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Original article

Chronic paracetamol treatment induces neuroinflammation and microglia activation in rat hippocampus

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Background: Several studies have demonstrated multidirectional effects of paracetamol (acetaminophen, APAP) treatment on the central nervous system. Recently, an alteration of learning and memory have been reported following long-term APAP exposure; however, the mechanism underlying these detrimental effects of APAP treatment is not fully clarified.

Objectives: To investigate the effect of chronic APAP treatment on the microglia activation and neuroinflammation in the hippocampus.

Methods: Male Wistar rats (weighting 250 - 300 g) in the APAP-treated group was once a day gavaged with 200 mg/kg bodyweight APAP for 30 days, while distilled water at the same volume was orally delivered to the rats in the control group. Expression of pro-inflammatory cytokines was evaluated using Western blotting analysis, while the ionized calcium-binding adaptor molecule 1 (Iba-1) and nuclear factor erythroid-2-related factor 2 (Nrf2) protein expressions were determined by using immunohistochemistry and immunofluorescence, respectively.

Results: As compared with the control rats, the expression of tumor necrosis factor-alpha (TNF-alpha) and interleukin-1beta (IL-1beta) were significantly higher in the APAP-treated rats then in the control rats. A significant increase in Iba-1 protein was demonstrated in rats with 30-day APAP exposure. In addition, an increment of Nrf2 protein expression was also observed in the APAP-treated group.

Conclusion: The present results suggest that chronic APAP treatment can induce microglia activation and upregulation of proinflammatory cytokines in the hippocampus. An increment of the Nrf2 expression may involve neuroinflammatory response following prolonged treatment with APAP.

Keywords: Paracetamol, pro-inflammatory cytokine, microglia activation, Nrf2, hippocampus.

Paracetamol (Acetaminophen, APAP), is commonly used for treatment of fever and pain throughout the world. Due to its properties of high availability, inexpensiveness, and minimal side-effects, there is a high possibility that the people who have chronic pain will select this drug as the first choice and use it for a long period.

APAP is still accepted as a safe drug, however, several recent reports demonstrated non-beneficial effects of APAP treatment in particular with long-term usage on several systems. A previous study in animal model revealed that sub-toxic concentration of APAP treatment could induce a reduction of intracellular glutathione (GSH) in pulmonary macrophages and type II pneumocytes suggesting that APAP treatment might be a risk factor for asthma morbidity.[1] The association between the APAP treatment and a higher risk of chronic obstructive pulmonary disease (COPD) has been confirmed by Nassini R, *et al*. in 2010. Their results showed that single and repeated treatment of APAP [15 - 60 mg/kg body weight (bw)] produced an elevated neutrophil population, myeloperoxidase activity, as well as cytokine and chemokine levels in the mice airway.[2] Furthermore, unwanted effect of this drug treatment has also been proposed in the cardiovascular system. Clinical studies in subjects who frequently took APAP at a dosage of 500 mg/day demonstrated almost 2-fold higher in the relative risk of incident hypertension than that observed in non-users.[3 - 5] Later in 2010, Sudano I, *et al*. have showed that treatment with APAP at a therapeutic dose (1 g/day) with standard cardiovascular therapy for 2 weeks in patients with coronary heart disease could induce an increase in both systolic and diastolic

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blood pressures.[6] Regarding the effect of APAP treatment on the central nervous system (CNS), the results obtained from the sibling-controlled study in Norwegian have previously demonstrated that the children exposed chronically to prenatal APAP (> 28 days) had poorer gross motor development and increase in the risk of attention deficit hyperactivity disorder and communication problems.[7, 8] A growing number of evidences suggest that generation of n-acetyl-p-benzoquinone imine (NAPQI) which is the active metabolite of APAP after being converted by cytochrome P450 family 2 subfamily E member 1 (CYP2E1) enzyme is a key factor involved with those unwanted effects of APAP.[1, 2, 6, 9]

It is known that CYP2E1 expresses in several parts of the brain including hippocampus[10] which is the brain area responsible for learning and memory formation.[11] For these reasons, the effect of APAP treatment on an alteration of learning and memory has been interested by several research groups. A number of studies has previously revealed that APAP treatment could manipulate the capacity of learning and memory.[12 - 14] However, the mechanism underlying these deteriorating effects is not yet fully understood.

