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Involvement of benzodiazepine receptors in neuroinflammatory and neurodegenerative diseases: evidence from activated microglial cells in vitro

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Abstract

Increased binding of a ligand for the peripheral benzodiazepine binding receptor is currently used in PET studies as an in vivo measurement of inflammation in diseases like multiple sclerosis and Alzheimer's disease. Although peripheral-type benzodiazepin receptors (PBRs) are abundant in many cell types and expressed in the CNS physiologically only at low levels, previous reports suggest that after experimental lesions in animal models and in human neurodegenerative/-inflammatory diseases upregulated PBR expression with increased binding of its ligand PK11195 is confined mainly to activated microglia in vivo/in situ. Because the functional role of the PBR is unknown, we confirm by immunohistochemistry and PCR (I) that this receptor is expressed on microglia in vitro and (II) that benzodiazepines modulate proliferation of microglial cells and the release of the inflammatory molecules nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α) in cell culture supernatants of primary rat microglia. Compared to lipopolysaccharide-activated controls the release of NO was markedly decreased in cultures treated with benzodiazepines (clonazepam, midazolam, diazepam) and the PBR ligand PK11195. Moreover, release of TNF-α and proliferation was significantly inhibited in the benzodiazepine-treated groups. These findings link the in vivo data of elevated PBR levels in neurodegenerative/-inflammatory diseases to a functional role and opens up possible therapeutic intervention targeting the PBR in microglia.

Keywords: Microglia; Peripheral benzodiazepine binding receptor; Nitric oxide; Tumor necrosis factor-α

Benzodiazepines (BZ) are widely used as tranquilizers, muscle relaxants, hypnotics, anticonvulsants, and sedatives. These effects are primarily mediated via the central BZ receptors (CBRs) located in the central nervous system (Braestrup and Squires, 1977; Tallman et al., 1980). CBRs are part of a macromolecular complex that also contains a γ-aminobutyric acid (GABA) receptor site and a chloride ion channel (DeLorey and Olsen, 1992). The GABA/BZ receptor complex is a hetero-oligomer composed of five subunits: BZ bind to the α-subunit and faciliate the inhibitory effect obtained by GABA (DeLorey and Olsen, 1992).

Benzodiazepine binding sites distinct from the CBR in the central nervous system have been detected in many peripheral tissues like kidney (Pedigo et al., 1981) and endocrine organs (De Souza et al., 1985), but are also present in a high density on microglial cells in the rat (Park et al., 1996; Banati et al., 1997). They have hence been called peripheral-type benzodiazepine receptors (PBR). In rats, PBR differ from CBR in their drug specifity: for example, the BZ clonazepam binds to CBR with high affinity, whereas the BZ Ro 5-4864 (4′-chlorodiazepam) as well as the non-BZ ligand PK11195 (an isoquinoline carboxamide derivative) bind to CBR with negligible affinity. The reverse is true with regard to the PBR: [^{3}H]PK 11195 binds to adrenal membranes of rats with a high affinity. The potency order of displacement of the [^{3}H]PK 11195 bound is PK 11195 > diazepam > clonazepam (Benavides et al., 1983). PBR are composed of at least three subunits: a binding site

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for isoquinolone (for instance PK 11195), a voltage-dependent anion channel, and an adenine nucleotide carrier, which all bind BZ (McEnery et al., 1992). Subcellular studies have indicated that PBR are mitochondrial in the rat brain (Basile et al., 1986), rat adrenal gland (Anholt et al., 1986), rat testis, lung, heart, skeletal muscle, and liver (Antkiewicz-Michaluk et al., 1988). It has also been reported that PBR are present in the plasma membrane of human erythrocytes, which lack mitochondria (Olson et al., 1988). In search for endogenous ligands for PBZ a brain neuropeptide termed diazepam binding inhibitor (DBI) was isolated and purified to homogeneity (Corda et al., 1984) that inhibited diazepam binding to the PBZ. Tryptic digestion of this peptide yielded an octaneuropeptide (ODN) that competed for [^{3}H]diazepam binding and antagonized the anticonflict actions of BZ in rats (Alho et al., 1985). The most commonly suggested functions for the PBR are regulation of steroid synthesis (Papadopoulos et al., 1990; Boujrad et al., 1993) and involvement in cell growth and differentiation (Wang et al., 1984; Camins et al., 1995).

