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Phospholipase A_2 Inhibitors or Platelet-activating Factor Antagonists Prevent Prion Replication*

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A key feature of prion diseases is the conversion of the cellular prion protein (PrP^C) into disease-related isoforms (PrP^{Sc}), the deposition of which is thought to lead to neurodegeneration. In this study a pharmacological approach was used to determine the metabolic pathways involved in the formation of protease-resistant PrP (PrP^{res}) in three prion-infected cell lines (ScN2a, SMB, and ScGT1 cells). Daily treatment of these cells with phospholipase A_2 (PLA_2) inhibitors for 7 days prevented the accumulation of PrP^{res}. Glucocorticoids with anti-PLA_2 activity also prevented the formation of PrP^{res} and reduced the infectivity of SMB cells. Treatment with platelet-activating factor (PAF) antagonists also reduced the PrP^{res} content of cells, while the addition of PAF reversed the inhibitory effect of PLA_2 inhibitors on PrP^{res} formation. ScGT1 cells treated with PLA_2 inhibitors or PAF antagonists for 7 days remained clear of detectable PrP^{res} when grown in control medium for a further 12 weeks. Treatment of non-infected cells with PLA_2 inhibitors or PAF antagonists reduced PrP^C levels suggesting that limiting cellular PrP^C may restrict prion formation in infected cells. These data indicate a pivotal role for PLA_2 and PAF in controlling PrP^{res} formation and identify them as potential therapeutic agents.

Prion diseases, or transmissible spongiform encephalopathies (TSEs),[1] are fatal neurodegenerative disorders that include Kuru, Creutzfeldt-Jakob disease (CJD), and Gerstmann-Sträussler-Scheinker (GSS) disease in man. Central to the pathogenesis of TSEs is the conversion of the host-encoded cellular prion protein (PrP^C) into β-sheet-rich disease-related isoforms (PrP^{Sc}) (1). The formation of PrP^{Sc} is accompanied by changes in biological and biochemical properties such as an increased resistance to proteases (2), the protease-resistant core of PrP^{Sc} designated PrP^{res}. This PrP^{Sc} self-aggregates and forms amyloidgenic fibrils and, in most prion diseases, aggregates of PrP^{Sc} are detected in the diseased brain before neuronal loss is observed (3).

The development of current therapeutic strategies is largely based on the belief that the deposition of amyloidgenic PrP^{Sc} fibrils leads to neurodegeneration and the clinical symptoms of prion diseases. Many compounds that interact directly with PrP to prevent PrP^{Sc} formation and/or disrupt preformed PrP^{Sc} aggregates have now been identified; these include large, flat multicyclic compounds and synthetic peptides specifically designed to disrupt the β-sheets in PrP^{Sc} (4–6). However, recent studies demonstrated that the propagation of PrP^{Sc} within prion-infected cells could be reduced following re-routing the trafficking of PrP^C following treatment with suramin (7). Other studies have also shown that restricting the supply, or alterations in the trafficking, of PrP^C can prevent the formation of PrP^{Sc} (8–11). In the present study we tested the hypothesis that the trafficking of PrP^C within cells, that is vital to PrP^{Sc} formation, is controlled by activation of specific signaling pathways. Previous studies have variously reported that PrP^C is associated with activation of the tyrosine kinases Fyn (12), with the cyclic AMP/protein kinase A pathway (13), or with the phospholipase A_2 (PLA_2)/cyclo-oxygenase (COX) pathway (14). Thus, in this study, a pharmacological approach was used to investigate the role of signal transduction mechanisms on levels of PrP^C in non-infected cells, and PrP^{Sc} in scrapie-infected neuroblastoma cell lines (ScN2a, ScGT1, or SMB cells). These studies indicate that activation of PLA_2 and the production of platelet-activating factor (PAF) (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), a bioactive phospholipid that is not stored in a preformed state (15) but rapidly synthesized in neurons in response to cell specific stimuli via the remodeling pathway (16), are essential factors in the production of PrP^{Sc}.

EXPERIMENTAL PROCEDURES

PrP^{res} Production—Scrapie-infected neuroblastoma cells (ScN2a cells; gift from Dr. M. Rogers, University College, Dublin, Ireland) that produce PrP^{Sc} and infectious agent, were grown in Hams F12 medium containing 2 mM glutamine, standard antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) and 2% fetal calf serum. SMB cells (TSE Resource Centre, Institute for Animal Health, Compton, UK), which also produce PrP^{Sc} and infectious agent, were grown in RPMI 1640 medium containing standard antibiotics, 2 mM glutamine and 2% fetal calf serum. ScGT1 cells (supplied by Dr. Sylvain Lehmann, CNRS-IGH, Montpellier, France), an immortalized murine hypothalamic neuronal cell line infected by the scrapie Chandler isolate and that persistently expresses PrP^{res}, were grown in Optimem supplemented with 2 mM glutamine, 5% fetal calf serum, and standard antibiotics. To measure the effect of drugs on PrP^{res} formation, cells were plated at 1×10^5 cells/well in 6-well microtiter plates in the presence or absence of drugs. Cells were then grown with daily changes of media and PrP^{res} production was evaluated after 7 days. Non-infected N2a cells or SMB cells

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[1] The abbreviations used are: TSE, transmissible spongiform encephalopathies; PAF, platelet-activating factor; CDP, cytidine-5-diphosphocholine; PrP, prion protein; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; PLA_2, phospholipase A_2; COX, cyclo-oxygenase; hexa-PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycerol-3-phospho-(*N*,*N*,*N*-trimethyl)-hexanolamine; PG, prostaglandin.

