ARTICLE
Journal of Cellular Biochemistry 111:922–932 (2010)

Journal of Cellular Biochemistry

Proteomic and Redox-Proteomic Evaluation of Homogentisic Acid and Ascorbic Acid Effects on Human Articular Chondrocytes

Daniela Braconi,[1] Marcella Laschi,[1] Adam M. Taylor,[2] Giulia Bernardini,[1] Adriano Spreafico,[3,4] Laura Tinti,[4] James A. Gallagher,[2] and Annalisa Santucci[1,3]*

[1]*Dipartimento di Biologia Molecolare, Università degli Studi di Siena, via Fiorentina 1, 53100 Siena (SI), Italy*

[2]*Department of Human Anatomy and Cell Biology, School of Biomedical Sciences, University of Liverpool, Sherrington Building, Ashton Street, Liverpool L69 3GE, UK*

[3]*Centro Interdipartimentale per lo Studio Biochimico delle Patologie Osteoarticolari, Università degli Studi di Siena, via Fiorentina 1, 53100 Siena (SI), Italy*

[4]*Dipartimento di Medicina Clinica e Scienze Immunologiche, Università degli Studi di Siena, 53100 Siena (SI), Italy*

ABSTRACT

Alkaptonuria (AKU) is a rare genetic disease associated with the accumulation of homogentisic acid (HGA) and its oxidized/polymerized products in connective tissues up to the deposition of melanin-like pigments (ochronosis). Since little is known on the effects of HGA and its metabolites on articular cells, we carried out a proteomic and redox-proteomic analysis to investigate how HGA and ascorbic acid (ASC) affect the human chondrocytic protein repertoire. We settled up an in vitro model using a human chondrocytic cell line to evaluate the effects of 0.33 mM HGA, alone or combined with ASC. We found that HGA and ASC significantly affect the levels of proteins with specific functions in protein folding, cell organization and, notably, stress response and cell defense. Increased protein carbonyls levels were found either in HGA or ASC treated cells, and evidences produced in this paper support the hypothesis that HGA-induced stress might be mediated by protein oxidation. Our finding can lay the basis towards the settling up of more sophisticated models to study AKU and ochronosis. J. Cell. Biochem. 111: 922–932, 2010.

KEY WORDS: ALKAPTONURIA; COMPARATIVE PROTEOMICS; OCHRONOSIS; OCHRONOTIC ARTHROPATHY; POST-TRANSLATIONAL MODIFICATIONS; PROTEIN CARBONYLS; PROTEIN OXIDATION; VITAMIN C

Alkaptonuria (AKU) is a rare autosomal recessive inborn error of metabolism of the aromatic amino acids phenylalanine and tyrosine due to a deficient activity of the enzyme homogentisate 1,2-dioxygenase (HGO, EC 1.13.11.5) in the liver [Phornphutkul et al., 2002]. As a consequence, the intermediate homogentisic acid (HGA) cannot be converted into fumaric acid and acetoacetic acid [Fernández-Cañón et al., 1996] and it is excreted with urine, which turns brown on standing or after alkalinization, or accumulated within the body as patients grow older. It is around the third or fourth decade of life that tissue injuries appear with the deposition of a dark (ochronotic) pigment on connective tissues [Zannoni et al., 1962], mainly joints, cardiac valves, kidney, and skin [Selvi et al., 2000; Phornphutkul et al., 2002; Helliwell et al., 2008; Taylor et al., 2010a, b]. In cartilages, ochronosis leads to the weakening of tissues, which become more brittle and prone to ruptures, chronic inflammation and, ultimately, osteoarthritis, a condition named ochronotic arthropathy [Selvi et al., 2000; Helliwell et al., 2008]. It has been shown in AKU case reports that both the bone and the cartilage are the primarily affected tissues, cartilage more severely than bone [Fisher and Davis, 2004]. As the single cellular constituent

Abbreviations used: AKU, alkaptonuria; ASC, ascorbic acid; BQA, benzoquinone acetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DNPH, 2,4-dinitrophenylhydrazine; DTE, 1,4-dithioerythritol; HGA, homogentisic acid; IAA, iodoacetamide; IEF, isoelectric focusing; MW, molecular weight; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Fondazione Monte dei Paschi di Siena Esercizio 2008 and Esercizio 2009; Grant sponsor: TLS Orphan_0108 and TELETHON GGP10058; Grant sponsor: AKU Society.

*Correspondence to: Prof. Annalisa Santucci, Dipartimento di Biologia Molecolare, Università degli Studi di Siena, via Fiorentina 1, 53100 Siena (SI), Italy. E-mail: santucci@unisi.it

Received 7 April 2010; Accepted 12 July 2010 • DOI 10.1002/jcb.22780 • © 2010 Wiley-Liss, Inc.

Published online 27 July 2010 in Wiley Online Library (wileyonlinelibrary.com).

of adult articular cartilage, chondrocytes are responsible for maintaining the cartilage matrix at a low turnover state of equilibrium. In mature cartilage, chondrocytes synthesize small proteoglycans such as biglycan and decorin and other specific and non-specific matrix proteins very slowly [Gartland et al., 2005].

The molecular characterization of AKU and ochronosis is far from being completed, mainly because of the lack of suitable models to study the disease. As an example, though HGO deficient mice exist, they do not develop ochronosis [Manning et al., 1999]. Nonetheless, oxidative stress generated by the oxidation of HGA into benzoquinone acetic acid (BQA) is believed to be one of the main factors for the development of ochronosis and arthropathies in AKU.

Ascorbic acid (ASC) was initially adopted to treat AKU in order to prevent the oxidation of HGA into BQA. [Kutty et al., 1974] However, results obtained with ASC are contradictory, since it was not proven to increase the urinary levels of HGA and, under certain conditions, it was demonstrated to act as a pro-oxidant, quickly co-oxidating with HGA and leading to additional production of reactive oxygen species (ROS) [Martin and Batkoff, 1987]. A low protein diet, with or without ASC supplementation, was also proposed [de Haas et al., 1998]; however, it is difficult to be maintained and beneficial effects have not been proven undoubtedly. The herbicide [2-(2-nitro-4-trifluoromethylbenzyl)-1,3-cyclohexandione] (nitisinone, Orfadin), which is used to treat hereditary type I tyrosinemia as it inhibits the enzyme p-hydrophenylpyruvate dioxygenase, was proposed as well to treat AKU. Nevertheless, further studies are needed to support this latter therapeutic strategy [Phornphutkul et al., 2002; Suwannarat et al., 2005].

In this work, a comparative proteomic approach was used on a human chondrocyte cell line that we chose as a model for the articular cartilage to study the effects of exogenous HGA and ASC supplementations. Also, because of the well-recognized importance of oxidative alterations in the progression of ochronotic arthropathy, a redox-proteomic analysis of carbonylated proteins was carried out to identify the molecular targets of oxidation.

We found that HGA and ASC significantly affect the levels of proteins with specific functions in assisting protein folding, with a role in cell organization and, notably, in the cell defense and stress response. Oxidative modifications of proteins could also be found and, specifically, ASC- and HGA-induced carbonylation was demonstrated, together with the production of carbonylated high molecular weight (MW) protein aggregates.

MATERIALS AND METHODS

REAGENTS

Unless otherwise indicated, all high quality reagents and antibodies were from Sigma–Aldrich (Milan, Italy). All water used was Milli-Q (Millipore, Bedford, MA).

CELL CULTURES

The immortalized human chondrocyte cell line C20 was cultured as a monolayer at 37.5°C in humidified air with 5% CO_2 in Dulbecco's Modified Eagle's medium (DMEM)/F12 + Glutamax (Invitrogen) containing 10% foetal calf serum and 1% Penicillin–Streptomycin. For experiments, cells were sub-cultured in 12-well plates with fresh standard medium. At cell confluence, the conditioned medium was removed and replaced with fresh standard medium without exogenous additions (control), or containing 0.1 mM ASC, 0.33 mM HGA, or 0.1 mM ASC together with 0.33 mM HGA. ASC and HGA were added to culture medium by using 0.1 M stock solutions in sterile PBS. Culture medium was changed after 3, 5, 7, and 9 days. Viability of C20 chondrocytes was evaluated in monolayer cultures and correlated to the number of living cells at each sampling using the MTT assay (Sigma–Aldrich).

