The role of platelet activating factor in prion and amyloid-β neurotoxicity

Clive Bate,[CA] Mario Salmona[1] and Alun Williams[2]

Department of Veterinary Pathology, Glasgow University Veterinary School, Bearsden Road, Glasgow G61 1QH, UK; [1]Department of Molecular Biochemistry and Pharmacology, Istituto di Ricerche Farmacologiche Mario Negri, Via Eritrea 62, 20157 Milano, Italy; [2]Department of Pathology and Infectious Diseases, Royal Veterinary College, Hawkshead Lane, North Mymms, AL9 7TA, UK

[CA]Corresponding Author: c.bate@vet.gla.ac.uk

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In the prion diseases, neurodegeneration is preceded by the accumulation of the disease-associated isoform of the prion protein (PrP^d). In the present study, neurones treated with three different phospholipase A_2 inhibitors were resistant to the toxic effects of PrP peptides or a synthetic miniprion (sPrPl106). Phospholipase A_2 inhibitors also protected neurones against a toxic peptide found in Alzheimer's disease (amyloid-β_{1-42}). Further studies showed that neurones pre-treated with platelet activating factor (PAF) antagonists were equally resistant to PrP peptides or amyloid-β_{1-42}. Moreover, both phospholipase A_2 inhibitors and PAF antagonists reduced the activation of caspase-3, a marker of apoptosis, and the production of prostaglandin E_2 that is closely associated with neuronal death in prion or Alzheimer's diseases. *NeuroReport* 15:509–513 © 2004 Lippincott Williams & Wilkins.

Key words: Amyloid-β; Neurotoxicity; Phospholipase A_2; Platelet activating factor; Prions; Prostaglandins

INTRODUCTION

The symptoms of the transmissible spongiform encephalopathies (TSEs), otherwise known as prion diseases, or Alzheimer's disease arise following the degeneration and subsequent loss of neurones. In these diseases aggregates of insoluble, misfolded, fibrillar proteins are thought to cause this neuronal dysfunction. In the case of the prion diseases, the host encoded prion protein (designated PrP^C) is converted into a disease-related isoform (PrP^d) in a process whereby a portion of the α-helix and random coil structure in PrP^C is refolded into a β-pleated sheet [1]. In Alzheimer's disease, fibrils consisting of amyloid-β peptides derived from the cleavage of the amyloid precursor protein by γ-secretases [2] are deposited in the brain to form senile plaques [3].

Standard techniques to study the mechanisms of neuronal loss *in vitro* include the addition of peptides derived from the PrP protein [4] or from amyloid-β [5] to neuronal cultures. The neuronal injury induced by these peptides includes characteristics of apoptosis such as surface blebbing, chromatin condensation and DNA fragmentation [4,6]. In the present study we examined the neurotoxicity of fibrillar peptides by exposing the human SH-SY5Y neuroblastoma cell line, or murine primary cortical neurones, to amyloid-β_{1-42}, to peptides derived from the human PrP protein (HuPrP106-126 or HuPrP82-146), or to a synthetic murine miniprion (sPrP106-126) [7]. Initial results demonstrated that neurones treated with phospholipase A_2 (PLA_2) inhibitors were protected, implicating this enzyme and its metabolites as essential factors in neurotoxicity. Further studies then identified platelet-activating factor (PAF) as a neurotoxin that is an essential component of the pathway that leads to neuronal death following application of PrP peptides or amyloid-β_{1-42}.

MATERIALS AND METHODS

Cell lines: The human neuroblastoma SH-SY5Y cell line was grown in RPMI-1640 medium supplemented with 2 mM glutamine, standard antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) and 5% fetal calf serum (FCS). Cells were plated at 3×10^4 cells/well into 96 well microtitre plates and allowed to adhere overnight before use. Cells were pre-treated with drugs for 3 h before the addition of peptides. After a further 24 h cell viability and prostaglandin E_2 content were determined.

Primary neuronal cultures: Primary cortical neurones were prepared from embryonic day 15.5. mice as described previously [8]. Cultures were drug-treated for 3 h before the addition of peptides. For toxicity assays medium was replaced after 48 h and cell viability was determined 4 days later. In some assays cells were collected 24 h after the addition of peptides and cell lysates were tested for caspase-3 activity.

