Chula Med J Vol. 59 No. 3 May - June 2015

Long-term paracetamol treatment induces interleukin-1β expression in human microglial cells

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Wongprom B, Maneesri-le Grand S, Thongtan T. Long-term paracetamol treatment induces interleukin-1β expression in human microglial cells. Chula Med J 2015 May - Jun; 59(3): 253 - 63

Background : *Paracetamol or acetaminophen (N-acetyl-p-aminophenol; APAP) is a widely used as an analgesic and antipyretic drug. This drug has long time been considered as a safe drug, when used in the therapeutic levels. However, the adverse effects concerning long-term APAP usage at the non-hepatotoxicity dosage regarding the human central nervous system, in particular microglia, remains unclear.*

Objective : *To investigate the effect of short-term (6, 12 and 24 hr.) and long-term APAP treatments (1, 2, 3 and 4 weeks) on the expression of the pro-inflammatory cytokine IL-1β in human microglia.*

Design : *Experimental research*

Setting : *Department of Biochemistry and Department of Pathology Faculty of Medicine, Chulalongkorn University*

Materials and Methods : *Human microglial (CHME-5) cells were cultured in the presence of APAP at the concentration determined by MTS assay for 6, 12 and 24 hr., representing short-term treatment. As for long-term APAP treatment, the cells were cultured with APAP for 1, 2, 3 and 4 weeks. Cell pellets were subsequently collected and the expression of the IL-1β in human microglia was determined by Western Blotting.*

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Results : *The study shows that long-term APAP treatment induces morphological changes in CHME-5 cells. An increase in the number of amoeboid phagocytic cells with shorten processes is demonstrated in the long-term APAP- treated cells since the second week of treatment. Also, the level of IL-1β expression is up-regulated in a time-dependent manner while the short-term treatment shows no alteration, compared to that of control cultured cells.*

Conclusions : *The results demonstrated that long-term paracetamol treatment activates human microglial cell morphological changes and up-regulates pro-inflammatory cytokine IL-1β expression.*

Keywords : *Paracetamol, microglia, IL-1β , long-term treatment.*

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Received for publication. December 2, 2014.

เบญจวรรณ วงษ์พรม, ศุภางค์ มณีศรี เลอกรองด์, ธนัญญา ทองตัน. การได้รับยาพาราเซตามอลในระยะยาวกระตุ้นการแสดงออกของอินเตอร์ลิวคิน-1β ในเซลล์ไมโครเกลียของมนุษย์. จุฬาลงกรณ์เวชสาร 2558 พ.ค. – มิ.ย.; 59(3): 253 – 63

เหตุผลของการทำวิจัย : *ยาพาราเซตามอลถูกใช้เป็นยาแก้ปวดและลดไข้อย่างแพร่หลาย เนื่องจากเป็นที่ยอมรับกันว่ามีความปลอดภัยหากใช้ในปริมาณที่ไม่เกินกว่าระดับที่ใช้ในการรักษา อย่างไรก็ตามยังไม่เคยมีการศึกษาถึงผลข้างเคียงจากการใช้ยาพาราเซตามอลในระยะยาวในปริมาณที่ไม่ก่อให้เกิดความเป็นพิษต่อตับที่มีต่อการทำงานของระบบประสาทส่วนกลาง โดยเฉพาะอย่างยิ่งเซลล์ไมโครเกลียของมนุษย์*

วัตถุประสงค์ : *เพื่อศึกษาผลของการได้รับยาพาราเซตามอลระยะสั้น (6, 12 และ 24 ชั่วโมง) และระยะยาว (1, 2, 3 และ 4 สัปดาห์) ที่มีต่อการสร้างสารไซโตไคน์กระตุ้นการอักเสบอินเตอร์ลิวคิน-1β ในเซลล์ไมโครเกลียของมนุษย์*

รูปแบบการวิจัย : *การศึกษาวิจัยเชิงทดลอง*

สถานที่ทำการศึกษา : *ภาควิชาชีวเคมี และภาควิชาพยาธิวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย*