PREPARATION OF CELL LYSATES

After 5, 7, and 9 days of culture, C20 cells were washed twice with sterile PBS and resuspended in 50 μl of a buffer containing 65 mM DTE, 65 mM CHAPS, 9 M urea, and 35 mM Tris-base. Cell disruption was achieved by sonicating for 1 min in an ice bath. Protein content in cell lysates was assessed according to Bradford [1976].

NUPAGE

Ten micrograms of C20 proteins were mixed with the NuPAGE LDS sample buffer and the NuPAGE reducing agent (Invitrogen). Samples were heated at 70°C for 10 min, then resolved through 4–12% gradient NuPAGE® Novex Bis-Tris pre-cast gels (Invitrogen) and stained with SimplyBlue Safestain (Invitrogen) following manufacturer's instructions.

TWO-DIMENSIONAL ELECTROPHORESIS (2D-PAGE) AND WESTERN BLOT

C20 cell lysates were first mixed with a buffer containing 8 M urea, 35 mM CHAPS, 10 mM DTE, and a trace of Bromophenol Blue. Proteins were adsorbed onto Immobiline Dry Strips (IPG 18 cm, non linear 3–10 pH range, Bio-Rad, Milan, Italy) and allowed to stand at room temperature for 10 h. One hundred micrograms (2D gels to be transferred onto NC membranes) or 50 μg (2D gels to be silver stained) of proteins were used. Then isoelectric focusing (IEF) was carried out with a Protean IEF cell (Bio-Rad). The voltage was linearly increased from 300 to 3,500 V during the first 3 h and then stabilized at 5,000 V for 22 h (total 110 kV h).

For the Western blot analysis of carbonylated proteins, after the IEF IPG strips were briefly rinsed with water and incubated at room temperature for 20 min with 10 mM DNPH in 5% (w/v) TFA to allow derivatization of protein carbonyls according to Reinheckel et al. [2000]. Strips were hence rapidly washed twice with a solution containing 8 M urea, 20% (v/v) glycerol, 9 M SDS and 150 mM Tris–HCl pH 6.8.

Prior to SDS–PAGE, IPG strips for both silver stained 2D-gels and 2D-gels to be transferred onto nitrocellulose (NC) membranes were equilibrated in 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.05 M Tris–HCl pH 6.8 containing first 2% (w/v) DTE and later 2.5% (w/v) IAA. SDS–PAGE was carried out applying 40 V per gel until the dye front reached the bottom of gels.

Silver ammoniacal staining was carried out according to Switzer et al. [1979] whereas gels to be transferred onto NC were washed and equilibrated in a transfer buffer [50 mM Tris, 40 mM glycine, 1.3 mM SDS, 20% (v/v) methanol] and protein transfer was carried out using a semidry Novablot transblot cell (Bio-Rad) applying 0.7 mA/cm^2 for 75 min. Protein transfer was checked by staining with 0.2% Ponceau

S in 3% (w/v) TFA for 3 min and de-staining with water. For the immunorevelation of protein carbonyls, NC sheets were incubated with rabbit anti-dinitrophenyl antibodies 1:10,000 (over-night at 4°C), followed by peroxidase-conjugated anti-rabbit antibodies 1:7,000 (2 h at room temperature) and revelation was achieved through chemiluminescence (ECL, Amersham Biosciences).

Protein spots of interest were identified by gel matching with proteomic reference maps, already produced, calibrated and characterized in our laboratories [Spreafico et al., 2006, 2009].

IMAGE ANALYSIS

Images of gels and films were acquired (Image Scanner, Amersham Biosciences) and analyzed with Image Master™ Platinum (Amersham Biosciences). For comparative proteomic analysis, spot % relative abundance was used; for multiple spots identified as different molecular species of a same protein, the average % relative abundance was calculated. For the quantitative analysis of immunorevealed protein carbonyls, the intensity of bands, which is automatically normalized by Image Master™ Platinum against the surrounding background, was chosen as the reference parameter.

STATISTICAL ANALYSIS

All of the experiments were carried out in triplicate, showing good reproducibility of results. For comparative proteomics and Western blot analysis, only representative gels and films are shown. Analysis of variance was followed by the Bonferroni-type multiple comparison to evaluate statistical differences. At least a P value ≤ 0.05 was considered significant.

RESULTS

CELL CULTURES

In this work, a human articular chondrocyte cell line was used as an in vitro model to study alkaptonuric ochronosis. Cells were challenged with 0.33 mM HGA, alone or concomitantly with 0.1 mM ASC. As a control, cultures without exogenous supplementations or added with 0.1 mM ASC alone were used, respectively. The tested HGA concentration was chosen on the basis of previous observations of ours, because it was shown to induce in vitro the deposition of ochronotic-like pigments in osteosarcoma and chondrocyte cell lines (data not shown), as well as in human articular primary chondrocytes [Tinti et al., 2010]. The tested HGA concentration was in the range of circulating HGA levels in alkaptonuria patients' serum (50–400 μM) [Angeles et al., 1989]. This concentration was also necessary for us to gain the HGA effects within the in vitro environment over a time which could allow the analysis, because in alkaptonuric patients HGA-induced ochronosis takes many years to produce evident clinical manifestations [Fisher and Davis, 2004].

The supplementation with ASC had a double reason. On the one hand, an in vitro ASC supplementation ranging 30–600 μM [Ibold et al., 2009] is needed for production and quality of cartilage, because it stimulates the production of both collagen and glycosaminoglycan [Frech and Clegg, 2007]. One the other hand, we investigated if it could exert anti-oxidative effects in protecting cells from HGA-induced toxicity. The tested ASC concentration was chosen because falling within the optimal experimental range [Ibold et al., 2009] and also on the basis of our preliminary results on the effects of HGA and ASC on various cell models [Selvi et al., 2000; Tinti et al., 2010]. Martin and Batkoff [1987], who investigated in vitro the oxidation pattern of HGA together with ASC, reported the ASC concentration in vivo to be within 40–150 μM, although lower ASC levels may be found in elderly people (reaching 20 μM) as a result of only dietary intake [VanderJagt et al., 1987]. Nonetheless, the extrapolation of the ASC concentration used in our in vitro experiments to an in vivo scenario may be made much more complicated by the fact that synovial fluid, and especially cartilage, tend to accumulate ASC; such a phenomenon may be reproduced hardly in in vitro cultures.

We carried out a temporal analysis sampling cell lysates after 5, 7, and 9 days of treatment, and evaluated both cell proliferation and protein expression profiles. At each time point, cultures containing HGA showed a darkener medium of growth (Fig. 1 of supplementary materials). Such a phenomenon resembled the urine darkening observable in AKU sufferers, which is a typical early clinical sign of the disease and is due to oxidation of the excreted HGA under aerobic conditions, which produces a ochronotic product and causes the urine to turn dark [Phornphutkul et al., 2002]. Moreover, once compared to the control, cell viability was significantly lowered by 9 days of treatment with HGA, as well as by 7 and 9 days of treatment with HGA + ASC (Fig. 1), confirming previous observation of ours [Tinti et al., 2010].

NUPAGE ANALYSIS OF C20 PROTEIN PROFILES

The protein profiles of C20 cells after 5, 7, and 9 days under the tested conditions showed only minor differences. Nonetheless, a noteworthy finding was that protein aggregates stacked in the wells of gels were found almost exclusively in HGA- and HGA + ASC-treated cultures at each time point (Fig. 2 of supplementary material).

