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RESEARCH PAPER

Nimodipine inhibits IL-1β release stimulated by amyloid β from microglia

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BACKGROUND AND PURPOSE

There is growing evidence that inflammation plays a major role in the pathogenesis of neural damage caused by deposition of amyloid β (Aβ) in the brain. Nimodipine has received attention as a drug that might improve learning and reduce cognitive deficits in Alzheimer's disease, but the mechanism of action is poorly known. In this study, we tested the hypothesis that nimodipine inhibited Aβ-stimulated IL-1β release from microglia.

EXPERIMENTAL APPROACH

Cultures of N13 microglia cells or primary mouse microglia were treated with nimodipine, and intracellular accumulation and release of IL-1β in response to Aβ or to the P2 receptor agonists ATP and benzoyl ATP (BzATP) were measured. Accumulation of IL-1β was measured *in vivo* after intrahippocampal inoculation of Aβ in the absence or presence of nimodipine. The effect of nimodipine on Aβ-triggered cytotoxicity was also investigated.

KEY RESULTS

We show here that nimodipine dose-dependently inhibited Aβ-stimulated IL-1β synthesis and release from primary microglia and microglia cell lines. Furthermore, nimodipine also inhibited Aβ-induced IL-1β *in vivo* accumulation at concentrations known to be reached in the CNS. Finally, nimodipine protected microglia from Aβ-dependent cytotoxicity.

CONCLUSION AND IMPLICATIONS

These data suggest that alleviation of symptoms of Alzheimer's disease following nimodipine administration might be due to an anti-inflammatory effect and point to a novel role for nimodipine as a centrally acting anti-inflammatory drug.

Abbreviations

Aβ, amyloid β; BBB, blood–brain barrier; BzATP, benzoyl ATP; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration

Introduction

Alzheimer's disease is the most common form of dementia in Western countries. This disorder is characterized by the presence of extracellular aggregates of the amyloid β (Aβ) peptide and intracellular neurofibrillary tangles, associated with progressive synaptic and neuronal loss (Ballard *et al*., 2011). It is generally accepted that Aβ accumulation plays a central role in the pathogenesis of Alzheimer' s disease. Aβ is normally produced in the ageing brain where it is cleared and removed by microglia, the resident immune cells of the brain (Naert and Rivest, 2011). In Alzheimer' s disease, continuous Aβ deposition initiates a chronic inflammatory reaction characterized by recruitment of microglia and secretion of a host of inflammatory mediators, including IL-1β, TNF-α, reactive oxygen species and metalloproteases (Michelucci *et al*., 2009; Eikelenboom *et al*., 2010). Several findings point to IL-1β as a main player in Alzheimer' s disease as this cytokine is elevated in brain specimens from Alzheimer' s disease patients and it has been associated with a clear-cut neurotoxic effect (Vandenabeele and Fiers, 1991).

The main source of IL-1β in the brain is microglia, although there is evidence that astrocytes might also be a source under pathological conditions (Hanisch and

Kettenmann, 2007). There is no doubt that Aβ itself can be an efficient stimulus for IL-1β release from resident microglia, but recent data suggest that the amyloid peptide acts in concert with ATP released from activated microglia (Sanz *et al.*, 2009). It is an established fact that ATP accumulates in the inflammatory microenvironment to concentrations that may reach hundred micromolar concentrations (Pellegatti *et al.*, 2008; Weber *et al.*, 2010; Wilhelm *et al.*, 2010), and thus sufficient to activate the low-affinity P2X7 receptor. As P2X7, a member of the P2 receptor family (receptor nomenclature follows Alexander *et al.*, 2011), is the most potent inducer of IL-1β maturation and release so far identified (Ferrari *et al.*, 1997; 2006), it is not surprising that accumulation of ATP at inflammatory foci triggers IL-1β release from mononuclear phagocytes. The mechanism by which Aβ triggers IL-1β release is as yet poorly understood; however, very recent evidence from our laboratory has demonstrated a key role for the P2X7 receptor. In fact, we have shown that intrahippocampal injection of Aβ did not produce IL-1β in mice with genetic deletion of the P2X7 receptor (P2X7-KO mice) (Sanz *et al.*, 2009). New evidence of the pathogenetic role of Aβ and a better understanding of its mechanism of action have rekindled interest in drugs that cross the blood–brain barrier (BBB) and may counteract the neurotoxic effects of Aβ.

Nimodipine is an L-type calcium channel blocker with antihypertensive properties that crosses BBB, thus reaching the brain and the CSF. It has been suggested that nimodipine might be beneficial in the treatment of chronic cerebral ischaemia, vascular cognitive impairment and Alzheimer's disease (Lopez-Arrieta and Birks, 2002). *In vitro* experiments have shown that nimodipine attenuated toxicity induced by Aβ in cortical and hippocampal neurons and in neuroblastoma cells (Weiss *et al.*, 1994; Ueda *et al.*, 1997). In mesencephalic neuron/glia co-cultures, pretreatment with nimodipine decreased, in a dose-dependent manner, death of dopaminergic neurons mediated by LPS-activated microglia. The protective effect of nimodipine has been assigned to inhibition of microglial activation and to the related decrease of NO, TNF-α and IL-1β production (Li *et al.*, 2009).

In the present study, we investigated the effects of nimodipine on Aβ-stimulated IL-1β release from microglia and showed that nimodipine was a powerful blocker of intracellular IL-1β accumulation and IL-1β secretion triggered by Aβ in the N13 microglia cell line as well as in primary microglia. Nimodipine also prevented Aβ-triggered IL-1β release *in vivo*. Furthermore, we showed that nimodipine also inhibited Aβ-dependent microglia cell death. Our results provide a mechanistic explanation for the observed beneficial effects of nimodipine and suggest potential novel therapeutic applications.

