Inhibitor of cyclooxygenase-2 protects against amyloid β peptide-evoked memory impairment in mice

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Abstract:
Alzheimer’s disease (AD) results in an impairment of memory and behavior. It is accepted that amyloid β (Aβ) peptides are responsible for the etiopathology of AD, but the precise signaling pathways leading to the disease have not been elucidated. In this study, we have investigated the role of cyclooxygenase-2 (COX-2) in Aβ(1–42)-evoked memory impairment in mice. Moreover, the effect of systemic inflammation on Aβ-dependent locomotor and memory disturbances has been evaluated. Twelve-month-old C57Bl6 mice were injected intracerebroventricularly (icv) with Aβ(1–42) alone or simultaneously with intraperitoneal (ip) administration of lipopolysaccharide (LPS). Some mice also received COX-2 inhibitor, NS-398. Another group of mice was pretreated with LPS at 4 and 7 months of age, and then injected with Aβ(1–42) at 12 months of age. All mice were subjected to behavioral tests one week after Aβ administration. COX-2 protein level was analyzed in the hippocampus using immunochemical method.

Our data demonstrated that Aβ enhanced COX-2 protein level and decreased the locomotion and exploration in mice. Systemic inflammation elevated COX-2 immunoreactivity at an early stage after injection and intensified behavioral disturbances. Moreover, the object recognition in Aβ-treated mice was significantly affected compared to control mice. The administration of LPS simultaneously with Aβ worsened recognition performance. A COX-2 inhibitor protected mice against memory deficit and locomotor disturbances. In LPS-pretreated animals, Aβ induced locomotor disturbances, but had no effect on memory and COX-2 level.

Our results indicate that Aβ evokes enhancement of COX-2 protein level and memory deficit. Systemic inflammation modulates Aβ effect on the brain function. The COX-2 inhibitor protects the brain against Aβ-induced memory disturbances.

Key words: cyclooxygenase, Alzheimer’s disease, systemic inflammation, memory

Introduction
Extracellular deposits of amyloid β (Aβ) peptides in the form of senile plaques with accompanying synaptic and neuronal loss in specific areas of the brain are
the hallmark of Alzheimer’s disease (AD). Recent reports indicate that Aβ oligomers accumulated intracellularly and extracellularly may be responsible for the initiation of the disease [26, 41], although the pathomechanism of AD is not fully understood. Inflammation has also been recognized to play a central role in the AD development. It was observed that the aggregated polymers of Aβ initiated a brain-specific inflammatory response which exaggerated neurodegeneration [14, 20, 28]. In the regions of neuropathological lesions, astrocytic and microglial activation is observed [2, 29]. In vitro studies confirmed that Aβ could activate microglia and astrocytes to induce the production of inflammatory cytokines, including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, IL-8 and reactive oxygen species, that may directly damage neurons [8, 44]. In addition, several epidemiological studies have indicated that a long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) may reduce the risk of developing AD [19, 25, 37].

Most NSAIDs are cyclooxygenase (COX) inhibitors, therefore, the role of COX, especially COX-2, the inducible form of COX, in the pathogenesis of AD receives increasing attention. It is known that the expression of COX-2, as well as the level of its product, PGE2, is increased in AD brain [6]. In vitro and in vivo studies suggest a possible link between COX-2 expression, degeneration and cell death in AD [15, 17]. Recent data indicate that COX-2 is activated in early phase of AD, before clinical symptoms of dementia appear and before microglia is activated [18]. Also, increases in free fatty acids, eicosanoids, and products of lipid peroxidation are well known to occur early during the progression of AD [38]. These data lead to the hypothesis that COX-2 activation is not only a secondary event related to the inflammation but it is also actively involved in the early stages of neurodegeneration. This early activation of COX-2 may be connected with Aβ oligomers accumulated in the brain, which receive growing attention as the main cause of AD. Neuronal pathology preceding plaque formation has been demonstrated in transgenic mice expressing human mutant presenilin 1, mutant amyloid precursor protein (APP), or both mutant proteins. Recently, it has been found that Aβ oligomers impair memory independently of plaques or neuronal loss [26] and that cytosolic phospholipase A2 (cPLA2) is directly involved in soluble Aβ-mediated release of arachidonic acid and subsequent neuronal apoptosis [24].

