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Inflammatory molecule release by β-amyloid-treated T98G astrocytoma cells: role of prostaglandins and modulation by paracetamol

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Abstract

Deposition of β-amyloid in the brain triggers an inflammatory response which accompanies the neuropathologic events of Alzheimer's disease and contributes to the destruction of brain tissue. The present study shows that β-amyloid can stimulate human astrocytoma cells (T98G) to secrete the proinflammatory factors interleukin-6 and prostaglandins. Furthermore, prostaglandins can stimulate T98G to secrete interleukin-6, which in turn triggers the formation of additional prostaglandins. Prostaglandins are, therefore, a key element in the induction and maintenance of a state of chronic inflammation in the brain which may exacerbate the fundamental pathology in Alzheimer patients. Paracetamol (0.01–1000 μM), an unusual analgesic/antipyretic drug which acts preferentially by reducing prostaglandin production within the central nervous system, and indomethacin (0.001–10 μM) caused a clear dose-dependent reduction of prostaglandin E_2 production by stimulated T98G cells whereas interleukin-6 release was not affected. These data provide further evidence of the involvement of non-steroidal anti-inflammatory drugs in the inflammatory processes that can be generated by glial cells in intact brain.

Keywords: Prostaglandin; Interleukin-6; Non-steroidal anti-inflammatory drug; Alzheimer's disease

1. Introduction

The pathologic elements typically found in the autopsy analysis of the brains of patients suffering from Alzheimer's disease are loss of nerve cells, fibers and synapses and the presence of neurofibrillary tangles and neuritic or senile plaques (Price, 1986). The plaques are formed following an excessive deposition of β-amyloid, a 39- to 43-amino acid peptide derived from alternative cleavage of a large transmembrane protein, the β-amyloid precursor protein (Masters et al., 1985; Hardy and Allsop, 1991; Selkoe, 1994).

Recent findings on increased in vitro neurotoxic effects of concentrated solutions of 'aged' β-amyloid and physico-chemical studies on β-amyloid peptide led to the hypothesis that the most important stage in the pathogenesis of Alzheimer's disease is the transformation of soluble β-amyloid into highly insoluble filamentous aggregates due to the formation of a β-sheet secondary structure (Pike et al., 1991; Cotman et al., 1992; Lorenzo and Yankner, 1994; Pollack et al., 1995). However, the presence of large β-amyloid deposits and neurofibrillary tangles in clinically nondemented subjects indicates that these hallmarks alone are insufficient to sustain the neurodegenerative process (Masliah et al., 1993). Thus, two main hypotheses have been advanced to explain the mechanism linking amyloid plaques to dementia: β-amyloid acts as a potent neurotoxic agent (Yankner et al., 1990) or, alternatively, the plaques elicit a cascade of cellular events leading to neurodegeneration (Davies, 1994; Guilian et al., 1995). Over the past few years, activated microglial cells have been detected within or around Alzheimer lesions and have been shown to produce several growth factors and inflammatory mediators (Eddleston and Mucke, 1993; McGeer and McGeer, 1995) as well as oxygen free radicals (Colton and Gilbert,

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1987). Furthermore, several inflammatory markers which have been found in close association with neuritic plaques (reviewed in McGeer and McGeer, 1995) are absent or weakly expressed in brain samples from nondemented elderly individuals. These observations provide the basis to consider inflammation as the final common pathogenetic pathway through which β-amyloid deposition, neurofibrillary tangle formation and other deleterious events lead to the loss of nerve cells and their connections in Alzheimer patients (Aisen and Davis, 1994; Rogers, 1995). In fact, according to this 'inflammation theory', β-amyloid would exacerbate local foci of inflammation that may result from trauma, ischemia or infections, by stimulating glial cells to release inflammatory mediators which are then found in Alzheimer autopsy samples.

Relevant support to this theory is provided by recent epidemiological studies which showed that prolonged anti-inflammatory therapy confers some degree of protection against the start of Alzheimer's disease (McGeer et al., 1990; Breitner et al., 1994; Andersen et al., 1995; Stewart et al., 1997). Furthermore, a double-blind clinical trial involving Alzheimer patients treated for 6 months with the non-steroidal anti-inflammatory drug (NSAID), indomethacin, showed positive results (Rogers et al., 1993).

Paracetamol or acetaminophen is a widely used antipyretic and analgesic drug with low peripheral anti-inflammatory activity (Clissold, 1986). This compound can easily penetrate the blood-brain barrier and, although its mechanism of action is still debated, there is evidence that it acts preferentially by reducing prostaglandin production within the central nervous system (Flower and Vane, 1972; Marshall et al., 1987). In fact, unlike the majority of the other NSAIDs, paracetamol blocks the cyclooxygenase enzyme only in an environment which is low in peroxides. This condition is present in the brain but not in sites of inflammation in the periphery, where recruited leukocytes generate high concentrations of cellular peroxides (Hertz and Cloarec, 1984). This unusual characteristic makes paracetamol a potentially interesting molecule for the treatment of the inflammatory components of Alzheimer-type neurodegeneration.