Although in the undamaged brain comparatively little binding of the PBR ligand PK11195 is found, PBR are a marker of neuronal damage: Intrastriatal injection of excitotoxic compounds (kainate, AMPA, *N*-methyl-D-aspartate) in rats provoked a dose-dependent increase in the level of PBR in rats as assayed by pharmacological binding data (Benavides et al., 1987). Quantitative receptor autoradiography was used to measure increased binding of [^{3}H]flunitrazepam to PBZ after transient cerebral ischemia in gerbils after transient cerebral ischemia.

The cellular source of the increased PK11195 binding in CNS pathology has remained controversial for long. However, it has recently been demonstrated by microaudiography and positron emission tomography (PET) imaging that activated but not resting microglial cells are the primary source of increased focal binding site densities for the PBR ligand [^{3}H][R]-PK11195 in vitro as well as in vivo in patients suffering from multiple sclerosis (Vowinckel et al., 1997; Banati et al., 2000) and Alzheimer's disease (Cagnin et al., 2001), providing a cellular marker of disease activity in vivo.

Microglial cells respond to any pathological event that directly or indirectly affects the CNS by the production and release of a battery of potent proinflammatory cytokines and reactive oxygen species (Banati et al., 1993; Rothwell and Hopkins, 1995; Kreutzberg, 1996). This inflammatory reaction is associated with inflammatory diseases as multiple sclerosis as well as neurodegenerative diseases like Parkinson's disease or Alzheimer's disease. However, it is so far uncertain whether this initiation of an inflammatory response is beneficial, detrimental or simply incidental.

It is so far, unknown if and how this increase of microglial [^{3}H][R]-PK11195 binding to the PBZ contributes to the pathogenesis of the CNS diseases mentioned above. Two major possible neurotoxic secretion products of microglial cells are nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α). NO is neurotoxic due to inhibition of complex 1 and 2 of the respiratory chain. Moreover, it reacts with superoxide anion to generate peroxynitrite, a highly reactive molecule capable of oxidizing proteins, lipids, and DNA, which causes striatal neurodegeneration in a mouse model in vivo (Mihm et al., 2001). The cytokine TNF-α is an important factor in the regulation of neuronal apoptotic cell death: TNF-immunoreactive glial cells have been detected in the substantia nigra and immunoreactivity for TNF receptors was found in cell bodies and processes of most dopaminergic neurons of parkinsonian patients (Boka et al., 1994). Immunocytochemical techniques demonstrated TNF-α-positive astrocytes and microglial cells as well as an induction of nitric oxide synthase in brain lesions of multiple sclerosis patients. Furthermore, amyloid-β and IFN-γ activate microglia to produce reactive nitrogen intermediates and TNF-α (Meda et al., 1995). Given this there is little doubt that activated microglia can inflict significant damage on neighboring neurons.

Our study therefore addresses the question if certain benzodiazepines might influence the release of the aforementioned microglial inflammatory mediators by binding at the PBR on activated microglial cells in vitro. This could open up a possible therapeutic intervention targeting the PBR in activated microglia. We here report that microglial proliferation and activation is decreased in the presence of different benzodiazepine receptor ligands. These data suggest that through their effects on microglia, PBR ligands could alter the pathogenesis of neuroinflammatory and -degenerative disorders.

Material and methods

Cell culture

For all experiments Wistar rats that were bred and held under constant conditions (12h/12h light/dark cycle) in the animal house of the University of Kiel were used Free-floating microglial cells were collected from the medium of primary cell cultures from neonatal rat cerebral cortex after 10 days, as detailed elsewhere (Sievers et al., 1994; Wilms et al., 1997; Wilms et al., 1999): Briefly, free-floating microglia was collected from the medium of primary cell cultures from neonatal rat cerebral cortex after 10 days (Giulian and Baker, 1986; Gebicke-Haerter et al., 1989). Contaminating astrocytes were removed by preplating the cells on plastic dishes for 5–10 min and discarding the unattached cells. This procedure increased the purity of the microglial preparation to more than 98% with very few astrocytes remaining. Prior to plating, cell number and viability was estimated by trypan blue exclusion. The microglial cells were seeded onto 96-well-culture plates (Falcon) or 6-well-culture plates (Costar, Bodenheim, Germany) in different densities depending on the experimental setup and cultivated in ACM up to 3 days at 37°C in humidified

