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Synergism in the repression of COX-2- and TNF α -induction in platelet activating factor-stressed human neural cells

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Abstract

Platelet activating factor (PAF; β -acetyl- γ -O-hexadecyl-L- α -phosphatidylcholine) triggers a rapid pro-inflammatory gene expression program in primary cultures of human neural (HN) cells. Two genes and gene products consistently induced after PAF treatment are the cytosoluble prostaglandin synthase cyclooxygenase-2 (COX-2) and the pro-apoptotic tumor necrosis factor alpha (TNF α). Both of these mediators are associated with the activation of inflammatory signaling, neural cell dysfunction, apoptosis and brain cell death, and both have been found to be up-regulated after brain injury *in vivo*. In this study we investigated the effects of the non-halogenated synthetic glucocorticoid budesonide epimer R (BUDeR), the novel PAF antagonist LAU-0901, and the electron spin trap and free radical scavenger phenyl butyl nitron (PBN), upon early COX-2 and TNF α gene activation and prostaglandin E₂ (PGE₂) release in PAF-stressed primary HN cells. The data indicate that these three biochemically unrelated classes of inflammatory repressors act synergistically in modulating PAF-induced up-regulation of COX-2, TNF α , and PGE₂ by quenching oxidative stress or inflammatory signaling, resulting in increased HN cell survival. These, or analogous classes of compounds, may be useful in the design of more effective combinatorial pharmacotherapeutic strategies in the treatment of complex neuro-inflammatory disorders.

Keywords

Budesonide epimer R; cyclooxygenase-2; human neural cells; LAU-0901; neuroinflammation; oxidative stress; phenyl butyl nitron; prostaglandin E₂; TNF α

INTRODUCTION

Neuro-inflammatory episodes associated with brain injury are initiated by inducible signaling mediators that drive neural cell dysfunction, apoptosis and brain cell death. These pathogenic signals include pro-inflammatory mediators such as platelet activating factor (PAF), a rapidly inducible cyclooxygenase-2 (COX-2) and tumor necrosis factor alpha (TNF α). PAF, a potent phospholipid and extracellular signaling G-protein-coupled receptor ligand, is generated via phospholipase A₂ over-activation during brain injury [1-3]. While basal levels of PAF are virtually undetectable in resting neural tissue, PAF rapidly accumulates in physiologically-

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stressed neural cells where it selectively activates a network of early response gene expression, including the rapid induction of COX-2 and TNF α [4-11]. COX-2 in turn, as the rate-limiting enzyme in prostaglandin biosynthesis, is pivotal in the up-regulation of prostaglandin E₂ (PGE₂) production, and in driving pro-inflammatory signaling in several types of brain injuries that include neurotrauma, cerebral ischemia and Alzheimer's disease (AD) [6-12]. The lipid mediators and prostaglandins generated by COX-2, such as PGE₂, that normally regulate neurophysiological aspects of thermoregulation, immune modulation, pain and fever, modulate the progression of neuro-inflammatory signaling in disease [6,11,13]. The 157 amino acid pro-inflammatory cytokine TNF α further induces COX-2 gene expression, apoptosis, neuronal damage and the secretion of mediators that perpetuate PAF-initiated pathogenic pathways, including up-regulation in the expression of adhesion molecules and the promotion of leukocyte infiltration in the cerebral vasculature [8-12]. Understanding the mechanism of effective repression of this PAF-induced TNF α and COX-2 pathogenic cascade thereby represents an informative approach in advancing our knowledge of the pharmacology of inflammation management.

