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Cytotoxic effect of hemin in colonic epithelial cell line: Involvement of 18 kDa translocator protein (TSPO)

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ABSTRACT

Aims: The aim of this study is to investigate the effect of hemin in colonic epithelial cells (Caco-2) cell proliferation and if this effect was due to a direct modulation of 18-kDa translocator protein (TSPO) and/or heme oxygenase type 1 (HO-1).
Main methods: The main methods are as follow: cell proliferation and cell cytotoxic assays on Caco-2 cell lines treated with hemin in the presence or not of 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195) and Sn-protoporphyrin IX (Sn-PPIX), and immunoblotting for TSPO and HO-1 protein analysis, siRNA directed against TSPO.
Key findings: Hemin was shown to be toxic for the Caco-2 cell line in a concentration and time dependent manner. Although hemin was able to induce HO-1 in a dose dependent manner, a specific HO-1 inhibitor, Sn-PPIX, was unable to interfere with the effect of hemin on Caco-2 cells. Instead, PK 11195, a specific TSPO ligand, was able to counteract the effect of hemin, suggesting an important role of TSPO in the hemin activity. Cell viability assay further confirms the high cytotoxic effects exerted by hemin on Caco-2 cells expressing TSPO compared to the siRNA-TSPO targeted cells. In addition, hemin was able to decrease significantly the TSPO protein density in a dose dependent manner after 24 h of incubation.
Significance: The interaction and the consecutive down regulation of TSPO by hemin played an important role in the control of Caco-2 cell viability. The presented data suggest that TSPO might contribute to protect cells from potential toxic compounds such as free tetrapyrroles, candidating this receptor as a survival receptor protein.

Introduction

A growing body of experimental evidence suggests that hemin could play a significant role in different human dysfunction such as colorectal cancer, bowel inflammation and neuronal cell injury (Goldstein et al., 2003). However, the molecular mechanism by which hemin exerts this effect is still under debate (Goldstein et al., 2003). Hemin is a lipid-soluble molecule that interacts rapidly with numerous cell constituents and its toxicity may be mediated by both oxidative and non-oxidative mechanisms (Hebbel and Eaton, 1989). At the high micromolar concentrations, hemin has been found to be toxic to a variety of cell types (Balla et al., 1992; Bhoite-Solomon et al., 1993; Braverman et al., 1995; da Silva et al., 1996). In this perspective, Noguchi et al. (1982) have reported that a considerable amount of hydrogen peroxide (H_2O_2) is produced during heme degradation, catalyzed by the reconstituted heme oxygenase (HO) system. Ferrous irons and H_2O_2 are able to cleave DNA through Fenton reaction at sequence-specific sites often found in telomere repeats (Henle et al., 1999), and therefore they might play a pivotal role in cell fate. Recently it has been proposed that heme is able to induce the production of H_2O_2 by the inducible heme oxygenase type 1 (HO-1) in colonic epithelial cells (Caco-2), which participates in DNA damage, cell proliferation, apoptosis, and the production of inflammatory cytokines (Ishikawa et al., 2010). Moreover, the finding that catalases and zinc-protoporphyrin inhibited the hyper proliferation in Caco-2 cells supports a key role of HO-1 and oxidative stress in cell damage (Nowis et al., 2008). Interestingly, hemin, thanks to its chemical configuration, is able to interact with an intracellular receptor mainly localized on the external mitochondrial membrane called 18 kDa translocator protein (TSPO) (Taketani et al., 1995). Although some cellular functions of TSPO are conserved, such as cholesterol binding and transport, its biological significance seems to have adapted to serve specific functions (Batarseha and Papadopoulos, 2010), such as cell proliferation (Corsi et al., 2008; Shoukrun et al., 2008; Zeno et al., 2012). Indeed, the knockdown of the protein resulted in a 60% reduction in the number of C6 glioma cells in the pre-G1 apoptotic phase, suggesting an anti-apoptotic role

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of TSPO (Levin et al., 2005). The finding that specific TSPO ligands such as the 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195) and the 4′-chlorodiazepam (Ro 5-4864) attenuate apoptosis in human glioblastoma cells suggests that TSPO activity is intimately related to cell type or tissue analyzed (Kugler et al., 2008). Nevertheless, the conservation of TSPO throughout evolution highlights the significance of this protein for proper cellular function and development.

Therefore, there is a substantial possibility that the effect of heme on cell fate might be related to an interaction with TSPO. The present study was planned to investigate the effect of heme on human colon adenocarcinoma Caco-2 cell proliferation through a direct modulation of TSPO and/or HO-1. For this purpose, we utilized serial treatments with hemin in association or not with the specific TSPO ligand PK 11195 or the specific HO-1 inhibitor Sn-protoporphyrin IX (Sn-PPIX).