TABLE I

PLA_2 inhibitors reduced the PrP^{res} content of prion-infected cell lines

To measure the effect of drugs on PrP^{res} formation, ScN2a, SMB, or ScGT1 cells were plated at 1×10^5 cells/well in 6-well plates. Cells were grown for 7 days, with daily changes of medium, in the presence or absence of the drugs shown. The levels of protease-resistant PrP in cellular lysates were then determined using an ELISA. Values shown are the mean PrP^{res} pg/1×10^7 cells ± S.D. of triplicate experiments repeated three times (9 observations).

Enzyme	Drug	Pr^{press} ScN2a	Pr^{press} SMB	Pr^{press} ScGTI
		pg/1 × 10^7 cells ± S.D.		
Control		1047 ± 202	2193 ± 249	6118 ± 332
Phospholipase C	Neomycin sulfate (10 μM)	955 ± 57	2245 ± 188	6238 ± 348
	U-73122 (5 μM)	1032 ± 93	2303 ± 204	6407 ± 404
	Ethyl-18-OCH_3 (10 μM)	1052 ± 115	2150 ± 138	6082 ± 429
Phospholipase A_2	CDP (1 μM)	<50[a]	<50[a]	<50[a]
	BEL (1 μM)	<50[a]	<50[a]	<50[a]
	Aristolochic acid (0.5 μg/ml)	<50[a]	100 ± 78[a]	167 ± 60[a]
	$AACOCF_3$ (1 μg/ml)	<50[a]	<50[a]	<50[a]

[a] PrP^{res} content of cells significantly less ($p < 0.05$) than that of untreated cells.

that had been "cured" of infectivity by serial passages in the presence of pentosan polysulphate (PS cells) (17) were used as controls. For time course experiments, ScN2a cells were plated at 5×10^6 cells/well in the presence or absence of dexamethasone and collected after 24, 48, or 72 h. At the end of the treatment, cells were detached and counted to establish cell numbers.

Evaluation of Infectivity—To challenge mice directly, cultured SMB cells were detached and counted, washed twice with phosphate-buffered saline then put through one rapid freeze-thaw cycle. The homogenate was precipitated, washed twice with phosphate-buffered saline, and finally homogenized in sterile 0.9% (w/v) saline at 2.5×10^6 cell equivalents/ml. Mice under halothane anesthesia were injected intracerebrally with 30 μl (7.5×10^4 cell equivalents) of this homogenate. Mice were monitored for clinical signs of scrapie until reaching a predefined clinical end point. All animal work was conducted strictly according to local and national guidelines.

Cell Lysates—Lysates were made from ScN2a, SMB, or ScGT1 cells to evaluate PrP^{res} content. Cells were detached and counted, washed twice in PBS and finally suspended in an extraction buffer containing 10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, and 0.5% sodium deoxycholate at 1×10^7 cells/ml. Samples were sonicated at 4 °C for 10 min, and cellular debris was removed by centrifugation at $5{,}000 \times g$ for 1 min. The supernatant was digested with proteinase K at 10 μg/ml for 1 h at 37 °C, digestion was blocked with 5 mM phenylmethylsulfonyl fluoride, and samples were then halved; one half was tested for PrP by an enzyme-linked immunosorbent assay (ELISA; see below) and the other examined by PrP Western blot. This second sample was centrifuged at $50{,}000 \times g$ for 4 h at 4 °C; the pellet was dissolved in 50 μl of Laemmli buffer (Bio-Rad), boiled for 5 min and 20 μl subjected to electrophoresis on a 15% polyacrylamide gel. Proteins were transferred onto a Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences) by semidry blotting. Membranes were blocked using 10% milk powder in Tris-buffered saline containing 0.2% Tween 20. PrP^{res} was detected by incubation with mAb SAF83 (a gift from J. Grassi, CEA, Saclay, France) for 1 h at room temperature, followed by a secondary anti-mouse IgG conjugated to peroxidase (1 h at room temperature). Detection of bound antibody was visualized using an enhanced chemiluminescence kit (Amersham Biosciences). Lysates were also made from the non-infected N2a cells to evaluate PrP^C content. Cells were treated as above except that proteinase K digestion was excluded.

PrP ELISA—PrP in lysates was measured using a PrP-specific ELISA as previously described (18). Briefly, Nunc Maxisorb Immunoplates were coated with antibodies isolated from rabbit antiserum raised to the ovine PrP100-111 peptide conjugated to keyhole limpet hemocyanin (gift from Dr. J. P. M. Langeveld, Central Institute for Animal Disease Control, Lelystad, The Netherlands). Cell lysates were applied, and specific binding was detected by mAb SAF83 (gift from Prof. J. Grassi, CEA, Saclay, France), followed by an anti-mouse IgG-alkaline phosphatase conjugate (Sigma) and an appropriate indicator. Results were calculated by reference to a standard curve of recombinant murine PrP (Prionics, Zurich, Switzerland). The detection limit of this assay is 50 pg/ml.

