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Research Article

Role of Hypotaurine in Protection against UVA-Induced Damage in Keratinocytes

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ABSTRACT

Photoageing and skin cancer are major causes of morbidity and are a high cost to society. Interest in the development of photoprotective agents for inclusion in topical cosmetic and sunscreen products is profound. Recently, amino acids with a sulfinic group, notably hypotaurine, have been included as ingredients in cosmetic preparations. However, the mechanism of action of hypotaurine as a possible anti-aging agent is unknown, despite its use as a free radical scavenger. To address this issue, we investigated hypotaurine uptake in a human keratinocyte model and examined its effect on UVR-induced cytotoxicity. Hypotaurine was taken up by keratinocytes in a time- and concentration-dependent manner, with levels remaining significantly above baseline 48 h after washout. A cytoprotective effect of pre-incubation with 2.5–5 mMhypotaurine was shown as indicated by increased cell viability when keratinocytes were irradiated with UVA at 5 or 10 Jcm^{-2}, with the level of hypotaurine also significantly reduced. These findings indicate a potential cytoprotective effect of hypotaurine against the deleterious effects of UVA irradiation. This provides support for further studies to evaluate the potential photoprotective benefits of hypotaurine supplementation of topical cosmetic and sunscreen products.

Abbreviations: PPG, propargylglycine; ROS, reactive oxygen species; TAUT, taurine transporter.

INTRODUCTION

Photoageing and skin cancer are consequences of cumulative cutaneous exposure to ultraviolet radiation (UVR) and are increasingly major causes of morbidity, placing huge burdens on society and health service provision and having important economic impact (1–3). In addition to the harmful effects of UVR on skin, induction of several protective mechanisms is also initiated, including production of enzymatic and non-enzymatic antioxidants that interact with reactive oxygen species (ROS) to minimize oxidative damage (4). However, UVR-induced oxidative stress may overwhelm natural cutaneous antioxidant capabilities, resulting in the induction of cell death or survival of aberrant cells that are in turn implicated in the processes of photocarcinogenesis and photoageing (5,6). UVA irradiation in keratinocytes increases hydrogen peroxide (H_2O_2) levels followed by conversion to hydroxyl radical, which seems to be a major contributor to UVA-induced damage in keratinocytes (7). In recent decades, considerable efforts have been made to prevent or limit oxidative stress-induced harmful effects of UVR on human skin, notably through supplementation with antioxidants, which are increasingly included in topical anti-aging, photoprotective and cosmetic products (8,9).

The sulfinic acid, hypotaurine has been identified as a potential active ingredient for inclusion in such preparations in order to minimize or prevent oxidative skin damage (10). Hypotaurine is a sulfur amino acid, characterized by the presence of a sulfinic group rather than a carboxyl group, which can be oxidized to taurine (11,12) through a non-enzymatic reaction upon interaction with ROS such as hydroxyl radical (13). The ability of hypotaurine to react with ROS or other radical species, such as nitrogen dioxide radicals or carbonate radical anions, has been shown in kinetic experiments using isolated superoxide dismutase enzyme or by pulse radiolysis (14,15). Hypotaurine has been shown to have a protective effect against oxidative stress-induced damage in rat placental trophoblasts (16) as well as in human skin fibroblast models of cigarette smoke-induced oxidation (17).

Whilst the potential roles of hypotaurine in human skin are largely unexplored, taurine has been more thoroughly investigated in different skin models, particularly in its roles as an osmolyte involved in maintaining skin barrier homeostasis and in protecting against epidermal and keratinocyte dehydration, in addition to protection against UV-induced apoptosis in epidermal keratinocytes and retinal ganglion cells (18–22). The photoprotective effect of taurine has been demonstrated by upregulation of both taurine and its transporter (TAUT) following UVB exposure of human keratinocytes (22) and in a TAUT deficient mouse model showing increased sensitivity to UVB-induced immunosuppression due to a role for taurine in regulating UVB-induced hydrolysis of membrane lipids and release of the immunosuppressive molecule platelet-activating factor (21).

The localization of taurine in skin has been investigated, with high levels found in the granular and upper spinous layers of rat

and dog epidermal keratinocytes, but absent in stratum corneum, basal and lower spinous layers (23). Taurine is not evenly distributed in the skin and its quantification in human excised skin layers has shown it to be most abundant in the epidermis (~250 µg taurineg^{-1} skin layer) and with lower concentrations in increasingly deeper layers of the dermis and in the stratum corneum (~50 µg taurineg^{-1}) (24). The distribution of the taurine transporter (TAUT) has also been investigated and has been shown to be detectable in the epidermis with a distribution gradient from the spinous to granular layers but not the dermis, using Western blot analysis (using a polyclonal anti-TAUT antibody) (20). Taurine uptake into keratinocytes against a concentration gradient has also been demonstrated and hypotaurine used as competitor has shown a reduction of taurine confirming that TAUT accept hypotaurine (25). Interestingly, hypotaurine has also been shown to be a substrate for TAUT/slc6a6 in keratinocytes (25) and GABA transporter family member (GAT2/Slc6a13) in human embryonic kidney (HEK293) cells (26), confirming that hypotaurine can be a substrate for TAUT in human skin. Moreover, hypotaurine uptake mediated by TAUT and GAT2 has been shown to contribute to the cellular resistance to oxidative stress (26).