Material and methods

Cell line

Human colon adenocarcinoma Caco-2 cells (RCB0988) from the Riken Cell Band (Tsukuba, Japan) were maintained in DMEM supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, 100 U/mL penicillin, and 1% nonessential amino acids. The cells were cultured in a humidified incubator at 37 °C with 95% atmospheric air/5% CO_2. Caco-2 cells were used between the 15th and 26th passages. For all experiments, the cells were seeded at a density of 3 × 104 cells/cm^2 in cell culture plates. The cells were carefully treated to avoid as much light exposure as possible.

Cell treatments

After 24-h growth in the appropriate culture plates, the growth medium was replaced with a medium containing reduced 5% fetal bovine serum. Hemin, Sn-PPIX and PK 11195 (Sigma, Italy) were prepared on a stock solution in 1 M sodium hydroxide and/or DMSO and further diluted in the non-supplemented DMEM. Caco-2 cells were exposed to different concentrations of hemin (0.1–1 mM) for 0–6–24 h in the absence and presence of various concentrations of PK 11195 (1–50 μM) or Sn-PPIX (5 μM). Control cells were maintained in no supplemented DMEM.

Cell viability

Caco-2 cells were plated on 96-well plates (Euroclone, Italy) at a concentration of 2000 cells/cm^2. After exposure to the desired concentrations of hemin in the presence or not of PK 11195 and Sn-PPIX, 20 μl MTS (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, Italy) was added to each well and incubated for a period of 2.5 h. Finally, absorption was measured at 492 nm using a Multiscan microplate reader (Labsystem Multiscan® MCC/340, Finland).

LDH assay

Hemin was directly added to differentiated cultures to the desired concentration. At the end of the exposure period (24 h), cell injury was estimated by examination of cultures under phase-contrast microscopy and was quantified by measuring lactate dehydrogenase (LDH) release into the culture medium following the manufacturer's instructions (Cytotoxicity Detection kit Roche-Applied-Sciences, Italy) Briefly, 100 μl sample of the medium was removed from each culture and was placed in a 96-well assay plate. Then 100 μl of the reaction mixture composed by Diaphorase/NAD +, tetrazolium chloride and sodium lactate was added to each well. The absorbance of the reaction mixture at 492 nm was determined using a multiskan plate reader (Labsystem Multiscan® MCC/340, Finland). The LDH signal that was associated with 100% cell death was determined by lysing cells with 0.2% Triton X-100.

Cell proliferation

Cell proliferation was measured by 5-bromo-20-deoxyuridine (BrdU) incorporation assay. This assay was performed with a BrdU cell proliferation ELISA kit (Roche-Applied-Sciences, Italy) according to the manufacturer's instructions. Briefly, Caco-2 cells were labeled with BrdU after the treatments and incubated for an additional 2 h. The labeling medium was removed, and the cells were dried. Thereafter, cellular DNA was denaturated by adding a fixative solution for 30 min at room temperature. After removal of the fixative solution, anti-BrdU antibody conjugated with a peroxidase solution was added to each well, and the cells were incubated for 90 min at room temperature. After three washes with PBS, tetramethylbenzidine solution was added to each well, and the cells were incubated for 15 min at room temperature. The reaction was terminated with 1 M H2SO4, and the absorbance at 450 nm (reference wavelength 655 nm) was read using the Multiscan microplate reader (Labsystem Multiscan® MCC/340, Finland).

Measurement of ROS

ROS were measured using CellROX® Green Reagent as described by the manufacturer's instructions. Briefly, CellROX® Green Reagent is a fluorogenic probe designed to reliably measure oxidative stress in living cells. The cell-permeable green dye is non fluorescent while in a reduced state and, upon oxidation, exhibits excitation/emission maxima at 485/520 nm. CellROX® Green Reagent is a DNA dye, and upon oxidation, it binds to DNA; thus, its signal is localized primarily in the nucleus and mitochondria. Caco-2 cells were seeded on 96-well plates at 20 × 103 cells/well and treated with hemin at different concentrations, for the indicated time intervals, and CellROX® Green Reagent was added at a final concentration of 5 μM to the cells and then incubated for 30 min at 37 °C. Subsequently, the medium was removed, and the cells were washed three times with PBS. The resulting fluorescence was measured using a fluorometer (Fluoroskan ascent FL). Menadione 100 μM was added 30 min before treatment with CellROX® as a reference ROS inducer drug.

