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Hydroxytyrosol prevents chondrocyte death under oxidative stress by inducing autophagy through sirtuin 1-dependent and -independent mechanisms

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Abbreviations: AMPK, AMP-activated protein kinase; ECM, extra-cellular matrix; GRO α , growth-related oncogene α ; HT, hydroxytyrosol; LC3, microtubule-associated protein 1 light chain 3; MDC, monodansylcadaverine; OA, osteoarthritis; qRT-PCR, quantitative RT-PCR; ROS, reactive oxygen species; siRNA, short interfering RNA; SIRT-1, sirtuin 1; TOM20, translocase of outer mitochondrial membrane.

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ABSTRACT

Background: Hydroxytytosol (HT), a major phenolic antioxidant found in olive oil, can afford protection from oxidative stress in several types of non tumoral cells, including chondrocytes. Autophagy was recently identified as a protective process during osteoarthritis (OA) development and critical for survival of chondrocytes. Therefore we have investigated the possibility to modulate chondrocyte autophagy by HT treatment.

Methods: DNA damage and cell death were estimated in human C-28/I2 and primary OA chondrocytes exposed to hydrogen peroxide. Autophagic flux and mitophagy were monitored by measuring levels and location of autophagy markers through western blot, immunostaining and confocal laser microscopy. Late autophagic vacuoles were stained with monodansylcadaverine. The involvement of sirtuin1 (SIRT-1) was evaluated by immunohistochemistry, western blot and gene silencing with specific siRNA.

Results: HT increases markers of autophagy and protects chondrocytes from DNA damage and cell death induced by oxidative stress. The protective effect requires the deacetylase SIRT-1, which accumulated in the nucleus following HT treatment. In fact silencing of this enzyme prevented HT from promoting the autophagic process and cell survival. Furthermore HT supports autophagy even in a SIRT-1-independent manner, by increasing p62 transcription, required for autophagic degradation of polyubiquitin-containing bodies.

Conclusions: These results support the potential of HT as a chondroprotective nutraceutical compound against OA, not merely for its antioxidant ability, but as an autophagy and SIRT-1 inducer as well.

General Significance: HT may exert a cytoprotective action by promoting autophagy in cell types that may be damaged in degenerative diseases by oxidative and other stress stimuli.

Keywords: Autophagy, Chondrocyte, Hydroxytyrosol, Osteoarthritis, Oxidative stress, SIRT-1

1. Introduction

Autophagy is recognized as an evolutionary conserved pathway, keeping cellular homeostasis through removal and recycling of damaged macromolecules and organelles [1]. The autophagic pathway entails sequestration of cytoplasmic components in double-membrane vesicles termed autophagosomes and proceeds through distinct phases. Cell energy stress and environmental stimuli impact on regulatory factors that signal to the autophagic machinery, which involves homologues of the proteins coded by autophagy-related genes (*Atg*) originally identified in yeasts. Stimulation of Beclin 1-VPS34-ATG14L complex promotes autophagosomal membrane nucleation, then two ubiquitin-like conjugation systems intervene for autophagosome elongation and maturation: the ATG5-ATG12 and the microtubule-associated protein light chain 3 (LC3/ATG8) conjugation system. In particular, the appearance of a membrane-associated, phosphatidylethanolamine-conjugated form of LC3 (LC3-II), detectable by western blot and visible as discrete puncta on immunofluorescence analysis, is a key regulatory step and indicator of autophagosome formation. In the last steps, autophagosomes fuse with lysosomes and finally the cargo is degraded, released in cytosol and recycled. Notably, the ubiquitin- and LC3-binding protein SQSTM1/p62 is critical for autophagic degradation of various cargos including ubiquitinated proteins, damaged mitochondria or peroxisomes, and itself undergoes selective turnover through autophagy [2].

Although the role of autophagy is context- and tissue-dependent and not fully understood, this process has been observed to decrease during aging and in several age-related diseases, including osteoarthritis (OA). In fact defective autophagy has recently emerged as a feature of articular cartilage in OA-affected joints in both humans and animal models [3]. Chondrocytes, which are responsible for maintaining a functional extra-cellular matrix (ECM) in healthy cartilage, are substantially post-mitotic cells that rely on a relatively high constitutive level of autophagy, and interference with the autophagic process has proved to cause augmented death of chondrocytes and the onset of OA [4].

Major risk factors for OA are aging, trauma and defective mechanical loading, obesity and metabolic syndrome, and related, distinct subtypes of OA can be envisaged [5]. Nevertheless, aging-associated molecular changes in joint cartilage as well as mechanical, inflammatory and metabolic stressors can all lead to increased levels of reactive oxygen species (ROS), which provoke oxidative stress and critically contribute to the damage of chondrocytes and their capability to regulate ECM remodeling [6]. Mitophagy, i.e. removing of mitochondria in autophagosomes, normally eliminates aged mitochondria, a main source of ROS and oxidative stress, thus exerting homeostatic control on OA-related gene expression in chondrocytes [7]. However it has been

reported that experimentally induced mitochondrial dysfunction associated with excessive ROS production led to a decline in autophagy in human T/C-28a2 chondrocytes [8]. Conversely pharmacological activation of autophagy protects these cells from dysfunctional mitochondria and cell death [8]. Therefore autophagy modulation has been proposed as a protection strategy for chondrocytes and cartilage from aging and OA.

A useful alternative to current, unsatisfactory drug treatment of OA, mainly based on NSAIDs, may be represented by the employment of food-derived molecules [9, 10]. Several compounds are promising, but detailed molecular and cellular studies are required to ascertain their potential and optimize their use. In particular information is lacking about the ability of these “nutraceuticals” to modulate chondrocyte autophagy, with the notable exception of glucosamine [11], a precursor of ECM glycosaminoglycans.

Phenolic components of olive oil, a main source of fat in the “Mediterranean diet”, may be added to the list of dietary factors that deserve to be tested. In fact some studies in animal models of arthritis [12-14] and a few pilot trials [15-17] indicate that treatments with olive oil or olive phenolics-enriched extracts may be beneficial for OA patients. Hydroxytyrosol (HT), as a powerful antioxidant and major phenol present in olive oil, has been the object of intensive investigation, mainly focused to discover health benefits to prevent or treat cardiovascular diseases, diabetes and cancer [18, 19]. Quite recently we have found that HT can afford protection against DNA damage, cell death and expression of OA-related markers in human primary chondrocytes stimulated by hydrogen peroxide (H_2O_2) or growth-related oncogene α (GRO α), a chemokine involved in OA pathogenesis [20]. In the present study we have unveiled the ability of HT to induce autophagy and identified some molecular events by which HT modulates the autophagic process and protects human chondrocytes under conditions of oxidative stress.

2. Material and Methods

2.1. Cell cultures and viability

C-28/I2 chondrocytes [21], kindly provided by Dr. Mary Goldring, are a human cell line representative of primary chondrocytes that has been used extensively as a reproducible “in vitro” model to study chondrocyte physiopathology in experiments requiring large numbers of cells. They were grown in DMEM-F12 medium supplemented with 10% fetal bovine serum as previously detailed [22]. With local Ethics Committee approval, primary cultures of chondrocytes were used

and prepared from fragments of articular cartilage obtained from two adult OA patients undergoing knee arthroplasty as described [22]. The cells were incubated in the absence or presence of 100 μ M H_2O_2 for 2 to 48 h as indicated in the various figures; 100 μ M HT (from Cayman Chemical) was added 30 min before H_2O_2 , on the basis of a published previous study [20]. Autophagy inhibitors 3-methyladenine and cloroquine and the autophagy inducer rapamycin (all from Sigma-Aldrich) were used at 10 mM, 50 μ M and 500 nM, respectively, and were added to cell medium 30' before other treatments. Control cells received the corresponding volume of vehicle. Viable cells were directly counted following the trypan blue exclusion test. Dead cells including the dye were reported as a percentage of the total number of cells. The use of the human cells in this study was in accordance with the Helsinki Declaration of 1975.

