Hydrogen Peroxide Regulates the Mitochondrial Content of Uncoupling Protein 5 in Colon Cancer Cells

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Key Words

Reactive oxygen species • Uncoupling proteins • Mitochondrial dysfunction • Energy metabolism • Colon cancer cells

Abstract

Uncoupling protein-2 (UCP2) and uncoupling protein-5 (UCP5) are ion carriers located in the inner mitochondrial membrane that mediate a regulated discharge of the proton gradient generated by the respiratory chain and are possibly involved in the protection against free radical production. Mitochondrial dysfunction is involved in the pathogenesis of colon cancer for which UCP2 has been proposed as a potential therapeutic target. The object of this study was to investigate the possible effects of mitochondrial dysfunction and oxidative stress on the mitochondrial content of UCP2 and UCP5 in colonic cells. Proliferation rate/index, antioxidant systems, reactive oxygen species, oxidative damage, mitochondrial markers and protein expression of UCP2 and UCP5 were analysed in non-metastatic HT-29 cells, metastatic SW-620 cells, and in a HT-29 differentiated derivative (Glu-R). In this study, we found a clear relationship between mitochondrial dysfunction in colonic epithelial cells and the levels of UCP2 and

UCP5 proteins. Furthermore, hydrogen peroxide was responsible for the coordinated activation of the antioxidant response and the increase of UCP5 protein levels. Our findings suggest that uncoupling activity could act as a local feedback mechanism to limit the enhanced steady-state H₂O₂ production in dysfunctional mitochondria of colonic cells.

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Introduction

Uncoupling proteins (UCPs) belong to a family of ion carriers located in the inner mitochondrial membrane. UCP1 is known to be a key element in thermogenesis of brown adipose tissue but the role of more recently identified homologues to UCP1, which are found in different tissues, are still largely unknown. The possible role of UCP2 to UCP5 in limiting mitochondrial reactive oxygen species (ROS) production [1-4] set these proteins as a potential therapeutic target, since many lines of evidence suggest that oxidative stress from mitochondrial origin and mitochondrial dysfunction are underlying mechanisms of several diseases and physiopathological situations [5-12]. Although UCPs have been associated to pathologi-

cal conditions in which mitochondrial dysfunction is present such as diabetes [13], obesity [14], idiopathic Parkinson's disease [15] and more recently, cancer [16], little is known about the intriguing relationship between mitochondrial dysfunction and the expression of UCPs. In particular, Derdak et al. proved that UCP2 overexpression in colon cancer cells promote their survival and facilitate their resistance against antitumor drugs [17]. Less is known about UCP5 levels in human colon cancer cells, only a previous study reported that UCP5 transcripts were found in lower levels in human adult intestinal tissue than in human colon cancer cells [18]. Despite UCP5 protein expression has been suggested to be a possible protective mechanism against oxidative cytotoxicity in neuronal cells [19], the expression of UCP5 in human colon adenocarcinoma cells has not been confirmed at the protein level and the possible factors involved in its regulation are still unknown.

Therefore, the aim of this study was to investigate the relationship between the levels of UCP2 and UCP5 and mitochondrial dysfunction in colonic cells, to establish the role of hydrogen peroxide as a possible regulator of UCP5 levels in dysfunctional mitochondria of colonic cells, and to define the relevance of UCPs in the control of elevated endogenous ROS production in dysfunctional mitochondria of colonic cells. For this purpose, we chose a model in vitro representing different stages of colon cancer progression: Glu-R cells (a HT-29 differentiated derivative), non-metastatic HT-29 cells and metastatic SW-620 cells.

Materials and Methods

Materials

Routine chemicals used were obtained from Sigma-Aldrich (St. Louis, USA), Panreac (Barcelona, Spain) and Amersham Pharmacia Biotech (Little Chalfont, UK). Real-time PCR reagents and primer sequences were supplied by Roche Diagnostics (Basel, Switzerland) and Amplex Red reagent by Molecular Probes (Paisley, UK). Cell culture reagents were purchased from Invitrogen (Carlsbad, USA) and Ractiva (Barcelona, Spain). The antibodies against UCP5/BMCP1 (cat. UCP51-A) and UCP2 (cat. UCP21-A) were purchased from Alpha Diagnostic International (San Antonio, USA), and alpha-tubulin antibody (cat. sc-5286) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA).

