Dehydroepiandrosterone triggers autophagic cell death in human hepatoma cell line HepG2 via JNK-mediated p62/SQSTM1 expression

Rolando Vegliante1, Enrico Desideri1,3, Luca Di Leo1 and Maria Rosa Ciriolo1,2,*

1Department of Biology, University of Rome ‘Tor Vergata’, Rome 00133, Italy and 2IRCCS San Raffaele, Rome 00166, Italy
3Present address: Department of Microbiology, Immunobiology and Genetics, University of Vienna, Vienna 1030, Austria

*To whom correspondence should be addressed. Tel: +39 06 72594369; Fax: +39 06 72594312; Email: ciriolo@bio.uniroma2.it

Abstract

Autophagy is a catabolic process that cancer cells usually exploit during stress conditions to provide energy by recycling organelles and proteins. Beyond its prosurvival role, it is well accepted that occurrence of autophagy is often associated with a particular type of programmed cell death known as autophagic cell death (ACD). Dehydroepiandrosterone (DHEA) is an endogenous hormone showing anticancer properties even if the underlying mechanisms are not fully clear yet. Here, we provide evidence that DHEA induces ACD in human hepatoma cell line, HepG2. Indeed, autophagy inhibitors (i.e. 3-methyladenine or Atg5 siRNA) significantly reduced the percentage of dead cells. DHEA induces p62-dependent autophagy, which turns detrimental and brings about death. DHEA stimulates reactive oxygen species-independent jun N-terminal kinase (JNK) phosphoactivation and the treatment with JNK inhibitor reduces p62 mRNA levels, as well as DHEA-induced ACD. The transcription factor nuclear factor (erythroid-derived-2)-like-2 (Nrf2) constitutes the link between JNK and p62 since its migration to the nucleus is suppressed by JNK inhibitor and its inhibition through a dominant negative Nrf2 plasmid transfection decreases p62 protein levels. Overall, our data indicate that DHEA induces ACD in HepG2 via a JNK–Nrf2–p62 axis. Thus, DHEA could represent a new appealing drug for eliminating tumor cells through autophagy particularly in apoptosis-resistant cases.

Introduction

Dehydroepiandrosterone (DHEA) is an endogenous steroid hormone synthesized in the adrenal cortex, brain, gonads and gastrointestinal tract in humans (1). DHEA and its sulfate ester (DHEA-S) are the most abundant serum steroids after cholesterol, reaching a maximum concentration between the age of 15 and 25 years, and progressively decreasing with age (2). This observation has paved the way to investigate a possible relationship between DHEA serum levels decline and diseases that are generally related to aging such as diabetes, obesity, cardiovascular diseases, reduction of the immune defense and cancer. For instance, it is well documented that DHEA has beneficial effects in animal models of non-insulin-dependent diabetes mellitus due to both insulin sensitivity and secretion (3,4). DHEA has also an immune stimulatory potential mainly achieved through the stimulation of cytokines production from both myeloid and lymphoid cells (5,6). Interestingly, growing lines of evidence demonstrated chemopreventive and antiproliferative properties of DHEA both in vitro and in vivo (7). Indeed, it is well accepted that DHEA is a strong uncompetitive inhibitor of glucose-6-phosphate dehydrogenase (G6PDH) leading to a decrease in the production of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and ribose-5-phosphate, which are essential for lipid and nucleotide biosynthesis, respectively. Thus, G6PDH inhibition by DHEA may account for its antiproliferative effects (8,9). In contrast, a report showed that DHEA inhibited cell growth in G6PDH-null Chinese hamster ovarian cells (CHO variant E89) (10). Moreover, DHEA has also been shown to induce G6PDH-independent cell-cycle arrest...
in human colonic adenocarcinoma cell lines, via intracellular depletion of mevalonate and its derivatives that are essential for cell-cycle progression and DNA replication (11,12). Actually, DHEA efficiently inhibited 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis whose direct product is mevalonate (13). Induction of apoptotic cell death by DHEA may also account for DHEA potential antitumor properties, as demonstrated in mouse thymocytes, microglial cells and lymphocytes, and human neuroblastoma cells (14–17). On the other hand, some reports demonstrated a prosurvival role of DHEA by increased expression of the antiapoptotic proteins B-cell lymphoma 2 (Bcl-2) and extra-large (Bcl-XL) (18,19). Thus, it appears that several mechanisms could be responsible for the antiproliferative effects of DHEA, impinging also in different cell death program commitment. Among the different types of programmed cell death, apoptosis is the better and first characterized and involves a complex proteolytic cascade that culminates in the activation of cytosine proteases known as caspsases (20). Another one, which has gained interest in the last years, is necrosis that is regulated by the activation of receptor-interacting kinase 1 (RIPK1) and often occurs under caspase deficiency (21).