Taurine does not directly react with ROS (27) and its protective activity in epidermal keratinocytes against dehydration or UV-induced apoptosis has been shown to be related to its role as an osmolyte (19,20). On the contrary, the taurine precursor, hypotaurine reacts efficiently and directly with ROS, including hydroxyl radicals, superoxide anions and singlet oxygen (15,27). Moreover, considering UVA induces ROS and intracellular levels of hydrogen peroxide, which can further generate hydroxyl radicals (7,28), hypotaurine could potentially play a major role in protecting against UVR-induced oxidative damage.

The aims of this study were to investigate hypotaurine uptake in cultured human keratinocyte (HaCaT) cells, to assess its potential in protection against UVR-induced oxidative damage and to evaluate its potential value for inclusion in sunscreen and cosmetic products.

MATERIALS AND METHODS

Cell culture and test treatments. HaCaT human keratinocytes (Interlab Cell Line Collection) were routinely cultured in DMEM with 10% FBS, 2% GlutaMAX™ and 1% Sodium Pyruvate in 5% CO_2 at 37°C. In all test conditions, cells were allowed to adhere for 24 h before any treatment and were seeded as follows: MTT assay 2.5×10^3 cells/well in 96-well plates; NRU assay 1×10^4 cells/well in 96-wells plates; HPLC 1×10^5 cells/well in 12-well plates. The test compounds hypotaurine, taurine, β-alanine and DL-Propargylglycine cystathionine γ-lyase inhibitor (PPG) were diluted in culture media to the concentrations and for the times indicated in the figure legends. Control cells were analyzed after 24 h in culture and were untreated.

Trypan blue. After treatment, the media was removed, and cells were incubated with 1mL EDTA for 10'. The ETDA was removed and 1 mL trypsin was added for 1' at 37°C. Cells were collected and centrifuged for 5' at 160 g at room temperature and resuspended in 1 mL media. 100 µL of trypan blue was added to 100 µL of cells and mixed. 10 µL of sample were added in the Neubauer chamber and cells were counted under microscope.

MTT assay. A Thiazolyl Blue Tetrazolium Bromide (MTT) stock solution (5 mg mL^{-1} in PBS) was prepared 24 h in advance and stored in freezer. The day of the assay, MTT aliquot was warmed in water-bath for 15' and 25 µL was added to each well at a final concentration of 1 mg mL^{-1} and incubated at 37°C for 2 h. After which the MTT was removed, the wells washed with 100 µL PBS and 100 µL DMSO added (29) and mixed with a plate shaker for 5' protected from light with tinfoil. Optical density was read at 570 nm with 690 nm subtraction using a BioTeck–Synergy 2 microplate reader.

NRU assay. A NR stock solution was prepared 24 h in advance at 37°C protected from light (1:10 dilution in PBS to 0.33 mg mL^{-1} from N2889-20ML Sigma) (30). The day of the assay, 20 µL of 0.33 mg mL^{-1} (NR solution) was added to each well directly into treatment media to have a final concentration of 55 µg mL^{-1} and incubated at 37°C for 2 h. Half an hour before the end of NR incubation, the NR destain solution was prepared as followed: 50% (v/v) EtOH in ddH$_2$O containing 1% (v/v) glacial acetic acid, and kept in ice. After the 2 h, the NR solution was removed and the wells washed with 100 µL EBSS, carefully removed within 1 min. The plate was left drying for 2' and 100 µL NR destain solution added and mixed with a plate shaker for 10' protected from light with tinfoil. After, optical density was read at 540 nm using a BioTeck-Synergy 2 reader.