Notwithstanding the evidence reported on the possible beneficial effects of anti-inflammatory therapy in Alzheimer patients, no systems have been so far proposed for the search for anti-inflammatory compounds to be specifically used in this pathology. The present paper describes the characterization and the pharmacological validation of an in vitro model which might be useful in the search for anti-inflammatory molecules for the treatment of Alzheimer's disease. The human astrocytoma cell line T98G was used to study the role of β-amyloid in the production of inflammatory mediators from astrocytic cells and to dissect the different steps of the inflammatory loop which may develop in an Alzheimer brain. This study allowed us to identify prostaglandins as possible key players in the induction and maintenance of an inflammatory state within the central nervous system. This model was then exploited to analyze the effects of paracetamol and indomethacin on the production of inflammatory mediators from astrocytoma cells treated with amyloid β protein or with other relevant stimuli. The overall results of our work indicate that NSAIDs may beneficially interfere with the inflammatory loop induced in the central nervous system by β-amyloid deposition, thus confirming the validity of the rationale on which the anti-inflammatory approach to Alzheimer's disease is based.

2. Materials and methods

2.1. Cell culture

T98G, a human glioblastoma cell line, was purchased from ECACC (Salisbury, UK). The astrocytic origin of this cell line was confirmed by assessing the expression of its specific marker, the glial fibrillary acidic protein (Bignami et al., 1972). T98G cells are routinely maintained in Dulbecco's modified Eagle's medium (DMEM) (Flow Labs, Irvine, Scotland) supplemented with 10% fetal bovine serum (HyClone, Logan, USA), 1% penicillin–streptomycin solution (HyClone), 1% non-essential amino acid solution (Sigma, St. Louis, USA), 1 mM sodium pyruvate (Sigma) and 2% L-glutamine (HyClone). The cultures were placed in a humidified 5% CO_2 atmosphere at 37°C. Cells were harvested for culture passages with 0.05% trypsin and 0.02% EDTA solution (Sigma) in Dulbecco's phosphate-buffered salt (PBS) without Ca^{2+} and Mg^{2+} (Flow).

2.2. Cell stimulation assay

Cells were plated at 1×10^6 cells/well in 24-well flat-bottomed plates (Falcon BD, Bedford, USA) and were incubated at 37°C in 1 ml of serum-free DMEM containing various concentrations of the different stimuli: 0.2 ng/ml recombinant human interleukin-1β (Genzyme, West Malling, UK), 35–3500 ng/ml prostaglandin E_2 (Sigma), 5–500 ng/ml recombinant human interleukin-6 (Genzyme), or 10–50 μM 'aged' β-amyloid (amino acids 1–40) (Bachem, Bubendorf, Switzerland). 'Aged' β-amyloid was obtained by incubating the peptide in sterile double-distilled water for 6 days at 37°C as a 1 mM stock solution. After 48 h, the supernatants were collected and immediately analyzed or frozen at −80°C until used. Paracetamol or indomethacin was used at the concentration of 0.01–1000 μM and 0.001–10 μM, respectively. Stock solutions of test molecules were prepared in dimethylsulfoxide (Merck, Darmstadt, Germany) at a concentration of 1 M. Parallel control experiments were performed in order to exclude cytotoxic effects of the drugs, which were found to be safe at the above concentrations. Each experiment was repeated three times and the treatments were carried out in duplicate.

2.3. Interleukin-1β, interleukin-6 and prostaglandin E_2 determination

The content of interleukin-1β, interleukin-6 and prostaglandin E_2 in the supernatant was measured by means of commercially available specific immunoassay kits (Amersham, Salisbury, UK) following the manufacturer's instructions. Optical density values were measured in a Titertek Multiskan Plus apparatus (Flow) at a wavelength of 450 nm. The assay sensitivity was <1 pg/ml for interleukin-1β and interleukin-6 and 40 pg/ml for prostaglandin E_2. All measurements were performed in duplicate.

2.4. Measurement of interleukin-1β, interleukin-6 and cyclooxygenase mRNA abundance

In order to evaluate the mRNA levels in treated and untreated cells, 10^7 T98G cells were plated in 25-cm^2 flasks and incubated in a humidified 5% CO_2 atmosphere at 37°C. After 24 h, the medium was removed and cells were incubated with 2 ml of medium containing different stimuli (3500 ng/ml prostaglandin E_2, 50 μM β-amyloid, 500 ng/ml interleukin-6, 0.2 ng/ml interleukin-1β) or medium alone. Treatments were carried out at 37°C for 4 h. The cells were then washed in PBS and the cellular pellet was stored at −80°C. The isolation of mRNA from T98G cells was performed by using a Micro-Fast Track kit (Invitrogen, Leek, The Netherlands) following the manufacturer's instructions.

