Nrf2KO MEFs, which could be due to up-regulation of some antioxidant genes to counteract the oxidative stress exerted by cellular metabolism, as previously reported [45]. Thus, the enhancement of basal apoptosis in Nrf2KO MEFs and tissues is not likely to be accounted by increased oxidative stress. Accordingly, levels of ROS only increased significantly in Nrf1–Nrf2 double KO MEFs in comparison with any of the single KOs [46]. Furthermore, the deleterious effects of Nrf2 deletion in lung of Nrf2KO mice is not observed under basal conditions, but only after challenging mice with bleomycin [47].

Apoptotic signaling is stimulated in Nrf2KO MEFs

The decisive event in the intrinsic apoptotic pathway is the permeabilization of the OMM that allows the release of several pro-apoptotic factors like cytochrome *c* and AIF to trigger the apoptotic cascade. Those factors are released through the Bcl-2 family-regulated mitochondrial apoptotic-induced channel [48]. The decrease of Bcl-2 levels in MEFs lacking Nrf2 we show here is in accordance with previous investigations demonstrating that the silencing of Nrf2 decreases Bcl-2 polypeptide in Hepa-1 and Hek-293 cells and, conversely, up-regulation of Nrf2 induces the expression of Bcl-2 and Bcl-xL [24, 25]. There is a direct relationship between Nrf2 and both Bcl-2 and Bcl-xL expression because these two antiapoptotic proteins are under transcriptional control by Nrf2 that binds to an ARE located in the promoter region of the corresponding genes [24, 25]. However, the existence of a direct link between Nrf2 and Bax expression is unclear. In a cellular model, it was previously shown that experimental augmentation of Nrf2 levels prevented Bax increase induced by treatment with etoposide. However, Bax levels were not significantly altered in cells that had not been treated with the apoptosis inducer [25], which agrees with the lack of Bax changes in Nrf2KO MEFs shown here. In addition, although Bax polypeptide was significantly increased in skeletal muscle from aged (> 24 months of age) Nrf2KO mice [27, 41], it is not clear if this Bax increase is a direct effect of Nrf2 deficiency or it is an indirect effect related with aging [27]. Strikingly, Bim, another proapoptotic member of Bcl-2 family, is positively regulated by Nrf2 as part of a protective response against hyperproliferative conditions that may prevail due to constitutive Nrf2 activation in liver from caNrf2 transgenic mice [42].

The significant decrease of Bcl-2 levels and Bcl-2/Bax ratio observed in Nrf2KO MEFs is consistent with an enhancement of the mitochondrial pro-apoptotic pathway in these cells. Release of cytochrome *c* to the cytosol together with dATP and apoptotic protease-activating factor-1 (Apaf-1) association promotes assembly of the apoptosome which triggers a caspase cascade *via* activation of caspase-9 [34], whereas the release of apoptosis-inducing factor (AIF) to the cytosol and its translocation to the nucleus is sufficient to facilitate DNA fragmentation through a caspase-independent pathway [49]. To our knowledge, this is the first demonstration that genetic deletion of Nrf2 increases cytosolic cytochrome *c* and nuclear AIF levels in mitotic cells. Furthermore, the increase of nuclear AIF observed in Nrf2KO MEFs agrees with the increase of AIF in skeletal muscle from aged Nrf2KO mice [27]. Despite the significant accumulation of AIF in nucleus, we did not find a decrease of AIF levels in mitochondria, which could reflect the necessity to sustain appropriate levels of mitochondrial AIF in Nrf2KO cells. Indeed, AIF can exert a cytoprotective role in the mitochondria and enable cells to cope more effectively with oxidative stress by acting as ROS scavenger and ensuring proper assembly and functionality of mitochondrial respiratory complex I [50]. On the other hand, since the procedure for mitochondrial isolation involves relatively harsh techniques of shearing, the possibility exists that the method leads to the enrichment of more robust mitochondria, which could mask a putative decrease of AIF levels in mitochondria isolated from Nrf2KO *versus* wildtype MEFs.

Apaf-1 levels were also significantly increased in Nrf2KO MEFs, which supports that Nrf2 may participate in the regulation of Apaf-1 levels by decreasing its abundance in cytosol.

Expression of the APAF-1 gene is subjected to a very complex regulation including transcriptional regulation by E2F-1 and p53, epigenetic regulation by histone deacetylases and methylation of the APAF-1 gene and suppression by several miRNAs [51–53]. How Nrf2 might interact with any of these systems known to regulate Apaf-1 levels remains for further investigation.