ตัวอย่างและวิธีการศึกษา : *เซลล์เพาะเลี้ยงไมโครเกลียของมนุษย์ (CHME-5) ถูกเพาะเลี้ยงในสภาวะที่ได้รับยาพาราเซตามอลที่ความเข้มข้น 100 μM ซึ่งศึกษาโดยเทคนิค MTS assay เป็นเวลา 6, 12 และ 24 ชั่วโมง เพื่อศึกษาผลของยาพาราเซตามอลระยะสั้น สำหรับการศึกษาผลของยาในระยะยาว เซลล์ถูกเพาะเลี้ยงในสภาวะที่ได้รับยาเป็นเวลา 1, 2, 3 และ 4 สัปดาห์ ก่อนที่จะทำการสกัดโปรตีนเพื่อศึกษาระดับการแสดงออกของสารไซโตไคน์กระตุ้นการอักเสบอินเตอร์ลิวคิน-1β ด้วยเทคนิค Western Blotting*

ผลการศึกษา : *การได้รับยาพาราเซตามอลในระยะยาวสามารถเหนี่ยวนำให้เซลล์เพาะเลี้ยงไมโครเกลียเกิดการเปลี่ยนแปลงรูปร่าง โดยพบว่ามีการเพิ่มขึ้นของจำนวนเซลล์ที่มีลักษณะรูปร่างเป็น amoeboid ตั้งแต่สัปดาห์ที่สองของการได้รับยาพาราเซตามอลอย่างต่อเนื่อง นอกจากนั้นปริมาณอินเตอร์ลิวคิน-1β เพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับเซลล์เพาะเลี้ยงควบคุม ทั้งนี้ปริมาณการแสดงออกจะเพิ่มสูงมากยิ่งขึ้นตั้งแต่สัปดาห์ที่สอง ในขณะที่การได้รับยาพาราเซตามอลในระยะสั้นไม่มีความแตกต่างกันของระดับอินเตอร์ลิวคิน-1β เปรียบเทียบกับเซลล์เพาะเลี้ยงควบคุม*

สรุป : *ผลการศึกษานี้แสดงให้เห็นว่าการได้รับยาพาราเซตามอลในระยะยาวสามารถกระตุ้นเซลล์ไมโครเกลียของมนุษย์ให้เปลี่ยนแปลงรูปร่าง และเพิ่มการแสดงออกของสารไซโตไคน์กระตุ้นการอักเสบอินเตอร์ลิวคิน-1β*

คำสำคัญ : *ยาพาราเซตามอล, เซลล์ไมโครเกลีย, อินเตอร์ลิวคิน-1β, การได้รับยาในระยะยาว.*

Paracetamol or acetaminophen (*N*-acetyl-p-aminophenol; APAP) is widely used as an analgesic and antipyretic drug. It has been considered safe when used within the therapeutic range. Hepatotoxicity is the major adverse effect regarding its overdoses, which may be fatal from acute liver failure. However, over the past decade, many adverse effects of APAP treatment, even in the non-hepatotoxic dosage, have been revealed in several systems such as circulatory,[1] respiratory[2]and central nervous systems.[3]

Anti-inflammatory activity of APAP is mild and dose-dependent. Recently, it has been shown that long-term treatment (30 days) with APAP at non-hepatotoxic dosage could enhance the levels of pro-inflammatory cytokines expression in the hippocampus of rat brain.[4] Still, the mechanism underlying the increment of pro-inflammatory cytokines is not yet identified.

