Cyclo-oxygenase inhibitors protect against prion-induced neurotoxicity *in vitro*

Clive Bate,[CA] Scott Rutherford, Mike Gravenor,[1] Stuart Reid[2,3] and Alun Williams

Institute of Comparative Medicine, and [2]Comparative Epidemiology and Informatics, Department of Veterinary Pathology, Glasgow University Veterinary School, Bearsden Road, Glasgow G61 1QH ; [1]Institute for Animal Health, Compton RG20 7NN; [3]Department of Statistics and Modelling Science, University of Strathclyde, Livingstone Tower, Glasgow G1 1XW, UK

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

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The mechanisms of neuronal loss during the course of the prion diseases are not fully understood. In this study, neurones treated with certain non-steroidal anti-inflammatory drugs (NSAIDs) were protected against the otherwise toxic effects of a peptide derived from the prion protein, or extracts containing infectious prions (PrP^{SC}). These NSAIDs inhibit the cyclo-oxygenase (cox) enzymes that metabolise arachidonic acid to prostaglandins (PG). Conversely, drugs that inhibited the metabolism of arachidonic acid to leucotrienes enhanced neurotoxicity. Studies with selective inhibitors highlighted the importance of the cox-1 isoform in prion-induced neurotoxicity. The cox-1 inhibitors also inhibited neuronal PGE_2 production and protected both neuroblastoma cells and primary cortical neurones against prions. They also reduced microglia-mediated killing of prion-treated neurones. *NeuroReport* 13:1933–1938 © 2002 Lippincott Williams & Wilkins.

Key words: Cyclo-oxygenase; Microglia; Neurotoxicity; Non-steroidal anti-inflammatory drugs; Prion; Prostaglandins

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) or prion diseases are invariably fatal neurodegenerative disorders that include scrapie, bovine spongiform encephalopathy and the variant form of Creutzfeldt–Jakob disease, (vCJD). A key event in prion disease pathogenesis is the conversion of a normal host protein, designated PrP^{C} [1] into a disease isoform, PrP^{SC}, via a process whereby a portion of the α-helix and random coil structure in PrP^{C} is refolded into a β-pleated sheet [2]. This PrP^{SC} is then able to self-aggregate into toxic fibrils that accumulate in areas of the brain and pathology, such as glial activation and neuronal loss [3,4] then develops.

Neuronal culture systems have been developed to investigate interactions between prions, neurones and microglia. Previous studies have demonstrated the neurotoxicity of either preparations containing PrP^{SC} [5] or defined peptides derived from either the human [6] or the mouse prion protein [7] *in vitro*, and that at least two processes appear to be involved in prion-induced neuronal death. In the absence of microglia, β-sheet-rich prion-derived peptides or prion preparations are directly toxic for neuroblastoma cells [8,9]. However, the addition of microglia to neurones significantly increased the neurotoxicity of PrP peptides, suggesting that microglia kill neurones sublethally damaged by PrP^{SC} [9,10].

In this study we report the development of a rapid, reliable model for investigating the neurotoxicity of prions by combining the murine neuroblastoma cell line NB4 1A3 in combination with either PrP peptides or prion preparations containing infectious prions. Toxicity assays conducted on both neuroblastoma cells and on primary cortical neurones indicated that arachidonic acid metabolism by cyclo-oxygenase-1 was crucial in prion-induced neurotoxicity. The effect of non-steroidal anti-inflammatory drugs (NSAIDs) on the killing of prion-damaged neuroblastoma cells by microglia was also examined.

MATERIALS AND METHODS

Cell lines: The mouse neuroblastoma NB4 1A3 cell line (European Collection of Cell Cultures) was grown as previously described. Cells were plated at 5×10^4 cells per well into 96-well microtitre plates (Costar) and allowed to adhere to plates overnight before use. Cells were pretreated with drugs for 3 h before the addition of either prion preparations or prion peptides for a further 24 h incubation, after which cell survival was assayed by an MTT assay.

Primary neuronal cultures: Primary cortical neurones were prepared from brains removed from embryonic day 15.5 mice as described previously [9]. After 2 days, the medium was changed to B27 neurobasal medium (Life Technologies, Paisley, UK) containing 2 mM glutamine, with 5 μM cytosine arabinoside to prevent the proliferation of astrocytes and with 5 mM L-leucine methylester (LLME) to reduce the number of contaminating microglia. Mature cultures were pretreated with drugs for 3 h before the addition of media containing peptides. Medium was

replaced after 48 h and cell viability was determined 4 days after the addition of peptides. In some experiments, microglia were added a further 3 h after the addition of PrP peptides.

