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Journal of Cellular Biochemistry 117:402–412 (2016)

Journal of Cellular Biochemistry

Cysteine Prevents the Reduction in Keratin Synthesis Induced by Iron Deficiency in Human Keratinocytes

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ABSTRACT

L-cysteine is currently recognized as a conditionally essential sulphur amino acid. Besides contributing to many biological pathways, cysteine is a key component of the keratin protein by its ability to form disulfide bridges that confer strength and rigidity to the protein. In addition to cysteine, iron represents another critical factor in regulating keratins expression in epidermal tissues, as well as in hair follicle growth and maturation. By focusing on human keratinocytes, the aim of this study was to evaluate the effect of cysteine supplementation as nutraceutical on keratin biosynthesis, as well as to get an insight on the interplay of cysteine availability and cellular iron status in regulating keratins expression *in vitro*. Herein we demonstrate that cysteine promotes a significant up-regulation of keratins expression as a result of *de novo* protein synthesis, while the lack of iron impairs keratin expression. Interestingly, cysteine supplementation counteracts the adverse effect of iron deficiency on cellular keratin expression. This effect was likely mediated by the up-regulation of transferrin receptor and ferritin, the main cellular proteins involved in iron homeostasis, at last affecting the labile iron pool. In this manner, cysteine may also enhance the metabolic iron availability for DNA synthesis without creating a detrimental condition of iron overload. To the best of our knowledge, this is one of the first study in an *in vitro* keratinocyte model providing evidence that cysteine and iron cooperate for keratins expression, indicative of their central role in maintaining healthy epithelia. J. Cell. Biochem. 117: 402–412, 2016.

KEY WORDS: CYSTEINE; KERATINS; KERATINOCYTES; IRON METABOLISM; IRON-RELATED PROTEINS; LABILE IRON POOL

Keratins are members of the intermediate filament family preferentially expressed in epithelial tissues. They are responsible of the mechanical integrity of the epithelia at both cellular and tissue level by forming a dense and stable network spanning from the cell periphery to the nucleus of keratinocytes. In addition to protecting epithelial cells from mechanical stress, keratins participate in the regulation of cell growth, proliferation, migration and apoptosis [Coulombe and Omary, 2002; Oshima, 2007; Vaidya and Kanojia, 2007; Kim and Coulombe, 2007]. More than 50 different intermediate filament proteins have been identified and classified into six groups based on their amino acid sequence. Epithelial cells express two specific groups of keratins, type I acidic (e.i. K9–K28 and K31–K40) and type II basic (e.i. K1–K8 and K71–K86) which copolymerize to form intermediate filaments (IF) [Schweizer et al., 2006; Moll et al., 2008; Lee and Coulombe, 2009; Bragulla and Homberger, 2009]. Hair and nails express specialized type I and type II keratin proteins which are extremely rich in cysteine, allowing an high degree of cross-linking between the keratins and keratin-associated proteins (KAPs) [Jones et al., 1997; Steinert, 2001]. The importance of cysteine and its oxidized form, cystine, in maintenance of the healthy structure of hair has been demonstrated in several studies in both humans and mice. Low levels of cysteine in hair have been found in patients affected by trichothiodystrophy, a genetic disorder characterized by hair fragility [Liang et al., 2006; Khumalo et al., 2010]. On the other hand, oral administration of L-cysteine combined with retinol has benefic effects on hair loss in humans [Hertel et al., 1989]. Moreover, L-cystine and vitamin B6 have been shown *in vivo* to inhibit the smoke-induced alopecia in a dose dependent manner [D'Agostini et al., 2007]. According to an early study carried out on sheep on low quality diet, cysteine infusion determined an increase of both keratin-associated proteins (KAP4) mRNA and hair keratinocytes expressing the cysteine-rich KAP4 protein, without interfering with mRNA and protein levels of KAPs with lower cysteine content [Fratini et al., 1994].

Maria Concetta Miniaci and Carlo Irace contributed equally to this work.

Grant sponsor: IDI Farmaceutici, Pomezia (Rome, Italy).

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Manuscript Received: 15 April 2015; Manuscript Accepted: 21 July 2015
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 24 July 2015
DOI 10.1002/jcb.25286 • © 2015 Wiley Periodicals, Inc.

As well as cysteine, iron represents another critical factor involved in many physiological processes of hair follicle, such as growth, maturation, and cell division, suggesting that iron deficiency could affect hair synthesis [Trost et al., 2006; Elston, 2010; St Pierre et al., 2010]. Indeed, several iron metabolism disorders, including the ones characterized by dietary reduced adsorption, are associated to anemia and hair loss, both resolved by iron supplementation therapy [Du et al., 2008]. However, to date studies concerning iron deficiency as a cause of hair loss have produced conflicting results. The importance of adequate amounts of iron for health is well known. This metal is essential for living cells as iron-containing proteins play a central role in oxygen transport, electron transport, and redox reactions. Moreover, the iron-dependent enzyme ribonucleotide reductase is critical for DNA synthesis, particularly in rapid turnover tissues—like epithelia—endowed with high proliferative cells [Yu et al., 2007]. Biological accessible iron in the cytoplasm is mainly divalent and potentially interacting with local counter-ions, mostly thiol-containing compounds like glutathione and cysteine itself. This pool—the "labile iron pool" (LIP)—is now accepted to be at the crossroad between cellular iron import and export, storage in ferritin and incorporation into proteins. The LIP is potentially harmful because of its ability to catalyze the formation of reactive oxygen species (ROS). Consequently, though iron deficiency is detrimental to cells, iron excess is equally toxic since ROS can damage several biomolecules and cellular structures (Papanikolaou and Pantopoulos, 2005; Meli et al., 2013). Therefore, to prevent the damaging consequences of both iron deficiency and iron overload, intracellular iron level must be maintained within a definite range. At cellular level, iron homeostasis is achieved by the coordinated expression of proteins involved in iron uptake, such as transferrin receptor 1 (TfR-1), storage, such as ferritin, and export, such as ferroportin [Santamaria et al., 2004; Hentze et al., 2010]. The expression of these proteins is mainly regulated at post-transcriptional level by the binding of Iron Regulatory Proteins (IRP1 and IRP2) to Iron Responsive Elements (IRE), located in the 5′untranslated region of ferritin and ferroportin mRNAs and in the 3′untranslated region of TfR1 mRNA. At systemic level, iron homeostasis is regulated by hepcidin, a liver-produced peptide hormone that promotes the degradation of the ferroportin in specific cell types, thus reducing iron influx in the extracellular space [Ganz and Nemeth, 2012].

Taking into consideration the critical role played by cysteine and iron in the maintenance of the biological functions of epithelial cells, the aim of this study was to evaluate the biological effects of cysteine on keratin biosynthesis, as well as to get an insight on the interplay of cysteine availability and iron status in regulating cellular keratins expression. For this reason we focused on the possible impact of cysteine administration, coupled or not to iron-overload/iron-deficiency, in keratins biosynthesis *in vitro* in human HaCaT keratinocytes. These cells are a commonly used model system for human epidermal keratinocytes since they retain an high differentiation capacity and functional properties besides expressing a wide spectrum of keratins [Breitkreutz et al., 1998]. Herein we show that cellular keratins content was significantly down-regulated by iron deficit and up-regulated by cysteine. Interestingly, cysteine treatment was able to revert the negative effect of iron deficiency on keratin synthesis, possibly *via* a regulation of the main proteins involved in iron homeostasis thereby affecting LIP magnitude. To the best of our knowledge, this is one of the first investigations showing the coupled effects of cysteine and iron on keratins biosynthesis in human keratinocytes.