It is well documented that inflammation in peripheral tissues induces the synthesis of cytokines, which affect the brain [22]. Systemic inflammation seems to be a very important factor which can modulate neuropathology in AD. It is suggested that the response of microglia is critically dependent on whether it has recently been activated by a previous stimulus. On the other hand, microglia activated during neurodegenerative processes, such as AD, may be more susceptible to the influence of peripheral stimuli [32]. Previously, we demonstrated that systemic inflammation induced oxidative stress that led to molecular damage, energy disturbances and translocation of apoptosis-inducing factor (AIF) from mitochondria to nucleus in the brain [10, 11], suggesting that some proapoptotic pathways may be activated in this condition. Little is known about how systemic inflammation affects the Aβ-induced alterations and whether it has long-lasting effects on disease progression and behavior. The aim of the present study was to elucidate the effect of systemic inflammation on Aβ-evoked locomotor and memory disturbances and the role of COX-2 in Aβ neurotoxicity.

Materials and Methods

Materials

LPS (from E. coli serotype 055:B5; toxicity 15 × 10^6 U/mg), Amyloid β(1–42), DMSO, Tri-zol, Acrylamid, APS, TEMED, BSA, Aβ(1–42), LPS, NS-398 and anti-rabbit IgG were obtained from Sigma, St. Louis, USA. Protease inhibitor cocktail Complete was from Roche Diagnostics GmbH. Vetbutal was from Biowet, Pu³awy, Poland. Anti-COX-2 IgG was from Cayman Chemical, anti-mouse IgG was from Amersham Biosciences, while anti-β-actin antibody was purchased from MP Biomedicals, Inc.

Animals

All the experiments were carried out on male C57BL/6 mice, 12 month old, supplied from Animal House of Medical Research Centre, Warszawa, Poland. The animals were maintained under controlled conditions of temperature and humidity with 12-h light/dark cycle. All experiments on animals were accepted by Polish
National Ethics Committee, and were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Aβ(1–42) was administered intracerebroventricularly (icv) at the dose of 1 nmol per mice. In brief, the mice were anesthetized by intraperitoneal (ip) injection of sodium pentobarbital solution (Vetbutal, 60 mg/kg) and placed in a stereotaxic frame (Stoeling Co., USA). A 1-mm hole was drilled 1 mm posterior to bregma and 1.3 mm lateral. A microsyringe with a 26-gauge stainless-steel needle (Hamilton) was inserted to a 2-mm depth, and Aβ suspended in 5 μl saline was slowly injected for 5 min. Control animals were injected with 5 μl of saline. Some mice also received LPS (1 mg/kg ip, in 100 μl of 0.9% NaCl) directly after Aβ injection. The animals were then returned to their home cage. For drug treatment, mice were administered a single daily injection of NS-398 (5 mg/kg, ip, dissolved in 50% DMSO:PBS mixture) for four days. In another set of experiments, mice were pretreated with LPS (1 mg/kg) two times, at 4 and 7 months of age, and then with 1 nmol of Aβ(1–42), at 12 months of age. One week after Aβ administration all mice were subjected to behavioral tests and decapitated on day 8.

Open field test

Mice were evaluated in an open field test as described by Carter et al. [4]. An individual mouse was placed in an open-topped plywood box 60 × 60 cm square × 30 cm high, with a gray floor marked with white grid lines (divided into 25 squares 12 × 12 cm). During each testing session, a mouse was placed in the central square of the open field and observed for a 5-min period. The following parameters were measured: (1) total number of squares entered (defined as three or more paws moving into a square); (2) total number of peripheral squares entered; (3) total number of central squares entered; (4) number of rears against the wall (standing up on hind legs using the wall for support); (5) number of rears in the periphery without the wall (standing up on hind legs) and (6) number of complete grooming cycles.