Microglia cultures: Primary microglia cultures were prepared from newborn mice as described previously [9]. Isolated microglia were added to neurones within 96-well microtitre plates at 5×10^3 cell per well (i.e. to give a neurone:microglia ratio of $\sim$10:1).

Peptides: Peptides containing the amino acid residues 105–132 of the murine prion protein (MoPrP105-132), a control consisting of the same amino acids as MoPrP105-132 but in a scrambled order (MoPrP105-132 scrambled), a peptide corresponding to amino acid residues 106 to 126 of the human prion protein (HuPrP106-126), and a control (HuPrP106-126 scrambled), were synthesized by solid-phase chemistry and purified by reverse-phase HPLC.

Prion preparations: Partially purified brain extracts containing prions (PrP^{SC}) were obtained from C57BL/6 mice infected with the C506M3 scrapie strain, killed at terminal stage of disease, using a standard protocol. SAF were incorporated into liposomes as previously described [5]. These prion preparations were interpreted into liposomes as described previously [5]. Liposomes containing similarly partially purified brains from age-matched, non-infected mice acted as controls.

Drugs: Glutamate, staurosporine, acetyl salicylic acid, indomethacin, nondihydroguaiacetic acid (NDGA), ibuprofen, ketorolac and DuP-697 were obtained from Sigma. DFU (5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl) phenyl-2(5H)-furanone) was a gift from Merck–Frosst, Canada, and caffeic acid was obtained from Novabiochem.

Toxicity assays: The survival of treated cells was determined by addition of the metabolic dye 3,[4,5 dimethylthiazol-2yl]-2,5 diphenyltetrazolium bromide (MTT) assay as previously described. Optical density was measured on a spectrophotometer at a wavelength of 595 nm and results calculated by reference to untreated cells. In co-cultures, the contribution of non-neuronal cells to the MTT assay of co-cultures was never $> 5\%$ of the total absorbance.

Prostaglandin E_2 (PGE_2) assay: Analysis of total, intracellular or extracellular PGE_2 levels was determined using a competitive enzyme immunoassay kit (Amersham Biotech, UK) according to the manufacturer's instructions.

Statistical analysis: Comparison of treatment effects was carried out using one and two-way ANOVA techniques as appropriate. *Post hoc* comparisons of means were performed as necessary. For all statistical tests, significance was set at the 5% level.

RESULTS

Neuroblastoma cells treated with NSAIDs are protected against the neurotoxicity of PrP peptides: Previous studies demonstrated that the addition of MoPrP105-132 to NB4 1A3 neuroblastoma cells resulted in a dose-dependent reduction in cell survival [9]. Cell viability, in cultures containing 40 μM MoPrP105-132 ($69 \pm 9\%$, mean ($\pm$ s.d.) percentage cell survival) was consistently less than the viability of cells incubated in control medium ($100 \pm 3\%$, $p < 0.05$) or cultures containing MoPrP105-132-scrambled ($99 \pm 94\%$, $p < 0.05$). To determine if the MoPrP105-132-induced neurotoxicity involved the metabolism of arachidonic acid, neuroblastoma cells were treated with drugs that inhibit either cyclo-oxygenases (cox), the enzymes that metabolize arachidonic acid to prostaglandins (PGs), or with drugs that inhibit lipoxygenase, the enzyme which converts arachidonic acid to leucotrienes, before the addition of MoPrP105-132. The survival of cells incubated with 40 μM MoPrP105-132 in the presence of cox inhibitors such as acetyl salicylic acid or ibuprofen was significantly greater than for cells incubated with 40 μM MoPrP105-132 alone ($p < 0.05$). In contrast cells, treated with lipoxygenase inhibitors (caffeic acid or NDGA) plus 40 μM MoPrP105-132 showed reduced neuronal survival when compared to neuroblastoma cells incubated with MoPrP105-132 alone ($p < 0.05$; Fig. 1).