MATERIALS AND METHODS

CELL CULTURES AND TREATMENTS

HaCaT cells, an immortalized, non-tumorigenic human keratinocyte cell line, were cultured in Dulbecco′s modified Eagle′s medium (DMEM, Invitrogen, Paisley, UK) containing high glucose (4.5 g/L), supplemented with 10% fetal bovine serum (FBS, Cambrex, Verviers, Belgium), L-glutamine (2 mM, Sigma, Milan, Italy), penicillin (100 units/ml, Sigma) and streptomycin (100 μg/ml, Sigma) at 37°C in a humidified 5% CO_2 atmosphere. At confluence, the complete culture medium was replaced with fresh complete medium and cells were treated with various concentrations of L-cysteine (Sigma) dissolved in sterile water for different time periods. Similarly, control cells were subjected to replacement with fresh complete medium.

For iron repletion/depletion experiments, cells were treated with 50 μg/ml ferric ammonium citrate (FAC) (Sigma) or with 100 μM desferrioxamine (DFO) (Desferal, Novartis, Origgio, Varese, Italy) in growth medium for 18 h.

ASSESSMENT OF CELL VIABILITY

HaCaT cells were seeded in 96-well plates at a density of 10^4 cells/well. After 24 h the medium was replaced with fresh medium and then the cells were treated for 24, 48, and 72 h with L-cysteine at different concentrations (from 1 nM to 100 μM). Celll viability was estimated with MTT assay, which measures the level of mitochondrial dehydrogenase activity using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as substrate [Fiorito et al., 2013]. This assay is based on the redox ability of living mitochondria to convert the tetrazolium salt, MTT, into insoluble formazan. After treatments, the medium was removed and the cells were incubated with 20 μl/well of an MTT solution (5 mg/ml) for 1 h in a humidified 5% CO_2 incubator at 37°C. The incubation was stopped by removing the MTT solution and adding DMSO (100 μl/well) to solubilize the formazan. The absorbance was monitored at 550 nm by using a microplate reader (iMark microplate reader, Biorad, Milan, Italy). Data are expressed as mean percentage of the viable cells *versus* the respective control.

Moreover, we evaluated the relative number of live and dead cells using a MultiTox-Fluor Multiplex Cytotoxicity Assay (Promega, Madison, WI). Briefly, cells were plated into 96-well plates (5×10^3/well) and after the treatment with cysteine, the solution containing the fluorogenic substrates for live-cell and dead-cell protease activities was added to the cells. Following 30 min at 37°C the fluorescence was measured in a Perkin Elmer microplate reader (Perkin Elmer LS 55 Luminescence Spectrometer, Beaconsfield, UK) using a filter combination with an excitation wavelength of 400/485 nm and an emission wavelength of 505/520 nm (slits 5 nm). Data are expressed as percentage of viable cell *versus* control cultures.

PREPARATION OF CELLULAR EXTRACTS

HaCaT cells were grown in the presence or absence of L-cysteine at different concentrations (from 1 to 100 μM). After treatment, protein extracts were prepared according to previously described procedure [Achtstaetter et al., 1986]. Briefly, cell pellets were lysed at 4°C for 10 min with buffer containing 1% Triton X-100, 5 mM EDTA, in PBS (pH 7.4) containing protease inhibitors. After centrifugation at 14,000*g* for 10 min at 4°C, the supernatant was collected as the soluble fraction and stored at −80°C. The pellet was homogenized at 4°C for 30 min in 1 ml of 10 mM Tris-HCl, pH 7.6, 140 mM NaCl, 1.5 M KCl, 5 mM EDTA, 0.5% Triton X-100, and protease inhibitors. After centrifugation at 14,000*g* for 10 min at 4°C, the resulting pellet (insoluble fraction) was resuspended in buffer containing 5 mM EDTA in PBS (pH 7.4) and stored at −80°C. To obtain cytosolic extracts for Western blot analysis of iron related proteins (TfR-1, ferritin and IRP1) cell pellets were lysed at 4°C for 30 min in a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5% (v/v) glycerol, 10 mM NP-40 and protease inhibitor tablets (Roche). The supernatant fraction was obtained by centrifugation at 15,000*g* for 10 min at 4°C and then stored at −80°C.

Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad, Milan, Italy).

WESTERN BLOT ANALYSIS

The same amounts (50 μg) of proteins from soluble and insoluble fractions were loaded on 10% SDS–PAGE and electrotransferred onto a nitrocellulose membrane (GE Healthcare, UK) using a Bio-Rad Transblot (Bio-Rad). Proteins were visualized by reversible staining with Ponceau-S solution and destained in PBS. Membranes were blocked at room temperature in milk buffer [1×PBS, 5–10% (w/v) non-fat dry milk, 0.2% (v/v) Tween-20] and then incubated at 4°C overnight with 1:250 monoclonal anti-Pan cytokeratin antibodies (mixture) (Sigma–Aldrich, Milan, Italy) which recognize the following human cytokeratins, according to their molecular weight: K1 (68 kDa), K4 (59 kDa), K5 (58 kDa), K6/K10 (56 kDa), K13 (54 kDa), K8 (52 kDa), K18 (45 kDa), and K19 (40 kDa). As a secondary antibody was used a goat anti-mouse IgG + IgM (1:5,000, Jackson ImmunoResearch Laboratories, Baltimore Pike, West Grove, PA). For Western blot analysis of TfR-1, ferritin and IRP1, samples containing 50 μg of proteins were loaded on 12% (for ferritin) or 8% (for IRP1 and TfR-1) SDS–PAGE and transferred to nitrocellulose membranes. After blocking at room temperature in milk buffer [1×PBS, 5–10% (w/v) non-fat dry milk, 0.2% (v/v) Tween-20], the membranes were incubated at 4°C overnight with 1:1,000 rabbit polyclonal antibody to human ferritin (Dako Cytomation, Glostrup, Denmark), or with 1:1,000 mouse monoclonal antibody to human transferrin receptor 1 (Zymed Laboratories Inc., CA), or with 1:250 goat polyclonal antibody to human IRP1 (Santa Cruz Biotechnology, Santa Cruz, CA), or with 1:250 goat polyclonal antibody to human IRP2 (Santa Cruz Biotechnology). Subsequently, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG, or peroxidase-conjugated goat antimouse IgG+IgM, or peroxidase-conjugated rabbit anti-goat IgG (all the secondary antibodies were purchased from Jackson ImmunoResearch Laboratories). The immunocomplexes were visualized by the ECL chemoluminescence method (ECL, Amersham Biosciences) and analyzed by an imaging system (ImageQuant^{TM} 400, GE Healthcare Life Sciences). Densitometric analyses were conducted using the GS-800 imaging densitometer (Bio-Rad). β-actin antibody (Sigma–Aldrich) was used to normalize the results.