Autophagy is a catabolic process aimed at maintaining the cellular homeostasis, since it degrades damaged organelles and protein aggregates, and at recycling cell components to gain macromolecules and energy during stress conditions (22). The autophagic process is carried out through the formation of a double-membrane vesicle called phagophore or isolation membrane, which evolves into a mature autophagosome containing the cargo to be degraded and fuses with lysosomes to form autolysosomes to enable the completion of autophagy (23). The main regulators of the first steps of autophagy are the autophagy-related proteins (Atg) implicated in the formation and maturation of the autophagosome, and the structural microtubule-associated protein 1 light chain 3 and its interactor p62/sequestosome-1, which specifically recognize and load the cargo into vesicles (23,24). Autophagy is generally considered a survival mechanism, although its occurrence has also been associated with a particular type of programed cell death known as autophagic cell death (ACD) or type II cell death, which is specifically inhibited by autophagy suppression (25). Despite poor characterization, it is well accepted that ACD occurs in a caspase-independent way (25).

Pharmacologically active compounds can modulate different types of cancer cell death depending on the cellular setting. Moreover, the discovery of fundamental interconnections between the different cell death mediators and signaling pathways, regulated by active compounds, could represent novel opportunities for targeting tumor cells. Since the mechanisms by which DHEA is able to induce cell death is still obscure, the aim of this work was highlighting its potential role for context-specific anticancer effect. We demonstrated that DHEA induces canonical apoptosis in carcinoma HeLa cell line, whereas it promotes ACD in HepG2 cells through the activation of jun N-terminal kinase (JNK) that in turn stimulates the nuclear translocation of transcription factor nuclear factor (erythroid-derived)-2-like-2 (Nrf2), thus enhancing p62 expression. We present several lines of evidence that p62 accumulation in response to DHEA stress stimuli positively regulates autophagy, which turns out toxic and leads to cell death. Overall, our work shed light on a new antitumor role of DHEA through the promotion of ACD, making this molecule a good candidate as antitumor drug particularly beneficial to treat apoptosis-resistant cancer.

Materials and methods

Materials

Cloroquine (CQ), dimethylsulfoxide (DMSO), TRI Reagent, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 3-methyladenine (3-MA), paraformaldehyde, iodoacetic acid, glucose-6-phosphate, 6-phosphogluconate, β-nicotinamide adenine dinucleotide phosphate disodium salt (NADP+), SP600125 and Triton X-100, H2O, and etoposide (ETO) were from Sigma–Aldrich. Trypan blue 0.4% solution was from Lonza. (H + 1)-horseradish peroxidase conjugated goat anti-mouse and anti-rabbit IgG were from Bio-Rad Laboratories. Dihydroetidium (DHE), Alexa Fluor® 568 donkey anti-rabbit IgG (H + L), LysoTracker® Red DND-99 and Hoechst 33342 were from Life Technologies Ltd. TurboFect Transfection Reagent and DharmaFECT 1 Transfection Reagent were from Thermofisher Scientific. Z-Val-Ala-Asp-(Ome)-fluoromethylketone (z-VAD) was from MP Biomedicals. Necrostatin-1 (nec-1) was from Roche Applied Science. DHEA was from LKT Laboratories.

Cell lines and cell culture

Human hepatocellular carcinoma HepG2 cells (ICCL HTL95005) and human cervix carcinoma HeLa cells (ICCL HTL95023) were obtained from the ‘Istituto Nazionale per la Ricerca sul Cancro’, Genova (Italy). HepG2 were grown in RPMI 1640 with 1-glutamine (Lonza) supplemented with 10% fetal bovine serum (EuroClone) and 10 μM penicillin/streptomycin (Lonza). HeLa were grown in Dulbecco’s modified Eagle’s medium 4.5 g/l (Lonza) supplemented with 10% fetal bovine serum (EuroClone), 2 mM 1-glutamine (Lonza) and 100 μg/ml ampicillin/streptomycin (Lonza). Cells were cultured at 37°C in an atmosphere of 5% CO2 in air. During the experiments, cells were plated at a density of 2 × 104 cells/ml. Both cell lines were authenticated and characterized by the supplier. The cells were expanded immediately and multiple aliquots were cryopreserved. Cells were used within 6 months of resuscitation.