Object recognition task

The object recognition task was performed according to Meziane et al. [29] with a slight modification. This task is based on the spontaneous tendency of rodents to explore a novel object [13]. Recognition performance is expressed by a recognition index (RI) representing the relative time spent exploring a novel object and a familiar one during the second trial and is highly dependent on the delay between the two trials. The apparatus was a Plexiglas box (40 × 30 cm) placed in a sound-isolated room with a constant illumination. The objects to be discriminated were china cups and plastic funnels. A day before the testing, mice were submitted to a habituation session, whereby they were allowed to explore the apparatus for 60 min. The next day, they were submitted to a 10-min acquisition trial during which they were individually placed in the open field in the presence of two objects A (cups). The time of exploration of objects A (when the animal’s snout was directed toward the object at a distance of 1 cm or less) was recorded. Five-minute retention trials were carried out 3 h later. During this trial, object A’ (a duplicate of the object A in order to avoid olfactory traits) and another object B (funnel) were placed in the open field, and the times (tA and tB) the animal took to explore the two objects were recorded. A recognition index (RI) was defined as (tB/(tA’ + tB)) × 100. When RI < 50, it was concluded that mouse did not recognize familiar object A’, if RI > 50 the mouse remembered the object.

Western blot analysis of COX-2

The brains were dissected quickly after decapitation and the hippocampus was isolated on ice-cold Petri dish. Hippocampus was homogenized in 10 mM Tris-HCl and mixed with 2x Laemmli sample buffer and denatured for 5 min at 95°C. After a standard SDS-PAGE on 10% polyacrylamide gel, proteins were transferred onto nitrocellulose membrane, and the membrane was washed for 5 min in TBS-Tween (0.05%) and blocked for 60 min in 5% non-fat milk solution in TBS-Tween. After blocking, the membrane was incubated at 4°C overnight in solution of monoclonal anti-COX-2 antibody (1:1000; 160126 Cayman) in TBS-Tween, and washed three times for 5 min in TBS-Tween. Then, the incubation with secondary antibody (1:8000; Anti-rabbit IgG A-0545, Sigma, St. Louis, USA) in 5% non-fat milk/TBS-Tween solution was performed for 60 min, followed by four washing steps (3 × 5 min TBS-Tween, 1 × 5 min TBS). Bound antibody was detected by chemiluminescent reaction (ECL) under standard conditions.
After stripping, β-actin, as loading control, was detected on membranes. A set of incubations was performed, followed by chemiluminescent reaction: 5 min in TBS-Tween, 60 min in 5% non-fat milk/TBS-Tween, 120 min in 0.1% BSA/TBS-Tween containing mouse monoclonal anti-β-actin antibody (1:400; Mab (C4); MP Biomedicals, Inc.), 3 × 5 min TBS-Tween, 60 min in 5% non-fat milk/TBS-Tween containing secondary antibody (1:4000; Anti-mouse IgG NA931V, Amersham Biosciences), 3 × 5 min in TBS-Tween, 5 min in TBS).

**Statistical analysis**

All experiments were carried out at least in triplicate except for behavioral tests for which every group consisted of six mice. The presented data are the means ± SEM. Densitometric analysis and size-markers based verification was performed with TotalLab4 software. For statistical comparison, Student’s t-test and one-way ANOVA followed by Newman-Keuls *post-hoc* test were used, p values < 0.05 were considered statistically significant.