METABOLIC LABELING WITH [^{35}S]-METHIONINE AND IMMUNOPRECIPITATION

Keratins biosynthesis was evaluated by immunoprecipitation after [^{35}S]-methionine metabolic labeling. In detail, HaCaT cells were treated with L-cysteine 10 μM for 48 h. During the final 4 h of treatment, cells were incubated first in a methionine-free medium (for 1 h) and then in methionine-free medium supplemented with 100 μCi/ml L-[^{35}S]-methionine (Perkin Elmer, Italy) for 3 h, as previously described [Miniaci et al., 2013, 2015]. Cells were washed three times with PBS and lysed to obtain the soluble and insoluble fractions. Aliquots (300 μg) of both fractions were incubated at 4°C for 16 h with 5 μg of anti-Pan cytokeratin antibody, previously conjugated with protein G PLUS-Agarose (Santa Cruz Biotechnology). After immunoprecipitation, the samples were washed three times with the appropriate lysis buffer and boiled with SDS loading buffer. Immunoprecipitated proteins were resolved using 10% SDS–polyacrylamide gel. After electrophoresis, the gel was treated with Amplify (Amersham Biosciences, UK) for 30 min, fixed in 50% methanol and 10% acetic acid, dried, and then exposed at –80°C for autoradiography. The intensity of the bands was quantified by densitometric analysis and the incorporation of [^{35}S]-methionine into total proteins was normalized according to the protein content.

CELLULAR LABILE IRON POOL (LIP) EVALUATION

The cellular labile iron content was estimated by a fluorimetric assay using the metalsensitive probe calcein (CA) and the iron chelator SIH (salicylaldehyde isonicotinoyl hydrazone), generously provided by Prof. Prem Ponka (McGill University, Montreal, QC, Canada), as previously reported [Breuer et al., 1995; Maffettone et al., 2008; Santamaria et al., 2011]. After incubation for 24 and 48 h with cysteine 10 μM, HaCaT cells, plated at a density of 1×10^4 cells/well, were loaded with 0.5 mM CA-AM (calcein-acetomethoxy, Molecular Probes, Invitrogen, Eugene, OR) for 30 min at 37°C in calcium- and bicarbonate-free modified Krebs Henseleit buffer (KHB), consisting of 20 mM HEPES, pH 7.4, 119 mM NaCl, 4.9 mM KCl, 0.96 mM KH_2PO_4, and 5 mM glucose. CA-AM rapidly penetrates across the plasma membrane and is intracellularly hydrolysed to release free CA. After loading, the cultures were washed two times with KHB. Cellular CA fluorescence was recorded in a Perkin Elmer microplate reader (Perkin Elmer LS55 Luminescence Spectrometer) using a filter combination with an excitation wavelength of 485 nm and an emission wavelength of 530 nm (slits 5 nm). Cell cultures without CA-AM were used as blank to correct non-specific autofluorescence. Trypan blue was added in all experiments to eliminate extra-cellular fluorescence. Once hydrolyzed, calcein becomes trapped in the cytoplasm and emits intense green fluorescence. The calcein-loaded cells have a fluorescence component (DF) that is quenched by intracellular iron and can be revealed by addition of 100 mM SIH. The rise in fluorescence is equivalent to the change in calcein concentration or to the amount of cellular iron originally bound to CA. Thus, the changes in CA fluorescence intensity were directly

proportional to the iron labile pool. To characterize the responsiveness of CA fluorescence toward different concentrations of intracellular iron, cells were preloaded for 18 h with ferrous ammonium sulphate (FAS), ferric ammonium citrate (FAC) or with the ferrous iron chelator desferrioxamine (DFO).

STATISTICAL ANALYSIS

All the experiments have been repeated four times. Data were presented as mean ± SEM and the statistical analysis was performed using Graph-Pad Prism (Graph-Pad software Inc., San Diego, CA), and ANOVA test for multiple comparisons followed by Bonferroni's test.

RESULTS

CYSTEINE IN VITRO BIOSCREEN

Preliminary biochemical and toxicological investigations were performed to screen the cysteine bioactivity *in vitro*. In particular, we first focused on the evaluation of cell growth and viability following the administration of various concentrations of cysteine (the range $10^{-3} \rightarrow 10^{2}$ μM has been explored) on human keratinocytes (HaCaT cell line). Data concerning the antiproliferative cisplatin (cDDP)—a positive control for cytotoxic effects—are included. Bioscreens were performed up to 72 h of incubations and have allowed to determine the range of concentration and incubation times to carry out *in vitro* treatments under optimal conditions of cell growth and protein biosynthesis. The experimental procedure has involved the evaluation of the cellular metabolic activity by the MTT assay, and the estimation of the live/dead cells ratio by a specific fluorimetric assay. The results are presented in Figure 1 and taken together show that cysteine, for all the screened concentrations and times, does not interfere with the HaCaT keratinocytes viability. Indeed, even at the higher cysteine concentration (100 μM), no significant changes in cell metabolic activity (Fig. 1A), as well as in the live/dead cell ratio (Fig. 1B), were detected with respect to control cultures. Similarly and to support, following *in vitro* exposure to high concentration of cysteine no morphological modification of HaCaT subconfluent monolayers appeared when examined by phase-contrast light microscopy for the dynamic cell population monitoring (data not shown). Therefore, these outcomes would suggest the theoretical safety of cysteine administration to human keratinocytes, thus allowing us to use it in a wide range of concentrations.

CYSTEINE TREATMENT RESULTS IN AN INCREASED BIOSYNTHESIS OF KERATINS IN HaCaT CELLS

To determine whether cysteine supplementation regulates keratin expression, HaCaT cells were incubated in a growth medium containing a range of cysteine concentrations (1, 10, and 100 μM) for 24, 48, and 72 h. After incubations, the levels of keratins in the

A

Hours (h)	Cysteine (μM)						cDDP (μM)
	10^{-3}	10^{-2}	10^{-1}	1	10	10^{2}	10
24	97 ± 4	101 ± 4	100 ± 6	99 ± 1	93 ± 7	100 ± 4	71 ± 7
48	95 ± 3	99 ± 4	98 ± 5	97 ± 3	96 ± 6	97 ±5	63 ± 5
72	95 ± 6	94 ± 4	94 ± 6	95 ± 8	95 ± 6	98 ± 5	43 ± 7