CO_2/air (7.5:92.5%). ACM was produced by incubating a confluent subculture of astrocytes with fresh culture medium (DMEM + 10% fetal bovine serum, both from Life Technologies, Karlsruhe, Germany) for 24 h. The medium was withdrawn, sterile filtered to remove cell debris, and stored at −70°C. For the observation of morphological changes in cell shape the cells were fixed with 2% glutaraldehyde in phosphate-buffered saline (PBS) and stained with Coomassie blue dye (Biorad, Hercules, CA, USA). For immunohistochemistry the cells were grown on glass coverslips and treated as described later. All cells were cultured in a humidified atmosphere enriched with 5% CO_2.

Drug treatment

For the induction of microglial activation lipopolysacchccaride (LPS; 200ng/ml, from Sigma), a bacterial endotoxin and generally accepted inducer of proinflammatory properties was added to the control groups. Moreover, the experimental groups were incubated with drugs acting on (i) the central-type benzodiazepine receptor, (ii) receptor-specific ligands with dual action on both the central and peripheral benzodiazepine receptor, and (iii) a ligand acting solely on the peripheral benzodiazepine receptor. The central benzodiazepine receptor agonists midazolam and clonazepam were used in concentrations from 1 to 100 μM, whereas the antagonist flumazenil was used in the range from 5 to 20 μM. Diazepam (1–100 μM), which shows affinity to both the central and the peripheral benzodiazepine receptor, and the selective PBR ligand 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquino-linecarboxamide (PK11195, 50–100 μM) were also used to discriminate between both receptors. To elucidate a possible involvement of endogenous benzodiazepines (endozepines) in control of microglial cell function, we also investigated the influence of the octadecaneuropeptide ODN in concentrations from 1 nM to 100 μM. ODN is a processing product of the endogenous diazepam-binding inhibitor (DBI). Twenty-four hours after stimulation/incubation with the above-mentioned drugs NO and TNF-α concentrations in the cell culture supernatants were determined. For each drug the concentration with the highest modulatory effect was determined (see Results). All cells were cultured at 37°C in a humidified atmosphere enriched with 5% CO_2.

Antibodies and immunohistochemistry

The following primary antibodies were used for the identification of contaminating astrocytes or the routine assessment of purity of microglia cell cultures: antiglial fibrillary acidic protein antibody (polyclonal anti-GFAP, 1:200; Dakopatts, Hamburg, FRG) for the detection of astrocytes and anti-OX42 antibody (1:200; Robinson et al., 1986; Seralab, Asbach, FRG) for the detection of microglia. For PBR immunohistochemistry, 50,000 microglia cells grown on glass coverslips over 24 h were fixed in ice-cold acetone (5 min) and stained for 1 h with a polyclonal anti-PBR antibody (1:200, clone FL-169, Santa Cruz) followed by goat antirabbit IgG (1:300, 1 h, Vector Laboratories, Burlingame, USA). The immunohistochemical staining was performed with the ABC technique according the manufacturers protocol (Vector). Negative controls were performed by omitting the primary antibody. Cells were embedded in saline–glycerol mixture (9:1) and photographed with a fluorescence microscope (Axioplan, Carl Zeiss, Germany) on Fuji 1600 (Fuji, Munich, Germany).

TNF-α ELISA

For the detection of TNF-α in the cell culture supernatants 200,000 microglia cells/well seeded in 96-well culture plates (Falcon) were incubated with LPS (200 ng/ml) in the presence or absence of PBR ligands. TNF-α was determined in culture supernatant of each well (six separate measurements/treatment) after a 24-h incubation period using a commercial ELISA-kit (TNF-α: Bender Medsystems, Vienna, Austria). ELISA was performed according to the manufacturer's protocol and read at a wavelength of 450 nm (reference wavelength 550 nm) with a SLT Reader 340 ATTC. Data were provided by four independent experiments.