In 2006, Fakunle PB, *et al*. have demonstrated that chronic treatment (6 weeks) with APAP (100 mg/kg bw) alone or in combination with alcohol intake could induce neuronal damage in the hippocampus.[15] Furthermore, several studies have demonstrated the association between APAP treatment and neuroinflammation in hippocampus. The results from our previous study have revealed that 30-day intraperitoneal injection with APAP (200 mg/kg bw) could induce an overexpression of proinflammatory cytokines (tumor necrosis factor alpha [TNF-alpha] and interleukin-1 alpha [IL-1alpha]) in hippocampus [16]; however, the mechanism underlying the neurotoxic effect of long-term APAP treatment is not yet clarified.

It is well known that microglia activation is implicated in neuroinflammation and many neurodegenerative disorders including depression, Alzheimer's disease (AD) and Parkinson's disease (PD).[17, 18] In the resting state, microglia are in the ramified form with long processes which use for monitoring the brain environment.[19, 20] In response to various pathological stimuli, the microglia can transform to an ameboid shape and can produce large amounts of various cytotoxic mediators including reactive oxygen species (ROS), chemokines, and pro-inflammatory cytokines leading to promote the neuronal inflammation and damage.[21, 22]

Additionally, it has been recently reported that the nuclear factor erythroid 2 related factor 2 (Nrf2) is one of the gene regulators involve in the inflammatory process in the central nervous system (CNS).[23] An activation of Nrf2 signaling pathway could prevent neuronal toxicity induced by inflammation.[23] Several research groups have suggested that overexpression of Nrf2 might be one potential therapeutic strategy for many neuropathological conditions including AD and PD. [24, 25]

Previously, we have reported that the rats with 30-day oral APAP treatment demonstrated an elevation of oxidative stress and synaptic impairment in the hippocampus.[26] This study aimed to investigate whether the inflammation and microglia activation involve in these deteriorations observed in hippocampus. In the present study, the effect of long-term APAP treatment at the dose of 200 mg/kg bw on the expression of pro-inflammatory cytokines (TNF-alpha and IL-1beta) as well as the expression of ionized calcium binding adaptor molecule 1 (Iba-1) protein which is a specific biomarker for activated microglia [23, 27] were monitored. Since the Nrf2 gene expression is participated in the neuroinflammatory process, the expression of the Nrf2 protein was as well determined by the immunofluorescence assay.

Materials and methods

Animals

Male Wistar rats (weighing 250 – 300 g) were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The animals were housed five per cage in a temperature- and humidity-controlled room with a 12-h dark/light cycle. Food and drinking water were available *ad libitum*. All the protocols used in this study were approved by the Ethics Committee for Animal Experiment of the Faculty of Medicine, Chulalongkorn University (ACUC-CU 23/2558).

To investigate the effects of long-term APAP treatment on neuroinflammation in the hippocampus, the rats were divided into two groups (ten animals each): control and APAP-treated (APAP200) groups. The rats in the APAP-treated group were daily gavaged once with 200 mg/kg bw APAP for 30 days, while the control rats were orally fed distilled water for the same period. The oral gavage was daily performed at a time between 9.00 - 10.00 a.m.

Tissue collection

All animals in both control and APAP200 groups were humanely killed 24 h after the last treatment. To perform the biochemical analysis, five rats in each group were intraperitoneally injected (i.p.) with an excessive dose of sodium pentobarbital (60 mg/kg bw) and transcardially perfused with 250 ml of 0.1 M phosphate buffered saline (PBS) pH 7.4 before decapitation. The hippocampus was rapidly dissected on ice and immediately frozen in liquid nitrogen before storing at – 80°C until protein extraction. For preparation of tissue homogenates, the frozen hippocampi were homogenized in RIPA buffer (Cell Signaling Technology®, MA, USA) containing proteinase inhibitors (Cell Signaling Technology®, MA, USA) and were then centrifuged at 12,000 g at 4°C for 15 min.[28] The supernatant was collected, and the protein concentration was measured by using a Pierce™ BCA assay kit (Thermo Scientific, IL, USA).

For the immunohistochemistry, the brain was removed out of the skull and immerged in the 4% paraformaldehyde at 4°C for 48 h before being further processed for embedding in the paraffin.