Methods

Cells and solutions

Microglial N13 cells, a kind gift of Prof. Paola Ricciardi-Castagnoli (Singapore Immunology Network, Agency for Science, Technology and Research, Biopolis, Singapore), were grown in RPMI-1640 medium supplemented with 2 mM glutamine, 10% fetal calf serum (FCS) (Gifco BRL, Basel, Switzerland), 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin. Primary mouse microglia cells were isolated from 2 to 4-day-old post-natal mice as described previously (Sanz *et al.*, 2009). More than 98% of cells were identified as microglia using a macrophage cell-specific F4/80 biotinylated mAb antibody (Serotec, Dusseldorf, Germany) followed by staining with Oregon Green 488 goat anti-rat IgG (Molecular Probes, Leiden, The Netherlands). All animal care and experimental procedures complied with institutional and national guidelines (see below).

Microglia were plated in astrocyte-conditioned medium (high glucose-DMEM supplemented with 2 mM glutaMAX™ (Gibco Life Technologies Europe BV, Monza, Milan, Italy), 10% FCS, 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin), and used for experiments 24 h after plating. Short-term experiments were run either in FCS-free RPMI medium or in a saline solution with the following composition: 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 20 mM HEPES, 5.5 mM glucose, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, pH 7.4. When indicated, $CaCl_2$ was omitted and 500 μM EGTA was added. Nimodipine, dissolved in dimethyl sulfoxide, was added at a concentration of 36 nM (15 ng·mL^{-1}) 2 min before the addition of the relevant agonist, unless otherwise stated. Long-term (chronic) experiments were performed in astrocyte-conditioned medium. All experiments were performed at 37°C.

Measurement of enzymic activity

Lactate dehydrogenase (LDH) activity was measured according to standard laboratory procedures. IL-1β was measured with a R&D kit (R&D, Minneapolis, MN, USA).

Changes in intracellular Ca^{2+} and in plasma membrane permeability

Changes in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ were measured with the fluorescent indicator fura-2/AM (Molecular Probes-Invitrogen, Life Technologies Europe BV, Monza, Italy), as described previously (Sanz *et al.*, 2009). Briefly, cells were loaded at 37°C with 4 μM fura-2/AM in 1 mM Ca^{2+}-containing, 250 μM sulfinpyrazone-supplemented, saline solution. The $[Ca^{2+}]_i$ was measured using the 340/380 nm excitation ratio at an emission wavelength of 505 nm. ATP-dependent increases in plasma membrane permeability were measured with the extracellular fluorescent tracer ethidium bromide (Molecular Probes-Invitrogen) at the wavelength pair 360/580nm as earlier described (Sanz *et al.*, 2009). Changes in plasma membrane potential were measured with the fluorescent dye bis (1,3-diethylthiobarbiturate) trimethineoxonal (bisoxonol, Molecular Probes, Inc., Eugene, OR, USA) at the wavelength pair 540/580 nm, as previously described (Ferrari *et al.*, 1996). Experiments were performed in a spectrofluorometer (LS50, Perkin-Elmer Ltd., Beaconsfield, UK) equipped with a thermostat-controlled (37°C) cuvette holder and magnetic stirrer.

Western blotting

Cells were lysed, run on 7.5% SDS-polyacrilamide gel and blotted onto a nitrocellulose paper (Amersham Italia, Milan, Italy). IL-1β was detected with a rabbit anti-IL-1β polyclonal Ab (Chemicon, Millipore, Billerica, MA, USA) followed by

staining with protein A labelled with horse-radish peroxidase and visualization by chemiluminescence (Amersham, GE Healthcare Europe GmbH, Milan, Italy).

Animals

Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D. L. no. 116, G. U., Suppl. 40, February 18, 1992, Circolare no. 8, G. U., July 14, 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). All efforts were made to minimize the number of animals used and their suffering. The results of all studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). This study was conducted in BALB/c female mice weighing 18–20 g. A total number of 32 mice were used (Harlan Laboratories, Uidne, Italy). They were housed at constant room temperature (22 ± 1°C) and relative humidity (55 ± 5%) under a 12 h light/dark cycle (lights on from 7:00 AM to 7:00 PM). Food and water were freely available.

Surgery

The Aβ peptide ($A\beta_{1–42}$) was dissolved in artificial CSF (aCSF, vehicle) at a concentration of 2.2 μM. Briefly, mice were anaesthetized with 3 mL·kg^{-1} Equithesin i.p. (1.2 g of sodium pentobarbital, 5.3 g of chloral hydrate, 2.7 g of $MgSO_4$, 49.5 mL of propylene glycol, 12.5 mL of ethanol and 58 mL of distilled water), and secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). The skin was shaved, disinfected and cut with a sterile scalpel to expose the skull. The mice were randomly divided into four groups. The control group was injected with vehicle, the second group received a solution freshly prepared of $A\beta_{1–42}$ (2.2 μM), the third group was injected with nimodipine (15 ng·mL^{-1}, 36 nM), and the fourth group received a solution freshly prepared containing $A\beta_{1–42}$ (2.2 μM) and nimodipine (36 nM) into the right dorsal hippocampus, using the following coordinates relative to the bregma: anteroposterior: –2.0; medial lateral: +1.8; dorsoventral: –2.3. The injections were delivered at an infusion rate of 0.5 μL·min^{-1} for a duration of 2.0 min. The injection needle was left in place for 5 min before withdrawal to allow diffusion from the tip and prevent reflux of the solution. Placement of needle track was visible and was verified at the time of dissection. Animals were kept on a warming pad until they had fully recovered from the anaesthetic and kept in individual cages to prevent damage to the scalp sutures until they were killed for tissue processing. Seven days after surgery, animals were killed by cervical dislocation and brains were removed. Right and left hippocampi were collected by tissue dissection and immediately frozen on dry ice. Tissues were stored frozen at –80°C until IL-1β quantification.