HPLC analysis. Hypotaurine uptake was analyzed by HPLC as described in (13). Briefly, after treatment, cells were collected by centrifugation for 5' at 160 g at room temperature following treatment with EDTA and trypsinization. The cell pellets were lysed with 500 µL of cold lysis buffer (0.1 M HCl with 0.2% TDGA), vortexed and incubated at 4°C for 5'. Lysed cells were centrifuged for 10' at 160 g and 10 µL of supernatant was taken and derivatize for 2' with 25% OPA-mercaptoethanol (0.2 M OPA in 1 mL methanol, 5 mL of borate buffer and 40 µL mercaptoethanol) and 65% borate buffer (0.81 M boric acid, 0.79 M potassium hydroxide in 100 mL dH$_2$O) in a 100 µL final volume. Total protein was quantified by BCA assay using bovine serum albumin standard. 50 µL of sample were analyzed by HPLC after 2' incubation. HPLC analysis was performed with Varian ProStar HPLC fluorimetric equipment and Phenomenex column (Gemini C18, 5 µM, 250 × 4.6 mm). The excitation was performed at 340 nm and the emission at 450 nm. The mobile phase A was prepared with 0.05 M sodium acetate at pH 5.5: methanol (80:20, v/v), and mobile phase B with methanol: H$_2$O (80:20, v/v). Flow rate was 1 mL min^{-1} at 20°C and the elution gradient was linear from phase A to 50% phase B in 5' followed by isocratic at 50% phase B (13).

UVA irradiation. Philips Cleo (100W-R) lamp with a 320–395 nm range (λ_{max}: 365 nm), with 320 nm cut off glass filtering was used to reach 3.4 mW cm^{-2} UVA intensity (as assessed using a calibrated UV light meter: IL-1400A International Light INC. Newburyport, Massachusetts) approximately 10 cm from the plates. UVA doses of 5, 10 and 15 Jcm^{-2} were reached with 24, 48 and 72 min of irradiation respectively. Before irradiation, cells were treated for 24 h ± hypotaurine, then washed with PBS and incubated in fresh FBS-free media for irradiation. After the irradiation, cells were washed with PBS and incubated for 24 h in fresh media. The % viability was calculated by comparison to control cells which were also placed under the light source but shielded (dark).

Statistical analysis. Statistical analysis was performed using GraphPad Prism-7.03, and data expressed as mean ± standard deviation (SD) with 3–6 replicates in 3 independent experiments for cell viability (MTT and NRUA) and 2–3 replicates in 2–3 independent experiments for HPLC. Statistical significances were analyzed using the two-tailed non-parametric t-test (Mann–Whitney's correction) for Figures 1,4 and 6 to compare the means between 2 groups or the one-way ANOVA (Dunnett's correction) for Figures 2, 3 and 5 between 3 and more groups.

RESULTS

Hypotaurine supplementation enhances HaCaT cell viability

To assess the effect of hypotaurine supplementation of culture media on intracellular levels, HaCaT cells were initially cultured for 24 h with ± 5 mM hypotaurine as this concentration has been used in taurine accumulation studies (20,23). The intracellular hypotaurine concentration was determined by HPLC analysis (Fig. 1a), which shows that hypotaurine levels were significantly increased in cells from 0.21 ± 0.2 nmol mg^{-1} protein in untreated cells to 9.87 ± 0.8 nmol mg^{-1} protein (*$P < 0.05$). Although the concentration decreased 24 and 48 h after hypotaurine washout, levels remained significantly higher than baseline values. The impact of 5 mM hypotaurine supplementation for 24 h on cell viability was assessed by MTT assay (Fig. 1b).
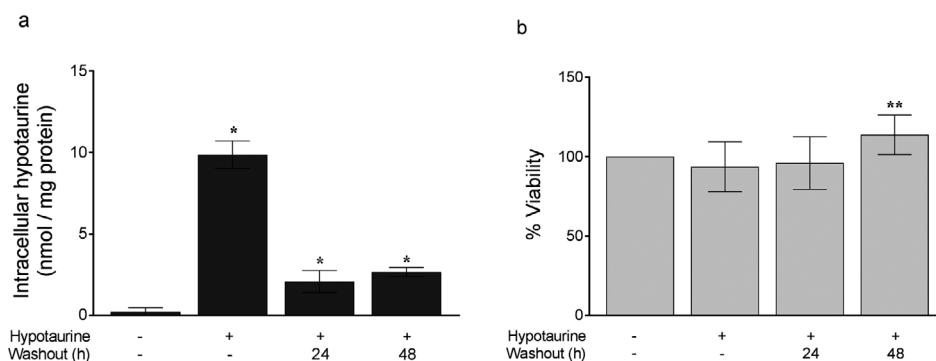
a

b

Figure 1. Hypotaurine supplementation enhances HaCaT cell viability. HaCaT cells were cultured with 0 or 5 mM hypotaurine for 24 h after which, they were washed with PBS to remove excess hypotaurine from media and incubated in fresh media for the indicated washout times (24 and 48 h). (a) Following cell lysis, intracellular hypotaurine levels were detected by HPLC and normalized to total protein. (b) Cell viability was determined by MTT assay and normalized to untreated cells. Graphs show the means and error bars represent the SD of 3 replicates in 3 independent experiments. For comparison of untreated and hypotaurine treated cells: *$P < 0.05$, **$P < 0.01$ (Student t-test).

