BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **249,** 182–186 (1998)
ARTICLE NO. RC989103

Prostaglandin E2 Induced Polymerization of Human α-1-Antichymotrypsin and Suppressed Its Protease Inhibitory Activity: Implications for Alzheimer's Disease

Federico Licastro,*[,1] Lizabeth Jane Davis,* Steve Pedrini,*
Douglas Galasko,† and Eliezier Masliah†

†*Department of Neuroscience and Pathology, University of California at San Diego; and*
**Dipartimento di Patologia Sperimentale, Università di Bologna, Italy*

Received June 16, 1998

Different molecular forms of α-1-antichymotrypsin (ACT) in sera and cerebrospinal fluids from patients with Alzheimer's disease (AD) were detected. Monomeric and polymeric ACT were observed by polyacrylamide gel electrophoresis of both sera and cerebrospinal fluids. ACT polymers were increased in AD patients with the apolipoprotein E (APOE) 4 allele. Increased levels of inactive ACT molecules were also detected in brain homogenates of patients with the APOE 4 allele. Experimental conditions promoting *in vitro* polymerization of ACT and the effect of polymerization on the biological activity of this serpin were also explored. Incubation of this serpin with prostaglandin of E series (PGE 2) induced ACT polymerization and decreased its activity. Amyloid β-peptide$_{1\text{-}42}$ did not significantly affected the biological activity of ACT. Inactivation of protease inhibitors by inflammatory molecules such as PGE 2 released from activated microglia in AD brains may promote amyloid deposition and neurodegeneration.

The serpin ACT [1-2] inhibits cathepsin G and chymotrypsin-like enzymes [3-5] and is an acute phase protein whose plasma concentration increases several fold after tissue injury [3]. Human activated astrocytes express and release ACT [6] and this serpin is a secondary component of amyloid deposits in senile plaques from the brain of patients with AD [7]. ACT binds the amyloid β peptides (Aβ) *in vitro*, and affects the fibril structures formed by the assembly of Aβ [8-11].

ACT concentrations are increased in brains [7,12], cerebrospinal fluids (CSF) [13] and sera of patients with AD [14-15]. Increased levels of both monomers and SDS stable ACT polymers in sera from patients with AD [16] have also been found. Reactive gliosis is associated with AD brains [17] and activated astrocytes surrounding neuropathological AD lesions overexpress ACT mRNA [7]. Moreover, ACT levels in AD brains correlate with the number of activated proliferating astrocytes and are increased in AD patients with APOE 4 alleles [12].

In the present study we show that: 1) Increased levels of polymeric ACT were present in AD patients. 2) Inactive ACT was increased in brain homogenates from AD patients with the APOE 4 allele. 3) *In vitro* PGE induced the polymerization of ACT and decreased its functional activity.

MATERIAL AND METHODS

AD biological samples were obtained from the Alzheimer's Disease Research Center at the University of California, San Diego; 13 were clinically and histopathologically diagnosed as AD and 5 as controls. Clinical and neuropathological criteria for AD diagnosis were as described elsewhere [18]. Apolipoprotein E (APOE) genotyping was performed by the polymerase chain reaction (PCR) as previously described [19].

Polyacrylamide gel electrophoresis (PAGE) and Western blot analysis. ACT solutions (Calbiochem, 1 mg/ml in 0.015 M TRIS HCl, 0.15 M NaCl pH 7.4) were dissolved and aliquots were kept at −80 °C until assayed. Protein samples (10 ml) contained 20% glycerol, 0.05M Tris HCl and 0.04% bromophenol blue and were loaded on 7.5 % or 10 % polyacrylamide precast minigels (Ready Gels, BioRad, USA) without denaturation (native-PAGE). Parallel samples were denatured by the method of Laemmli [20] and heated for 4 minutes at 95 °C (SDS-PAGE). Electrophoresis was performed using a Mini-Protean II cell (BioRad, USA) and molecular weight standards from 29 to 205 KDa (Sigma, USA) were included on each gel. Monomeric and polymeric bovine serum albumin (BSA, Sigma) were used as molecular weight standards in native PAGE. Then, proteins were electrotransferred to reinforced nitrocellulose membranes (Amer-

[1] Correspondence, proof and reprint requests: Prof. Federico Licastro, Dipartimento di Patologia Sperimentale , Università di Bologna, Via S. Giacomo 14, 40126 Bologna, Italy.