Measurement of nitrite production

Approximately 200,000 microglial cells/well seeded in 96-well culture plates (Falcon) were treated with LPS (200ng/ml) in the presence or absence of PBR ligands for 24 h. The generation of nitric oxide was determined by measuring nitrite accumulation in the medium using Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 5% H_3PO_4; Sigma, Germany): 100 μl of culture supernatant of each well and 100 μl of Griess reagent were mixed and incubated for 5 min; the absorption was estimated in an automated plate reader (SLT reader 340 ATTC) at 540 nm. Sodium nitrite ($NaNO_2$, Merck, Darmstadt, Germany) was used to generate a standard curve for quantification. Background NO_2 was subtracted from the experimental values. The results were obtained from six separate measurements of identical treated wells/drug, and the data were provided by six independent experiments.

Reverse transcription–polymerase chain reaction (RT–PCR) for PBR

About 500,000 microglial cells were seeded into two 9.6-cm^2 culture dishes and maintained with or without LPS treatment (200 ng/ml) for 24 h in culture. Total RNA from glial cells was isolated using the Trizol reagent, a monophasic solution of phenol and guanidine isothiocyanate, according to the manufacturer's recommended protocol (Gibco BRL, Life Technologies). RNA samples were reverse-tran-

scribed by MMLV reverse transcriptase (Superscript RT) (Gibco BRL, Life Technologies) and oligo-$(dT)_{15}$ primers (Promega, Madison, WI). The cDNA products were used immediately for PCR. PCR amplifications of the cDNAs for the peripheral benzodiazepine receptor (PBR) and the housekeeping gene β-actin were carried out. The primers used for amplification of rat PBR cDNA had the following sequences: 5′-CCT CGC CGA CCA GAG TTA TCA C-3′ and 5′-GGC TCC TAC ATA ATC TGG AAA GAG-3′, yielding a 302-bp product. Primer design for the PBR gene using the Gene-Fisher software (Giegerich et al., 1996) was based on the published sequence M84221 (NCBI, Nucleotide database). The primers used for amplification of rat β-actin cDNA had the sequences 5′-AGA GGG AAA TCG TGC GTG ACA- 3′ and 5′-CAC TGT GTT GGC ATA GAG GTC-3′ (Westenfelder et al., 1999), yielding a 281-bp product. Amplification of β-actin cDNA served as control of intactness of RNA. Furthermore, all primers spanned an intron in order to detect any DNA contamination in the PCR reaction and were purchased from MWG-Biotech AG (Ebersberg, Germany). The PCR conditions were as follows: 94°C for 4 min and then 40 cycles of 94°C/30 s, 58°C/60 s, and 72°C/60 s followed by one cycle 72°C/10 min. cDNA products and a 100-bp DNA ladder (Gibco BRL, Life Technologies) to provide size standards were electrophoresed on 2% agarose gels and visualized with ethidium bromide incorporation under UV light.

Proliferation assay

250,000 microglia cells were incubated in fresh medium containing [^{3}H]thymidine (0.5 μCi/well, Amersham) in the absence or presence of test substances. At the end of the incubation, cells were washed twice with ice-cold phosphate-buffered saline (PBS), followed by methanol for 10 min, distilled water for 5 min, 10% trichloroacetic acid (Sigma) for 5 min, and distilled water for 5 min. After solubilization in 1 M NaOH, the cells were transferred to scintillation-counting vials, and incorporated radioactivity was measured, after neutralization with HCl and the addition of scintillation fluid, using a liquid scintillation counter (Wallac, Turku, Finland). Data were obtained from four separate experiments with fivefold determinations.

Western blot analysis

For Western blot analysis, 750,000 microglia cells were incubated with or without LPS (200 ng/ml). Cells were harvested after 24 h in 100 μl of lysis buffer (128 mM Tris/HCl, 4.6% SDS, 10% glycerine, 0.005% bromphenolblue, 25 μl/ml mercaptoethanol, pH 6.8) and boiled for 5 min (95°C), and insoluble material was removed by centrifugation at 10,000*g* at 4°C for 5 min. Protein aliquots (5 μg each) were resolved by 12.5% SDS–PAGE and Western blotted with a polyclonal primary antibody against the PBR (1:400, a kind gift from Dr. V. Papadopoulos, Georgetown University Medical Center, Washington, DC, USA; Amri et al., 1996) overnight at 4°C and subsequent detection with peroxidase-labeled secondary antibodies was performed. Antibody binding was detected via enhanced chemiluminescence (Amersham Pharmacia Biotech, Essex, UK).

