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The Apoptotic Effects of Methylparaben and Ultraviolet B Light on M624 Human Melanoma Cells

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Abstract

Methylparaben is a commonly used antimicrobial in cosmetics that has been shown to have negative effects on mammalian cells. Human melanoma M624 cells were treated with 1 and 5 mM methylparaben in the presence and absence of 25 mJ/cm² ultraviolet B (UV-B) light. Cell proliferation assays showed that 5 mM methylparaben was toxic to M624 cells after 24 hours. Apoptotic signaling pathways were analyzed via isolation of separate cellular compartments and protein analysis via western blot. Upon 5 mM methylparaben treatment, PARP I was cleaved indicating apoptosis, which was mediated by the TNF- α receptor activated in the lipid rafts of the M624 cells. Upon 25 mJ/cm² UV-B radiation, PARP II was activated indicating cellular damage, cytochrome c was released from the mitochondria, and caspase-3 was expressed. Upon combinatory treatment with 5 mM methylparaben and 25 mJ/cm² UV-B, apoptosis was induced through mitochondrial release of cytochrome c, expression of caspase-3 and cleavage of PARP I, while methylparaben-induced TNF- α receptor activation and UV-B-induced PARP II activation was inhibited., demonstrating that antimicrobial methylparaben in cosmetics can cause damage to cells.

Keywords: methylparaben, ultraviolet light, apoptosis

1. Introduction

Paraben compounds are antimicrobials used in cosmetics as preservatives due to their broad antimicrobial functions and their ability to meet the criteria for an ideal preservative [1].

However, the side effects of paraben use include increased estrogenic activity of receptors, sensitization of broken skin, endocrine disruptive effects and adverse reproductive effects [2]. The paraben group of compounds includes methyl, ethyl, butyl, heptyl, and benzyl parabens. Methylparaben, a methylester of *p*-hydroxybenzoic acid, is the most widely used paraben in topical skin products [3]. Studies have recently shown that small amounts of methylparaben remain unhydrolyzed in the epidermis [4]. Handa et al. has been the first to describe the effects of methylparaben and ultraviolet B (UV-B) radiation as detrimental to human skin HaCaT keratinocytes [3].

Handa et al. studied HaCaT keratinocytes treated with UV-B and methylparaben. HaCaT keratinocytes were cultured in methylparaben-containing medium concentrations of 19.7, 1.97, and 0.197 mM (0.3, 0.03, and 0.003%) for 24 hours, exposed to UV-B (15 or 30 mJ/cm²) and further cultured for another 24 hours. The use of parabens in cosmetic products is permitted up to 0.8% (w/w), however the concentration in cosmetics is typically less than 0.32% [4]. Cellular viability, cell death, oxidative stress, nitric oxide production, cellular lipid peroxidation, and activation of nuclear factor kappa B and activator protein-1 were studied, demonstrating that methylparaben treatment increases cell death when the cells are exposed to UV-B and that the detrimental potential of methylparaben is dose and time dependent [3].

Ultraviolet B radiation (290–320 nm) is the main cause of tumor initiation and promotion [5]. UV-B is 1000 times more likely than UVA to cause sunburn and is also known to cause melanoma: while some of the harmful UV-B rays are absorbed by the ozone layer, some still penetrate the atmosphere, causing sunburns that lead to skin cancer.

Melanoma is the deadliest of the skin cancers and accounts for approximately three fourths of all skin cancer deaths [6]. Forms of melanoma currently being diagnosed and treated include superficial spreading melanoma (most common), lentigo maligna, lentiginous melanoma and nodular melanoma. The mechanism for UV-B-induced cancer can be attributed to the dysregulation of apoptosis, or programmed cell death. Apoptosis is a fundamental mechanism characterized by cell shrinkage, membrane blebbing, nuclear breakdown, and DNA fragmentation that is needed for embryonic development, tissue homeostasis, immune defense, and elimination of harmful cells [7]. Ultimately, the cell and its contents are broken down to membrane bound fragments that are phagocytosed by adjacent cells [8]. The apoptotic pathway is regulated by death receptor-mediated extrinsic pathways and mitochondria-mediated intrinsic pathways [9]. The dysregulation of apoptosis can result in pathophysiological states and diseases, such as cancer [7]. Dysregulation in the UV-B-induced apoptosis may also have a major impact on photocarcinogenesis [10].