COMPARATIVE PROTEOMICS

As shown by the results of cell viability assays, 9 days treated cells were the most interesting to be further analyzed in order to obtain insights into the C20 proteome responses to ASC and HGA. Whole C20 cell lysates collected after 9 days of treatment were hence subjected to 2D-PAGE. We used for the first dimension a non-linear 3.0–10.0 pH range, while for the second dimension a 9–16% acrylamide range was adopted. Silver staining allowed the resolution of about 1,800 spots in each 2D gel (Fig. 2). Once digitalized, the images of 2D gels were analyzed qualitatively and quantitatively, and proteins whose relative abundance was found to be significantly different (threshold set at 2.0) were detected. The majority of these proteins were hence identified by gel matching with reference master 2D-gels produced in our laboratory and already characterized [Spreafico et al., 2006, 2009]. Identified proteins were also clustered into functional groups; their complete list is provided in Table I.

The comparative proteomic analysis undertaken in our work allowed us to highlight and distinguish the effects of HGA and ASC, when added singularly to cell cultures, as well as those effects

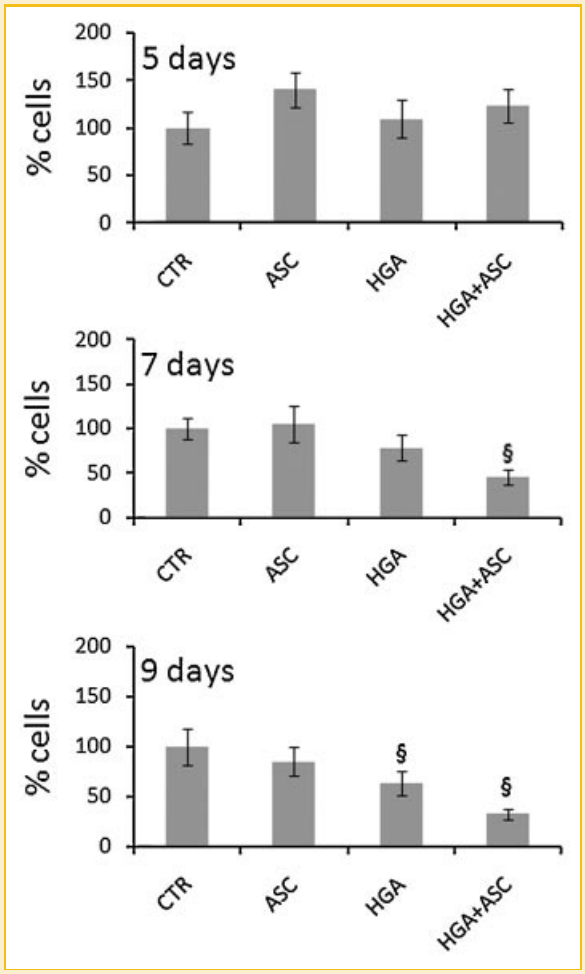

Fig. 1. MTT viability assay. % viable C20 cells under control conditions or in the presence of 0.1 mM ASC, 0.33 mM HGA, and 0.1 mM ASC + 0.33 mM HGA after 5, 7, and 9 days of treatment. Experiments were performed in triplicate; data are presented as average values ± standard deviation. Statistical significance compared to the control is indicated ($^{\S}P \leq 0.05$).

exerted when they were co-administered (Fig. 3 of supplementary material). Globally, we identified 32 proteins differently expressed in the tested conditions. Of these, 22 were proteins whose synthesis was altered by ASC (2D maps of C20 treated with ASC vs. the control, and C20 treated with HGA + ASC vs. HGA), 19 proteins whose synthesis was altered by HGA (2D maps of C20 treated with HGA vs. the control, and C20 treated with HGA + ASC vs. ASC), and 11 proteins whose synthesis was altered by HGA co-administered with ASC (2D map of C20 treated with HGA + ASC vs. the control). Results obtained could be reported schematically as follows.

EFFECTS OF ASC

A general under-expression of proteins was induced by ASC. Transgelin (TAGL), one of the proteins remodeling actin, and vinculin (VINC), which mediates cell adhesion and plays a fundamental role in cell morphology and motility, were both under-expressed.

In particular, cells were found to have decreased levels of proteins whose roles in the response to oxidative stress is crucial. These are:

(i) catalase, whose role is to counteract oxidative stress by decomposing H_2O_2;
(ii) mitochondrial superoxide dismutase (mSOD), whose activity is to inactivate superoxide radicals very toxic for cells
(iii) peroxiredoxin 1 (PRDX1), which regulates the cell redox state and eliminates peroxides.

Importantly, peroxiredoxin 3 was found to be under-expressed in osteoarthritic chondrocytes [Guo et al., 2008], and deficiency of mSOD is believed to be one of the reasons why osteoarthritic cartilage deteriorates, with an inverse the correlation between activity and chondrocytes ageing. mSOD was indeed proposed as a marker for osteoarthritis [Guo et al., 2008].

EFFECTS OF HGA

A general over-expression of proteins belonging to the protein fate group was induced by HGA together with a concomitant under-expression of proteins with structural functions. TAGL and VINC were both under-expressed by HGA, as already observed for ASC. Notably, HGA-treated cells showed under-expression of alpha-crystallin (CRYAB), which has anti-apoptotic functions and is involved in the cell defense from thermal shocks. On the contrary, HGA induced the expression of transketolase 1 (TKT), a protein belonging to the pentose-phosphate shunt with a protective role against oxidative stress [Kletzien et al., 1994; Pandolfi et al., 1995], calreticulin (CALR), which is required for assembly and quality control of proteins, and the disulfide isomerase PDIA1, a pivotal enzyme in triple helix collagen formation. An over-expression of PDIA1 has been already found in osteoarthritic chondrocytes in vivo, where PDIA1 is thought to contribute to the proper functions of load bearing joints and to the structural integrity of this highly specialized cartilage tissue, particularly to its ability to absorb and respond to mechanical stress [Grimmer et al., 2006]. HGA also induced the expression of the 78 kDa glucose-regulated protein (GRP78), and 75 kDa glucose-regulated protein (GRP75). GRP78 is a protein of the endoplasmic reticulum (ER) belonging to a super-family of stress-inducible proteins involved in many cell processes [Freyria et al., 1995], which also play a role in the response to glucose starvation and has anti-apoptotic functions. Increased levels of GRP78 have been shown to characterize the unfolded protein response [Harding et al., 2000] and were recently found in human advanced osteoarthritis as a marker of non-homeostatic ER function [Nugent et al., 2009]. GRP75 is a mitochondrial protein with anti-apoptotic functions induced by several stresses, when it is expected to help the protein folding [Wadhwa et al., 2002].

COMBINED EFFECTS OF HGA AND ASC

The under-expression of proteins with various functions was a common finding in cells treated with HGA + ASC, including the protein SERPIN H1. Serpins are cartilage-relevant differentiation markers [Boeuf et al., 2008], and specifically SERPIN H1 acts as a molecular chaperone for pro-collagen [Ruiz-Romero et al., 2005];