The neurotoxicity of MoPrP105-132 is reduced by cox-1 inhibitors: Two isoforms of the cox enzyme exist that are thought to have different roles [11] and to be expressed in different cell types [12]. To determine the involvement of these two isoforms in PrP-induced neurotoxicity, we compared the effects of inhibitors selective for either cox-1 or cox-2. Neuroblastoma cells were treated with either the cox-1 inhibitors acetyl salicylic acid or ketorolac, or with the

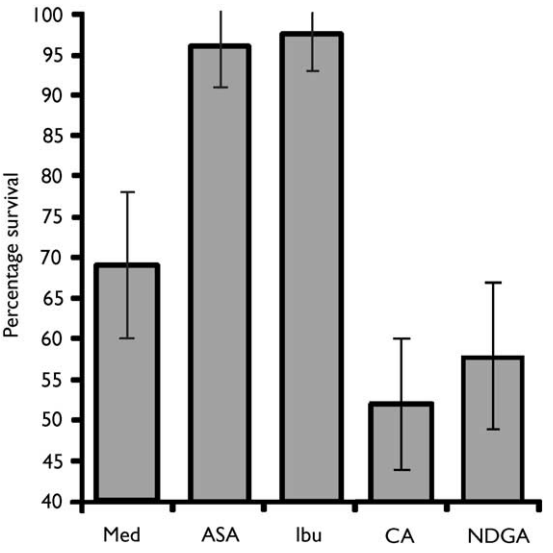

Fig. 1. Neuroblastoma cells pretreated with NSAIDs are protected against the toxic effects of MoPrP105-132. The survival of untreated neuroblastoma cell (Med), neuroblastoma cells treated with 1 μM of the cox inhibitors acetyl salicylic acid (ASA) or ibuprofen (Ibu), or cells treated with 1 μM of the lipoxygenase inhibitors caffeic acid (CA) or nordihydroguaiaretic acid (NDGA), and incubated with 40 μM MoPrP105-132. Each bar represents the mean $\pm$ s.d. from experiments conducted in triplicate and repreated at least ten times (a minimum of 30 observations).

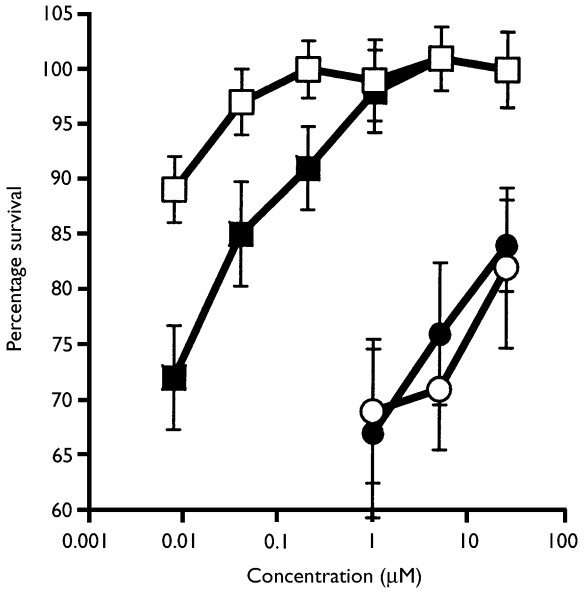

Fig. 2. Protection against the neurotoxicity of MoPrP105-132 correlates with inhibition of cox-1. The survival of neuroblastoma cells treated with different concentrations of acetyl salicylic acid (filled squares), ketorolac (open squares), DFU (open circles) or DuP-697 (closed circles). Each point shown represents the mean ± s.d. From experiments conducted in triplicate and repeated four times (12 observations).

cox-2 selective inhibitors DFU or DuP-697 before the addition of 40 μM MoPrP105-132. The survival of cells treated with acetyl salicylic acid or ketorolac was significantly greater than cells treated with DFU or DuP-697 ($p < 0.05$). Acetyl salicylic acid inhibited 50% of MoPrP105-132-induced neurotoxicity at < 0.1 μM, a concentration that selectively inhibits cox-1 [13]. Cells treated with ketorolac at concentrations that selectively inhibit cox-1 [14] were also protected against PrP neurotoxicity. In contrast, cells treated with 5 μM of either DFU or DuP-697, which are more potent inhibitors of cox-2 than cox-1, remained sensitive to MoPrP105-132-induced neurotoxicity (Fig. 2).