When compared to untreated cells, hypotaurine supplementation was associated with a small but significant increase in cell viability at the 48 h washout time (**$P < 0.01$).

Uptake of supplemental hypotaurine in HaCaT cells occurs in a time-dependent manner

To investigate whether increasing the time of hypotaurine supplementation resulted in increased cellular uptake, the intracellular concentration in HaCaT cells cultured with ± 5 mM hypotaurine for 2–72 h was determined by HPLC analysis (Fig. 2). Hypotaurine levels were significantly increased in cells from 2 h and reached a maximum at 10 h (14.2 ± 0.1 nmol mg^{-1} protein), with levels remaining significantly increased and stable until 72 h (Fig. 2). As the level of hypotaurine remained significantly elevated from 8h to 72h but without a statistical difference between these timepoints, subsequent experiments were performed following 24 or 48 h incubation to enable the longer-term effects to be assessed.
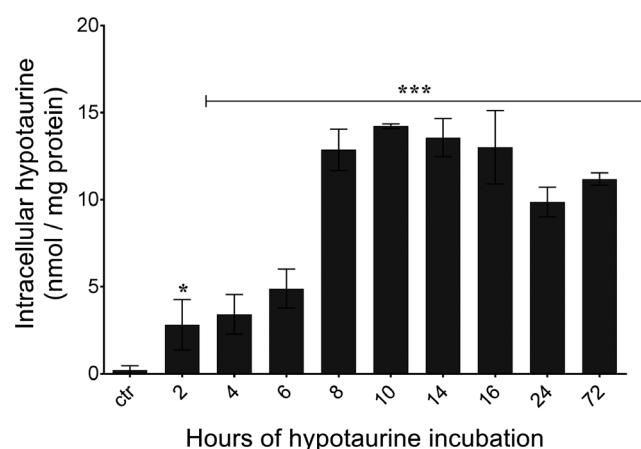

Figure 2. Uptake of supplemental hypotaurine in HaCaT cells occurs in a time-dependent manner. HaCaT cells were treated with 5 mM hypotaurine up to 72 h. Control cells were analyzed after 24 h in culture and were not treated with hypotaurine. Following cell lysis, intracellular hypotaurine was detected by HPLC and levels normalized to total protein. Graph shows the means and error bars represent SD of 2 replicates in 2–3 independent experiments. For comparison using ctr as control: *$P < 0.05$, ***$P < 0.005$ (ANOVA test).

Uptake of supplemental hypotaurine in HaCaT cells occurs in a concentration-dependent manner

To determine whether increasing the concentration of supplemental hypotaurine resulted in increased cellular uptake, HaCaT cells were treated for 24 h with hypotaurine (0.05–40 mM) and the intracellular hypotaurine concentrations were determined by HPLC analysis (Fig. 3a). This range of hypotaurine concentrations was selected for analysis as the lower levels reflect the amount of taurine present in epidermal tissue (23), whilst the higher levels are in line with the hypotaurine concentrations present in cosmetic cream (~18 mM) (10). Whilst increased intracellular hypotaurine was detectable at all levels of supplementation compared to untreated cells (ctr), the uptake was only significantly increased at concentrations above 2.5 mM, after which an exponential increase in hypotaurine accumulation was seen. The highest concentration investigated was 40 mM hypotaurine, with cellular uptake of 32.7 ± 2.0 nmol mg^{-1} protein (Fig. 3a). The impact of hypotaurine uptake on cell viability was assessed by both MTT and NRU assay following the treatment of cells with hypotaurine (0.05–40 mM) for 24 h (Fig. 3b). The NRU assay was performed and used as dark control for following UVA experiments. Hypotaurine supplementation above 5 mM was associated with a reduction in cell viability that was significantly decreased at 20 and 40 mM (83.7 ± 8.1 % and 69.7 ± 6.4 %; Fig. 3b (MTT)). In contrast, when viability was assessed by neutral red uptake (NRU) assay, no significant impact on cell viability was observed (Fig. 3b). Using trypan blue exclusion as an indicator of live cell number after hypotaurine treatment at 0, 1, 5 and 20 mM did not show a reduction in viable cell number ($1.6 \; 10^5 \pm 0.06$ cells mL^{-1} at 20 mMvs $1.56 \; 10^5 \pm 0.08$ cells mL^{-1} at 0 mM, similar results for 1 and 5 mM).