Statistical analysis

Data are expressed as mean ± SEM of (n) independent experiments. One-way ANOVA test was used for comparison of the NO and TNF-α groups. An α-level of 0.05 was used for statistical significance and all tests were performed two-sided.

Results

Cell morphology and expression of PBR

Within 3 days the seeded microglial cells developed the characteristic ramified shape of resting microglia in vitro with branched cellular processes and a small cell soma (Fig. 1A). After treatment with LPS the microglial cells adopted an amoeboid phenotype characteristic of activated myelomonocytic cells (Fig. 1B). Both resting and activated cells could be immunostained with a specific antibody directed against the PBR (Fig. 1D and E). However, it should be noted that resting microglia (Fig. 1D) showed only faint cytoplasmatic staining, whereas in LPS-activated cells (Fig. 1E) the staining intensity was greatly enhanced. Unspecific staining was not detectable (Fig. 1C, omitting of the primary antibody). Moreover, the presence of the PBR could be verified by RT–PCR (Fig. 1F).

Western blot analysis

To test a possible mechanism of posttranscriptional upregulation of the PBR Western immunoblot assays were performed. There was an increase in the steady-state level of PBR in LPS-activated microglia compared to untreated control cultures (Fig. 2). Adrenal gland tissue served as a positive control for PBR occurence.

Proinflammatory molecules: NO

Various concentrations (see “Drug Treatment”) of the indicated drugs showed effects on microglia NO- and TNF-α production. The concentrations with the highest modulatory effect are given below. Generation of nitrite was significantly higher ($P < 0.01$) when LPS stimulation was used to prime the cells [21.59 μM ± 1.22] as compared to native controls [1.63 μM ± 0.16; Fig. 3]. A significant inhibition ($P < 0.01$) of the LPS-induced increase of NO levels in the microglial culture medium was seen after cotreatment with the PBR ligand PK11195 (100 μM; 6.57 μM ± 0.88; Fig. 3). Likewise, 5 μM clonazepam [9.33 μM