**Results**

Aβ enhanced COX-2 protein level in the hippocampus. COX-2 immunoreactivity was elevated in the hippocampus of mice on day 8 after Aβ(1–42) icv injection. However, COX-2 protein level was unchanged compared to control in the animals which received LPS immediately after Aβ (Fig. 1). This event may be connected with initial LPS-induced COX-2 elevation, which reached four times higher value at 3 h after LPS administration compared to control and decreased almost to control level at 48 h after LPS injection (Fig. 2).

![Fig. 1. COX-2 level in the hippocampus of mice 8 days after Aβ and LPS treatment. Aβ(1–42) (1 nmol/mice) was injected icv alone or together with LPS (1 mg/kg, ip). COX-2 level in the hippocampus was analyzed by Western blot method 8 days after Aβ treatment. Results were normalized to β-actin level, and are presented as the mean ± SEM of three independent experiments. * p < 0.05 compared to control; # p < 0.05 compared to Aβ-treated group](image1)

![Fig. 2. COX-2 level in the hippocampus of mice after LPS treatment. LPS (1 mg/kg) was injected ip. COX-2 level was analyzed by Western blot method 3, 6, 12, 48 h and 8 days after LPS treatment. Results were normalized to β-actin level, and are presented as the mean ± SEM of three independent experiments. *** p < 0.001 compared to control](image2)
Aβ and LPS reduce locomotion and exploration in the open field test

The study indicated the differences in the locomotion and in the level of anxiety between Aβ-treated and untreated animals. Mice treated with Aβ(1–42) alone or together with LPS were significantly less active (Fig. 3A; p < 0.001) and spent less time in central area of box than control animals (Fig. 3B, p < 0.01; p < 0.001, respectively). LPS administration had no effect on Aβ-induced changes. A specific COX-2 inhibitor, NS-398, significantly enhanced the locomotion and exploration of mice treated with Aβ and LPS (Fig. 3A and B, p < 0.001).

The COX-2 inhibitor protected mice from Aβ- and LPS-evoked impairment of object recognition

The effect of Aβ and LPS on memory was analyzed using an object recognition task. The retention was lower in Aβ-treated mice compared to the control mice (Fig. 4, p < 0.05). The injection of LPS immediately after Aβ significantly worsened recognition performance, as revealed by RI lower than 50, which means that the time spent exploring the new object was not significantly different from the exploration time of the familiar object, indicating that animals did not remember the familiar object (Fig. 4, p < 0.05 compared to Aβ-treated group). The selective COX-2 inhibitor significantly improved the memory of mice treated with Aβ and LPS (Fig. 4, p < 0.05).
The effect of LPS pretreatment on locomotion, exploration and recognition of mice after icv administration of Aβ

To analyze whether transient systemic inflammatory episodes during life may influence susceptibility of the brain to Aβ neurotoxicity, systemic inflammation in mice was induced two times, at 4 and 7 months of age, and then at 12 months of age, Aβ(1–42) was injected icv. The effect of Aβ(1–42) icv administration on COX-2 protein level in the hippocampus, locomotion, exploration and recognition of mice pretreated with LPS was analyzed and compared with animals untreated with LPS. COX-2 immunoreactivity in LPS-pretreated mice was unchanged after Aβ administration compared to appropriate control, in contrast to mice untreated with LPS, in which it was significantly enhanced (Tab. 1). LPS-pretreated mice showed similar decrease in open field behavior after Aβ injection as mice untreated with LPS. However, recognition index (RI) of mice pretreated with LPS remained unchanged after Aβ injection, whereas RI of LPS-untreated mice was decreased significantly by Aβ(1–42) (p < 0.05, Tab. 1). These data indicated that mice pre-treated with LPS were resistant to Aβ-induced memory dysfunction.