Peptides: Peptides containing amino acid residues 106–126 of the human PrP protein (HuPrP106-126) and a peptide consisting of the same amino acids in a scrambled order (HuPrP106-126scrambled) were synthesised by solid-phase

chemistry and purified by reverse-phase HPLC. A longer peptide containing amino acids 82–146 of the human PrP protein (HuPrP82-146) corresponding to a PrP fragment found in certain prion-infected human brains [9], a control peptide (HuPrP82-146scrambled), and a synthetic mini-prion (sPrP106) derived from the murine PrP sequence [7] were also used. A peptide corresponding to amino acids 1–42 of the amyloid-β protein (amyloid-$\beta_{1\text{-}42}$) and a control peptide (amyloid-$\beta_{42\text{-}1}$) were obtained from Bachem (St Helens, UK).

Cell viability assays: To determine cell survival cultures were treated with WST-1 (Roche Diagnostics Ltd, Lewes, UK) for 3 h and optical density was read on a spectrophotometer at a wavelength of 450 nm. WST-1 is cleaved to formazan by mitochondrial dehydrogenases and the amount of dye formed correlates to the number of metabolically active cells. Percentage cell survival in cultures was calculated by reference to untreated cells incubated with WST-1 (100%).

Caspase-3 activity: Caspase-3 activity was measured using a flourometric immunosorbent enzyme assay (FIENA) kit as per the manufacturer's instructions (Roche Diagnostics, Lewes, UK).

Prostaglandin E_2 assay: Analysis of total prostaglandin E_2 levels was performed using an enzyme-immunoassay kit Amersham Biotech (Amersham, UK).

Drugs: $AACOCF_3$ and aristolochic acid were obtained from Sigma (Poole UK). Cytidine-5-diphosphocholine (CDP), 1-O-hexadecyl-2-acetyl-sn-glycerol-3-phosphocholine (PAF) and a PAF-antagonist, 1-O-hexadecyl-2-acetyl-sn-glycerol-3-phospho-(N,N,N-trimethyl)-hexanolamine (Hexa-PAF) were obtained from Calbiochem (Nottingham, UK). CV-6209, U73122 and ethyl-18-OCH_3 were obtained from Biomol (Exeter, UK).

Statistical analysis: Comparison of treatment effects were carried out using one and two-way ANOVA as appropriate. *Post hoc* comparisons of means were performed as necessary.

RESULTS

PLA_2 inhibitors protect neurones against PrP peptides: The effects of drugs that inhibit some of the common signal transduction pathways on the neurotoxicity of PrP peptides were investigated. SH-SY5Y cells grown in the presence of three different PLA_2 inhibitors, 1 μM CDP, 1 μg/ml aristolochic acid or 1 μg/ml $AACOCF_3$ were resistant to the otherwise toxic effects of 40 μM HuPrP106-126 or 10 μM HuPrP82-146. The toxicity of PrP peptides was not significantly affected by treatment with inhibitors of phospholipase C (U73122 or ethyl-18-OCH_3; Table 1). The concentrations of the drugs used were chosen with reference to published inhibitory constants for the appropriate enzymes. None of the drugs used affected cell survival, even when tested at 10 times the concentration used in these assays. Cells treated with CDP, aristolochic acid or $AACOCF_3$ were also resistant to the otherwise toxic effects of 10 μM amyloid-$\beta_{1\text{-}42}$, a peptide that is associated with neurodegeneration in Alzheimer's disease [2]. The addition of control peptides did not affect cell survival (data not shown).

PAF antagonists block the neurotoxicity of PrP or amyloid-β peptides: Activation of PLA_2 results in the release of arachidonic acid and the production of PAF [10] and the addition of exogenous PAF caused a dose-dependent reduction in the survival of SH-SY5Y cells (unpublished data). In the present studies, SH-SY5Y cells pre-treated with PAF antagonists (1 μM Hexa-PAF or 1 μM CV-6209) were protected against the toxic effects of 40 μM HuPrP106-126, 10 μM HuPrP82-146 or 10 μM amyloid-$\beta_{1\text{-}42}$ (Fig. 1a). The protective effect of Hexa-PAF and CV-6209 against HuPrP82-146 were dose-dependent with inhibitory constants (IC_{50}) ~0.01 μM (Fig. 1b).