Statistical analysis

All results are expressed as mean ± SD and analysed by ANOVA with Bonferroni *post hoc* test.

Materials

Aβ 1–42 and inactive scrambled Aβ peptide (iAβ) 42-1 were purchased from Bachem (Bubendorf, Switzerland). Aβ peptides were dissolved in dimethyl sulfoxide at a final concentration of 10 mM. Freshly prepared Aβ solution mainly consists of monomers/oligomers, as witnessed by staining. Nimodipine, a kind gift by Bayer (Leverkusen, Germany), was dissolved in dimethyl sulfoxide at 10 mg·mL^{-1}.

Results

Nimodipine inhibits IL-1β release

The release of IL-1β is a two-step process requiring, first, IL-1β gene transcription and pro-IL-1β accumulation, and then pro-IL-1β cleavage and secretion. According to the established experimental protocols, pro-IL-1β accumulation is driven by LPS-priming, and release by P2X7 stimulation (Ferrari *et al.*, 1996). Scattered reports in the past (Lorton *et al.*, 1996) and data from our own laboratory (Sanz *et al.*, 2009) showed that Aβ is an efficient stimulus for IL-1β release from LPS-activated human monocytes and microglia. Thus, we have applied this protocol to investigate the effect of nimodipine on IL-1β production by microglia. Figure 1A shows that ATP, benzoyl ATP (BzATP) or Aβ triggered IL-1β release from the microglial cell line N13 primed with LPS. Cytokine release in response to any of these stimuli was inhibited by nimodipine. In N13 cells, the effect of nimodipine was rather small, especially with ATP as an agonist, but nonetheless statistically significant. Stimulation by nucleotides also caused IL-1β release from primary microglia, but to a lesser extent than that from N13 cells (Figure 1B). Nimodipine decreased cytokine release from primary microglia to a larger extent than from the N13 cell line. Interestingly, in primary microglia, LPS itself in the absence of the second stimulus caused some IL-1β release which was partially inhibited by nimodipine, although this effect was not statistically significant. The iAβ was not a stimulus for IL-1β accumulation and release either in N13 cells or in primary microglia.

IL-1β is synthesized as inactive pro-IL1β (34 kDa) that is cleaved to mature Il-1β (17 kDa) by caspase-1 and other proteases. Thus, we analysed supernatants to identify the IL-1β form secreted from N13 cells in the absence or presence of nimodipine. As shown in Figure 1C, ATP, benzoyl ATP or Aβ caused release of both the 34 kDa and the 17 kDa IL-1β forms. Interestingly, Aβ was a better stimulus than ATP or BzATP for secretion of both forms. Release of the 17 kDa as well as of the 34 kDa form in response to the three stimuli was inhibited by incubation in the presence of nimodipine. Inhibition by nimodipine of ATP or BzATP-stimulated IL-1β release suggested a possible interference at the level of the P2X7 receptor, thus we tested whether nimodipine might interfere with P2X7 activation. A possible interference of Aβ at the P2X7 receptor is also hinted to by the recent demonstration that Aβ may directly activate P2X7 receptors (Sanz *et al.*, 2009). The P2X7 receptor is a non-canonical receptor that, depending on the level of activation, behaves as a cation-selective ion channel or as non-selective pore permeable to low MW aqueous solutes. Nimodipine, at the concentration effective to inhibit IL-1β release, had no effect on ATP-stimulated Ca^{2+} increases (Figure 2A), nor did it affect BzATP-stimulated ethidium bromide uptake, an assay currently used to monitor opening of the P2X7 large conductance pore (Figure 2B).