B

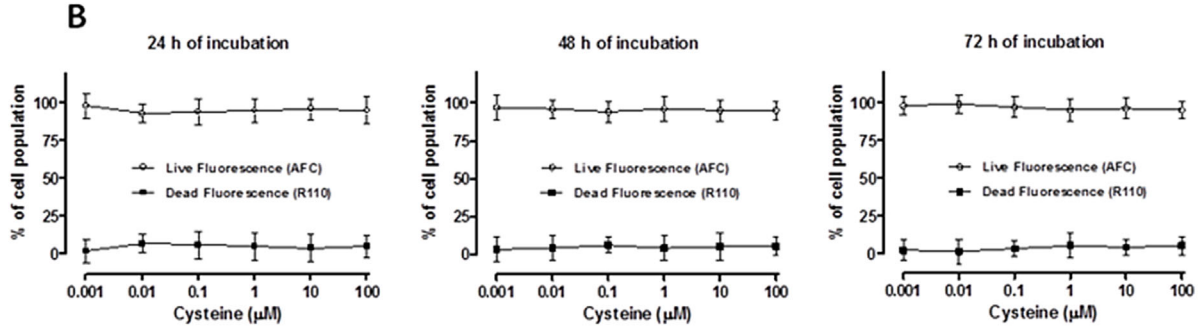

Fig. 1. Cysteine *in vitro* bioactivity. (A) Cell viability was evaluated by the MTT assay. HaCaT cells were treated for the indicated times (24, 48, and 72 h) with a range of concentrations of cysteine ($10^{-3} \rightarrow 10^{2}$ μM). Results are expressed in table as the percentage of cell viability to untreated control cultures and are reported as mean ± SEM (n = 5) of four independent experiments. Cisplatin is the positive control for cytotoxicity (cDDP, cis-diamminedichloroplatinum). (B) Inversely correlated fluorescent measures for live and dead cells counting after cysteine treatments. HaCaT cells were incubated with cysteine for the indicated times and concentrations, and then the MultiTox-Fluor Multiplex Cytotoxicity Assay (Promega) was performed according to the manufacturer's instructions. Results are reported in line graphs as mean percentage ± SEM (n = 5) of live and dead cells of four independent experiments.

soluble and insoluble fractions were evaluated by Western blot analysis using a broad spectrum anti-Pan keratin antibodies, as described in the experimental section. *In vitro* treatments with cysteine resulted in a significant increase of keratin expression within both the soluble and insoluble fraction. As reported in Figure 2, the cellular content of cytokeratins in the soluble fraction was clearly enhanced at the all examined times, reaching the maximum following 48 h of cysteine exposure. In the same experimental conditions, an equal marked increase in the keratins insoluble fraction was observed, with the more evident effects after 48 and 72 h (Fig. 3). Notably, some cytokeratin isoforms—i.e., K5, K6/K10, K18, and K19—reached an expression level close to 400% with respect to control cultures (Fig. 3B). It is noteworthy that keratin cellular content remained high even after 72 h of treatment, albeit at lower levels with respect to 48 h—an effect probably due to protein turnover.

By assuming that cysteine enhances the keratins cellular content, we next assessed *de novo* protein synthesis to further investigate cysteine role in the regulation of keratins expression. For that reason, following HaCaT exposure to 10 μM cysteine for 48 h we measured by immunoprecipitation the [^{35}S]-methionine incorporation into cytokeratins. The results are depicted in Fig. 4 and provide clearly evidence that the incorporation rate of ^{35}S-methionine into keratins increased after cysteine supplementation. As shown by densitometric analysis (Fig. 4C and 4D), ^{35}S-labelling was more pronounced for the keratin insoluble fraction, particularly for K1, K4, K5, K6/K10, K18 keratin isoforms. Consequently, we can state that in human keratinocytes cysteine is able to induce an increase of keratins biosynthesis at a relatively low concentration (10 μM), albeit by different protein translation rates.

CYSTEINE TREATMENT PREVENTS THE REDUCTION OF KERATIN EXPRESSION CAUSED BY IRON DEFICIENCY

Next, we investigated the interplay of cysteine supplementation and cellular iron status in regulating the cytokeratin expression. To modify the intracellular iron level, HaCaT cells were pre-treated with ferric ammonium citrate (FAC), as a source of iron, or with

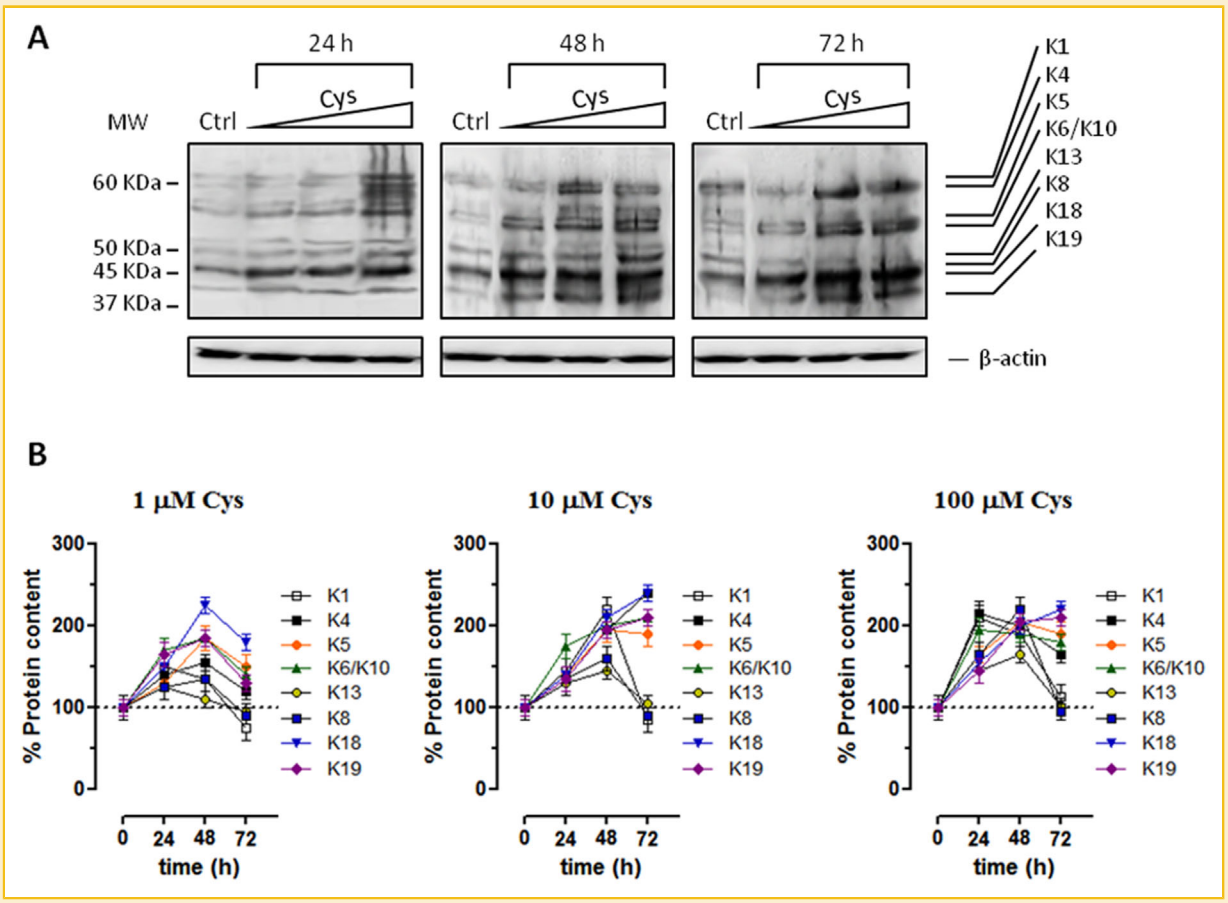

Fig. 2. Cysteine effect on keratins expression in soluble fraction of HaCaT cells. (A) Western blot analysis showing the keratin levels in soluble fraction of HaCaT cells exposed to cysteine (1, 10, and 100 μM) for 24, 48, and 72 h. Equal amounts of proteins (50 μg) were separated on a 10% SDS–polyacrylamide gel and subjected to Western blot analysis using monoclonal anti-Pan cytokeratin antibodies that recognize the following human cytokeratins according to their molecular weight: K1 (68 kDa), K4 (59 kDa), K5 (58 kDa), K6/K10 (56 kDa), K13 (54 kDa), K8 (52 kDa), K18 (45 kDa), and K19 (40 kDa). Molecular weight marker position (MW) is reported. Shown are blots representative of four independent experiments. (B) After chemoluminescence, the corresponding bands were quantified by densitometric analysis and plotted in line graphs as percentage of control. The anti-β-actin antibody was used to standardize the amounts of proteins in each lane. Shown are the averages ± SEM values of four independent experiments.