Generally, pro-inflammatory cytokines can be secreted mainly by astrocytes and microglia.[5,6] Microglia, the resident brain macrophages, respond to immunologic challenges by production of many mediators such as nitric oxide (NO), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β).[7,8] Microglial activation is beneficial to tissue homeostasis, but chronic activation of microglial cells results in neuroinflammation.[9] Over-activated microglia play major roles in many neurodegenerative disorders through their sustained release of pro-inflammatory mediators.[10]

IL-1β is a pro-inflammatory cytokine that can bind to IL-1 receptors both in paracrine and autocrine manners. IL-1β is initially expressed as an inactive pro-IL-1β which needs to be activated following cleavage by a pro-inflammatory Caspase-1. It has been previously reported that incubation of human neuroblastoma cells with APAP (2mM) induced ROS production and elevated Caspase-1 activity. The level of IL-1β was also increased after 18 hr. of APAP exposure to neuroblastoma cells.[11]

Since APAP can easily cross the blood-brain barrier,[12,13] thus after reaching the blood circulation, it can directly expose to the cells embedded around the blood vessels including the endothelium, neurons, astrocytes and microglia. It should be noted that not only the microglial cells but also other cell types in the brain (i.e. astrocytes, endothelial cells) are sources of inflammatory proteins. However, up to now, there is no study regarding human microglia in response to APAP treatment. This study sought to determine the effects of short-term (6, 12 and 24 hr.) and long-term APAP treatments (1, 2, 3 and 4 weeks.) on human microglia. APAP dosage was determined using MTS assay and the levels of IL-1β were assessed by Western Blotting.

Materials and Methods

Cell culture

Human embryonic fetal microglial (CHME-5) cells (a gift from Prof. Dr. Duncan R. Smith, Institute of Molecular Biosciences, Mahidol University) were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, USA),100 units/ml of penicillin and 100 μg/ml of streptomycin (Hyclone, USA). CHME-5 cells were maintained at 37°C in a humidified incubator in the atmosphere of 5% CO_2.

Cytotoxicity assay by MTS

Human microglial (CHME-5) cells were seeded onto 96-well plates (5×10^3 cells per well) and cultured for 12 hr. before time. Culture medium was later replaced by 100 μl of medium containing various concentrations of APAP for 24 hr. before analysis of cell viability using the CellTiter 96® Aqueous One Solution Cell Proliferation assay (Promega, USA) according to the manufacturers' recommendations. Briefly, 100 μl of fresh medium was added into each well and 20 μl of MTS reagent was added, followed by incubation in 5% CO_2 incubator at 37°C for 2 hr. Absorbance was measured at 490 nm. Optical density (OD) was determined and presented as % cell survival. IC_{50} curves were generated using the program Graph Pad Prism version 5.0. Percent survival (Y axis) versus log concentration of APAP (X axis) was plotted.

APAP treatment

In short-term treatment, CHME-5 cells were seeded at a density of 4×10^6cells in 75 cm^2 culture flask and maintained for 24 hr. Then, the complete medium was removed. The control cultured cells were refilled with complete medium while the APAP-treated cells were incubated with complete medium containing 100 μM APAP for 6, 12 and 24 hr. before the samples were collected at appropriate time points. As for long-term treatment, the cells were seeded at a density of 3×10^5 cells in 75 cm^2 culture flask and maintained for 24 hr. Then, the complete medium was removed. The control cultured cells were refilled with complete medium while the APAP-treated cells were incubated with complete medium containing 100 μM APAP for 72 hr. After that, the cells were trypsinized, counted and seeded onto a new flask at a density of 3×10^5 cells per flask. The control cultured cells and APAP-treated were cultured for 1, 2, 3 and 4 weeks before samples were collected at appropriate time points.