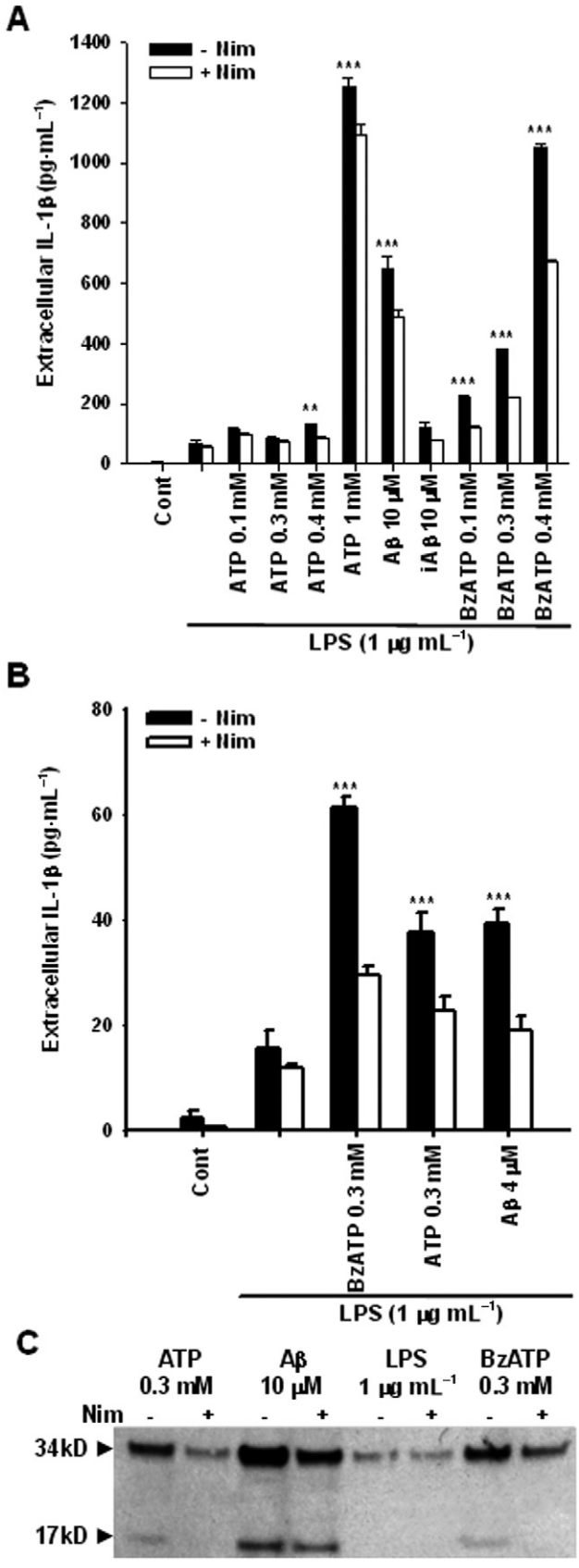

Figure 1

Nimodipine (Nim) inhibits IL-1β release induced by ATP, Bz-ATP or Aβ. N13 cells (panels A and C) or primary microglia (panel B) were plated in 24 (A and C) or 48 (B) well plates and incubated at 37°C in FCS-free RPMI for 5 h with 1 μg mL^{-1} LPS in absence or presence of 36 nM nimodipine. Then cells were stimulated for 30 min with ATP, BzATP or Aβ and supernatants were withdrawn for ELISA (A and B) or Western blot analysis. Cell concentration was 75 × 10^3 cells per well in A and C, and 15 × 10^3 cells per well in B. In A and B, data are means ± SD of triplicate determinations from a representative experiment replicated on three separate occasions. $^{**}P < 0.01$; ***<0.001 for data obtained with or without nimodipine.

Only with a much more sensitive readout, that is, plasma membrane depolarization, was it possible to detect a slight (5–10%) inhibition of BzATP effect (Figure 2C). Altogether, these results suggest that nimodipine had little, if any, effect on the P2X7 receptor itself.

Aβ causes nimodipine-sensitive intracellular accumulation of IL-1β

Previous experiments show that Aβ is a potent stimulus for IL-1β release from LPS-primed microglia *in vitro*, but our *in vivo* data suggest that Aβ might be a full stimulus for IL-1β release, even in the absence of LPS priming (Sanz *et al.*, 2009). Thus, we investigated whether Aβ alone might be sufficient to drive IL-1β release during a chronic, several day, incubation. As shown in Figure 3A, Aβ dose-dependently stimulated intracellular IL-1β accumulation, while iAβ was fully inactive. Rather interestingly and unexpectedly, ATP, an agonist thought to function only as a stimulus for IL-β maturation and release, was also able to cause intracellular IL-1β accumulation in the absence of LPS priming. Intracellular accumulation of IL-1β stimulated by Aβ or ATP was completely prevented by nimodipine. Optimal nimodipine concentration was 36 nM. At higher concentrations (72 nM = 30 ng·mL^{-1}), nimodipine was ineffective (Figure 3B). We also tested two other Ca^{2+} channel blockers, the related dihydropyridine nifedipine and the unrelated ω-conotoxin GVIA (Figure 3C). Nifedipine was active in the same concentration range as nimodipine, while ω-conotoxin GVIA had a small, though statistically significant effect, only at the highest concentration tested (500 nM).

A time course (3h-7days) of Aβ-stimulated IL-1β accumulation and release showed that this cytokine could be detected both intra- and extracellularly as early as 3 h after Aβ challenge, and that nimodipine was an effective blocker beyond the 3 h time point (Figure 4A,B). Analysis of cell lysates (Figure 4C) showed that during the 7 day incubation, Aβ dose-dependently stimulated intracellular pro-IL-1β accumulation, which was reduced by nimodipine. The scrambled Aβ peptide was inactive. ATP triggered pro-IL-1β accumulation as well as formation of mature IL-1 β. Pro-IL-1-β was detected in the supernatants from unstimulated controls and in those from microglia challenged with Aβ or ATP. By contrast, mature IL-1β was found only in the supernatants from stimulated cells, a clear proof of the specificity of the process (Figure 4D). Mature cytokine release was inhibited by nimodipine. In lysates from control and in those from stimulated cells, an incompletely processed IL-1 form was also present.