The PARP protein is an indicator of apoptosis. PARP I cleavage is an indication of induced apoptosis, while PARP II expression is an indicator of advanced cellular damage. PARP I and PARP II are activated by DNA interruptions and are involved in cell survival/death, transcription, DNA repair, and cell division [11, 12]. They act as both damage sensors and signal transducers to down-stream effectors [11, 13]. PARP I and PARP II also function to signal the cell to undergo apoptosis when the amount of DNA damage is beyond repair capacity [13]. PARP I will cleave into 89- and 24-kDa fragments that contain the active site and the DNA-binding domain of the enzyme during apoptosis. The caspase cascade is an important

apoptotic signaling pathway, and caspase-3 is known to be responsible for the cleavage of PARP I during cell death. The sequence at which caspase-3 cleaves PARP I is well conserved in distant species, indicating an important role for PARP I cleavage in apoptosis [14].

One major regulator of apoptosis is the mitochondria through release of apoptotic factors, such as cytochrome c, through permeabilization of the outer mitochondrial membrane. The rapid increase in permeability causes depolarization, uncoupling of oxidative phosphorylation, and mitochondrial swelling [8]. The mitochondrial pathway of apoptosis through the mitochondrial permeability transition is important for the elimination of UV-B-damaged human keratinocytes [15]. Cytochrome c is a soluble protein that participates in the electron transfer between complex III and complex IV of the respiratory chain in a normal functioning cell [16]. However, an apoptotic signal can lead to the release of cytochrome c from the intermembrane space of the mitochondria into the cytosol. Cells undergoing apoptosis may have an elevation of cytochrome c in the cytosol and a corresponding decrease in the mitochondria [17]. Once released, cytochrome c can bind to the adaptor molecule apoptotic protease activating factor-1 (Apaf-1) and can subsequently activate caspase-9 or caspase-8. Finally, caspase-3 can be activated which is the irreversible point in the mitochondrial apoptosis pathway [9].

Another important regulator of apoptotic induction in the cell is contents of the lipid raft domains. Lipid rafts contain plasma membrane proteins compartmentalized into sphingolipid- and cholesterol-rich microdomains and function as platforms for signal transduction [18]. These lipid raft domains are dynamic, float freely within the bilayer and coalesce upon clustering of their components [19]. Sphingolipids and cholesterol are necessary for the assembly of the lipid raft compartments [20]. Lipid rafts and associated proteins are known to be important in the pathogenesis of several diseases, including cancer progression. In the lipid rafts, caveolin-1 functions through direct protein–protein interactions to regulate diverse cellular processes, including raft-mediated endocytosis, vesicular transport, cell migration, and signal transduction [21]. An upregulation of caveolin-1 expression in clinical studies was associated with the occurrence of metastasis [22]. Caveolin-1 is a lipid raft indicator protein because it is a cholesterol binding protein. This is important because the integrity of the lipid raft is dependent on the ability of cholesterol to pack tightly with the saturated sphingolipids [23]. TNF- α binds to the TNF receptor (TNFR) to initiate apoptosis [24]. TNF- α is a pleiotropic cytokine that can signal for proliferation, stress, inflammation, and cell death [8]. The TNF pathway occurs in a number of different cell types [25]. Cytochrome c is located downstream in the tumor necrosis factor α (TNF- α) apoptotic pathway.

