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Platelet activating factor receptor expression is associated with neuronal apoptosis in an *in vivo* model of excitotoxicity

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Abstract

Platelet activating factor (PAF), an endogenous proinflammatory agent, mediates neuronal survival, glutamate release, and transcriptional activation following excitotoxin challenge. To determine whether PAF receptor (PAFR) expression is altered during excitotoxicity, changes in PAFR mRNA localization were compared with markers of neuronal apoptosis and reactive gliosis following systemic injection of kainic acid. Data from semi-quantitative RT-PCR, *in situ* hybridization, DNA fragmentation, cellular morphology analysis, and immunohistochemistry demonstrate that the localization of PAFR mRNA is altered during kainic acid-induced neurodegeneration. While PAFR mRNA is normally exhibited by neurons and microglia in rat hippocampus, expression becomes restricted to apoptotic neurons and to glia involved in phagocytosing apoptotic debris following treatment with excitotoxin. PAFR mRNA is rarely detected in surviving neurons. These data provide the first indication that PAFR-expressing neurons may be preferentially susceptible to excitotoxic challenge.

Keywords: platelet activating factor; apoptosis; neurodegeneration; kainic acid; epilepsy; excitotoxicity

Abbreviations: CNS, central nervous system; BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; H/E, hemotoxylin and eosin; PAF, platelet activating factor; PAFR, platelet activating factor receptor; RT-PCR, reverse transcriptase-polymerase chain reaction

Introduction

In vivo models of excitotoxicity produce a well-defined sequence of neuronal death in brain regions with high levels of glutamate receptors. Following systemic injection of kainic acid, neuronal necrosis is observed in rat hippocampus within hours of excitotoxin administration while animals are still undergoing epileptiform seizures (Ben-Ari, 1983; Lassman *et al*, 1984; Smeyne *et al*, 1993; Bennett *et al*, 1995). The loss of plasma and nuclear membrane integrity and leakage of cytoplasmic and nuclear material into brain parenchyma triggers a cerebral inflammatory response characterized by reactive gliosis and activation of microglia (Bennett *et al*, 1995; Taniwaki *et al*, 1996; Boschert *et al*, 1997). Morphological changes defining neuronal apoptosis appear only after epileptiform activity has subsided and excitotoxin has been cleared from brain tissue. Apoptotic neurons are identified by their pyknotic, condensed, and hyperchromatic appearance, blebbing of extracellular and nuclear membranes, intranucleosomal fragmentation of DNA, and compartmentalization of cytoplasmic and nuclear components into smaller 'apoptotic' bodies without compromise of membrane integrity (Smeyne *et al*, 1993; Sakhi *et al*, 1996; Boschert *et al*, 1997). Comparable kinetics of cell death are observed in experimental models of ischemia/reperfusion and following direct intracerebral injection of glutamate analogs (Dragunow *et al*, 1994; van Lookeren Campagne *et al*, 1995; Nitatori *et al*, 1995; Choi, 1996).

Protracted exposure to proinflammatory ligands is characteristic of a number of clinical conditions linked to excitotoxicity, including Alzheimer's disease, Huntington's disease, ischemia, and epilepsy (Kalaria, 1993; Giulian *et al*, 1994; Majno and Joris, 1995; Steinberg *et al*, 1996). How a cerebral inflammatory response affects the kinetics of neuronal loss is not well understood although proinflammatory agents such as platelet activating factor (PAF: 1-O-alkyl-2-acetylglycero-3-phosphocholine) have been directly linked to peripheral pathogenesis. PAF is a proinflammatory phospholipid made up of predominant molecular species $C_{16}H_{33}$ and $C_{18}H_{35}$ with biological activity at fM concentrations (Beusenberg *et al*, 1994). PAF interacts with a G protein-linked receptor protein (PAFR) cloned from guinea pig lung (Honda *et al*, 1991), rat spleen (Bito *et al*, 1994), human heart (Sugimoto *et al*, 1992), HL60 cells (Ye *et al*, 1991), and U937 cells (Kunz *et al*, 1992) and expressed at low-levels in central nervous system (CNS) (Bito *et al*, 1994). PAF regulates peripheral cytokine and inflammatory networks (Bonavida and Mencia-Huerta, 1994) with prolonged signal transduction implicated in the pathogenesis of hypotensive shock, bronchial asthma, ulcerative colitis, Crohn's disease, and tumour progression (Sobhani *et al*, 1992; Yamamoto *et al*, 1993; Ferraris *et al*, 1993; Bennett *et al*, 1993; Graham *et al*, 1994). In CNS, sustained PAF stimulation is observed during ischemia, encephalitis, meningitis, and human immunodeficiency virus-1 infection (Kumar *et al*, 1988; Gelbard *et al*, 1994; Marcheselli and Bazan, 1994). *In vitro* activation of PAFR in neurons and glia elicits calcium

transients (Yue *et al*, 1992), release of arachidonic acid metabolites (Petroni *et al*, 1994), phosphoinositide turnover (Yue *et al*, 1992; Petroni *et al*, 1994), and proto-oncogene expression (Squinto *et al*, 1989). With respect to excitotoxicity, PAF enhances glutamate release and increases expression of transcriptional factors associated with apoptosis (i.e. c-*fos*, c-*jun*) (Bazan *et al*, 1993; Marcheselli and Bazan, 1994). PAF antagonists attenuate excitotoxic cell death *in vitro* and protect neurons from ischemic injury *in vivo* (Braquet *et al*, 1989; Feurstein *et al*, 1990). Thus, PAF appears to influence excitotoxic cell loss.

To determine whether pivotal PAF signal transduction molecules are implicated in excitotoxic apoptosis, we examined the distribution of PAFR mRNA in damaged neurons and reactive glia of the rat hippocampus following systemic injection of kainic acid.