NSAIDs reduce PGE2 production in PrP-treated neuroblastoma cells: The production of PGs in response to PrP peptides in both untreated and NSAID-treated cells was examined. Neuroblastoma cells incubated with 40 μM MoPrP105-132 produced greater amounts of PGE_2 (324 ± 24 pg/ml) than did untreated neuroblastoma cells (< 20 pg/ml; $p < 0.05$) or neuroblastoma cells treated with 40 μM MoPrP105-132 scrambled (< 20 pg/ml; $p < 0.05$). The production of total PGE_2 in response to 40 μM MoPrP105-132 was reduced in neuroblastoma cells that had been pretreated with acetyl salicylic acid, ketorolac, DFU or DuP-697 when compared to untreated cells incubated with MoPrP105-132 (Table 1). When the production of intracellular and extracellular PGE_2 was investigated separately, intracellular PGE_2 production was found to be inhibited by pretreatment with cox-1 inhibitors and extracellular PGE_2 production was reduced by both cox-1 and cox-2 inhibitors (Table 1).

Specificity of neuroprotection: To determine if the neuroprotective effect of NSAIDs was specific for PrP peptides, neuroblastoma cells treated with acetyl salicylic acid, indomethacin or ibuprofen were exposed to MoPrP105-132, HuPrP106-126 (the excitatory amino acid glutamate) or the apoptosis-inducing drug staurosporine. The treated neuroblastoma cells were resistant to the toxic effects of both MoPrP105-132 and HuPrP106-126, but remained sensitive to the toxicity of high concentrations of glutamate or to staurosporine (Table 2). In a further series of experiments the survival of neuroblastoma cells treated with indomethacin, acetyl salicylic acid or ibuprofen and exposed to three different prion preparations was significantly greater than the survival of untreated neuroblastoma cells exposed to SAF ($p < 0.05$). In contrast, the survival of neuroblastoma cells treated with caffeic acid and SAF preparations was significantly reduced when compared to cells treated with SAF alone ($p < 0.05$). Preparations made from non-infected mouse brains were not toxic for neuroblastoma cells.

Primary cortical neurones are protected against the neurotoxicity of MoPrP105-132 or prion preparations by NSAIDs: In order to determine whether the above results were indicative of the process in non-transformed cells, experiments were also conducted on primary murine cortical neurones. Neurones were treated with various drugs for 3 h before exposure to 40 μM MoPrP105-132. There was a significant difference between the survival of cells treated with different drugs, which was due to increased survival of cells treated with 1 μM acetyl salicylic

Table 1. PGE_2 production in neurones treated with MoPrP105-132. The amounts of PGE_2 produced (pg/ml) by neuroblastoma cells incubated for 24 h with 40 μM MoPrP105-132 in the presence or absence of the cox inhibitors. Total PGE_2, intracellular and extracellular PGE_2 were measured in separate assays. Each value given represents the mean ± s.d. from triplicate experiments repeated twice (6 observations). Background levels of PGE_2 from untreated neuroblastoma cells, or neuroblastoma cells incubated with 40 μM MoPrP105-132 scrambled, were below the minimum detection limit of 20 pg/ml (data not shown).

Drug treatment	Total PGE_2	Intracellular PGE_2Extracellular	PGE_2
Control medium	324 ± 24	182 ± 27	202 ± 24
Ketorolac 0.1 μM	35 ± 17	< 20	54 ± 14
Acetyl salicylic acid 0.1 μM	84 ± 19	< 20	102 ± 16
DFU 0.1 μM	172 ± 35	174 ± 35	58 ± 17
DuP-697 0.1 μM	198 ± 27	190 ± 22	82 ± 16

Table 2. Neurones treated with NSAIDs are protected against the toxicity of prion extracts or PrP peptides. Survival of neuroblastoma cells incubated with 40 μM PrP peptides, staurosporine, glutamate or with three different prion preparations, in the presence of drugs that affect arachidonic acid metabolism (all at 1 μM).