The goal of this study was to examine the early kinetics of PAF-induced TNF α COX-2 expression, and PGE₂ release, and to investigate the effects of 3 different drug compounds on TNF α , COX-2 and PGE₂ levels in PAF-stressed primary human neural (HN) cells. TNF α and COX-2 RNA C and protein levels, as well as PGE₂ abundance, provide a useful index for the inflammatory status of brain cells [2,6,13]. In this study we show that specific combinations of inflammatory repressors, including the glucocorticoid BUDeR [13,14], the novel PAF antagonist LAU-0901 [15,16], and the electron spin trap and free radical scavenger PBN [17-19], are effective in repressing PAF-triggered pathogenic events in an HN cell model used to study inflammatory and neurodegenerative mechanisms [2,13,14,20]. While each of these compounds were found to repress PAF-induced TNF α , COX-2 and PGE₂ up-regulation, their combinatorial use together was found to exhibit significant synergism in modulating PAF-induced pathogenic signals.

MATERIALS AND METHODS

The experimental design for this study is described schematically in Fig. 1. Briefly, neuronal-glial co-cultures of HN cells (Cambrex Cell Systems, Walkersville, MD) were grown to ~70% confluence (~50,000 cells per well) in 3.5-cm diameter well COSTAR plates at 37°C, 5% CO₂/20% O₂/75% N₂ in a neural progenitor maintenance medium (NPMM; CC-4241; Cambrex) [14,18,20]. NPMM contained human epidermal and fibroblast growth factors, gentamicin/ amphotericin B, and neural survival factor-1; at source HN cells tested negative for HIV-1, hepatitis B and C, mycoplasma, bacteria, yeast, and fungi (Cambrex). Under low light levels, HN cells were fixed with 4% paraformaldehyde for 10 min, washed 3 times in phosphate buffered saline (PBS, pH 7.2) for 10 min and were then blocked using 10% goat serum plus 0.1% Triton X100 (T9284; Sigma) for 1 hr, followed by 3 washes in PBS. Primary neuron-specific monoclonal antibody β -tubulin III (β TIII) and glial-specific polyclonal antibody glial fibrillary acidic protein (GFAP), both diluted 1:1500 in 1% bovine serum albumen (Chemicon, Temecula, CA) were applied to the cells for 4 h at 4°C, followed by 3 washes in PBS. Goat-anti-human conjugated with Cy3 (Molecular Probes, Eugene, OR) at 1:2000 dilution, and goat-anti-human conjugated with FITC (Molecular Probes), at 1:1500 dilution, were used to visualize primary antibody binding. Signals were digitized using an Axioplan 2 microscope equipped with a high speed digital camera (SensiCam, Zeiss Corporation, Thornwood, NY; Fig. 2a) [14,18,20]. Hoechst 33258 bis-benzimide (H-1398; 50 nM, Molecular Probes, Eugene, OR) was used as a nuclear stain to monitor HN cell viability via the apoptotic state of neurons and glia using electronic digital microscopy [14,20].

Previous dose-response studies *in vitro* have shown significant inhibition of amyloid- and cytokine-induced COX-2 RNA levels by BUDeR [13,14], LAU-0901 [15,16] and PBN [17, 18] at concentrations of 100 nM, 100 nM, and 10 μ M, respectively. Therefore, these same, already optimized, concentrations were used in this *in vitro* study. PAF (10 ng/ml; P4904; Sigma Chemical Company, St. Louis, MO) and PBN (B7263; Sigma) were solubilized, respectively, in DMSO and distilled water (as drug carriers) [13,16,18]. LAU-0901 [tetrahydro-4,7,8,10-methyl-1-(chloro-2-phenyl)-6-(methoxy-4-phenyl-carba-moyl)-9-pyrido [4',3'-4,5] thieno[3,2-f]triazolo-1,2,4 [4,3-a]diazepine-1,4], a novel compound that has superseded LAU-8080 in solubility and efficacy [15,16], and budesonide epimer R [BUDeR; 16,17-butyldienebis(oxy)-11,21-di-hydroxypregna-1,4-diene-3,20-dione], solubilized in 99% 2-hydroxypropyl- β -cyclodextrin (as carrier; C0926, Sigma), were kind gifts from Dr N.G. Bazan, Louisiana State University [13,14]. Controls received the drug carriers DMSO, distilled water or cyclodextrin alone; all drug compounds were added as small aliquots to cell culture NPMM [13-15].