Results

Kainic acid-induced seizures

Rats were injected with 10 mg/kg kainic acid. Seizure progression was assessed according to previously published criteria (McIntyre *et al*, 1982). Rats exhibited stage 1 'wet-dog shakes' 15–30 min after treatment, stage 2 rearing and weak clonic convulsions 60 min after injection, stage 3 loss of postural control and increasingly severe convulsions 90 min after treatment, and stage 4 generalized limbic seizures 120–180 min after excitotoxin administration. Seizures were stopped 5 h after kainate injection by administration of a sub-anesthetic dose of sodium pentobarbitol (25 mg/kg).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of PAFR

A 381 bp fragment of PAFR was amplified from whole brain RNA with appropriate restriction digest characteristics (Figure 1A). Control reactions, carried out in the absence of RNA or RT, failed to produce cDNA demonstrating the specificity PAFR template transcript (Figure 1A). Changes in PAFR expression were standardized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression to establish that alterations in PAFR message, particularly decreases in steady-state mRNA, were not the result of random variations in total RNA template concentration. GAPDH was chosen as an internal standard based on previously published results (Szaflarski *et al*, 1995). Because the relative abundance of GAPDH mRNA is significantly greater than PAFR in brain, optimal cycling times for PAFR amplification (30 cycles) and GAPDH amplification (23 cycles) were determined (date not shown). Based on these results, GAPDH primers were added to ongoing PCR reactions at the onset of cycle 8. To confirm that PCR amplification fell within a linear range of detection, varying amounts of template RNA were co-amplified using this protocol (Figure 1B). Two μg of template RNA was sufficient to detect both increases and decreases in PAFR and GAPDH mRNA.

Total RNA was isolated from the hippocampal formation of six animals per time point at 0, 5, 24, and 168 h following kainate injection and subjected to RT-PCR analysis. Southern analysis confirmed the identity of the amplified fragments as PAFR and GAPDH (Figure 1C). The kinetics of PAFR expression were quantitated by ELISA of biotinylated (PAFR) and fluoresceinated (GAPDH) PCR

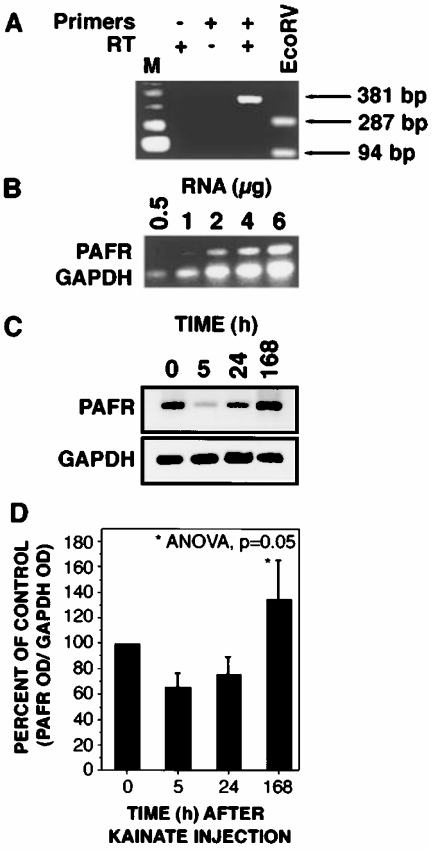

Figure 1 Semi-quantitative RT-PCR analysis of PAFR expression following systemic injection of kainic acid. (A) Total RNA was extracted from whole brain tissue of control animals and RT-PCR performed as described in Materials and Methods. Two μg of total RNA was reverse transcribed in a 20 μl volume. Ten μl of cDNA was PCR amplified. Primers were 5′-biotin-CACTTATAACCGC-TACCAGGCAG-3′ (forward) and 5′-biotin-AAGACAGTGCAGACCATCCA-CAG-3′ (reverse) defining a 381 bp PAFR fragment. Amplification produced the appropriate PAFR cDNA product with the correct restriction enzyme digest sites. RT-PCR reactions processed in the absence of RNA or primers did not amplify PAFR cDNA. (B) The optimal concentration of template RNA for semi-quantitative analyses was established by co-amplifying varying concentrations of rat brain RNA with biotinylated PAFR primers and fluoresceinated GAPDH primers. GAPDH primers were 5′-FITC-TGGTGCTGAGTATGTCGTGGAGT-3′ (forward) and 5′-FITC-AGTCTTCTGAGTGGCAGTGATGG-3′ (reverse) defining a 292 bp GAPDH fragment. PCR reactions were carried out at 95°C (30 s), 55°C (90 s) and 72°C (120 s) for 30 cycles (PAFR) and 23 cycles (GAPDH). Two μg of template RNA fell within a linear range of amplification sufficient to detect both increases and decreases in relative mRNA expression. (C) The kinetics of PAFR expression in rat hippocampus at 0, 5, 24, and 168 h after exposure to kainic acid were determined. Total RNA was extracted from dissected hippocampi. The identity of the 381 bp PAFR and 292 bp GAPDH product was confirmed by Southern blotting using a 1 Kb *Not*I/*Hind*III fragment of pCDM8/PAFR and a 1.5 Kb *Pst*I fragment of pGAPDH. Blots were probed for PAFR, stripped, and re-probed for GAPDH. (D) Semi-quantitative analysis of relative PAFR expression following kainate-induced seizures. The amount of biotinylated PAFR RT-PCR product was standardized against the amount of co-amplified fluoresceinated GAPDH RT-PCR product by ELISA as described in Materials and Methods. Data represent the mean $\pm$ s.e.m. of six animals per time point and are expressed as the ratio of PAFR to GAPDH expression

products as described in Materials and Methods. PAFR signal was standardized against GAPDH expression. Relative PAFR expression decreased to 65% that of control levels at 5 and 24 h after kainic acid injection (Figure 1D). mRNA levels increased to 135% that of control at 168 h after treatment (Figure 1D).

In situ localization of PAFR mRNA

PAFR expression was examined in more detail by radioactive *in situ* hybridization at 0, 24 and 168 h after kainic acid administration. In control animals, PAFR mRNA was detected throughout CA pyramidal cell fields of the hippocampal formation and granule cells of the dentate gyrus (Figure 2A). A decrease in the number of PAFR-positive neurons was evident 24 h after kainic acid injection (Figure 2B). Hybridization was restricted to isolated groups of cells in the CA layers and to crest cells of the dentate gyrus. An increase in PAFR hybridization was detected 168 h after kainate administration (Figure 2C). Elevated expression was localized to CA1, CA3b, and CA3c pyramidal cell fields, the dentate gyrus (particularly in cells immediately adjacent to CA3b and CA3c neurons), and cells of the oriens layer, stratum radiatum, and lacunosum molecular layer.