	Control	Indomethacin	Acetyl salicylic acid	Ibuprofen	Caffeic acid
MoPrP105-132	69 ± 5	98 ± 3	97 ± 4	97 ± 4	54 ± 5
MoPrP105-132 scrambled	100 ± 3	101 ± 4	99 ± 5	100 ± 2	99 ± 3
HuPrP106-126	67 ± 7	98 ± 3	99 ± 5	98 ± 4	51 ± 3
HuPrP106-126 scrambled	99 ± 3	98 ± 4	100 ± 3	101 ± 3	101 ± 4
Staurosporine 40 nM	35 ± 3	36 ± 2	36 ± 3	37 ± 4	33 ± 4
Glutamate 100 mM	64 ± 3	64 ± 3	64 ± 3	63 ± 8	62 ± 5
Prion preparation 1	54 ± 7	96 ± 5	94 ± 4	96 ± 3	45 ± 5
Prion preparation 2	59 ± 5	95 ± 4	92 ± 4	97 ± 3	37 ± 5
Prion preparation 3	62 ± 6	99 ± 2	95 ± 5	98 ± 2	45 ± 5
Normal mouse brain 1	99 ± 3	100 ± 4	101 ± 2	101 ± 3	98 ± 4
Normal mouse brain 2	101 ± 4	101 ± 3	101 ± 3	102 ± 3	101 ± 4
Normal mouse brain 3	97 ± 6	102 ± 4	100 ± 4	100 ± 4	99 ± 5

Extracts from non-infected mouse brains did not kill neuroblastoma cells. Each value represents the mean ± s.d. from experiments conducted in triplicate and repeated at least eight times.

acid ($p < 0.05$) or with 1 μM ibuprofen ($p < 0.05$). In contrast, neurones pretreated with either 1 μM DFU or 1 μM DuP-697 remained susceptible to the toxic effects of MoPrP105-132 (i.e. survival of cells was not significantly different from untreated cells). The survival of neurones that had been pretreated with caffeic acid before the addition of 40 μM MoPrP105-132 was reduced compared with untreated cells ($p < 0.05$; Fig. 3a). In addition, pretreated neurones were exposed to prion preparations. The survival of cells treated with acetyl salicylic acid ($p < 0.05$) or with ibuprofen ($p < 0.05$) was significantly greater than that of untreated cells incubated with prion preparations ($p < 0.05$). In contrast, neurones pretreated with DFU, DuP-697 or caffeic acid remained susceptible to the toxic effects of prions (Fig. 3b).

NSAIDs protect PrP-damaged neurones against microglia-mediated killing: Previous studies have shown that microglia selectively kill neurones treated with PrP peptides [10]. The survival of cortical neurones treated with 40 μM PrP105-132 and co-cultured with microglia was greatly increased in co-cultures containing either ibuprofen or acetyl salicylic acid compared with untreated co-cultures ($p < 0.05$). Co-cultures containing DuP-697 or DFU exhibited similar levels of cell survival to control cultures and co-cultures containing caffeic acid showed reduced cell survival when compared with untreated co-cultures (Fig. 4).

DISCUSSION

In these studies, a number of the common NSAIDs were shown to protect against the neuronal loss that would have otherwise occurred after the addition of PrP-derived peptides, or PrP^{SC} preparations. Neuroblastoma cells were used in most of these studies since the absence of