After 21 days of growth HN cells (Fig. 2a) were exposed to PAF for 0, 1, 2, 3, 6 and 24 h. Total RNA was rapidly isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA isolation reagents, containing RNase inhibitors and 0.2- μ M filtered, RNase-free water (Ambion 9915G) [13,14,20]. Total RNA samples were analyzed for concentration and spectral purity using RNA 6000 Nano LabChip analysis (Caliper Technologies, Mountainview, CA) and a 2100 Spectral Bioanalyzer (Agilent Technologies, Palo Alto, CA). In the extracted total RNA 28S/18S ratios all exceeded 1.2 and $A_{260/280}$ values were typically ≥ 2.0 . COX-2, TNF α and RNA levels β -actin in the same HN cell sample were determined using Northern assay [13,18,20]. Basal COX-2 and TNF α RNA was first detected at about day 7 of HN cell growth and remained relatively unchanged at low levels throughout 21 days of growth, at which point neuronal and glia cells were present in about equal populations (Fig 2) [14,20]. The 21 day time-point of HN cell growth was used for all subsequent experiments. HN cells were next exposed to BUDeR, LAU-0901 and/or PBN 1 h before PAF treatment as described (Fig. 1), and controls received only drug carrier minus the drug compound. At 3 h PGE₂ levels were analyzed in HN cell media using ELISA and a PGE₂-acetylcholinesterase conjugate (PGE₂ tracer) with a detection limit of 35 pg/ml (Cayman Chemicals, Ann Arbor, MI) [14,18]. Similarly, TNF α protein levels at 3 h were quantified in HN cell media using a human Chemikine TNF α ELISA kit with a detection sensitivity of 5 pg/ml (Chemicon). HN cell survival was determined using a Hoescht 33258 apoptosis assay [5,20] (Table 1). Northern and immunoassay data were quantified using data-acquisition software on a GS-250 molecular imager (Bio-Rad, Hercules, CA) or a Fluoroskan Ascent FL/Labsystems 96-well plate reader (Thermo Electron Corporation, San Jose, CA). The means of COX-2 and TNF α over β -actin RNA, TNF α protein and PGE₂ levels in the same sample for each experimental condition, were averaged for 5 independent experiments and compared. Means and standard errors were plotted using Microsoft Excel SP3 algorithms (Microsoft Corporation, Redmond, WA; Figs. 2 & 3). Statistical significance was determined using a two-way factorial analysis of variance (*p*, ANOVA; Statistical Analysis System; SAS Institute, Cary, NC).

RESULTS

As depicted in the experimental design, HN cells were exposed to BUDeR, LAU-0901 and/or PBN 1 h prior to treatment with PAF and total RNA and protein were isolated at 0 time and 1, 2, 3, 6 and 24 h later (Fig.1). Both COX-2 and TNF α RNA exhibited significant induction after 3 h of PAF treatment after which both inflammatory markers declined, in contrast to β -actin RNA levels which were not found to change at any time point examined (Fig. 2b). After 3 h of PAF treatment, PAF was found to induce COX-2 and TNF α RNAs to a mean of 2.7- and 2.1-fold, respectively, over basal levels. Confocal microscopy using neuronal-, glial- and nuclear-specific staining (β TIII, GFAP, and Hoescht 33258, respectively) showed no