Drugs—Dexamethasone, prednisolone, prednisone, hydrocortisone, ibuprofen, acetyl salicylic acid, nordihydroguaiaretic acid (NDGA), AACOCF$_3$, aristolochic acid, bromoenol lactone (BEL), and neomycin sulfate were obtained from Sigma. Cytidine-5-diphosphocholine (CDP), caffeic acid, 1-*O*-alkyl-2-acetyl-*sn*-glycerol-3-phosphocholine (PAF), and

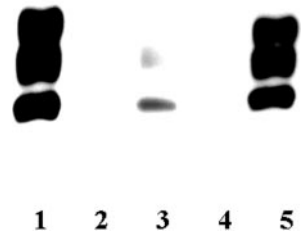

FIG. 1. **PLA_2 inhibitors reduce the PrP^{res} content of ScGT1 cells.** ScGT1 cells were grown for 7 days in the presence of control medium (*lane 1*), 1 μg/ml $AACOCF_3$ (*lane 2*), 0.5 μg/ml aristolochic acid (*lane 3*), 1 μM CDP (*lane 4*), or 10 μM ethyl-18-OCH_3 (*lane 5*). Cellular lysates were digested with 10 μg/ml proteinase K for 1 h at 37 °C and protease-resistant PrP was visualized by immunoblot with mAb SAF83 using enhanced chemiluminescence.

1-*O*-alkyl-2-acetyl-*sn*-glycerol-3-phospho-(*N*,*N*,*N*-trimethyl)-hexanolamine (hexa-PAF) were obtained from Novabiochem (Nottingham, UK). C-PAF, CV-6209, SQ-22536, U73122, and ethyl-18-OCH_3 were obtained from Biomol (Exeter, UK).

Prostaglandin (PG)E_2 Assay—Analysis of cellular PGE_2 levels was determined in cells by using an enzyme immunoassay kit (Amersham Biosciences) according to the manufacturer's instructions. This assay is based on competition between unlabeled PGE_2 in the sample and a fixed amount of labeled PGE_2 for a PGE_2-specific antibody. The detection limit of this assay is 20 pg/ml.

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

RESULTS

PLA_2 Inhibitors Reduce the PrP^{res} Content of Three Prion-infected Cell Lines—In an initial screening experiment, the effects of drugs that inhibit some of the common signal transduction pathways were investigated for their effects on the PrP^{res} content of ScN2a cells. ScN2a cells treated daily for 7 days with one of four different PLA_2 inhibitors (1 μM CDP, 1 μg/ml aristolochic acid, 1 μM BEL, or 1 μg/ml $AACOCF_3$) contained significantly less PrP^{res} than did untreated cells. In contrast, the levels of PrP^{res} in ScGT1 cells were not significantly affected by treatment with three inhibitors of phospholipase C (Table I and Fig. 1). To confirm the effects of PLA_2 inhibitors on PrP^{res} production, two other prion-infected neuroblastoma cell lines (SMB and ScGT1 cells) were also treated with these drugs. The PrP^{res} content of SMB or ScGT1 cells, treated with CDP, aristolochic acid, BEL, or $AACOCF_3$ was also greatly reduced. Even at concentrations 10 times higher than those used in these experiments, the drugs used did not alter cell survival or cell growth (data not shown).

TABLE II
Glucocorticoids reduce the PrP^{res} content of prion-infected cell lines

The effects of glucocorticoids on PrP^{res} formation were determined by plating ScN2a, SMB or ScGT1 cells at 1×10^5 cells/well in 6 well plates. Cells were grown for 7 days in the presence of the 1 μM glucocorticoids as shown. The levels of protease-resistant PrP in cellular lysates were then determined using an ELISA. Values shown are the mean PrP^{res} pg/1 $\times 10^7$ cells ± S.D. of quadruplicate experiments repeated three times (12 observations).

Drug	PrP^{res}		
	ScN2a	SMB	ScGT1
	pg/1 $\times 10^7$ cells ± S.D.		
None	1104 ± 148	2004 ± 261	5848 ± 435
Dexamethasone (1 μM)	<50[a]	<50[a]	<50[a]
Hydrocortisone (1 μM)	<50[a]	<50[a]	<50[a]
Prednisolone (1 μM)	<50[a]	<50[a]	<50[a]
Prednisone 1 (μM)	1142 ± 98	1983 ± 224	5985 ± 389

[a] PrP^{res} content of cells significantly less ($p < 0.05$) than that of untreated cells.

Corticosteroids Reduce the PrP^{res} Content of Prion-infected Cell Lines—In the present study, ScN2a, SMB, or ScGT1 cells treated with 1 μM dexamethasone, 1 μM hydrocortisone, or 1 μM prednisolone contained undetectable amounts of PrP^{res}, whereas cells treated with 1 μM prednisone, an inactive prodrug that is converted to active prednisolone in the liver, did not affect PrP^{res} levels (Table II). In further studies, the inhibitory effects of dexamethasone on PrP^{res} content of ScN2a cells was shown to be dose-dependent (Fig. 2). SMB and ScGT1 cells treated with dexamethasone also demonstrated a dose-dependent reduction in PrP^{res} (data not shown).