Western blot analysis

Cultured CHME-5 cells were collected by trypsinization and centrifuged at 1,500 rpm for 5 min at 4°C. Cell pellets were washed twice with ice-cold PBS (phosphate buffered saline, pH 7.4, Sigma) and resuspended in RIPA buffer (Cell Signaling, USA) containing protease and phosphatase inhibitor cocktails (Thermo, USA). Then, the lysates were sonicated three times for 5 sec and incubated on ice for 30 min. The samples were centrifuged at 12,000 ×g for 15 min at 4°C. The supernatants were collected as protein samples and stored at -80°C until used. The concentration of protein was determined by using BCA protein assay kits (Thermo, USA). 10 μg of the whole protein extracts were separated onto 15% SDS-PAGE and transferred to a nitrocellulose membrane using Mini Trans-Blot® Electrophoresis Transfer Cell (BioRad, USA). Non-specific protein binding was blocked by incubating the membranes in 0.1% Tween20/Tris-buffered saline (0.1% TBS-T) containing 5% skimmed milk for 1 hr. at room temperature. Then, membranes were incubated with mouse monoclonal anti-IL-1β (1:2,000, Cell signaling, USA) overnight at 4°C. Afterwards, the blots were washed with 0.1% TBS-T three times before incubation with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hr. at room temperature, or mouse monoclonal anti-β-actin (1:3000, Sigma, USA) overnight at 4°C followed by a HRP-conjugated

secondary antibody for 1 hr. at room temperature. Immunoreactive bands were visualized using chemiluminescence system (ECL, SuperSigna® lWest Pico Chemiluminescence Substrate Kits, GE Health care Life Science). Analysis the intensity of bands was performed by using the Image J software (Scion crop; Frederick, MD). The results were expressed as protein of interest/β-actin ratio.

Statistical analysis

Data are expressed as mean ± SEM. All statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). Statistically significance between means was determined by using two-way ANOVA followed by Bonferroni post hoc test. A $p< 0.05$ was considered statistically significant.

Results

Cytotoxicity assay of APAP on CHME-5 cells

The results demonstrate that APAP treatment for 24 hr., at the concentration of 6,000 μM, can induce 50% cell death (IC_{50}). At the concentration lower than 1,000 μM, APAP induces less than 10% cell death (Figure 1).

Effect of long-term APAP treatment on the morphological changes in CHME-5 cells

To determine whether human microglial (CHME-5) cells underwent activation in response to non-toxic dosage of paracetamol treatment or not, APAP-treated cells are examined for morphological changes in comparison to control cultured cells under light microscope. Ramified morphology with small soma and fine cellular processes, indicating resting microglia, are observed both in the control cultured cells and APAP-treated cells for short-term exposure (data not shown).Interestingly, we observe the transformation of microglial morphology from ramified resting cells to the amoeboid phagocytic appearance with shorten processes in the long-term APAP- treated cells since the second week of treatment (Figure 2).

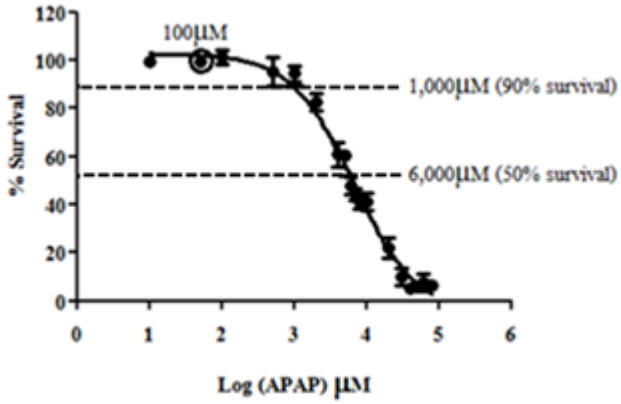

Figure 1. Cytotoxicity of paracetamol to CHME-5 cells as determined by MTS assay after 24 hr. of exposure. Data are shown as mean ± SEM of three independent experiments in duplicate assay.