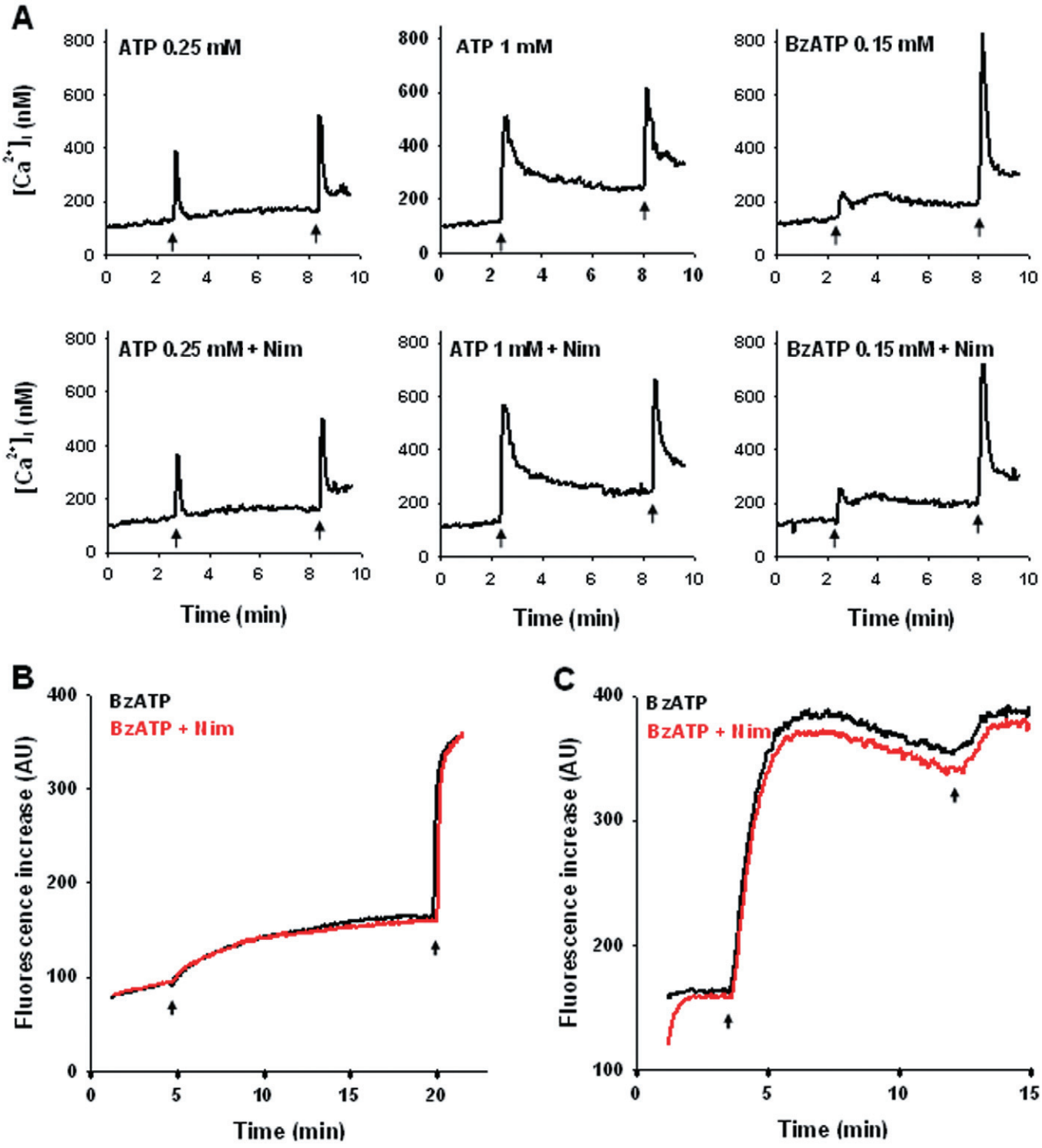

Figure 2

Nimodipine (Nim) does not affect ATP or BzATP-dependent activation of P2X7 receptors. In panel A, fura-2-loaded N13 cells were incubated at a concentration of 10^6 mL^{-1} in a thermostated and magnetically stirred fluorimeter cuvette in the Ca^{2+}-containing saline solution described in Methods and challenged (first arrow) with the indicated nucleotide concentrations and with ionomycin (1 μM, second arrow). Separate traces representative of three similar experiments performed for each condition are shown. In panel B, N13 cells were incubated in a thermostated and magnetically stirred fluorimeter cuvette at the concentration of 2×10^5 mL^{-1} in Ca^{2+}-containing, 100 nM bis-oxonol-supplemented saline solution and challenged with 150 μM BzATP (first arrow) and 30 mM KCl to collapse membrane potential (second arrow). In panel C, 5×10^5 mL^{-1} N13 cells were incubated in a thermostated and magnetically stirred fluorimeter cuvette in 20 μM ethidium bromide-containing saline solution from which Ca^{2+} was omitted and 500 μM EGTA was added, and challenged with 150 μM BzATP (first arrow) and 100 μM digitonin (second arrow).