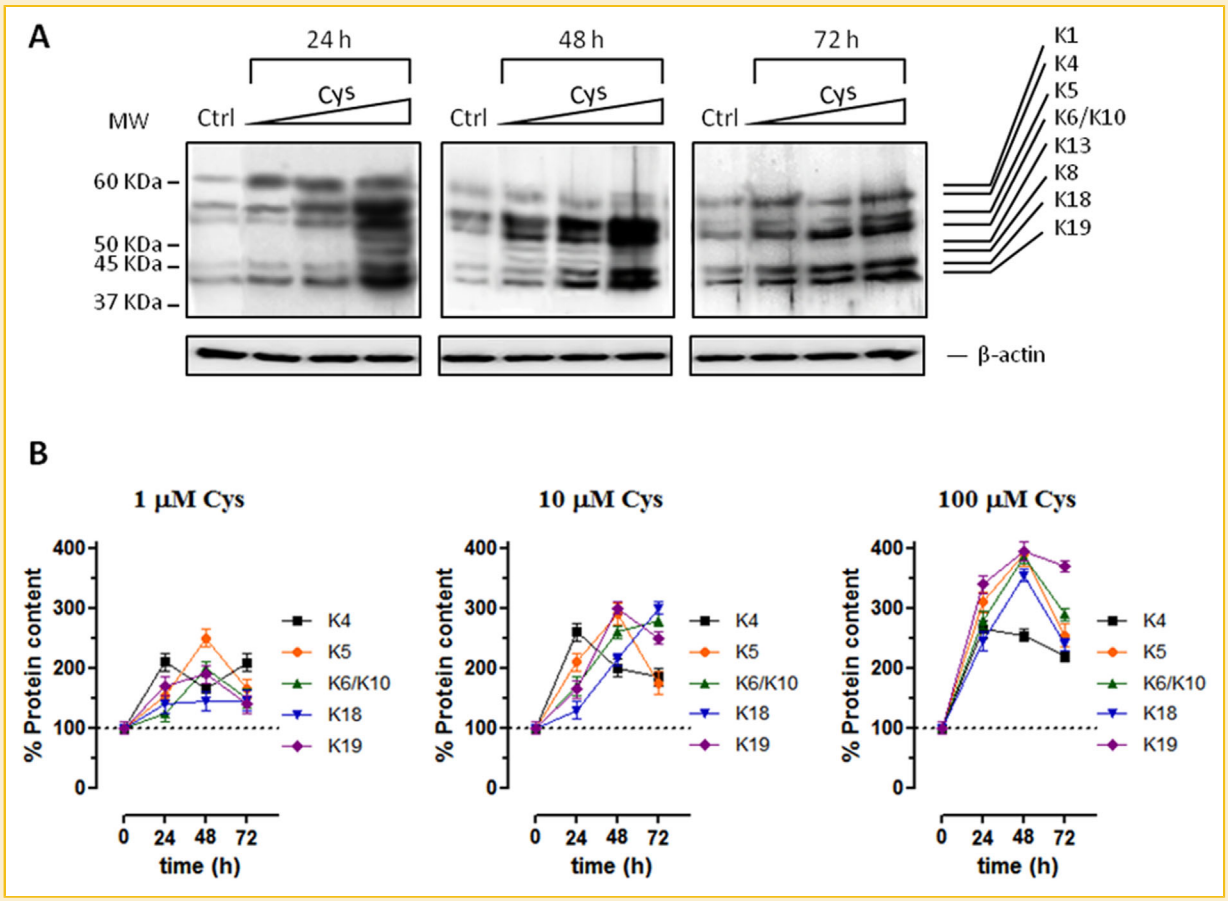

Fig. 3. Cysteine effect on keratins levels in insoluble fraction of HaCaT cells. (A) Western blot analysis showing the keratin levels in insoluble fraction of HaCaT cells exposed to cysteine (1, 10, and 100 μM) for 24, 48, and 72 h. Equal amounts of proteins (50 μg) were separated on a 10% SDS–polyacrylamide gel and subjected to Western blot analysis using anti-Pan mixture, as indicated in the legend of Figure 2. Molecular weight marker position (MW) is reported. Shown are blots representative of four independent experiments. (B) After chemoluminescence, the bands detectable in control lines (K4, K5, K6/10, K18, K19) were quantified by densitometric analysis and plotted in line graphs as percentage of control. The anti-β-actin antibody was used to standardize the amounts of proteins in each lane. Shown are the averages ± SEM values of four independent experiments.

desferrioxamine (DFO), an iron chelator, for 18 h *via* standardized protocols to avoid generating iron-dependent oxidative stress or impairment of cell viability, respectively. Firstly, Western blot analysis in Figure 5A and 5C demonstrates that *in vitro* iron overload by FAC treatment induced a significant increase in cellular keratin content with respect to control cells. Conversely, iron deficiency by DFO significantly decreased keratins levels. As clearly depicted in the related bar graphs (Fig. 5B and 5D), these effects were evident for both the soluble and the insoluble keratin fractions and could result plausibly by an imbalance of cellular iron homeostasis. Indeed, the estimation by the calcein fluorimetric assay has shown important perturbations in intracellular LIP amounts following iron repletion/depletion (see Fig. 6C). Notably, the concurrent cysteine supplementation (10 and 100 μM for 48 h) was able to significantly counteract, in a concentration-dependent manner, the reduction in keratins synthesis induced by iron depletion, mainly at the lower cysteine concentration (10 μM) in the insoluble fraction. Moreover, cysteine did further increase the keratin expression induced by iron repletion, albeit in a non-fully additive manner.