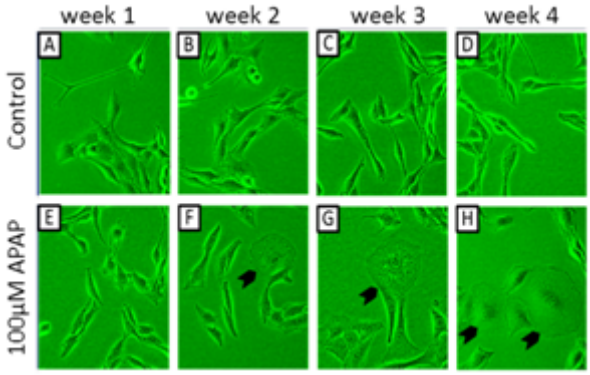

Figure 2. Morphological changes of long-term APAP-treated CHME-5 cells. Amoeboid microglial cells (arrowheads) were observed in long-term APAP-treated cultures (F-H). All cultured cells were observed under a light microscope (×200).

Effect of APAP treatment on the expression of pro-inflammatory cytokine IL-1β in CHME-5 cells

The results show that, for short-term APAP treatment, there is no alteration of IL-1β expression comparing between APAP-treated and control cultured cells. Interestingly, for long-term APAP treatment, the levels of IL-1β expression are up-regulated in APAP-treated CHME-5 cells for ≥ 2 weeks in a time-dependent manner (Figure 3).

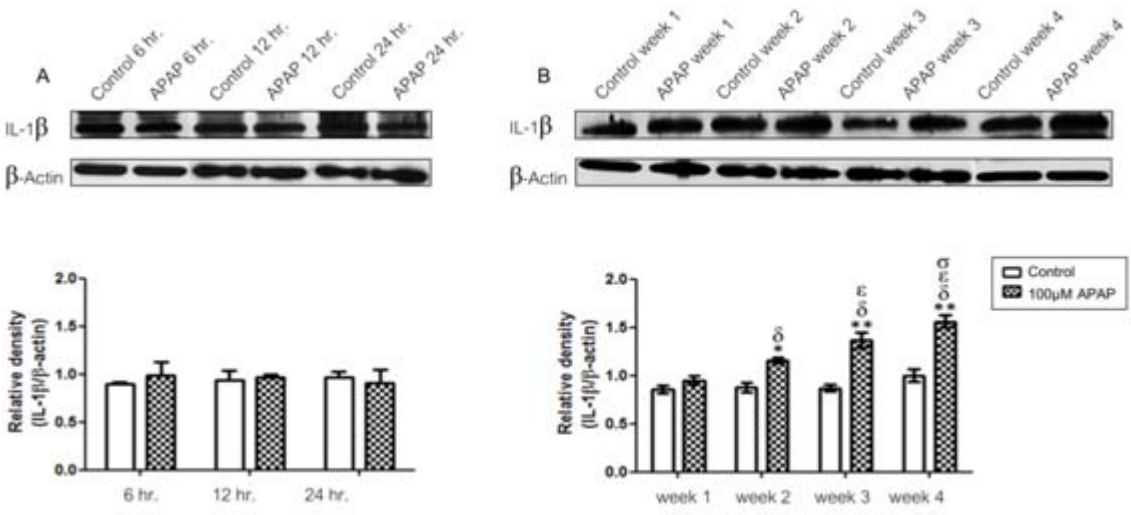

Figure 3. Expression of IL-1β in response to paracetamol treatment. CHME-5 cells were cultured with 100 μM APAP for short-term (A) *vs.* long-term treatment (B). Quantitative data are expressed as relative densities compared to β-actin. Data are expressed as mean ± SEM of three independent experiments in duplicate assay. $^{*}P < 0.01$ compared to the control cultured cells, $^{**}P < 0.001$ compared to the control cultured cells, $^{\delta}P < 0.05$ compared to the APAP-treated cells in the first week, $^{\varepsilon}P < 0.05$ compared to the APAP-treated cells in the second week, $^{\varepsilon}P < 0.05$ compared to the APAP-treated cells in the third week.