Immunoblotting

The culture medium was aspirated, and the monolayer was immediately lysed in 100 μl of buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), and a protease inhibitor cocktail (Sigma, Italy). Protein concentration was determined by the Bio-Rad Protein Assay kit. Protein samples from Caco-2 cells treated or not for 24 h with 10 and 200 μM of hemin were solubilized in Nu-Page sample buffer (141 mM Tris base, 106 mM Tris–HCl pH 7.4 SDS 2%, 0.51 mM EDTA, 0.22 mM serva blu, 175 mM phenol red, and 0.05 M DTT) (Invitrogen, Italy), boiled for 5 min and loaded onto a pre-cast 12% SDS-PAGE. Separated proteins were electrophoretically transferred to a nitrocellulose membrane (Invitrogen, Italy). The membrane was blocked in TBST (20 mM Tris–HCl, 0.5 M NaCl and 0.05% Tween 20) buffer containing 5% non-fat dried milk overnight at 4 °C or 1 h at room temperature and probed with specific primary polyclonal antibody anti-TSPO (Anti-TSPO polyclonal antibody Trevigen, USA), anti-HO-1 (Rabbit polyclonal to heme oxygenase-1, Abcam, UK) and anti-tubulin (Mouse monoclonal antibody β-tubulina, Santa Cruz, USA) (1:1000) for 3 h under gentle agitation. The membrane was then washed 3 times in TBST for 10 min each time, incubated for 1 h with HRP-conjugated anti-rabbit antibody (Santa Cruz, USA) or with HRP-conjugated anti-mouse antibody (Santa Cruz, USA) and visualized using chemiluminescence (Amersham, UK). The specificity of the band recognized by the anti-TSPO antibody was demonstrated using pre-adsorbed antibody prepared by incubating the antibody with

recombinant TSPO fusion protein used also as a control (Trevigen, USA) as previously described (Corsi et al., 2004).

Gene silencing

TSPO silencing was achieved in Caco-2 colon cancer cells using a 100 nM concentration of a mixture of three pre-designed siRNA duplexes (Sigma Aldrich, St. Louis, MO, USA; Oligo name: SASI_Hs01_00054088; SASI_Hs01_00054087; SASI_Hs01_00054085) and 15 μl of Lipofectamine RNAimax reagent (Life Technologies). Two rounds of "Reverse Transfection", performed according to the supplied protocol, were carried out on 0.4×10^6 cells every 24 h and, following a 48 h period of incubation, a dose of 50 μM of hemin was added for additional 24 h. Each experiment included a negative control represented by a non-targeting siRNA synthesized by Sigma-Aldrich in order to exclude the non-specific effects of siRNA transfection.

Statistical analysis

All data are presented as the mean ± SD of at least three different experiments done in quadruplicate. One-way ANOVA analysis of variance and Dunnett's and Newmann–Keuls' test were performed to compare differences between the groups, and *t*-test was performed to compare two groups, as indicated in the figures (Graph-Pad 5 Software Inc, San Diego, CA, USA). *P* values <0.05 were considered significant.

Results

Hemin toxicity at different time points

Fig. 1 summarizes the results on cell viability as measured by MTS assay at 6 and 24 h. Hemin decreases the cell viability in a dose and time dependent manner. In particular the effect was greater after 24 h of incubation, where the inhibition of cell viability was significant at all the concentrations tested, reaching a maximum effect at 500 μM and 1 mM concentrations ($IC_{50} = 1$ mM). After 6 h, the incubation with hemin resulted in a marked decrease of cell growth which reached significant values only at the higher concentrations tested.

Continuous exposure to hemin for 24 h produced cell toxicity, as determined by LDH release, which increased dramatically between 50 and 200 μM hemin (Fig. 2). Exposure to 10 μM hemin did not produce any variation of LDH release by Caco-2 cell line.

Effect of hemin on Caco-2 cell proliferation

The cells were treated for 6 to 24 h with various concentrations of hemin ranging from 0.1 to 200 μM, and cell proliferation was determined by the BrdU incorporation assay. The rationale for the choice of these concentrations ranges comes from the results obtained on Caco-2 cell toxicity.

As expected (Fig. 3), the cell proliferation was significantly inhibited in a dose dependent manner, confirming the results obtained on cell toxicity. After 6 and 24 h of incubation, hemin peaked at 200 μM and the decrease being 71 and 87% respectively. The IC_{50} at 6 and 24 h were 150 and 50 μM respectively.

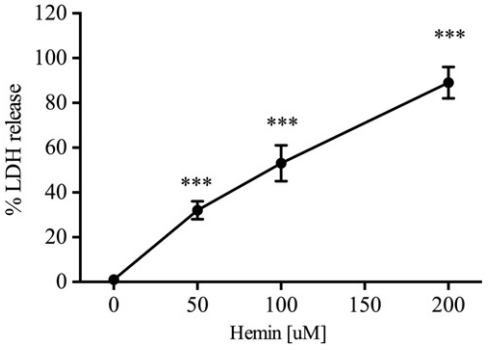

Fig. 2. Hemin is toxic at low micromolar concentrations. Cultures (*n.* 6–8 per condition) were exposed to the indicated concentrations of hemin for 24 h. LDH values, expressed as the mean ± SD of three independent experiments, are scaled to those in untreated cultures exposed continuously to 0.1% Triton X-100, which releases 100% of culture LDH. The low mean LDH values in the medium of untreated cultures from the same plating were subtracted from all values. ***$P < 0.001$ vs. control cells exposed to the culture medium only. One way ANOVA and Dunnett's as post test.