Research supported with funds from Associazione Italiana di Ricerca e Assistenza agli Alzheimer (A.R.A.D.) and Italian MURST 40% and 60% (to FL, LJD) and NHI grant AG 05131 (to EM).

0006-291X/98 $25.00

sham, UK). After the electrotransfer, proteins were stained with 0.5% Ponceau solution (Sigma). Nitrocellulose membranes were then saturated in phosphate-buffered saline (PBS) with 5% non-fat dry milk and 0.05% Tween 20 (buffer A) for 1 hour. Affinity purified polyclonal antibodies specific for ACT (Calbiochem, USA; final dilution 1:1000) were diluted in buffer A, added to membranes and incubated for 2 hours at room temperature. Membranes were then washed two times with PBS containing 5% nonfat milk and 0.5% Tween 20 (buffer B) and one in buffer A.

Anti-rabbit IgG conjugated with horseradish peroxidase (Boehringer Mannheim, USA) was diluted 1:200 in buffer A and added to membranes for 1 hour. After several washings with buffer B, and then with PBS, peroxidase DAB substrate (3,3′diaminobenzidine tetrahydrochloride; SIGMA Fast; SIGMA,) was added and incubated for 3-5 minutes. Finally, the reaction was stopped by washing membranes with distilled water.

Brain homogenates. Mid frontal cortex homogenates were prepared as previously described [12]. Aliquots of brain homogenates were dialyzed against a 1000 fold dilution of 100 mM Tris HCl, 150 mM NaCl, pH 7.5 three times.

Cathepsin G inhibition assay. Cathepsin G (Phoenix Pharm, USA) was dissolved in 200 mM Tris HCl, pH 7.4, then diluted 1:10 in 50 mM NaOAc pH 5.5, 150 mM NaCl. The inhibition of cathepsin G activity was measured by a spectrofluorimetric kinetic assay. Briefly, final concentrations of assay components were: 20 mU/ml cathepsin G, 100 mM Tris HCl pH 7.5, 150 mM NaCl, 5 mM NaAcOOH. The above mixture was plated in microtiter plates with 5, 25 or 50 ml of brain cytosolic fractions or equivalent volumes of bidistilled water to a final volume of 90 ml and incubated for 5 min at room temperature. Finally, 10 ml of substrate (500 mM succinyl-L-alanyl-L-alanyl-l-prolyl-L-phenylalanine-4-methyl-coumaryl-7-amide; Penninsula Laboratories, USA) was added and plate was incubated in the dark at 37 °C for 30 min. Formation of fluorescent products was measured at 385 nm excitation and 450 nm emission.

Spectrophotometric assay for chymotrypsin inhibitory activity. Chymotrypsin (Fluka, dissolved in 1 mM HCl, then diluted in 100 mM $CaCl_2$, 80 mM Tris; final concentration 2 ng/ml) was incubated for 5 min. with or without ACT (Sigma, 8 nM), PGE 2 (Sigma, 150 nM), Aβ1-42 (Sigma, 150 or 525 nM) or ascorbic acid (Sigma, 800 nM). The chymotrypsin substrate, N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-4-nitroanilide (Fluka, dissolved in DMSO and then diluted in 10 mM CaCl2, 80 mM Tris; final concentration 15 mM) was added to the different reagent mixtures and spectrophotometric continuous reading was performed for 5 min. at 410 nm. Control tubes consisted of chymotrypsin and its substrate without other reactants (100% of enzyme activity).

Cerebrospinal fluid concentration. CSF from patients with AD were concentrated using microncentrators (Microcon, Amicon, USA). Briefly, CSF samples (0.5 ml) were applied on the concentrator, centrifuged (10,000 g) at 4 °C for 50 minutes and concentrates were collected by washing the membranes as indicated by the manufacturer. Aliquots of CSF concentrates were then separated by native PAGE and analyzed by Western blot as described above.

ACT immunoreactivity. ACT (9 mM) was incubated without and with PGE 2 (180 mM) at 37°C for different time periods. Control tubes contained comparable concentrations of ethanol (1.7%), since PGE 2 stock solutions contained 95% ethanol. In parallel experiments ACT was incubated with A$\beta_{1\text{-}42}$ (American Peptides, 90 mM). At the end of incubation, tubes were centrifuged for 5 min. at 12,000 g and ACT immunoreactive materials in the supernatants were detected by commercially immunodiffusion plates specific for human ACT (ACT Nanorid, The Binding Site, USA).