Cell viability

The percentages of dead and viable cells were evaluated by Trypan blue staining procedure.

Treatments

The caspase inhibitor z-VAD and the RIPK1 inhibitor nec-1 were both prepared at a concentration of 10 mM and used at a final concentration of 10 μM. A 500 mM solution of the autophagy inhibitor 3-MA was prepared just before the experiments and used at a final concentration of 5 mM.
JNK inhibitor SP600125 was used at a final concentration of 25 μM starting from a 25 mM stock solution. ETO was used at a final concentration of 50 μM starting from a 100 mM stock solution. The antioxidant Trolox was used at a final concentration of 200 μM starting from a 20 mM solution. All these compounds were used with a pretreatment of 1h before DHEA treatments. A 100 mM solution of DHEA was prepared just before the experiments by dissolving the lyophilized compound in DMSO and used at the indicated final concentrations. DHEA was dissolved in DMSO at a 5 mM concentration and used at a final concentration of 50 μM. The inhibitor of the late phase of autophagy CQ was dissolved in H₂O and used at a final concentration of 50 μM. Both DHE and CQ were added to the medium 30 min before the end of the indicated times. Treatments were all performed in serum-supplemented media and maintained throughout the experiments for the indicated times.

**Western blot analyses**

Total protein lysates were obtained as reported previously (26), electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane. Primary antibodies used are as follows: anti-caspase 9, anti-Atg5 and anti-p-c-Jun (Ser63) were from Cell Signaling Technology; anti-LC3 and anti-Tubulin were from Sigma–Aldrich; anti-p-62/SQSTM1 was from MBL; anti-Actin, anti-Beclin-1, anti-p-JNK (Thr183/Tyr185), anti-JNK, anti-Nrf2, anti-β-Actin, anti-LAMP-1 and anti-G6PDH were from Santa Cruz Biotechnology. The specific protein complex, formed upon incubation with specific secondary antibodies, was identified using a Fluorchem Imaging System (Alpha Innotech) after incubation with LiteAblot® TURBO (EuroClone).

**Plasmids, siRNAs and transfections**

Knockdown of Atg5, p62/SQSTM1 and G6PDH was performed by transfecting cells with On-Target Plus Smart Pool small interference RNA (siRNA) (Dharmacon). Controls were transfected with a scrambled siRNA duplex, which does not present homology with any other human mRNA (siCtrl) (Santa Cruz Biotechnology). For overexpression, cells were transiently transfected with pmxEGFP™-Green-C empty vector or pmxEGFP™-Green-C containing the dominant negative Nrf2 protein (D/N Nrf2), eGFP–LC3-containing plasmid (a kind gift of Prof. F. Cecconi—Department of Biology, University of Rome ‘Tor Vergata’, Rome), pMXs-puro GFP encoding p62 (provided by Dr. C. Rodolfo, Department of Biology, University of Rome ‘Tor Vergata’, Rome), pMXs-puro GFP empty vector and pMXs-puro GFP encoding p62, respectively, according to the manufacturer’s instructions. Plasmids and siRNAs were transfected using TurboFect Transfection Reagent and DharmaFECT I Transfection Reagent, respectively, according to the manufacturer’s instructions.

**Cell fractionation**

To obtain nuclear extracts, cells were lysed in nucleic buffer (1 mM K₂HPO₄, pH 6.4, 150 mM NaCl, 14 mM MgCl₂, 1 mM ethyleneglycol-bis(aminomethylene)-tetraacetic acid, 0.1 mM dithiothreitol, 0.3% Triton X-100), centrifuged at 450g for 10 min at 4°C. After 3 washes with a buffer lacking nucleic acid, the nuclear fractions obtained were further lysed with radioimmunoprecipitation assay buffer (50 mM Tris–HCL, pH 8, 150 mM NaCl, 1% sodium dodecyl sulfate).