Effect of hemin on heme oxygenase expression

Heme oxygenase expression was assessed at base-line (Ctrl) and after exposure to hemin at two different concentrations. In these experiments, cultures were exposed to 10 and 200 μM hemin concentration for 24 h; untreated controls were kept in the same serum-free culture medium. At the former time point, hemin-exposed cells decrease they proliferative activity but are still viable in particular at the lower concentration tested. As shown in Fig. 4a in untreated cultures, HO-1 immunoreactivity was minimal, but after hemin exposure, a significant increase in HO-1 was observed in particular for the 200 μM concentration treatment (Fig. 4b). However, as shown in Fig. 5, Sn-PPIX (5 μM) a selective inhibitor of HO-1, which did not alter cyclin D1 activity, was unable to reverse or rescue Caco-2 cells after 24 h hemin treatments at all the concentrations tested.

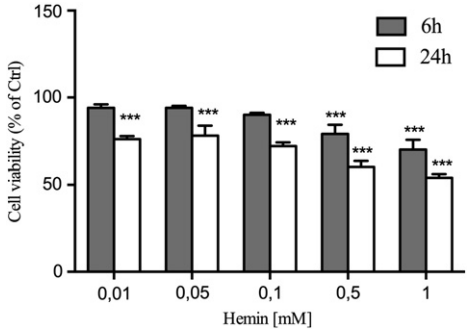

Fig. 1. Effect of hemin Caco-2 cell viability. Cells were treated with various concentrations (0.01 to 1 mM) of hemin for 6 (grey bars) and 24 h (white bars). Cell viability was determined by the MTT assay. The cell viability was directly proportional to the production of formazan, which was measured spectrophotometrically at 492 nm. Values are of three independent experiments. ***$P < 0.001$, compared with the untreated control. One-way ANOVA with Dunnett's as post test.

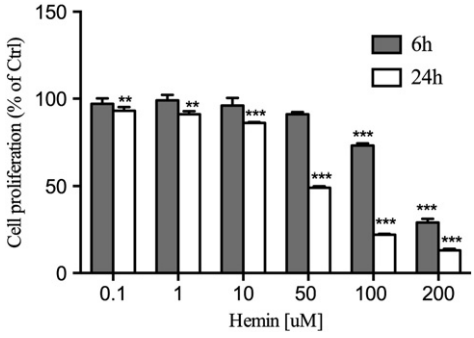

Fig. 3. Effect of the concentration of hemin on the proliferation of human colonic epithelial Caco-2 cells. The cells were treated for 6 (white bars) and 24 h (grey bars) with various concentrations of hemin (0.1 to 200 μM), and cell proliferation was determined by the BrdU incorporation assay which was measured at 450 nm. The values are expressed as the mean ± SD of four independent experiments. ** $P < 0.01$ and***$P < 0.001$, compared with the untreated control. One-way ANOVA with Dunnett's as post test.

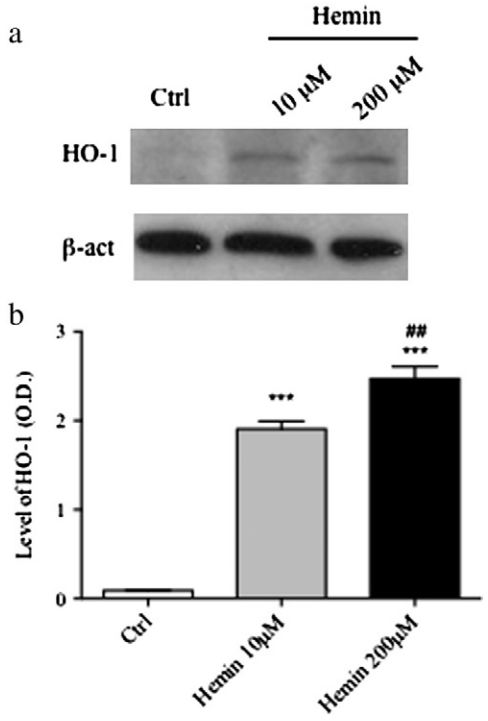

Fig. 4. Effect of hemin on HO-1 in human colonic epithelial Caco-2 cells. a): Representative western blots and b): densitometric analyses of protein levels of HO-1 of Caco-2 cell lysate after incubation with 10 and 200 μM of hemin for 24 h. Densitometry values were normalized to the protein loading control, beta-actin. The values are expressed as the mean ± SD of three independent experiments ($n = 6$ per group). *** $P < 0.001$ vs untreated cells (Ctrl); ## $P < 0.01$ vs hemin 10 μM, using one-way ANOVA with and Newmann–Keuls as post test.