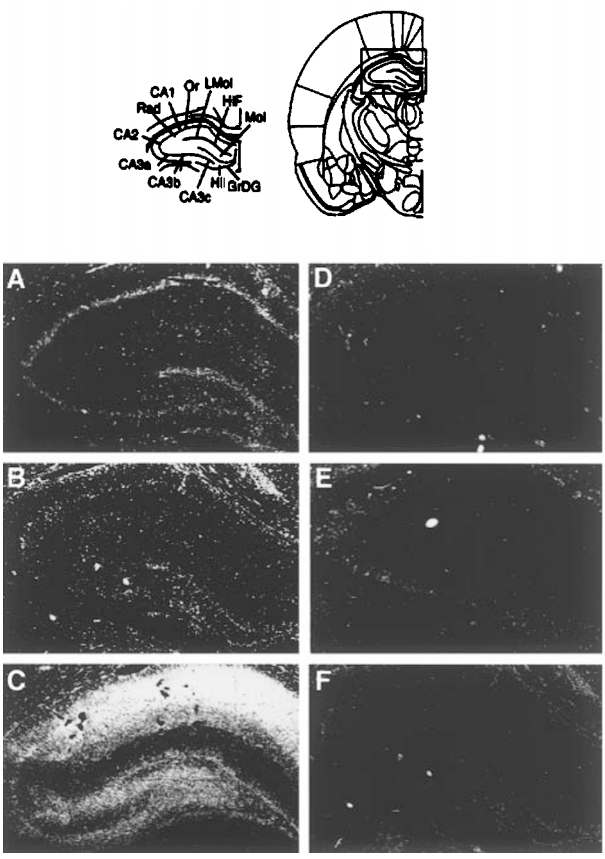

Figure 2 *In situ* hybridization for PAFR mRNA in hippocampal formation at 0, 24, and 168 h following systemic injection of kainic acid. (A,D) Control (0 h). (B,E) 24 h after kainate injection. (C,F) 168 h after kainate injection. Panels D, E, and F are adjacent sections, pretreated with RNase, and processed for PAFR hybridization, demonstrating the specificity of labeling in A, B, and C. Details are as described in Materials and Methods. Abbreviations are as follows: CA1, CA1 pyramidal cell field; CA2, CA2 pyramidal cell field, CA3a-c, CA3a-c pyramidal cell fields; DG, granule cells of the dentate gyrus; HiF, hilar fissure; Hil, hilus of the dentate gyrus; LMol, lacunosum molecular layer of the hippocampal formation; Mol, molecular layer of the dentate gyrus; Or, oriens layer of the hippocampal formation; Rad, stratum radiatum of the hippocampal formation

Non-radioactive *in situ* hybridization was used to identify cells expressing PAFR. Identification of cell type was based on morphological evaluation. In control tissue, mRNA was detected in pyramidal and granule neurons, microglia, vascular endothelial cells, and ependymal cells lining cerebral ventricles (Figure 3A, G and H). By 24 h after kainic acid injection, a reduction in both the intensity of label and in the number of PAFR-expressing neurons was observed (Figure 3B). PAFR-positive neurons were localized in discrete groups primarily in CA1, CA3, and the hilar region of the dentate gyrus (Figure 3B). Some cells were surrounded by PAFR-expressing microglia and astrocytes (data not shown). By 168 h after kainate administration, PAFR mRNA was primarily localized to activated astrocytes and microglia infiltrating the CA1 and CA3 pyramidal cell fields, the oriens layer, and the stratum radiatum (Figure 3C). This shift in localization from neurons to glia corresponds with the increase in PAFR mRNA observed 168 h after treatment by RT-PCR and radioactive *in situ* hybridization. Non-radioactive *in situ* analysis demonstrates that the elevated expression is due to an increase in the overall number of PAFR-expressing glia localized to hippocampus and not to increased transcription given that hybridization is detected in multiple glia but at low levels (Figure 3C).

Excitotoxic apoptosis

Apoptosis was identified at 0 h, 24 h, and 168 h after kainate injection on the basis of both hemotoxylin and eosin (H/E) morphology and TUNEL as described in (Bennett *et al*, 1998). In control tissue, apoptotic characteristics were detected consistently in some ependymal cells and cells of the choroid plexus. Apoptotic neurons were observed rarely (approximately three cells per 10 μm coronal section). Necrotic neurons were not detected. Systemic treatment with kainic acid resulted in substantial cell loss in the hippocampus. Neuronal apoptosis (Figure 3I) and necrotic debris was observed 24 h after treatment in pyramidal cells of CA3a, CA3b, CA3c and the lateral reaches of CA1. Crest cells of the dentate gyrus and neurons in the ventral reaches of the superior limb and dorsal reaches of the inferior limb of the dentate gyrus (i.e. in close proximity to CA3c neurons) demonstrated apoptotic characteristics. Dentate granule cells outside of thse regions were spared. Astrocytes and microglia were seen infiltrating damaged tissue. Glia proximal to neurons exhibiting DNA damage appeared morphologically to be involved in phagocytosis (data not shown).

It should be noted that TUNEL-positive neurons were more numerous than cells identified on the basis of H/E staining aberrations. In the hippocampal formation, sporadic interneurons in the stratum radiatum, lacunosum molecular, and molecular layer of the dentate gyrus as well as cells within the oriens layer immediately surrounding damaged