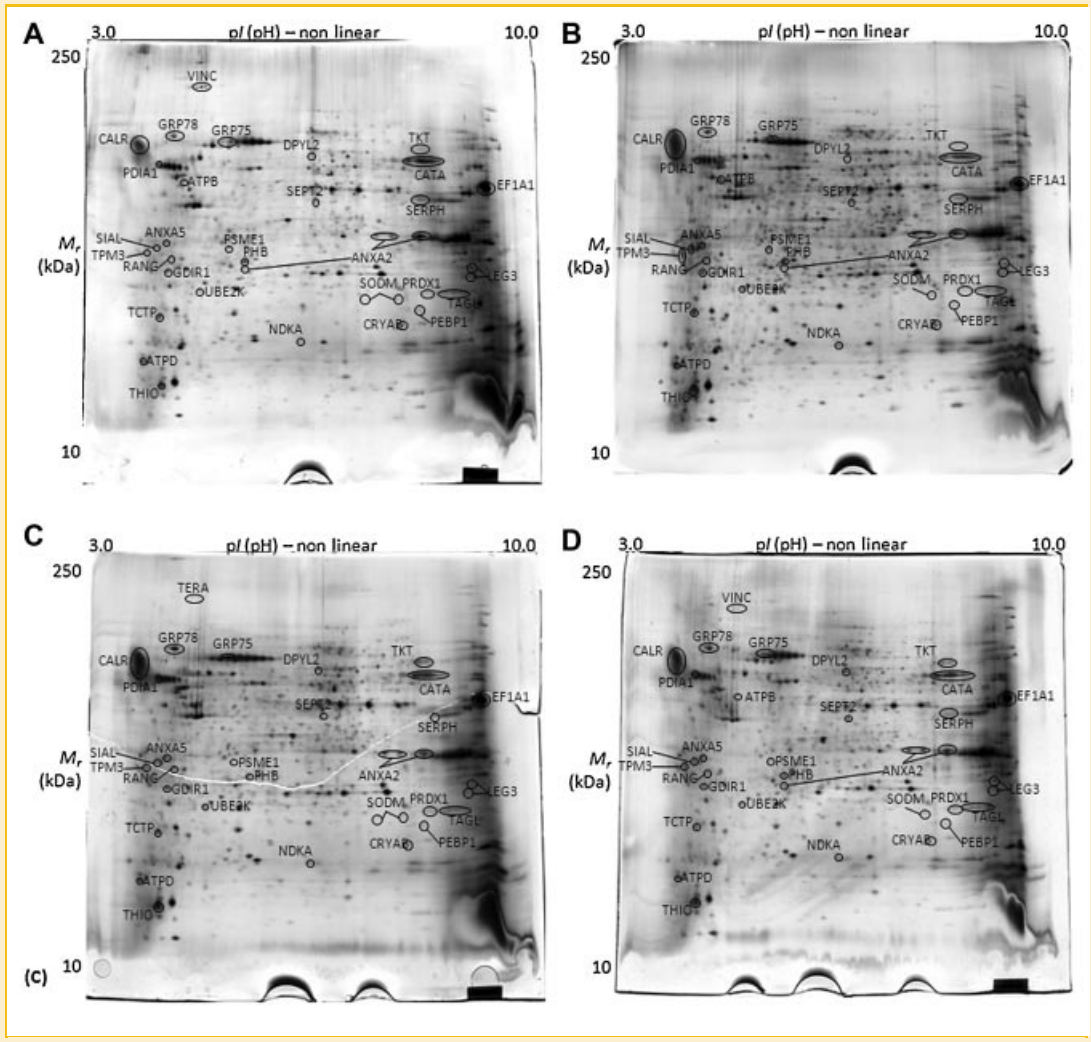

Fig. 2. Proteomic analysis. Silver stained 2D maps of C20 cells under control conditions (A), or in the presence of 0.1 mM ASC (B), 0.33 mM HGA (C), and 0.1 mM ASC + 0.33 mM HGA (D) after 9 days of treatment. Representative images from a triplicate set are shown.

together with GRP78, can bind denatured collagen [Freyria et al., 1995]. On the contrary, a minor set of proteins was found to be over-expressed. This set included PDIA1 and GRP75, like observed previously in the study of HGA-induced effects. This finding thus reinforced the hypothesis of HGA-induced negative effects on chondrocytic protein folding. The under-expression of phosphatidylethanolamine-binding protein 1 (PEBP1) was also found. PEBP1 inhibits Raf kinase, and it was identified among the chondrocytic proteins whose synthesis is altered in osteoarthritis, though with mechanisms still to be fully elucidated [Guo et al., 2008].

An interesting finding was the under-expression of mSOD and PDX1 (already observed in ASC-treated cells), as well as the over-expression of thioredoxin (THIO). THIO is a thioredoxin catalyzing disulphide exchange reactions and plays a role in S-nitrosylation/denitrosylation of cysteines [Benhar et al., 2009]. Since THIO mediates the cell response to nitric oxide, its over-expression could in turn indicate that NO release is one of the mechanisms of HGA-induced alterations.

REDOX-PROTEOMICS

A redox-proteomic approach was used to identify C20 proteins undergoing carbonylation after the treatment with HGA, ASC, or HGA + ASC. Patterns of carbonylated proteins after 9 days of treatment are reported in Figure 3. A comprehensive list of carbonylated proteins is reported in Table II.

We found immunoreactive high MW aggregates, non-resolvable through the gels, in HGA- and HGA + ASC-treated cells. 4 immunoreactive spots in the control, 27 in ASC-treated C20 cells, 22 in HGA-treated C20 cells, and 5 in cells treated with HGA + ASC were resolved. The majority of these immunoreactive spots were identified by gel matching with the corresponding silver stained 2D map and hence with the chondrocyte reference proteomic maps characterized in our laboratory [Spreafico et al., 2006, 2009].

Under control conditions, we identified as carbonylated the proteins SERPH and EF1A1. In either ASC- or HGA-treated cells we found as carbonylated CALR, PDIA1, HSP7C, GRP75, ACTB, G3P2, and CATA. The proteins VINC and VIME were oxidized only in ASC-treated cells, while DJ1 and ANXA2 only in HGA-treated cells. The

TABLE I. Differentially Expressed Proteins in ASC-, HGA-, and HGA + ASC-Treated Chondrocytes

Spot	AN	Gene	Protein	Biological processes[1]	Loc.[2]	Fold-change[3] ASC	HGA	HGA + ASC
Metabolism								
Nucleotide, nucleoside and nucleic acid								
DPYL2	Q16555	*DPYSL2*	Dihydropyrimidinase-related protein 2	Cell differentiation, remodeling of cytoskeleton, signal transduction	C, Cs	−2.6		
NDKA	P15531	*NME1*	Nucleoside diphosphate kinase A	Nucleotide metabolism, cell differentiation, regulation of apoptosis	C, N	+2.1		−2.2[b]
Energy								
Pentose phosphate pathway								
TKT	P29401	*TKT*	Transketolase	Metabolic process	C	−4.3		+4.8[b]
Respiration and fermentation								
ATPB	P06576	*ATP5B*	ATP synthase subunit beta, mitochondrial	ATP synthesis, regulation of intracellular pH	Mt		A	P[c]
ATPD	P30049	*ATP5D*	ATP synthase subunit delta, mitochondrial	ATP synthesis from ADP in the presence of a proton gradient across the membrane	Mt		−2.2	
Transcription, protein synthesis and turnover								
EF1A1	P68104	*EEF1A*	Elongation factor 1-alpha 1	Protein biosynthesis, translational elongation	C	−2.0	+2.0	+3.7[b]
PSME1	Q06323	*PSME1*	Proteasome activator complex subunit 1	Protein degradation	C, Pr		−2.2	−2.1[a] −2.1[b]
UBE2K	P61086	*UBE2K*	Ubiquitin-conjugating enzyme E2 K	Post-translational modification of proteins, ubiquitin-dependent protein catabolic process	C	+2.2		
Protein fate (folding, maturation, transport)								
CALR	P27797	*CALR*	Calreticulin	Molecular chaperone, protein maturation and stabilization, regulation of apoptosis	ER, C, EM		+2.3	−2.6[c]
GRP75	P38646	*HSPA9*	Stress-70 protein, mitochondrial	Anti-apoptosis, protein folding, unfolded protein binding	Mt	−2.3		+3.2[a] +7.4[b]
GRP78	P11021	*HSPA5*	78 kDa glucose-regulated protein	Anti-apoptosis, protein folding, unfolded protein binding, cellular response to glucose starvation	ER, Mel			+2.0[b]
SERPH	P29043	*SERPINH1*	Serpin H1	Stress response, unfolded protein binding, binds specifically to collagen and could be involved as a chaperone in the biosynthetic pathway of collagen	ER	−4.6		−3.0[a]
PDIA1	P07237	*P4HB*	Protein disulfide-isomerase	Catalyzes the formation, breakage and rearrangement of disulfide bonds. At the cell surface, seems to act as a reductase that cleaves disulfide bonds of proteins attached to the cell. May therefore cause structural modifications of exofacial proteins. Inside the cell, seems to form/rearrange disulfide bonds of nascent proteins. At high concentrations, functions as a chaperone that inhibits aggregation of misfolded proteins. At low concentrations, facilitates aggregation (anti-chaperone activity) [Mezghrani et al., 2000]	ER, Mel, M		+4.9	+4.4[a]
TERA	P55072	*VCP*	Transitional endoplasmic reticulum ATPase	ER-associated protein catabolic process, endoplasmic reticulum unfolded protein response, protein ubiquitination, retrograde protein transport (ER to cytosol)	C, N		P	+5.1[b] A[c]
Signal transduction								
GDIR1	P52565	*ARHGDIA*	Rho GDP-dissociation inhibitor 1	Rho protein signal transduction, cell motion, anti-apoptosis, regulation of cell adhesion	C			−2.1[b]
PEBP1	P30086	*PEBP1*	Phosphatidylethanolamine-binding protein 1	Serine protease inhibitor which inhibits thrombin, neuropsin and chymotrypsin but not trypsin, tissue type plasminogen activator and elastase	C	−3.7		−2.8[a]
RANG	P43487	*RANPB1*	Ran-specific GTPase-activating protein	Signal transduction	N	−2.1		−2.2[a] −2.4[c]
TCTP	P13693	*TPT1*	Translationally-controlled tumor protein	Microtubule stabilization, calcium homeostasis	C			−2.1[b]
Cellular organization								
Cytoskeleton and microtubules								
TAGL	Q01995	*TAGLN*	Transgelin	Actin cross-linking/gelling protein. Involved in calcium interactions and contractile properties of the cell that may contribute to replicative senescence. Over-expressed in human senescent fibroblasts	C	−2.8	−2.1	
VINC	P18206	*VCL*	Vinculin	Cell adhesion, morphology and motility	CJ, M, C, Cs, S	A	A	−2.8[a] P[b] P[c]
Cell cycle								
PHB	P35232	*PHB*	Prohibitin	Inhibition of DNA synthesis, regulation of apoptosis and proliferation	M, Mt		−2.9	
SEPT2	Q15019	*SEPT2*	Septin-2	Cell division, mitosis	C		−3.4	+3.5[c]