While Handa et al. demonstrated the apoptotic effects of methylparaben in normal skin cells, this study examines the apoptotic signaling pathways activated by methylparaben in cancerous melanoma cells, as well as the role (or absence of role) of the mitochondria and lipid raft domains in these signaling pathways. In this study, we have analyzed the expression and activation of the nuclear apoptotic indicator PARP I, PARP II, and cleaved PARP proteins, as well as the expression of TNF- α receptor in the lipid rafts in UV-B and methylparaben-treated human M624 melanoma cells. In addition, the expression of caspase-3 and the expression and location of cytochrome c were also analyzed under these cellular conditions in order to fully characterize the cell signaling pathway activated by the cosmetic antimicrobial agent methylparaben.

2. Materials and methods

2.1. Cell culture and treatment

Transformed M624 human melanoma cells were obtained from Dr. Shiyong Wu's laboratory (Edison Biotechnology Institute, Ohio University, Ohio, USA) and originally generated by the National Institute of Health (NIH) (USA). Cells were cultured in DMEM supplemented with fetal bovine serum (FBS) (10%) and penicillin/streptomycin (1%). At 80% cell confluency, cells were treated with methylparaben and UV-B. Cells were incubated with 1 or 5 mM methylparaben in media including supplements for 2 hours. The methylparaben media was removed while treating the cells with 25 mJ/cm² UV-B radiation and replaced after radiation, and then incubated for another 4 hours before cell lysis.

2.2. Cell proliferation assays

Cells were plated at approximately 25% confluency and then treated with methylparaben and UV-B radiation as described above, except paraben solutions were allowed to incubate for 24 hours and then viable cells were stained using 0.5% crystal violet solution. Results are averaged from three independent trials.

2.3. Protein samples

Whole Cell Lysate Preparation Cells were washed three times with cold, 1X Phosphate Buffered Saline (PBS) (10 mM phosphate). Cells were then placed in a -20°C freezer for at least 2 hours, removed, and washed once more with PBS. TNET lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.1% Triton X-100) was mixed with protease inhibitors and added to the cells. The solution containing cells was homogenized with 23 gauge needles and syringes and centrifuged at low speed after which supernatant was collected.

2.4. Lipid raft isolation

Lipid rafts were prepared as previously described [26]. Briefly, 3,3'-dithiobis (sulfosuccinimidyl propionate) (DTSSP) (1.25 mM) was added to each plate of cells after UV-B and methylparaben treatment and incubated for 1 hour at 4°C. The cells were then collected and frozen overnight. Cells were washed and lysed as described above to prepare samples and placed in an iodixanol solution gradient and ultra-centrifuged for 5 hours. Fractions were collected and the lipid rafts were present in fraction two, indicated by the presence of caveolin-1 protein.

2.5. Mitochondrial isolation

Cells were washed with PBS and suspended in an isotonic buffer (10 mM Hepes, pH 7.4, 0.2 M mannitol, 0.07 M sucrose) supplemented with protease inhibitors. Samples were homogenized and then centrifuged at 900× g for 5 minutes. Then cells were centrifuged at 10,000× g for 30 minutes at 4°C to obtain the heavy membrane pellet. Samples were re-suspended in an SEM buffer (250 mM Sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2).

2.6. Sample preparation

Samples were prepared by adding protein loading buffer (0.25 M Tris, pH 6.8, 10% sodium dodecyl sulfate, 0.05% bromophenol blue, 50% glycerol, 10 mM β -mercaptoethanol). The samples were vortexed, boiled for 5 minutes, vortexed and then stored at -20°C .

2.7. SDS-page

SDS-PAGE was performed using a 15% separating gel and 4% stacking gel to create a gel with 1.0 mm thickness. Protein samples were separated at 200 volts for 45 minutes and then transferred to a nitrocellulose membrane via wet transfer at 100 volts for 35 minutes. After the transfer, the nitrocellulose membrane was blocked using dried non-fat milk in $1\times$ tris buffered saline with 0.05% Tween (TBS-T) for 20 minutes.