Western blot analysis

Equal amounts of protein (40 μg) were electrically separated in 15% SDS polyacrylamide gels and were then transferred onto a nitrocellulose membrane with a 0.2-μm pore size (GE Healthcare Life Sciences, Buckinghamshire, UK). The blotted membranes were incubated at room temperature for 1 h with 5% w/v of skimmed milk (Sigma, St. Louis, MO, USA) in tris-buffered saline pH 7.4 containing 0.1% Tween-20 (TBS-T) for TNF-alpha and beta-actin detections or 5% bovine serum albumin (BSA) diluted in TBS-T for IL-1beta detection. The membranes were then incubated overnight at 4°C with rabbit anti-TNF-alpha antibody (1:1,000 dilution; Santa Cruz, California, USA), mouse anti-IL-1beta antibody (1:2,000 dilution; Cell Signaling Technology®, MA, USA) or mouse anti-beta-actin antibody (1:3,000 dilution; Sigma, St. Louis, MO, USA). After three times washing with TBS-T, the membranes were incubated with anti-rabbit antibody conjugated with horseradish peroxidase (HRP) (1:10,000 dilution; Sigma, St. Louis, MO, USA) for detecting TNF-alpha expression or anti-mouse antibody conjugated with HRP (1:10,000 dilution; Sigma, St. Louis, MO, USA) for detecting IL-1beta and beta-actin expressions. After that the membranes were washed for three time in TBS-T. Immunoreactive bands were then visualized by using an enhanced chemiluminescence system (Amersham™ ECL™ Prime Western Blotting Detection Reagent, GE Healthcare Life Sciences, Buckinghamshire, UK). The densities of the immunoreactive bands were determined using ImageJ software (National Institute of Health, Bethesda, MD, USA). The results are reported as the ratio of the densities of TNF-alpha and IL-1beta to β-actin.

Immunohistochemistry

The paraformaldehyde-fixed hippocampi (five per group) were paraffinized was coronally sectioned at the thickness of 5 μm. All sections were deparaffinized and incubated with an antigen retrieval solution (citrate buffer pH 6.0, Dako, Glostrup, Denmark), 3% hydrogen peroxide, and 3% normal horse serum (PAN Biotech GmbH, Aidenbach, Germany) in PBS. The sections were incubated with primary rabbit anti-Iba-1 antibody (1:400 dilution; Abcam, Cambridge, UK) at 37°C for 30 min. The Iba-1 immunoreactivity was detected using an ultraView Universal DAB Detection Kit (Ventana Medical Systems, Inc., Arizona, USA). The entire process was conducted with an automatic slide staining machine (Benchmark XT, Ventana Medical Systems, Inc., USA). All slides were counterstained with hematoxylin, dehydrated in ethanol series, mounted, and cover-slipped with a mounting media. All sections were scanned using a slide scanner (Aperio ScanScope, Aperio, Vista, California, USA). In order to quantitative analyze, the Iba-1 immunoreactivity in the hippocampus (approximately -3.14 to 3.30 from bregma) was evaluated by using the positive pixel counting algorithms (v9.1, Aperio, Vista, California, USA) and the immunoreactivity was reported as positive pixel per square millimeters (pixel intensity/mm^2).

Immunofluorescence assay

To determine the expression and localization of the Nrf2 protein in the hippocampus, the paraffin-embedded tissue sections were prepared as described previously. All the sections were deparaffinized and rehydrated and then further incubated with an antigen retrieval solution (citrate buffer pH 6.0, Dako, Glostrup, Denmark) and 3% normal horse serum (PAN Biotech GmbH, Aidenbach, Germany) in PBS. The sections were treated overnight at 4°C with primary rabbit anti-Nrf2 antibody (1:200 dilution;

Abcam, Cambridge, UK). After rinsing in PBS, the sections were further incubated with Alexa Fluor® 488 anti-rabbit IgG secondary antibody for 2 h. Then, the sections were incubated with 4; 6-diamidino-2-phenylindole (DAPI) for nuclear counterstaining. Finally, the sections were cover slipped with a cover glass and further examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis

The data are represented as the mean ± standard error of the mean (SEM). The statistical analyses were performed by using unpaired Student's t – test. A P - value less than 0.05 was considered to indicate statistical significance.

Results

To exclude the involvement of hepatotoxicity in any alterations observed in the hippocampus following long-term APAP treatment, the hepatic morphology and three main enzymes associated with the liver function (aspartate aminotransferase [AST], alanine aminotransferase [ALT] and alkaline phosphatase [ALP]) were monitored in this study. The results demonstrated that neither the morphology of the liver nor the level of those three enzymes obtained from rats treated with 200 mg/kg bw APAP for 30 days were different from the control rats (data not shown). With these results, we can ensure that the involvement of hepatotoxicity is not included to all the alteration observed in the present study.