2.2. Western blotting

In western blotting procedure equal amounts of cell extract were subjected to electrophoresis in 15% gels, blotted onto nitrocellulose membranes as previously described [23], and probed with primary antibody at 4°C overnight. The following antibodies were used: anti-LC3A/B (Cell Signaling Technology), anti-p62 (Santa Cruz Biotechnology), anti- β -actin (Sigma-Aldrich), anti-SIRT-1 (Santa Cruz Biotechnology).

After washes, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit (Cell Signaling Technology) or anti-mouse (Santa Cruz Biotechnology) IgG for 1 h. The chemiluminescent signals were detected using an ECL system (LuminataTM Crescendo, Millipore). β -actin was used as loading control. A representative image of visualized immunoreactive bands and densitometric analysis show the relative intensity of protein expression.

2.3. Detection of γ H2AX

The extent of oxidative DNA damage and in particular the amount of double strand breaks tagged by the phosphorylation of the histone H2AX were evaluated by flow cytometer on cells previously fixed with 2% PFA and then post-fixed with 90% methanol on ice, as described before [20]. After a treatment with 0.02 U/ml chondroitinase ABC (Sigma) in Tris/HCl pH 8, 20' at 37°C in a humid chamber and a step of blocking of nonspecific bindings (with 150 μ l 5% BSA in TBS with the addition of 0.1% Triton), a mouse anti- γ H2AX monoclonal antibody (Millipore 05-636, IgG1, at 5 μ g/ml) was incubated overnight at 4°C and then revealed by an Alexa Fluor 647 Donkey anti-mouse IgG (Jackson ImmunoResearch). Specificity was checked by running an isotypic control

for each sample under analysis: cells were probed with normal mouse immunoglobulins of the same isotype (IgG1) and at the same concentration of the anti- γ H2AX antibody. Analyses were performed by using a FACS Canto II flow cytometer (BD) on at least 5000 cells per sample. For each sample the level of “median channel of fluorescence intensity (MCFI) increment” of γ -H2AX staining was calculated as the difference between the median channel of fluorescence intensity of the samples stained for γ H2AX and that of the same sample probed with the isotypic control.

2.4. Immunocytochemical detection of autophagy and mitophagy

To carry out immunocytochemical studies chondrocytic (C-28/I2 or primary chondrocytes) cells were seeded in chamber slides. Autophagy was assessed by staining the cells with monodansylcadaverine (MDC) (Sigma-Aldrich), a lysosomotropic agent that accumulates in late autophagic vacuoles as a function of the acidity of their content. MDC was used at 50 μ M on viable cells 1 hour at 37°C. Then cells were washed, fixed with 2% PFA 10 min at RT and then few drops of an anti-fading agent (50 mg/ml 1,4-diazabicyclo[2.2.2]octane, SIGMA, in 90% glycerol in Tris-HCl, 0.1 M pH 8.0) were added before mounting the coverslips. Pictures were taken with a Nikon ELIPSE 90i Fluorescence Microscope equipped with a NIS software using the UV-2A filter set. Filter settings were as follows: excitation (EX): 330-380 nm, dichroic mirror (DM) at 400 nm and barrier filter (BA) for emission at 420 nm.

Immunofluorescence on cells grown on glass slides was used to detect the extent of “mitophagy” as assessed by colocalization of LC3 A/B positive autophagic vacuoles and the mitochondrial marker TOM20. At the end of the treatment, C-28/I2 cells were carefully washed, gently fixed with 2% PFA for 10 min and then the slides were stored at 4°C in PBS until processing.

Each condition was prepared in duplicate to allow for comparison of the cells stained with antibodies to LC3 A/B and TOM20 with cells stained with control antibodies. In the immunocytochemical protocol primary and secondary antibodies were diluted in 3% BSA in TBS with the addition of 0.1% Triton. Between each step, two washings with 3%BSA TBS left for 5' at RT under continuous stirring were performed to remove unbound reagents.

Before immunocytochemistry, further steps of permeabilization were carried out using a 10' treatment with ice-cold 90% methanol (100 μ l per well) followed by a treatment with 0.02 U/ml chondroitinase ABC (Sigma) in Tris/HCl pH 8, 20' at 37°C in a humid chamber. After a step of blocking of nonspecific bindings (with 150 μ l 5% BSA in TBS with the addition of 0.1% Triton), primary antibodies were delivered at the same time and left overnight at 4°C: a rabbit polyclonal

anti-LC3A/B antibody (Cell signaling) and a mouse monoclonal anti-TOM20 antibody (Santa Cruz Biotechnology). Immunodetection was performed by addition to all wells (sample and controls) of a biotinylated goat anti-rabbit polyclonal antiserum (DAKO) that was left for 30 min. Then, to reveal the stained proteins, the cells underwent addition of an Alexa Fluor 555 conjugated streptavidin (Molecular Probes) together with an Alexa Fluor 488 polyclonal donkey anti-mouse IgG (H+L) (Molecular Probes). At the end of a 30' incubation at RT, cells were washed and then underwent nuclear counterstaining with DAPI (Molecular Probes) left for 30' at RT. Finally, ProLong® Diamond Antifade Mountant (Molecular Probes) was applied to fluorescently labeled cells before image acquisition at the Nikon ELIPSE 90i Fluorescence Microscope. Distinct images were acquired for the green (TOM20), red (LC3A/B) and blue (nuclei) fluorescence and then merged to evidence sequestering of damaged mitochondria into autophagosomes. TOM20 green fluorescence was acquired with a FITC filter set (Ex=465-495; DM=505; BA=515-555); LC3A/B red fluorescence was acquired with a TRITC filter set (Ex=540/25; DM=565; BA=605/55); DAPI blue nuclear counterstaining was acquired with a UV-2A filter set as described above. Confirmation of mitophagy occurrence, was also carried out exploiting confocal laser scanning microscopy, using a NIKON confocal microscope system A1 equipped with a Nikon Eclipse Ti microscope and an Argon Ion laser for a 488 nm line and a DPSS laser for a 561 nm line. Emission signals were detected by a photomultiplier tube (DU4) preceded by emission filters BP 525/50 nm and BP 595/50 nm for AlexaFluor 488 and AlexaFluor 555 respectively. Laser scanning, image acquisition and processing were performed with Nikon Imaging Software NIS Elements AR-4 (Nikon Inc., USA). Fields of 210 µmx210µm (acquired with a Nikon plan apo 60x1.40 oil objective) were acquired and analyzed.

2.5. Immunocytochemical analysis of SIRT-1 protein

The level and subcellular localization of SIRT-1 were investigated by a colorimetric detection system, using the Supersensitive link-label (biotin based) IHC kit (Biogenex) and Fast red substrate (Biogenex), essentially as described by the manufacturer. The immunodetection was carried out on cells treated, fixed and permeabilized with both methanol and chondroitinase ABC as described above. SIRT-1 was detected with a mouse monoclonal anti-SIRT-1 antibody (LS-Bio) and signal specificity was checked by comparison with isotype controls, i.e. mouse IgG1 monoclonal (MAB002). At the end, to facilitate their automatic counting, the cells were treated with a DAPI nuclear counterstaining. Then, an automatic analysis procedure was employed exploiting the NIS software that combined colorimetric and fluorescent staining to easily count the cells. At least four fields (with 40-160 cells each) were counted for each condition. For each field the percentage of

cells with SIRT-1 localized in the nucleus was assessed. This experiment was carried out 4 times. To compare the different culture conditions, the average values of the four fields were taken into account for each experiment.