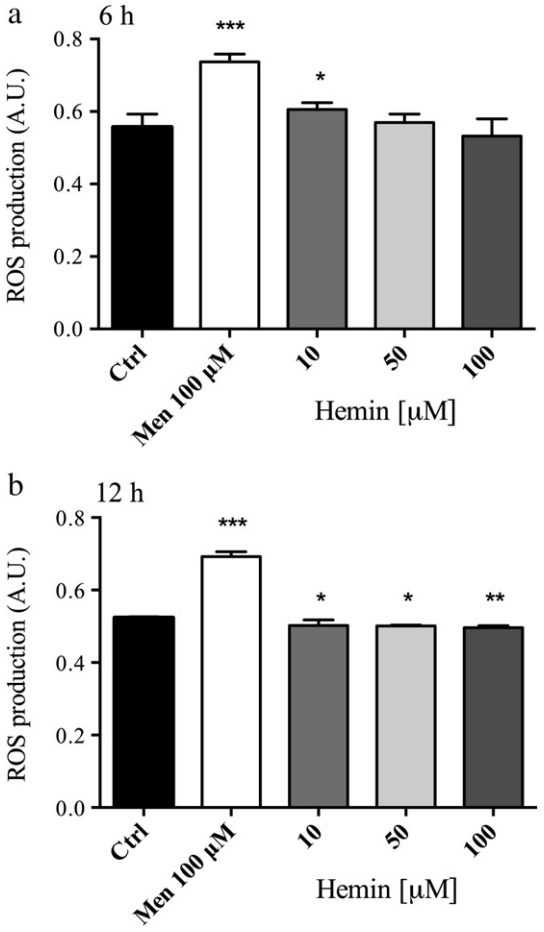

Fig. 6. Effects of hemin on intracellular ROS production. Caco-2 cells were treated with different concentrations of hemin (50–100–200 μM) at 6 h (a) and 12 h (b). ROS production was assessed by CellROX® green reagent assay and expressed as arbitrary units (A.U.). The values are expressed as the mean ± SD of three independent experiments. 100 μM of Menadione (Men) was used as positive control. The values are expressed as the mean ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs untreated cells (Ctrl) using one-way ANOVA with Dunnett's as post test.

Modulation of intracellular ROS

We focalized our attention on the ability of hemin to induce of ROS in nuclei, which might generate DNA damage, using a specific CellROX® green reagent. Caco-2 cells were treated with different concentrations of hemin for 6 (Fig. 6a) and 12 h (Fig. 6b). After 6 h of incubation time, hemin significantly induced ROS production only at 10 μM concentration whereas at 50 and 100 μM was unable to modulate the ROS levels. Interestingly, after 12 h of incubation time, hemin significantly decreased the ROS production at all the concentrations tested.

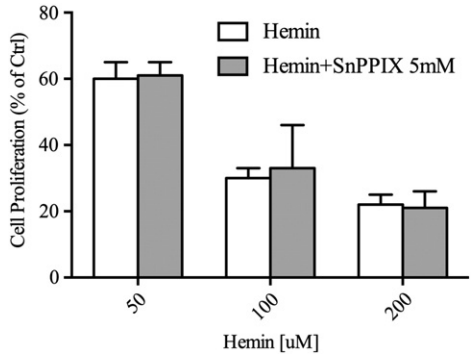

Fig. 5. Effects of SnPPIX on hemin-antiproliferative activity in human colonic epithelial Caco-2 cells. The cells were treated for 24 h with various concentrations of hemin (50–100–200 μM) alone or in combination with 5 μM of SnPPIX and the cell proliferation was measured by BrdU assay. The values are expressed as the mean ± SD of three independent experiments.

Interaction of hemin with TSPO

Since the hemin is able to bind TSPO with relatively high affinity, we analyzed the ability of the specific TSPO ligand PK 11195 to interfere with hemin antiproliferative activity. Fig. 7a shows the effect of PK 11195 (1 μM) on the hemin-antiproliferative activity on Caco-2 cells. The specific TSPO ligand was able to significantly reduce the inhibition of cell proliferation elicited by hemin at all the concentrations tested. However PK 11195 was unable to restore the values to the level of control. We therefore examined the effect of increasing concentration of PK 11195 on 50 μM hemin antiproliferative activity. As clearly shown in Fig. 7b, although PK 11195 counteracts significantly the effect of hemin, its effect was not in a dose dependent manner. We then evaluate the potential ability of hemin to modulate the amount of TSPO protein. We analyzed the TSPO protein density in Caco-2 cells after 24 h incubation with hemin at different concentrations. As shown in Fig. 8a the pharmacological treatments of hemin at 10 and 200 μM had different effects on the relative amount of TSPO detected by immunoblotting. Compared with control cells, both treatments down-regulated TSPO protein levels; in particular, 200 μM of hemin caused about a 55% reduction in detectable TSPO (Fig. 8b).