Effect of chronic APAP treatment on the expression of TNF-alpha and IL-1beta proteins

Using the western blot analysis, the results demonstrated that treatment with 200 mg/kg bw APAP for 30 days could increase the expression of proinflammatory cytokines in the rat hippocampus. The results revealed that the expression of hippocampal TNF-alpha protein was significantly higher in the rats with chronic APAP treatment than that observed in the control group ($P < 0.01$, Figure 1). In parallel with the finding of an increment of TNF-alpha expression, the results also demonstrated a significant increase in the hippocampal IL-1beta protein in the rats received with 30-day APAP treatment as compared with that in the control group ($P < 0.05$, Figure 2).

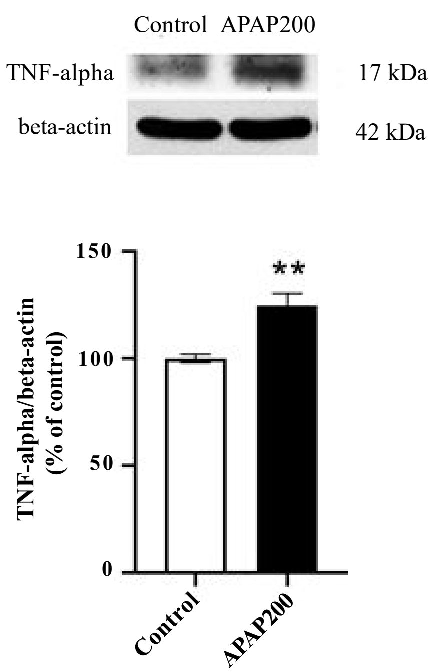

Figure 1. Effect of chronic APAP treatment on the expression of TNF-alpha protein. The expression of TNF-alpha protein in the hippocampus obtained from the control and 200 mg/kg bw APAP-treated (APAP200) rats were determined by using Western blot analysis. The histogram bars are represented as mean ± SEM. **$P < 0.01$ compared to the control group.

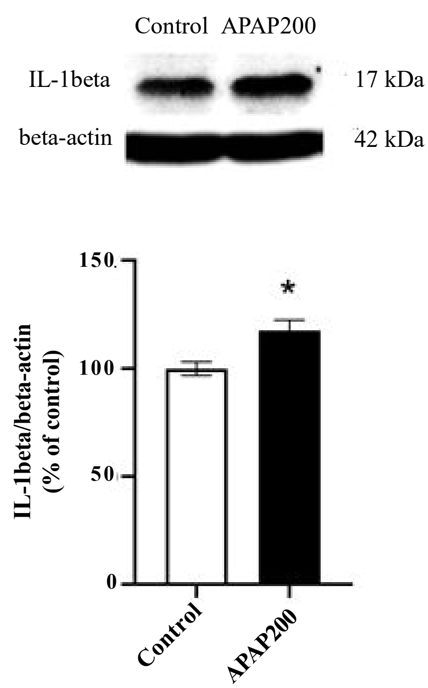

Figure 2. Effect of chronic APAP treatment on the expression of IL-1beta protein. The expression of IL-1beta protein in the hippocampus obtained from the control and 200 mg/kg bw APAP-treated (APAP200) rats were determined by using Western blot analysis. The histogram bars are represented as mean ± SEM. *$P < 0.05$ compared to the control group.

Effect of chronic APAP treatment on the expression of hippocampal Iba-1 protein

The results from the Iba-1 immunohistochemistry demonstrated that the Iba-1 immunoreactivity was higher in the hippocampus of rats treated with 30-day APAP as compared to the rats in the control group (Figure 3A). The significant increase in the intensity of Iba-1 immunoreaction was demonstrated in the chronic APAP-treated rats ($P < 0.05$, Figure 3B).

Effect of chronic APAP treatment on the expression of Nrf2

In the present study, the results obtained from the immunofluorescence assay demonstrated that the expression of total Nrf2 protein (cytoplasmic and nuclear proteins) was higher in the rats that received 200 mg/kg bw APAP for 30 days. An increment of the total Nrf2 protein was observed in the region of CA1, CA3 and dentate gyrus of the hippocampus obtained from the rat treated with 30-day APAP as compared with those observed in the control group (Figure 4).