**Quantitative real-time PCR**

Cells were homogenized in TRI Reagent, and RNA was extracted according to manufacturer’s instructions. Total RNA was resuspended in ribonuclease-free water and 1 μg of total RNA was used to generate first-strand cDNA using the GoScriptReverse Transcription System (Promega). In order to hybridize unique regions of the appropriate gene sequence, specific sets of primer pairs were designed and tested with primer-BLAST (NCBI). Primers used were obtained from Sigma–Aldrich and are as follows: p62/SQSTM1, forward: 5′-GGGAAAGGCGCTGCAGCAGGG-3′, reverse: 5′-CTGGCCACCCCGAAGTGTCCG-3′; Actin, forward: 5′-GCGGAGGACCTTTGATTGCA-3′, reverse: 5′-GGGACTTCCTGTAACAACGGA-3′. Real-time PCR was performed using the iTAQ universal SYBR Green Supermix (Bio-Rad) on a StepOne real-time PCR System (Applied Biosystems). All reactions were run as triplicates. Data were analyzed by the StepOne™ Software (v2.3) using the second-derivative maximum method. The fold changes in mRNA levels were relative to a control after normalization to the internal standard Actin.

**Reactive oxygen species evaluation**

Thirty minutes before the end of the experimental time, cells were incubated with 50 μM DHE at 37°C. Cells were then washed, resuspended in ice-cold phosphate-buffered saline (PBS) and collected. The fluorescence intensities of 2-hydroxyethidium, formed by the reaction of DHE with reactive oxygen species (ROS), were analyzed cytofluorimetrically by recording FL-2 fluorescence by means of a FACSCalibur instrument.

**Fluorescence microscopy analyses**

After the described treatments, cell medium was removed and cells were washed in PBS and fixed with 4% paraformaldehyde for 10 min, permeabilized with a solution of PBS/0.4% Triton X-100 for 10 min, blocked with PBS/10% bovine serum for 30 min, incubated overnight with an anti-Nrf2 antibody and then for 1 h with an Alexa Fluor® 488 dye-conjugated secondary antibody; nuclei were stained with 1 μg/ml of Hoechst 33342 for 10 min. To detect autophagolysosomes, cells were transfected with EGF–LC3 plasmid for 24 h and, after the described treatments, prepared for immunofluorescence analysis as described for Nrf2 but, in this case, fixed cells were incubated overnight with an anti-LAMP-1 antibody and for 1 h with an Alexa Fluor® 568 dye-conjugated secondary antibody; nuclei were stained with 1 μg/ml of Hoechst 33342 for 10 min. Fluorescent images of cells were digitized with a Delta Vision Restoration Microscopy System (Applied Precision, Issaquah, WA) equipped with an Olympus IX70 fluorescence microscope (Olympus Italia, Segrate, Milano, Italy).

**G6PDH enzyme activity assay**

G6PDH enzyme activity was determined in an Eppendorf BioSpectrometer® spectrophotometer by measuring the rate of increase in fluorescence at 340 nm due to the conversion of NADP⁺ to NADPH by either G6PDH or 6-phosphogluconate dehydrogenase. First, the combined activity of G6PDH and 6-phosphogluconate dehydrogenase was measured in the presence of glucose-6-phosphate and 6-phosphogluconate. The activity of 6-phosphogluconate dehydrogenase alone was determined in the presence of 6-phosphogluconate. The rate of change was measured over a period 6 min observation period. G6PDH activity was calculated as the difference of the two activities. Cell lysates were added to the reaction buffer containing 50 mM Tris, 1 mM MgCl₂, pH 8.1. Substrate concentrations were as follows: glucose-6-phosphate and 6-phosphogluconate (200 μM) and NADP⁺ (100 μM). For calculations of specific activity, a molar extinction coefficient of 6220 for NADPH was used. Enzyme activities were normalized based on protein concentration determined by Lowry method.

**Protein carbonyls detection**

Carbonylated proteins were detected using the Oxyblot® Kit (Millipore) according to the manufacturer’s protocol.

**Measurement of glutathione**

Measurement of glutathione was assayed by high-performance liquid chromatography as described previously (27). Briefly, intracellular glutathione was assayed upon formation of 5-carboxymethyl derivatives of free thiols with iodoacetic acid, followed by the conversion of free amino groups to 2,4-dinitrophenyl derivatives by the reaction with 1-fluoro-2,4-dinitrobenzene as described by Reed et al. (28). Cells were washed with PBS, resuspended and lysed by repeated cycles of freezing and thawing under liquid nitrogen.