EFFECT OF CYSTEINE ON CELLULAR IRON HOMEOSTASIS

To explore the molecular mechanisms by which cysteine counteracts the negative effect of iron deficiency on keratin biosynthesis, we examined the cellular content of the main proteins involved in iron metabolism, such as ferritin, TfR-1, IRP1, and IRP2, following *in vitro* cysteine supplementation. Toward this aim, HaCaT cells were exposed to 10 μM cysteine for 24 and 48 h and the expression of ferritin, TfR-1, IRP1, and IRP2 was evaluated by Western blot analysis. As shown in Figure 6A and 6B, cysteine treatment induced a significant increase of the iron related-protein expression, which was more evident after 24 h of treatment. In particular, ferritin content increased of about 1.6 and 1.4-fold, whereas TfR-1 levels increased of about 1.8 and 1.2-fold after 24 h and 48 h of cysteine treatment with respect to control cultures. With regard to Iron Regulatory Proteins, the bifunctional *c*-Aconitase/IRP1 showed

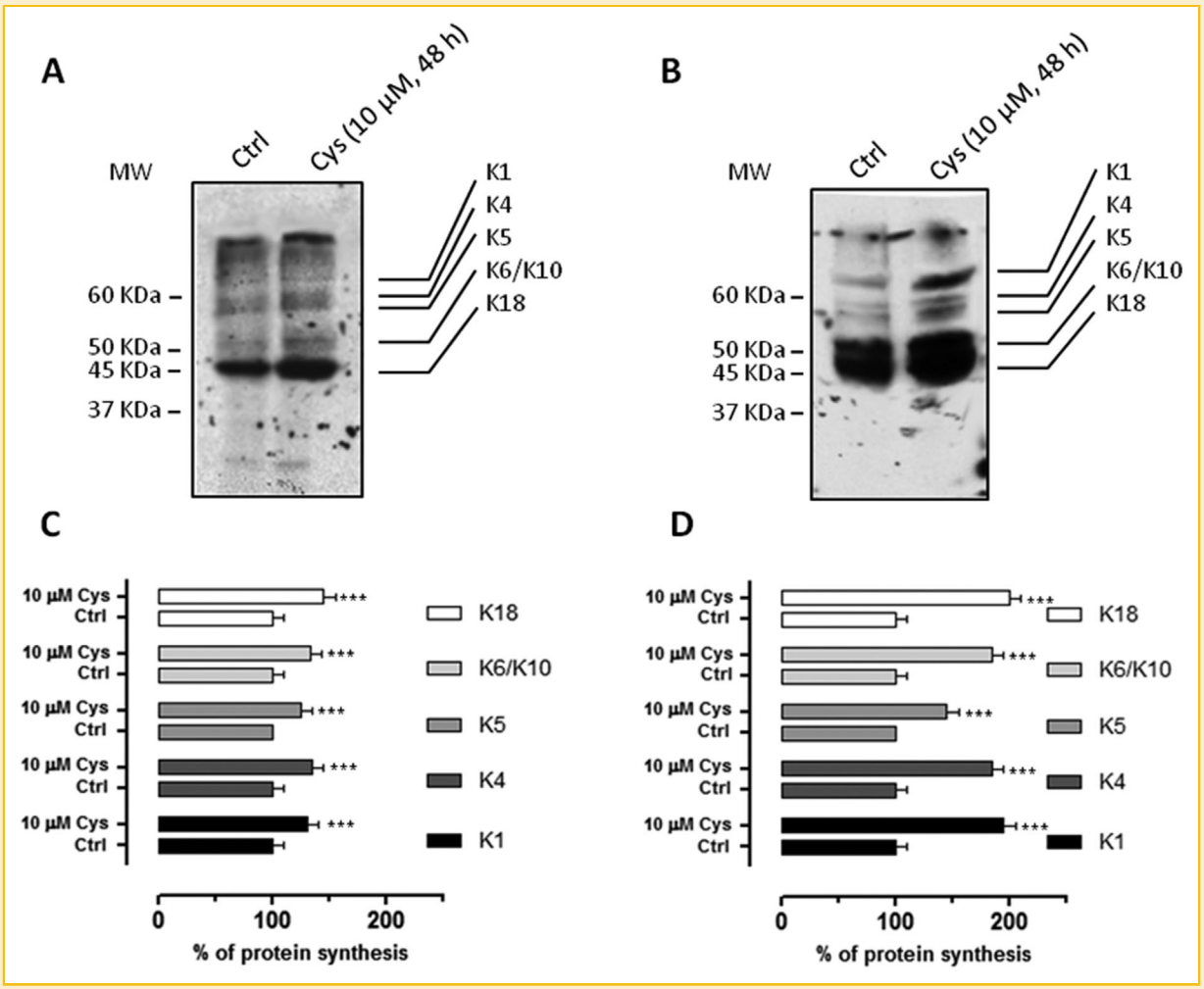

Fig. 4. Cysteine promotes keratin synthesis in HaCaT cells. (A, B) Cells were labeled with L-[^{35}S]-methionine during the last 3 h of cysteine in vitro exposure (10 μM for 48 h). Aliquots from soluble (A) and insoluble (B) fractions were immunoprecipitated with anti-Pan cytokeratin antibodies and proteins were then subjected to 10% SDS–PAGE analysis and autoradiography. Molecular weight marker position (MW) is reported. The autoradiograms shown are representative of four independent experiments. (C, D) The bands corresponding to cytokeratins 1, 4, 5, 6/10, and 18 from soluble (C) and insoluble (D) fractions were quantified by densitometric analysis and plotted in bar graphs as mean percentage of untreated control cultures ± SEM of four independent experiments. *** $P < 0.001$ vs. control cells.

a slight but significant increase following 24 h of cysteine treatment as compared to untreated cells, whereas IRP2—expressed at very low levels—resulted substantially unaffected by cysteine supplementation.

Finally, to verify whether the effects of cysteine on the expression of the proteins involved in iron metabolism may lead to variations in the free cellular iron content, we measured LIP amount using the calcein fluorimetric assay. The results obtained in HaCaT cells treated for 24 and 48 h with 10 μM cysteine are represented in line graph (Fig. 6C) and demonstrate that LIP extent remained essentially unaltered compared to control cells. On the other hand, variations in LIP magnitude were triggered by iron repletion/depletion, using FAC and DFO, respectively. Overall, the effect of cysteine supplementation on the cytosolic chelatable iron pool is correlated with the concomitant up-regulation of TfR-1 and ferritin detected in human keratinocytes.

Interestingly, cysteine treatment did not change significantly the LIP extent in iron-repleted cells, while it was able to partially restore the cellular metal homeostasis in iron-depleted cells, giving rise in the latter case to a LIP extent similar to that of untreated cells. This effect is likely due to the cysteine-mediated induction of ferritin but especially of TfR-1 at the cell membrane.