Activation of the mitochondrial caspase cascade is blunted in Nrf2KO MEFs

Release of mitochondrial proapoptotic factors leads to activation of a caspase cascade for the proteolytic degradation of many cellular substrates [54]. Apoptosome-dependent activation of caspase-9 and the subsequent cleavage and activation of the executioner caspase-3 are key factors of mitochondrial (intrinsic) pathway apoptosis which takes part in cell removal after ionizing radiation exposure, DNA and mitochondrial damage or growth factor withdrawal [34, 55]. On the other hand, caspase-8 is the prototypic caspase that is activated through the plasma membrane-dependent extrinsic pathway, typical of immune-system tumor removal, immune system education and development [56]. Since cytosolic cytochrome *c* and Apaf-1 were substantially increased in Nrf2KO MEFs, it was reasonable to expect that activities of caspases-9 and -3 would be also upregulated. However, proteolytic activities of caspases-9 and -3 were not increased significantly in Nrf2KO MEFs in comparison with the control cells. Instead, a significant increase of caspase-8 activation was found in Nrf2KO MEFs. These results were obtained not only by performing caspase enzymatic assays with fluorogenic peptide substrates, which have been criticized in some instances because of their low specificity [57], but also after western blot determination of the activated cleaved caspases and the full-length uncleaved procaspases. In addition, this latter technique allowed us to evidence that procaspase-9 was significantly elevated and procaspase-8 decreased in Nrf2KO MEFs. Because the components of the caspase activation system are constitutively expressed in the majority of cells and the activation of caspases is a post-translational process, substantial interest has been paid to the factors controlling proteolytic activation of procaspases, but the mechanisms involved in transcriptional regulation of caspases gene expression are not fully understood [58]. Interestingly, the increase of procaspase-9 levels in Nrf2KO MEFs is in agreement with the increase of Apaf-1 levels in the same cells because these proteins are transcriptionally controlled by the same factors, including E2F1 and p53 [58].

The existence of lower levels of procaspase-8 in Nrf2KO MEFs is in agreement with the increase of its cleaved forms. An increase of caspase-8 activation was the most prominent difference between wild-type and Nrf2KO MEFs regarding the activation of the caspase cascade. Thus, we investigated if activation of this caspase was related with the increase of basal apoptosis in Nrf2KO MEFs. Cleavage of Bid by caspase-8 is the link between extrinsic apoptotic pathway and activation of the mitochondrial apoptosis in type II cells, whereas caspase-8 directly activates executioner caspase-3 in type I cells [35, 37, 56, 59]. Although MEFs are well characterized type II cells [35], the levels of tBid were even decreased in Nrf2KO in comparison with wild-type MEFs. Furthermore, apoptotic DNA fragmentation was not decreased by Z-IETD-FMK, a non-toxic irreversible inhibitor of caspase-8, at concentrations ranging from 5 to 25 μ M, even when full inhibition of caspase-8 in fibroblasts by Z-IETD-FMK has been reported at concentrations above 2 μ M [60]. Taken

together, these observations support that activation of caspase-8 is not related with the elevation of basal apoptosis in Nrf2KO MEFs. Caspase-8 plays a number of non-apoptotic roles in cells, such as promoting activation NF- κ B signaling, regulating autophagy and altering endosomal trafficking, and enhancing cellular adhesion and migration [61]. How these cellular functions might have been altered in Nrf2KO cells and the putative involvement of caspase-8 activation remain for further investigation.

One possibility to explain the lack of caspase-3 activation in Nrf2KO MEFs would involve the participation of the Inhibitors of Apoptotic Proteases (IAPs). One of the main IAPs is the X-chromosome linked Inhibitor of Apoptotic Proteases (XIAP), known to behave as a potent inhibitor of caspase-9 and effector caspases [37]. We found no change of XIAP levels among wild-type and Nrf2KO MEFs but when cells were treated with BV-6, a Smac/DIABLO mimetic and XIAP inhibitor, MEFs from the two genotypes behaved in a different way. The induction of cell death by BV-6 specifically in Nrf2KO MEFs is likely due to the fact that cells lacking Nrf2 but not wild-type MEFs have a very high content of pro-apoptotic factors, although the intrinsic caspase pathway is blunted in Nrf2KO MEFs because endogenous XIAP might be high enough to block the caspase cascade. It is thus very likely that the increase of apoptotic DNA fragmentation observed in Nrf2KO MEFs could be mediated by increased nuclear levels of AIF, as it has been demonstrated that AIF is able to induce chromatin condensation and fragmentation in a caspase-independent pathway [49].