The effects of 1 μM dexamethasone on ScN2a cells were not immediate as levels of PrP^{res} in treated cells were not significantly different from control cells after 24 h (1009 pg/ml ± 48 in treated cells *versus* 1112 pg/ml ± 97 in untreated cells, $n = 8$ independent observations, mean PrP^{res} ± S.D.) but they were significantly reduced after 48 h (561 pg/ml ± 116 *versus* 1129 pg/ml ± 76, $n = 8$, $p < 0.05$) and further reduced after 72 h (66 pg/ml ± 55 *versus* 1076 pg/ml ± 77, $n = 8$, $p < 0.05$). We were unable to detect PrP^{res} in cells that had been treated for 4 days or more with 1 μM dexamethasone. When ScN2a cells that had been treated with 1 μM dexamethasone for 7 days were then grown for a further 12 weeks in drug-free medium these cells remained clear of detectable amounts of PrP^{res} (<50 pg/ml). Similarly, SMB or ScGT1 that had been treated with 1 μM dexamethasone for 7 days remained clear of detectable amounts of PrP^{res} when grown in drug-free medium for 12 weeks.

PLA_2 Inhibitors Reduce PGE_2 Production in Prion-infected Cells—PrP peptides increase PLA_2 activity resulting in the production of PGE_2 (14). In the present study the levels of PGE_2 were significantly raised in prion-infected cells when compared with their non-infected counterparts, which suggests that prion infection activates PLA_2 pathways in neurons. Prion-infected cells treated with 1 μM CDP, 1 μg/ml $AACOCF_3$, or 1 μM dexamethasone produced significantly less PGE_2 than did untreated cells showing that drug treatment did indeed inhibit PLA_2 (Fig. 3).

PAF Antagonists Block PrP^{res} Formation—The effects of downstream pathways following PLA_2 activation on the formation of PrP^{res} were investigated. Arachidonic acid, released from membrane phospholipids by PLA_2, is converted to leucotrienes and prostaglandins by the lipoxygenase (LOX) and COX enzymes respectively. Because the PrP^{res} content of ScN2a, SMB or ScGT1 cells was not affected by treatment with the LOX or COX inhibitors, other factors produced following PLA_2 activation were therefore examined. PAF is generated in neurons by the remodeling pathway following PLA_2 activation (16)

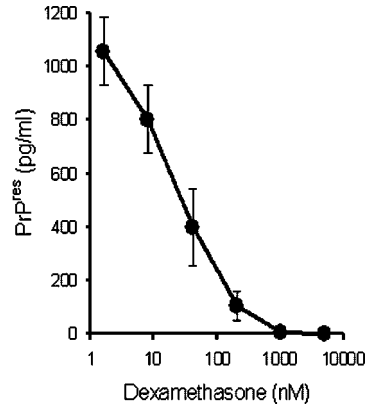

FIG. 2. **Dexamethasone causes a dose-dependent reduction in the PrP^{res} content of ScN2a cells.** To determine the efficacy of dexamethasone, ScN2a cells were grown for 7 days in the presence of different concentrations of dexamethasone as shown. The levels of protease-resistant PrP in cellular lysates were then determined using an ELISA. Values shown are the mean PrP^{res} pg/1 $\times 10^7$ cells ± S.D. of quadruplicate experiments repeated three times (12 observations).

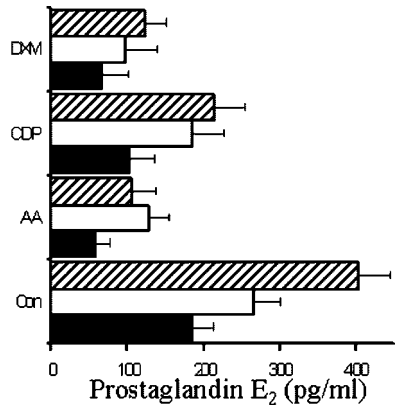

FIG. 3. **PLA_2 inhibitors reduced PGE_2 production in prion-infected cells.** ScN2a cells (*shaded bars*), SMB cells (*open bars*), or ScGT1 cells (*striped bars*) were incubated in control medium (*Con*), 1 μg/ml $AACOCF_3$ (*AA*), 1 μM CDP (*CDP*), or 1 μM dexamethasone (*DXM*). Levels of PGE_2 were measured after 24 h. Values shown are the mean level of PGE_2 pg/ml ± S.D. produced during quadruplicate experiments repeated three times (12 observations).

and the PrP^{res} content of cells was reduced following treatment with the PAF antagonists hexa-PAF, CV-6209, ginkgolide A, or ginkgolide B (Table III). These PAF antagonists did not affect the survival, or growth rates of prion-infected cells. All four PAF antagonists caused a dose-dependent reduction in the PrP^{res} content of ScN2a cells (Fig. 4), and there was a highly significant relationship between the level of PrP^{res} and the concentration of PAF antagonist ($p < 0.05$) for all 4 drugs. We noted that at each concentration, PrP^{res} levels were greater in cells treated with gingkolide A compared with gingkolide B ($p < 0.05$). Furthermore, the PrP^{res} content of ScN2a cells was reduced to below detectable levels following 7 days of treatment with 2 μM PAF antagonists (<50pg/1 $\times 10^7$ cells). Treatment with the PAF antagonists also caused a dose-dependent reduction in the PrP^{res} content of SMB and ScGT1 cells. Furthermore, ScN2a, SMB, or ScGT1 cells treated with 2 μM hexa-PAF, or with 2 μM ginkgolide B, for 7 days remained free of detectable PrP^{res} when grown in drug-free medium for a further 12 weeks (data not shown).