2.6. RNA interference

For transient transfection experiments, C-28/I2 cells were seeded at a density of 20000/cm² in 6-well plates in antibiotic-free growth medium. When the cells reached 70% confluence, they were transfected with 25nM ON-TARGETplus Human Sirt1 siRNA or ON-TARGETplus non-targeting pool (Dharmacon) by Lipofectamine® RNAiMAX Reagent in Opti-MEM® Medium (Life Technologies) according to manufacturer's instructions. The cells were incubated for 24 hours before evaluating mRNA expression or protein expression.

2.7. RNA isolation, cDNA synthesis and Real-Time PCR

Total cellular RNA was extracted with 700 µl TRIZOL (Invitrogen), according to manufacturer's instructions. The RNA pellets were treated with DNase (DNA-free, Ambion, Austin, TX) and, after buffer exchange, were quantified by using RiboGreen RNA quantitation reagent (Molecular Probes, Eugene OR). The same quantity of total RNA (100ng) was reverse transcribed by using random primers and the reagents provided with the Superscript VILO System for RT-PCR (Invitrogen). The cDNA mixture (2 µl) was used in Real time PCR analysis in a LightCycler Instrument (Roche Molecular Biochemicals) by means of the QuantiTect SYBR Green PCR kit (TaKaRa, Japan) with the following protocol: initial activation of HotStart Taq DNA polymerase at 95°C for 10", followed by amplification (40 cycles: 95°C for 5" followed by appropriate annealing temperature for each target as detailed below kept for 20"). The protocol was concluded by melting curve analysis to check amplicon specificity. The following primers were used: human p62 forward AAG-CCG-GGT-GGG-AAT-GTT-G and reverse GCT-TGG-CCC-TTC-GGA-TTC-T (Sigma-Aldrich); human LC3 forward AGC-ATC-CAA-CCA-AAA-TCC-CG and reverse TGA-GCT-GTA-AGC-GCC-TTC-TA (Sigma-Aldrich); human GAPDH forward TGG-TAT-CGT-GGA-AGG-ACT-CA and reverse GCA-GGG-ATG-ATG-TTC-TGG-A (Invitrogen). Primers were annealed at 59°C, except GAPDH at 56°C. The amount of mRNA was normalized for GAPDH expression in each sample and referred to untreated, control sample.

2.8. Statistical analysis

All the experiments shown were performed independently at least three times with comparable results. All the data presented in graphs are expressed as means \pm SEM. Means of groups were compared with GraphPad Prism 5 statistical software (GraphPad Software, Inc.) and analyzed for statistical significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. *Hydroxytyrosol leads to autophagy induction in C-28/I2 chondrocytes*

In a previous work with primary chondrocyte cultures exposed to oxidative stress, we found that HT protects against DNA damage and cell death [20]. To investigate the cellular and molecular mechanisms involved in these HT beneficial effects, we have performed a new study with C-28/I2 chondrocytes, which are widely used as representative of primary chondrocytes and allow abundant availability of cell material. First we have evaluated the ability of HT to stimulate autophagy. In order to monitor the autophagic process, two proteins widely used as markers of autophagy have been considered: LC3 and SQSTM1/p62 [2]. LC3 can be detected by western blot in non-lipidated and lipidated forms, termed LC3-I and LC3-II, respectively. This protein participates in phagophore assembly and, conjugated with phosphatidylethanolamine, in autophagosome formation. On the other hand, p62 operates as a molecular link between LC3 and ubiquitinated substrates, thus docking them to autophagy degradation. Decreased p62 levels, in combination with increased LC3-II, are commonly associated with autophagy activation [24]. In the current study, C-28/I2 cells were exposed to oxidative stress by the treatment with hydrogen peroxide for 2 h, in presence or absence of HT in the culture medium. HT induced LC3-II expression markedly and independently of the condition of oxidative stress (Fig. 1A and B). At the same time the level of p62 protein was reduced by HT treatment (Fig. 1A and C). These findings indicate that HT is able to stimulate the autophagic process.

Monodansylcadaverine (MDC) is an autofluorescent compound that stains late autophagic compartments (in particular autolysosomes). In our experiments we observed a reduced MDC fluorescence in C-28/I2 chondrocytes under oxidative stress conditions, 2 h after treatment with hydrogen peroxide (Fig. 1D). An opposite effect was shown in presence of HT. Moreover in this condition we assessed the increase in LC3-II puncta that colocalize with mitochondria, highlighted

with an antibody specific for the translocase of outer mitochondrial membrane, a protein with a Mw of 20 kDa (TOM20). Confocal laser scanning microscopy confirmed these findings, via a marked increase of these colocalized signals, evident as a yellow staining (Fig. 1E). Thus HT treatment promotes mitophagy along with autophagy in C-28/I2 cells.

3.2. Autophagy protects C-28/I2 cells from oxidative stress-induced DNA damage and cell death

Next we evaluated the ability of HT and autophagy to protect C-28/I2 chondrocytes against DNA damage and cell death under conditions of oxidative stress. In order to assess the degree of DNA damage and cell death, a phosphorylated form of histone H2AX referred to as γ H2AX was detected as a marker of DNA double strand breaks, while the percentage of dead cells was estimated by trypan blue exclusion test. We found that HT as well as the autophagy inducer rapamycin significantly reduced the DNA damage elicited by hydrogen peroxide after 2 h treatment (Fig. 2A). Moreover both compounds were able to decrease the percentage of dead C-28/I2 cells after 24 h (Fig. 2B). Conversely the pro-survival effect of HT was abolished in presence of autophagy inhibitors, in particular we used 3-methyladenine and cloroquine, operating at different steps in the autophagic process. These compounds alone did not change cell viability significantly. Thus, these data indicated that HT exerts its cell protective action by autophagy induction. It should be noted that the autophagy inhibitors were not able to impede the HT effect on DNA damage completely (not shown). Since rapamycin mimics HT in preventing DNA damage, it may be inferred that HT-stimulated autophagy counteracts both H_2O_2 -induced DNA damage and cell death. However in conditions of autophagy inhibition, HT can still contrast DNA damage presumably by the known, direct antioxidant action of HT (i.e. not mediated by autophagy), which instead is not sufficient to prevent cell death, a more tardive and irreversible effect.

3.3. HT modulates autophagy by SIRT-1-dependent and –independent mechanisms

SIRT-1, belonging to the sirtuin family of proteins, is considered a “longevity” and “anti-aging” factor and known to play a regulatory role in a variety of cellular processes, including autophagy [25]. Moreover, according to recent evidence, it can exert a pivotal role in the protection of cartilage from OA [26]. Therefore we have investigated the involvement of SIRT-1 in HT action. Immunostaining showed that this protein is localized both in the nucleus and cytosol of control, untreated C-28/I2 chondrocytes (Fig. 3A). Oxidative stress induced in cells by hydrogen peroxide

reduced nuclear signal compared with control, whereas HT, alone or in association with hydrogen peroxide, was able to significantly increase SIRT-1 level and nuclear localization (Fig. 3A and B).