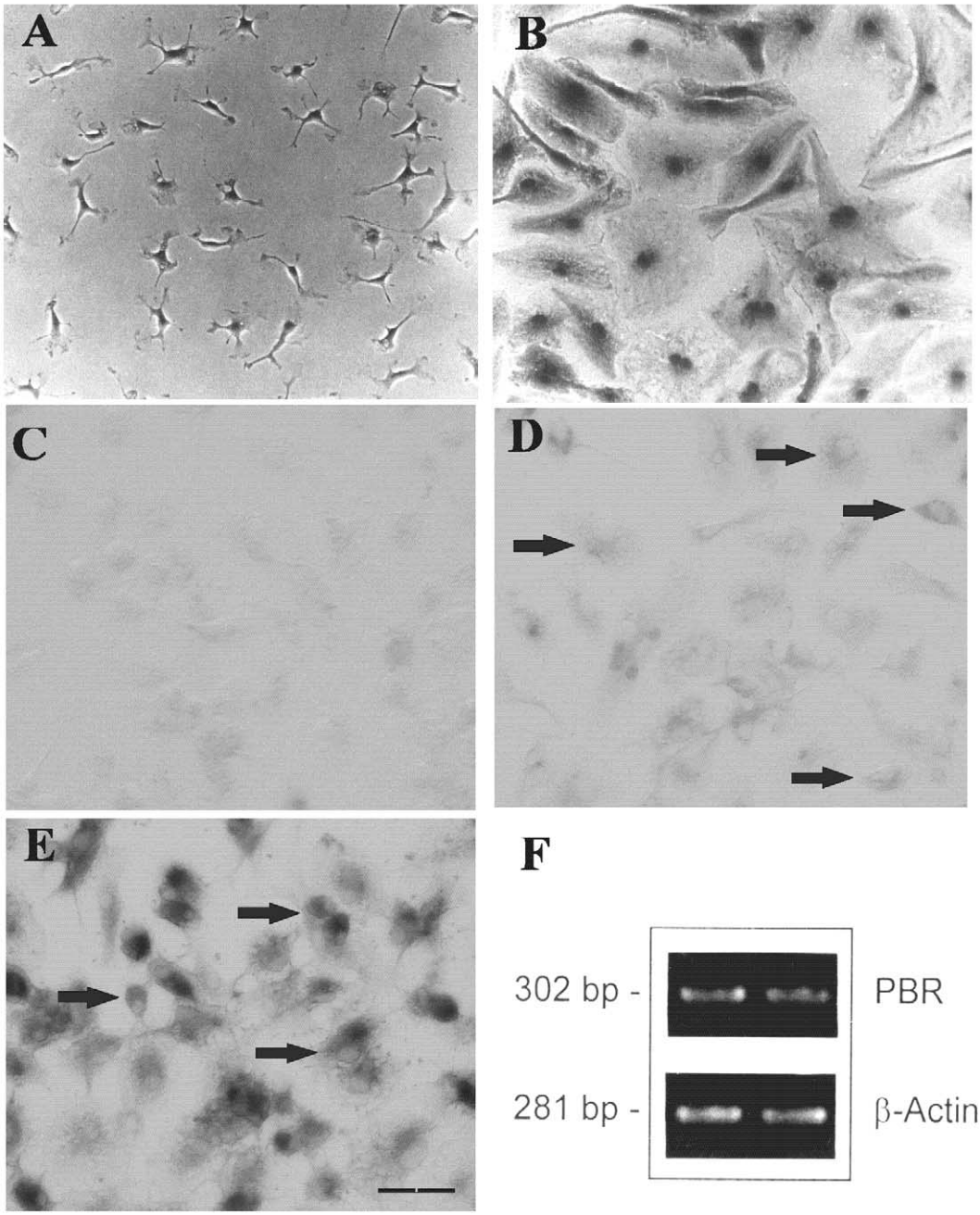

Fig. 1. Morphology of microglia cells cultivated in ACM (A, control, ramified phenotype) and after addition of LPS (B, 100 ng/ml, amoeboid phenotype). The expression of PBR is seen in immunohistochemistry: no staining was observed in the method control (C, omitting of the primary antibody), whereas a faint cytoplasmatic staining is visible in untreated cultures (arrows in D). This cytoplasmatic staining is greatly increased in LPS-treated cultures (E, arrows). Furthermore, the presence of PBR was verified by RT–PCR (F). RT–PCR products were visualized with agarose gel electrophoresis in the presence of ethidiumbromide. Electrophoresis of RT–PCR products yielded bands of expected size of 302 bp for the PBR. β-Actin housekeeping gene was used as an internal control. Bar = 15 μm.

$\pm$ 2.28; Fig. 3], 100 μM midazolam [3.16 μM $\pm$ 0.17; Fig. 3], and 100 μM diazepam [11.17 μM $\pm$ 0.96; Fig. 3] inhibited the LPS-induced increase in NO secretion, too. The addition of either flumazenil (5–20 μM) or ODN in various concentrations (1 nM–100 μM) was ineffective in influencing the LPS-induced NO production (data not shown).

Proinflammatory molecules: TNF-α

Concentration of TNF-α (Fig. 4) increased gradually in LPS-stimulated microglial cell cultures [305.68 pg/ml $\pm$ 148,66] as compared to native controls [0.06 pg/ml $\pm$ 0.01]. The addition of benzodiazepines inhibited the LPS-induced increase in cell culture supernatants in TNF-α (see Fig. 4) as

Fig. 2. LPS stimulates an increase in PBR protein expression in microglia. Microglia were stimulated with 100 ng/ml LPS for 24 h. Cell lysates with equal amounts of proteins were analyzed by Western blot analysis using a polyclonal anti-PBR antibody (1:400; a kind gift from Dr. V. Papadopoulos, Georgetown University Medical Center, Washington, DC, USA). Unstimulated microglia cells (Co) showed basal levels of PBR protein expression. In the presence of LPS the signal was greatly enhanced. Adrenal gland tissue (AG) served as positive control.

follows: +5 μM clonazepam [113.66 pg/ml ± 18.36], 10 μM (100 μM) midazolam [120.34 pg/ml ± 17.41], and 100 μM diazepam [149.68 pg/ml ± 85.57].