PAF Increases PrP^{res} Formation—To compliment the PAF antagonist studies, prion-infected cells were grown in medium containing PAF agonists. The PrP^{res} content of cells treated with PAF agonists (2 μM PAF or 2 μM C-PAF) were significantly higher than untreated cells (Table IV and Fig. 5). The PAF

TABLE III
PAF antagonists prevent PrP^{res} formation in prion-infected cells

ScN2a, SMB, or ScGT1 cells were grown for 7 days, with daily changes of medium, in the presence of control medium, in media supplemented with COX inhibitors (1 μM acetyl salicylic acid (aspirin) or 1 μM ibuprofen), with LOX inhibitors (5 μM NDGA or 5 μM caffeic acid), or with PAF antagonists (1 μM hexa-PAF, 1 μM CV-6209, 1 μM ginkgolide A or 1 μM ginkgolide B). The levels of protease-resistant PrP in cellular lysates were then determined using an ELISA. Values shown are the mean PrP^{res} pg/1 $\times$ 10^7 cells $\pm$ S.D. of triplicate experiments repeated three times (9 observations).

Enzyme	Drug	PrP^{res} ScN2a	PrP^{res} SMB	PrP^{res} ScGT1
		pg/1 $\times$ 10^7 cells $\pm$ S.D.		
Control		1047 ± 202	2193 ± 249	6118 ± 388
COX	Aspirin	955 ± 57	2245 ± 188	6238 ± 348
	Ibuprofen	1032 ± 93	2303 ± 204	6407 ± 404
LOX	NDGA	1052 ± 115	2150 ± 138	6082 ± 429
	Caffeic acid	985 ± 148	1894 ± 268	5958 ± 482
PAF antagonists	Hexa-PAF	<50[a]	<50[a]	<50[a]
	CV-6209	55 ± 25[a]	100 ± 78[a]	167 ± 60[a]
	Ginkgolide A	80 ± 34[a]	159 ± 38[a]	<50[a]
	Ginkgolide B	<50[a]	<50[a]	<50[a]

[a] PrP^{res} content of cells significantly less ($p < 0.05$) than that of untreated cells.

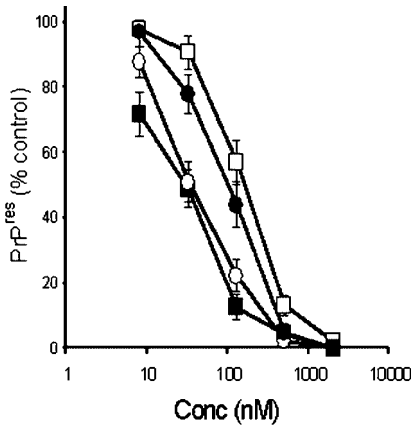

FIG. 4. **PAF antagonists cause a dose-dependent reduction in the PrP^{res} content of prion-infected cells.** ScN2a cells were grown in the presence of different concentrations of PAF antagonists: hexa-PAF (*open circles*), CV-6209 (*closed circles*), ginkgolide A (*open squares*), or ginkgolide B (*closed squares*) for 7 days. The levels of protease-resistant PrP were subsequently determined in an ELISA. Values shown are the mean PrP^{res} pg/1 $\times$ 10^7 cells $\pm$ S.D. of triplicate experiments repeated three times (9 observations).

TABLE IV
PAF agonists increase the PrP^{res} prion-infected cells

Incubating ScN2a, SMB, or ScGT1 cells with PAF agonists showed the effect of PAF agonists on the formation of PrP^{res}. Cells were grown for 7 days, with daily changes of medium, in the presence of control medium, or in media supplemented with PAF agonists (2 μM PAF or 2 μM C-PAF). The levels of protease-resistant PrP in cellular lysates were then determined using an ELISA. Values shown are the mean PrP^{res} pg/ml $\pm$ S.D. of triplicate experiments repeated 4 times ($n = 12$).

	PrP^{res} ScN2a	PrP^{res} SMB	PrP^{res} ScGT1
	pg/1 $\times$ 10^7 cells $\pm$ S.D.		
Control	1047 ± 202	2193 ± 249	6079 ± 542
PAF (2 μM)	1371 ± 90	3584 ± 332	18528 ± 3325
C-PAF (2 μM)	1320 ± 64	3169 ± 404	12484 ± 1842

agonists had no effect on the survival, growth rates, or protein concentration of ScN2a, SMB, or ScGT1 cells. While PAF caused an increase in the PrP^{res} content of all cell types, the increase in ScN2a cells was only 30%. PAF agonist had a greater effect on SMB cells, nearly doubling PrP^{res} content, and, on ScGT1 cells, the PrP^{res} content was increased 2–3-fold. To determine if the addition of PAF could restore PrP^{res} production to prion-infected cells treated with PLA_2 inhibitors,

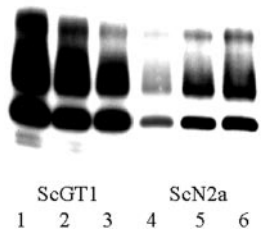

FIG. 5. **PAF increases the PrP^{res} content of ScN2a and ScGT1 cells.** ScN2a or ScGT1 cells were grown for 7 days in the presence of control medium (*lanes 3* and *4*), 2 μM C-PAF (*lanes 2* and *5*), or 2 μM PAF (*lanes 1* and *6*). Protease-resistant PrP was demonstrated by immunoblot with mAb SAF83.