RESULTS

SDS stable polymeric forms of ACT have been detected by Western blot analysis of sera from patients

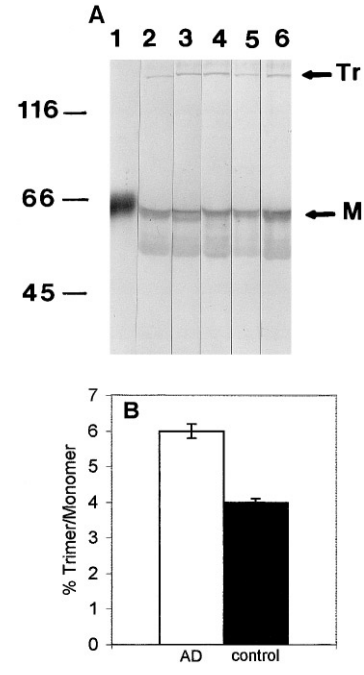

FIG. 1. (Panel A) Monomeric and polymeric ACT forms detected in sera of AD patients; 10% PAGE under denaturating and reducing conditions and Western blot analysis of purified ACT (lane 1) or serum ACT from AD patients (lanes 2-6). M = monomeric form, Tr = trimeric form; molecular weights standards are reported on the left side. (Panel B) Percentage of trimeric ACT in sera from AD and controls; absolute densitometric values of band from monomeric and trimeric ACT forms were calculated [(OD trimer/OD monomer)x100] and the relative representation of these two bands in sera from 6 AD and 4 controls is presented.

with AD and results are presented in Figure 1, panel A. Two different ACT molecular forms were immunoprecipitated. The first band was the ACT monomer (≈65 KDa), the second band of higher apparent MW (≈195 KDa) consisted of a trimeric form. Band densitometry was evaluated by a scanner and a computerized imaging analyzer, then the absolute values for each band was evaluated and the percentage between monomeric and trimeric ACT forms in each AD patient and 4 controls were calculated (Figure 1; panel B). Trimeric ACT represented 6 ± 0.1 % of monomeric ACT in AD sera, while control values were 4 ± 0.2 % ($p < 0.05$).

Non-denaturating PAGE of serum samples were also performed and SDS unstable polymeric forms of ACT were detected by Western blot analysis. Results obtained from patients with AD and a non-demented control are reported in Figure 2. Monomeric, dimeric, and tetrameric ACT forms were present in control serum (lane 6). Purified serum ACT added as standard (lane 1) also was resolved in different bands. Monomeric ACT was detectable in sera from only one patient with AD (lane 5), while dimeric ACT were detected in two patients (lane 4 and 5). Sera from the other two AD pa-

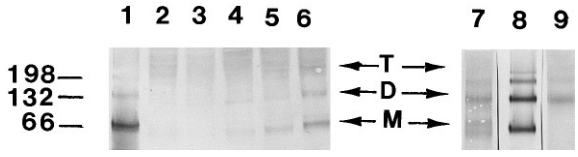

FIG. 2. Monomeric and polymeric ACT forms from sera and CSF of AD patients: 7.5% PAGE under non-denaturating and non-reducing conditions and Western blot analysis of purified ACT (lane 1) and serum ACT from AD patients (lanes 2-5) and a non demented controls (lane 6). Western blot analysis of purified ACT (lane 8) or CSF concentrates from AD patients (lane 7 and 9). M = monomeric ACT, D = dimeric ACT, T = tetrameric ACT; molecular weights are on the left side.

tients (lanes 2 and 3) showed only ACT polymers of high MW (>200 kDa). It is of interest that lane 5 referred to a serum sample from an AD patient with APOE 3/3 alleles, while the other three AD patients had APOE 4/4, 3/4 and 4/4 alleles (lanes 2-4, respectively).

Polymeric ACT forms were also present in CSF concentrates from AD patients as shown by Western blot of native PAGE (Figure 2). It is of interest that monomeric ACT was not detected in one patient with APOE 3/4 alleles (Fig. 2, lane 9) and high absolute levels (6.6 mg/l) of ACT as detected by the radial immune diffusion assay. Both monomeric and polymeric forms were detected in the other AD patient with APOE 3/3 (lane 7) and lower absolute ACT levels (1 mg/l).