Prostaglandin E_2 production in SH-SY5Y cells: Neuronal death induced by PrP peptides is accompanied by the production of prostaglandin E_2 in SH-SY5Y cells [8]. In this study the levels of prostaglandin E_2 were significantly raised in cells treated with 10 M HuPrP82-146, with 10 μM amyloid-$\beta_{1\text{-}42}$ or with 10 μM arachidoonic acid. The production of prostaglandin E_2 was reduced in cells that had been pre-treated with 1 μM CDP, 1 μM $AACOCF_3$, 1 μM Hexa-PAF or 1 μM CV-6209. The PLA_2 inhibitors had no effect on prostaglandin E_2 production in response to arachidonic acid, which was reduced in cells pre-treated with PAF antagonists (Table 2).

PLA_2 inhibitors or PAF antagonists reduce caspase-3 activity in primary cortical neurones: In further experiments primary cortical neurones were incubated with

Table 1. PLA_2 inhibitors protect neurones against toxic peptides from PrP or amyloid-β.

	Untreated	HuPrP106-126	HuPrP82-146	Amyloid-$\beta_{1\text{-}42}$
Medium	100±3	69±4	64±6	66±6
1 μM CDP	101±5**	99±3**	101±2**	98±5**
1 μg/ml Aristolochic acid	99±3	99±5**	98±3**	100±3**
1 μg/ml $AACOCF_3$	101±4	100±4**	101±2**	102±3**
5 μM U-73122	101±7	67±3	65±3	69±4
10 μM Ethyl-18-OCH_3	100±4	66±4	66±5	64±4

The survival of SHSY-5Y cells treated with PLA_2 inhibitors (CDP, aristolochic acid or $AACOCF_3$), or PLC inhibitors (U-73122 or Ethyl-18-OCH_3) and subsequently incubated for 24 h with 40 μM HuPrP106-126, 10 μM HuPrP82-146 or 10 μM amyloid-$\beta_{1\text{-}42}$. Each value is the mean±s.d. percentage cell survival from triplicate experiments repeated three times (nine observations).
**Cell survival significantly greater ($p < 0.05$) than in cells incubated with peptides in the absence of drugs.