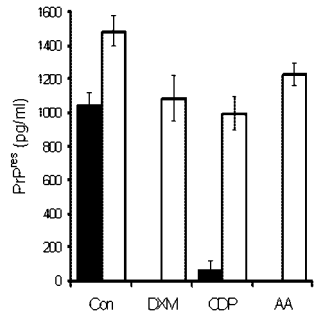

FIG. 6. **PAF reverses the inhibition of PrP^{res} formation by PLA_2 inhibitors.** ScN2a cells were grown in control medium (*Con*), in 1 μM dexamethasone (*DXM*), 1 μM CDP, or 1 μg/ml aristolochic acid (*AA*) in the absence (*shaded bars*) or presence of 1 μM PAF (*open bars*). Values shown are the mean PrP^{res} pg/1 $\times$ 10^7 cells $\pm$ S.D. of triplicate experiments repeated four times ($n = 12$).

ScN2a cells were treated with a mixture containing PLA_2 inhibitors (1 μM CDP, 1 μg/ml $AACOCF_3$, or 1 μM dexamethasone) and 2 μM PAF. The addition of PAF was able to restore PrP^{res} levels in ScN2a cells co-treated with PLA_2 inhibitors or dexamethasone (Fig. 6).

PAF Antagonists Reduce PrP^C Levels in Non-infected Cells—Since the production of PrP^{res} is dependent on the presence of PrP^C, the effect of PLA_2 inhibitors, PAF antagonists or PAF on PrP^C levels in non-infected cells was investigated. The PrP^C content of untreated N2a cells (33.2 $\pm$ 2.9 ng/1 $\times$ 10^7 cells) was significantly higher than that of cells treated with PLA_2 inhibitors (1 μM CDP: 12.8 $\pm$ 3.2, $n = 9$, $p < 0.05$; 1 μg/ml $AACOCF_3$: 2.5 $\pm$ 2.1, $n = 9$, $p < 0.05$; or 1 μM dexamethasone: 8.4 $\pm$ 2.4, $n = 9$, $p < 0.05$), or with the PAF antagonists 2 μM hexa-PAF (1.1 $\pm$ 1.2, $n = 9$, $p < 0.05$), 1 μM CV-6209 (6.8 $\pm$ 1.4, $n = 9$, $p < 0.05$), 1 μM ginkgolide A (1.5 $\pm$ 1.7, $n = 9$, $p < 0.05$), or 1 μM

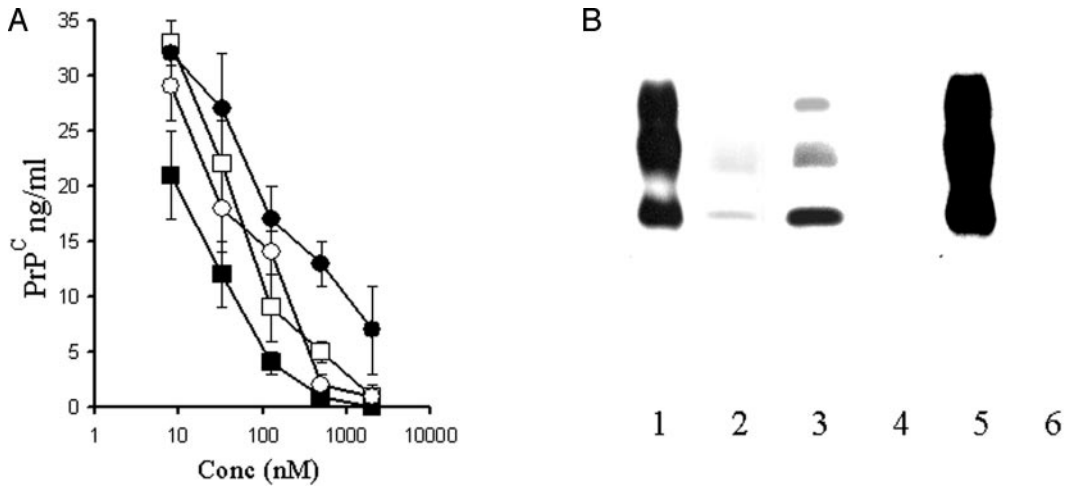

FIG. 7. **PAF antagonists cause a dose-dependent reduction in the PrP^C content of N2a cells.** *A*, N2a cells were grown in the presence of different concentrations of PAF antagonists: hexa-PAF (*open squares*), CV-6209 (*closed squares*), ginkgolide A (*open circles*), or ginkgolide B (*closed circles*) for 24 h. The levels of PrP^C/1 × 10^7 cells were subsequently determined in an ELISA. Values shown are the mean ± S.D. of quadruplicate experiments repeated two times (8 observations). *B*, N2a cells were grown in the presence of control medium (*lane 1*), 2 μM hexa-PAF (*lane 2*), 1 μM CV-6209 (*lane 3*), 1 μM ginkgolide B (*lane 4*), 2 μM PAF (*lane 5*), or 2 μM PAF (*lane 6*) after digestion with proteinase K. PrP was visualized by immunoblot with mAb SAF83 using enhanced chemiluminescence.