MAPK and Akt pathways are altered in Nrf2KO MEFs

Mammalian MAPK pathways can be activated by a wide variety of different stimuli through diverse receptor families and are responsible of a number of functions including apoptosis [62]. It is well known that the Nrf2-Trx1 axis controls the function of stress-activated protein kinases since Trx1, one of the main transcriptional targets of Nrf2, binds and inhibits Apoptosis signal-regulating kinase 1 (Ask1), a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family involved in both the stress response and apoptosis that activate JNK and the p38 MAPK family [63, 64]. Interestingly, previous studies have documented that the levels of Ask1 are increased in skeletal muscle from old Nrf2KO mice, and this increase is exacerbated by exercise [27, 41]. However, how the lack of Nrf2 may affect the phosphorylation state of the main MAPK families, and if these putative changes in MAPK activation participate or not in apoptosis engagement in cells without Nrf2 function have not been addressed to date. Within this context, we studied if the lack of Nrf2 altered phosphorylation state of the stress protein kinases p38 and JNK1. In addition, we also analyzed the phosphorylation state of ERK1/2 since, besides being activated by mitogenic signals, this MAPK can be also activated by environmental stress such as oxidative stress, [62]. In addition, our study also included Akt, a protein involved in a pathway related to proliferation and survival, protecting cells against apoptosis [40]. Genetic deletion of Nrf2 produced significant phosphorylation of ERK1/2 and p38, but phosphorylation states of JNK1 remained unaltered and Akt showed a trend to decrease.

Many apoptotic responses have been shown to be dependent on p38 activation [32, 65, 66]. Fibroblasts lacking p38 are more resistant to apoptosis induced by different stimuli [32]. On

the other hand, ERK1/2 signaling is also an important regulator of apoptosis, and influences the expression and/or activity of many members of the Bcl-2 protein family, in many cases to promote survival [67]. However, induction of apoptosis may be also mediated by ERK1/2 in other experimental models [68, 69]. Our results with ERK1/2 phosphorylation are consistent with previous observations carried out in immature dendritic cells, in which enhanced ERK1/2 phosphorylation state was found when Nrf2 was deleted [70]. However, the fact that apoptotic DNA fragmentation was not affected by inhibitors of p38 and ERK1/2 pathways suggests that activation of these kinases might not be decisive in determining cell fate towards survival or death in Nrf2KO MEFs.

Mitochondria show ultrastructural alterations in Nrf2KO MEFs

In our ultrastructural analysis focused on mitochondria we have found that Nrf2KO MEFs mitochondria were smaller when compared with wild-type MEFs. However, at a cellular level mitochondria were more abundant and there was a higher surface of cytoplasm occupied by these organelles. A higher number of mitochondria in Nrf2KO MEFs could be a consequence of mitochondrial fragmentation, which is known to potentiate mitophagy and cell death [71]. In accordance, the axis formed by p62 (which takes part in a non-canonical Nrf2 activation pathway), Nrf2 and NQO1 (one of the best defined Nrf2 targets) has been previously suggested as a way to assure mammalian longevity through mitochondrial integrity [72].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Nrf2 genetic deletion increases basal apoptosis both *in vivo* and *in vitro*

ERK and p38 activation in Nrf2KO MEFs does not participate in apoptosis engagement

Cytosolic cytochrome *c* and nuclear AIF levels significantly increase in Nrf2KO MEFs

Inefficient caspase activation supports caspase-independent pathways through AIF

Deletion of Nrf2 produces ultrastructural alterations of mitochondria in MEFs

Figure 1. Apoptotic DNA fragmentation is increased by genetic deletion of Nrf2

A) Abundance of oligonucleosomal fragments (Apoptotic Index) in MEFs. We found out that apoptosis was increased in the Nrf2KO immortalized MEFs when compared to their wild-type counterparts (** $p < 0.01$). Data are means $\pm$ SEM ($n=6$). **B)** Phosphorylated γ H2A.X (Ser139). Representative images of DAPI and P-H2A.X. Nuclei of Nrf2KO MEFs are more strongly stained with an antibody raised against P-H2A.X. **C)** Quantification of fluorescence intensity: Nrf2KO MEFs showed a higher mean grey value of γ H2A.X staining than wild-type MEFs (* $p < 0.05$). Data are means $\pm$ SEM ($n=60$). **D)** Abundance of oligonucleosomal fragments in liver. Apoptotic Index was higher in Nrf2KO liver (** $p < 0.01$). Data are means $\pm$ SEM ($n=5$). **E)** Abundance of oligonucleosomal fragments in

lung. Apoptotic DNA fragmentation was also significantly increased in lung tissue from Nrf2KO mice (* $p < 0.05$). Data are means $\pm$ SEM (n=5).

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Figure 3. Enzymatic activities of caspases

A) Caspase-9 activity: Proteolytic activity of caspases-9 was not increased significantly in Nrf2KO MEFs in comparison with the control cells. **B)** Caspase-3 activity: Enzymatic activity of caspase-3 did not change in Nrf2KO cells in comparison with wild-type counterparts. **C)** Caspase-8 activity: when we analyzed the enzymatic activity of caspase-8, a significant increase was observed for Nrf2KO cells compared with their wild-type counterparts (**p < 0.01). Data are means $\pm$ SEM (n=5).