2.8. Western blot

Western blot was performed using β -actin primary antibody (122 M4782, Sigma, St. Louis, MO) for 3 hours followed by three rinse wash cycles and then probed with anti-mouse secondary antibody (Santa Cruz Biotechnology, sc-2371) for 1 hour. PARP protein expression and cleavage was detected by probing overnight with PARP primary antibody (MA5-15031, Thermo Fisher Scientific, Waltham, MA) and anti-rabbit secondary antibody (32,260, Thermo Fisher Scientific, Waltham, MA). Caveolin-1 protein expression was detected by probing overnight with caveolin-1 primary antibody (sc-894, Santa Cruz Biotechnology, Dallas, TX) and anti-rabbit secondary antibody. TNF R1 protein expression was detected by probing overnight with TNF R1 primary antibody (sc-7418, Santa Cruz Biotechnology, Dallas, TX) and anti-mouse secondary antibody. Cytochrome c protein expression was detected by probing with cytochrome c HRP-conjugated primary antibody (sc-13,156, Santa Cruz Biotechnology, Dallas, TX) overnight. Caspase-3 protein expression was detected by probing overnight with Caspase-3 primary antibody (sc-7418, Santa Cruz Biotechnology, Dallas, TX) and anti-rabbit secondary antibody. All overnight incubations were performed shaking at 5°C ; all other incubations were performed shaking at room temperature. Protein expression was visualized via chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate).

3. Results

The toxicity of methylparaben and UV-B light to human melanoma M624 cells was initially analyzed using a clonogenic cell proliferation assay (**Figure 1**). The effects of methylparaben and UV-B light on apoptotic pathways in M624 cells were then investigated by analyzing expression of PARP and caspase-3 and cleavage of PARP in the whole cell lysate samples, TNF- α expression in the lipid rafts, and cytochrome c expression in both mitochondrial and regular lysate samples, via SDS-PAGE and western blot (**Figures 2–4**).

Human M624 melanoma cells were treated with methylparaben (1 and 5 mM) and $25\text{ mJ}/\text{cm}^2$ UV-B. The 5 mM concentration of methylparaben and the $25\text{ mJ}/\text{cm}^2$ UV-B were each shown

Figure 1. Phototoxicity studies: Crystal violet proliferative assay: cells were treated with methylparaben and UV-B radiation and then stained with a 0.5% crystal violet solution after 24 hours.

Figure 2. Western blot of whole cell lysate protein samples of PARP I, and cleaved PARP I, PARP II, cytochrome c and caspase 3. Samples were standardized using β -actin western blot.

to be toxic to the M624 human melanoma cells after 24 hours (**Figure 1**). The combination of methylparaben treatment and UV-B radiation showed amplified toxicity, even at the 1 mM paraben concentration level after 24 hours; 1 mM methylparaben alone was nontoxic to the cells (**Figure 1**).

Results showed that 5 mM methylparaben induced PARP I cleavage (lane 3), while 25 mJ/cm² UV-B induced PARP II expression instead (lane 4). When cells were treated with UV-B and

Figure 3. Western blot of mitochondrial protein samples of cytochrome c. Samples were standardized using β -actin western blot.

Figure 4. Western blot of lipid raft protein samples of TNF- α . Samples were standardized using caveolin-1 western blot.

5 mM methylparaben, UV-B-induced PARP II expression was completely inhibited (lane 6) while UV-B and 1 mM methylparaben partially inhibited the PARP II expression. Overall, methylparaben at 5 mM had an impact on PARP cleavage with and without UV-B and was able to inhibit UV-B-induced PARP II expression (**Figure 2**).

Cytochrome c protein expression in the whole cell and mitochondrial lysate samples was analyzed using β -actin standardized loading amounts. Cytochrome c expression was reduced in the whole cell lysate samples treated with 5 mM methylparaben and 5 mM methylparaben plus UV-B (**Figure 2**, lanes 3 and 6 versus lane 1) while cytochrome c expression in the mitochondrial lysate 5 mM methylparaben sample was relatively unchanged compared to the expression seen in the control mitochondrial lysate sample (**Figure 3**, lane 3 versus lane 1). UV-B radiation caused a decrease in cytochrome c in the mitochondrial samples treated with or without methylparaben (**Figure 3**, lanes 4, 5 and 6 versus lane 1).