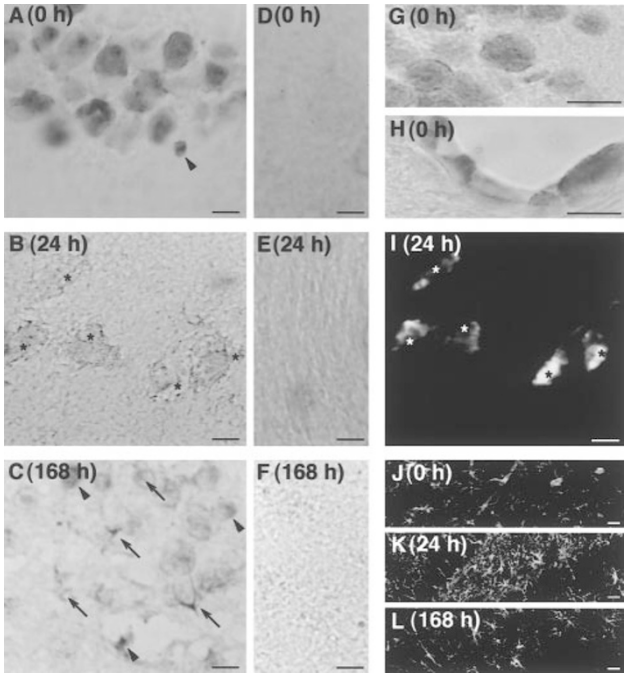

Figure 3 Cellular localization of changes in PAFR expression following systemic injection of kainic acid. All photomicrographs are of CA3 pyramidal cell field except panel G (dentate gyrus) and panel H (third ventricle). (A-H) PAFR *in situ* hybridization. (D, E, and F) are adjacent sections, pretreated with RNase, and processed for PAFR hybridization, demonstrating the specificity of labeling in (A, B, and C). (I) TUNEL performed on tissue presented in (B) following PAFR *in situ* hybridization (double-label). Asterisks in panel B and I indicate alignment of photomicrographs. (J-L) GFAP-labeled astrocytes. Details are as described in Materials and Methods. In control (0 h) sections, PAFR mRNA is detected in pyramidal neurons (A) and microglia (arrowhead in A), in granule cells of the dentate gyrus (G), and in ependymal cells lining ventricular walls (H). By 24 h after kainate injection, PAFR mRNA is detected almost exclusively in apoptotic neurons double-labeled with TUNEL (B and I). Note that the level of mRNA expressed by dying neurons (B) is less than that expressed by healthy cells in untreated animals (A). Non-apoptotic neurons in degenerating pyramidal cell fields do not express PAFR mRNA (B). By 168 h after treatment, low levels of PAFR transcript are rarely detected in surviving pyramidal neurons (C). A marked increase in the number of PAFR-expressing astrocytes (arrows in C) and microglia (arrowheads in C) infiltrating pyramidal cell fields is observed. This enhanced frequency of PAFR-positive glia cannot be attributed to an overall increase in reactive gliosis 168 h after excitotoxic insult given that labeling of reactive astrocytes with GFAP peaks at 24 h and is reduced 168 h after kainate injection (J-L). Scale bar, 20 μm

CA1 pyramidal neurons were consistently TUNEL-reactive. These data are in keeping with enhanced sensitivity of the *in situ* technique for detecting very early nuclear changes (Gavrieli *et al*, 1992). However, three notable exceptions in regions outside of the hippocampal formation emphasize the difficulty in differentiating necrotic from apoptotic cell death on the basis of TUNEL alone. Neurons in the amygdaloid nuclei, the ventromedial thalamus, and the medial regions of the ventrolateral thalamus were TUNEL-positive. Close examination of H/E stained sections demonstrated dense, ubiquitous eosinophilic staining of cell cytoplasm with hemotoxylin-impregnated nuclei indicative of a necrotic rather than an apoptotic process (data not shown). These data underlie the difficulty in relying exclusively on the TUNEL reaction to identify apoptosis in cell systems exhibiting complex temporal patterns of both apoptotic and necrotic death given the capacity of the technique to detect apoptotic DNA fragmentation, late-stage necrotic DNA destruction, and transient DNA repair. In the present study, only those brain regions exhibiting both TUNEL reactivity and H/E indices of active cell death were characterized as apoptotic.

Cell loss was quantified at 0, 24, and 168 h in CA3c pyramidal neurons (Table 1). A 46% reduction in cell number was observed 24 h after treatment compared to cell density in control animals. An additional 13% reduction was apparent 168 h after treatment. This 13% decrease could be accounted for by the loss of apoptotic neurons detected 24 h after kainate injection (Table 1).

PAFR expression and neuronal apoptosis

In control animals, PAFR was detected in healthy neurons. By 24 h afer kainate administration, PAFR expression was restricted either to neurons undergoing apoptosis or to glia located in close proximity to apoptotic debris. Double-labeling for PAFR *in situ* hybridization and TUNEL is depicted in Figure 3B and I. Expression in undamaged neurons was very low or absent. By 168 h after treatment, neuronal labeling was replaced by extensive glial expression in CA1 and CA3 pyramidal fields (Figures 2C and 3C). Thus, the expression of PAFR was seen to shift from healthy cells in control animals, to apoptotic neurons 24 h after systemic injection of kainic

Table 1 Cell number, PAFR expression, and apoptosis in CA3c pyramidal neurons following systemic injection with kainic acid

Time (h) after kainate injection	Total number of neurons[a]		Number of PAFR-expressing neurons[b]		Number of neurons exhibiting DNA strand breaks[c]	
	Mean ± S.E.M.	% of control (0 h)	Mean ± S.E.M.	% of existing cell number	Mean ± S.E.M.	% of existing cell number
0	136+8	100	82+6	60	0.3+0.3	0.2
24	74+3	54	27+2	36	24+1	33
168	56+4	41	7+2	12	0.7+0.7	1

[a]The total number of neurons in the CA3c pyramidal cell field was counted in replicate H/E-stained adjacent coronal sections from three animals per time point. Healthy neurons and cells exhibiting morphological indices of apoptosis were included in counts. Necrotic debris was not quantified. [b]The number of PAFR-expressing cells was quantified in replicate adjacent sections processed for non-radioactive *in situ* hybridization from three animals per time point. The number of PAFR-positive glia was not recorded. [c]Sections processed for *in situ* hybridization were double-labeled with TUNEL and the number of neurons exhibiting DNA strand breaks was quantified. Data represent mean+S.E.M. Details are as described in Materials and Methods

acid, to activated glia 168 h after treatment. This shift corresponded to a 59% reduction in the total number of CA3c pyramidal neurons; a loss that could be accounted for by the 91% reduction in PAFR-expressing neurons, a discrete subpopulation of neurons in the pyramidal cell fields (Table 1). If this decrease in PAFR-positive neurons was simply due to overall cell loss, then approximately 44 of the remaining 74 neurons (60%) at 24 h and 33 of the remaining 56 neurons (60%) at 168 h should have exhibited PAFR mRNA. Instead, only 36% of neurons exhibited transcript at 24 h and 12% of remaining pyramidal cells expressed PAFR message at 168 h. Each of these remaining PAFR-expressing neurons exhibited morphological or biochemical indices of neuronal apoptosis (Figure 3B and I and Table 1). These data indicate selective loss of PAFR mRNA-positive neurons and/or selective down-regulation of PAFR mRNA in surviving neurons.