The evaluation of mRNA abundance was performed by reverse-transcription polymerase chain reaction (RT-PCR). In detail, 1 μg of each mRNA was reverse transcribed into cDNAs using random hexamers included in the First-Strand cDNA Synthesis kit (Pharmacia, Uppsala, Sweden) following the instructions supplied by the manufacturer, and 2 μl of each cDNA was used for PCR amplification. Specific primers for interleukin-1β (sense 5′-AAACAgATgAAgTgCTCCTTCCAgg-3′, antisense 5′-TggAgAACACCACTTgTTgCTCCA-3′), interleukin-6 (sense 5′-ATgAACTCCTTCTCCACAAgCgC-3′, antisense 5′-gAAgAgCCCTCAggCTggACTg-3′), cyclooxygenase-1 (sense 5′-TgCCCAgCTCCTggCCCgCCgCTT-3′, antisense 5′-gTgCATCAACACAggCgCCTCTTC-3′), cyclooxygenase-2 (sense 5′-TTCAAATgAgATTgTgggAAAATTgCT-3′, antisense 5′-AgATCATCTCTgCCTgAgTATCTT-3′) and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (sense 5′-TgAAggTCggAgTCAACggATTTggT-3′, antisense 5′-CATgTgggCCATgAggTCCACCAC-3′) were synthesized by Primm (Milan, Italy). The RNA for G3PDH was amplified and used as internal control. In order to avoid a plateau effect preliminary studies were carried out with 21, 25, 27, 29, and 33 cycles. Amplifications were performed with Taq-polymerase (Promega, Madison, USA) in a DNA Thermal Cycler (Perkin Elmer-Cetus, Beaconsfield, UK). Interleukin-1β and interleukin-6 were amplified for 25 cycles (30 s at 95°C, 30 s at 55°C, 60 s at 72°C) while cyclooxygenase-1 and -2 were amplified for 27 cycles (90 s at 95°C, 90 s at 58°C, 90 s at 72°C).

Amplified products were electrophoresed on 2% agarose gels in order to evaluate the amounts of specific cDNAs with respect to the housekeeping gene G3PDH. Band intensity was measured and quantified using a densitometer (Molecular Dynamics, Sunnyvale, USA) and analyzed using the software Image Quant 3.3. The relative rates of G3PDH PCR specific products and test products were determined by the ratio of integrated volumes. Data were then analyzed using Excel software.

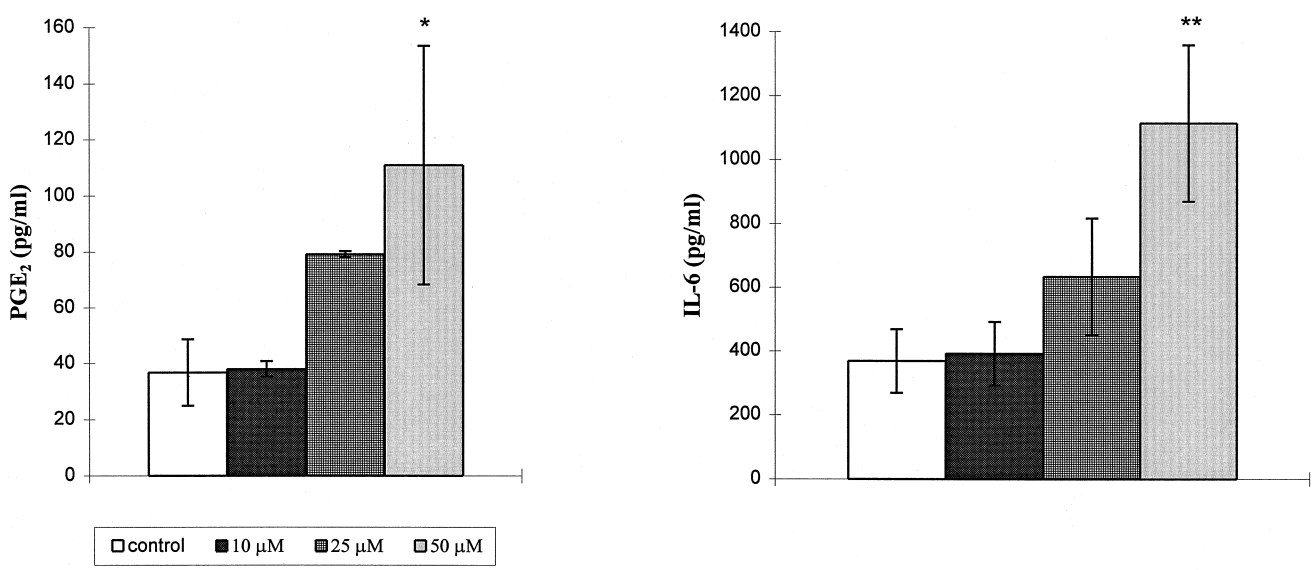


Fig. 1. Secretion of prostaglandin E_2 (PGE_2) and interleukin-6 (IL-6) by T98G cells incubated with increasing concentrations of 'aged' β-amyloid. Data are presented as the means ± S.D. of three different experiments. Asterisks denote statistically significant differences between untreated control cells and cells incubated with β-amyloid. $*\ P < 0.05$, $**\ P < 0.01$(Tukey's test).

Table 1
Effect of fresh β-amyloid (Aβ) on prostaglandin E_2 (PGE_2), and interleukin-6 (IL-6) production by T98G cells[a]

	PGE_2 (pg/ml)	IL-6 (pg/ml)
Medium	40 ± 15.1	312 ± 129.6
Fresh Aβ (50 μM)	41 ± 27.6	459 ± 179.3

[a]Results are presented as the mean ± S.D. of two separate experiments.

2.5. Data analysis

The differences between the amount of factors released by the cells in the presence of the different stimuli and that measured in untreated control samples were statistically analyzed by Tukey's test according to Tallarida and Murray (1987), using the computer program PHARM/PCS.