Caspase-3 expression was analyzed in whole cell lysate samples using β -actin standardized loading amounts. It was shown that caspase-3 expression was decreased when the M624 human melanoma cells were treated with 5 mM methylparaben, 1 mM methylparaben plus UV-B radiation, and 5 mM methylparaben plus UV-B radiation when compared to the control

sample (**Figure 2**, lanes 3, 5 and 6 versus lane 1). On the other hand, caspase-3 expression was greater in the sample treated with UV-B radiation when combined with 5 mM methylparaben treatment compared to 5 mM methylparaben treatment alone (**Figure 2**, lane 6 versus lane 3).

After standardizing the lipid raft samples via caveolin-1, TNF- α receptor expression in the lipid raft fraction was analyzed. Western blot analysis revealed that the expression of TNF- α receptor was increased in the cells treated with 5 mM of methylparaben compared with control cells (**Figure 4**, lane 3 versus lane 1). This methylparaben-induced increase in expression was inhibited in cells treated with UV-B light (**Figure 4**, lane 6 versus lane 3).

4. Discussion

Melanoma is the deadliest of the skin cancers and accounts for approximately three fourths of all skin cancer deaths [6]. Previous research performed by Handa et al. has shown the detrimental effects of methylparaben and UV-B treatment on HaCaT cells [3]. This study is the first to demonstrate the effects of methylparaben and UV-B in M624 human melanoma cells.

Phototoxicity studies performed on M624 human melanoma cells have shown that 5 mM methylparaben is toxic to M624 cells and UV-B exposure increases this toxicity (**Figure 1**). The PARP protein is an indicator of apoptosis: DNA interruptions activate PARP I and PARP II; PARP I cleavage is an indicator that apoptosis has been induced and PARP II expression is an indicator of cellular damage. PARP I and PARP II function to signal the cell to undergo apoptosis when the amount of DNA damage is beyond the repair capacity [13]. Results indicate that apoptosis occurred after 5 mM methylparaben and 5 mM methylparaben plus UV-B treatment due to the increased cleavage of PARP I. PARP II expression was induced after 25 mJ/cm² UV-B treatment indicated cellular damage. UV-B-induced PARP II expression was partially inhibited in the cells treated with 1 mM methylparaben and completely inhibited in the cells treated with 5 mM methylparaben, indicating that PARP II is not involved in the signaling pathway activated by 5 mM methylparaben in combination with UV-B radiation and that methylparaben-induced apoptosis is concentration-dependent (**Figure 2**).

Since cleavage of PARP I indicates that apoptosis was occurring in cells treated with methylparaben, and activation of PARP II indicates cellular damage was occurring in cells treated with methylparaben and UV-B radiation, the involvement of the mitochondria and the lipid rafts in the signaling pathways was then analyzed.

The apoptotic signal can be propagated through an intrinsic mitochondrial response that causes permeabilization of the outer mitochondrial membrane in order to release cytochrome c into the cytosol [7]. Analysis of cytochrome c in both whole cell and mitochondrial isolated samples enabled determination of the movement of cytochrome c under different treatment conditions. Most notably, there were changes in cytochrome c expression in the cells treated with 5 mM methylparaben compared to the cells treated with UV-B light radiation with or without methylparaben. In the 5 mM methylparaben treated sample, cytochrome c expression remained unchanged in the mitochondrial lysate while there was decreased expression of

cytochrome c in the whole cell lysate sample upon 5 mM methylparaben treatment indicating no release of cytochrome c from the mitochondria in these cells. On the other hand, UV-B light treatment showed a decreased cytochrome c expression in the mitochondrial isolated sample while the whole cell lysate sample showed an increased or unchanged cytochrome c expression in cells treated with or without methylparaben indicating a UV-B-induced apoptotic release of cytochrome c from the mitochondria.