ginkgolide B (0.4 ± 0.8, $n = 9$, $p < 0.05$). All PAF antagonists used caused a dose-dependent reduction in the levels of PrP^C in N2a cells, and there was a highly significant relationship between PAF antagonist concentration and PrP^C levels ($p < 0.05$). In addition, the type of ginkgolide used had a significant effect on PrP^C levels ($p < 0.05$) with cells treated with ginkgolide A containing more PrP^C than cells treated with the same concentration of ginkgolide B (Fig. 7). Time course studies showed that PrP^C levels were reduced within 24 h and remained low in the presence of any of the PAF antagonists for up to 7 days. However, even after prolonged treatment (7 days), removal of the PAF antagonists resulted in PrP^C levels returning to normal within 24 h. Conversely, the PrP^C content of N2a cells treated with PAF agonists, 2 μM PAF (74.6 ± 4.8, $n = 9$, $p < 0.05$), or 2 μM C-PAF (58.9 ± 3.8, $n = 9$, $p < 0.05$) was significantly higher than that of untreated N2a cells. The PrP^C formed in N2a cells treated with PAF agonists remained sensitive to digestion with proteinase K.

Dexamethasone Reduces the Infectivity of SMB Cells—Dexamethasone is a glucocorticoid widely used in medical practice. It has various mechanisms of action, including inhibition of PLA_2. To determine if dexamethasone-treated SMB cells retained infectivity, C57/Bl mice were inoculated via the intracerebral route with homogenates from untreated SMB cells or SMB cells treated for 7 days with 200 nM dexamethasone. The mean incubation period in mice inoculated with lysates from dexamethasone-treated cells (206 ± 8 days) (incubation period ± S.D.) was significantly longer than in mice inoculated with lysates from untreated SMB cells (179 ± 6 days; $n = 8$ mice in each group, $p < 0.05$).

DISCUSSION

In the present study we utilized a pharmacological approach to determine the metabolic pathways that underlie the formation of PrP^{res} in three prion-infected neuroblastoma cell lines (ScN2a, ScGT1, and SMB cells). In a broad screen of compounds we found that 4 different drugs that inhibit PLA_2 (aristolochic acid, $AACOCF_3$, BEL, and CDP) reduced the PrP^{res} content of prion-infected cells. The concentrations of the PLA_2 inhibitors used were at least 10 times less than the concentration of these drugs that had a toxic effect and treatment with PLA_2 inhibitors did not affect total cellular protein levels.[2] We confirmed that the drugs used inhibited PLA_2 by measuring levels of PGE_2 (a marker of PLA_2 activity). In the present study prion-infected cells treated with CDP, aristolochic acid or $AACOCF_3$ produced significantly less PGE_2 than untreated cells. It is of interest to note that none of the drugs completely inhibited PLA_2 activity, possibly because there exist several distinct enzymes with PLA_2 activity including cytosolic ($cPLA_2$) and secretory ($sPLA_2$) isozymes (19). Although aristolochic acid and CDP inhibit both $cPLA_2$ and $sPLA_2$, low concentrations of $AACOCF_3$ or BEL, which are reported to selectively inhibit $cPLA_2$ (20), inhibited PrP^{res} formation (Table I) indicating that $cPLA_2$ may be the isozyme of interest.

PLA_2 can also be inhibited by the lipocortins, a family of proteins that are produced in response to the glucocorticoids (21). In the present study cells treated with the active glucocorticoids: dexamethasone, hydrocortisone, and prednisolone showed a reduced PrP^{res} content, whereas the inactive precursor prednisone had no effect. The effect of dexamethasone was dose-dependent, and PrP^{res} was reduced to below detectable levels at nanomolar concentrations of dexamethasone. A significant effect on PrP^{res} content was not seen until 2 days after the commencement of treatment with dexamethasone, and cells were not clear of PrP^{res} until 4 days after treatment. Nevertheless, ScN2a cells that had been treated with 1 μM dexamethasone for 7 days remained free of detectable PrP^{res} when grown in drug-free medium for a further 12 weeks. Our *in vivo* observations showed that SMB cells treated with 200 nM dexamethasone for 7 days contained reduced levels of infectivity. Such observations are consistent with previous reports that transient steroid administration immediately postinfection reduced the susceptibility of mice to scrapie after peripheral challenge (22). However, the use of glucocorticoids in prion diseases should be treated with caution due to the observation that chronic administration of glucocorticoids can itself lead to neuronal atrophy (23).

Since PLA_2 and many of its metabolites play important roles in signal transduction, it is possible that altered levels of second messengers could cause the decrease in the PrP^{res} content of cells indirectly. Although the activation of PLA_2 is functionally associated with the production of prostaglandins the PrP^{res}

[2] C. Bate and A. Williams, unpublished data.