**Protein determination**

Proteins were determined by the method of Lowry et al. (29).

**Data analysis**

Data were from at least 3 independent experiments, unless otherwise indicated. The results are presented as means ± SD. Statistical evaluation was conducted by using Student’s t-test. Comparisons were considered significant at P ≤ 0.05 and extremely statistically significant at P ≤ 0.01.
Results

DHEA differently affects viability of HepG2 and HeLa cell lines

The mechanisms underlying the well-established antitumor properties of DHEA are still undefined and even controversial. In particular, it remains unclear whether DHEA induces or inhibits cell death and if this effect is related to G6PDH inhibition. To this aim, we chose two different types of human carcinoma cell lines, HepG2 and HeLa, and treated them for 24 h with different concentrations of DHEA to evaluate the percentage of dead cells. Direct cell counting by Trypan blue exclusion test revealed that HepG2 cells were significantly resistant to treatment up to 300 µM and only 50% cells died at the highest dose (Figure 1A, left) compared with HeLa. Indeed, the sensitivity of HeLa cells to DHEA was evident already at lower concentrations (Figure 1A, right). To dissect the molecular determinants of such different sensitivity, we looked at caspase 9 activation as a marker of apoptotic cell death. Upon treatment with DHEA, we observed the presence of caspase 9 cleavage in HeLa cells treated with DHEA (Figure 1B, right) and in HepG2 treated with the ETO, used as a positive control of caspase-dependent apoptosis (Figure 1B, left). Strikingly, z-VAD in combination with DHEA reduced the percentage of dead cells in HeLa (Figure 1C, right), whereas it exacerbated cytotoxicity in HepG2 cells (left). Thereafter, we focused on HepG2 cell line since our findings revealed a previously uncovered mechanism of DHEA cytotoxicity, which was independent from apoptosis.

DHEA induces ACD in human hepatoma cell line HepG2

The observation that the combination of DHEA with z-VAD increased the susceptibility of HepG2 to DHEA treatment prompted us to investigate whether necroptosis or ACD could be responsible for DHEA-induced cell death. Indeed, these two death processes are often activated when the caspases activity is compromised (21,30). Necroptosis, classically induced by apoptogenic compounds (e.g. staurosporine) in presence of caspase inhibitors (31), is reverted by the use of nec-1 that inhibits RIPK1. Results depicted in Figure 2A showed that nec-1 failed to protect cells from death, indicating that HepG2 did not die by necroptosis. Next, we looked at ACD and the use of 3-MA and siRNA against Atg5, both of which suppress autophagy at its early stage, protected cells from DHEA cytotoxicity, suggesting that autophagy was responsible for cell death (Figure 2B and C). Besides being efficiently reverted by autophagy inhibitors, another sine qua non hallmark of ACD is the presence of autophagic flux. To assess this issue, we evaluated the protein levels of canonical autophagic markers and substrates. Results depicted in Figure 2D demonstrated a substantial increase of the lipidated form of LC3 and of Beclin-1 and p62 levels, which are consistent with DHEA triggering stress-mediated autophagy. Moreover, immunofluorescence analysis confirmed the presence of EGFP–LC3 puncta, representative of autophagosomes, co-localizing with lysosomes (stained by LAMP-1) in DHEA-treated cells (Figure 2E). Taken together these results indicate that DHEA activates ACD in HepG2 cells.

p62 is required for DHEA-mediated autophagy and ACD

The increase of p62 levels under DHEA treatment was unexpected since the protein is normally degraded by the autophagic machinery. To discriminate between an increase and a block of the autophagic flux, we co-treated cells with DHEA and chloroquine, a widely used inhibitor of the fusion of autophagosomes and lysosomes and of lysosomal protein degradation. Under these conditions, we observed a further increase of LC3-II and p62 suggesting that DHEA did not lead to defective autophagy and did not impair p62 lysosomal degradation (Figure 3A). Thus, we moved at investigating whether other mechanisms could induce p62. Quantitative PCR analysis revealed a significant increase of p62 mRNA levels in treated cells compared with controls, indicating that p62 expression, rather than its turnover, was affected (Figure 3B). Importantly, we found that p62 knockdown by specific RNA interference strongly reverted DHEA-induced cell death suggesting that p62 accumulation is implicated in the promotion of ACD in HepG2 cells (Figure 3C). In order to dissect the underlying mechanisms, we checked for autophagy induction upon siRNA against p62 and discovered that LC3-II levels were decreased (Figure 3D). Altogether, these findings highlighted the pivotal role of p62 in the activation of DHEA-triggered autophagy and consequent ACD.