Reactive gliosis

The kinetics of reactive gliosis were examined to establish whether detection of PAFR mRNA in glia at 168 h was due to an increase in the overall number of proliferating astrocytes and microglia at this time point or reflects the specific expression of PAFR transcript by glia 168 h after kainic acid injection. Reactive gliosis was assessed by glial fibrillary acidic protein (GFAP) immunoreactivity (Figure 3J, K and L). We have previously demonstrated that increases in GFAP immunoreactivity are observed 5 h after kainic acid administration, with maximal gliosis detected 24 h after seizure induction, and a return to near-normal levels observed by 168 h (Bennett *et al*, 1995). Consistent with these data, GFAP-immunoreactive glia were noted in control sections at low numbers throughout hippocampus with preferential localization around blood vessels and ventricular walls (Figure 3J). Maximal reactive gliosis was detected 24 h after treatment within interneuronal layers of the hippocampal formation with pronounced accumulation in the lacunosum molecular layer and in the hilus of the dentate gyrus. Astrocytic process could be seen infiltrating CA pyramidal cell fields and dentate gyrus granular cells (Figure 3K). Morphological indications of glia phagocytosing both apoptotic bodies and necrotic debris could be observed in GFAP/TUNEL double-labeled sections and by H/E evaluation (data not shown). By 168 h after treatment, the number of glia infiltrating damaged hippocampus was reduced compared to earlier time points but elevated compared to control levels. GFAP-immunoreactive cells were restricted to the oriens and radiatum cell layers with processes extending into the pyramidal cell field, to the hippocampal hilus, and to cells immediately ventral to the dentate gyrus. Only GFAP-labelled glia at 168 h exhibited enhanced PAFR expression indicating that detection of astrocytic transcript was not simply the result of enhanced glial numbers normally expressing PAFR but represents a specific induction of PAFR message in proliferating astrocytes at this time point (Figure 3C and 3L).

Discussion

Sustained increases in the regional levels of the proinflammatory autacoid PAF have been demonstrated in a number of *in vivo* excitotoxic disorders and PAF antagonists have been shown to reduce ischemia/reperfusion cell loss while protecting neurons from glutamate toxicity *in vitro* (Braquet *et al*, 1989; Feurstein *et al*, 1990; Marcheselli and Bazan, 1994; Gelbard *et al*, 1994). To determine whether pivotal PAF signal transduction molecules are modulated during neuronal apoptosis, we analyzed changes in PAFR expression following systemic injection of kainic acid. Our results indicate that PAFR mRNA, normally expressed by a subpopulation of healthy hippocampal neurons and microglia, becomes restricted to neurons undergoing apoptosis and to glia involved in phagocytosing apoptotic debris subsequent to an excitotoxic challenge. Our data also suggest that 91% of all PAFR-expressing CA3 pyramidal cells apparently die or reduce PAFR mRNA levels within 168 h of treatment with kainic acid. These data provide the first indication that PAFR expression is modulated during excitotoxicity and leads to speculation that PAFR-expressing cells may be more susceptible to delayed excitotoxic apoptosis *in vivo*.

Normal PAFR expression in healthy brain tissue

PAFR transcript in human and rodent brain tissue has been demonstrated by RT-PCR analysis, Northern analysis, and *in situ* hybridization (Bito *et al*, 1994; Mori *et al*, 1996). Ligand binding studies indicate a maximum density of PAF binding sites in the cortex (posterior and neocortex), hippocampus and cerebellum; a lower density in the striatum and hypothalamus; and minimal radioligand labeling in thalamic nuclei (Bito *et al*, 1992; Domingo *et al*, 1994). Our data confirm that PAFR mRNA is expressed in the hippocampal formation. *In situ* localization of transcript indicates that PAFR is expressed primarily by a subpopulation of hippocampal neurons and microglia. Hybridization to nerve cells is consistent with pharmacological localization of PAF binding sites to primary neurons *in vitro* (Bito *et al*, 1992) and subcellular localization of [^{3}H]PAF binding to presynaptic nerve terminals, intracellular membranes, and microsomes in cultured cells (Squinto *et al*, 1989; Marcheselli and Bazan, 1993).

Changes in PAFR expression during acute and chronic excitotoxicity

The time-course of PAFR cellular redistribution parallels the known genetics of acute and chronic neuropathology following kainate-induced epileptiform seizure (Ben-Ari, 1983; Lassman *et al*, 1984; Bennett *et al*, 1995). Acute neuropathology is observed 0–12 h after kainic acid administration and is characterized by prolonged neuronal depolarization, sustained increases in the expression of immediate early gene products (c-*fos*, c-*jun*, *zif*-268), marked reactive gliosis, and a breakdown in blood-brain barrier function (Ben-Ari, 1983; Lassman *et al*, 1984; Bennett *et al*, 1995). Cell loss is primarily necrotic in nature and is evident in pyramidal neurons of the hippocampal formation and cells of the entorhinal and piriform cortices and amygdaloid nuclei (Pollard *et al*, 1994). Our data indicate that PAFR mRNA expression is reduced during this time period. This finding is consistent with the immediate reduction in [^{3}H]PAF binding

sites observed following other excitotoxic insults (Domingo *et al*, 1994).