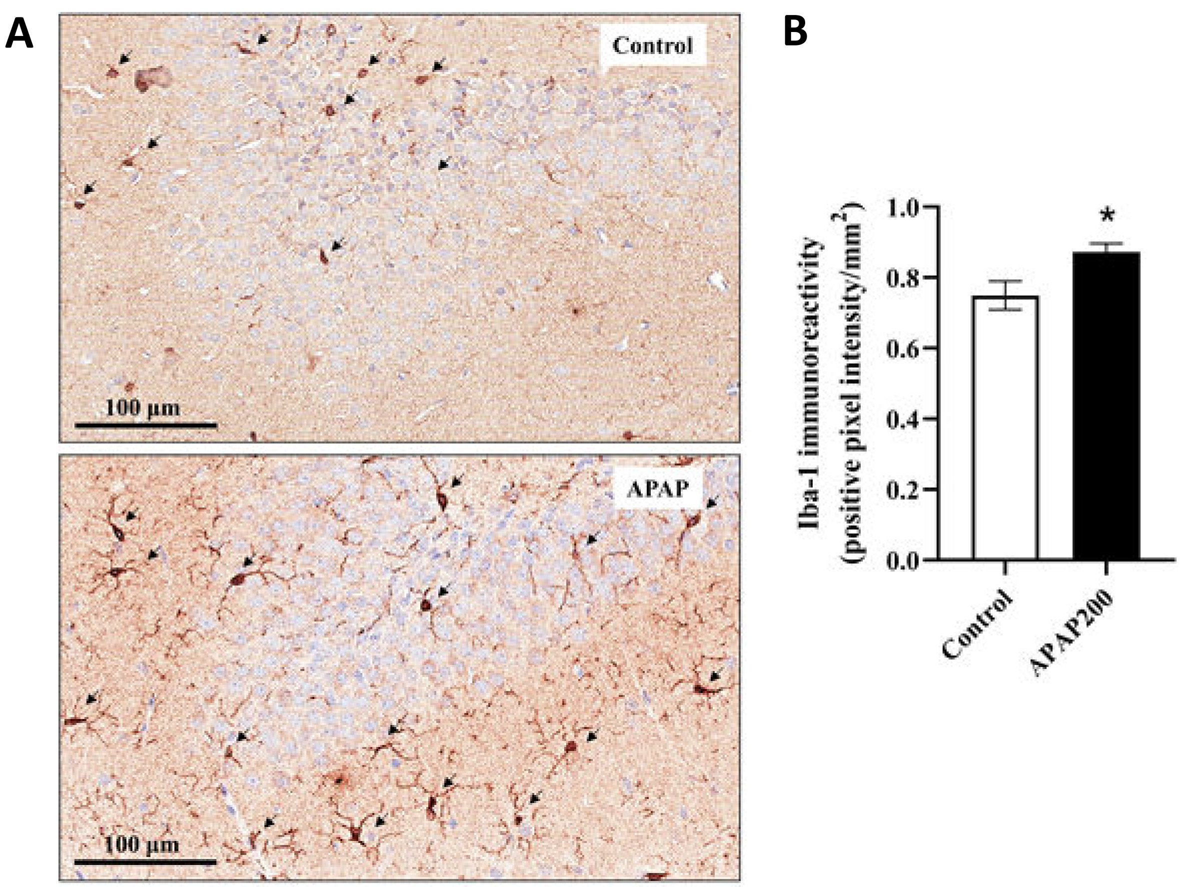

Figure 3. Effect of chronic APAP treatment on the expression of Iba-1 protein. **(A)** The images of immunohistochemical staining for Iba-1 protein in the hippocampus obtained from the control and 200 mg/kg bw APAP-treated (APAP200) rats were determined by using immunohistochemistry. The Iba-1 immunopositive cells are indicated by the arrows. Scale bars, 300 µm. **(B)** The quantitative data of Iba-1 immunoreactivity is represented as mean ± SEM. *$P < 0.05$ compared to the control group.