Inhibition of hypotaurine transport or of taurine metabolic synthesis impacts on cellular hypotaurine uptake and viability

Hypotaurine has previously been shown to be a substrate for the taurine transporter TAUT (25,26), so we first investigated whether the presence of the TAUT substrates, taurine or β-alanine (25), affected hypotaurine uptake and cell viability. Incubation of HaCaT cells with or without β-alanine prior to supplementation with hypotaurine or hypotaurine plus taurine
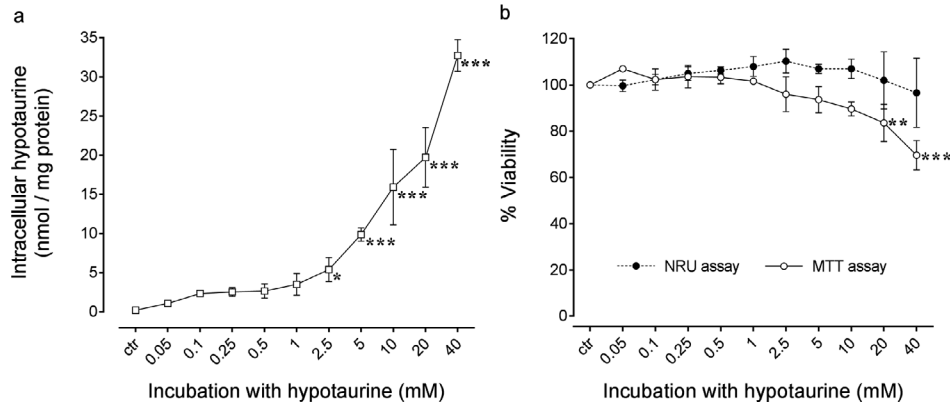
Figure 3. Uptake of supplemental hypotaurine in HaCaT cells occurs in a concentration-dependent manner. HaCaT cells were treated for 24 h with increasing concentrations of hypotaurine from 0.05 to 40 mM. (a) Following cell lysis, intracellular hypotaurine was detected by HPLC and levels normalized to total protein quantified. (b) Cell viability was determined by MTT and NRU assay and normalized to untreated cells (ctr). Graphs show the means and error bars represent SD of 3 replicates in 3 independent experiments. For comparison using the respective assay ctr as control: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.005$ (ANOVA test).

showed no significant impact on cell viability (Fig. 4a left and middle). The presence of taurine resulted in a significant decrease in the uptake of hypotaurine (5.8 ± 0.8 nmol mg^{-1} protein) compared to hypotaurine alone (9.87 ± 0.8 nmol mg^{-1} protein; Fig. 4b, left). HaCaT cells were treated for 24 h in presence of 5 mM β-alanine or 2 mM propargylglycine (PPG), washed in PBS and incubated for a further 24 h with hypotaurine or hypotaurine plus taurine. Incubation with β-alanine prior to supplementation with hypotaurine enhanced hypotaurine uptake into cells (18.6 ± 1.2 nmol mg^{-1} protein; Fig. 4b, middle) to levels above those seen with hypotaurine alone (Fig. 4b, left), but this was prevented in the presence of taurine where levels were comparable to hypotaurine alone.

Propargylglycine (PPG) inhibits the metabolic synthesis of taurine by blocking the decarboxylation of cysteinesulfinate to hypotaurine by acting on the cysteinesulfinate decarboxylase enzyme responsible for this reaction (31). We investigated whether the presence of PPG affected hypotaurine uptake and cell viability, where HaCaT cells pre-treated with PPG are

capable of reducing hypotaurine levels (31). As with β-alanine, incubation with PPG for 24 h prior to supplementation with hypotaurine enhanced hypotaurine uptake into cells (17.9 ± 1.2 nmol mg^{-1} protein; Fig. 4b, right) above hypotaurine alone (Fig. 4b, left). Again, this enhancement was prevented by co-incubation with taurine, where levels of intracellular hypotaurine were comparable to hypotaurine alone, and significantly higher than with hypotaurine plus taurine. In addition, cell viability was reduced for both hypotaurine alone and hypotaurine plus taurine after pre-incubation with PPG (81.0 ± 3.5 % and 77.3 ± 1.5 % respectively; Fig. 4a).

Hypotaurine supplementation protects against UVA-induced cell damage

To investigate whether hypotaurine supplementation could protect against UVA-induced cell damage, keratinocytes were UVA irradiated at three different doses in the absence or in the presence of hypotaurine. Oxidative damage caused by intracellular
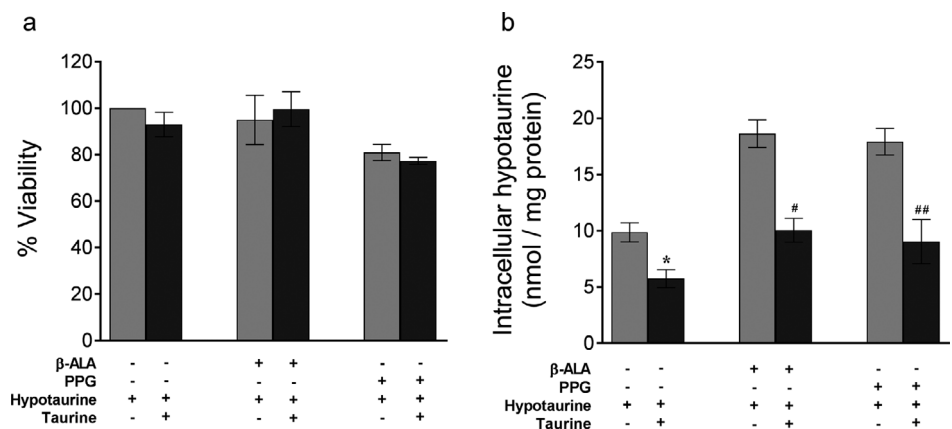