Effects of benzodiazepines on microglial proliferation

[^{3}H]Thymidine incorporation in cells treated with diazepam plus LPS [819 pmol/mg ± 378] was significantly lower than in those cells treated with LPS only [4193 pmol/mg ± 852, Fig. 5] at 6h.

Discussion

Peripheral-type benzodiazepine receptors are widely distributed receptors that recognize a subset of benzodiazepine receptors. Although they are expressed in the undamaged CNS at only a low level, it appears that the relative density of PBR is a reliable marker for neuroinflammation and gliosis with neuronal damage (Banati, 2002): Animal studies indicate that neuronal damage experimentally induced by excitoxicity (Altar and Baudry, 1990), ischemia (Stephenson et al., 1995), experimental facial nerve axotomy

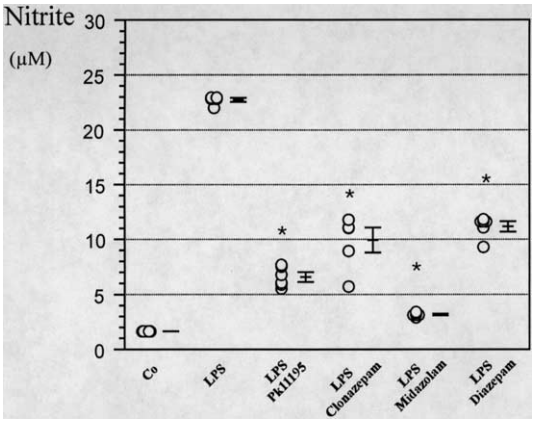

Fig. 3. Concentration of nitrite in microglial cell cultures after 24 h of coincubation with different ligands at the peripheral-type benzodiazepine receptor. Vertical lines are mean ± SEM (bars). *$P < 0.05$ compared with controls using the ANOVA test. The results were obtained from six separate measurements of identical treated wells per drug, and the data were provided by six independent experiments.

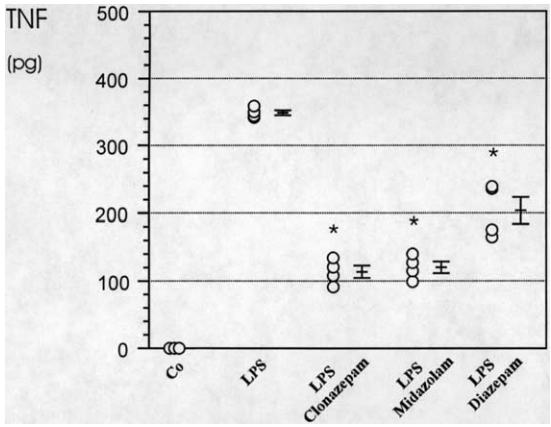

Fig. 4. Concentration of TNF-α in microglial cell cultures after 24 h of coincubation with different ligands at the peripheral-type benzodiazepine receptor. Vertical lines are mean ± SEM (bars). *$P < 0.01$ compared with controls using the ANOVA test. Data were provided by four independent experiments respectively.

(Banati et al., 1997), or the neurotoxin trimethyltin (Kuhlmann and Guilarte, 2000) causes a marked increase in PBR expression in situ. Using PET this increase is seen in humans in vivo suffering from multiple sclerosis (Banati et al., 2000) or Alzheimer's disease (Cagnin et al., 2001). The cellular source of this increased PBR expression in the CNS is controversial: Although binding of the PBR ligand PK11195 has been described in astrocytic (Itzhak et al., 1993) and microglial cell cultures (Stephenson et al., 1995), observations in in vivo cranial nerve axotomy models that leave the blood–brain barrier intact suggest that activated microglia is the main source of increased binding of PBR ligands (Banati et al., 1997). These findings could be sustained by our immunohistochemical results with the highest staining intensity in LPS-treated (activated) microglia in vitro (compare Fig. 1). Furthermore, Western blotting experiments served as evidence of an increased expression of