DISCUSSION

By virtue of its ability to form disulfide bonds, cysteine plays a crucial role in protein structure and in protein-folding pathways, so that it is currently recognized as a conditionally essential amino acid. Moreover, it is crucial in metabolic pathways involving methionine, taurine and glutathione [Brosnan and Brosnan, 2006]; as a rate-limiting substrate for GSH production, by triggering

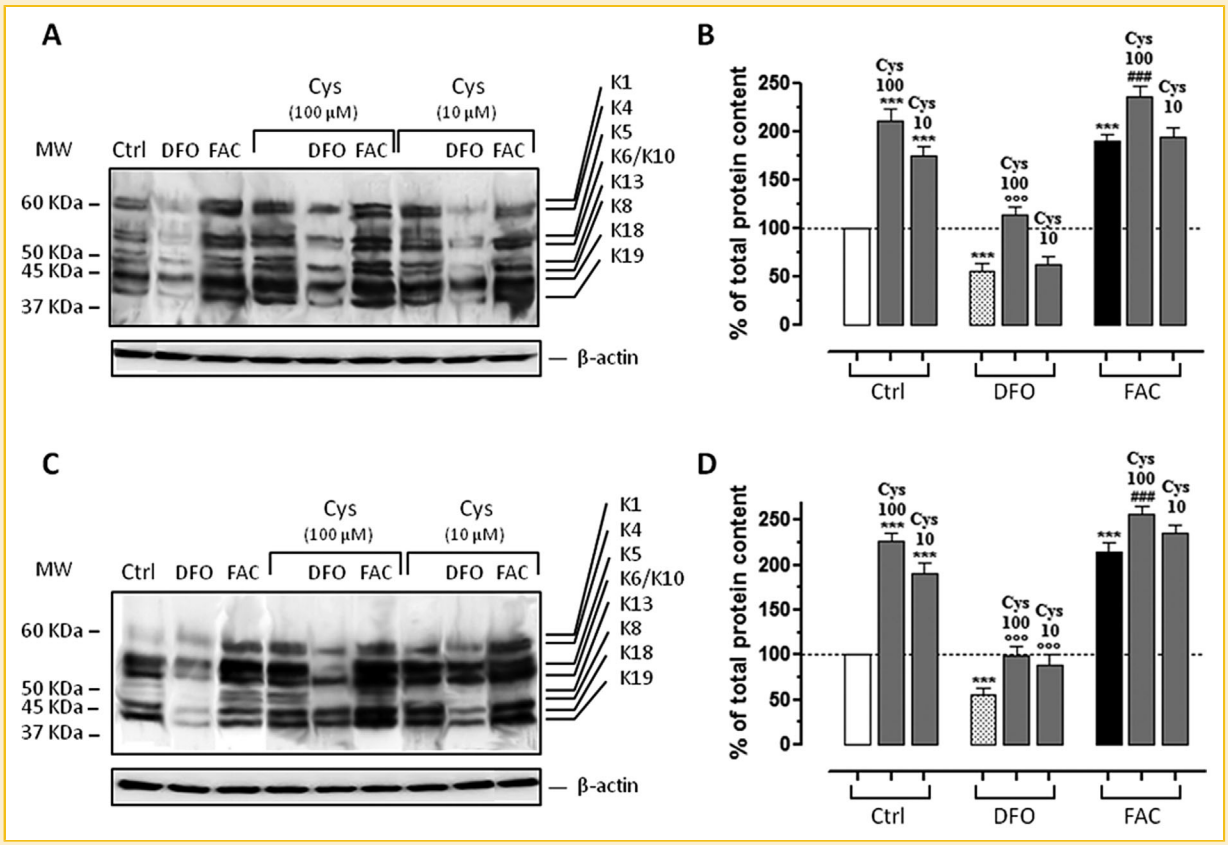

Fig. 5. Cysteine supplementation and cellular iron status in regulating the cytokeratin expression. (A, C) Western blot analysis showing the keratin levels in soluble (A) and insoluble (C) fraction from iron depleted (DFO) or iron repleted (FAC) HaCaT cells exposed or not to cysteine (10 and 100 μM) for 48 h, as indicated in the figure. Equal amounts of proteins (50 μg) were separated on a 10% SDS–polyacrylamide gel and subjected to Western blot analysis using the anti-Pan cytokeratin antibodies. Molecular weight marker position (MW) is reported. Shown are blots representative of four independent experiments. The anti-β-actin antibody was used to standardize the amounts of proteins in each lane. (B, D) Total cytokeratins cellular content emerging from each Western blot lane of soluble (B) and insoluble (D) fractions, quantified by densitometric analysis and plotted in bar graphs as mean percentage of untreated control cultures ± SEM of four independent experiments. *** $P < 0.001$ vs. control cells; °°° $P < 0.001$ vs. iron depleted cells (DFO); ### $P < 0.001$ vs. iron repleted cells (FAC).

antioxidant defences, cysteine is also engaged in several stressed and inflammatory states [McPherson and Hardy, 2011].

Among the cysteine-rich proteins, keratins are fundamental in epidermal development and homeostasis, in which cysteine is required for the disulfide bridges that confer additional strength and rigidity by permanent thermally stable cross-linking. Accordingly, over recent years the use of dietary cysteine and/or its derivatives as functional food supplements has greatly increased. In this context, we have herein demonstrated that cysteine supplementation promotes a significant up-regulation of keratins expression in human keratinocytes. The resulting *de novo* protein synthesis involved keratins accumulation within cells, thereby determining a remarkable increase in their cellular content.

The increase of keratin synthesis by cysteine is conceivable taking into account that this amino acid is an important substrate for protein translation. Indeed, as is the case of some amino acids (leucine, isoleucine, and valine) in human skeletal muscle, cysteine has anabolic effects on protein metabolism by increasing protein synthesis and decreasing protein degradation [Blomstrand et al., 2006]. For example, an exogenous supply of cysteine was needed to maintain maximal rates of protein synthesis in the perfused rat heart, suggesting an effect of cysteine on protein synthesis [Chua et al., 1984]. It is well known that amino acids regulate metabolic pathways involved in protein synthesis, such as mammalian target of rapamycin (mTOR), ribosomal S6 kinase ($p70^{S6K}$), eukaryotic initiation factor 2B (eIF2), and eukaryotic initiation factor 4E binding protein (4E-BP1) [Meijer and Dubbelhuis, 2004; Prod'homme et al., 2004; Proud, 2004]. Indeed, a stimulatory effect of oral administration of amino acids, particularly leucine, on mTOR, p70 S6 kinase, and the eukaryotic initiation factors has been demonstrated *in vivo* [Kimball and Jefferson, 2004]. Moreover, many of the cysteine biological effects at cellular level are closely associated to its role in promoting glutathione (γ-glutamyl-cysteinyl-glycine, GSH) synthesis, the major intracellular antioxidant. As a key functional component of GSH, cysteine provides a reactive thiol group that binds directly to different types of free radicals, including reactive oxygen and nitrogen species (ROS and RNS, respectively), thereby playing a variety of roles in detoxification, redox regulation and cellular signaling [Lushchak,