Cell culture and treatments

HT-29 and SW-620 human cell lines were obtained from the American Type Culture Collection (ATCC). Both cell lines were grown in Dulbecco's modified Eagle's medium (4.5 g/L of

glucose) supplemented with 10% (v/v) fetal bovine serum, 90 units/mL penicillin and 90 µg/mL streptomycin, in a 5% CO₂ humidified atmosphere at 37 °C. According to previously described protocols [20], HT-29 cells were grown in a inosine-containing medium with a reduced content of glucose to induce enterocytic differentiation. In the present experiments, a medium-containing high glucose was used to reach subconfluency before glucose-deprivation; cells were then rinsed with PBS, and medium-containing 2.5 mM inosine without glucose/pyruvate was added. The final concentration of glucose in the medium, due to the serum, was 0.5 mM. After 72 hours in culture, only adherent cells were harvested. Colonic cells which exhibited phenotypical features compatible with differentiation are referred as Glu-R. Colon cancer cells were treated with a medium containing stabilized H₂O₂ at a final concentration of 40 µM for 24 hours; this solution of hydrogen peroxide was monitored by Amplex red to be stable (at varying concentrations) for at least one hour at 37 °C.

Mitochondrial Isolation

6 X 10⁶ cells were harvested by trypsinization, centrifuged at 1600 rpm for 5 min, washed with PBS and resuspended in 1 mL of cold isolation buffer (0.25 M sucrose, 10 mM Tris-HCl, and 0.5 mM EDTA-K⁺, pH 7.4). Samples were then immediately homogenized at 4 °C; nuclei and cell debris were sedimented by differential centrifugation at 600 x g for 10 min and the pellets were discarded. Supernatant was subjected to a further centrifugation at 7000 x g for 10 min and the crude mitochondrial pellet obtained was resuspended with 0.1 mL of isolation buffer.

Quantification of protein, DNA and mitochondrial DNA content

The protein content in each sample was determined by Bradford's method [21]. The measurement of DNA content was performed using the diaminobenzoic acid method, as previously described by Thomas P. & Farquhar M. [22].

Mitochondrial DNA content was measured in pretreated cell lysates by real-time PCR as described before [23]. PCR was performed to amplify a 192-nts fragment of the human mitochondrial NADH dehydrogenase subunit 4 gene. The primer sequences were 5'-CCTGACTCCTACCCCTCACA-3' and 5'-ATCGGGTGATGATAGCCAAG-3'. The PCR reactions were cycled 32 times after initial denaturation (95 °C, 10 min), with the following parameters: denaturation at 95 °C for 10 s, annealing at 68 °C for 12 s, extension at 72 °C for 12 s.

Cell proliferation/viability assay

Cell proliferation was assessed by colorimetric analysis of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) reduction every 24 hours. After treatment to induce differentiation, Glu-R cells attached to the culture dishes were collected by trypsin-EDTA treatment. Suspended cells (1 X 10⁴) were transferred to each well of 24 well microplates and were allowed to adhere for 24 h, after which, cells were exposed to the vehicle or the treatment. MTT solution in phosphate-buffered saline (5 mg/mL) was added 1/10 to the culture medium in each well. The plates were incubated for 1 hour at 37°C, after

which the medium was replaced with dimethyl sulfoxide (DMSO) to dissolve any formazan crystals formed by the cells. The absorbance was recorded at 570 nm using a microplate reader. Results at different times were expressed as % viable cells $\pm$ SEM versus the initial number of viable cells (100%).

Determination of cell survival using colony formation assay

For clonogenic assays, colon cancer cells were plated in six-well plates and treated the day after with hydrogen peroxide (final concentration 40 μ M) for a period of 1 h. After removal of the medium containing H₂O₂, cells were trypsinized and plated at low density (2,000 cells per 60-mm plate). Cells were then cultivated for 12 days. Colonies were stained with crystal violet and clones were counted for each condition.

Measurement of cellular respiration

Respiration was measured polarographically as described previously by Partridge et al., with some modifications [24]. Cells at a concentration of 1.4 mg protein/mL were incubated in a water-thermostatically regulated chamber with a computer-controlled Clark-type O₂ electrode (Oxygraph; Hansatech, Norfolk, UK) in respiration buffer (145 mM KCl, 30 mM HEPES, 5 mM KH₂PO₄, 3 mM MgCl₂, 0.1 mM EGTA, and 0.1% bovine serum albumin; pH 7.4; 37 °C). In order to compare respiration of control cells and glucose-deprived cells, a pyruvate-malate (5 mM/2.5 mM) mix was used as a substrate instead of glucose.