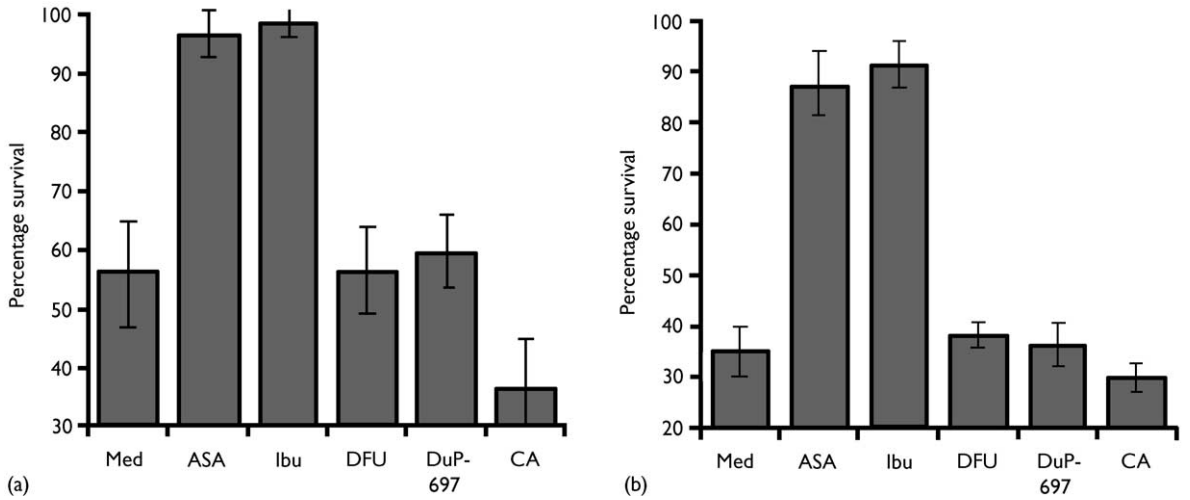

Fig. 3. (**a**) Cox-1 inhibitors protect primary cortical neurones against the neurotoxicity of MoPrP105-132. Survival of LLME-treated primary cortical neurones pretreated with 1 μM of cox-1 inhibitors (acetyl salicylic acid (ASA) or ibuprofen (Ibu)), cox-2 inhibitors (DFU or DuP-697) or a lipoxygenase inhibitor (caffeic acid (CA)), and subsequently incubated with 40 μM MoPrP105-132. Each bar represents the mean ± s.d. from experiments conducted in triplicate and repeated at least 10 times (30 observations). (**b**) Cox-1 inhibitors protect primary cortical neurones against the neurotoxicity of prions. Survival of LLME-treated primary cortical neurones pretreated with 1 μM of cox-1 inhibitors (acetyl salicylic acid (ASA) or ibuprofen (Ibu)), cox-2 inhibitors (DFU or DuP-697) or a lipoxygenase inhibitor (caffeic acid (CA)), and subsequently incubated with prion extracts. Each bar represents the mean ± s.d. from experiments conducted in triplicate and repeated at least 10 times (30 observations).

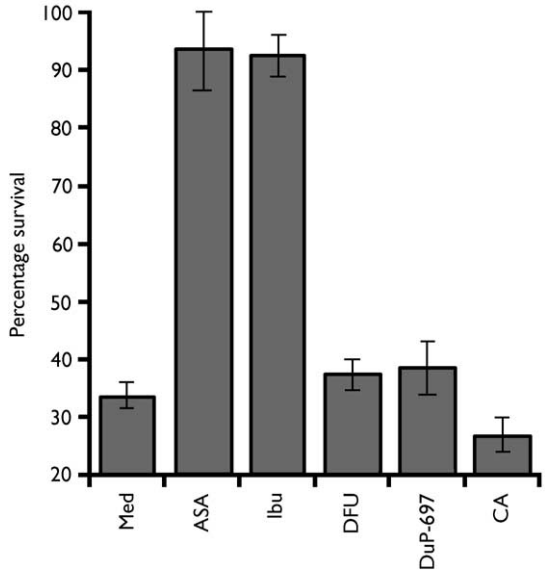

Fig. 4. Cox-1 inhibitors protect MoPrP105-132-treated primary cortical neurones against microglia-mediated killing. Survival of LLME-treated primary cortical neurones pretreated with 1 μM cox-1 inhibitors (acetyl salicylic acid (ASA) or ibuprofen (ibu)), cox-2 inhibitors (DFU or DuP-697) or the lipoxygenase inhibitor (caffeic acid (CA) and 40 μM MoPrP105-132 before the addition of 5×10^3 microglia/well. Each bar shown represents the mean from experiments conducted in triplicate and repeated at least 10 times.

contaminating microglia allowed us to examine the direct effect of PrP peptides on neurones. However, experiments were also conducted using primary cortical neurones to ensure that the process of prion-induced neurotoxicity was similar in non-transformed cells. The protective effect of NSAIDs was observed for both neuroblastoma cells and primary cortical neurones. Moreover, neurones pretreated with NSAIDs were not only resistant to the effects of the synthetic PrP peptide, but also to extracts containing infectious PrP^{SC}.

Since, at high concentrations ibuprofen and acetyl salicylic acid inhibit both cox-1 and cox-2, studies were performed with selective inhibitors to determine which isoform was involved in PrP-induced neurotoxicity. High levels of the cox-2 selective inhibitors DFU and DuP-697 were required to inhibit MoPrP105-132-mediated neurotoxicity. For example, ~50 μM DFU was required to reduce PrP-induced toxicity by 50%, a concentration much greater than the published inhibitory constant (IC_{50}) of DFU for cox-2 (41 nM) [13]. The concentration of acetyl salicylic acid that inhibited MoPrP105-132-mediated neurotoxicity, ~0.05 μM, was at a level that inhibits the cox-1, but not the cox-2 enzyme [15]. Similar results were obtained with the cox-1-selective inhibitor ketorolac. Furthermore, primary cortical neurones were protected against MoPrP105-132-induced neurotoxicity by acetyl salicylic acid or ketorolac at concentrations (1 μM) that inhibited cox-1 [14] but were still killed by MoPrP105-132 when treated with either DFU or DuP-697 at concentrations that inhibited cox-2. Initially, these results were considered surprising in the light of previous studies that had shown that the expression of cox-2 is increased in glutamate-treated hippocampal neurones [16] and in Alzheimer's disease (AD) [17].