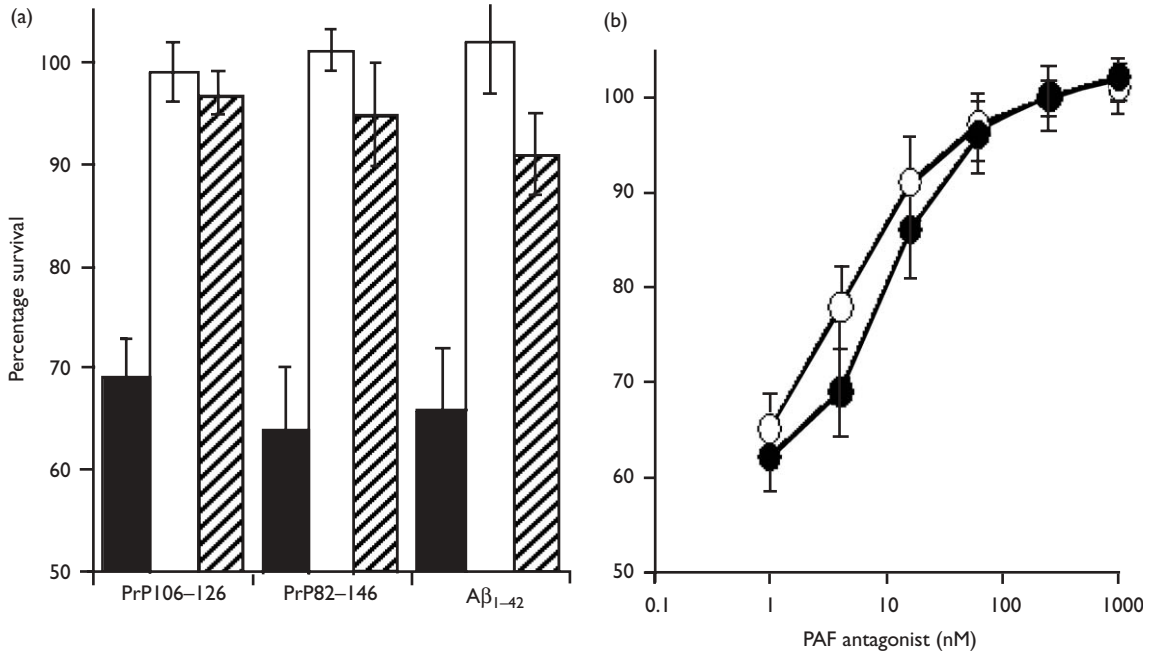

Fig. 1. PAF-antagonists protect neurones against amyloidogenic peptides. (**a**) The survival of SH-SY5Y cells pre-treated with control medium (shaded bars), with 1 μM Hexa-PAF (open bars) or with 1 μM CV-6209 (striped bars) for 3 h and thereafter incubated with 40 μM HuPrPI06-126, 10 μM HuPrP82-146 or 10 μM amyloid-β_{1-42}. (**b**) The survival of SH-SY5Y cells pre-treated with varying concentrations of Hexa-PAF (open circles) or with CV-6209 (closed circles) and thereafter incubated with 10 μM HuPrP82-146. Cell survival was measured 24 h later using the WST-1 method. Each value represents the mean percentage cell survival ± s.d. from triplicate experiments repeated four times (12 observations).

Table 2. Prostaglandin E_2 production in SH-SY5Y cells.

	Untreated	HuPrP82-146	Amyloid-β_{1-42}	Arachidonic acid
Medium	<20	362±46	281±27	203±19
1 μM CDP	<20	66±40**	63±37**	205±23
1 μg/ml $AACOCF_3$	<20	<20**	<20**	218±42
1 μM Hexa-PAF	<20	<20**	<20**	<20**
1 μM CV-6209	<20	<20**	<20**	<20**

Levels of prostaglandin E_2 (pg/ml) produced by SH-SY5Y cells pre-treated with CDP, $AACOCF_3$, Hexa-PAF or CV-6209 and thereafter incubated for 24 h with 10 μM HuPrP82-146, 10 μM amyloid-β_{1-42} or 10 μM arachidonic acid as shown. Each value is the mean ± s.d. level of prostaglandin E_2 produced by cells from triplicate experiments repeated twice (six observations).
**Prostaglandin E_2 production significantly less ($p < 0.05$) than in cells incubated with peptides or arachidonic acid in the absence of drugs.

5 μM sPrP106, 10 μM HuPrP82-146 or 10 μM amyloid-β_{1-42} in the presence or absence of 1 μM CDP, 1 μM $AACOCF_3$, 1 μM Hexa-PAF or 1 μM CV-6209. After 24 h cellular lysates were tested for caspase-3 activity as a measure of apoptosis. Caspase-3 activity was increased in cells treated with sPrP106, with HuPrP82-146 or with amyloid-β_{1-42}. However, caspase-3 activity was significantly reduced in cells pre-treated with CDP, $AACOCF_3$ or with PAF antagonists (Table 3). In further studies cortical neurones treated with 1 μM CDP, 1 μM $AACOCF_3$, 1 μM Hexa-PAF or 1 μM CV-6209 were resistant to the toxic effects of 5 μM sPrP106, 10 μM HuPrP82-146 or 10 μM amyloid-β_{1-42} (data not shown).

DISCUSSION

The present study utilized a pharmacological approach to determine the metabolic pathways that underlie neuronal loss in prion and Alzheimer's diseases. The observation that selective PLA_2 inhibitors prevented caspase-3 activation and neurodegeneration in response to a synthetic miniprion, to PrP peptides or to amyloid-β_{1-42} suggests that these fibrillar peptides activate PLA_2 initiating a cascade of events that ultimately result in neuronal death. A role for PLA_2 in neurodegeneration was suggested by the observation that PLA_2 is raised in the Alzheimer's diseased brain [11], and that amyloid-β peptides stimulate PLA_2 [12]. The precise role of PLA_2 in neurodegeneration is not clear since there are several distinct enzymes with PLA_2 activity including cytosolic ($cPLA_2$) and secretory ($sPLA_2$) isoenzymes. A previous study suggested that $AACOCF_3$ selectively inhibits $cPLA_2$ [13], and in the studies reported here low concentrations of $AACOCF_3$ inhibit neurotoxicity. It is noteworthy that the PLA_2 inhibitor CDP (clinically known as citicoline) improves cognitive function in Alzheimer's disease patients [14].