Analysis of enzymatic activities

All spectrophotometric and fluorimetric determinations were assayed in microtiter plate and were performed as previously described protocols with some modifications. Lactate dehydrogenase (LDH; EC 1.1.1.27) [25], citrate synthase (CS; EC 2.3.3.1) [26], ferrocycytochrome-c: oxygen oxidoreductase (COX or complex IV; EC 1.9.3.1) [27] and F₁F₀-ATP synthase (ATPase or complex V; EC 3.6.1.3) [28] activities were measured in cell lysates. Superoxide dismutase (SOD; EC 1.15.1.1) [29], glutathione peroxidase (GPx; EC 1.11.1.9) [30], glutathione reductase (GRd; EC 1.8.1.7) [31] and catalase (EC 1.11.1.6.) [32, 33] antioxidant activities were determined in the supernatant of samples previously disrupted by sonication (10,000 x g for 10 min).

Glutathione assay

Glutathione levels were measured in the supernatant of deproteinized cell lysates by a modified 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) - GSSG reductase recycling assay [34]. Reduced-/oxidized glutathione ratio (GSH/GSSG) was then calculated.

Detection of ROS levels by confocal microscopy

Intracellular ROS levels were detected using dichlorofluorescein diacetate (DCFH-DA) as previously described protocols [35, 36]. Cells were incubated for 35 min at 37 °C with DCFH-DA 1 μ M, and were then fixed in ice-cold methanol for 8 min. The sections were mounted with DakoCytomation Fluorescent Mounting Medium (Cat. Num. S3023) and examined on a Leica TCS SP2 confocal microscope (Leica Microsystems, Spain) using the appropriate selective barrier

filters (488-nm excitation and 510-nm emission). The intensity of the laser was held constant to allow quantitative comparisons of relative fluorescence intensity between studied groups; the fluorescent images of cells were acquired by using Leica TCS-NT software.

Measurement of cellular H₂O₂ production capacity

H₂O₂ production was determined as previously described by Campian et al. [37] with some modifications. Fluorescence values were measured in a microplate reader (FLx800; Bio-Tek Instruments, Winooski, VT) with 530 nm excitation and 590 nm emission wavelengths. After adding the substrate pyruvate-malate (5 mM/2.5 mM), colon cancer cells (0.5 mg protein/mL) were incubated for 25 minutes at 37 °C in respiration buffer containing 0.1 U/mL horseradish peroxidase and 50 μ M Amplex Red; background fluorescence was measured in parallel in wells containing all reactants except samples. Fluorescence was expressed as H₂O₂ levels using a standard curve generated with known concentrations of H₂O₂ stabilized solution. The fluorescent probe Amplex Red is a specific reagent to measure hydrogen peroxide because it does not form resorufin in the presence of other reactive oxygen or nitrogen species [38]. To confirm the specificity of Amplex Red to measure hydrogen peroxide in our model, colon cancer cells were pretreated with PEG-catalase (100 U/mL) for 5 hours at 37 °C. ROS production measured in pretreated cells was suppressed compared to controls (treated with the same vehicle), indicating that the differences observed in ROS production of colonic cells are due to hydrogen peroxide.

Analysis of mitochondrial hydrogen peroxide production

Mitochondria (0.12 mg protein/mL) were added to respiration buffer supplemented with 0.1 U/mL horseradish peroxidase and 50 μ M Amplex red reagent. The assays were performed in respiratory State 4 (absence of ADP) and in the presence of succinate (5 mM) as a substrate, or in the presence of this substrate plus 500 μ M GDP as inhibitor of mitochondrial chain uncoupling activity.

Measurement of oxidative damage

Lipid peroxidation was determined in cell lysates as malondialdehyde-thiobarbituric acid adducts according to previously described methods [39, 40]. Butylated hydroxytoluene (BHT) was added to homogenizing buffer at a final concentration of 4 mM to stop autooxidation during the heating step. The assay was performed spectrophotometrically at 532 nm using a molar extinction coefficient of 1.56x10⁵ M⁻¹·cm⁻¹.

Protein carbonyl groups were quantified using the Oxyblot™ protein oxidation detection kit (Chemicon, Chandlers Ford, UK). 2,4-dinitrophenylhydrazine (DNPH) was used to derivatize 5 μ g of cell lysate protein for 15 min, following the manufacturer's instructions. Proteins were then transferred to nitrocellulose filters by means of a slot-blot system (Bio-Rad, CA, USA). Oxidized proteins provided by the kit were included as a positive control and the samples treated with the control solution served as a negative control to the DNPH derivatization.