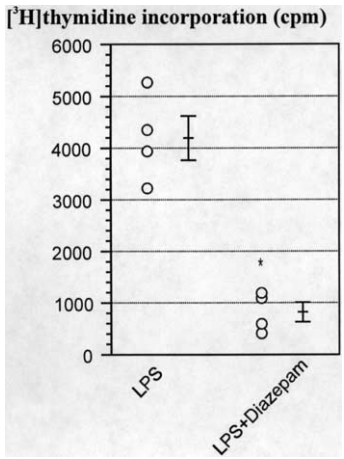

Fig. 5. Effect of 100 μM diazepam plus 100 ng/ml LPS on cell proliferation compared to LPS-only treated cell cultures. Results represent the mean ± SD from four separate experiments with fivefold determinations.

the PBR on the protein level after LPS treatment (compare Fig. 2). The possible functional role these microglial PBR receptors may play in the response to neuronal injury is unknown. In a previous study benzodiazepines bound to human microglial cells and inhibited HIV-p24-antigen expression in a concentration-dependent manner (Lokensgard et al., 1998). The present study provides unequivocal evidence that the PBR ligands PK11195, midazolam, clonazepam, and diazepam, but not the central-type benzodiazepine receptor antagonist flumazenil or the endogenous octadecaneuropeptide ODN, a processing product of the endogenous diazepam-binding inhibitor (DBI), interfere with the synthesis and release of proinflammatory and neurotoxic molecules generated by activated microglia in vitro, because they suppressed LPS-induced microglial NO and TNF-α secretion. This could lead to a reduction of the proinflammatory response of activated microglia with subsequent neuroprotection, because it is well known that both molecules are potent neurotoxins. For example, NO is neurotoxic due to inhibition of complex 1 and 2 of the respiratory chain. Moreover, it reacts with superoxide anion to generate peroxynitrite, a highly reactive molecule capable of oxidizing proteins, lipids, and DNA, which causes striatal neurodegeneration in a mouse model in vivo (Mihm et al., 2001). The cytokine TNF-α is an important factor in the regulation of apoptotic cell death: TNF-α-immunoreactive glial cells have been detected in the substantia nigra and immunoreactivity for TNF receptors was found in cell bodies and processes of most dopaminergic neurons of parkinsonian patients (Boka et al., 1994).

Increased binding sites for BZ ligands have been described in various brain tumors (Ferrarese et al., 1989; Black et al., 1990; Ikezaki and Black, 1990). Numerous observations indicate that macrophages are often observed to be clustered around tumors, and their presence is often correlated with tumor regression. BZ have been found to bind to specific receptors on macrophages and to modulate in vitro their metabolic oxidative responsiveness (Lenfant et al., 1986). It was shown that the capacity of macrophages to produce IL-1, TNF-α, and IL-6 was inhibited by i.p. injection of the PBZ ligand Ro5-4864. The results demonstrate in vivo immunosuppressive properties of PBZ mediated by, among others, TNF-α (Zavala et al., 1990). Furthermore, in our study addition of PBR ligands to microglial cell cultures inhibited microglial proliferation in vitro. An antiproliferative action of benzodiazepines was shown in cancer cells (Wang et al., 1984). Microglial proliferation and migration to the site of injury/neurodegeneration is one of the histopathological hallmarks of neurodegenerative diseases. Therefore, inhibition of microglial proliferation may also contribute to the reduction of inflammatory reactions associated with activated microglial cells.

We postulate that the presence of a high density of PBR in human neuroinflammatory and neurodegenerative diseases might be an adaptive response to neuronal damage with subsequent decreased release of neurotoxic microglial mediators. This hypothesis is supported by findings of Lacor and co-workers (Lacor et al., 1996, 1999) who demonstrated by immunohistochemistry that PBR expression is increased during Wallerian degeneration after peripheral nerve degeneration in rodents, with PBR returning to normal levels when regeneration was complete. A possible source of endogenous ligands at the PBR are astrocytes, which release substantial amounts of so-called endozepines (Patte et al., 1999). However, the precise role of endozepines in targeting the PBR is still unclear. A possible neuroprotective pharmacological strategy might target the PBR in microglia, because diazepam is neuroprotective in experimental models of stroke (Schwartz-Bloom et al., 2000).

In summary, the presence of a high density of PBR in microglia establishes the basis for additional investigations into their possible functional role, if any, in the microglial response to neuronal injury and subsequent degeneration.

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