Chronic neuropathology is elicited 24–168 h after kainate treatment and occurs in the absence of continued glutaminergic stimulation. Neurodegeneration is characterized by a renewed increase in c-*fos* transcriptional activation and delayed neuronal apoptosis in c-*fos*-expressing cells within the hippocampal formation (Pollard *et al*, 1994). Blood-brain barrier integrity is restored (Bennett *et al*, 1995). Reactive gliosis peaks 24 h after excitotoxin exposure (Bennett *et al*, 1995). Our data indicate that, during this time period, PAFR expression is restricted to apoptotic neurons and to a specific subpopulation of glia involved in phagocytosis of apoptotic debris. We also demonstrate that the percentage of PAFR-expressing CA3c neurons at 24 (36%) and 168 h (12%) after treatment with excitotoxin is substantially less than that observed in control tissue (60%). Systemic injection of kainic acid results in a 59% reduction in the average number of CA3c neurons. This reduction correlates with the almost complete loss (91%) of PAFR-positive CA3c neurons. Thus, PAFR expression may be specifically down-regulated in neurons destined to survive kainate toxicity or PAFR-expressing neurons are preferentially susceptible to excitotoxic challenge.

Protracted exposure to proinflammatory agents may influence subsequent neuronal fate

In support of the latter hypothesis, the kinetics of PAFR mRNA changes closely parallel previous reports of the redistribution of PAF ligand following epileptic seizure and ischemic insult (Kumar *et al*, 1988; Marcheselli and Bazan, 1994). This correlation suggests that PAF synthesis and PAFR expression are simultaneously present in degenerating tissue during excitoxicity. Expression of PAFR by apoptotic neurons at 24 h after kainate injection and the redistribution of PAFR mRNA to apoptosis-activated glia at 168 h after treatment provides circumstantial support for the idea that PAF receptor/ligand interaction is involved in mediating delayed neuronal apoptosis observed after excitotoxin challenge and may be responsible for directing the subsequent clearance of apoptotic debris from damaged tissue. PAF has already been shown to induce expression of transcription factors associated with neuronal apoptosis following exposure to excitotoxins through interaction with high affinity intracellular PAF binding sites (Marcheselli and Bazan, 1994). Our data are also consistent with reports that prolonged exposure to PAF *in vitro* elicits apoptosis in immature thymocytes (El Azzouzi *et al*, 1993), triggers cell death in neurons (Kornecki and Ehrlich, 1988; Lustig *et al*, 1992; Gelbard *et al*, 1994), and activates astrocytes (Brodie, 1994) in receptor-mediated fashion. While a role for PAF in neuronal apoptosis has yet to be established, definitive evidence has been presented that administration of PAF antagonists attenuates excitotoxic cell death *in vivo* (Braquet *et al*, 1989; Doly *et al*, 1993). These data lead us to speculate that the lack of PAFR transcription in neurons destined to survive prolonged exposure to kainic acid and the localization of mRNA to apoptotic neurons and activated glia infiltrating apoptotic tissue may reflect an endogenous program in CNS designed to promote cell death in (and target glia to) irreversibly damaged cells.

Relevance to *in vitro* excitotoxicity

The kinetics of excitotoxic cell death have been well-defined in experimental models of *in vivo* neurodegeneration. Protracted stimulation of excitatory neurotransmitter receptors induces immediate neuronal necrosis and delayed neuronal apoptosis (Smeyne *et al*, 1993; Pollard *et al*, 1994; Portera-Cailliau *et al*, 1995). However, while cultured neurons undergo rapid DNA fragmentation following treated with glutaminergic agents, cells do not consistently exhibit the morphological characteristics of apoptosis in *in vitro* cell death paradigms (Dessi *et al*, 1993; Ikeda *et al*, 1996). Delayed neuronal apoptosis following ligand removal is rarely observed in glutamate-treated cultures. *In vivo* and *in vitro* models of cell death differ in that neurons subjected to excitotoxic challenge *in vivo* are coincidentally exposed to high concentrations of proinflammatory agents. This exposure is also implicated in a number of clinical neurodegenerative conditions such as Alzheimer's disease, Huntington's disease, ischemia, and epilepsy (Kalaria, 1993; Giulian *et al*, 1994; Majno and Joris, 1995; Steinberg *et al*, 1996). What role do proinflammatory agents play in mediating excitotoxic cell loss? Our data raise the intriguing possibility that neurons expressing pivotal proinflammatory signal transduction molecules (i.e. PAFR) may be more likely to undergo delayed excitotoxic apoptosis. Thus, we speculate that coincident sustained exposure to excitatory neurotransmitter and proinflammatory ligands may precipitate an apoptotic cascade in damaged neurons. While specific PAFR antibodies are not currently available, analysis of PAFR protein during excitotoxicity will be required to substantiate this conclusion.

Summary

PAFR transcript is primarily expressed *in vivo* by neurons and microglia in healthy tissue. Expression is restricted to apoptotic neurons and to activated glia targeting apoptotic debris following excitotoxic challenge. These data implicate PAF in neuronal apoptosis and support the hypothesis that endogenous proinflammatory agents may play a role in the etiology of excitotoxic cell loss. We suggest that PAFR belongs to a family of proinflammatory mediators associated with excitotoxic and ischemic damage, including inducible nitric oxide synthase and cyclooxygenase-2. These proinflammatory agents may potentially modulate neuronal fate and mediate the cerebral phagocytic response to neuronal apoptosis.

Materials and Methods

Induction of epileptiform seizures

Status epilepticus was induced in 24 adult male Wistar rats (Charles River) by intraperitoneal injections of 10 mg/kg kainic acid (Sigma). Control rats were untreated littermates (n=6). Animals weighed between 300–400 g at the time of injection. Rats were monitored

for 5 h following drug administration and behaviour was rated according to (McIntyre *et al*, 1982): 0=normal activity, 1=increased frequency of 'freezing' and 'wet-dog shakes', 2=weak clonic convulsions with rearing, 3=increasingly severe convulsions with loss of postural control, 4=generalized limbic seizures, and 5=severe limbic seizures with respiratory difficulty. Animals were injected with a sub-anesthetic dose of sodium pentobarbitol (25 mg/kg) after underoing 2 h of stage 4 seizures (approximately 5 h post kainate injection). We have previously demonstrated that this protocol is sufficient to induce delayed neuronal loss while minimizing the amount of time animals are required to undergo epileptiform activity (Bennett *et al*, 1995). Food and water intake was monitored closely over the following 48 h to ensure recovery. All manipulations were performed in compliance with approved institutional protocols and according to the strict ethical guidelines for animal experimentation established by the Medical Research Council (Canada) and National Institute of Health (USA).