Fig. 1. Growth rate of colonic cells and morphological features of Glu-R and HT-29 cells. A, cell proliferation was assessed in Glu-R cells and cancer cell lines using the MTT assay as described in Materials and Methods section. B, the photomicrographs show morphological differences between Glu-R cells and HT-29 cell line: following treatment to induce differentiation, HT-29 adenocarcinoma cells underwent changes in growth pattern and morphology; these changes included an increase in cell size and the appearance of flattened star-shapes from the spherical parenteral population. All data were expressed as mean values $\pm$ SEM from at least three independent experiments performed in duplicate. Statistical significance ($P < 0.05$): *, Glu-R cells versus HT-29 cells; †, SW-620 cells versus HT-29 cells.

Fig. 2. Impaired mtDNA copy number and mitochondrial energy metabolism in colon cancer cells. A, DNA content was quantified using diamino-benzoic acid method. B, mtDNA content was measured in pretreated cell lysates by real-time PCR. C, phosphorilative capacity was measured as the activity ratio ATPase/CS. D, cellular respiration was measured polarographically. E, mitochondrial oxidative function was measured as the activity ratio cytochrome *c* oxidase/CS. F, glycolytic activity was measured as lactate dehydrogenase activity. All the results were expressed per milligram of cellular protein. All data were expressed as mean values $\pm$ SEM from at least three independent experiments performed in duplicate. Statistical significance ($P < 0.05$): *, Glu-R cells versus HT-29 cells; †, SW-620 cells versus HT-29 cells. COX, cytochrome *c* oxidase; CS, citrate synthase; LDH, lactate dehydrogenase.

Western blot analysis

Cells were rinsed with ice-cold PBS, scraped from plates, resuspended in ice-cold lysis buffer (20 mM Tris-HCl, 1.5 mM MgCl₂, 140 mmol/L NaCl, 0.5% Nonidet P-40, 10% glycerol, 1 mmol/L EGTA, 1 mmol/L NaVO₃, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μ g/mL leupeptin, and 10 μ g/mL pepstatin; pH 7.4) and then sonicated. Proteins (30 μ g) were eletrophoresed on

10% SDS-PAGE and were then electrotransferred onto a nitrocellulose filter (Bio-Rad, CA, USA). Ponceau S staining was performed in order to check correct loading and electrophoretic transfer. Blots were incubated with the corresponding antibodies. Immunoblot development was performed using an enhanced chemiluminescence western blotting analysis system

Fig. 3. Mitochondrial dysfunction is associated to greater endogenous ROS production in colonic cells. A, images obtained by confocal microscopy show intracellular ROS levels in colonic cells stained with the fluorescent probe dichloro-fluorescein diacetate (DCFH-DA). B, cellular H_2O_2 production was determined using the fluorescent probe Amplex Red. C, lipid peroxide levels were measured as thiobarbituric acid-reactive substances (TBARS) in whole cell lysates. D, protein carbonyl levels were assessed in whole cell lysates using immunoblot detection; the relative percentage was then calculated. All data were expressed as mean values $\pm$ SEM from at least three independent experiments performed in duplicate. Statistical significance ($P < 0.05$): *, Glu-R cells versus HT-29 cells; †, SW-620 cells versus HT-29 cells.

(Amersham, UK). Films were quantified by photodensitometric analysis (Kodak 1D Image Analysis Software). The apparent molecular weights of UCP5 (36 kDa), UCP2 (33 kDa) and tubulin (55 kDa) were estimated using protein molecular-mass standards (Bio-Rad, CA, USA). The expression of UCPs was normalized to tubulin expression.

Statistical analysis

All data were expressed as mean values $\pm$ SEM from at least three independent experiments performed in duplicate. Statistical analysis was carried out using the Statistical Program for the Social Sciences software for Windows (SPSS, Version 16.0). Statistical differences between experimental groups were assessed by Student's t-test. A level of $P < 0.05$ was accepted as significant.

Results

Growth rate and morphological features of Glu-R cells

As shown in Fig. 1A, we found a lower proliferation activity in Glu-R cells in comparison with HT-29 cells. Following treatment to induce differentiation, HT-29 adenocarcinoma cells underwent changes in growth pattern and morphology. These changes included an increase in cell size and the appearance of flattened star-shapes from the spherical parenteral population (Fig. 1B), as a result, cells became more tightly attached to the culture dish. In order to further investigate causes of lower proliferation rate of Glu-R cells, the cell cycle profile was

measured by flow cytometry and most of the viable cells were blocked at the G_0 - G_1 phase (69.6 ± 8.2) compared to HT-29 cells (52.2 ± 0.7). The percentage of viable cells in S and G_2 -M phases was significantly lower in Glu-R cells (S: 18.9 ± 2.7 ; G_2 -M: 11.5 ± 2.8) than in HT-29 cells (S: 25.5 ± 1.3 ; G_2 -M: 22.3 ± 1.8).