The role of this modulation was evaluated by silencing the gene and testing the cell survival ability after oxidative stress in presence or absence of HT. The level of SIRT-1 protein in C-28/I2 chondrocytes was reduced by more than 60% after silencing procedure compared with control cells (Fig. 4A and B). The knockdown of SIRT-1 did not affect cell survival significantly under basal conditions or after hydrogen peroxide treatment, but it was able to abolish the protective effect of HT after oxidative stress (Fig. 4C).

Then, to assess the role of SIRT-1 in autophagy stimulation, C-28/I2 cells were treated with cloroquine for 24 h, so as to block autophagy flux and allow accumulation of the autophagic protein LC3-II, thus affording a better observation by western blot analysis. Figure 5A and 5B show that LC3-II accumulated in C-28/I2 chondrocytes treated with HT, but not in SIRT-1 silenced cells. LC3-II level was significantly higher in HT-treated cells with respect to correspondent SIRT-1 knockdown cells already in the absence of cloroquine, and increased further in the presence of cloroquine. SIRT-1 silencing alone did not affect LC3-II content. Therefore the ability of HT to induce LC3-II formation depends on SIRT-1 expression.

Subsequently, we tested the effect of HT on p62 and LC3 gene expression in order to investigate an additional, possible mechanism of autophagy modulation employed by SIRT-1. While no notable change in LC3 transcript content was observed (data not shown), HT significantly induced an up-regulation of p62 mRNA (Fig. 5C). However the modulation is not dependent on SIRT-1 because the HT ability to induce p62 expression was preserved in SIRT-1 knocked-down C-28/I2 cells. Therefore HT appears to stimulate the autophagic process by affecting both SIRT-1 dependent and independent pathways.

3.4. HT-induced autophagy protects human primary OA chondrocytes

In order to validate the findings on the role of autophagy in the HT protective effects in C-28/I2 cell line, some experiments were performed with human primary chondrocytes isolated from OA patients. Essentially similar results were found in primary chondrocytes treated with HT and exposed to oxidative stress, as documented in Fig. 6. In fact accumulation of LC3-II protein together with reduction of p62 protein was visualized by Western Blot following a brief HT treatment (Fig. 6A). Although differences were not statistically significant due to large variability (Fig. 6B,C), the ability of HT to stimulate the autophagic process was confirmed by

immunofluorescence images showing increase in LC3-II puncta colocalizing with mitochondria (Fig. 6D), regardless of H₂O₂ exposure, which, alone, has an inhibiting effect on autophagy.

Next it was evaluated the ability of HT and autophagy to protect primary chondrocytes against DNA damage and cell death following oxidative stress. As well as described before with C-28/I2 cells, HT and the autophagy inducer rapamycin were able to reduce the DNA damage elicited by H₂O₂ (Fig. 6E) and the percentage of dead cells significantly (Fig. 6F). Again the pro-survival effect of HT was eliminated by pre-treatment with autophagy inhibitors, Thus, these data indicated that HT exerts its cell protective action in C-28/I2 line and human OA chondrocytes by the same modalities.

4. Discussion

In a recently published paper [20], we have reported how HT prevented or reduced ROS accumulation, DNA damage, expression of proinflammatory-, ECM degrading- and other OA-related genes, as well as cell death of human chondrocytes treated with stressors involved in OA pathogenesis, i.e. H₂O₂ and GRO α . In the present study we have investigated some cellular and molecular events mechanistically involved in the protective action of HT in human chondrocytes treated with H₂O₂. The main findings of the present work are the following: 1) HT induces autophagy activation; 2) autophagy stimulation by HT mediates its protective action against cell death under oxidative stress; 3) autophagy stimulation by HT occurs through SIRT-1-dependent and -independent mechanisms.

It has been reported that HT can exert anti-oxidant, anti-inflammatory, anti-apoptotic and other protective effects in a variety of non-tumoral cells and biological systems, which may prevent or slow progression of degenerative diseases [18, 19]. However the mode of action of HT is largely undefined and can be only partly ascribed to its free-radical scavenging activity and direct anti-oxidant power. It has been shown in some experimental settings that HT can enhance the cellular anti-oxidant and detoxifying defence systems through modulation of signaling pathways impacting on key transcription factors, such as Nrf2 or FOXO3 [27, 28]. However the role of autophagy in these events was not investigated. To our knowledge this is the first study showing that HT can activate the autophagic process, thus protecting from cell death. This ability of HT to induce

autophagy may not be limited to chondrocytes and would be interesting to define whether it can mediate some protective effects shown by HT in other cell types and experimental models.

SIRT-1 is a protein deacetylase that plays a role in chondrocyte survival and cartilage homeostasis [26]. The overexpression of SIRT-1 [29] or its activation by resveratrol [30] protects chondrocytes and inhibits OA-like gene expression changes *in vitro*, whereas selective knockout of Sirt1 in chondrocytes accelerates OA development in mice [31]. However the involvement of autophagy in the chondroprotective effects of SIRT-1 has not been reported. Actually it is known that SIRT-1 may regulate autophagy in different ways [32]. In fact SIRT-1 can modulate the expression of genes involved in autophagy by deacetylating key transcription factors or directly the activity of autophagic proteins by deacetylation. We have previously found that HT may enhance SIRT-1 expression in human chondrocytes [20]. Other research groups also reported an increased expression of SIRT-1 mRNA or protein in the heart of SAMP8 mice fed with a diet rich in olive oil phenolics [33], in the heart of HT-treated Sprague-Dawley rats [34], and in the brain of HT-treated db/db mice [35], but the functional role of SIRT-1 in the HT action was not investigated. In the present paper we show by mechanistic experiments that HT induces autophagy and protects from oxidative stress-induced cell death in a SIRT-1 dependent manner. In fact LC3-II formation, a critical event in autophagy, increased in C-28/I2 chondrocytes treated with HT, but not in SIRT-1 silenced cells. Instead LC3 gene expression was not affected by HT. Huang et al. [36] have demonstrated that LC3 deacetylation by SIRT-1 in the nucleus is required for LC3 export to cytosol where it is able to bind Atg7 and other autophagy factors and undergoes phosphatidylethanolamine conjugation to preautophagic membranes (and can be detected by western blot as LC3-II). The nuclear localization of SIRT-1 has proved to be critical also for its pro-survival and anti-apoptotic action [37, 38]. In H₂O₂-treated C-28/I2 cells nuclear localization of SIRT-1 decreases, confirming previous findings of reduced SIRT-1 levels in a variety of cell types under oxidative stress [39], whereas in HT-treated C-28/I2 cells SIRT-1 becomes concentrated in the nucleus. Interestingly an increase of nuclear localization in the hearts of HT-treated rats has also been reported [34]. We may therefore hypothesize that following HT treatment SIRT-1 accumulates in the nucleus where catalyzes the deacetylation of LC3, which in its turn translocates to the cytosol to drive the autophagic process. Indeed SIRT-1 silencing *per se* did not affect LC3-II accumulation, sustaining the view that HT-induced nuclear translocation is necessary for SIRT-1 effect on LC3-II formation. Moreover, HT supports autophagy even by increasing transcription of p62, required for autophagic degradation of polyubiquitin-containing bodies [2]. Thus, the enhanced expression of SQSTM1/p62 gene may sustain the increased p62 protein turnover. Accordingly, it has been reported that SQSTM1/p62 can be transcriptionally up-regulated in some situations of augmented autophagic

flux. Notably HT induced an increase of p62 mRNA also in siSIRT-1 cells, indicating an additional, SIRT-1 independent mechanism for autophagy activation. In this regard it should be considered that p62/SQSTM1 is a target gene for the stress and anti-oxidant responsive transcription factor Nrf2, which in its turn can be stimulated by HT as already mentioned [27]. Overall, by promoting key steps, HT appears able to increase the autophagic flux in chondrocytes.