significant changes in neuronal or glial cell apoptosis over 0 to 3 h, suggesting that the rapid up-regulation of COX-2 RNA, TNF α RNA and protein, and PGE₂ abundance are transient and early pre-apoptotic events (Table 1). After 6 h of PAF treatment both COX-2 mRNA and TNF α mRNA abundance were found to return to near basal levels (Fig. 2b). After 24 h of PAF treatment 71% of all HN cells showed signs of nuclear apoptosis, an event known to precede neural cell death (Table 1) [14,20]. After BUDeR, LAU-0901 and/or PBN pre-incubation, and then incubation with PAF for 3 h, compared to β -actin RNA levels in the same sample, both COX-2 and TNF α RNA abundance were found to be variably decreased (Fig. 2 and 3a), and this paralleled with increased HN cell viability at 24 h post-PAF treatment (Table 1). Levels of PGE₂ and TNF α protein in these same samples were found to be similarly down-regulated after drug treatment (Fig. 3b). According to the experimental design (Fig. 1), BUDeR, LAU-0901 and PBN repressed COX-2 RNA relative signal strength to 0.81, 0.72 and 0.56 of the maximum PAF-induced COX-2 RNA signal, repressed TNF α RNA signal to 0.71, 0.55 and 0.45 of the maximum PAF-induced TNF α signal, repressed TNF α protein signal to 0.66, 0.50, and 0.34 of the maximum PAF-induced TNF α protein signal, and repressed PGE₂ to 0.81, 0.61 and 0.51 of the maximum PAF-induced PGE₂ signal (Fig. 3).

Combinations of BUDeR+LAU-0901, LAU-0901+PBN and BUDeR+PBN were found to be more effective in the repression of COX-2 RNA, TNF α RNA and protein, and PGE₂ levels (Table 1, Fig 3). Interestingly, the highly mobile water- and lipid-soluble free radical scavenger PBN, alone or in combination, showed the most efficient repression of PAF-induced COX-2 RNA, TNF α RNA, TNF α protein or PGE₂ levels (Fig. 3b). This suggests that a significant pathogenic component within the PAF signaling axis may be the generation of excessive free radicals and oxidative stress. The combination of BUDeR, LAU-0901 and PBN together was found to repress COX-2 RNA signal to 0.24 of controls, TNF α RNA signal to 0.14 of controls, PGE₂ levels to 0.16 of controls, and TNF α protein 0.20 of controls. The use of BUDeR, LAU-0901 and PBN in combination was therefore found to be the most effective repressor of both COX-2 and TNF α expression and PGE₂ levels using the experimental protocol described (Fig. 1).

DISCUSSION

PAF serves multiple roles in brain cell signaling in both health and disease, including activating the glycogen synthase kinase 3 beta system [21], increasing NMDA receptor/nitric oxide synthase activity and expression [22], acting as a sensor in neurotrophin signaling, modulating neuronal-microglial interactions during development and brain cell death [23,24], enhancing kinase 1/2 phosphorylation, and in activating the binding of the pro-inflammatory transcription factor NF-kBp50/p65 to the immediate 5' regulatory regions of gene promoters [6,8,13,14, 18]. Both the COX-2 and TNF α promoters contain NF-kBp50/p65 binding sites and are responsive to transcriptional up-regulation via this pro-inflammatory transcription factor [5, 18,25]. The neuroprotective mechanism of BUDeR, and LAU-0901 against this multitude of pathogenic PAF actions are not completely understood, however each have been shown to impede some aspect of NF-kBp50/p65-mediated gene up-regulation [13-16]. For example, the pharmacokinetics of BUDeR, its epimerization, hepatotoxicity, metabolism and efficacy as an anti-asthmatic and anti-inflammatory, while first described about 20 years ago [12], have since been shown to be effective in repressing inflammatory gene up-regulation via modulation of glucocorticoid receptor binding and by blocking productive NF-kBp50/p65-DNA interactions [13,14]. The diazepine-based PAF receptor antagonist LAU-0901 has been shown to inhibit apoptosis, to repress the chemotaxis of inflammatory cells, and to inhibit the inflammatory response in retinal and neural cells, in part by augmenting NF-kBp50/p65-DNA signaling [13-18]. PBN functions as a small, omni-soluble, highly mobile, free radical scavenger and potent quencher of free radical-mediated signaling [5,17-19]. In these experiments, the fact that PBN alone elicited the most potent quenching of COX-2, TNF α and PGE₂ suggests that