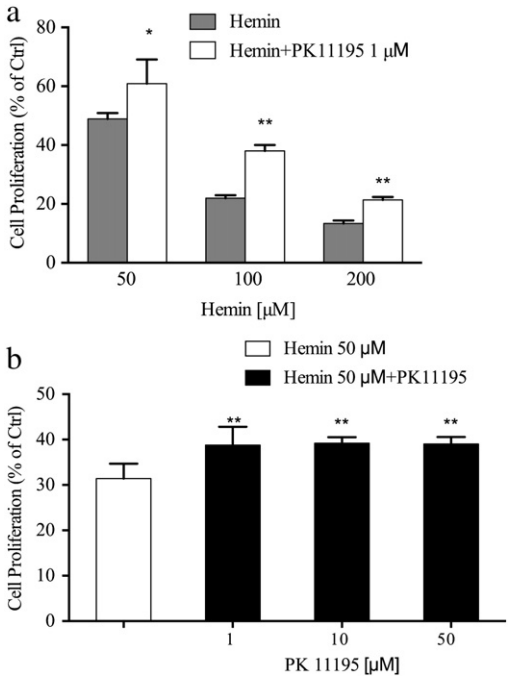

Fig. 7. Effects of PK11195 on hemin- antiproliferative activity in human colonic epithelial Caco-2 cells. a): The cells were treated for 24 h with various concentrations of hemin (50–100–200 μM) alone or in combination with 1 μM of PK11195 and the cell proliferation measured by BrdU assay. The values are expressed as the mean ± SD of three independent experiments. * $P < 0.05$ vs hemin 50 μM alone and ** $P < 0.01$ vs hemin 100 and 200 μM alone *t*-test. b): PK 11195 dose-dependent inhibition of hemin antiproliferative activity. The cells were treated for 24 h with 50 μM of hemin in the presence or not of various concentrations of PK 11195 (1–10–50 μM). The values are expressed as the mean ± SD of three independent experiments. ** $P < 0.01$ vs 200 μM hemin alone using one-way ANOVA with Dunnett's as post test.

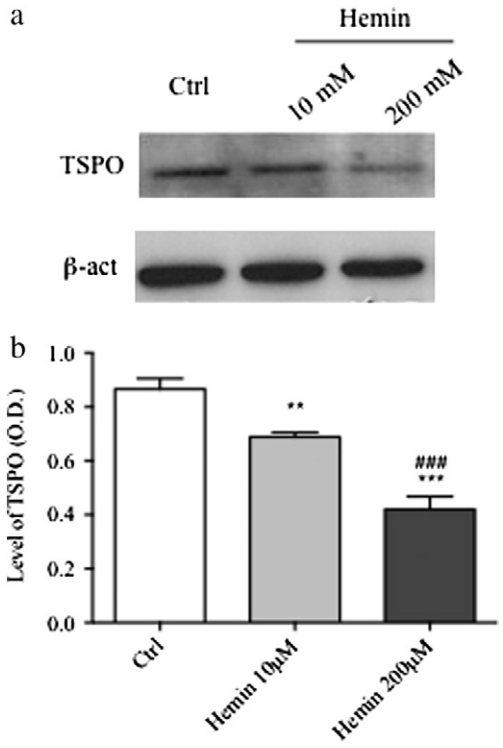

Fig. 8. Effect of hemin on TSPO protein in human colonic epithelial Caco-2 cells. a): Representative western blots and b): densitometric analyses of protein levels of TSPO of Caco-2 cell lysate after incubation with 10 and 200 μM of hemin for 24 h. Densitometry values were normalized to the protein loading control, beta-actin. The values are expressed as the mean ± SD of three independent experiments ($n = 6$ per group). ** $P < 0.01$ and *** $P < 0.001$ vs untreated cells (Ctrl); ### $P < 0.001$ vs hemin 10 μM, using one-way ANOVA with Newmann–Keuls as post test.