In our cell model autophagy activation mediated protection from cell death as demonstrated by experiments carried out with either inhibitors or activators of autophagy, including HT and SIRT-1. Our findings, documented by immunocytochemistry, support previous reports indicating that mitophagy is effective in protecting chondrocytes from apoptosis induced through the mitochondrial pathway [7, 8]. It is also worthy to underline that reducing the extent of oxidative DNA damage in chondrocytes, HT appears promising as a therapeutic tool to limit chondrocyte senescence, a major problem in OA as well as in aging cartilage [6].

It should be noted however that in some contexts prolonged or sustained autophagy may not have a protective outcome. For instance, even if autophagy is required to maintain muscle mass [40], excessive autophagy is believed to aggravate muscle wasting in animal models. Interestingly HT supplementation to rats inhibited the negative effects of exhaustive exercise on muscle together with the deregulated increase in autophagy [41]. . Therefore HT may have a homeostatic effect, activating autophagy to the extent required to limit cell damage after an acute insult, but also preventing excessive autophagy.

HT appears to resemble resveratrol, a polyphenol found in red wine and grapes, under some aspects, such as the multiple cellular targets and the ability to induce autophagy in a SIRT-1 dependent manner but also through other pathways [32, 39]. Interestingly resveratrol can directly induce autophagy via activation of AMPK [32], and HT is also known to stimulate AMPK in some experimental models [28, 35]. Actually AMPK and SIRT-1 may regulate their activities reciprocally, albeit indirectly, and contribute to autophagy induction [25, 39]. Thus AMPK might be involved in the stimulation of autophagic process by HT in chondrocytes. Finally resveratrol, as mentioned above for HT, has been reported to attenuate excessive autophagy [32, 39].

5. Conclusions

In a recent review, Liu-Bryan and Terkeltaub [42] conclude that the bioenergy sensors AMPK and SIRT1, by contrasting oxidative stress, pro-inflammatory, and ECM degrading processes in

chondrocytes “might provide a pragmatic ‘entry point’ for novel strategies to limit OA progression”. Accordingly, the present study supports the potential role of HT as a nutraceutical compound for chondroprotection against OA, not merely because of its antioxidant power, but as an autophagy and SIRT-1 inducer as well. Further investigation will allow a better characterization of molecular mechanisms underlying these effects.

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LEGENDS TO FIGURES

Fig. 1. Hydroxytyrosol stimulates autophagy in C-28/I2 chondrocytes. C-28/I2 cells were pre-incubated in the absence or in the presence of hydroxytyrosol (HT) for 30 min before addition of H₂O₂. After a further 2h incubation, cells were harvested for LC3, p62 and β -actin detection by western blotting. Representative images (A) and relative quantification for LC3-II/ β -actin (B) and p62/ β -actin (C) ratios are shown. Values are expressed as means \pm SEM, * p <0.05, ** p <0.01. Alternatively in immunocytochemical studies, C-28/I2 cells were stained at the end of incubation with monodansylcadaverine (D: 200x, original magnification), with antibodies to LC3 A/B and TOM20 (E). DAPI was used as a nuclear counterstaining. The labeled cells were visualized by fluorescence microscopy (left pictures, showing separate and merged TOM20 and LC3 signals, 400x, original magnification) and confirmation of mitophagy was also carried out with confocal laser scanning microscopy (CLSM, right column, 180x, original magnification:). Representative fields of control, non-stimulated cells (NS) or cells treated with HT and/or H₂O₂ are shown.

Fig. 2. Autophagy protects C-28/I2 cells from oxidative stress-induced DNA damage and cell death. C-28/I2 cells were pre-treated with or without hydroxytyrosol (HT), rapamycin (RAPA), 3-methyladenine (3MA) or cloroquine (CQ) for 30 min before addition of H₂O₂. After a further 2h incubation, cells were evaluated for the extent of DNA damage by detection of γ H2AX and calculation of the increment of the mean channel fluorescence intensity (MCFI) over basal fluorescence (A). Alternatively after 24 h incubation with or without H₂O₂, cells were counted to assess cell viability by trypan blue exclusion test (B). Values are expressed as means \pm SEM, * p <0.05, ** p <0.01, *** p <0.001.

Fig. 3. Hydroxytyrosol increases SIRT-1 nuclear localization. C-28/I2 cells were pre-incubated in the absence or in the presence of hydroxytyrosol (HT) for 30 min before addition of H₂O₂. After a further 2h incubation, cells were processed for immunodetection of SIRT-1 and finally treated with a DAPI nuclear counterstaining. The labeled cells were visualized and representative fields of control cells (NS) or cells treated with HT and/or H₂O₂ are shown (A: 400x, original magnification). Quantitative analysis of the percentage of cells with SIRT-1 localized in the

nucleus was assessed as described in Materials and Methods (**B**). Data are means \pm SEM, $*p<0.05$, $**p<0.01$.

Fig. 4. SIRT-1 is required for the cytoprotective effect of hydroxytyrosol under oxidative stress. (**A**, **B**) C-28/I2 cells were transfected for 48h with SIRT1-siRNA (siSIRT-1) or siRNA negative control (NC). SIRT-1 levels were determined by Western blotting. Representative images (**A**) and relative quantification for SIRT-1/ β -actin ratio (**B**) are shown. (**C**) 24h after transfection, C-28/I2 cells were treated with H_2O_2 for 24h. Hydroxytyrosol (HT) was added 30 min before H_2O_2 . At the end of incubation, cells were counted to assess cell viability by trypan blue exclusion test. Values are expressed as means \pm SEM, $*p<0.05$, $**p<0.01$.

Fig. 5. Hydroxytyrosol stimulates autophagy by SIRT-1-dependent and -independent mechanisms. (**A**,**B**) 24h after transfection with SIRT1-siRNA (siSIRT-1) or siRNA negative control (NC), C-28/I2 cells were treated with hydroxytyrosol (HT) for 24h. Chloroquine (CQ) was added 30 min before HT. At the end of incubation, cells were harvested for LC3 and β -actin detection by western blotting. Representative images (**A**) and relative quantification for LC3-II/ β -actin ratio (**B**) are shown. (**C**) 24h after transfection with SIRT1-siRNA (siSIRT-1) or siRNA negative control (NC), C-28/I2 cells were treated with hydroxytyrosol (HT) for 24h. Then cells were harvested and analysed by qRT-PCR for the amount of p62 mRNA. Values are expressed as means \pm SEM, $*p<0.05$.