In AD, a disease that shares many pathological features with prion disease, epidemiological evidence has shown a strong reduction in the incidence and onset of clinical disease in patients treated with NSAIDs [18]. Given that it is generally considered that the cox-2 isoform is induced by disease, there were expectations that the newly introduced cox-2 inhibitors would benefit AD patients without causing the adverse (cox-1 related) gastrointestinal effects of traditional NSAIDs. However, a recently reported clinical trial of a cox-2 selective NSAID in AD indicated that it was not protective [19], suggesting that the protective effect of traditional NSAIDs may be through inhibition of cox-1. Our current findings, that cox-1 inhibitors prevent prion-induced neuronal injury, support the view that cox-1 is the important isoform in chronic neurodegenerative disease. The neuroprotective effect of NSAIDs would not appear to be due to any direct effect on PrP^{SC} as electron microscopy studies failed to demonstrate any disruption of preformed prion fibrils or inhibition of fibril formation (data not shown).

Previous studies have shown that PGE_2 increased in brain areas showing neuronal death in murine scrapie [20] and raised levels of PGE_2 were detected in the cerebrospinal fluid of patients with Creutzfeldt–Jacob disease [21,22]. In this study, the production of PGE_2 from neuroblastoma cells incubated with MoPrP105-132 was reduced by pretreatment with either cox-1 or cox-2 inhibitors. The pretreatment of neurones with DFU or with DuP-697, at concentrations that did not affect PrP-induced neurotoxicity, reduced extracellular PGE_2 production but had no effect on intracellular production. In contrast, pretreatment of neurones with ketorolac or with acetyl salicylic acid, at concentrations that selectively inhibited cox-1, inhibited intracellular PGE_2 production. However, neuroblastoma cells survived the incubation with high concentrations of PGE_2 (data not shown) suggesting that PGE_2 may simply be a marker of the pathological process. Nevertheless, by-products of PG pathways may be important in prion disease neuropathogenesis as the metabolism of the cyclic endoperoxides (PGG_2 and PGH_2) results in the release of toxic free oxygen radicals that can cause neuronal damage [10]. It is not known whether PGs interact with caspase-8, which has been shown to required for the toxicity of prion peptides in the human SK-N-BE neuroblastoma cell line [23].

The lipoxygenases have been previously implicated in the toxicity of prion peptides [24]. However, in our assays, neurones that were pretreated with lipoxygenase inhibitors before the addition of PrP105-132 showed a significant decrease in survival when compared to untreated cells incubated with PrP105-132. The reasons underlying this difference are not clear. We note that those previous studies used cerebellar granule neurones and it is possible that arachidonic acid metabolism is different in different neuronal populations. However, the protective effect of the cox-1 inhibitors was not only found in the cortical neurones and neuroblastoma cell line reported here but also in two other murine, and one human cell line (data not shown).

In the present, study the presence of NSAIDs in co-cultures containing prion-damaged neurones and microglia was associated with greater levels of neuronal survival

compared with untreated co-cultures, suggesting that NSAIDs interfere with the ability of microglia to recognise or respond to prion-damaged neurones. Microglia respond to changes induced in neuronal cells by PrP peptides [9] and the current study suggests that NSAIDs may prevent these changes from occurring. The NSAIDs also inhibit PG production from microglia [25], which might affect their ability to kill damaged neurones. In this respect it is worth noting that the levels of cox-1 are increased in microglia in the AD disease brain [17].

CONCLUSION

This study showed that drugs which block the production of PGs affect some of the processes that are involved in neuronal death following prion infection. These observations provide insights into the metabolic pathways that are involved during neuronal death in response to prions or prion-derived peptides. Moreover, they raise the intriguing possibility that NSAIDs might be used in combination with drugs primarily targeted at the structure of PrP^{SC} to ameliorate the symptoms of prion infection.

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