3. Results

3.1. Effects of inflammatory mediators on interleukin-1β, interleukin-6 and prostaglandin E_2 secretion

T98G astrocytoma cells were incubated with different concentrations of β-amyloid, interleukin-1β, prostaglandin E_2 and interleukin-6 and the release of interleukin-1β, prostaglandin E_2 and interleukin-6 was determined in the supernatants.

β-Amyloid was 'aged' for 6 days at 37°C in order to promote the formation of aggregates, whose appearance was confirmed by microscope observations (Shearman et al., 1994). As in preliminary experiments, β-amyloid toxicity was observed, starting at the concentration of 100 μM. Confluent T98G cells were incubated for 48 h with 10, 25 and 50 μM of the 'aged' 1–40 fragment of β-amyloid and the release of inflammatory factors was assessed by immunoenzymatic assay. Fig. 1 shows the increase in prostaglandin E_2 and interleukin-6 secretion observed over basal values in 'aged' β-amyloid-treated cells in comparison to untreated controls. The aggregated 1–40 fragment induced a dose-dependent increase in prostaglandin E_2 and interleukin-6 release from T98G cells, an increase which was statistically significant at 50 μM. The specificity of this effect was confirmed by the observation that the cells cultured for 48 h in the presence of 'fresh' β-amyloid (50 μM) released prostaglandins and interleukin-6 in amounts comparable to basal release (Table 1). As expected, similar results were obtained when T98G cells were stimulated with 0.2 ng/ml of recombinant human interleukin-1β, which significantly ($p < 0.01$) increased basal levels of prostaglandin E_2 (from 66 to 2309 pg/ml) and interleukin-6 (from 345 to 35 864 pg/ml).

The finding that β-amyloid can stimulate prostaglandin secretion prompted us to study the role of prostaglandins in the release of certain inflammatory mediators from glial cells. The capacity of prostaglandin E_2 to stimulate the production of interleukin-6 was thus investigated. Confluent T98G cells were treated for 48 h with graded doses of purified prostaglandin E_2 (35, 350 and 3500 ng/ml) and the level of interleukin-6 was assayed in the supernatants. This treatment induced T98G cells to increase the basal secretion of interleukin-6 in a dose-dependent and statistically significant way (Fig. 2, panel A).

As prostaglandin E_2 was consistently observed to stimulate interleukin-6 production, T98G cells were treated for

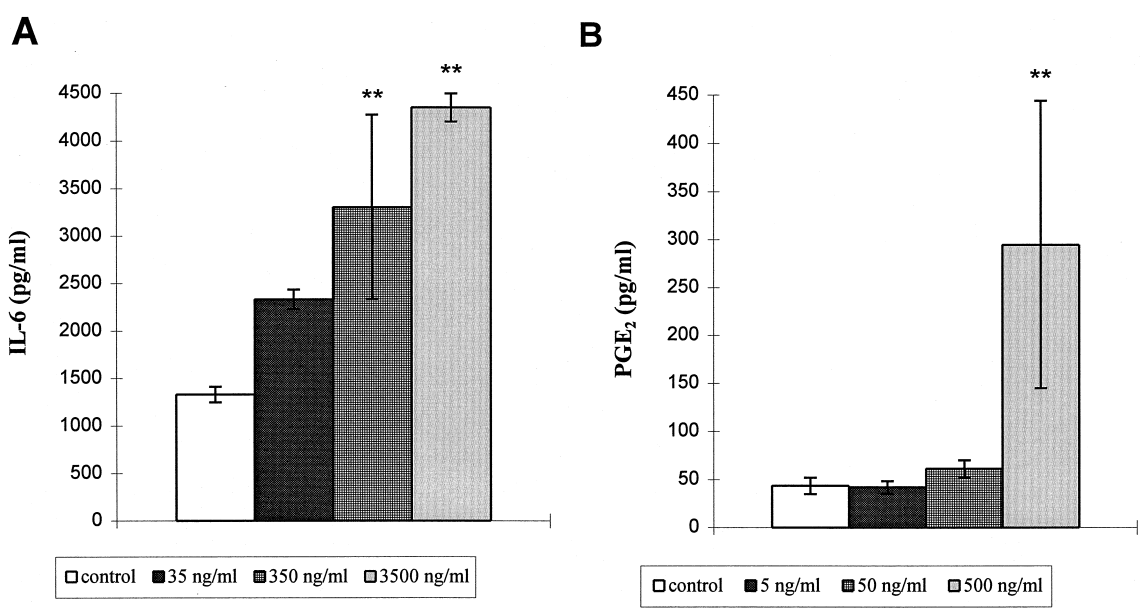
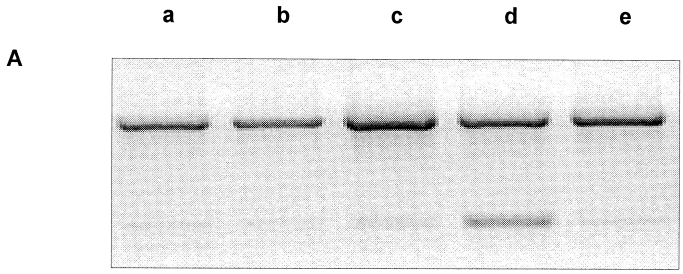
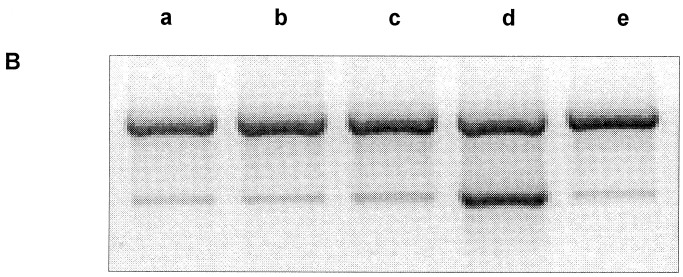