DHEA induces ACD independently of oxidative stress and G6PDH inhibition

In searching for the possible mechanism(s) by which DHEA induced autophagy, we focused on oxidative stress since DHEA ability to inhibit G6PDH could lead to a decrease of NADPH levels with consequent disruption of the cellular redox homeostasis. We confirmed that DHEA efficiently inhibited G6PDH activity by 50–60% (Supplementary Figure 1A, available at Carcinogenesis Online). DHEA staining analysis by flow cytometry revealed that DHEA induced an early and transient burst of ROS, which was restored to the control levels by the use of the antioxidant Trolox (Figure 4A). Consistent with this result, we observed time-dependent protein oxidation (in the form of carbonylation) (Figure 4B), which was restored by Trolox (Supplementary Figure 1B, available at Carcinogenesis Online). As a positive control for protein carbonylation, we took advantage of three different concentrations of H2O2 (namely 250, 500 and 1000 µM) and confirmed that Trolox reverted H2O2 (250 µM)-induced protein oxidation (Supplementary Figure 1B, available at Carcinogenesis Online). Moreover, DHEA caused a decrement of the reduced form of the antioxidant tripeptide glutathione, paralleled by the increase of its oxidized form (GSSG) (Figure 4C). However, the effects of DHEA that we observed in our experimental model were independent from the inhibition of G6PDH since the genetic knockout of the enzyme did not lead to an accumulation of p62 protein levels (Figure 4D). Accordingly, we observed that Trolox was unable to revert both p62 increase and LC3 lipidation (Figure 4E). On the other hand, H2O2 promoted a normal autophagic flux, characterized by LC3 lipidation and p62 degradation (Supplementary Figure 1C, available at Carcinogenesis Online). Taken together, these results indicate that DHEA induction of cell death is independent of G6PDH inhibition and oxidative stress.

JNK controls p62 expression under DHEA treatment

There is general consensus that the activation of JNK mitogen-activated protein kinase is required for the triggering of ACD (25,32). Thus, we investigated whether JNK was involved in DHEA-promoted death. We observed a transient and early accumulation of JNK-phosphorylated active form (Thr183/Tyr185), which correlated with a concomitant phosphoactivation of its downstream target c-Jun (Ser63) (Figure 5A). Puissant et al. (33)
recently revealed a direct role of JNK in the induction of p62, and this prompted us to question whether DHEA could activate the JNK–p62 axis resulting in ACD. To this aim, we used the JNK-specific inhibitor SP600125, which efficiently inhibited phosphorylation of both JNK and c-Jun (Supplementary Figure 1D, available at Carcinogenesis Online). Figure 5B and C show that pretreatment with SP600125 attenuated p62 protein accumulation and, notably, decreased p62 mRNA levels indicating that JNK controlled p62 expression at the transcription level. To further confirm that JNK was upstream of p62-mediated ACD, we evaluated the percentage of dead cells that was significantly reduced by the use of SP600125 (Figure 5D). These data suggest that DHEA activated JNK that, in turn, positively regulated p62 transcription. We also confirmed that oxidative stress was not the answer to explain how JNK was activated under DHEA treatment since, as shown in Figure 5E, preincubation with Trolox failed to prevent JNK and c-Jun phosphorylation. Furthermore, we could infer that DHEA specifically induces p62-mediated ACD since p62 overexpression alone, up to 48 h (the efficiency of transfection is shown in Figure 5F), did not lead to cell death (data not shown). While investigating the mechanisms of JNK activation, Geeraert et al. (34) work gave us hint about a possible involvement of hyperacetylation of tubulin. Indeed, we found a rapid increase of acetyl-tubulin levels under DHEA treatment consistent with the times of JNK activation (Supplementary Figure 1E, available at Carcinogenesis Online). The precise mechanisms of tubulin acetylation increase require deeper study.