Figure 4. Inhibition of TAUT or of taurine metabolic synthesis impacts on cellular uptake and viability. HaCaT cells were treated for 24 h in presence and absence of 2 mM propargylglycine (PPG) or 5 mM β-alanine (β-ALA), washed in PBS and incubated for a further 24 h with 5 mM hypotaurine (▭) or 5 mM each of hypotaurine plus taurine (▬). (a) Cell viability was determined by MTT assay and normalized to treatment with hypotaurine alone. (b) Intracellular hypotaurine was detected by HPLC and normalized to total protein. Graphs show the means and error bars represent SD of 3 replicates in 3 independent experiments. For comparison using presence of hypotaurine as control: *$P < 0.05$, for comparison using presence of hypotaurine and taurine as control: #$P < 0.05$, ##$P < 0.01$ (Student *t*-test).

ROS on cell viability was analyzed by Neutral Red Uptake (NRU) assay, which shows the highest predictability of human phototoxicity of all the *in vitro* assays evaluated (30,32). HaCaT cells were irradiated with UVA at 5, 10 or 15 Jcm^{-2} alone (Fig. 5), with the two higher doses showing a statistically significant cytotoxic effect on cell viability 91.7 $\pm$ 1.9 % ($^{\#}P < 0.05$) and 84.1 $\pm$ 2.4 % ($^{\#\#\#}P < 0.005$), respectively. Dark control (no UVA) with hypotaurine (0.05–40 mM) for 24 h was performed (Fig. 3b ($\bullet$)) showing no significant effect on cell viability. The pre-incubation of cells with hypotaurine before UVA irradiation significantly reduced the cytotoxic effects of UVA at hypotaurine concentrations between 0.5 and 20 mM at all the three UVA doses investigated and at all hypotaurine concentrations used, when irradiated with 15 Jcm^{-2} UVA. The maximum viability reached ($^{***}P < 0.005$) following irradiation with 5 Jcm^{-2} UVA was at 2.5 mM of hypotaurine (112.8 $\pm$ 2.6 %), at 10 Jcm^{-2} was at 5 mM of hypotaurine (104.7 $\pm$ 2.2 %), and at 15 Jcm^{-2} was at 5 mM of hypotaurine (97.6 $\pm$ 2.8 %; Fig. 5).

Intracellular hypotaurine levels decrease after UVA irradiation

To assess hypotaurine levels in cells after UVA irradiation, HaCaT cells, which had been pre-treated for 24 h with 5 mM hypotaurine, were irradiated with 0, 5, 10 or 15 Jcm^{-2} UVA and intracellular hypotaurine and taurine concentrations were determined by HPLC analysis 24 h after irradiation. The level of hypotaurine was reduced at all three UVA doses compared to the non-irradiated control (9.87 $\pm$ 0.8 nmol mg^{-1} protein; Fig. 6). The level of taurine was reduced at highest doses of UVA (10 and 15 Jcm^{-2}) compared to the non-irradiated control (102 $\pm$ 10 nmol mg^{-1} protein; Fig. 6 insert).

DISCUSSION

Hypotaurine has already been included as an ingredient in cosmetic preparations despite a lack of published evidence to support its mechanism of action and role as an anti-photoageing agent (8,9). The current study sought to evaluate the effect of supplemental hypotaurine uptake in HaCaT cells on cytotoxicity in the presence and absence of UVA irradiation, in order to assess whether there was evidence to support the rationale for the addition of hypotaurine to topical sunscreen and cosmetic preparations. The role of taurine, the oxidative form of hypotaurine, has been shown to have a positive effect on cell survival attributed to its osmolyte activity (19,20) and to have a photoprotective effect against UV-induced damage *via* regulating the release of platelet-activating factor from the membrane of UVB-irradiated cells (21). To our knowledge, this is the first study that has investigated whether hypotaurine, which has been well studied for its antioxidant effects *in vitro*, including kinetic experiments using enzyme and radiolysis analysis and *ex vivo* experiments in rat placental trophoblasts and human skin fibroblasts (14–17), could have photoprotective effects. Approaches to the prevention of photoageing and skin cancer have included a prominent focus on limiting ROS production generated by UVA and counteracting oxidative damage, through the use of antioxidant agents, such as vitamins E and C, β-carotene and retinoids, which are capable of interacting with ROS and reducing their formation and impact (8,33).

