EFFECT OF PARACETAMOL ON MELANIZATION PROCESS IN HUMAN EPIDERMAL MELANOCYTES

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Abstract: Paracetamol (acetaminophen) is commonly used a drug of choice for treatment of pain and fever. Unlike non-steroidal anti-inflammatory drugs (NSAIDs) it does not cause gastrointestinal damage or untoward cardiac effects, however cutaneous adverse effects have been reported. It is known that paracetamol binds to melanin biopolymers, but the relation between the affinity of this drug to melanin and its toxicity is not documented. The aim of this work was to examine the impact of paracetamol on melanogenesis in cultured human normal epidermal melanocytes (HEMn-DP). The effect of paracetamol on cell viability was determined by WST-1 assay, melanin content and tyrosinase activity were measured spectrophotometrically. It has been demonstrated that paracetamol induced concentration-dependent loss in melanocytes viability. The value of EC50 was found to be ~ 20.0 mM. The analyzed drug inhibited melanin biosynthesis in a concentration-dependent manner by decreasing the melanin content as well as the tyrosinase activity. The demonstrated inhibitory effect of paracetamol on melanization process in normal epidermal melanocytes in vitro may explain the potential role of melanin biopolymer in the mechanisms of undesirable side effects of this drug in vivo, as a result of its accumulation in pigmented tissues.

Keywords: paracetamol, melanocytes, viability, melanization, tyrosinase

Paracetamol (acetaminophen) is an analgesic and antipyretic agent (1), commonly present in both prescription and over-the-counter (OTC) products. It is the most popular drug which is used for the treatment of pain and fever in children. The mechanisms of action of paracetamol is multidirectional but pharmacodynamics is still not well known. It is available in single-ingredient drug products and in fixed-dose combination drug products (2). This aspect may easily lead to overdosage of this drug. Oxidative metabolism of paracetamol results in the formation of toxic intermediate N-acetyl-p-benzoquinone imine (NAPQI), which is usually conjugated to glutathione and excreted in the bile. Paracetamol overdosage or lack of glutathione result in accumulation of NAPQI and apoptosis or necrosis of hepatocytes (3). The main possible side effects are liver and kidney failures (4). However, cutaneous adverse drug reactions due to paracetamol administration have also been reported like Stevens-Johnson syndrome, toxic epidermal necrolysis overlap and baboon syndromes (5, 6), urticaria (7), fixed dermatitis (8), cellulitis like and bullous fixed drug eruptions (9-11), acute generalized exanthematous pustulosis localized in the neck region in pregnant woman (12) and eczema (13, 14).

Melanin is the main surface pigment in vertebrates and in humans (15). It is categorized into two distinct classes, yellow-reddish pheomelanin which contains sulfur and brown – black eumelanin (16). Both eumelanin and pheomelanin are derived from the common precursor dopaquinone, which is formed following the oxidation of tyrosine by tyrosinase (17). The degree of pigmentation of the human skin, hair and eyes is, to a large extent, determined by the ability of specialized cells – melanocytes, to synthesize melanin (18). Various toxins, drugs and chemicals are bound to melanin and retain in pigmented tissues such as the skin, eye and pigmented parts of the brain for long periods of time (19).

Melanin protects the pigmented cells and adjacent tissues by adsorbing potentially harmful substances, which then are slowly released in nontoxic...
concentrations. On the other hand, the long-term exposure may build up high levels of noxious chemicals stored in the melanin, which ultimately may cause degeneration in melanin-containing cells and secondary lesions in surrounding tissues (20).

Previously, we have documented that various drugs suppressed melanin biosynthesis in human light (ciprofloxacin (21), lomefloxacin (22), amikacin (23), netilmicin (25), streptomycin (26), nicotine (27), gentamicin (28)) and dark (nicotine (29), chlorpromazine (30), gentamicin (28)) pigmented melanocytes. Moreover, we have also documented that paracetamol forms stable complexes with model synthetic melanin (31). The effect of paracetamol on melanin biosynthesis in melanocytes has not been studied so far. This work is a continuation of our earlier studies and may be significant for the assessment of toxic reactions produced by this drug in human organism.

The aim of this work was to examine the effect of paracetamol on melanization process in cultured human epidermal melanocytes (HEMn-DP).

EXPERIMENTAL

Chemicals

Phosphated-buffered saline (PBS), 3,4-dihydroxy-L-phenylalanine (L-DOPA), synthetic melanin and amphotericin B were purchased from Sigma-Aldrich Inc. (USA). Paracetamol was obtained in the form of solution – Perpalgan (10 mg/1 mL) from Bristol-Meyers Squibb (Poland). Neomycin sulfate was obtained from Amara (Poland). Penicillin was acquired from Polfa Tarchomin (Poland). Growth medium M-254 and human melanoyp growth supplement-2 (HMGS-2) were obtained from Cascade Biologics (UK). Trypsin/EDTA was obtained from Cytogen (Poland). Cell Proliferation Reagent WST-1 was purchased from Roche GmbH (Germany). The remaining chemicals were produced by POCH S.A. (Poland).