Effect of siRNA targeting TSPO on Caco-2 cell viability

In order to evaluate the role of TSPO in the maintenance of cell viability, we performed TSPO silencing experiments in Caco-2 cells. TSPO inhibition was obtained according to "Reverse Trasfection" protocol supplied by Lipofectamine RNAimax. After 48 h of incubation, the cells were treated or not with 50 μM concentration of Hemin for 24 h and cell viability was determined. TSPO down-regulation was firstly assessed by Western blot analysis. The result, reported in Fig. 9a, clearly demonstrated the strong inhibition of TSPO protein compared to Caco-2 cells treated with a non-targeting siRNA, referred as a control. Subsequently, the data achieved by MTS assay, highlighted several interesting findings (Fig. 9b). These results firstly revealed a strong reduction of cell viability in the absence of TSPO expression. In addition, in the hemin treated samples, MTS assay further confirms the high cytotoxic effects exerted by this molecule on Caco-2 cells expressing TSPO as compared to the siRNA-TSPO targeted cells (Fig. 9b). Indeed, in the presence of low levels of TSPO transcript, Caco-2 cells were able to counteract the toxicity exerted by hemin, partially restoring cell proliferation and viability.

Discussion

Epidemiological studies have shown that Western-style diet (high meat, high fat, low fiber) is a high risk factor for colon cancer. Incidence increases in countries with a high meat consumption (Armstrong and Doll, 1975). It has been shown that hemoglobin is genotoxic in human colon cells, and that this is associated with free radical mechanisms and with cytotoxicity, especially for hemin (Glei et al., 2006). However, several lines of evidence indicate that hemin possesses both the

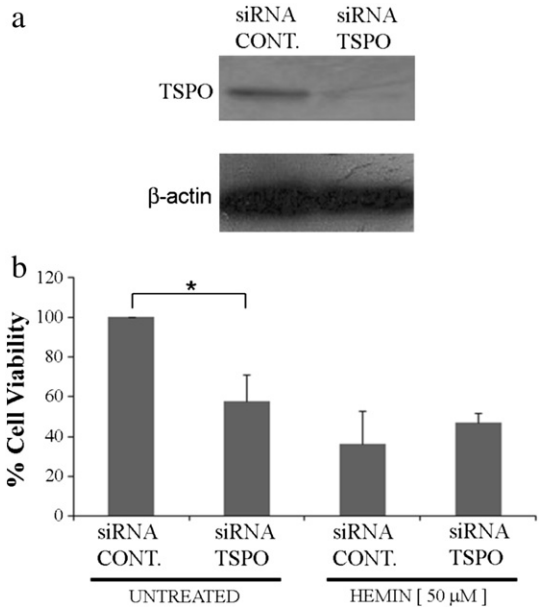

Fig. 9. Evaluation of Caco2 cell viability under TSPO silencing condition. a): Western blot analysis of TSPO protein expression upon transient siRNA transfection. The analyzed protein and samples are respectively indicated on the top and on the left of the panel. Expression of beta-actin was also evaluated to normalize the amount of protein extract loaded in the various lanes. b): MTS assay was carried out on siRNA transfected cells and upon hemin treatment. The histogram reports the cell viability data achieved in Caco2 cells transfected with a control or with TSPO-targeting siRNA and treated with a 50 μM of hemin for 24 h. Untreated samples were also reported. The data are indicated as mean ± SD of three independent experiments. * $P < 0.05$ using paired *t*-test.