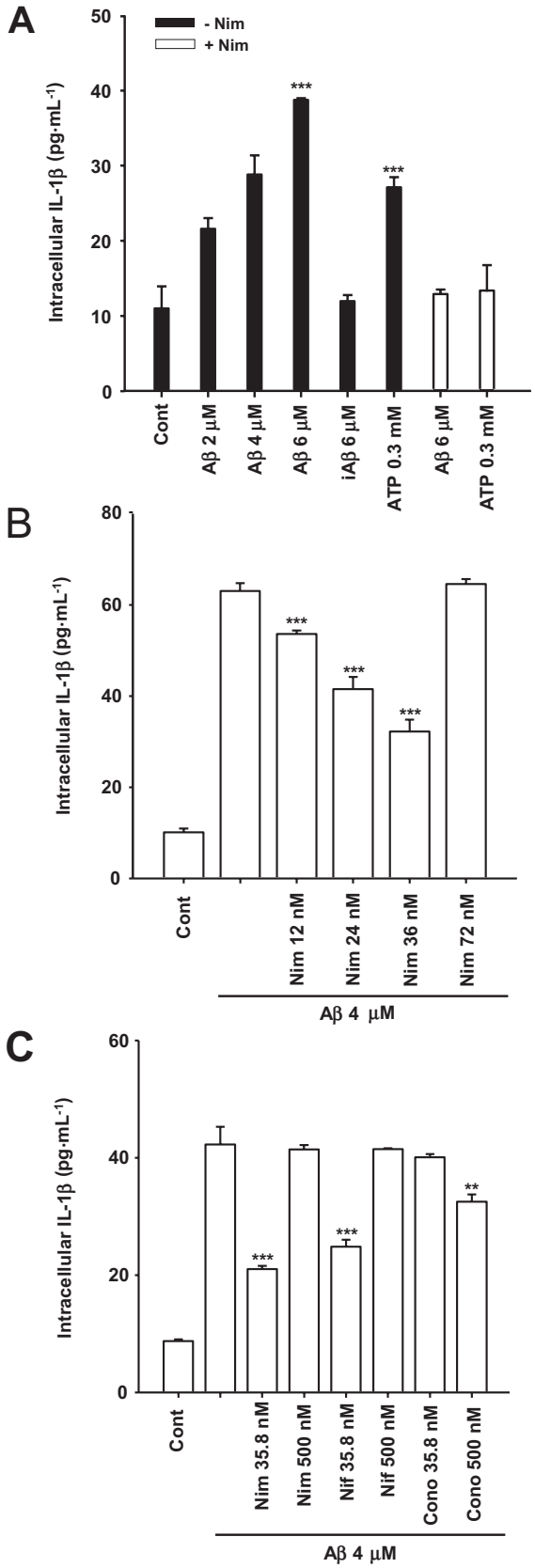

Figure 3

Nimodipine (Nim) inhibits intracellular accumulation of IL-1β triggered by chronic treatment with Aβ. Cells, 15×10^3 per well, were incubated for 7 days in a thermostated (37°C), humidified, CO_2 incubator in the astrocyte-conditioned medium described in Methods in the absence or presence of either Aβ, iAβ or ATP. In panel A, nimodipine (36 nM, open bars) was also present in some samples throughout the experiment. In panel B, the effect of increasing nimodipine concentrations was tested. In panel C, the effect of nimodipine was compared with that of nifedipine and ω-conotoxin GVIA. At the end of the 7 days incubation, supernatant was removed, cells lysed RPMI containing 0.1% Triton X100 and protease inhibitors, and intracellular IL-1β assayed by ELISA. Immunoreactive cytokine comprises both immature and mature IL-1β. Data are means ± SD of triplicate determinations from a representative experiment replicated on three separate occasions. ***$P < 0.001$, **$P < 0.01$ samples treated without blockers versus samples treated with blockers.

To fully assess the potential anti-inflammatory effect of nimodipine, we tested its effect *in vivo*. As shown in Figure 5, intra-hippocampal injection of nimodipine (36 nM) together with Aβ abolished IL-1β accumulation. In contrast to the *in vitro* experiments, *in vivo* nimodipine itself caused a small, albeit barely statistically significant, increase in IL-1β accumulation.

Nimodipine protects microglia Aβ-induced cell death

Among the different mechanisms of neurotoxicity due to Aβ, direct injury of neuronal and glial cells is thought to play an important role. Figure 6A shows that chronic incubation of primary microglia together with Aβ caused a significant decrease in cell number. Cell count performed in eight microscopic fields from three different culture wells for each condition showed that an average of 23 ± 3 cells per field were present in controls, while 15 ± 2 cells per field were present in cultures treated for 7 days with Aβ. In the presence of Aβ plus nimodipine or of iAβ, 23 ± 4 or 20 ± 2 cells per wells were present, respectively. Morphological analysis showed that cells exposed to Aβ exhibited rounding, retraction of pseudopods and shrinkage, and lost adherence with the substrate. None of these changes occurred in the presence of nimodipine. As an independent assay, we monitored release of LDH, an index of plasma membrane injury. Figure 6B,C shows the kinetics of LDH accumulation into cell supernatant over a 7 day incubation and Aβ dose dependency, respectively. Aβ, but not iAβ, caused a steady increase in the supernatant content of LDH which was abolished by nimodipine. Microglia were very sensitive to toxic effects of Aβ because as little as 0.5 μM was sufficient to induce LDH release (Figure 6C).

Discussion

Alzheimer' s disease is a major neurodegenerative disorder affecting an increasing and progressively younger population (Ballard *et al.*, 2011). No treatment for halting or delaying