TABLE I. (*Continued*)

Spot	AN	Gene	Protein	Biological processes[1]	Loc.[2]	Fold-change[3] ASC	HGA	HGA + ASC
Annexin family								
ANXA2	P07355	*ANXA2*	Annexin A2	Calcium-regulated membrane-binding protein. May be involved in heat-stress response	BM, EM, Mel			+2.2[b]
ANXA5	P08758	*ANXA5*	Annexin A5	Calcium-regulated membrane-binding protein, anti-apoptosis	C, N, M	+2.1		
Other functions								
LEG3	P17931	*LGALS3*	Galectin-3	Cell differentiation	N, C, M	−2.4		
SIAL	P21815	*IBSP*	Bone sialoprotein 2	Biomineralization, cell adhesion	EM	+3.3		−3.1[b]
TPM3	P06753	*TPM3*	Tropomyosin alpha-3 chain	Stabilization of cytoskeleton and actin filaments, cell motion	C, Cs	+2.3		+2.1[c]
Cell rescue, defense, and stress								
CATA	P04040	*CAT*	Catalase	Hydrogen peroxide catabolic process, negative regulation of apoptosis, positive regulation of cell division	Pe	−2.0		
CRYAB	P02511	*CRYAB*	Alpha-crystallin B chain	Anti-apoptosis, protein folding, unfolded protein binding, response to heat	–		−2.4	
PRDX1	Q06830	*PRDX1*	Peroxiredoxin-1	Cell redox-homeostasis, peroxide catabolic process, cell proliferation	C, N, Mel	−5.7		−4.0[a] −3.0[c]
SODM	P04179	*SOD2*	Superoxide dismutase [Mn], mitochondrial	Response to ROS	Mt	−2.6		−2.1[a]
THIO	P10599	*TXN*	Thioredoxin	Cell redox homeostasis, cell motion, cell proliferation	C			+2.2[a]

AN, accession number.
[1]Protein biological processes as retrieved by UniProt knowledgebase.
[2]Protein sub-cellular localization: cytosol (C); cytoskeleton (Cs); cell junction (CJ); membrane (M); basal membrane (BM); extracellular matrix (EM); melanosome (Mel); mitochondrion (Mt); nucleus (N); peroxisome (Pe); proteasome (Pr); endoplasmic reticulum (ER); secreted (S). Retrieved by UniProt knowledgebase (http://www.uniprot.org/).
[3]Fold-change in protein % relative abundance (as average values in case of multiple spots); (+) over-expressed proteins, (−) under-expressed protein according to the following comparisons. Under the column "ASC," the fold change values calculated in ASC-treated cells versus the control (CTR) are reported. Under the column "HGA," the fold change values calculated in HGA-treated cells versus CTR are reported. Under the column "HGA + ASC," the fold-change values were calculated as follows: [a]HGA + ASC versus CTR; [b]HGA+ASC versus ASC; [c]HGA + ASC versus HGA. "A" and "P" indicate the absence or presence of proteins, respectively.

simultaneous treatment with HGA + ASC induced the carbonylation of EF1A1 and G3P2.

DISCUSSION

Though AKU has been well characterized clinically, little is known on the molecular mechanisms leading to ochronosis. This is due mainly to the rarity of the disease [Phornphutkul et al., 2002], to the difficulties and ethical issues of collecting ochronotic samples from AKU sufferers, and also to the lack of suitable in vitro or in vivo models reproducing the pathological conditions. In the present work, we used a chondrocytic model to evaluate the effects of HGA, which is the compound accumulated during life in AKU sufferers, and ASC, which was the first drug hypothesized to ameliorate AKU and ochronosis, although its efficacy is still controversial [Phornphutkul et al., 2002].

Several theories have been proposed for the development of ochronosis, involving either direct or indirect effects of HGA. On the one hand, HGA can act as a chemical irritant, cause inflammation [Keller et al., 2005] and bind to collagen [Ellaway et al., 2001]. On the other hand, HGA undergoes spontaneous oxidation, yielding the quinone intermediate BQA that, in vivo, may polymerize to form ochronotic pigment and bind to collagen fibers [Ellaway et al., 2001], increasing intermolecular cross-linking with a process similar to that observed in aging or tanning hides to make leather [Milch, 1961]. BQA is very toxic and contributes to the production of ROS, such as O_2^- and H_2O_2 [Martin and Batkoff, 1987]. Furthermore, either BQA or its semiquinone can catalyze a rapid co-oxidation of ASC, generating O_2^- and H_2O_2 during the reaction [Martin and Batkoff, 1987]. Additionally, the ochronotic pigment itself may contribute to propagate oxidative alterations [Hegedus, 2000]. Both HGA and BQA can thus deplete anti-oxidants, resulting in oxidative damage of macromolecules. This can promote inflammation and contributing to tissue damage [Hegedus and Nayak, 1994]. The generated oxidative imbalance, which should be read as repeated insults throughout patients' life initiated by HGA auto-oxidation, may at some point overwhelm the anti-oxidant defenses and let ochronosis develop.

It is fundamental to settle up models to evaluate the effects of HGA and its metabolites. Our work is the first adopting a proteomic approach for an in vitro AKU model based on a C20 chondrocytic cell line. As the only cells synthesizing articular cartilage matrix, the ability of chondrocytes to tolerate and counteract external stress is important to the health and maintenance of proper joint functions [Oliver et al., 2005]. This makes fundamental the knowledge on chondrocytes' response to HGA in alkaptonuric ochronosis.

To gain an insight into HGA and ASC effects on C20 protein repertoires, we undertook the proteomic and redox-analysis of 9 days-treated cells. At this sampling, HGA and HGA + ASC negatively affected cell proliferation.

Our comparative analysis highlighted specific effects of ASC and HGA in chondrocytic proteomes and confirmed the pro-oxidant action of ASC. In this light, ASC was found to negatively affect proteins whose roles are vital in the response to oxidative stress.