Discussion

It is generally accepted that APAP can distribute throughout the body without binding to tissues. Then, the concentrations of APAP *in vitro* setting can be directly correlated with its concentrations *in vivo* without corrections for tissue uptake. In this study, the dosage of 100 μM APAP determined by MTS assay was chosen to represent the peak plasma concentrations of paracetamol after a therapeutic dose (1g, four times daily).[14] In fact, this selected dose is about 10 times lower than the previous study of Posadas et al., using human neuroblastoma cell line as a model to study the mechanism involved APAP–mediated toxicity.[11] Our present results clearly showed that, with the long-term treatment of 100 μM APAP, CHME-5 cells underwent activation and released the pro-inflammatory cytokine IL-1β whereas the short-term treatment with the same dosage had no effect on the cells. Our results also revealed that CHME-5 cells responded differently to the APAP treatment between short-term and long-term treatments. While long-term treatment with APAP could induce the alterations in both morphology and pro-inflammatory cytokine expression, short-term treatment showed no effect.

APAP can be metabolized into *N*-acetyl-p-benzoquinone imine (NAPQI) by enzyme cytochrome P-450 isoform CYP2E1 which expressed in several brain cells including microglia. NAPQI is a highly reactive toxic intermediate. So, after being produced, it is normally conjugated with glutathione (GSH) resulting in non-toxic products. This might be the reason why there was no change in CHME-5 cells in response to short-term APAP exposure. However, with long-term treatment, NAPQI can be continuously produced which consequently leads to the accumulation of NAPQI and the depletion of GSH. GSH depletion can result in the increase of oxidative stress and finally induce damage to the cells. Besides, NAPQI itself can bind to sulfhydryl groups in cellular proteins, including mitochondrial proteins. This can result in a defective in ATP synthesis. Based on these cumulative data, it can be suggested that the increment of NAPQI formation, due to the long duration of the APAP treatment, can be at least one mechanism underlying those morphological changes in long-term APAP-treated CHME-5 cells observed in this study.

Regarding to the anti-inflammatory effects of APAP, it was reported that this drug could decrease prostaglandin E_2 production in the central nervous system.[15] Interestingly, with the difference of the treatment dosage and duration, APAP demonstrates the different effects on the inflammatory processes. At a low dose, treatment with APAP inhibited inflammation by decreasing the levels of many pro-inflammatory cytokines including TNF-α, IL-1α, IL-1β and macrophage inhibitory protein-1 alpha (MIP-1α).[16,17] With a high dose, however, APAP could increase both pro- and anti-inflammatory cytokine (IL-10, IL-13) expressions in several animal models.[18-22]Concerning the duration of APAP treatment, our previous study have demonstrated the different effect of APAP treatment *in vivo*, at non-hepatotoxic dosage, on the expression of TNF-α and IL-1α in the hippocampus of rat brain. While short-term treatment with APAP had no effect on those pro-inflammatory cytokines expression, the long-term APAP treatment (30 days) could increase their expression.[4] These results are in line with the study of chronic APAP (6 weeks) usage in combination with

alcohol intake by Fakunle et al. That chronic treatment induced the hippocampal neuron damage.[23] Our present results clearly showed that the level of pro-inflammatory cytokine IL-1β was up-regulated in the long-term APAP - treated CHME-5 cells since the second week of the treatment whereas the short-term treatment, with the same non-hepatotoxic dosage, had no effect on the cells.

Taken together, our data demonstrates that, in opposite to short-term treatment, long-term APAP exposure activates human microglia to release pro-inflammatory cytokine IL-1β in a time-dependent manner. This finding is well correlated with a previous study in rat brain.[4] The results imply that long-term APAP treatment may cause NAPQI accumulation and oxidative stress which can further activate microglia to release pro-inflammatory cytokines. Given that IL-1β can induce the activation of NF-kB, the involvement of NF-kB signaling in response to long-term APAP exposure is of particular interest.

Acknowledgements

This study was supported by THE 90th ANNIVERSARY OF CHULALONGKORN UNIVERSITY FUND (Ratchadaphiseksomphot Endowment Fund) Grant No. 41 (3/2556) and HEALTH CLUSTER: Grant No.RES560530206-HR.

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