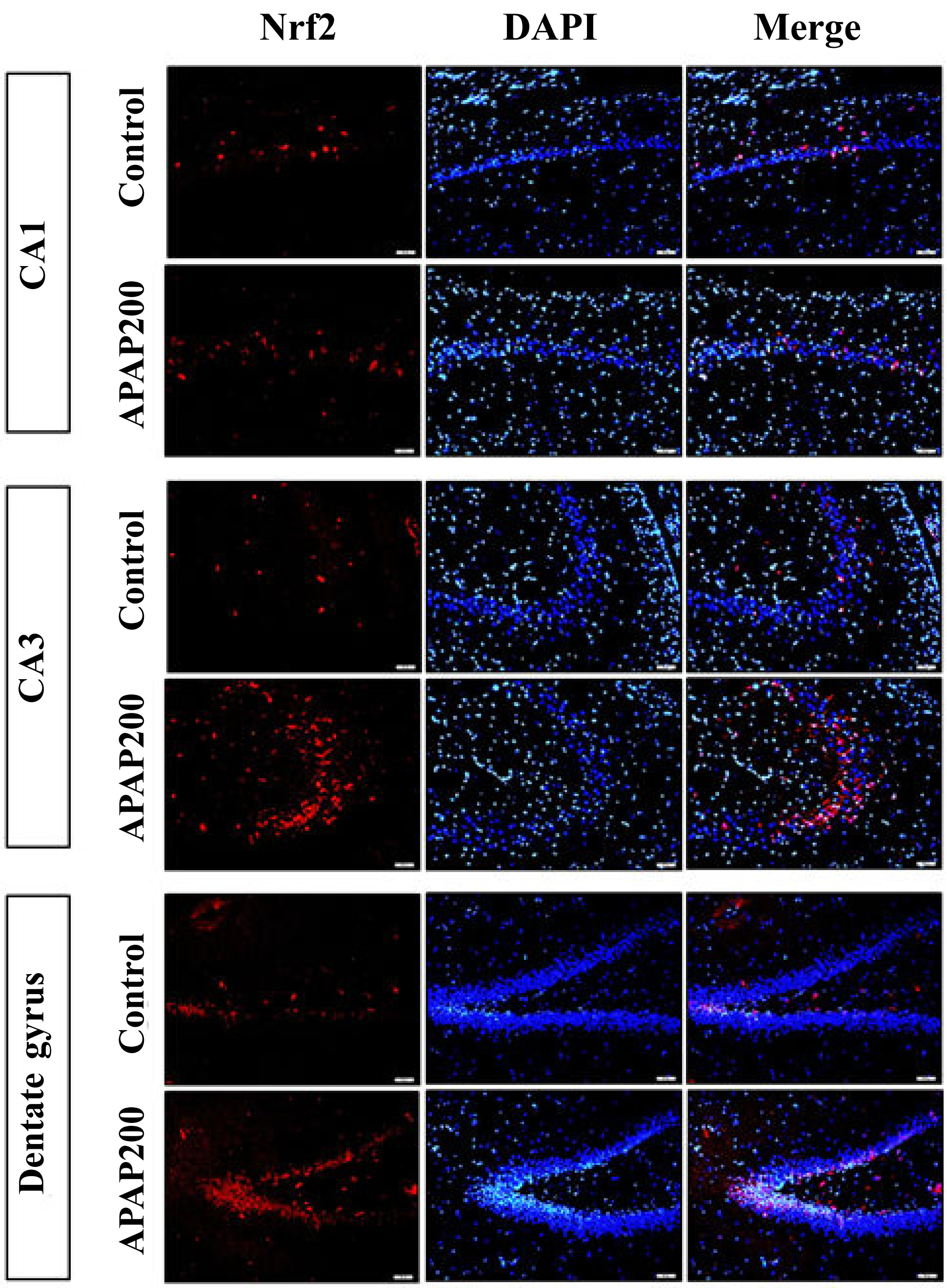

Figure 4. Effect of chronic APAP on the expression of Nrf2 protein. The expression of the Nrf2 protein in the hippocampus obtained from the control and 200 mg/kg bw APAP-treated (APAP200) rats were determined by using immunofluorescence assay. The photographs of Nrf2 immunoreaction in the region of CA1, CA3 and dentate gyrus of the hippocampus are shown. Scale bars, 50 μm.

Discussion

The present study have revealed that long-term treatment with APAP could induce an upregulation of pro-inflammatory cytokines (TNF-alpha and IL-1beta) as well as the microglia activation in hippocampus. This alteration was observed in parallel with the increment of an expression of Nrf2 protein in the same brain area.