Fig. 6. Hydroxytyrosol-induced autophagy protects human primary OA chondrocytes from oxidative stress. Human primary chondrocytes were pre-incubated in the absence or in the presence of hydroxytyrosol (HT) and/or other compounds as indicated, for 30 min before addition of H_2O_2 . After a further 2h incubation, cells were harvested for LC3, p62 and β -actin detection by western blotting. Representative images (**A**) and relative quantification for LC3-II/ β -actin (**B**) and p62/ β -actin (**C**) ratios are shown. Cells were also stained at the end of incubation with antibodies to LC3 A/B and TOM20 (**D**). The labeled cells were visualized by fluorescence microscopy, showing separate and merged LC3 and TOM20 signals (400x, original magnification). Representative fields of control, non-stimulated cells (NS) or cells treated with HT and/or H_2O_2 are shown. DAPI was used as a nuclear counterstaining. Alternatively cells were evaluated for the extent of DNA damage

by detection of γ H2AX (**E**). Finally, after 24 h incubation with or without H₂O₂, cells were counted to assess cell viability by trypan blue exclusion test (**F**). Values are expressed as means \pm SEM, * p <0.05, ** p <0.01, *** p <0.001

ACCEPTED MANUSCRIPT

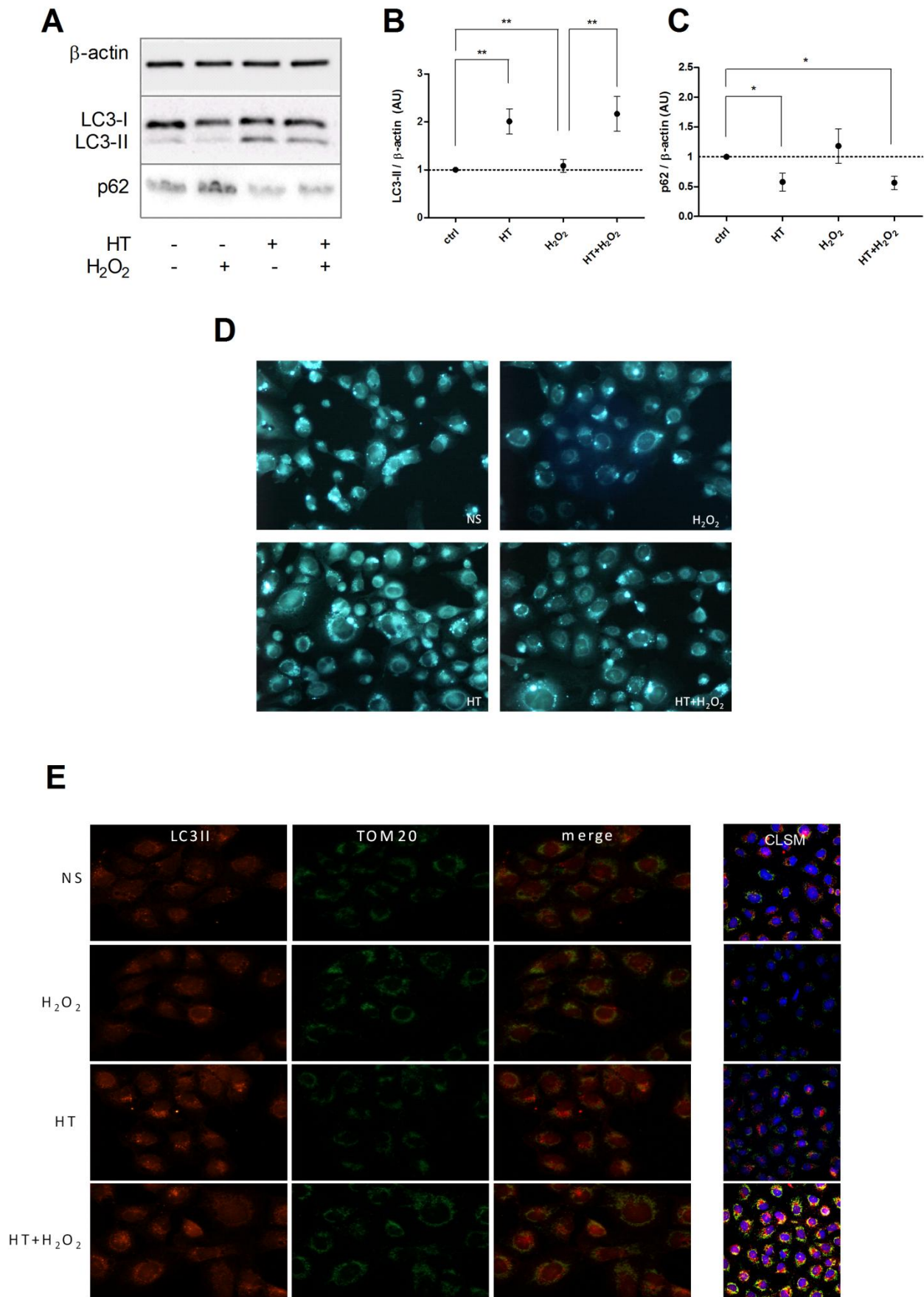


Figure 1

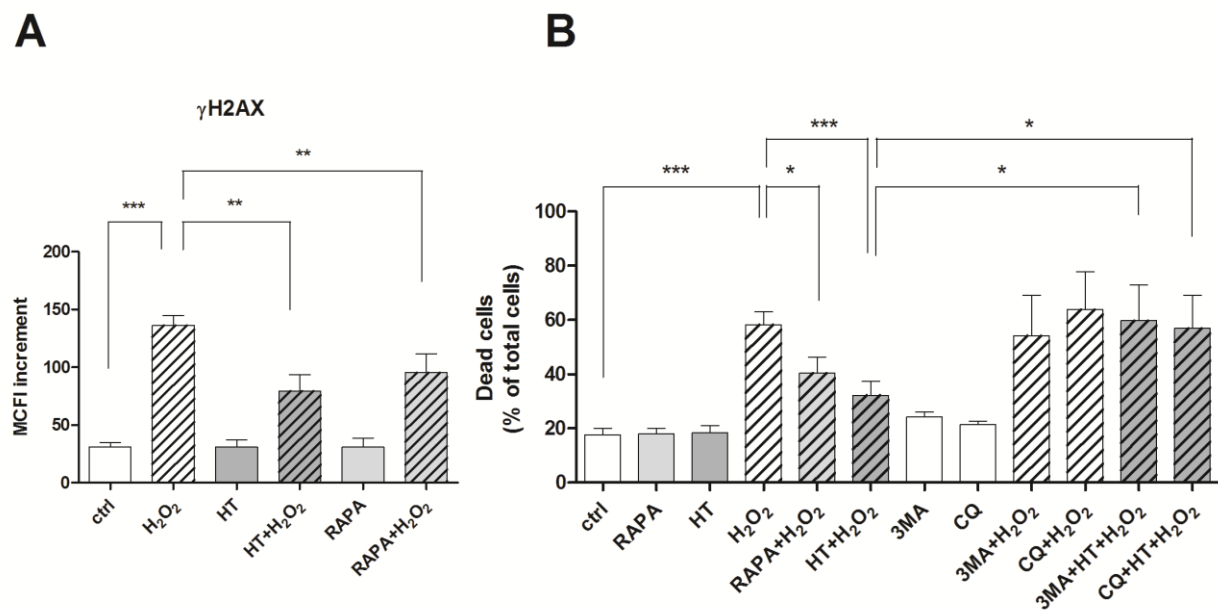
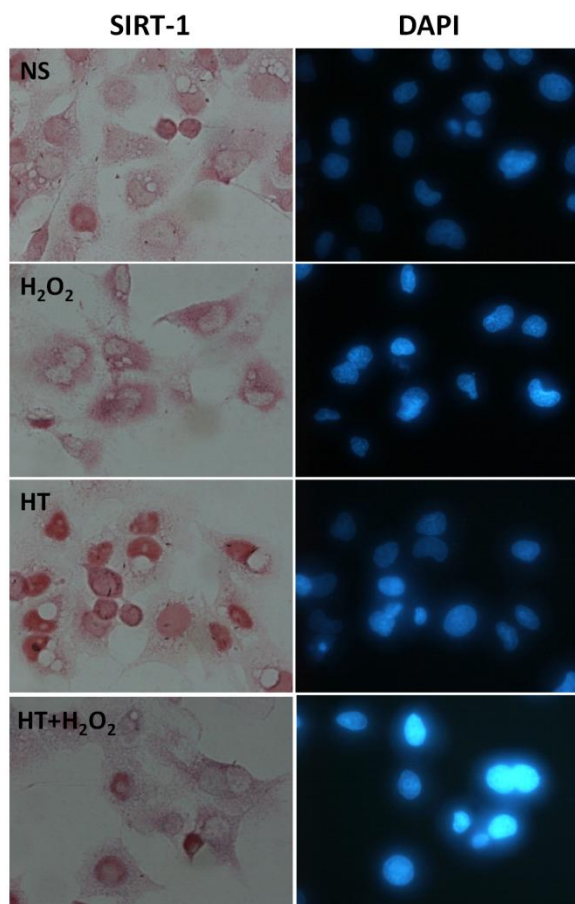