The same pattern of expression was seen with caspase-3, indicating caspase-3 activation upon release of cytochrome c from the mitochondria in cells treated with UV-B and UV-B with 5 mM methylparaben. However, 5 mM methylparaben treatment alone inhibited caspase-3 expression. These results suggest that both UV-B light and UV-B light radiation in addition to the 5 mM methylparaben treatment results in M624 human melanoma cells taking a different apoptotic pathway in comparison to cell treatment with only 5 mM methylparaben. When human melanoma M624 cells are exposed to UV-B light or both 5 mM methylparaben and UV-B light, apoptosis occurs through the intrinsic pathway as indicated with the release of cytochrome c from the mitochondria and expression of caspase-3. In comparison, treatment with only 5 mM methylparaben does not result in the release of cytochrome c from the mitochondria into the cytosol or caspase-3 expression (**Figures 2 and 3**).

The TNF- α receptor expression in the lipid rafts of M624 melanoma cells induced by methylparaben and UV-B radiation was analyzed by western blot analysis of lipid raft samples. Results show that apoptosis was initiated through the TNF- α receptor when treated with 5 mM methylparaben, but the involvement of the TNF- α receptor was inhibited in the presence of UV-B light. The integrity of the lipid raft is dependent on the ability of cholesterol to pack tightly with the saturated sphingolipids [23]. Caveolin-1 in the lipid rafts binds to cholesterol, which is necessary for the assembly of the lipid raft components [20]. Caveolin-1 expression was used to standardize the lipid raft samples in order to examine the expression of the TNF- α receptor in the lipid rafts of cells. TNF- α commonly initiates apoptosis by binding to the TNF- α receptor [24]. The activation of TNF- α receptor can lead to formation of TNFR1-associated death domain protein (TRADD). TRADD recruits Fas-associated protein with death domain (FADD) and leads to caspase activation and apoptosis [27]. Death domain formation occurs only when TNF- α receptor is translocated into lipid raft domains [19]; therefore, the expression of the receptor in the lipid rafts was analyzed. Results showed expression of TNF- α receptor in the lipid rafts of cells treated with 5 mM methylparaben, which was inhibited in the presence of UV-B radiation (**Figure 4**). These results suggest an alternate TNF- α apoptotic pathway that does not involve the release of cytochrome c from the mitochondria into the cytosol or expression of caspase-3.

TNF- α apoptotic pathways exist that do not cause the release of cytochrome c from the mitochondria that should be investigated in future studies. One of these TNF- α apoptotic pathway involves inhibition of NF- κ B to allow JNK to induce caspase 8-independent cleavage of Bid to produce jBid. jBid then translocates into the mitochondria to induce the release of Smac, but not cytochrome c. [27, 28]. This research and future steps in this project are important to the understanding of the apoptotic pathway in human M624 melanoma cells when treated with methylparaben and UV-B radiation.

5. Conclusion

This study has demonstrated cell damage, cellular apoptosis and cellular damage upon exposure to the cosmetic antimicrobial methylparaben. In summary, results show that upon 5 mM methylparaben treatment, PARP I was cleaved indicating apoptosis, which was mediated by the TNF- α receptor activated in the lipid rafts of the M624 cells (**Figure 5a**). Upon 25 mJ/cm²

Figure 5. Apoptotic pathways: (a) apoptotic proteins activated by 5 mM methylparaben; (b) apoptotic proteins activated by 25 mJ/cm² UV-B; (c) apoptotic proteins activated by combination of 5 mM methylparaben and 25 mJ/cm² UV-B.

UV-B radiation, PARP II was activated indicating cellular damage, cytochrome c was released from the mitochondria, and caspase-3 was expressed in the cell (**Figure 5b**). Upon combinatory treatment with 5 mM methylparaben and 25 mJ/cm² UV-B, apoptosis was induced through mitochondrial release of cytochrome c, expression of caspase-3 (and the caspase cascade) and cleavage of PARP I (**Figure 5c**). Differences in signaling pathways treated with 1 versus 5 mM methylparaben suggest concentration-dependent activation of apoptosis in M624 cells. Future studies will further elucidate the details of the signaling pathways through analysis of additional apoptotic proteins and lipids of the cells.

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Conflict of interest

No conflicts of interest.

Author details

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