Rats were deeply anesthetized with sodium pentobarbital at 0, 5, 24, or 168 h after kainate injection and euthanized by decapitation (n=6/time period). Brains were rapidly removed and dissected down the midline. The hippocampal formation was removed from one hemisphere and total cellular RNA was isolated using TriReagent (Molecular Research Center) according to the protocol provided by the manufacturer. Coronal sections, 10 μm in thickness, were cryostat cut from the other hemisphere and thaw-mounted on sterile gelatin-coated microscope slides. Sections were post-fixed for 30 min in 4% paraformaldehyde in 100 mM phosphate-buffered saline (PBS: 100 mM sodium phosphate buffer pH 7.2, 154 mM NaCl).

Semi-quantitative RT-PCR analysis of PAFR transcripts

Template RNA was random-primed with 10 pmol pdN_6 (Life Science Technologies). RNA and primer were heated for 10 min at 70°C. dNTPs (0.125 mM each, Promega), dithiothrietol (DTT: 10 mM), 1 × RT buffer (Life Sciences Technologies), and superscript RT (200 U; Life Sciences Technologies) were added. Reactions were incubated at 25°C for 10 min, 42°C for 1 h, and 50°C for 30 min. Two μl of the RT reaction mix was PCR amplified with hot-start in the presence of dNTPs (0.3 mM each), 1 × PCR buffer (Life Sciences Technologies), 2.5 U DNA Taq polymerase (Life Sciences Technologies), 20 pmol PAFR primers, and 10 pmol GAPDH primers. The reaction mixture was denatured at 95°C for 30 s, annealed at 55°C for 90 s, and extended at 72°C for 120 s. Samples were co-amplified for both PAFR and rat GAPDH. PAFR priming occurred over 30 cycles. GAPDH primers were added at the onset of cycle 8 and amplified over the remaining 23 cycles. PAFR primer sequences were 5′-C A C T T A T A A C C G C T A C C A G G C A G-3′ (forward) and 5′-AAGACAGTGCAGACCATCCACAG-3′ (reverse), defining a 381 bp fragment of the rat spleen cDNA (Bito *et al*, 1994). PAFR primers were biotinylated at the 5′ end. GAPDH primer sequences were 5′-TGGTGCTGAGTATGTCGTGGAGT-3′ (forward) and 5′-AGTCTTCT-GAGTGGCAGTGATGG-3′ (reverse) defining a 292 bp fragment of the rat GAPDH cDNA (Piechaczyk *et al*, 1984). GAPDH primers were fluoresceinated at the 5′ end. Labeled primers were synthesized by the Molecular Biology Core Facility at Adirondack Biomedical Research Institute. PAFR PCR products were digested with *EcoR*V to demonstrate appropriate restriction enzyme digestion characteristics.

PCR products were size-fractionated by electrophoresis on a 1.2% (w/v) agarose gel, transferred to Hybond N membrane (Amersham), baked at 80°C for 20 min, and irradiated with ultraviolet light for 2 min for Southern analysis. Membranes were washed for 30 min at 65°C in 2 × SSC wash solution ((1 × SSC: 150 mM NaCl, 15 mM sodium citrate)/0.1% SDS), prehybridized for 1 h at 65°C in pre-hybridization solution (50 μg/ml sonicated salmon DNA, 6 × SSC, 5 × modified Denhardt's solution (0.1% ficoll, 0.1% polyvinylpyrolidone, 0.1% bovine serum albumin, 50 μM trans-1,2,-diamine-cyclohexane-NNN′N′,-tetraacetic acid), 0.5% SDS, 50 mM sodium phosphate) and hybridized for 18 h at 65°C in fresh pre-hybridization solution containing 5% dextran sulfate and a denatured ^{32}P-labeled 1 Kb *Not*I/*Hind*III fragment of the human leukocyte PAFR gene in pCDM8/PAFR (Kunz *et al*, 1992) kindly provided by Dr. N. Gerard, Harvard Medical School, Boston MA or a ^{32}P-labeled 1.5 Kb *Pst*I fragment of the rat GAPDH gene in pGAPDH (Piechaczyk *et al*, 1984). The cDNA insert was purified from agarose using the Qiaex II Agarose Gel Extraction kit (Qiagen) and multiprimed using a random primer labeling kit (Pharmacia) according to the protocols provided by the manufacturers. Following hybridization, membranes were washed twice in wash solution for 15 min at 65°C and twice in high stringency solution (0.16 × SSC, 10 mM Na Citrate, 0.1% SDS). Membranes were air-dried and exposed to X-ray film (Kodak) for 48 h at −70°C. Blots were probed for PAFR, stripped, and re-probed for GAPDH.

Semi-quantitative analysis was performed by ELISA of biotinylated and fluoresceinated RT-PCR products from six animals per time point processed in duplicate. Biotinylated (PAFR) and fluoresceinated (GAPDH) RT-PCR products were amplified simultaneously in the same reaction. Oligonucleotide primers were separated from PCR product by centrifugation through a 500 μl Sephacryl S-300 (Pharmacia) column pre-equilibrated with 1 × PCR buffer. PAFR and GAPDH amplicons were immobilized on 96 well ELISA plates coated with anti-DNA primary antibody (1 : 1000; Boehringer Mannheim) in 10 mM PBS, pH 7.5 containing 0.05% NaN_3 and blocked with 170 mM boric acid pH 8.5, 120 mM NaCl, 0.05% Tween-20, 0.2% bovine serum albumin (BSA), 0.05% NaN_3. Products were detected by reaction with extravidin-peroxidase (1 : 10 000; Sigma) and alkaline phosphatase anti-FITC (1 : 33; Boehringer Mannheim). Wells were incubated with 50 μl peroxidase blue substrate (Boehringer Mannheim) according to the protocol provided by the manufacturer. Absorbance was read at 450 nm. Wells were washed repeatedly, incubated with p-nitrophenylphosphate (Boehringer Mannheim) according to the protocol provided by the manufacturer, and absorbance read at 405 nm. In duplicate reactions, p-nitrophenylphosphate absorbance was determined before peroxidase blue absorbance. PAFR (peroxidase blue) optical density was standardized against GAPDH (p-nitrophenylphosphate) optical density to ensure that changes in PAFR signal accurately reflected changes in mRNA expression and were not the result of unequal template concentration or gel-loading error.