Impaired mtDNA copy number and mitochondrial energy metabolism in colon cancer cells

As shown in Fig. 2, Glu-R cells showed higher mtDNA copy number than untreated malignant cells. Conversely, mtDNA copy number was similar between both cell lines (Fig. 2B). Although HT-29 cells showed higher total DNA content than that observed in metastatic SW-620 cells, a similar ratio between mitochondrial genome and cellular genome was conserved in both cell lines, HT-29 cells ($100\% \pm 14$) and SW-620 cells ($104\% \pm 28$).

Mitochondrial respiratory chain activities were normalized to mitochondrial abundance by using CS activity [41]. Glu-R cells had higher phosphorylative capacity (measured as ATPase activity) than untreated malignant cells (Fig. 2C). Although COX activity in Glu-R cells was lower than HT-29 cells, oxygen consumption showed a trend to be higher in Glu-R cells compared to HT-29 cells ($P = 0.059$). Additionally, Glu-R cells showed lower glycolytic activity than HT-29 cells (Fig. 2F). Citrate synthase, ATPase and oxygen consumption were similar between HT-29 and SW-620 cell lines, however, we found

Fig. 4. Antioxidant capacity in colonic cells. A, cellular catalase activity. B, cellular glutathione peroxidase activity. C, cellular glutathione reductase activity. D, cellular superoxide dismutase activity. E, cellular content of reduced glutathione. F, balance between reduced-/oxidized glutathione. Antioxidant enzymatic activities and glutathione levels were measured by spectrophotometry. Values were expressed per milligram of cellular protein and represent the mean values $\pm$ SEM from at least three independent experiments performed in duplicate. Statistical significance ($P < 0.05$): *, Glu-R cells versus HT-29 cells; †, SW-620 cells versus HT-29 cells. GPx, glutathione peroxidase; GRd, glutathione reductase; SOD, superoxide dismutase; GSH, reduced glutathione; GSSG, oxidized glutathione.

a drop in COX activity in SW-620 cells (36.5%). According to the results of oxidative function, we found that lactate dehydrogenase activity was higher in metastatic SW-620 cells than in HT-29 cells.

Mitochondrial dysfunction is associated to greater endogenous ROS production in colonic cells

Glu-R cells exhibited significantly lower intracellular ROS levels (Fig. 3A) and lower H_2O_2 production capacity (Fig. 3B) than HT-29 cells. Interestingly, intracellular ROS levels and H_2O_2 production capacity were greater in SW-620 cells than HT-29 cells. Increased cellular H_2O_2 production (measured by Amplex Red in the presence of pyruvate/malate as substrates) was observed in cells pretreated for 1 hour with respiratory chain inhibitors such as rotenone (complex I inhibitor) or antimycin (complex III inhibitor) at a final concentration of 10 μ M, reflecting that endogenous H_2O_2 production in colon cancer cells has mainly mitochondrial origin (data not shown).

Cellular oxidative damage markers in colonic cells

Glu-R cells showed a significant reduction of cellular oxidative damage (lipid peroxidation and protein carbonylation) in comparison with HT-29 cells (Figs. 3C and D). Metastatic SW-620 cells had lower levels of lipid peroxidation and a trend towards a lower protein oxidation in comparison with cells derived from the primary tumor (HT-29).

Antioxidant capacity and levels of UCPs in colonic cells

Although Glu-R cells exhibited lower glutathione levels, the activities of catalase, glutathione peroxidase and SOD antioxidant enzymes were similar to those observed in HT-29 cells (Fig. 4). Moreover, there was a higher glutathione reductase activity in Glu-R cells compared to HT-29 cells. The activity of GPx and catalase antioxidant enzymes was higher in metastatic SW-620 cells (Fig. 4A and B). Conversely, SW-620 cells showed lower SOD activity - enzyme which detoxifies superoxide anions in-

Fig. 5. Western blot of mitochondrial uncoupling protein-5 and uncoupling protein-2 in colonic cells. A, levels of UCP5 in colonic cells. B, levels of UCP2 in colonic cells. Whole-cell lysates (30 μ g) were fractioned in 10% SDS-PAGE; α -tubulin was used as a loading control; representative bands are shown. Values were expressed as mean $\pm$ SEM from at least three independent experiments performed in duplicate. Statistical significance ($P < 0.05$): *, Glu-R cells versus HT-29 cells; †, SW-620 cells versus HT-29 cells.