To investigate the biological activity of ACT in brains from AD patients with different APOE genotypes, brain homogenates were prepared, ACT levels and inhibitory activity of these samples upon cathepsin G were then measured (Table 1). ACT absolute levels as

TABLE 1

Inhibition of Cathepsin G Activity by Aliquots of Brain Homogenates from Three Patients with AD

		ACT				
AD	APOE	mg/l§	%tot.pr.#	Homogenate ml	Cat.G %activity	ACT ng*
#1	3/3	0.1	0.01	0	100	0
				5	100	0.5
				25	90	2.5
				50	81	5
#2	3/4	4.5	0.16	0	100	0
				5	70	22
				25	55	112
				50	43	225
#3	4/4	1.7	0.10	0	100	0
				5	100	8.5
				25	71	42
				50	46	85

Note. § = ACT concentration in brain homogenate; # = ACT % of total protein in brain homogenate; * = Absolute levels of ACT in brain homogenate aliquots used for the enzyme assay.

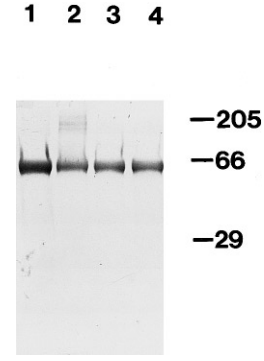

FIG. 3. Polymerization induced by PGE2; 10% PAGE under denaturing conditions and Western blot analysis. ACT was incubated for 40 hrs at 37 °C without (lane 1) or with PGE 2 (lane 2) or at time 0 without and with PGE 2 (lane 3 and 4, respectively).

well as the total maximal inhibitory activity were higher in patients with the APOE 4 allele. However, lower absolute concentrations of ACT in the brain homogenates from the patient with APOE 3/3 in comparison to patients with 4/4 or 3/4 alleles inhibited cathepsin G activity.

These findings suggested that in AD patients with the APOE 4 allele and high concentrations of ACT a large proportion of the serpin was not biologically active.

Incubation of purified ACT with PGE 2 for 24 hours at 37 °C resulted in polymerization of the serpin, as shown by SDS PAGE and Western blot analysis (Figure 3). Polymer formation was also indirectly detected by radial immunoprecipitation of supernatants from test tubes where ACT was incubated with PGE 2 or the amyloid peptide $A\beta_{1\text{-}42}$ for 0-48 hours at 37 °C (Table 2). This assay measured the progressive disappearance of ACT immunoreactivity in supernantants incubated with PGE 2or $A\beta_{1\text{-}42}$. Only 8 % of non aggregated monomeric ACT was detected after 48 hrs incubation of the serpin with PGE 2, while 75 % of ACT was present in monomeric form after the incubation with $A\beta_{1\text{-}42}$.

Polymerization of the serpin was associated with a loss of functional activity. In fact, coincubation of PGE 2 for 24 or 48 hours with ACT also decreased the biological activity of the serpin as assessed by spectrophotometric measurement of chymotrypsin activity (Figure

TABLE 2

Immunoreactivity of Supernatants from Tubes Containing Purified Human ACT Incubated with PGE 2 or $A\beta_{1-42}$ at 37°C for 0, or 48 Hours (T_0 or T_{48})

	Immunoreactivity (%)		Immunoreactivity (%)
ACT + PGE 2 T_0	100	ACT + $A\beta$ T_0	100
ACT + PGE 2 T_{48}	8	ACT + $A\beta$ T_{48}	75