In situ hybridization

In situ hybridization was performed using a *Not*I/*Hind*III fragment of the human leukocyte PAFR gene (Kunz *et al*, 1992). The fragment was either multiprimed with [α-^{35}S]dCTP (Dupont) to a specific activity of approximately 4×10^8 c.p.m./μg using Multiprime Labeling Kit (Amersham) or with FITC-labeled dNTPs using DNA Colour Kit (Amersham). Fresh-frozen (non-radioactive analysis) and fixed sections (radioactive analysis) were rehydrated for 10 min in 10 mM PBS containing 5 mM $MgCl_2$. Fresh-frozen tissue was fixed for 20 min in 4% paraformaldehyde in 10 mM PBS. Sections designated as negative controls were rinsed briefly in 2 × SSC and incubated for 1 h at 37°c with 100 μl RNase A (100 μg/ml). Experimental slides were maintained in fresh 10 mM PBS and 5 mM $MgCl_2$ for 1 h at 37°C. For radioactive analysis, sections were rinsed five times in 10 mM PBS with 10 mM DTT for 2 min per wash, two times in 1 × SSC with 10 mM DTT for 5 min per wash, and prehybridized for 1 h at room temperature in 40 μl 50% formamide, 1 × SSC, 0.4% BSA, 20 mM DTT, 20 mM

vanadyl-ribonucleoside complex, 10 μg/ml yeast tRNA (heat-denatured), and 200 μg/ml salmon testes DNA (heat-denatured). Hybridization was performed at 44°C overnight in 40 μl of prehybridization solution supplemented with 10% dextran sulfate and ^{35}S-labeled probe. Sections were washed twice in 50% formamide, 1 × SSC, and 10 mM DTT at 50°C for 30 min per wash, three times in 50% formamide, once in 1 × SSC, 10 mM DTT at 44°C for 20 min, and three times in 0.5 × SSC with 10 mM DTT at room temperature for 20 min per wash. Sections were dehydrated through progressively higher concentrations of ethanol. Slides were dipped in NBT-2 nuclear-track photographic emulsion (Kodak) and exposed for 14 days at 4°C. Autoradiograms were developed by incubating slides for 5 min in Kodak D-19 developer, 30 s in 2% acetic acid, 5 min in 30% sodium thiosulphate, and 30 min in running water. Sections were lightly counterstained with H/E. For non-radioactive analysis, sections were incubated successively in 20 mM HCl for 10 min at room temperature, in 10 mM PBS containing 0.1% Triton-X for 90 s at 4°C, and in cold 20% glacial acetic acid for 15 s at 4°C. Sections were rinsed in PBS between incubations. Denatured FITC-labeled probe was added to hybridization buffer (Amersham) diluted 1 : 1 with deionized formamide and sections were heated for 1 min at 90°C before incubation at 42°C overnight. Following hybridization, sections were washed twice in 1 × SSC containing 0.1% SDS and twice in 0.2 × SSC containing 0.1% SDS at 42°C for 5 min with shaking. Sections were rinsed in PBS and exposed to peroxidase anti-FITC (1 : 1000, Boehringer Mannheim) in 10 mM PBS containing 3% BSA for 1 h at room temperature. Probe binding was visualized colorimetrically by incubation in 1 mg/ml diaminobenzidine in 50 mM Tris-HCl containing 0.003% H_2O_2. In some cases, sections were lightly counterstained with H/E. Slides were dehydrated and coverslipped with Permount (Fisher). The total number of H/E-stained CA3c neurons and the number of neurons exhibiting non-radioactive PAFR *in situ* hybridization in dorsal hippocampus from sections at bregma −4.6 were counted at 250 × magnification by light microscopy. Counts were averaged over two adjacent sections per animal.

Reactive gliosis

Activated astrocytes were identified by immunoreactivity with a monoclonal mouse anti-GFAP antibody (1 : 40; Boehringer Mannheim). Sections were incubated with primary antibody overnight at 4°C, washed with 10 mM PBS, and reacted for 30 min at 37°C with FITC-labeled or Texas-Red labeled goat anti-mouse IgG antibodies (1 : 20 and 1 : 40 respectively; Amersham). Both primary and secondary antibodies were diluted in Ab buffer (10 mM PBS, 0.3% Triton X-100, 3% BSA). Sections were coverslipped under glycerol containing 0.1% *p*-phenylenediamine (Sigma). To assess specificity of immunoreaction, adjacent sections were processed as described above but in the presence of primary antibody.

In situ detection of apoptotic cells

Apoptotic cells were identified by evaluation of cell ultrastructure following hemotoxylin and eosin (H/E) staining and by biochemical analysis of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) of DNA strand breaks. H/E stained cells with oval nuclei and prominent nucleoli without eosinophilic cytoplasm were considered to be normal and healthy. Pyknotic cells with multiple eosinophilic inclusions and amorphous nuclei were defined as apoptotic cells. Swollen or membrane-compromised neurons with dense, ubiquitous eosinophilic staining of cell cytoplasm or spillage of eosinophilic and hyperchromatic cell cytoplasm into extracellular space were defined as necrotic. Sections (10 μm) reacted for TUNEL were permeabilized by a 15 min incubation in 0.1% Triton X/0.1% sodium citrate on ice. Sections were rinsed for 2 min in 10 mM PBS on ice prior to reaction FITC-labeled dUTP in TDT buffer (30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) and TdT. Labeling was carried out according to the protocol supplied by the manufacturer (Boehringer Mannheim). Sections were coverslipped under glycerol containing 0.1% *p*-phenylenediamine (Sigma) and labeling assessed by immunofluorescence. Negative controls included sections incubated with FITC-labeled dUTP in the absence of TdT. In some cases, fresh-frozen sections were processed for non-radioactive PAFR *in situ* hybridization prior to TUNEL or reacted with anti-GFAP following TUNEL. The total number of CA3c TUNEL-positive neurons were counted as described above under fluorescent microscopy. Counts were averaged over two adjacent sections per animal.

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