We have shown that supplemental hypotaurine is taken up by keratinocytes with an increase of ~ 30–fold above basal levels following 24 h incubation and that this remained elevated for at least 48 h after removal of supplemental hypotaurine from the culture media (Fig. 1a). Hypotaurine supplementation was associated with a small but significant increase in cell viability at the 48 h washout timepoint. A possible reason for this could be due to effects on antioxidant expression regulated by Nrf2 downstream of hypotaurine. It has been reported that in HT22 cells, taurine promotes Nrf2 nuclear translocation and induces heme
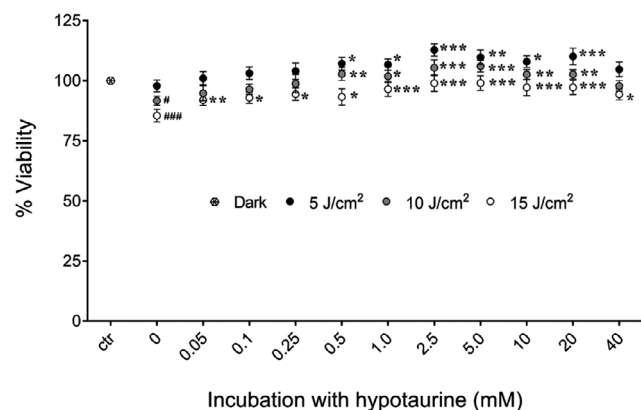

Figure 5. Hypotaurine supplementation protects HaCaT cells during UVA irradiation. HaCaT cells were treated for 24 h $\pm$ hypotaurine from 0.05 to 40 mM and irradiated $\pm$ UVA at 5, 10 and 15 Jcm^{-2}. Cell viability was determined by NRU assay 24 h after UVA irradiation and normalized to ctr. Graph shows the means, and error bars represent SD of 6 replicates in 3 independent experiments. Dark control (no UVA) with hypotaurine from 0.05 to 40 mM in Fig. 3b ($\bullet$). For comparison using for each UVA dose 0 mM as control *versus* hypotaurine from 0.05 to 40 mM: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$, for comparison using ctr as control *versus* 0 mM only: $^{\#}P < 0.05$, $^{\#\#\#}P < 0.005$ (ANOVA test).

Figure 6. Intracellular hypotaurine levels decrease after UVA irradiation. HaCaT cells were treated for 24 h with hypotaurine 5 mM and irradiated $\pm$ UVA at 5, 10 or 15 Jcm^{-2}. 24 h after irradiation, intracellular hypotaurine and taurine (insert) was detected by HPLC and levels normalized to total protein. Graph shows the means and error bars represent SD of 2 replicates in 2 independent experiments. For comparison using dark as control: $P > 0.05$ (Student *t*-test).

oxygenase-1 expression and can improve resistance to oxidative damage induced by glutamate (34). Although this pathway has been shown to be effective following taurine supplementation in HT22 cells, it remains to be investigated and verified for hypotaurine. Hypotaurine uptake into cells was time-dependent and reached a maximum level after 10 h of incubation and remained significantly above control levels for the duration of the 72 h incubation period (Fig. 2). We have also demonstrated for the first time that hypotaurine uptake in keratinocytes is con-centration-dependent, with intracellular levels increasing expo-nentially at supplementation concentrations in excess of 2.5 mM (Fig. 3a).

Hypotaurine supplementation showed a small but significant positive impact on cell viability 48 h after its removal from media (Fig. 1b), although this was not statistically significant at 24 h. In contrast, higher concentrations of hypotaurine supple-mentation negatively impacted on cell viability, especially above 20 mM (Fig. 3b), when determined using the MTT assay. How-ever, when using the NRU assay as an endpoint, there was no significant hypotaurine dose-dependent impact on cell viability seen when compared with untreated cells. In addition, using try-pan blue exclusion as an indicator of live cell number after hypotaurine treatment did not show a change in viable cell num-ber indicating that cells were alive and possessed intact cell membranes. The discrepancy in the results obtained using MTT and NRU assays could be explained through the mechanism of action for each assay: the MTT assay assesses both cell survival and cell growth by measuring the activity of mitochondrial dehy-drogenases (29), whilst the NRU assay is based on the incorpo-ration of neutral red into the lysosomes of viable cells (32). These results suggest that high concentrations of hypotaurine could affect mitochondrial dehydrogenase activities without causing cell death and therefore show a negative effect on the cell viabil-ity that is not apparent when a different assay is used.