Nrf2 is responsible for JNK-promoted p62 accumulation under DHEA treatment

Finally, in searching for the molecular determinants of JNK-induced p62 expression, we focused on the transcription factor Nrf2, a master regulator of the antioxidant response (35, 36). Indeed, there is evidence that Nrf2 can be activated by JNK and translocates into the nucleus to promote the transcription of its target genes (37, 38). Moreover, other studies identified p62 as a target gene of Nrf2 (39, 40), and this was rather appealing in our study since Nrf2 could make the perfect bridge between JNK and p62. To verify this hypothesis, we checked for Nrf2 intracellular localization under DHEA treatment and found that Nrf2 translocated into the nucleus (Figure 6A). Moreover, immunofluorescence analysis revealed that Nrf2 migration to the nucleus was...
suppressed by the presence of SP600125 (Figure 6B), strengthening our model of JNK activation upstream of Nrf2. On the contrary, Trolox failed to prevent Nrf2 translocation into the nucleus (Supplementary Figure 2B, available at Carcinogenesis Online). Finally, we transfected cells with a D/N Nrf2 plasmid (the efficiency of transfection is showed in Supplementary Figure 2A, available at Carcinogenesis Online) to assess whether the inhibition of Nrf2 could affect p62 and LC3-II levels, and
as expected, results depicted in Figure 6C show that D/N Nrf2 caused a decrease of both proteins. Strikingly, D/N Nrf2 rescued DHEA-induced cell death as well (Figure 6D). These results shed light on a mechanism of ACD induced by DHEA mediated by the JNK–Nrf2–p62 axis.

Discussion

Metabolic adaptations of cancer cells are hallmarks of neoplastic transformation, but at the same time, they represent potential target for antitumor drugs. Moreover, pharmacological active compounds can modulate different programmed cell death pathways, depending on the cellular setting. In line with this concept, we observe a different cell death commitment by treating carcinoma cells with DHEA, with HeLa activating apoptosis, while HepG2 dying with the features of ACD. This represents a previously uncovered aspect of DHEA-mediated anticancer effects setting the stage for its clinical use against apoptosis-resistant/defective malignancies.

Bringing further insights into the mechanism of action of DHEA on autophagy, we showed that the characteristic lipidated form of LC3 and increased levels of autophagolysosomes depict the process. The exacerbation of ACD in the presence of z-VAD is supported by the observation that the caspase inhibitor alone can trigger ACD via JNK and extracellular signal-regulated kinase activation (41). Interestingly, we also observed an increase of the levels of Beclin-1, which has been associated with ACD occurrence. Indeed, it was reported that Beclin-1 knockout prevented ceramide-induced ACD in Hep3B and CNE2 cells (42). The authors also demonstrated that the upstream inducer of Beclin-1 was JNK, the inhibition of which allowed autophagy inhibition, strengthening the growing consensus depicting JNK as an ACD mediator.

We showed that, upon DHEA treatment, JNK contributes to the execution of ACD because its inhibition significantly rescued HepG2 cells from the induced cell death. Although we did not investigate the genuine mediator of JNK activation, we provide evidence that it was not related to induction of oxidative stress. Indeed, DHEA treatment primed cells to a higher intracellular ROS burst thus resulting in a sustained metabolic oxidative stress that culminates in protein carbonyl production and glutathione oxidation, in agreement with its capacity to function as a competitive inhibitor of G6PDH. However, our study indicated
pretreatment with antioxidants such as Trolox was ineffective in inhibiting JNK activation and its downstream effect on ACD. The unrelated link between oxidative stress and ACD induction was clearly confirmed by the results obtained from experiments performed upon G6PDH downregulation, where we did not observe any increase of p62 levels.

The capability of JNK to regulate autophagy commitment finds support in studies documenting its activation following tubulin hyperacetylation. Indeed, it has been found that, under nutrient deprivation, tubulin hyperacetylation on lysine 40 promotes the activation of JNK which, in turn, liberates Beclin-1 from its inhibitory boundary to B-cell lymphoma 2 (Bcl-2) thus facilitating autophagosome formation and transport along the microtubules (34). However, the upstream sensor/mediators for such observed process were not identified and the authors just speculated on a derangement between acetyltransferase/deacetylase activities. Our results show a very significant hyperacetylation of tubulin after DHEA treatment. Therefore, we can hypothesize that tubulin hyperacetylation could be responsible for JNK activation also in our system. Work is presently underway...
in our laboratory to find the missing link between DHEA treatment and tubulin acetylation and to fill the gap between tubulin acetylation and JNK activation.