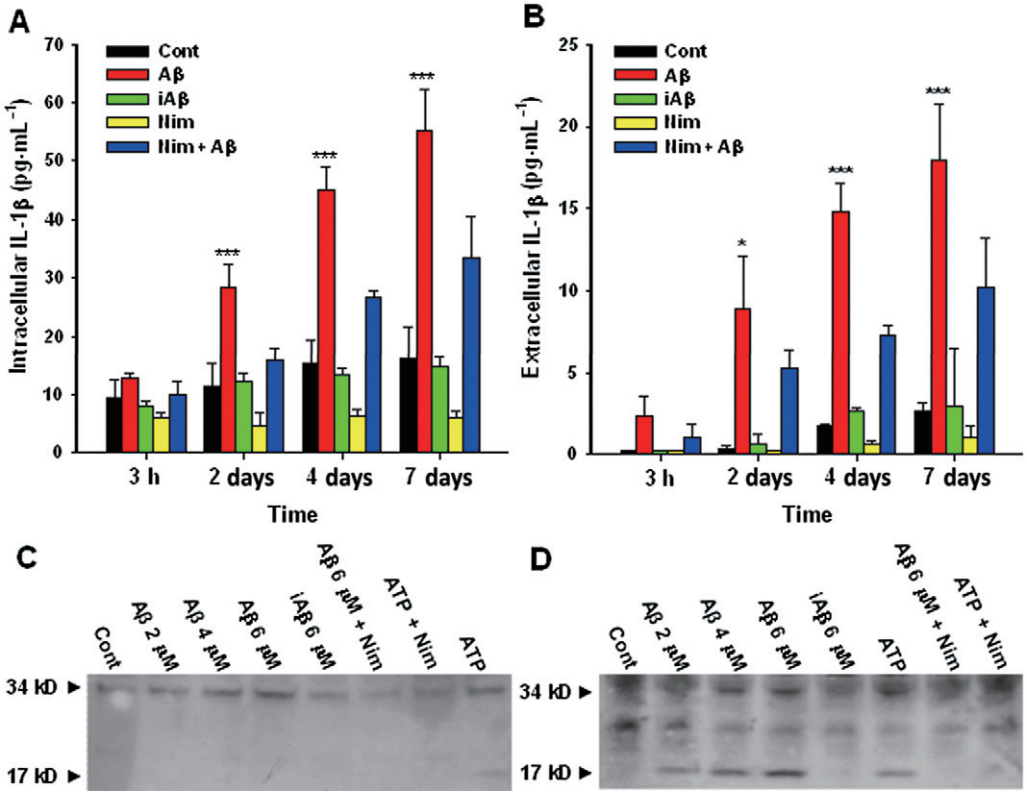

Figure 4

Time course of Aβ-triggered IL-1β accumulation and release in the presence or absence of nimodipine (Nim). Primary microglia was incubated with 4 μM Aβ or iAβ in absence or presence of nimodipine for the indicated time as described in the legend to Figure 3. Cellular lysates (A and C) and supernatant (B and D) were used to measure IL-1β by ELISA (A and B) or Western blot (C and D). In A and B, data are means ± SD of triplicate determinations from a representative experiment replicated on three separate occasions. *$P < 0.5$, ***$P < 0.001$ for Aβ versus Aβ plus nimodipine.

progression of this disease is currently available, with the main intervention for alleviating patients' sufferings being almost exclusively based on treating the cognitive and behavioural manifestations. However, there are few doubts that brain Aβ deposits have a major role in AD and that inflammation is an important factor in amplifiing, if not in initiating, the neuronal damage (Vandenabeele and Fiers, 1991; Eikelenboom *et al.*, 2010). On this basis, therapeutic approaches to block deposition of the Aβ peptide, for example, Aβ vaccination, or neuroinflammation (e.g. administration of anti-inflammatory drugs), have been proposed (Lambracht-Washington *et al.*, 2011). Among other approaches, interventions aimed at ameliorating brain perfusion have also been explored, and among these, treatment with nimodipine (Weiss *et al.*, 1994; Lopez-Arrieta and Birks, 2002), a blocker of L-type voltage-dependent Ca^{2+} channels. Scattered experiments in the past have shown that administration of nimodipine might be beneficial in Alzheimer' s disease, but the mechanistic basis of this effect has remained obscure.

Due to the increasing attention that is being paid to inflammation in the pathogenesis of Alzheimer's disease, we investigated whether nimodipine might interfere with the secretion of the key pro-inflammatory cytokine IL-1β. IL-1β production is a complex process subjected to multiple checkpoints at the level of transcription, maturation and secretion. The minimum level of complexity assumes a two-step release process: transcription of the IL-1β gene and cytoplasmic accumulation of pro-IL-1β, and caspase-1-driven cleavage of pro-IL-1β into mature IL-1β (Ferrari *et al.*, 2006). Most pro-inflammatory agents are incomplete stimuli for IL-1β secretion in that they cause pro-IL-1β accumulation, but not maturation and release or, *vice versa*, trigger maturation and release, but are unable to drive gene transcription. In a previous study, we showed that Aβ replicates many of the effects of extracellular ATP as a second stimulus for IL-1β release, but we also showed that, *in vivo*, Aβ acting at P2X7 receptors is a full stimulus for IL-1β release (Sanz *et al.*, 2009). These results were further extended in the present study showing that, even *in vitro*, Aβ is a full stimulus for IL-1β release, in the absence of LPS priming. These observations underline the importance of agents targetting IL-1β for the reduction of neuroinflammation in Alzheimer's disease.

The protective effect of nimodipine in Alzheimer's disease has been, in general, assigned to its ability to ameliorate brain perfusion, thanks to its activity as a Ca^{2+} channel blocker. However, it has also been suggested that nimodipine might directly blunt inflammation by inhibiting LPS-mediated microglia activation (Li *et al.*, 2009). We show here that two

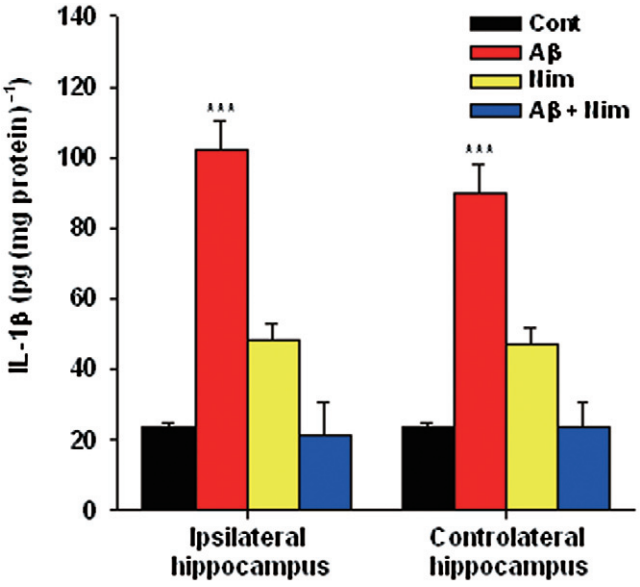

Figure 5

Nimodipine (Nim) inhibits Aβ-stimulated IL-1β accumulation *in vivo*. Mice were injected with CSF, Aβ, Aβ plus nimodipine,or nimodipine alone, as described in Methods. After 7 days, the animals were killed, brains were dissected, homogenates of hippocampus prepared and their IL-1β content measured. Data are means ± SD (n = 8).

dihydropyridines, nimodipine and nifedipine, and to a much smaller extent another Ca^{2+} channel blocker such as ω-conotoxin, exerted an important protective activity against Aβ-dependent brain injury acting at least at two levels: (i) at the level of IL-1β accumulation and release; (ii) protecting microglia cells against Aβ-mediated damage. Both actions are of benefit to brain physiology as inhibition of IL-1β secretion will down-modulate inflammation and protection of microglia will enable these cells to carry on their Aβ-scavenging activity, which is thought to be of relevance in protecting the brain against damage due to chronic accumulation of the amyloid peptide (Paresce *et al.*, 1996).