Figure 2

A



B

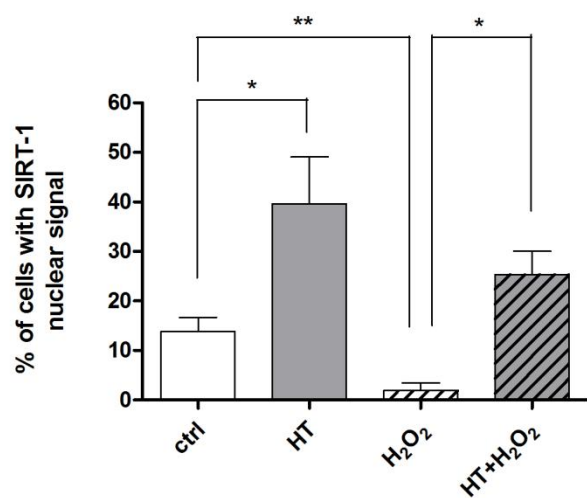
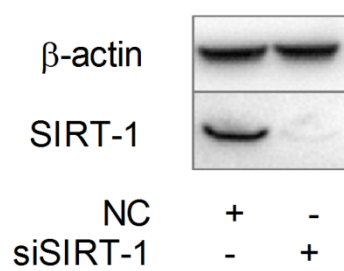
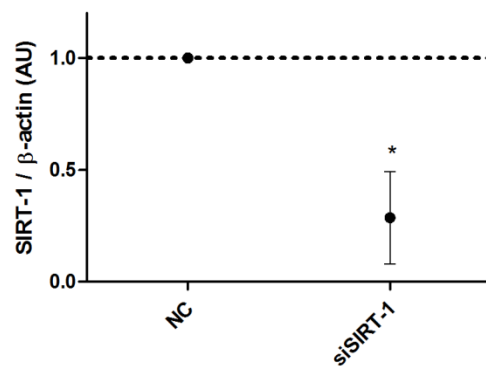


Figure 3

A



B



C

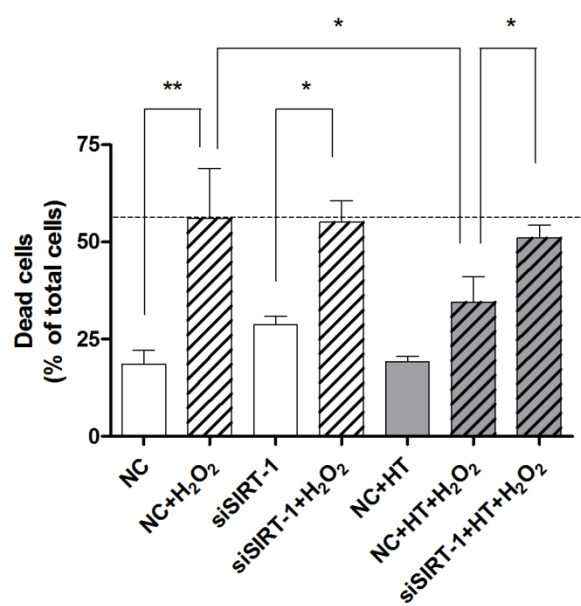
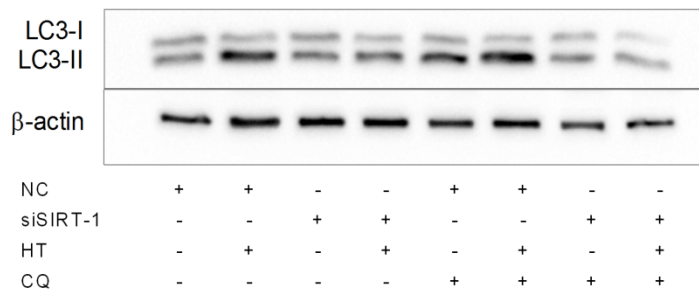
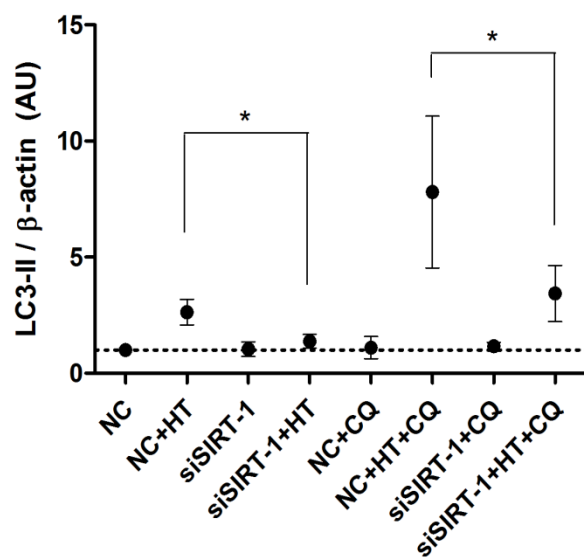