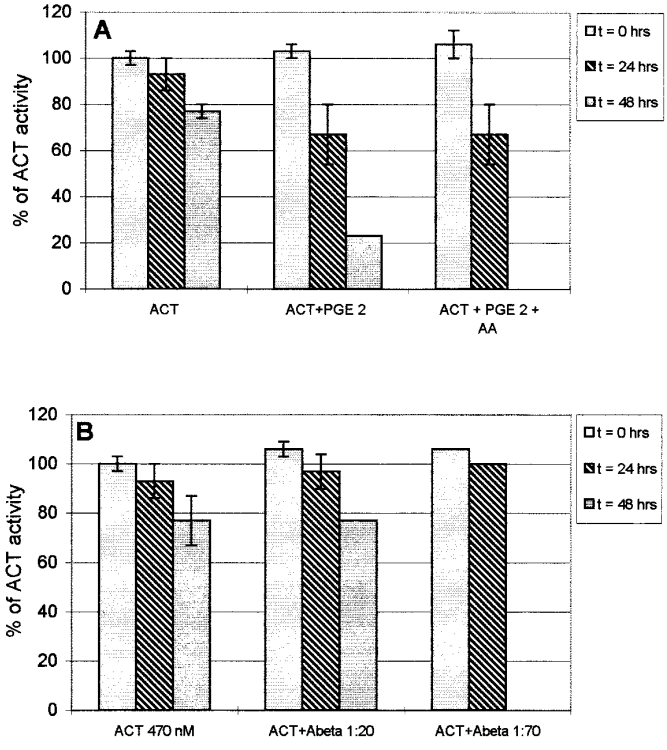

FIG. 4. ACT inhibition upon chymotrypsin activity; (panel A) ACT inhibitory activity decreased when the serpin alone was pre-incubated at 37 °C up to 48 hrs. The addition of PGE to ACT significantly decreased the inhibitory activity of the serpin and this decrement was not affected by 50 mM ascorbic acid (AA). (Panel B) $A\beta_{1\text{-}42}$ did not significantly decreased ACT inhibitory activity.

4; panel A). Incubation mixtures containing PGE 2/ ACT with ascorbic acid not maintain ACT activity. Incubation of ACT with fresh $A\beta_{1\text{-}42}$ did not significantly interfere with ACT activity (Figure 4; panel B).

DISCUSSION

Serpins are vulnerable to mutations affecting the reactive center of the molecule, and are sensitive to mild denaturating conditions which result in polymer formation and loss of function [21]. Polymerization takes place when the reactive center loop of one serpin inserts into a β-sheet of another serpin [21,22].

Our findings showed that both monomeric and polymeric forms of ACT were detectable in human sera and CSF and increased relative levels of ACT polymeric forms were found in AD patients. These data confirm a previous report where increased polymeric serum ACT was found in patients with AD [16]. In this report Western blot of serum proteins resolved by native PAGE also showed in some AD patients a decrement of monomeric ACT.

In a previous report we have found that absolute ACT levels from brain homogenates of AD patients correlates with APOE 4 alleles, ACT concentrations being higher in patients with APOE 4/4 and 3/4 that those from patients with APOE 3/3 [12]. Here we have shown that a higher proportion of inactive ACT was present from brain homogenates of patients with APOE 4/4 and 3/4 than that with APOE 3/3 alleles.

Polymerization of this protease inhibitor was also induced by increasing temperature (data not shown) or by incubation of the purified serpin with PGE 2. It is interesting to note that corticosteroid binding globulin, a molecule structurally related to serpins, was also shown to bind hydrophobic compound such as cortisol [23], and antitrypsin has been shown to form non-covalent complexes with cholesterol [24]. In our experimental conditions incubation of purified ACT with cholesterol did not induce SDS stable polymers or affect SDS-labile polymerization (data not shown). Moreover, addition of ascorbic acid (50 or 100 mM) did not preserve ACT activity. These data suggested that: 1) PGE 2 bound with ACT by hydrophobic interactions. 2) PGE 2 induced the molecular rearrangement of the serpin. 3) Oxygen radicals production by PGE 2 was not involved in ACT polymerization and inactivation.

Interactions between ACT and PGE 2 appeared to be of great interest, since both compounds are produced during inflammation. Our data suggested a possible metabolic regulation of inflammatory hydrophobic compounds over some serpins. In fact, during brain inflammation, high levels of PGE were produced by activated microglia [25], and PGE also induced interleukin-6 synthesis by astrocytes [26]. Other compounds released by activated microglia, such as interleukin-1 and tumor necrosis factor resulted in astrocyte activation and ACT release by these cells [8]. Therefore, in AD brains the release of PGEs might affect the metabolic fate of ACT, promoting either a regional or local cytokine imbalance or a functional inactivation of the serpin.

Another hydrophobic substance, such as $A\beta_{1\text{-}42}$ peptide, induced a very low level of ACT polymerization. These findings are partially in accordance with other reports [11-12] suggesting specific interaction between $A\beta_{1\text{-}42}$ and ACT. However, our findings showed that $A\beta_{1\text{-}42}$ up to a molar ratio of 1:70 did not inactivate ACT. Aged $A\beta_{1\text{-}42}$ was also used and it did not interfere with the activity of ACT (data not shown).

Inflammation [27] is associated with the neurodegenerative alterations of AD brains, arachidonate metabolites promoted the release of amyloid precursor protein [28] and PGE 2 promoted β-amyloid peptide accumulation in neuroblastoma cell cultures [29]. Our findings suggest that in AD brains PGE 2 release by activated microglia might promote the inactivation of protease inhibitors. Impaired proteolytic activity might further promote amyloidogenic peptide generation and accumulation.