We also investigated the possible mechanisms involved in the uptake of hypotaurine in HaCaT cells. The presence of TAUT in different tissues, especially in human skin (25,26), suggests a possible mechanism for tissue-specificity of hypotaurine trans-portation into cells. Our results indicate that intracellular hypotaurine concentration was detected after 24 h of incubation (9.87 ± 0.8 nmol mg^{-1} protein), albeit at much lower levels than in the culture media (5 mM; Figs. 1a and 3a), although for-mal comparison was not feasible due to the differences in units of measurement. Interestingly, when taurine was present with hypotaurine in the incubation medium, the hypotaurine uptake detected in cells was significantly lower than with hypotaurine alone (Fig. 4b), suggesting competition for the transporter between taurine and hypotaurine and a consequent decrease in hypotaurine uptake. This would be in line with the results reported by Grafe and colleagues, where [3H]taurine uptake in keratinocytes was inhibited equally by unlabeled hypotaurine, taurine or β-alanine (25). The residual amount of hypotaurine uptake in the presence of taurine was still significant compared to basal hypotaurine levels in cells, suggesting that taurine pres-ence does not fully inhibit the hypotaurine uptake. This would be worth studying further through the use of *in vivo* models, such as the taurine transporter-knockout mouse, which exhibits extensive taurine depletion in tissue (35). The 24 h pre-incuba-tion of cells with PPG, a cysteinesulfinate decarboxylase inhibi-tor that inhibits the decarboxylation of cysteinesulfinate to hypotaurine (31), followed by washout before hypotaurine

addition, enhanced the uptake of hypotaurine into HaCaT cells compared to incubation with hypotaurine alone (~2-fold; Fig. 4b). Similarly, the 24 h pre-incubation of cells with β-ala-nine, an amino acid that has a similar affinity to TAUT as hypotaurine and taurine (25), followed by washout before hypotaurine addition, showed a ~ 2-fold increment of the uptake of hypotaurine *vs* hypotaurine alone (Fig. 4b). These findings suggest that 24 h pre-incubation with inhibitor of the metabolic synthesis as well as with competitor of TAUT increases the demand for external hypotaurine uptake.

Exposure of cells to UVA generates ROS and consequently leads to oxidative damage that can result in cell death (28). Our data demonstrate that the dose-dependent decrease in cell viability caused by UVA irradiation of HaCaT cells can be prevented by pre-exposure supplementation with hypotaurine. This protective effect of hypotaurine against UVA-induced cytotoxicity was apparent at all three UVA doses tested, and a hypotaurine dose-dependent effect was also observed. Interest-ingly, the hypotaurine protective effect was most significant at 2.5 mM both ± UVA irradiation (Fig. 5), with statistically sig-nificant protection against UVA-induced cytotoxicity when cells were pre-incubated with hypotaurine between 0.5 and 20 mM at all the UVA doses examined. These findings suggest that despite the reduction in viability seen at the higher concentra-tions of hypotaurine supplementation in the absence of UVA exposure (Fig. 3b), there is potential for a beneficial effect of supplementing with hypotaurine to protect against UVA-in-duced cell damage. Our data also demonstrate a reduction in intracellular hypotaurine levels after UVA irradiation, suggest-ing that it is depleted in response to UVA exposure (Fig. 6). As hypotaurine itself does not absorb radiation in the UVA region (36), this cannot be a consequence of a direct photo-chemical interaction between hypotaurine and UVA. On the contrary, hypotaurine is well known to interact with ROS and act as an antioxidant (15,27). The taurine level detected did not significantly change in the context of hypotaurine depletion, probably due to lack of sensitivity of detection at these levels, as there were 10-fold higher levels of intracellular taurine com-pared to hypotaurine present in the cells after treatment with 5 mM hypotaurine and moreover, taurine levels are reduced at higher UVA doses. This decrease seems to be related to the well-known taurine interaction with UV as osmolyte (18-22). Our data therefore support an interaction between hypotaurine and ROS generated by UVA irradiation of cells, resulting in its oxidation to taurine as the underlying mechanism of protec-tion and further investigations such as intracellular ROS detec-tion using a fluorescent probe would help clarify the role of hypotaurine as a protective agent.

CONCLUSION

In conclusion, the data we present here demonstrate that sup-plemental hypotaurine is taken up by cultured human ker-atinocytes and has a positive effect on cell viability and a protective effect against UVA-induced cytotoxicity, indicating a possible interaction between hypotaurine and ROS produced following UVA exposure. These results support the potential for hypotaurine to be an effective photoprotective ingredient in sunscreen and cosmetic formulations in order to reduce UVA-induced cell damage and prevent photoageing and this warrants further investigation.

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