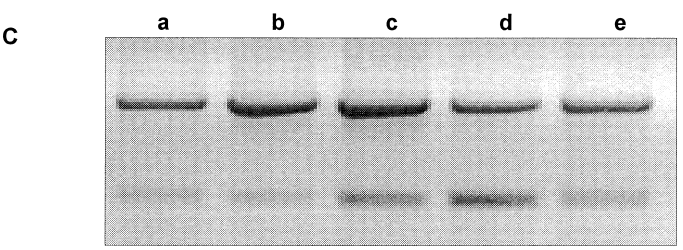
Fig. 2. Secretion of interleukin-6 (IL-6) and prostaglandin E_2 (PGE_2) by T98G cells treated with increasing concentrations of prostaglandin E_2 (panel A) or interleukin-6 (panel B). Data are presented as the means ± S.D. of three different experiments. Asterisks denote statistically significant differences between untreated control cells and cells incubated with the relevant stimuli. ** $P < 0.01$(Tukey's test).



d.u.	17241	16619	33026	24051	24680	G3PDH
d.u.	1914	1813	3095	9958	2362	IL-1 β
ratio	1.00	0.98	0.84	3.73	0.86	IL-1 β increase



d.u.	23529	29415	30979	30503	30992	G3PDH
d.u.	2855	2933	4423	27280	2669	IL-6
ratio	1.00	0.82	1.18	7.37	0.71	IL-6 increase



d.u.	20813	31575	37341	20534	19225	G3PDH
d.u.	4114	3717	13738	16929	6407	COX-2
ratio	1.00	0.60	1.86	4.17	1.69	COX-2 increase

Fig. 3. Effects of different stimuli on levels of mRNA for interleukin-1β (IL-1β—panel A), interleukin-6 (IL-6—panel B) and cyclooxygenase-2 (COX-2—panel C) in T98G cells. (Lane a) Untreated cells; (lane b) β-amyloid (50 μM); (lane c) prostaglandin E_2 (3500 ng/ml); (lane d) interleukin-1β (0.2 ng/ml); (lane e) interleukin-6 (500 ng/ml). Corresponding G3PDH mRNA levels are shown for each sample. Arbitrary densitometry units (d.u.) are reported under each picture. mRNA increase for each sample was determined by calculating the ratio between the specific d.u. and the corresponding G3PDH d.u. after normalization vs. untreated cell d.u. Representative of two separate experiments.

48 h with increasing concentrations of purified human recombinant interleukin-6 (5, 50 and 500 ng/ml). Results shown in Fig. 2 (panel B) indicate that interleukin-6, at the highest concentration, induced T98G cells to release also consistent prostaglandin E_2 in concentrations statistically higher than those released by untreated control cells.

In these same experiments (except those in which interleukin-1β was used as stimulus), the interleukin-1β concentration in the supernatants was also measured but the levels of this cytokine were constantly found to be well below the detection limits of the assay (< 1 pg/ml).

3.2. Effects of inflammatory mediators on interleukin-1β, interleukin-6 and cyclooxygenase-1 and -2 mRNA levels

To determine whether the increase in interleukin-6 and prostaglandin E_2 secretion depended on de novo protein synthesis or whether it was the result of release from intracellular stores, the expression of mRNA for interleukin-1β, interleukin-6, cyclooxygenase-1 and cyclooxygenase-2 was analyzed by RT-PCR using mRNA extracted from cells stimulated with 50 μM β-amyloid, 0.2 ng/ml interleukin-1β, 3500 ng/ml prostaglandin E_2, or 500 ng/ml interleukin-6 and from untreated controls. G3PDH mRNA levels, as indicated by a specific 983 bp band, were used as internal controls.

The results of RT-PCR experiments shown in Fig. 3A indicate that T98G cells constitutively express basal levels of interleukin-1β mRNA because an interleukin-1β-specific band (388 bp) could be visualized after 25 PCR cycles in untreated cells. Furthermore, the interleukin-1β mRNA concentration was markedly increased following treatment with interleukin-1β whereas β-amyloid, prostaglandin E_2 or interleukin-6 stimulation did not modify the basal level of interleukin-1β mRNA. The results of the analysis of interleukin-6 mRNA abundance after 25 cycles are illustrated in Fig. 3B. Again, T98G cells appeared to express constitutive amounts of interleukin-6 mRNA (628 bp) and this expression was increased after stimulation with interleukin-1β. No effects on interleukin-6 mRNA concentration were observed following treatment with interleukin-6, prostaglandin E_2 or β-amyloid. Densitometric analysis of the gels confirmed that only interleukin-1β stimulation induced an increase in interleukin-1β and interleukin-6 mRNA levels. When the mRNA levels for cyclooxygenase-1 were analyzed, no transcripts could be visualized either in resting or treated cells after up to 29 PCR cycles, indicating that the cyclooxygenase-1 enzyme is absent or very weakly expressed in T98G cells (data not shown). In contrast, cyclooxygenase-2 mRNA (Fig. 3C) was present in unstimulated cells because a specific band could be observed after 27 cycles at 305 bp. Cyclooxygenase-2 mRNA levels were consistently increased in interleukin-1β-treated cells whereas only minor modifications were observed following exposure to interleukin-6, prostaglandin E_2 or β-amyloid. However, it is worth noting that the cyclooxygenase-2 mRNA levels determined by densitometric analysis in the different samples correlated with the amounts of prostaglandin E_2 measured in the corresponding supernatants: interleukin-1β ≫ interleukin-6 > β-amyloid > untreated cells.