content of cells was not affected by treatment with inhibitors of either COX or LOX. The activation of PLA_2 also leads to the synthesis of the bioactive phospholipid PAF in neurons via the remodeling pathway (16). PAF is not stored in a preformed state, but rather is rapidly synthesized in response to cell-specific stimuli (15) and in this study four different PAF antagonists all reduced the PrP^{res} content of ScN2a, ScGT1, or SMB cells. The effects of PAF antagonists were dose-dependent with an IC_{50} ~50 nM, and at a concentration of 2 μM two PAF antagonists (hexa-PAF and ginkgolide B) were able to reduce PrP^{res} to below detectable levels. The finding in the present study that ginkgolide B had a greater effect on PrP^{res} formation than ginkgolide A is consistent with previous reports that ginkgolide B a more potent PAF antagonist than ginkgolide A (24). The role of PAF in prion replication was supported by two further complementary studies. Firstly, the addition of PAF agonists (PAF or C-PAF) increased the production of PrP^{res} in all 3 prion-infected cell lines without affecting total cellular protein concentrations. The magnitude of the effects of the PAF agonists were cell type-dependent, with a greater increase in PrP^{res} content seen in ScGT1 cells than in SMB cells and both showing greater effects than the ScN2a cells. Secondly, the addition of PAF restored PrP^{res} production in dexamethasone or CDP-treated ScN2a cells. Collectively, these results suggest that the effect of dexamethasone or the PLA_2 inhibitors on PrP^{res} formation is mediated via a reduction in PAF formation.

The observation that PrP^{C} is essential for the development of prion diseases (25) suggests that the density and cellular location of PrP^{C} may influence PrP^{res} production. Both the PLA_2 inhibitors and the PAF antagonists reduced cellular PrP^{C} levels indicating that these drugs may prevent the formation of PrP^{res} by limiting the supply of the PrP^{C} substrate. Ginkgolide B, a more potent PAF antagonist than ginkgolide A (24), had a greater effect on PrP^{C} levels in N2a cells than ginkgolide A. In contrast, PAF agonists increased cellular PrP^{C} levels, further indicating the importance of PAF in controlling PrP^{C} expression. The PrP^{C} in PAF treated cells remained sensitive to proteinase K digestion, unlike PrP^{C} species induced in N2a cells treated with proteasome inhibitors (26). The regulation of PrP^{C} expression is poorly understood, previous studies have shown that in neuronal cell lines PrP^{C} expression was increased after treatment with insulin, nerve growth factor, epidermal growth factor, or tumor necrosis factor α (27, 28).

There are a number of possible mechanisms for the exact manner by which PAF antagonists could affect PrP^{res} formation. PrP^{C} is found in lipid rafts or caveolae (29), specialized membrane compartments that contain high levels of cholesterol and sphingomyelin (30). Since the formation of these lipid rafts is cholesterol-dependent (31), and drugs that affect cholesterol levels influence the formation of PrP^{res} (8, 18), it is possible that PAF may regulate the composition and hence the function of lipid rafts. In this respect it should be noted that PAF induces sphingomyelinase which itself has been shown to increase the formation of PrP^{res} in ScN2a cells (32). PAF has been demonstrated to increase sterol synthesis (34) and to inhibit cholesterol esterification (33), while PAF antagonists inhibit cholesterol biosynthesis from lanosterol (35). Collectively, these data suggest that PAF may be involved in the maintenance of cholesterol-dependent lipid rafts.

The conversion of PrP^{C} to PrP^{Sc} is thought to occur after PrP^{C} has reached the plasma membrane and subsequently been re-internalized for degradation (36–38). These observations raise the possibility that the activation of PLA_2 seen in prion infected cells and the production of PAF may encourage the formation of PrP^{res} by enhancing propitious trafficking and sorting pathways. In some cell lines PAF antagonists prevent endocytosis (39), while in other studies, $cPLA_2$ inhibitors (AACOCF$_3$ or BEL) prevent the maintenance of the Golgi network (40), endosome fusion, and endocytosis (41), and modulate the intracellular trafficking of some proteins (42). Together with the observation that the Golgi and the endosomal compartments are involved in the trafficking of a GFP-tagged PrP^{C} (43), these observations suggest that treatment of neurons with PLA_2 inhibitors or PAF antagonists may inhibit PrP^{res} formation by altering the intracellular trafficking of PrP^{C}.

Currently, the development of therapeutic strategies to combat prion disease is largely based on the identification of drugs that bind to and disrupt aggregated PrP^{Sc}. This strategy is based on the belief that PrP^{Sc} is a major, if not the only, component of the infectious agent (44), and that the formation of fibrillar aggregates of PrP^{Sc} leads to neurodegeneration. Thus, it is thought that inhibiting PrP^{Sc} formation, or disrupting pre-formed PrP^{Sc}, will prevent the establishment of disease. The data presented here support the view that PLA_2 and PAF regulate the formation of PrP^{res}, and thus presumably the propagation of infectious prions since dexamethasone-treated SMB cells showed reduced levels of infectivity. The effects of the PAF antagonists were dose-dependent and caused a 50% reduction in PrP^{res} content at nanomolar concentrations. Both PLA_2 inhibitors and PAF antagonists caused a rapid reduction in the PrP^{C} content of N2a cells. Thus, the effects of PLA_2 inhibitors and PAF antagonists on PrP^{res} formation may result from reducing the supply of PrP^{C} to sites conducive to conversion of PrP^{C} to PrP^{res}. While PrP^{res} formation is undoubtedly a complex process, these observations provide insight into the signaling processes that initiate the formation of PrP^{res} and presumably prions. We therefore propose that PAF antagonists may have a role in preventing neurodegeneration in prion diseases when used in combination with drugs targeted at the structure of PrP^{Sc} itself.

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Mechanisms of Signal Transduction:

Phospholipase A_2 Inhibitors or Platelet-activating Factor Antagonists Prevent Prion Replication

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