the generation of reactive oxygen species (ROS) may play a contributory role to activation of these pathogenic signaling pathways that are in addition to productive NF-kBp50/p65-DNA binding. The testing of additional free radical scavengers such as superoxide dismutase, catalase or L-NAME in future experiments should further clarify the role of ROS in this test system. It should also be pointed out that while informative, these *in vitro* studies have limitations, such as the aqueous solution pharmacokinetics of BUDeR, LAU-0901 and PBN when used together in combination, and the potential for *in vivo* interactions among these bioactive compounds [13,14,17]. These data however represent a proof in principal of synergism in combinatorial pharmacological approaches to complex neuro-inflammatory signaling.

In summary, the incubation of PAF with primary HN cells provides a useful *in vitro* brain cell injury model that recapitulates the rapid induction of COX-2 and TNF α mRNA and TNF α protein to abundances several-fold above their basal levels, as is observed *in vivo* [5-10]. COX-2 mRNA up-regulation by PAF translates into a significantly increased production of PGE $_2$ (Fig. 3). Preliminary investigations have revealed that multiple doses of PAF in the HN cell incubation medium over longer time points exhibits only an initial (up to 3 h) and non-perpetuated induction of COX-2, TNF α PGE $_2$ and in the HN cell model used (Fig. 2b; data not shown). It may be that PAF is more important as an initial pathogenic inducer after acute brain cell injury, and that once initiated, downstream effectors of PAF or other pro-inflammatory mediators, such as TNF α , COX-2 and PGE $_2$ may perpetuate pathogenic signaling during more chronic forms of brain injury that lead to neural degeneration. In support of this are the observations that TNF α , -2 COX and PGE $_2$ are all up-regulated in AD brain and in pro-inflammatory models of neurodegenerative disease [3-6,18,20]. Whatever the cause, in the PAF-stressed HN cell model inflammation and oxidative stress appear to coexist as overlapping pathogenic entities, and hence provide multiple therapeutic targets [14,26,27]. We maintain that it is important conceptually to appreciate that pharmacological strategies directed at each of these inducible pathological pathways may interact synergistically to provide a more efficacious treatment for complex neurological disorders with both oxidation- and inflammation-driven components.

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REFERENCES

- [1]. Ohmori T, Hirashima Y, Kurimoto M, Endo S, Takaku A. In vitro hypoxia of cortical and hippocampal CA1 neurons: glutamate, nitric oxide, and platelet activating factor participate in the mechanism of selective neural death in CA1 neurons. *Brain Res* 1996;743:109–115. [PubMed: 9017237]
- [2]. Zhu P, DeCoster MA, Bazan NG. Interplay among platelet-activating factor, oxidative stress, and metabotropic glutamate receptors modulates neuronal survival. *J Neurosci Res* 2004;77:525–531. [PubMed: 15264222]
- [3]. Bazan NG. Lipid signaling in neural plasticity, brain repair, and neuroprotection. *Mol Neurobiol* 2005;32:89–103. [PubMed: 16077186]
- [4]. Djaladetti R, Lev N, Melamed E. Neuroprotection in progressive brain disorders. *Isr Med Assoc J* 2003;5:576–580. [PubMed: 12929297]
- [5]. Lukiw WJ, Bazan NG. Survival signaling in Alzheimer's disease. *Biochem Soc Trans* 2006;34:1277–1282. [PubMed: 17073801]
- [6]. Cui JG, Hill JM, Zhao Y, Lukiw WJ. Expression of inflammatory genes in the primary visual cortex of late-stage Alzheimer's disease. *Neuroreport* 2007;18:115–119. [PubMed: 17301674]