3.3. Effects of paracetamol on β-amyloid-, interleukin-1β- and interleukin-6-induced inflammatory markers

Confluent T98G cells were incubated with several concentrations of paracetamol (0.01–1000 μM) or indomethacin (0.001–10 μM) in the presence of interleukin-1β (0.2 ng/ml), β-amyloid (50 μM), or interleukin-6 (500 ng/ml). After 48 h, the supernatants were collected and utilized for the determination of prostaglandin E_2 and interleukin-6 levels. The results of the experiments in which the inhibiting effects of indomethacin and paracetamol on prostaglandin E_2 release were evaluated are sum-

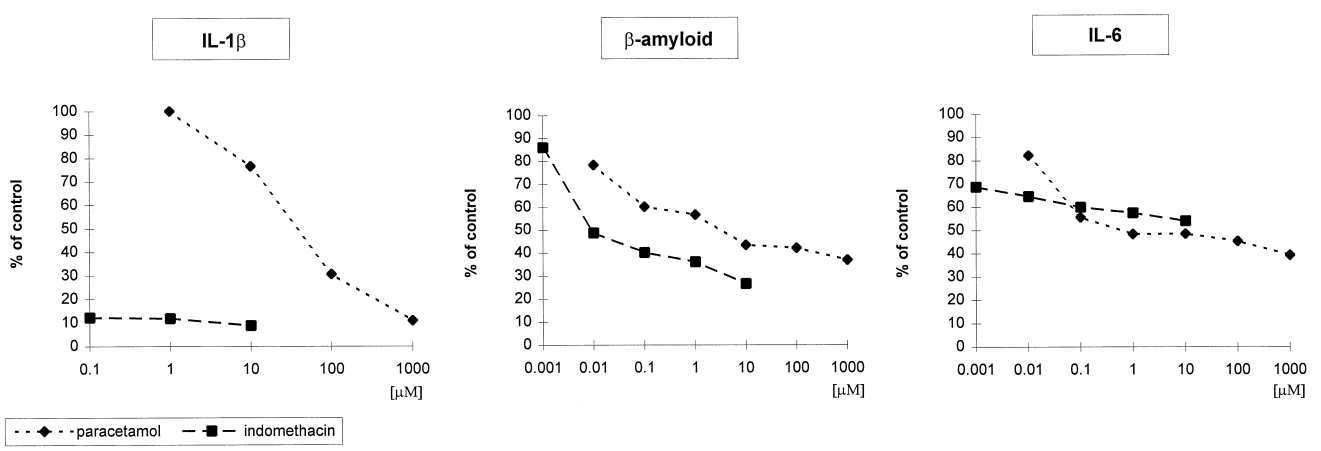


Fig. 4. Effects of indomethacin and paracetamol on prostaglandin E_2 production by stimulated T98G cells. Prostaglandin E_2 was measured in the supernatants of T98G stimulated with interleukin-1β (IL-1β—0.2 ng/ml), β-amyloid (50 μM) or interleukin-6 (IL-6—500 ng/ml) with or without graded concentrations of the drugs. Data are presented as the percentage release with respect to the amount of prostaglandin E_2 released by the cells stimulated in the absence of the drugs and represent the mean of three different experiments (S.D. < 15%).

marized in Fig. 4. The results are expressed as the percentage release with respect to the amount of prostaglandin E_2 released by cells stimulated in the absence of drugs. Data show that paracetamol and indomethacin had a similar pattern of inhibitory activity, even though these effects were exerted at different concentrations. Both drugs, in fact, consistently reduced prostaglandin E_2 production and their effect appeared to be more pronounced when prostaglandin E_2 release was stimulated by interleukin-1β than by β-amyloid or interleukin-6. No significant effects were exerted by indomethacin and paracetamol on interleukin-6 production although a slight inhibition (about 30% of the control) was seen with both drugs at the highest concentration assayed (data not shown). Parallel control experiments were set up to exclude toxic effects of the drugs: no reduction in cell viability was measured by crystal violet staining after a 48-h incubation with either drug in the presence of the different stimuli.