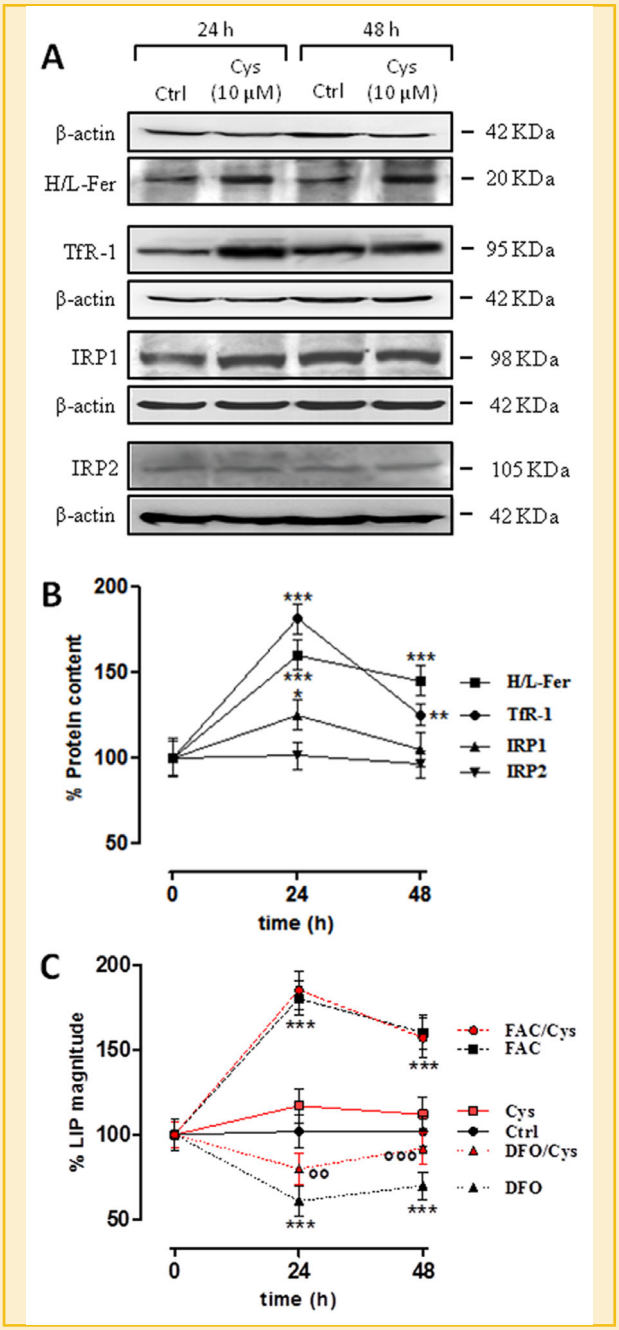

Fig. 6. Cysteine effect on iron homeostasis in keratinocytes. (A) Western blot analysis showing ferritin, TfR-1, IRP1 and IRP2 levels in HaCaT cells incubated with 10 μM cysteine for 24 and 48 h, as described in the experimental section. (B) Densitometric analysis of the bands corresponding to ferritin, TfR-1, IRP1, and IRP2, reported in line graphs as percentage of control. The anti-β-actin antibody was used to standardize the amounts of proteins in each lane. Shown are the averages ± SEM values of four independent experiments. *** $P < 0.001$ vs. control cells; ** $P < 0.01$ *vs* control cells; * $P < 0.05$ vs. control cells. (C) LIP extent variations estimated by the CA fluorescent method in iron depleted (DFO) or iron repleted (FAC) HaCaT cells incubated or not with 10 μM cysteine for 24 and 48 h. Additional control cultures were exposed only to 10 μM cysteine for 24 and 48 h. Shown are the average ± SEM ($n = 5$) values of three independent experiments plotted in a line graph as percent of control untreated cultures. *** $P < 0.001$ vs. control cells; °°° $P < 0.001$ vs. iron depleted cells (DFO); °° $P < 0.01$ vs. iron depleted cells (DFO).

2012]. Several biological processes regulated through GSH are critical to proliferating cell systems—like epithelia—and include cell growth, differentiation and apoptosis. GSH also operates as cofactor for antioxidant enzymes, such as glutathione peroxidases, glutathione transhydrogenases and glutathione transferases. In accordance, several studies have demonstrated that cysteine supplementation is able to preserve healthy state of skin and hair, reducing the symptoms of androgenic alopecia, and protecting hair follicles against oxidative stress [Trüeb, 2009; Wagener et al., 2013].

By analyzing in the interplay of cysteine supplementation and cellular iron status in the regulation of cytokeratin expression, we found that the simultaneous exogenous supplementation of both cysteine and iron results in a further increase of keratins levels. As well as cysteine, intracellular iron seems to be another key factor in regulating keratins expression in epidermal tissues [Hirobe, 2009]. Indeed, we have shown that a cellular iron deficiency status by desferrioxamine-mediated metal chelation negatively affects keratins expression. This effect can be explained according to the special role played by iron, particularly in cells endowed with a very high proliferative activity, as an essential component of fundamental biochemical activities. One of the most surprising findings emerging from our study is that cysteine supplementation seems able to revert the adverse effect of iron deficiency on cellular keratin expression. One of the reasons may lie in the regulatory role of this amino acid on protein biosynthesis and turnover. As reported for keratins, cysteine availability leads to a synthesis induction of many other proteins, including those involved in iron metabolism. Indeed our results have shown a significant cysteine-induced up-regulation of TfR-1, ferritin and IRP1, among the most important cellular proteins required for the maintenance of iron homeostasis. This induction is particularly detectable after 24 h of cysteine treatment, and mainly concerns the TfR-1, required for iron intake into the cell. The TfR-1 overexpression at the cell membrane allows metal entry, thereby restoring iron content. The concomitant increase in ferritin protein—the major regulator of the cellular iron content—may allow safe storage of any excess iron, thus limiting the potential oxidative stress induction by free iron within cells. In fact, by acting as a buffer against iron deficiency and iron overload, ferritin provides protection against oxidative stress. This result is consistent with the estimation of the cellular free iron content, showing no substantial changes in labile iron pool (LIP) following cysteine supplementation, and a trend toward a recovery of LIP when cysteine was administered in iron depleted cells. LIP is fundamental in preserving cellular biochemical activities since it represents the catalytic and redox-active iron; its level must be maintained within a limited range that assures the iron supplies for the cell and prevents iron excess causing oxidative damage [Kakhlon and Cabantchik, 2002]. Consequently, as evidenced by the recovery of the keratin levels in iron depleted cells, the restoration of iron homeostasis following treatment with cysteine should occur without creating cellular dangerous pro-oxidant conditions. This is an important endpoint. Actually, these findings further reinforce the idea of a regulatory role in protein synthesis for cysteine, in conjunction with its antioxidant protective functions.

In addition, but not less relevant for rapid proliferating epidermal cells, the enhanced iron metabolic availability induced by cysteine should make possible its incorporation into the ribonucleotide reductase, where it plays a crucial role in the reduction of ribonucleotides to deoxyribonucleotides for DNA biosynthesis, thus allowing cell proliferation [Cooper et al., 1996]. Furthermore, a recent study has demonstrated that DNA polymerases activity is strictly dependent by an iron–sulphur cluster, which plays an essential role in their biological function and whose assembly directly depends on cellular iron availability [Netz et al., 2011].

In conclusion, the herein presented study provides convincing evidence of the interplay between cysteine and cellular iron homeostasis. Cysteine and iron cooperate in human keratinocytes for keratins expression, suggesting their central role in maintaining healthy epithelia. *Via* induction of iron-related proteins, the enhanced metabolic iron availability by cysteine supplementation reflects this relationship and suggests that cysteine can balance the trophic and toxic properties of iron.

ACKNOWLEDGEMENTS

We thank IDI Farmaceutici, Pomezia (Rome, Italy) for the financial support. The authors wish to dedicate this manuscript to Prof. Alfredo Colonna who prematurely passed away.

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