Cell culture

The normal human epidermal melanocytes, neonatal, dark pigmented (HEMn-DP) from Cascade Biologics (UK) were grown according to the manufacturer’s instruction. The cells were cultured in M-254 basal medium supplemented with HMGS-2, penicillin (100 U/mL), neomycin (10 µg/mL), and amphotericin B (0.25 µg/mL) at 37°C in 5% CO₂. All experiments were performed using cells in the passages 5–8.

Cell viability assay

The viability of melanocytes was evaluated by the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazoliol]-1,3-benzene disulfonate) colorimetric assay. WST-1 is a water soluble tetrazolium...
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salt, the rate of WST-1 cleavage by mitochondrial dehydrogenases correlates with the number of viable cells. In brief, 5000 cells per well were placed in a 96-well microplate in a supplemented M-254 growth medium and incubated at 37°C and 5% CO₂ for 48 h. Then, the medium was removed and cells were treated with paracetamol solutions in a concentration range from 0.001 to 20.0 mM. After 21-h incubation, 10 µL of WST-1 was added to 100 µL of culture medium in each well, and the incubation was continued for three hours. The absorbance of the samples was measured at 440 nm with a reference wavelength of 650 nm, against the controls (the same cells but not treated with paracetamol) using a microplate reader UVM 340 (Biogenet, Poland). The controls were normalized to 100% for each assay and treatments were expressed as the percentage of the controls.

Measurement of melanin content

The melanocytes were seeded in T-25 flasks at a density of 1 × 10⁵ cells per flask. Paracetamol treatment in a concentration range from 0.2 to 20.0 mM began 48 h after seeding. After 24 h of incubation, the cells were detached with trypsin/EDTA. Cell pellets were placed into Eppendorf tubes, dissolved in 100 µL of 1 M NaOH at 80°C for 1 h, and then centrifuged for 20 min at 16,000 × g. The supernatants were placed into a 96-well microplate and absorbance was measured at 405 nm - a wavelength at which melanin absorbs light (32). Melanin content in paracetamol-treated cells was expressed as the percentage of the controls (untreated melanocytes).

Tyrosinase activity assay

Tyrosinase activity in HEMn-DP cells was determined by measuring the rate of oxidation of L-DOPA to DOPAchrome, according to the method described earlier (21, 28). The cells were cultured at a density of 1 × 10⁵ cells in T-25 flasks for 48 h. After 24-h incubation with paracetamol (concentration range from 0.2 to 20.0 mM), cells were washed three times with PBS, lysed and clarified by centrifugation at 10,000 × g for 5 min. A tyrosinase substrate L-DOPA (2 mg/mL) was prepared in the same lysis phosphate buffer. Hundred microliters of each lysate was put in a 96-well plate, and the enzymatic assay was initiated by the addition of 40 µL of L-DOPA solution at 37°C. Absorbance was measured every 10 min for at least 1.5 h at 475 nm using a microplate reader. Tyrosinase activity was expressed as the percentage of the controls (untreated melanocytes).

Statistical analysis

In all experiments, mean values of at least three separate experiments performed in nine replications (n = 9) ± standard error of the mean (S.E.M.) were calculated. The results were analyzed statistically using GraphPad Prism 6.01 software by means of one-way ANOVA and Tukey’s multiple comparison test. A value of p < 0.05 (*) or p < 0.01 (**), obtained by comparing the data with those for control (cells without paracetamol), was considered statistically significant.

RESULTS

To assess the influence of paracetamol on the viability of melanocytes, cells were treated with paracetamol in a range of concentrations from 0.001 mM to 20.0 mM for 24 h (Fig. 1). The cell viability was determined by the WST-1 test assay. For paracetamol concentrations from 0.001 mM to 1.0 mM, changes were not statistically significant. After treatment of cells with 5.0, 7.5, 10.0 and 20.0 mM of paracetamol, the loss in cell viability by 10.9, 11.6, 34.6 and 48.7%, respectively, was observed. The value of EC₅₀ (i.e., the concentration of a drug that produces loss in cell viability by 50%) was ~ 20.0 mM.

The effectiveness of melanization process was estimated by measuring the melanin content and cellular tyrosinase activity in melanocytes treated with paracetamol in EC₅₀ concentration as well as 10-fold and 100-fold lower concentrations, for 24 h. After determining a calibration curve, the melanin content per cell was determined as 55.2±58.0 pg/cell for melanocytes treated with the paracetamol and 60.8 ± 1.7 pg/cell for a control samples. The obtained results, recalculated for the culture (1 × 10⁵ cells), were finally expressed as a percentage of the controls (Fig. 2). Paracetamol in concentration of 0.2 mM had no effect on melanin content. In cells treated with paracetamol in concentrations of 2.0 and 20.0 mM for 24 h, melanin production decreased by about 7 to 11%, respectively.