We think that these effects are not likely to be due to blockade of Ca^{2+} channels because the effective nimodipine doses were much lower than those currently used to block

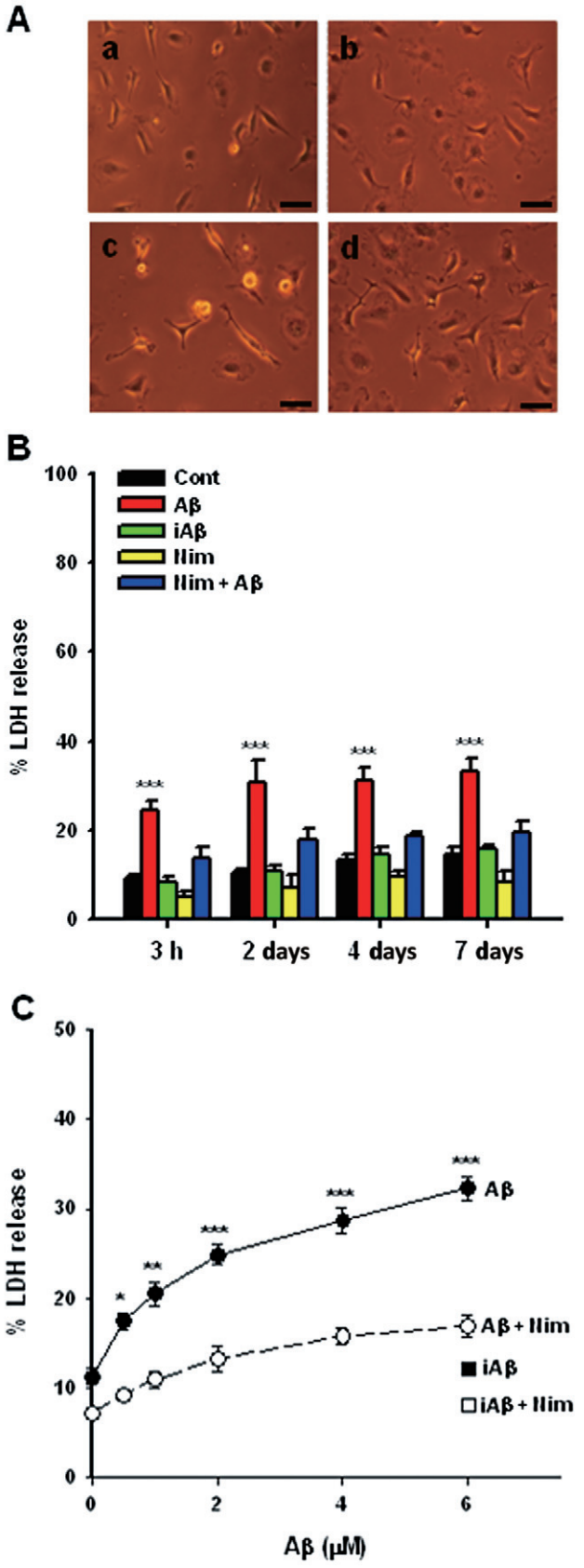

Figure 6

Nimodipine (Nim) inhibits cell death triggered by Aβ in primary microglia. Cells were incubated as described in the legend to Figure 3 with Aβ or iAβ in the absence or presence of nimodipine. In A, cell number was determined by microscopic examination of eight different fields in photographs taken from three separate wells at the end of the 7 day incubation. Panel a, control cells; panel b, cells incubated in the presence of nimodipine alone; panel c, cells incubated in the presence of 4 μM Aβ; panel d, cells incubated in the presence of 4 μM Aβ + nimodipine. Bar = 25 μm. Panels B (time course) and C (Aβ dose dependency) show lactic dehydrogenase (LDH) content of supernatants from the different experimental conditions. Data are means ± SD of triplicate determinations from a representative experiment replicated on three separate occasions. *P < 0.5, **P < 0.01, ***P < 0.001 for Aβ versus Aβ plus nimodipine.

Ca^{2+} channels, and microglia cells do not express voltage-activated Ca^{2+} channels. As there is increasing evidence supporting a key role of P2X7 receptors in Aβ-dependent IL-1β release, and on the basis of the observation that nimodipine inhibits ATP- as well as Aβ-stimulated release, we tested the hypothesis that this Ca^{2+} channel blocker might act as a P2X7 receptor blocker. However, this was not the case as nimodipine had a small, if any, inhibitory activity on P2X7-dependent responses upstream of IL-1β-secretion. Thus, it is likely that nimodipine acted at several steps in the chain of events leading to IL-1β secretion downstream of P2X7 receptors, such as gene transcription, caspase-1 activation and cytokine release. Furthermore, the ability of nimodipine to inhibit both ATP and Aβ-stimulated IL-1β release suggests that this Ca^{2+} blocker acts at a common step in the intracellular transduction cascade, downstream of plasma membrane receptors, such as TLR-4, and possibly at the level of NF-κB. An interference at the inflammasome level might also be postulated, but this seems to be less relevant in view of the strong inhibition on pro-IL-1 accumulation caused by nimodipine. Of particular relevance in the present study is the ability of nimodipine to inhibit Aβ-stimulated IL-1β release *in vivo* and in the same range of concentrations shown to be active *in vitro*. This suggests that nimodipine might be a useful tool to counteract Aβ toxicity *in situ* at concentrations achieved in the blood or in the CSF following oral administration of the drug.

In conclusion, our data show that nimodipine strongly inhibited IL-1β release and suggest that its anti-inflammatory activity might be beneficial in AD.

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Conflict of interest

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