Discussion

Our data indicated that Aβ(1–42) peptides administered icv induced the increase in COX-2 protein level exclusively in the hippocampus but had no effect on other brain parts (data not shown). Moreover, it was found that systemic inflammation evoked by LPS enhanced COX-2 protein level in the hippocampus 3 h after ip injection and had significant modulatory influence on Aβ-evoked memory deficit. Two different effects of LPS were observed depending on the time of LPS administration in relation to Aβ. LPS administered directly after Aβ injection significantly potentiated the memory impairment evoked by Aβ peptides. A COX-2 inhibitor protected mice from memory deficit and behavioral changes induced by simultaneous injection of Aβ(1–42) and LPS. Moreover, our data indicate that LPS injected 2 times (5 and 8 month before Aβ administration) had an ameliorating effect on Aβ-induced alteration of recognition.

It was previously suggested that systemic inflammation can play a role in the development of AD and brain injury. Although the central nervous system contains the blood-brain barrier (BBB) that obstructs the entry of inflammatory cells, pathogens and some macromolecules into the brain, it has been suggested

<table>
<thead>
<tr>
<th>Analyzed parameters</th>
<th>Untreated with LPS</th>
<th>Pretreated with LPS</th>
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<tbody>
<tr>
<td>COX-2 immunoreactivity</td>
<td>209.30 ± 32.65**</td>
<td>86.25 ± 8.64#</td>
</tr>
<tr>
<td>Total number of squares entered</td>
<td>70.76 ± 12.06*</td>
<td>60.84 ± 13.33*</td>
</tr>
<tr>
<td>Total number of central squares entered</td>
<td>60.91 ± 14.68*</td>
<td>69.58 ± 18.03***</td>
</tr>
<tr>
<td>Recognition index (RI)</td>
<td>77.02 ± 4.45*</td>
<td>95.86 ± 15.98</td>
</tr>
</tbody>
</table>

Two groups of mice, one untreated with LPS and another pretreated with LPS (1 mg/kg, ip) received icv injection of Aβ(1–42) or saline for control (for details, see Materials and Methods) at 12 months of age. Seven days later mice were subjected to behavioral tests: open field test and object recognition task. Mice were decapitated on day 8 and COX-2 level in the hippocampus was analyzed by Western blot method. The table presents the results of COX-2 immunoreactivity analysis, open field test results (total number of squares entered and total number of central squares entered) and object recognition task performance (recognition index RI = t_f/(t_f + t_n) × 100, with t_f and t_n, respectively, representing the time of exploration of familiar and the novel object). Data are presented as percent of appropriate control ± SEM. n = 3 for COX-2 analysis and n = 6 for behavioral tests. *, **, *** p < 0.05, p < 0.01, p < 0.001 respectively, compared to appropriate control. ## p < 0.01 compared to LPS-untreated Aβ-injected group
that proinflammatory cytokines released during systemic inflammation may influence inflammatory processes in the brain and modulate the response to Aβ peptides. There are several routes by which a systemic inflammatory response may influence the central nervous system: cytokines may enter the brain via circumventricular organs, where BBB is absent, they may communicate with microglia through endothelium in intact BBB and they may be actively transported thought BBB [3]. Systemic inflammation seems to be able to switch the cytokine profile in the brain from an anti-inflammatory profile to a pro-inflammatory profile [32]. There are several epidemiological studies that support this hypothesis [7, 33]. It was also observed that cognitive function of AD subjects can be impaired after the resolution of a systemic infection [16]. There are also some studies indicating that an increased production of proinflammatory cytokines and a decreased production of anti-inflammatory cytokines increase the risk of developing AD [40].

Several animal models have been developed to investigate the course of AD pathology. Acute injection or chronic infusion of amyloid peptides into the brain activates astrocytes and microglia [9, 35, 36, 39, 44] and produces profound neurodegeneration [9, 12, 23, 31]. The acute injection model is especially appropriate for the investigation of potential mechanisms of inflammatory response to amyloid β peptides.