Tyrosinase activity in HEMn-DP cells treated with paracetamol also decreased in a manner correlating well with the inhibitory effect on melanin formation (Fig. 3). After 24-h incubation with paracetamol, tyrosinase activity was suppressed to 87% at 0.2 mM, 83% at 2.0 mM and to 80% at 20.0 mM, when compared with the controls.

DISCUSSION

Paracetamol is extensively used for the treatment of pain and fever. Moreover, it is a drug of
choice when application of non-steroidal anti-inflammatory drugs are contraindicated, e.g., in the case of gastric ulcers, hypersensitivity to aspirin, impairments in blood coagulation, in pregnant women, breastfeeding mothers and children with fever accompanying a disease (2).

Previously, we have documented that paracetamol forms stable complexes with synthetic DOPA-melanin characterized by the association constants $K_1 \sim 4.5 \times 10^5 \text{ M}^{-1}$ and $K_2 \sim 2.45 \times 10^4 \text{ M}^{-1}$. The total number of binding sites was estimated to be 8.9 (nmol drug/mg melanin) (31).

The ability of melanin to bind many drugs and chemical substances may have concurrently beneficial or negative effects on the organism. This interaction protects melanocytes and other cells against excessive exposure to potentially dangerous compounds through their previous accumulation and further elimination in non-toxic concentrations. However, a chronic exposition to xenobiotics with high affinity for melanin may cause degeneration of cells containing melanin. It is believed that the process of drug-induced damages of tissues containing melanin takes place when the detoxifying capacities of melanin are exhausted (20, 33). This effect is mainly connected with high dose and/or long-term exposure to drugs. The onset of the adverse effects may be delayed, and the entire manifestation of the lesions may occur even years after cessation of the offending substance (33).

Based on the previous study concerning the interaction of paracetamol with synthetic melanin (31), it can be concluded that paracetamol binds to melanin strongly but in low amounts. Thus, the unbound drug is able to cause the skin adverse reactions in vivo, e.g., drug eruption (4), urticaria, rash-es or blisters (7). The most interesting side effect is the ability of paracetamol to cause eczema at school age children which were treated with this drug at early childhood (13, 14, 34, 35). It is an example of cutaneous adverse effect, which may be connected with delayed or slow release and dislocation of paracetamol from complexes with melanin to circula-tory system.

In the present study, the effect of paracetamol on melanocytes viability, as well as on melanization process in pigmented cells was analyzed. We used the culture of normal human melanocytes HEMn- DP as an in vitro experimental model system.

The obtained results have shown that paracetamol in a range of concentrations from 0.001 mM to 1.0 mM had no effect on cell viability (Fig. 1).

![Figure 2. The effect of paracetamol on melanin content in melanocytes. Cells were cultured with 0.2, 2.0 or 20.0 mM of paracetamol for 24 h and melanin content was measured as described in Material and Methods. Results are expressed as percentages of the control samples. Data are the mean ± S.E.M. of at least three independent experiments performed in triplicate. * p < 0.05 vs. the control samples; ** p < 0.01 vs. the control samples.](image-url)
Higher concentrations of the drug above 1.0 mM, resulted in a concentration-dependent loss in melanocytes viability. The value of EC$_{50}$ was ~ 20.0 mM.

The changes of melanin content and tyrosinase activity in melanocytes were analyzed for paracetamol concentrations of 0.2 mM, 2.0 mM and 20.0 mM. The obtained results have shown that paracetamol in concentration of 0.2 mM does not significantly affect the melanin content in melanocytes (Fig. 2). Treatment of cells with paracetamol in concentrations of 2.0 mM and 20.0 mM caused decrease in melanin content to 93% and 89%, respectively.

The melanization process in melanocytes was also determined by the activity of tyrosinase, a key enzyme responsible for the activation of the pigmentation machinery. This enzyme can be inhibited by different substances and drugs, e.g., ketoprofen (36), gentamicin (28), kanamycin (24). In our study we have demonstrated that paracetamol in concentrations of 0.2 mM, 2.0 mM and 20.0 mM suppressed tyrosinase activity to 87, 83 and 80%, respectively (Fig. 3), what may explain the observed decrease in melanin content in melanocytes.

To summarize, our study shows that paracetamol in higher concentrations (above 1.0 mM) reduces melanocytes viability. This drug also inhibits tyrosinase activity leading to reduction in melanin content.

**CONCLUSION**

The current study has shown that paracetamol inhibits melanization process in normal human epidermal melanocytes in vitro, what may be a reason for the adverse skin reactions observed in vivo (2, 5, 6). These side effects may be explained by the demonstrated inhibition of tyrosinase activity and decrease in melanin content in melanocytes treated with paracetamol. Thus, melanin is not able to protect melanocytes and surrounding tissues against high concentrations of paracetamol.

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