REFERENCES

1. Travis, J. M., Garner, D., and Bowen, J. (1978) *Biochemistry* **17,** 5647–5651.

2. Rubin, H., Wang, Z. M., Nickbarg, E.B ., McLarney, S., Naidoo, N., Schoenberger, O. L., Johnson, J. L., and Cooperman, B. S. (1990) *J. Biol. Chem.* **265,** 1199–1207.
3. Travis, J., and Salvesen, G. S. (1983) *Ann. Rev. Biochem.* **52,** 655–709.
4. Morii, M., and Travis, J. (1983) *J. Biol. Chem.* **258,** 12749–12752.
5. Cooperman, B. S., Stavridi, E., Nickbarg, E., Rescorla, E., Schechter, N. M., and Rubin, H. (1993) *J. Biol. Chem.* **268,** 23616–23625.
6. Abraham, C. (1992) *Res. Immunol.* **143,** 631–636.
7. Abraham, C. R., Selkoe, D. J., and Potter, H. (1988) *Cell* **52,** 487–501.
8. Fraser, P. E., Nguyen, J. T., McLachlan, R., Abraham, C. R., and Kirschner, A. D. (1993) *J. Neurochem.* **61,** 298–305.
9. Ma, J., Yee, A., Brewer, H. B., Das, S., and Potter, H. (1994) *Nature* **372,** 92–94.
10. Eriksson, S., Janciauskiene, S., and Lannfelt, L. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2313–2317.
11. Janciauskiene, S., Eriksson, S., and Wright, H. T. (1996) *Nature Struc. Biol.* **3,** 668–671.
12. Licastro, F., Mallory, M., Hansen, L. A., Masliah, M. (1998) *J. Neuroimmunol.* (in press).
13. Licastro, F., Davis, L. J., Morini, M. C., Cucinotta, D., and Savorani, G. (1994) *Alzh. Dis. Ass. Disord.* **8,** 241–249.
14. Licastro, F., Parnetti, L., Morini, M. C., Davis, L. J., Cucinotta, D., Gaiti, A., and Senin, U. (1995) *Alzh. Dis. Ass. Disord.* **9,** 112–118.
15. Licastro, F., Morini, M. C., Polazzi, E., and Davis, L. J. (1995) *J. Neuroimmunol.* **57,** 71–75.
16. Licastro, F., Sirri, V., Trerè, D., and Davis, L. J. (1997) *Dementia Geriatr. Cogn. Disord.* **8,** 337–342.
17. Mancardi, G. L., Liwnicz, B. H., and Mandybur T. I. (1983) *Acta Neuropathologica.* **61,** 76–84.
18. Hansen, L. A., Masliah, E., Galasko, D., and Terry, R. D. (1993) *J. Neuropathol. Exp. Neurol.* **52,** 648–654.
19. Galasko, D., Saitoh, T., Xia, Y., Thal, L., Katzman, R., Hill, L., and Hansen, L. (1994) *Neurology* **44,** 1950–1951.
20. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
21. Stein, P. E., and Carrell, R. W. (1995) *Nature Struc. Biol.* **2,** 96–113.
22. Schulze, A. J., Baumann, U., Knof, S., Jaeger, E., Huber, R., and Laurell, C. B. (1990) *Eur. J. Biochem.* **194,** 51–56.
23. Hammond, G. L., Smith, C. L., and Underhill, D. A. (1991) *Steroid Biochem. Mol. Biol.* **40,** 755–762.
24. Janciauskiene, S., and Eriksson, S. (1993) *FEBS Lett.* **316,** 269–272.
25. Hartung, H. P., Schafer, B., Heininger, K., Toyka, K. V. (1989) *Brain Res.* **489,** 113–119.
26. Fiebich, B. L., Hull, M., Lieb, K., Gyufko, K., Berger, M., and Bauer J. (1997) *J. Neurochem.* **68,** 704–709.
27. McGeer, P. L., Rogers, J., and McGeer E. G. (1994) *Alzh. Dis. Ass. Dis.* **8,** 149–158.
28. Kinouchi, T., Ono, Y., Sorimachi, H., Ishiura, S., Suzuki, K. (1995) *Biochem. Biophys. Res. Com.* **209,** 841–849.
29. Prasad K. N., La Rosa F. G., Prasad J. E. (1998) *In vitro Cell Dev. Biol. Anim.* **34,** 265–274.