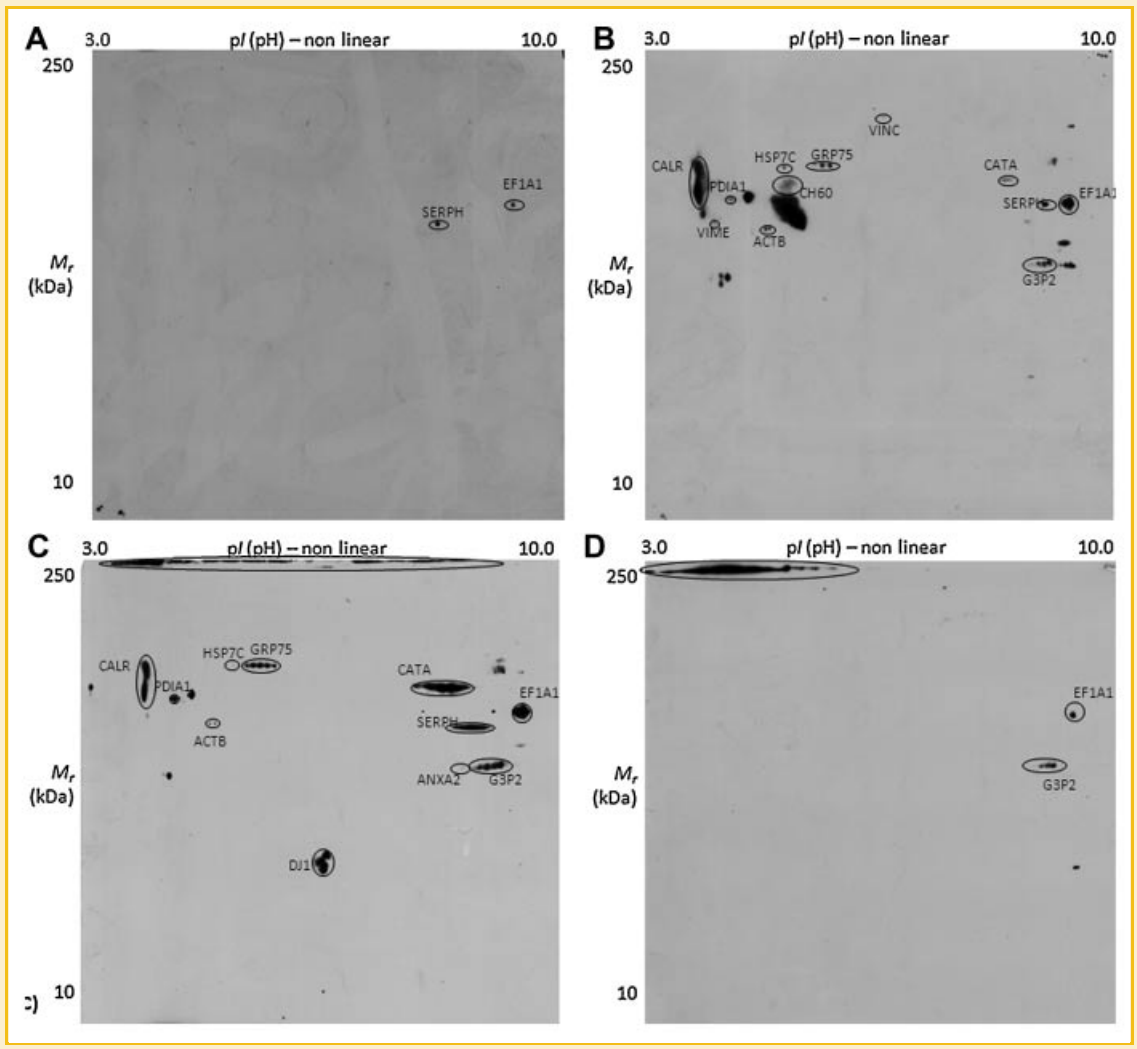

Fig. 3. Redox-proteomic analysis. Carbonylated proteins in C20 cells under control conditions (**A**), or in the presence of 0.1 mM ASC (**B**), 0.33 mM HGA (**C**), and 0.1 mM ASC + 0.33 mM HGA (**D**) after 9 days of treatment. Representative images from a triplicate set are shown.

HGA was shown to induce proteomic alterations that are partially overlapping with proteomic studies on osteoarthritic cartilage and, specifically, HGA induced alteration of protein folding, in particular, it induced the expression of PDIA1, which is fundamental in load bearing joints. Load bearing joints are the first to be affected by ochronosis with negative relapses for AKU patients' mobility and hence quality of life. HGA-induced specific alterations of this protein may let us speculate of a fundamental role of PDIA1 also in the alkaptonuric disease. Additionally, the HGA-induced over-expression of TKT1 suggested the generation of an oxidative

TABLE II. Identification of Carbonylated (+) Proteins in ASC-, HGA-, and HGA + ASC-Treated Chondrocytes

Spot	Protein	CTR	ASC	HGA	HGA + ASC	Functions
ACTB	Actin		+	+		Structural constituent of cytoskeleton, cell motion
ANXA2	Annexin 2			+		Cytoskeletal protein binding
CALR	Calreticulin		+	+		Cell cycle, senescence, cell proliferation, apoptosis
CATA	Catalase		+	+		Response to oxidative stress
CH60	Heat shock protein 60 kDa		+			Protein folding and maturation, response to unfolded protein
DJ1	Protein DJ-1			+		Protein binding
EF1A1	Translation elongation factor 1α1	+	+	+	+	Translational elongation
G3P2	Glyceraldehyde-3-phosphate dehydrogenase		+	+	+	Glycolysis, response to oxidative stress
GRP75	75 kDa glucose-regulated protein		+	+		Protein folding, anti-apoptosis
SERPH	Serpin H1			+		Response to unfolded protein, collagen binding
HSP7C	Heat shock cognate 71 kDa protein		+	+		Protein folding, stress response
PDIA1	Protein disulfide-isomerase		+	+		Cell redox homeostasis
VIME	Vimentin		+			Structural constituent of cytoskeleton, cell motion

imbalance that cells try to counteract through the activation of the pentose phosphate pathway as an alternative metabolic route.

The effects generated by the concomitant treatment of cells with HGA and ASC reinforced the hypothesis of an oxidative imbalance and an altered protein folding generated by the tested compounds while failed to highlight any beneficial effect of ASC in the protection of cells from HGA-induced stress.

Redox-balance is one of the main regulators of cartilage degradation/stimulation, as ROS play a role either in mediating the loss of cartilage or stimulating the production of chondrocyte matrix. Hence, chondrocytes anti-oxidant systems need to be finely tuned in coordinating the responses to ROS. Oxidative stress may impair chondrocytes response to growth factors, migration to sites of cartilage injuries, interactions with extracellular matrix, as well as apoptosis [Hitchon and El-Gabalawy, 2004]. Moreover, ROS can cause low levels of collagen fragmentation and enhance collagen fibril cross-linking together with damage of the extracellular matrix components [Hitchon and El-Gabalawy, 2004].

Protein carbonylation is widely accepted as a reliable and reproducible biomarker of oxidative stress for many human diseases [Dalle-Donne et al., 2003]. Increases in protein carbonyls formation have been already reported for rheumatoid arthritis and osteoarthritis, two pathologies that share some features with AKU [Chapman et al., 1989].

Globally, major targets of oxidation were structural components (such as ACTB and VIME), as well as proteins with functions in the stress response (such as CATA, CH60, GRP75, SERPH, HSP7C, and PDIA1). Under control conditions, only two carbonylated proteins were identified: SERPH and EF1A1, and this phenomenon was considered as a sort of physiological ''basal'' signal. The treatment of C20 either with ASC or HGA resulted in a marked increase of carbonylated proteins. A common protein carbonylation signature was found, as the same proteins were found to be oxidized in both treatments. Nonetheless, VINC and VIME were specifically oxidized in ASC-treated cells, while DJ1 and ANXA2 only in HGA-treated cells. Interestingly, in HGA-treated C20 we found high MW carbonylated protein aggregates that were not resolved in SDS–PAGE. Analogous high MW carbonylated protein aggregates were observed in HGA + ASC C20 and this may be related to the contemporaneous observation of only a small number of carbonylated protein spots.