4. Discussion

Although consensus on the primary mechanism of neuronal degeneration in Alzheimer's disease has not been reached, it has been recently observed and it is now generally accepted that β-amyloid, once aggregated into amyloid fibrils, can trigger a series of events which result in the activation of microglial cells and the subsequent production and release of a series of classical inflammatory mediators which have the capacity to damage neuronal cells (Eikelenboom et al., 1994). Thus, microglial cells have become a possible cellular target for pharmacological approaches to Alzheimer's disease (Wood, 1994), and compounds able to reduce the local abnormal inflammatory response mediated by activated glial cells and by the upregulated complement system may have a potential role in the treatment of the disease (Aisen and Davis, 1994).

Breitner (1996) proposed that cyclooxygenase inhibition is the key process of anti-inflammatory interventions in Alzheimer's disease. In vitro findings, epidemiological observations and clinical trials, in fact, support the hypothesis that inhibition of cyclooxygenase activity may decrease microglial activation and antagonize the cytokine signal transduction cascades that result in the activation of astrocytes subsequent to microglial activation. Since prostaglandin production is the main target of the prophylactic anti-inflammatory treatments reported to be potentially effective in delaying the onset of Alzheimer's disease (McGeer et al., 1990; Breitner et al., 1994), the aim of the present work was to clarify the role of prostaglandins in a β-amyloid-dependent model of central nervous system inflammation and to study the effects of paracetamol, an atypical cyclooxygenase inhibitor that preferentially acts in the central nervous system, on the release of inflammatory mediators in this model.

The importance of interleukin-1β in triggering and sustaining inflammatory processes within the central nervous system has been clearly demonstrated. High levels of interleukin-1β have been measured in autopsy brain samples from Alzheimer patients (Griffin et al., 1989), and treatment of human astrocytoma and glioma cell lines with interleukin-1β results in the synthesis of interleukin-6 (Cinque et al., 1992; Cadman et al., 1994), tumor necrosis factor-α (Bethea et al., 1992), interleukin-8 and monocyte chemotactic and activating factor (MCAF) (Kasahara et al., 1991). Moreover, interleukin-1β can induce the expression of adhesion molecules for leukocytes on glial cells (Moynagh et al., 1994; Rosenmaln et al., 1995) and the secretion of several factors of the complement system (Walker and McGeer, 1993; Barnum et al., 1993) as well as prostaglandin production (Watanabe et al., 1990). Our results confirm these observations and, in addition, they indicate that β-amyloid per se can directly trigger an inflammatory response, since β-amyloid-treated T98G cells released interleukin-6 and prostaglandins. Consequently, they would also allow us to speculate that β-amyloid deposits in Alzheimer brains may directly activate astrocytes and microglial cells to release inflammatory factors which, in turn, contribute to neurodegeneration. Our data are not conclusive on this point because it cannot be completely excluded that the effects of β-amyloid are mediated through the release of a very small amount of interleukin-1β; however, the absence of detectable interleukin-1β in the supernatants and the lack of a detectable increase in interleukin-1β mRNA levels in β-amyloid-treated cells allow us to speculate that β-amyloid has direct proinflammatory activity on T98G cells. In this case, the release of interleukin-6 and prostaglandins would be subsequent to the activation of different types of mechanisms, such as direct phospholipase A_2 activation or Ca^{2+} mobilization. This hypothesis is in partial agreement with the observations of Gitter et al. (1995) that showed that β-amyloid can potentiate interleukin-1β-induced interleukin-6 secretion and that this phenomenon is mediated by Ca^{2+} fluxes because the potentiating effect of β-amyloid can be inhibited by ethylene glycol tetraacetic acid (EGTA) whereas EGTA treatment does not affect the interleukin-1-stimulated secretion of interleukin-6. Ca^{2+} mobilization was thus suggested to be the initial event in the signalling mechanism of β-amyloid. In agreement with this report, our data on mRNA levels seem to exclude the capacity of β-amyloid to directly induce mRNA synthesis and tend to indicate that a different mechanism is activated.

These findings strongly support the involvement of prostaglandins in the induction and maintenance of an inflammatory state in Alzheimer patients. The T98G glioma cells consistently released prostaglandin E_2 upon stimulation with interleukin-1β or with 'aged' β-amyloid, together with the proinflammatory cytokine interleukin-6. Furthermore, this interleukin-1β- or β-amyloid-dependent

interleukin-6 secretion might be at least partially mediated by prostaglandins since prostaglandin E_2 induced interleukin-6 release by T98G cells. In addition, stimulation with high concentrations of interleukin-6 (500 ng/ml) triggered the release of prostaglandin E_2. Even though the amounts of prostaglandin E_2 or interleukin-6 released by stimulated T98G were 10- to 100-fold lower than those necessary to elicit inflammatory factor release in our system, it is worth stressing that in inflammatory processes, the in situ concentration of the different mediators can reach very high values. It is, therefore, conceivable that at the site of inflammation, interleukin-6 and prostaglandin E_2 can accumulate in concentrations high enough to trigger the release of additional factors which, in turn, can act as new stimuli. An overall analysis of these results allows us to hypothesize the presence, in Alzheimer patients, of an inflammatory loop in which prostaglandins produced by stimulated glial cells play a central role by modulating the release of the proinflammatory cytokine interleukin-6, which, in turn, can induce T98G to secrete additional prostaglandins. A vicious cycle is thus set up which maintains glial cells in an activated state, a situation in which they produce and release a series of potentially toxic products, such as glutamate (Bezzi et al., 1998), that can be the cause of the neuronal damage typically found in Alzheimer's disease. The release of prostaglandins by activated glial cells and their capacity to induce the secretion of inflammatory cytokines represent intermediate steps which fit in well with the proposed inflammation theory for the pathogenesis of Alzheimer's disease.