In the present study, we used the animal model of memory deficit induced by icv administration of Aβ(1–42) into 12-month-old mice and the model of systemic inflammation induced by ip injection of LPS to determine whether systemic inflammation can exacerbate behavioral and biochemical changes evoked by Aβ peptides. It was observed that mice treated with Aβ(1–42) showed a reduced locomotion and exploration and the decrease was more pronounced in the group treated simultaneously with Aβ and LPS. The recognition was significantly lower in Aβ-treated mice than in the control group, and injection of LPS immediately after Aβ substantially worsened recognition performance. These results clearly demonstrate that the systemic inflammation evoked coincidentally with Aβ-induced pathology can exacerbate Aβ(1–42) induced changes in the brain. The results are consistent with those obtained by Kitazawa et al. [21] demonstrating that microglia activation during systemic inflammation exacerbated neuropathological features in an animal model of AD. It confirms that inflammatory processes in the periphery may modulate brain response to Aβ peptides. However, the effect depends on severity and duration of inflammation. Data suggest that short moderate inflammatory episodes before Aβ peptides neurotoxicity may have some protective effect. Mice pretreated with LPS two times, 5 and 8 month before Aβ administration, showed some behavioral disturbances in open field test but their recognition performance remained unchanged. These results indicate that short inflammation episodes preceding Aβ-induced pathology may exert some type of preconditioning action on the brain.

The mechanism of the observed modulatory effect of systemic inflammation remains to be clarified. An important role of COX-2 is supported by our results, showing that NS-398, a selective COX-2 inhibitor, exerts protective effect against behavioral changes and memory deficit in mice treated with Aβ and simultaneously with LPS. Several epidemiological studies, showing an inverse correlation between treatment with COX inhibitors and the risk of developing AD, supported the role of COX in AD development [19, 25, 37]. However, clinical trials failed to confirm the beneficial effect of most inhibitors [1]. It has been suggested that COX inhibitors are protective only when used at an early stage of AD. Furthermore, non-demented patients treated with NSAIDs show a reduced number of activated microglia when compared to non-treated patients [27], indicating that COX inhibition exerts its action on AD brains through the suppression or prevention of microglial activation. It is also possible that a long-lasting anti-inflammatory treatment during epidemiologic research protects the brain from inflammatory processes in periphery and decreases the number of inflammatory factors affecting the brain. It was previously observed that hippocampal levels of pro-inflammatory markers (IL-1β, TNF-α, PGE2) increase with the age and chronic oral treatment with celecoxib, a selective COX-2 inhibitor prevented age-dependent augmentation of inflammatory processes [5].

In our model, the level of COX-2 in the hippocampus analyzed at 8 days after Aβ injection was increased, but was not altered in the group treated simultaneously with Aβ and LPS, suggesting a modulatory effect of systemic inflammation on Aβ-evoked changes. However, a protective effect of the COX-2 inhibitor on memory deficit in mice treated with Aβ and LPS confirms the role of COX-2. Our data demonstrated that LPS induced a pronounced increase in COX-2 level in the brain 3 h after the treatment,
which returned to the control level after 48 h. The increase in COX-2 activity could accelerate and enhance the inflammatory response to Aβ peptides and contribute to molecular alterations and memory deficit. Recent data indicate that COX-2 is activated in the early phase of AD, before clinical symptoms of dementia appear and before microglia is activated, and its level decreases in advanced stages of AD [18]. It was also observed that COX-2 mediates microglial activation and induces neuronal loss [34, 42]. Interestingly, we did not observe an increase in COX-2 protein level after Aβ injection in the groups of animals pretreated with LPS, which also showed a lack of memory deficit after Aβ(1–42) icv injection. It suggests that some protection against molecular events leading to memory disturbances was associated with earlier processes induced by systemic LPS administration.

In conclusion, on the basis of our results, we suggest that systemic inflammation leads to the exaggeration of Aβ toxicity or to brain protection depending on time, duration and severity of inflammation. NS-398 prevents memory deficit induced in mice by Aβ treatment together with LPS.

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