We have already mentioned the pro-oxidant action of ASC, and others have already reported that ASC represents a potential source of protein carbonyls [Miyata et al., 1998; Blackburn et al., 1999]. These findings were confirmed by the proteomic and redox-proteomic analysis of the effects of ASC in our C20 cell model.

It has been demonstrated that within reducing environments, as the case of rheumatoid joint [Blake et al., 1994], chronic oxidative stress increases the formation of dehydroascorbic acid. The inability to reduce it back to ASC results, in turns, in an enhanced and rapid production of highly reactive carbonyls from dehydroascorbate [Linetsky et al., 2008]. Specifically, Fan et al. [2006] showed that an increased uptake of ASC into mouse lenses caused increased formation of modified lenticular proteins exhibiting characteristic browning and fluorescence. Linetsky et al. [2008] showed that ASC can increase, in a concentration-dependent fashion, protein cross-linking and production of high MW protein aggregates un-resolvable through SDS–PAGE gels under reducing conditions. ASC was also found to cause the precipitation of crystallins very quickly, probaly because of glycation and oxidation. Alpha-crystallins play fundamental chaperoning activity and, if their activity is overwhelmed, high MW protein aggregates occur. Hence, results found in our chondrocytic model found optimal agreement and were validated by these previous observations in other biological systems. In fact, we proved a significant under-expression of alpha-crystallin either in HGA- or HGA + ASC-treated C20 cells, a direct correlation with an increased production of oxidized proteins and, in the case of HGA + ASC, also with a significant presence of oxidized high MW protein aggregates.

HGA-induced high MW aggregates provided an indirect evidence of HGA oxidation to BQA. In fact, it has been recently demonstrated that quinones can produce cross-linked protein aggregates that cannot migrate in SDS–PAGE [Liebeke et al., 2008]. Quinone-induced aggregation of proteins was thought to be mediated by S-thiolation; our results suggest that also carbonylation, already known to induce protein cross-linking, may contribute to this phenomenon.

Notably, to the best of our knowledge, we were the first to investigate carbonylated proteins in human chondrocytes using a redox-proteomic approach. Here, we also showed that protein carbonylation was induced either by treating chondrocytic cells with ASC or HGA in a common panel of proteins (protein signature). These include well-known targets of oxidation shared by a variety of systems, from unicellular organisms to human tissues, such as cytoskeletal components (ACTB, ANXA2, VIME), glycolytic enzymes (G3P2) and stress-induced proteins and molecular chaperones (CATA, CH60, GRP75, HSP7C). In turn, since carbonylation is often accompanied by protein function loss, it is conceivable to hypothesize that cell organization and proper response to stress may be altered or even impaired in ASC- and HGA-treated chondrocytes.

Globally, our findings suggest that HGA may alter the protein folding and reinforced the hypothesis of both ASC- and HGA-mediated imbalance of redox homeostasis in chondrocytes (under-expression of specific anti-oxidant proteins together with the induction of protein carbonylation). More specifically, the co-administration of both ASC and HGA greatly enhanced the production of high MW carbonylated protein aggregates that may help, in vivo, the production of the ochronotic pigment. Since ASC was proposed for the treatment alkaptonuric ochronosis, our evidences suggest caution to be taken when considering ASC as a beneficial drug for AKU sufferers.

ACKNOWLEDGMENTS

This work was supported by Fondazione Monte dei Paschi di Siena Esercizio 2008 and Esercizio 2009, Toscana Life Sciences Foundation project Orphan_0108 and TELETHON GPP10058 to Annalisa Santucci, and by the AKU Society (www.alkaptonuria.info) to James A Gallagher. Adam Taylor was supported by a PhD studentship from the University of Liverpool.

REFERENCES

Angeles AP, Badger R, Gruber HE, Seegmiller JE. 1989. Chondrocyte growth inhibition induced by homogentisic acid and its partial prevention with ascorbic acid. J Rheumatol 16(4):512–517.

Benhar M, Forrester MT, Stamler JS. 2009. Protein denitrosylation: Enzymatic mechanisms and cellular functions. Nat Rev Mol Cell Biol 10(10):721–732.

Blackburn AC, Doe WF, Buffinton GD. 1999. Protein carbonyl formation on mucosal proteins in vitro and in dextran sulfate-induced colitis. Free Radic Biol Med 27(3–4):262–270.

Blake DR, Winyard PG, Marok R. 1994. The contribution of hypoxia-reperfusion injury to inflammatory synovitis: The influence of reactive oxygen intermediates on the transcriptional control of inflammation. Ann NY Acad Sci 723:308–317.

Boeuf S, Steck E, Pelttari K, Hennig T, Buneb A, Benz K, Witte D, Sültmann H, Poustka A, Richter W. 2008. Subtractive gene expression profiling of articular cartilage and mesenchymal stem cells: serpins as cartilage-relevant differentiation markers. Osteoarthritis Cartilage 16(1):48–60.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Chapman ML, Rubin BR, Gracy RW. 1989. Increased carbonyl content of proteins in synovial fluid from patients with rheumatoid arthritis. J Rheumatol 16(1):15–18.

Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta 329(1–2):23–38.

de Haas V, Carbasius Weber EC, de Klerk JB, Bakker HD, Smit GP, Huijbers WA, Duran M, Poll-The BT. 1998. The success of dietary protein restriction in alkaptonuria patients is age-dependent. J Inherit Metab Dis 21(8):791–798.

Ellaway CJ, Holme E, Standing S, Preece MA, Green A, Ploechl E, Ugarte M, Trefz FK, Leonard JV. 2001. Outcome of tyrosinaemia type III. J Inherit Metab Dis 24(8):824–832.

Fan X, Reneker LW, Obrenovich ME, Strauch C, Cheng R, Jarvis SM, Ortwerth BJ, Monnier VM. 2006. Vitamin C mediates chemical aging of lens crystallins by the Maillard reaction in a humanized mouse model. Proc Natl Acad Sci USA 103(45):16912–16917.

Fernández-Cañón JM, Granadino B, Beltrán-Valero de Bernabé D, Renedo M, Fernández-Ruiz E, Peñalva MA, Rodríguez de Córdoba S. 1996. The molecular basis of alkaptonuria. Nat Genet 14(1):19–24.

Fisher AA, Davis MW. 2004. Alkaptonuric ochronosis with aortic valve and joint replacements and femoral fracture: A case report and literature review. Clin Med Res 2(4):209–215.

Frech TM, Clegg DO. 2007. The utility of nutraceuticals in the treatment of osteoarthritis. Curr Rheumatol Rep 9(1):25–30.

Freyria AM, Ronzière MC, Boutillon MM, Herbage D. 1995. Effect of retinoic acid on protein synthesis by foetal bovine chondrocytes in high-density culture: Down-regulation of the glucose-regulated protein, GRP-78, and type II collagen. Biochem J 305(Pt 2):391–396.

Gartland AJ, Buckley KA, Dillon JP, Curran JM, Hunt JA, Gallagher JA. 2005. Isolation and culture of human osteoblasts. In: Picot J, editor. Human cell culture protocols. 2nd edition. Totowa, New Jersey: Humana Press. pp 29–54.

Grimmer C, Balbus N, Lang U, Aigner T, Cramer T, Müller L, Swoboda B, Pfander D. 2006. Regulation of type II collagen synthesis during osteoarthritis by prolyl-4-hydroxylases: Possible influence of low oxygen levels. Am J Pathol 169(2):491–502.

Guo D, Tan W, Wang F, Lv Z, Hu J, Lv T, Chen Q, Gu X, Wan B, Zhang Z. 2008. Proteomic analysis of human articular cartilage: Identification of differentially expressed proteins in knee osteoarthritis. Joint Bone Spine 75(4):439–444.

Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, Ron D. 2000. Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell 6(5):1099–1108.

Hegedus ZL. 2000. The probable involvement of soluble and deposited melanins, their intermediates and the reactive oxygen side-products in human diseases and aging. Toxicology 145(2–3):85–101.

Hegedus ZL, Nayak U. 1994. Homogentisic acid and structurally related compounds as intermediates in plasma soluble melanin formation and in tissue toxicities. Arch Int Physiol Biochim Biophys 102(3):175–181.

Helliwell TR, Gallagher JA, Ranganath L. 2008. Alkaptonuria—A review of surgical and autopsy pathology. Histopathology 53:503–512.

Hitchon CA, El-Gabalawy HS. 2004. Oxidation in rheumatoid arthritis. Arthritis Res Ther 6(6):265–278.

Ibold Y, Lübke C, Pelz S, Augst H, Kaps C, Ringe J, Sittinger M. 2009. Effect of different ascorbate supplementations on in vitro cartilage formation in porcine high-density pellet cultures. Tissue Cell 41(4):249–256.

Keller JM, Macaulay W, Nercessian OA, Jaffe IA. 2005. New developments in ochronosis: Review of the literature. Rheumatol Int 25(2):81–85.

Kletzien RF, Harris PK, Foellmi LA. 1994. Glucose-6-phosphate dehydrogenase: A "housekeeping" enzyme subject to tissue-specific regulation by hormones, nutrients, and oxidant stress. FASEB J 8(2):174–181.

Kutty MK, Iqbal QM, Teh EC, Lumpur K. 1974. Ochronotic arthropathy: An electron microscopical study with a view on pathogenesis. Arch Pathol 98(1):55–57.

Liebeke M, Pöther DC, van Duy N, Albrecht D, Becher D, Hochgräfe F, Lalk M, Hecker M, Antelmann H. 2008. Depletion of thiol-containing proteins in response to quinones in *Bacillus subtilis*. Mol Microbiol 69(6):1513–1529.

Linetsky M, Shipova E, Cheng R, Ortwerth BJ. 2008. Glycation by ascorbic acid oxidation products leads to the aggregation of lens proteins. Biochim Biophys Acta 1782(1):22–34.

Manning K, Fernández-Cañón JM, Montagutelli X, Grompe M. 1999. Identification of the mutation in the alkaptonuria mouse model. Mutations in brief no. 216. Online. Hum Mutat 13(2):171.

Martin JP, Jr., Batkoff B. 1987. Homogentisic acid autoxidation and oxygen radical generation: Implications for the etiology of alkaptonuric arthritis. Free Radic Biol Med 3(4):241–250.

Mezghrani A, Courageot J, Mani JC, Pugniere M, Bastiani P, Miquelis R. 2000. Protein-disulfide isomerase (PDI) in FRTL5 cells. pH-dependent thyroglobulin/PDI interactions determine a novel PDI function in the post-endoplasmic reticulum of thyrocytes. J Biol Chem 275(3):1920–1929.

Milch RA. 1961. Studies on alkaptonuria: Collagenase degradation of homogentisinic-tanned hide powder collagen. Proc Soc Exp Biol Med 107:83–90.

Miyata T, Inagi R, Asahi K, Yamada Y, Horie K, Sakai H, Uchida K, Kurokawa K. 1998. Generation of protein carbonyls by glycoxidation and lipoxidation reactions with autoxidation products of ascorbic acid and polyunsaturated fatty acids. FEBS Lett 437(1–2):24–28.

Nugent AE, Speicher DM, Gradisar I, McBurney DL, Baraga A, Doane KJ, Horton WE, Jr. 2009. Advanced osteoarthritis in humans is associated with altered collagen VI expression and upregulation of ER-stress markers Grp78 and bag-1. J Histochem Cytochem 57(10):923–931.

Oliver BL, Cronin CG, Zhang-Benoit Y, Goldring MB, Tanzer ML. 2005. Divergent stress responses to IL-1beta, nitric oxide, and tunicamycin by chondrocytes. J Cell Physiol 204(1):45–50.

Pandolfi PP, Sonati F, Rivi R, Mason P, Grosveld F, Luzzatto L. 1995. Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress. EMBO J 14(21):5209–5215.

Phornphutkul C, Introne WJ, Perry MB, Bernardini I, Murphey MD, Fitzpatrick DL, Anderson PD, Huizing M, Anikster Y, Gerber LH, Gahl WA. 2002. Natural history of alkaptonuria. N Engl J Med 347(26):2111–2121.

Reinheckel T, Körn S, Möhring S, Augustin W, Halangk W, Schild L. 2000. Adaptation of protein carbonyl detection to the requirements of proteome analysis demonstrated for hypoxia/reoxygenation in isolated rat liver mitochondria. Arch Biochem Biophys 376(1):59–65.

Ruiz-Romero C, López-Armada MJ, Blanco FJ. 2005. Proteomic characterization of human normal articular chondrocytes: A novel tool for the study of osteoarthritis and other rheumatic diseases. Proteomics 5(12):3048–3059.

Selvi E, Manganelli S, Mannoni A, Benucci M, Minacci C, Marcolongo R. 2000. Chronic ochronotic arthritis: Clinical, arthroscopic and patholoc findings. J Rheumatol 27(9):27–29.

Spreafico A, Frediani B, Capperucci C, Chellini F, Paffetti A, D'Ambrosio C, Bernardini G, Mini R, Collodel G, Scaloni A, Marcolongo R, Santucci A. 2006. A proteomic study on human osteoblastic cells proliferation and differentiation. Proteomics 6(12):3520–3532.

Spreafico A, Chellini F, Frediani B, Bernardini G, Niccolini S, Serchi T, Collodel G, Paffetti A, Fossombroni V, Galeazzi M, Marcolongo R, Santucci A. 2009. Biochemical investigation of the effects of human platelet releasates on human articular chondrocytes. J Cell Biochem 108(5):1153–1165.

Suwannarat P, O'Brien K, Perry MB, Sebring N, Bernardini I, Kaiser-Kupfer MI, Rubin BI, Tsilou E, Gerber LH, Gahl WA. 2005. Use of nitisinone in patients with alkaptonuria. Metabolism 54(6):719–728.

Switzer RC III, Merril CR, Shifrin S. 1979. A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. Anal Biochem 98(1):231–237.

Taylor AM, Wilson PJ, Ingrams DR, Helliwell TR, Gallagher JA, Ranganath LR. 2010a. Calculi and intracellular ochronosis in the submandibular tissues from a patient with alkaptonuria. J Clin Pathol 63(2):186–188.

Taylor AM, Wlodarski B, Prior IA, Wilson PJ, Jarvis JC, Ranganath LR, Gallagher JA. 2010b. Ultrastructural examination of tissue in a patient with alkaptonuric arthropathy reveals a distinct pattern of binding of ochronotic pigment. Rheumatology (Oxford) 47(7):1412–1414.

Tinti L, Spreafico A, Braconi D, Millucci L, Bernardini G, Chellini F, Cavallo G, Selvi E, Galeazzi M, Marcolongo R, Gallagher J, Santucci A. 2010. Evaluation of antioxidant drugs for the treatment of ochronotic alkaptonuria in an in vitro human cell model. J Cell Physiol 225(1):84–91.

VanderJagt DJ, Garry PJ, Bhagavan HN. 1987. Ascorbic acid intake and plasma levels in healthy elderly people. Am J Clin Nutr 46(2):290–294.

Wadhwa R, Taira K, Kaul SC. 2002. An Hsp70 family chaperone, mortalin/mthsp70/PBP74/Grp75: What, when, and where. Cell Stress Chaperones 7:309–316.

Zannoni VG, Malwista SE, La Du BN. 1962. Studies on ochronosis II. Studies on benzoquinoneacetic acid, a probable intermediate in the connective tissue pigmentation in alkaptonuria. Arthritis Rheum 5:547–556.