	HT-29		SW-620	
	H ₂ O ₂	-	-	+
Glutathione reductase (μ IU/mg)		3.19 $\pm$ 0.18	5.82 $\pm$ 0.19*	3.53 $\pm$ 0.26
Glutathione peroxidase (μ IU/mg)		5.10 $\pm$ 0.67	4.98 $\pm$ 0.17	8.70 $\pm$ 0.23
Catalase (mIU/mg)		1.63 $\pm$ 0.08	2.07 $\pm$ 0.05*	2.10 $\pm$ 0.17
Superoxide dismutase (mIU/mg)		2.57 $\pm$ 0.41	2.51 $\pm$ 0.09	1.08 $\pm$ 0.12
GSH content (nmol/mg)		6.28 $\pm$ 1.66	6.20 $\pm$ 0.18	11.2 $\pm$ 1.7
GPx/SOD (μ IU/mIU)		2.09 $\pm$ 0.17	1.99 $\pm$ 0.08	5.58 $\pm$ 0.06
Catalase/SOD		0.44 $\pm$ 0.03	0.49 $\pm$ 0.01	1.62 $\pm$ 0.30
				0.19 $\pm$ 0.01*†

Table 1. Hydrogen peroxide induces the antioxidant response in colon cancer cells. Colon adenocarcinoma cell lines were treated with a medium containing stabilized hydrogen peroxide (40 μ M) for 24 hours, an equal volume of vehicle was added to controls. Values were expressed per milligram of cellular protein and represent the mean $\pm$ SEM from at least three independent experiments performed in duplicate. Statistical significance ($P < 0.05$): *, treated cells versus control cells; †, treated HT-29 cells versus treated SW-620 cells.

creasing cellular content of hydrogen peroxide - than HT-29 cells. The GSH/GSSG ratio (Fig. 4F), GSH levels and GRd activity were similar in both cancer cell lines.

As shown in Fig. 5, Glu-R cells exhibited one fourth of the protein levels of UCP5 compared to HT-29 cells. UCP5 levels were four-fold higher in the more malignant cells compared to HT-29 cells. Although UCP2 levels were similar between both cancer cell lines, Glu-R cells exhibited significantly lower UCP2 content in comparison with HT-29 cells.

Hydrogen peroxide activates the endogenous antioxidant response and increases the mitochondrial content of UCP5 in colon cancer cells

Hydrogen peroxide treatment (40 μ M) induced an increase in the activity of catalase and glutathione reductase antioxidant enzymes in HT-29 cells; similarly, hydrogen peroxide increased the activity of SOD and glutathione reductase and also augmented the content of reduced glutathione in SW-620 cells (Table 1). Interestingly, there was a differential response between both cell

Fig. 6. Effects of hydrogen peroxide on cellular viability, long-term survival and UCP5 protein expression in colon cancer cells. A, cell viability was measured after treating cells with a medium containing stabilized hydrogen peroxide (40 μ M) for 24 hours and then results were expressed as % viable cells against control. B, clonogenic analysis of colon cancer cell lines were performed after treating cells with a medium containing hydrogen peroxide (40 μ M) for 1 h; cells were then trypsinized, and plated in duplicates at low density (2,000 per 60-mm plate); after 2 weeks, formed colonies were stained with crystal violet and clones were counted for each condition; represented is the percentage of viable clones after hydrogen peroxide treatment relative to untreated cells. C, UCP5 protein expression in HT-29 cells treated with hydrogen peroxide. D, UCP5 protein expression in SW-620 cells treated with hydrogen peroxide. Human colon adenocarcinoma cells were treated with a medium containing stabilized hydrogen peroxide (40 μ M) for 24 hours, an equal volume of vehicle was added to controls; α -tubulin was used as a loading control; representative bands are shown. Values were expressed as mean $\pm$ SEM from at least three independent experiments performed in duplicate. Statistical significance ($P < 0.05$): *, treated SW-620 cells versus treated HT-29 cells; †, treated cells versus control cells.