Figure 4

A



B



C

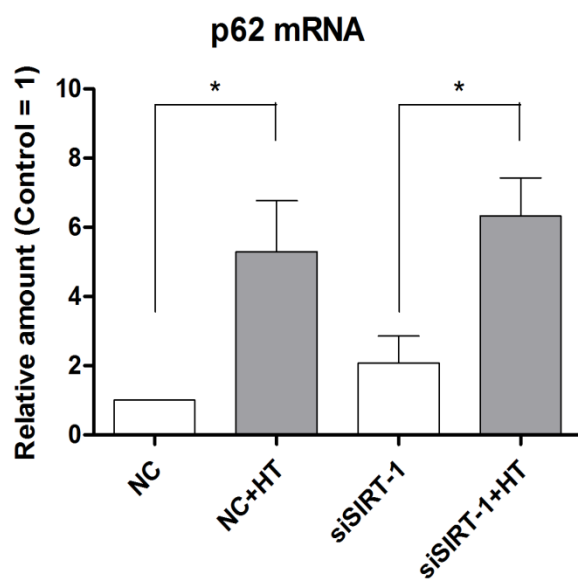


Figure 5

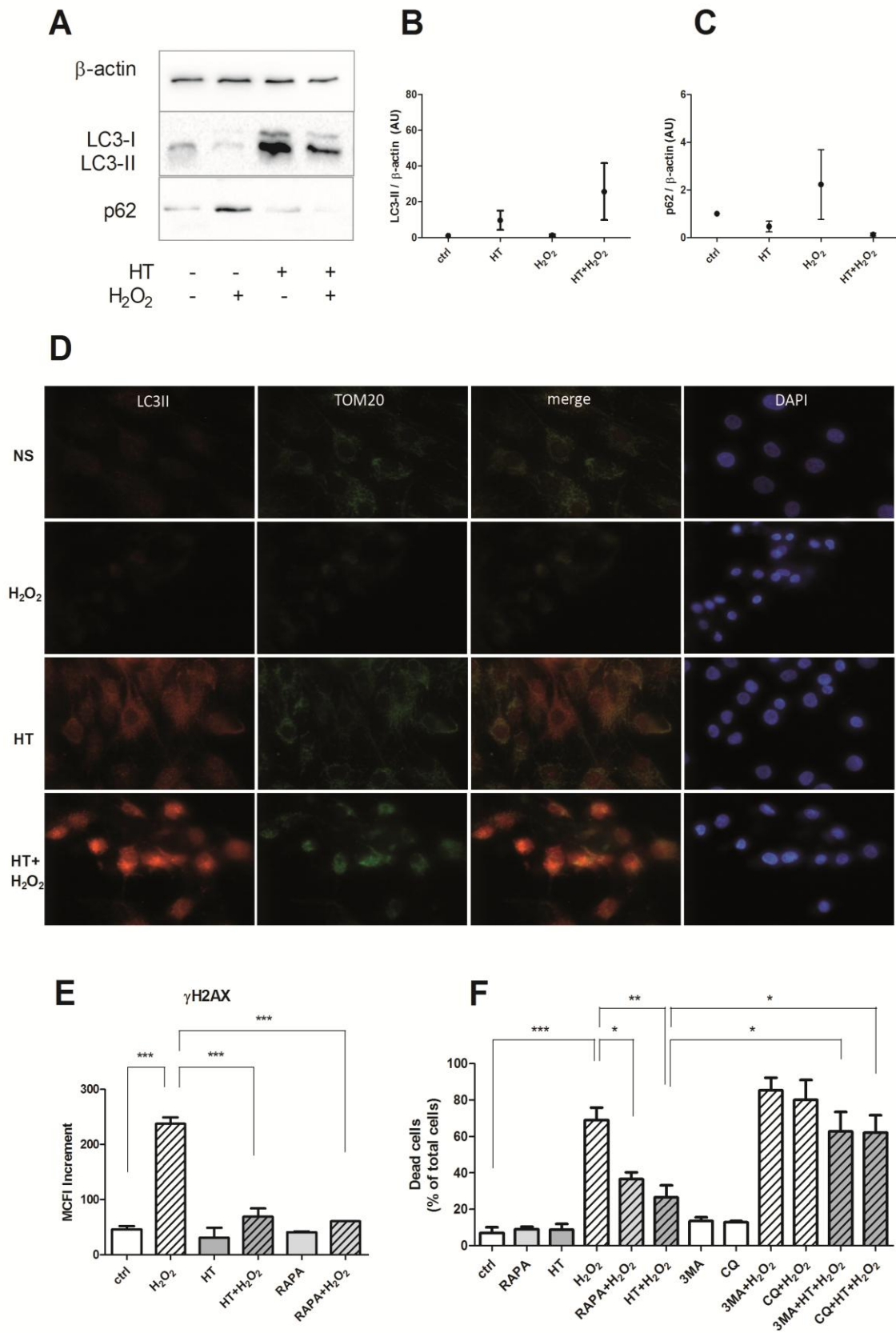
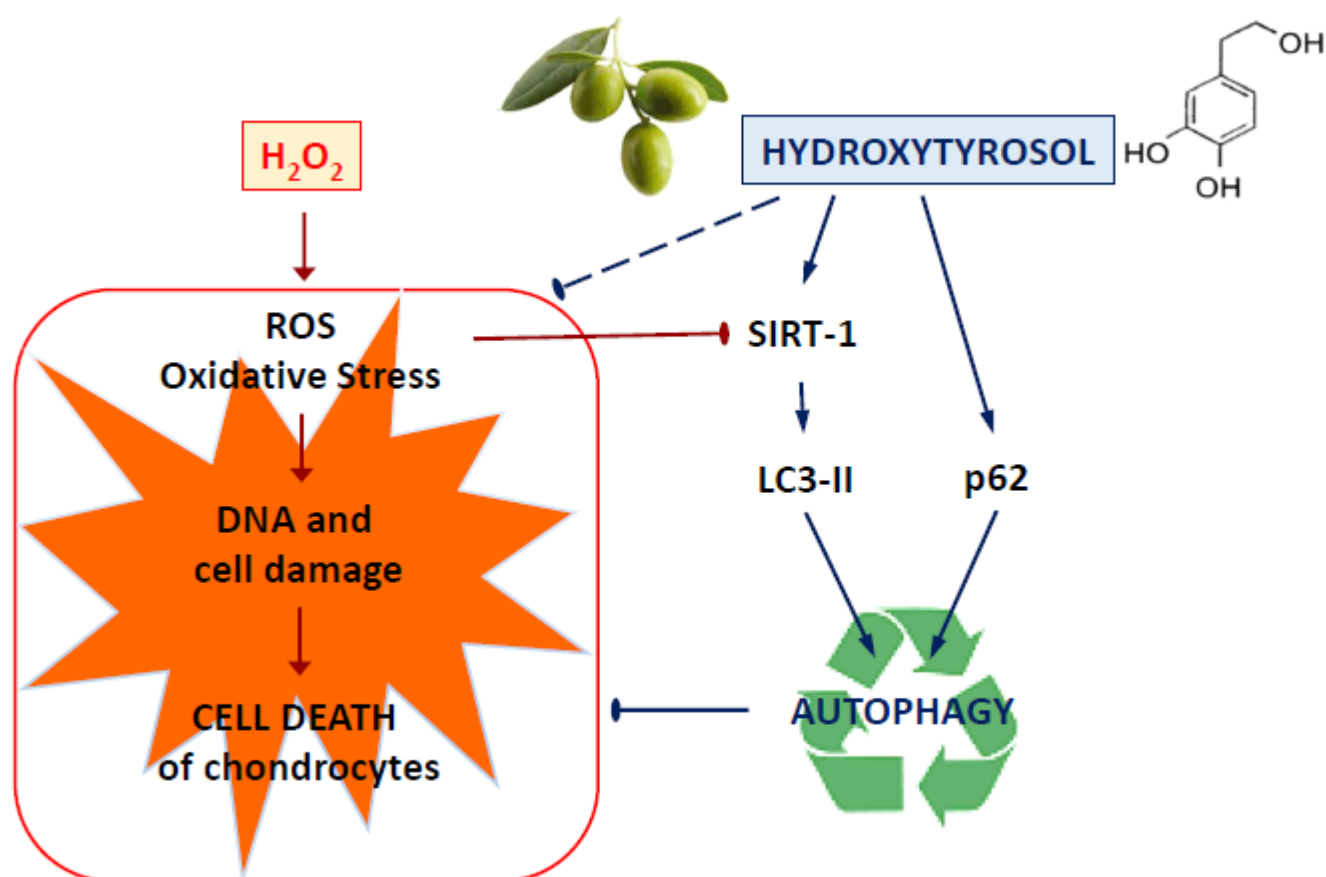


Figure 6



Graphical abstract

HIGHLIGHTS

- Hydroxytyrosol protects chondrocytes from H₂O₂ - induced DNA damage and cell death
- Hydroxytyrosol induces autophagy activation
- Autophagy mediates hydroxytyrosol protective action under oxidative stress
- Hydroxytyrosol stimulates autophagy by SIRT1-dependent and -independent mechanisms