In search of the molecular process that links JNK activation to ACD stimulation, we focused on the high expression of p62 under DHEA treatment. This was an unexpected result because p62 represents the interactor that recognizes and loads the cargo into the autophagosomes, a process finally leading to its degradation. Indeed, we found that DHEA induced JNK-mediated p62 expression and this led to the commitment of cells to ACD: in fact, p62 knockdown decreased LC3 lipidation (and so autophagy occurrence) and, more importantly, rescued cells from death. Our findings are in accordance with Puissant et al. (33) who demonstrated that natural compound resveratrol (RSV) promotes ACD in chronic myelogenous leukemia cells via JNK-dependent p62 expression. Data shown in this article encourage us to believe that JNK/p62 pathway could be a canonical way of induction of ACD, and thus, our study may support comprehension of
Figure 6. DHEA promotes p62 expression through the JNK–Nrf2 axis. (A) HepG2 cells were treated for the indicated times with 300 µM DHEA and nuclear fractions were collected. Western blot analyses of Nrf2 were performed. Lactate dehydrogenase (LDH) and Lamin A/C were used as cytosol and nuclei purity control, respectively. Numbers indicate relative Nrf2/LDH ratio (first three lanes) and Nrf2/Lamin A/C ratio (last three lanes). (B) HepG2 cells were pretreated for 1h with either vehicle or 25 µM of JNK inhibitor SP600125 and then treated for 6h with 300 µM DHEA. Fluorescence microscope analyses were performed to analyze Nrf2 (green fluorescence) localization. Hoechts 33342 (nuclei). (C) HepG2 cells were transfected with either the empty vector or a plasmid containing D/N Nrf2. Twenty-four hours after transfection, cells were treated for the indicated times with 300 µM DHEA. Western blot analyses of p62 and LC3 were performed. Actin and Nrf2 were used as loading and expression controls, respectively. (D) HepG2 cells were transfected with either the empty vector or a plasmid containing D/N Nrf2. Twenty-four hours after transfection, cells were treated for 24h with 400 µM DHEA and percentage of dead cells was assayed by Trypan blue staining procedure. Data are presented as mean ± SD of n = 3 independent experiments. *P < 0.05 against empty vector DHEA. Western blots and immunofluorescence images are from one experiment representative of three that gave similar results.
this poorly characterized death process. Indeed, we also found that p62 overexpression alone was not sufficient to induce ACD, indicating that cell death is a specific effect of DHEA-activated pathway.

In our model, DHEA-activated JNK positively regulates Nrf2 translocation into the nucleus, and this is not the first evidence of the interplay between the kinase and the transcription factor. The activation of this pathway has usually been observed following treatment with compounds inducing phase II detoxifying and antioxidant stress genes in different cell types, including HepG2 cells (37,38,43,44). According to the authors, the chemopreventive effects of these molecules were due to the induction of Nrf2-mediated antioxidant response that rescued cells from oxidative stress. On the other hand, there is growing and compelling evidence that a persistent activation of Nrf2 could be detrimental to cells, especially in the liver (45,46). Indeed, it is largely accepted that p62 is able to bind the C-terminal of Kelch-like ECH-associated protein 1 (Keap1), which is responsible for Nrf2 proteasome-dependent degradation under normal conditions, thus allowing Nrf2 to migrate into the nucleus and exert its transcriptional activity (45,47). p62 is itself a target of Nrf2, and its transcription could, thus, give rise to a positive feedback loop with Nrf2 that culminates in a lethal accumulation of p62-containing transcriptional activity (45,48). It is well demonstrated that excess of p62 levels dampened the degradation of ubiquitinated proteins via proteasome leading to increased intracellular aggregates that eventually led to cell death.

Overall, our work highlights a new non-aptotic cytotoxic effect of DHEA, which could explain the potent antitumor properties of the molecule. Moreover, our results strengthen the feasible application of therapeutic regimens exploiting autophagy as an effective cancer cell-killing strategy, particularly to target apoptosis-resistant cells.

**Supplementary material**

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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**References**