Fig. 7. GDP effect on mitochondrial hydrogen peroxide production in State 4 in colonic cells. H₂O₂ production from isolated mitochondria was measured fluorimetrically using the Amplex red reagent. Succinate (5 mM) was used as substrate with or without 500 μ M GDP, an inhibitor of mitochondrial chain uncoupling. Values were expressed as mean $\pm$ SEM from at least three independent experiments performed in duplicate. Statistical significance ($P < 0.05$): *, Glu-R cells versus HT-29 cells; †, SW-620 cells versus HT-29 cells. # Significant difference between mitochondrial H₂O₂ production without GDP and with GDP ($p < 0.05$); mt protein, mitochondrial protein.

lines. Whereas HT-29 cells treated with H₂O₂ sustained the steady-state balance GPx/SOD or increased the ratio between catalase and SOD activities, SW-620 cells treated with H₂O₂ showed a marked decrease in catalase/SOD and GPx/SOD ratios compared to controls.

Both cell lines treated with exogenous hydrogen peroxide at a dose of 40 μ M did not display a significant reduction of cell viability compared to controls after 24 hours (Fig. 6A). SW-620 cells exhibited significantly lower viability than HT-29 cells at a dose of 40 μ M, reflecting

higher steady-state levels of oxidative stress. Accordingly, HT-29 cells showed greater viability in comparison with SW-620 cells under the higher doses tested after 24 hours.

As shown in Fig. 6B, the percentage of viable clones, relative to untreated, formed by cells treated with hydrogen peroxide was 78.8% for HT-29 cells and 72.5% for SW-620 cells. These results suggest that survival of colonic cells containing dysfunctional mitochondria depends on the capacity to sustain an efficient control of oxidative stress.

Hydrogen peroxide caused an increase of 56.6% and 29.0% in UCP5 levels of HT-29 cells (Fig. 6C) and SW-620 cells (Fig. 6D), respectively. This finding is in accordance with the less effective antioxidant response developed by SW-620 cells in comparison with HT-29 cells to counteract exogenous oxidative stimuli, reflecting that antioxidant response in SW-620 cells was notably activated in the basal state.

A regulatory role of UCPs on mitochondrial ROS production of colonic cells

In the presence of succinate (5 mM) as a substrate, resting mitochondria (State 4 respiration) from Glu-R cells showed the lowest rate of hydrogen peroxide production. The rate of mitochondrial H_2O_2 production was lower in HT-29 cells than in metastatic SW-620 cells (see Fig. 7).

To confirm the regulatory role of UCPs on mitochondrial ROS production of colonic cells, we analyzed H_2O_2 production of isolated mitochondria in State 4 respiration in the absence or presence of guanosine 5'-diphosphate (GDP) as a UCP function inhibitor [42]. As shown in Fig. 7, GDP addition at a final concentration of 500 μ M caused a significant rise in the rate of mitochondrial H_2O_2 production in colonic cells; the increase was higher in mitochondria from colon cancer cell lines (41.5% for HT-29 cells and 46.4% for SW-620 cells) than in those from Glu-R cells (8.11%).

Discussion

In this study we used an in vitro model to assess the influence of oxidative stress and mitochondrial dysfunction on the expression of UCPs in colonic cells. To validate the three different phenotypes of colonic epithelial cells, we determined metabolic parameters and redox state in HT-29 cells induced to differentiate (Glu-R), non-metastatic HT-29 cells and metastatic SW-620 cells.

Compared to the parenteral cell line, Glu-R cells exhibited clear features of functional colonic differentiation. Glu-R cells showed an increase in mitochondrial functionality and mitochondrial content as indicated by the higher mtDNA copy number [43]. The improved mitochondrial function is in accordance with their decreased glycolytic activity as well as their better redox homeostasis, reflected by a marked decrease of endogenous H_2O_2 production, intracellular ROS levels and cellular oxidative damage. According to the results obtained in Glu-R cells, differentiated colonic cells have been described to show the same morphological changes, a markedly slower growth and similar changes in cell cycle profile. In relation to this, ultrastructural examination of cells induced to differentiate show morphologic features of epithelial differentiation, including formation of a microvillar apical membrane and lateral desmosome adhesions; as the differentiation process continues, a marked accumulation of mitochondria becomes increasingly visible by electron microscopy [44]. Growth arrest, differentiation and apoptosis are reported to proceed sequentially starting at early time points (24h). However, the presence of the brush border, which represents a maximal degree of structural colonic differentiation, is usually studied after longer periods in culture (14-21 days) [45]. Our results are in accordance with previous studies which report that the redox state of the GSH system becomes oxidized when cells are induced to differentiate by chemical agents as a consequence of progression from proliferation to contact inhibition and differentiation in colon carcinoma cells [46]. Notably, this change in the GSH/GSSG redox system has been shown to be inherently associated with differentiation and is not a function of manipulations such as the addition of a differentiating agent or removal of a required growth factor. The aforementioned functional characteristics identified in Glu-R cells, together with their acquired morphology and slow growth pattern are attributed to normal cells and such features are compatible with the ability of HT-29 cells to differentiate in vitro [5, 20].