Figure 4. Levels of procaspases

A) Procaspase-9: The level of procaspase-9 was substantially increased in Nrf2KO MEFs (***p < 0.001). **B)** Procaspase-3: Levels of procaspase-3 were maintained in Nrf2KO MEFs in comparison with the wild-type controls. **C)** Procaspase-8: Levels of procaspase-8 were slightly decreased in Nrf2-null MEFs (**p < 0.01). Quantification of intensity reaction was normalized to Ponceau S staining.

Figure 5. Levels of activated cleaved caspase 8

Cleaved, activated caspase-8 levels were significantly elevated, both for the 24/26 kDa (A) and the 18 kDa (B) caspase-8 fragments (* $p < 0.05$). Data are means $\pm$ SEM (n=4). Quantification of intensity reaction was normalized to Ponceau S staining.

Figure 6. Caspase-8 activation is not involved in the augmentation of basal apoptosis in Nrf2KO MEFs

A) Bid and B) tBid: Levels of full-length Bid were unchanged among wild-type and Nrf2KO MEFs whereas the abundance of tBid was decreased in the Nrf2KO MEFs (* $p < 0.05$). Data are means $\pm$ SEM ($n=3$). Quantification of intensity reaction was normalized to Ponceau S staining. **C) Caspase-8 inhibitor treatment:** No effect on DNA fragmentation was achieved by treating wild-type or Nrf2KO MEFs with the caspase-8 inhibitor Z-IETD-FMK, and basal apoptosis levels remained equally increased in the KOs both in the absence and in the presence of the inhibitor at any of the tested concentrations (** $p < 0.01$ and *** $p < 0.0001$). Data are means $\pm$ SEM ($n=4$).

Figure 7. Role of XIAP in the inhibition of caspase-dependent apoptosis in Nrf2KO MEFs

A) XIAP levels: Levels of XIAP did not change between Nrf2KO and wild-type MEFs. Data are means $\pm$ SEM (n=3). **B)** Effect of XIAP inhibition on cell viability: Treatment with BV-6 decreased viability in Nrf2KO MEFs from the lowest tested concentration in a concentration-dependent manner (**p<0.001). However, wild-type MEFs treated with BV-6 did not show any effect on cell viability. **C)** Effect of XIAP inhibition on procaspase-3 processing: We observed no changes in wild-type MEFs either without treatment or after being treated with BV6 at 12.5 μ M. However, the abundance of procaspase-3 was significantly lower in Nrf2KO MEFs when treated with the XIAP inhibitor (*p<0.05). Data

are means $\pm$ SEM (n=4). In A and C, quantification of intensity reaction was normalized to Ponceau S staining.

Figure 8. Alterations of MAPK and Akt pathways in Nrf2KO MEFs

A) Western blots showing phosphorylated and total levels of MAPK and Akt: **B to E)** Calculations of kinase activation by phosphorylation. We measured phosphorylated and total levels p38, JNK1, ERK1/2 and Akt. Putative alterations in the activity of each pathway were estimated from the ratio between the signals corresponding to phosphorylated and total polypeptide levels for each kinases. **B)** p38 activation: We found an increase in the activated form of p38 in Nrf2KO immortalized MEFs ($***p < 0.001$). **C)** JNK1 activation: We found no significant differences between the two genotypes. **D)** ERK1/2 activation: The results showed an increase of the phosphorylated proteins in Nrf2KO ($*p < 0.05$). Data are means $\pm$ SEM (n=3). **E)** Akt activation: Changes of phosphor-Akt did not reach significant differences with the two-tailed t-test ($p = 0.084$), although they tended to decrease in Nrf2KO and were significant with a one-tailed t-test. Data are means $\pm$ SEM (n=3). Data of reaction intensity obtained with each phosphor-specific antibody were normalized to those obtained with the antibody recognizing total levels of the corresponding protein.

Figure 9. Ultrastructural alterations of mitochondria in Nrf2KO MEFs

A) Representative micrograph of wild-type MEFs. **B)** Representative micrograph of Nrf2KO MEFs. **C)** Mitochondrial area: We have found that mitochondria are significantly smaller in Nrf2KO MEFs when compared to their wild-type counterparts (*** $p < 0.001$). **D)** Mitochondrial circularity: Circularity did not change among genotypes. **E)** Number of mitochondria per area of cytoplasm: There is a higher number of mitochondria per μm^2 of cytoplasm in Nrf2KO MEFs when compared to their wild-type counterparts (** $p < 0.01$). **F)** Portion of cytoplasm occupied by mitochondria: The portion of cytoplasm occupied by mitochondria is also higher in Nrf2KO MEFs (* $p < 0.05$).