antiproliferative and proliferative effects on Caco-2 cell lines (Ishikawa et al., 2010; Noda et al., 2001) This apparent discordance might rely on the concentration of hemin end/or the intracellular ROS production through heme oxygenase-1 induction. In this context, our data indicates that hemin induced cell toxicity in Caco-2 cell line in a dose and time dependent manner, reaching a maximum effect after 24 h of incubation. Indeed, the inhibition of cell proliferation elicited by hemin did not merge the LDH production at the same concentrations and times tested. This evidence suggests a possible different molecular mechanism involving the regulation of cell viability. It has been reported that the toxic effect of hemin is mostly due to the production of ROS (Ishikawa et al., 2010; Noda et al., 2001) through HO-1 induction. However, in this context, the role of HO-1 is still controversial. Several researchers on neuronal cells showed that iron release from hemin after HO-1 activity was responsible of the cell toxicity (Goldstein et al., 2003; Dwyer et al., 1998; Huang et al., 2002). However, Regan et al. (2000) previously observed that HO-1 induction was protective in cultured astrocytes exposed to hemoglobin (Regan et al., 2000). A beneficial effect of HO-1 has also been observed by Doré and Snyder (1999) in hippocampal neurons exposed to hydrogen peroxide and in systems of non-neural origin (Vile et al., 1994; Abraham et al., 1995; Dennery et al., 1997; Ishikawa et al., 2010). Our result on ROS generations indicates that hemin increased ROS production only at the low concentration and at the shorter incubation time. The evidence that high concentrations of hemin failed to induce the production ROS is probably due to the induction of HO-1, which mediated antioxidant activity. However, our data showed that despite the induction of HO-1 produced by hemin, the specific inhibition of HO-1 with Sn-PPIX was unable to restore Caco-2 cell proliferation. One explanation could be that the beneficial effect of converting hemin to an antioxidant (biliverdin) might be overcome by other molecular mechanisms able to inhibit cell viability of Caco-2 cells. Another explanation might be addressed to the type of assay used. In order to verify the presence of ROS in the nuclei, and their potential genotoxicity, we used a specific assay able to detect ROS in the nucleus and mitochondria, but not in the cytoplasm, probably underestimating the total production of ROS elicited by hemin. However, Menadione, used, as positive control of ROS generator, was able to significantly increase the levels of ROS in the nuclei and/or mitochondria, suggesting that most likely the induction of HO-1 has played an important role on the lack of hemin pro-oxidant activity at the highest concentrations tested. The finding that hemin is able to bind the intracellular mitochondrial receptor TSPO, with nanomolar affinity, strongly suggests a possible involvement of this protein in the hemin antiproliferative and/or cytotoxic effect. TSPO is involved in a number of cellular functions such as cholesterol transport, cell proliferation and apoptosis (Papadopoulos et al., 2006). Although the pharmacological modulation of TSPO has been extensively described, and it is known the ability of hemin to bind TSPO (Taketani et al., 1995), there are no evidence on the modulation of cell growth after the binding of hemin to TSPO. We then evaluated the antiproliferative effect of hemin in the presence or not of the specific TSPO ligand PK 11195, which per se did not alter Caco-2 cell proliferation (data not shown). The ability of PK 11195 to inhibit partially the hemin effect suggests an involvement of TSPO on hemin activity and a possible modulation of the receptor by this molecule. In particular, our data suggest that the binding of hemin to TSPO elicits a cytotoxic effect rather than an inhibition of cell proliferation, which might be a logical unspecific consequence of cytotoxicity. The latest assumption is sustained also by the transient siRNA targeting TSPO transfection data, where the cytotoxic effect of hemin in Caco-2 cells expressing TSPO was higher than the one observed in the siRNA-TSPO directed cells. It must be remarked that the transient siRNA directed against TSPO transfection alone resulted in a significant decrease of Caco-2 cell viability compared to the control, indicating that TSPO is a crucial protein for the cell viability. Indeed, it is known that its functional inactivation induces an early embryonic-lethal phenotype in mouse (Papadopoulos et al., 1997). In addition, it has been demonstrated that stable overexpression of TSPO, in mammary epithelial MCF10A acini, drives proliferation and provides partial resistance to luminal apoptosis (Wu and Gallo, 2013). The discrepancy between our results and those obtained on HT 29 colon cancer cell line, where TSPO knock down produced a decrease of apoptosis and increase of cell proliferation (Shoukrun et al., 2008), should be addressed to the different cell line adopted in the experiments. Although Caco-2 cells are derived from a human colon adenocarcinoma, they retain the ability to polarize and form a transporting epithelial monolayer in cell culture, which has many characteristics of polarized intestinal enterocytes in situ (Grasset et al., 1985). In such cell system, the TSPO density is far less than in HT 29, and this difference might be of relevance in the modulation of biological processes involving cell proliferation and/or cytotoxicity.

Moreover the results obtained by immunoblotting on TSPO protein clearly showed a down regulation of the receptor protein. Taking into account that a knock down of TSPO is related to an increase of cell death, and that the receptor is fundamental for the cell growth (Levin et al., 2005), we could surmise that the inhibition of Caco-2 cell viability might be due, at least in part, to the hemin-related TSPO degradation. In this context, recently, in *Arabidopsis thaliana*, it has been shown that the interaction of heme with the tryptophan-rich sensory protein (At-TSPO), a related membrane protein belonging to protein/peripheral-type benzodiazepine receptor protein family, produced a down regulation of the receptor, suggesting a potential role of At-TSPO as a scavengers of free tetrapyroles (Vanhee et al., 2011). In addition, it has been reported that the heme binding regulates At-TSPO degradation, through an active autophagy pathway, suggesting that the physiological consequence of active At-TSPO down regulation may be heme scavenging (Vanhee et al., 2011). Our results strongly support this evidence, and reinforce the idea that TSPO might act as a scavenger of porphyrin-based compounds also in eukaryotic model such as Caco-2 cell line.

Conclusion

Finally, our data showed a new mechanism by which hemin modulates cell viability and proliferation on Caco-2 cell line. The interaction and the consecutive down regulation of TSPO by hemin might play an important role in the control of cell viability. In addition, the data presented suggest that TSPO might contribute to protecting cells from potential toxic compounds such as free tetrapyrroles, candidating the TSPO as a survival receptor protein.

Conflict of interest statement

The author declares no conflict of interest.

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