RT-PCR results established that the prostaglandin E_2 measured in the medium of untreated and stimulated T98G cells was almost exclusively produced through the action of cyclooxygenase-2. This observation was confirmed by Northern blot analysis, which revealed very low levels of mRNA for cyclooxygenase-1 both in resting and in stimulated cells (data not shown). The lack, or at least the very low amounts, of cyclooxygenase-1 is in agreement with studies showing that the basal level of the cyclooxygenase-2 isoform in the brain is much higher than in most other tissues (Yamagata et al., 1993). mRNA analysis of the cyclooxygenase-2 gene in stimulated cells indicated that whereas interleukin-1β, interleukin-6 and, very interestingly, prostaglandin E_2 themselves induced the de novo synthesis of cyclooxygenase-2, the prostaglandin E_2 release observed following β-amyloid treatment was not related to an increase in cyclooxygenase-2 mRNA but probably depended on disturbances exerted by β-amyloid at the membrane level.

Over the past few years, preclinical, epidemiological and clinical evidence has been presented (Ferrari et al., 1990; McGeer et al., 1990; Rogers et al., 1993; Breitner et al., 1994) that nonsteroidal anti-inflammatory agents, whose main effect is the reduction of prostaglandin production by inhibition of cyclooxygenase-1 and/or -2, may significantly delay the onset or, alternatively, slow the progress of Alzheimer's disease. On the basis of these data and our experimental results for T98G cells, we investigated the effects of indomethacin and paracetamol on the different steps of the inflammatory loop which might be established in the central nervous system of Alzheimer patients. Paracetamol is an unusual analgesic and antipyretic compound which penetrates the central nervous system and reportedly reduces prostaglandin production in the brain but not in spleen homogenates (Flower and Vane, 1972; Marshall et al., 1987). Indomethacin was chosen as the reference drug since it is the only NSAID successfully tested in a clinical trial involving Alzheimer patients (Rogers et al., 1993). The data in the present work indicated that paracetamol (0.01–1000 μM) is effective in inhibiting prostaglandin production induced by stimulation with interleukin-1β, β-amyloid or interleukin-6. Similar results were obtained when the cells were treated with indomethacin at lower concentrations (0.001–10 μM). In this regard, it should be stressed that the relatively high concentrations of paracetamol required to inhibit prostaglandin production are normally found in brain tissues following the administration of the drug at therapeutic doses (1–4 g/day) (Prescott, 1980). Indomethacin and paracetamol completely inhibited prostaglandin E_2 production when the cells were stimulated with interleukin-1β and this effect was dose-dependent. In contrast, the reduction in prostaglandin E_2 concentration observed in the supernatant of interleukin-6- or β-amyloid-treated T98G cells did not exceed 70%. Several different explanations can be advanced for the treatment-related behavior of paracetamol and indomethacin: the inhibitory potential of the drugs may be more pronounced when the rate of prostaglandin E_2 production is elevated or when cyclooxygenase-2 is maximally induced by prostaglandin E_2 in a self-sustained fashion. It cannot be excluded that paracetamol might directly affect the levels of cycloxygenase-2 protein induced by interleukin-1β in T98G cells, as reported by Tordjman et al. (1995) for mouse resident peritoneal macrophages. In addition, the shape of the indomethacin and paracetamol inhibition curves for β-amyloid- or interleukin-6-induced prostaglandin E_2 production suggests the possible involvement of a non-cyclooxygenase-dependent mechanism for these compounds at higher doses. Paracetamol, for instance, may act as a 'free-radical scavenger' or as an oxidizable substrate for peroxides, and by trapping these radicals paracetamol may also lower cyclooxygenase activity (Hertz and Cloarec, 1984). As expected, neither paracetamol nor indomethacin consistently modified interleukin-6 release from T98G cells. Thus, the inhibitory effect of paracetamol on prostaglandin production induced by different stimuli, including β-amyloid, makes this drug potentially capable of interrupting the inflammatory loop which develops following β-amyloid deposition in the brain and, therefore, makes the drug potentially interesting for the treatment of Alzheimer's disease. Although no association was recently

found between the use of paracetamol and the risk of Alzheimer's disease (Stewart et al., 1997), such epidemiological data do not exclude a priori the hypothesis of the therapeutic potential of long-term consecutive treatment with paracetamol in reducing symptoms and progression of Alzheimer's disease. Only clinical trials will allow us to verify the clinical efficacy of paracetamol in Alzheimer's disease.

In conclusion, the present study shows that: (1) β-amyloid, by itself or by possibly inducing the release of extremely low amounts of interleukin-1β, can stimulate T98G human astrocytoma cells to secrete a series of different proinflammatory factors, such as interleukin-6 and prostaglandin E_2; (2) prostaglandins have a central role in the maintenance of T98G cells in an activated state characterized by the additional release of inflammatory mediators; (3) paracetamol, and probably other NSAID, may interrupt the inflammatory loop induced in the central nervous system by β-amyloid deposition by inhibiting cyclooxygenase-dependent prostaglandin production.

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