Metastatic SW-620 cell line showed higher intracellular ROS levels and H_2O_2 production, higher glycolytic activity and lower mitochondrial oxidative capacity than HT-29 cells, reflecting greater mitochondrial dysfunction. Despite the elevated steady-state levels of hydrogen peroxide in SW-620 cells, their cell viability was not compromised (Fig. 1A) and paradoxically, their cellular protein and lipid oxidation was even lower than HT-29 cells. UCP5 levels in concert with catalase and GPx antioxidant activities (key H_2O_2 detoxifying enzymes) were increased

in SW-620 cells. In this sense, the activation of antioxidant mechanisms, including UCP5, appeared to effectively counteract steady-state oxidative injuries and consequently, allowing tumor cells to sustain their viability.

Reactive oxygen species, whether produced endogenously as a consequence of normal cell functions or derived from external sources, pose a constant threat to cells living in an aerobic environment. Transient fluctuations in ROS perform important regulatory functions, but when present at high and/or sustained levels, ROS can cause severe damage and compromise cell survival [47]. To mimic a transient change in intracellular H_2O_2 concentration, both cancer cell lines were exposed to an acute oxidative stimulus and were cultured for 24 h to allow maximal protein expression of UCPs. As a result of H_2O_2 addition in culture medium, cancer cells activated the antioxidant response, which was cell type-dependent. Our experiments revealed that colon cancer cells respond to an oxidative stimulus that compromises their survival by activating compensatory mechanisms that involve not only antioxidant systems but also UCP5 proteins.

UCPs have been described as downregulators of mitochondrial ROS production in several tissues due to their dissipating effect on the proton gradient [48–50], in which even mild uncoupling significantly reduces the ROS production [51]. In this study we analysed UCP2 and UCP5 protein expression in colonic cells, but in previous experiments we also confirmed the expression of UCP4 by western blot in both HT-29 and SW-620 colon cancer cells (data not shown). Therefore, we considered interesting to measure total uncoupling activity in isolated mitochondria, which was assayed indirectly as a change in mitochondrial ROS production in the presence of GDP as a specific inhibitor of uncoupling proteins. Our experiments revealed that GDP-dependent inhibition of mitochondrial ROS production was significantly higher in colon cancer cells than in Glu-R cells, probably reflecting the importance of uncoupling proteins' function in limiting the enhanced steady-state mitochondrial H_2O_2 production in colon cancer cells.

In the tumoral microenvironment, higher expression of UCP2 has been associated with malignant transformation [52], increased aerobic glycolysis (Warburg effect) [53] and augmented cancer cell survival [17]. Moreover, enhanced resistance against chemotherapeutic agents has been described in cancer cells overexpressing UCP2 [54]. We propose in the present study a possible explanation to those observations focusing UCPs as elements of a complex coordinated antioxi-

dant response in colon cancer cells, probably activated against the high levels of hydrogen peroxide released by dysfunctional mitochondria. However, further research concerning UCPs and colon cancer is needed, given the possible implications of UCPs in the cytoprotection of cancer cells against oxidative stress, such as the one caused by antitumor drugs.

In conclusion, this study has proven that enhanced levels of UCP2 and UCP5 are associated to mitochondrial dysfunction in colonic cells. In accordance with this, functional enterocytic differentiation is characterized by higher mitochondrial function and better oxidative balance, which is accompanied by a down-regulation of uncoupling protein levels and uncoupling activity. In addition, this study evidences the regulatory role of UCPs on mitochondrial ROS production of colonic cells and also reveals for the first time that the increase in UCP5 levels is involved in a compensatory antioxidant response against oxidative stimulus that compromises colonic cell survival. Hence hydrogen peroxide released in higher amounts by dysfunctional mitochondria of colonic cells might stimulate the coordinated activation of antioxidant systems and UCPs to counteract oxidative damage and perhaps facilitate cell survival.

Abbreviations

UCPs (uncoupling proteins); SOD (superoxide dismutase); GPx (glutathione peroxidase); GRd (glutathione reductase); GSH (reduced glutathione); GSSG (oxidized glutathione); ROS (reactive oxygen species); TBARS (thiobarbituric acid-reactive substances); COX (cytochrome *c* oxidase); ATPase (ATP synthase); CS (citrate synthase); LDH (lactate dehydrogenase); mtDNA (mitochondrial DNA).

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