The dose of APAP employed in the present study was 200 mg/kg bw. This dose, after converting by the human equivalent dose guideline distributed by US Food and Drug Organization (32.25 mg/kg), could be considered as a dose within a therapeutic dose range in a human. [26, 28, 29] We demonstrated that neither the liver enzymes nor hepatic morphology were affected by 200 mg/kg bw APAP treatment for

30 days. Therefore, it can be ensured that the hepatotoxicity is not involved in all the alterations observed in the present study.

In this study, an increment of hippocampal TNF-alpha and IL-1beta protein expression were demonstrated in rats orally treated with APAP for 30 days. The association between APAP treatment and inflammation has been previously reported in several cell cultured studies. In 2012, Posadas et al. reported that neuroblastoma cells which being exposed to the APAP could show an upregulation of IL-1beta protein expression.(30) Studies in astrocytes and microglial cells culture have as well demonstrated that long-term APAP treatment could induce an increment of pro-inflammatory cytokines productions in those cells. (31, 32)

In 2013, Chantong C, *et al*. demonstrated for the first time that intraperitoneal injection with APAP (200 mg/kg) for 30 days could induce an increase of TNF-alpha and IL-1alpha immunoreactivity in the hippocampus of the rat.(16) Our current results are consistent with this previous report. Based on these results, it indicate that long-term oral consumption of APAP which is the major route of treatment for APAP, can induce a neuroinflammation in the hippocampus.

Moreover, the microglia activation following long-term APAP treatment is clearly observed in this study. The significant increase in Iba-1 expression was demonstrated in the rat hippocampus obtained from the APAP treated group. It is well recognized that microglia activation has a vital role in inflammatory response in the CNS. Moreover, activated microglia is also reported to induce the detrimental effect on neurobehaviors including learning and memory impairment.(33, 34) Hyperreactivity of microglia can be stimulated by various conditions such as autoimmune injuries, toxic insults as well as oxidative stress induction. (35 - 38)

Our research group has recently reported that 30-day gavage treatment with APAP could induce elevation of protein carbonyl oxidation and reduction of GSH levels in the hippocampus. Besides an increment of oxidative stress, the results demonstrated the synaptic degeneration in the animal treated with APAP for a long period. An increase of NAPQI, the toxic metabolite of APAP, due to the long-term exposure with APAP has been proposed to be the cause of those alterations.(26)

It is known that microglia cells are the brain residual microphage which are sensitive for various brain insults and act as a first line of defense.(39, 40) Several studies have demonstrated that an increase of oxidative stress in the brain can easily activate microglia.(37, 38) After activation, microglia can release several cytotoxic molecules including ROS, chemokines and pro-inflammatory cytokines.(21, 22) Therefore, we assumed that the increment of pro-inflammatory cytokine expression observed in the present study might be associated with oxidative stress induced microglia activation following long-term treatment with APAP.

Apart from the neuroinflammation, the present study demonstrated an overexpression of the Nrf2 protein in the rats with oral 30-day APAP treatment. Nrf2 is accepted as a transcription factor which can potentially regulate the defense mechanism of oxidative stress- and inflammation-mediated neuronal toxicity. The activation of Nrf2/antioxidant response element (ARE) signaling can disrupt the NF-kB pathway resulting in inhibition of pro-inflammatory cytokine generation.(23) In addition, stimulated Nrf2 signaling can lead to enzymatic degradation of pro-inflammatory free heme and generation of anti-inflammatory compounds due to the promotion of heme oxigenase-1 activity.(23) However, the expression of Nrf2 is complicated in various disease progressions. For example, a decrease in the Nrf2 level was demonstrated in AD patients despite the presence of oxidative stress (25), whereas an increase in the expression of the ARE-related genes was observed in patients with mild cognitive impairment.(41, 42) In the present study, an upregulation of Nrf2 was well demonstrated in long-term APAP treatment. With these results, it can be assumed that an increased Nrf2 expression can indicate the neural compensatory response against neuroinflammation through prolonged APAP exposure.

Conclusion

The data obtained from this study indicate that oral long-term APAP treatment even with the dose within a therapeutic dose range can induce neuroinflammation in the hippocampus. The microglia activation might be, at least, one mechanism involving in detrimental effect of this drug. It is known that the hippocampus is a key brain region responsible for learning and memory process. Therefore, an alteration of learning and memory behaviors could probably be expected in the case of long-term APAP treatment.

Acknowledgements

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Conflict of interest

The authors, hereby, declare no conflict of interest.

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