The activation of PLA_2 leads to the synthesis of PAF in neurones via the remodeling pathway [10]. PAF has been

Table 3. PLA_2 inhibitors reduce caspase-3 activity in neurones treated with peptides from PrP or amyloid-β.

	None	sPrPI06	HuPrP82-146	Amyloid-β_{1-42}
Medium	0.5 ± 0.5	6.9 ± 1.1	5.8 ± 1.1	5.7 ± 1.2
1 μM CDP	0.6 ± 0.9	1.5 ± 0.8**	2.1 ± 0.6**	1.3 ± 1.1**
1 μg/ml $AACOCF_3$	0.4 ± 0.5	1.2 ± 1.3**	1.8 ± 0.8**	1.5 ± 0.5**
1 μM Hexa-PAF	0.5 ± 0.6	0.6 ± 0.9**	0.4 ± 0.7**	0.7 ± 0.8**
1 μM CV-6209	0.6 ± 0.8	1.1 ± 0.7**	0.9 ± 0.3**	1.2 ± 1.0**

Primary cortical neurones were treated with CDP, $AACOCF_3$, Hexa-PAF or CV-6209 for 3 h and thereafter incubated with 5 μM sPrPI06, 10 μM HuPrP82-146 or 10 μM amyloid-β_{1-42} for a further 24 h. Each value is the mean ± s.d. caspase-3 activity (AFC) of neuronal cells from triplicate experiments repeated twice (six observations).

** Caspase-3 activity significantly less ($p < 0.05$) than in cells incubated with peptides in the absence of drugs.

shown to be a mediator of neurodegeneration following ischaemia [15], and in the present study the addition of 10 μM PAF killed SH-SY5Y cells and primary cortical neurones (data not shown). The role of PAF in neurodegeneration was confirmed by showing that cells pre-treated with two different PAF antagonists were resistant to both PrP and amyloid-β peptides. The protective effect of the PAF antagonists were dose-dependent with an IC_{50} ~10 nM. Pre-treatment with the PLA_2 inhibitors, or the PAF antagonists also rendered neuronal cells resistant to the toxicity of partially purified prion preparations (data not shown). Neurones treated with inhibitors of cyclo-oxygenases (COX), the enzymes that metabolise arachidonic acid to prostaglandins, are also resistant to the neurotoxic effects of PrP peptides [16] or amyloid-β_{1-42} [17], suggesting that these peptides activate the production of toxic prostaglandins. This is supported by the findings that levels of prostaglandin E_2 are increased in Creutzfeldt-Jakob disease [18] and Alzheimer's disease [19]. The presence of PLA_2 inhibitors reduced prostaglandin E_2 production in response to both PrP and amyloid-β peptides (activation of PLA_2 causes the release of arachidonic acid from membrane phospholipids), but had no effect on prostaglandin E_2 production after the addition of exogenous arachidonic acid (showing that the PLA_2 inhibitors, CDP or $AACOCF_3$, had no direct effect on the COX enzymes). In contrast, a PAF antagonist reduced prostaglandin E_2 production in response to both PrP and amyloid-β peptides, and after the addition of exogenous arachidonic acid. Moreover, a PAF antagonist greatly reduced the neurotoxicity of arachidonic acid (unpublished data). These results are compatible with the hypothesis that, following the release of arachidonic acid by PLA_2, PAF can modulate COX-related production of toxic prostaglandins, or toxic free oxygen radicals produced as by-products of prostaglandin metabolic pathways.

CONCLUSION

We present evidence that identifies PLA_2 and the release of PAF as important components in the process of neuronal death induced by either PrP peptides or by amyloid-β_{1-42}. While we are aware that neuronal death *in vivo* is a complex process that may involve a number of mechanisms, the results of the neuroprotection studies reported here suggest that the use of specific PLA_2 inhibitors such as CDP may be beneficial for patients with either TSEs or Alzheimer's disease. PAF, generated following PLA_2 activation, appears to be a potent neurotoxin and PAF antagonists may also be able to prevent neurodegeneration in TSEs or Alzheimer's disease.

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