- [7]. Zador Z, Benyo Z, Lacza T, Hortobagyi T Sr, Harkany T, Hortobagyi T. Neuroprotection in brain ischemia. *Ideggyogy Sz* 2004;57:81–93. [PubMed: 15133909]
- [8]. He J, Bazan HE. Synergistic effect of platelet-activating factor and tumor necrosis factor-alpha on corneal myofibroblast apoptosis. *Invest Ophthalmol Vis Sci* 2006;47:883–891. [PubMed: 16505020]
- [9]. Rossi D, Brambilla L, Valori CF, Crugnola A, Giaccone G, Capobianco R, Mangieri M, Kingston AE, Bloc A, Bezzi P, Volterra A. Defective tumor necrosis factor-alpha-dependent control of astrocyte glutamate release in a transgenic mouse model of Alzheimer disease. *J Biol Chem* 2005;280:42088–96. [PubMed: 16253995]
- [10]. Fehrenbacher JC, Burkey TH, Nicol GD, Vasko MR. Tumor necrosis factor alpha and interleukin-1beta stimulate the expression of cyclooxygenase II but do not alter prostaglandin E2 receptor mRNA levels in cultured dorsal root ganglia cells. *Pain* 2005;113:113–122. [PubMed: 15621371]
- [11]. Quadros A, Patel N, Crescentini R, Crawford F, Paris D, Mullan M. Increased TNFalpha production and COX-2 activity in organotypic brain slice cultures from APPsw transgenic mice. *Neurosci Lett* 2003;353:66–68. [PubMed: 14642439]
- [12]. Pedersen S, Steffensen G, Ekman I, Tonnesson M, Borga O. Pharmacokinetics of budesonide. *Eur J Clin Pharmacol* 1987;31:579–582. [PubMed: 3549321]
- [13]. Lukiw WJ, Pelaez RP, Martinez J, Bazan NG. Budesonide epimer R or dexamethasone selectively inhibit platelet-activating factor-induced or interleukin 1beta-induced DNA binding activity of cis-acting transcription factors and cyclooxygenase-2 gene expression in human epidermal keratinocytes. *Proc. Natl. Acad. Sci. USA* 1998;95:3914–3919. [PubMed: 9520467]
- [14]. Boedker M, Boetkjaer A, Bazan NG, Cui JG, Zhao Y, Pelaez RP, Lukiw WJ. Budesonide epimer R, LAU-8080 and phenyl butyl nitron synergistically repress cyclooxygenase-2 induction in [IL-1beta+Abeta42]-stressed human neural cells. *Neurosci Lett* 2005;380:176–180. [PubMed: 15854773]
- [15]. Cortina MS, Gordon WC, Lukiw WJ, Bazan NG. Oxidative stress-induced retinal damage up-regulates DNA polymerase gamma and 8-oxoguanine-DNA-glycosylase in photoreceptor synaptic mitochondria. *Exp Eye Res* 2005;81:742–750. [PubMed: 15979612]
- [16]. Esquenazi S, He J J, Bazan HE, Bazan NG. Prevention of experimental diffuse lamellar keratitis using a novel platelet-activating factor receptor antagonist. *J Cataract Refract Surg* 2004;30:884–891. [PubMed: 15093656]
- [17]. Butterfield DA, Koppal T, Howard B, Subramaniam R, Hall N, Hensley K, Yatin S, Allen K, Aksenov M, Aksenova M, Carney J. Structural and functional changes in proteins induced by free radical-mediated oxidative stress and protective action of the antioxidants N-tert-butyl-alpha-phenylnitron and vitamin E. *Ann. N.Y. Acad. Sci* 1998;854:448–462. [PubMed: 9928452]
- [18]. Bazan NG, Lukiw WJ. Cyclooxygenase-2 and presenilin-1 gene expression induced by interleukin-1beta and amyloid beta 42 peptide is potentiated by hypoxia in primary human neural cells. *J. Biol. Chem* 2002;277:30359–30367. [PubMed: 12050157]
- [19]. Lin S, Rhodes PG, Lei M, Zhang F, Cai Z. alpha-Phenyl-n-tert-butyl-nitron attenuates hypoxic-ischemic white matter injury in the neonatal rat brain. *Brain Res* 2004;1007:132–141. [PubMed: 15064144]
- [20]. Lukiw WJ, Cui JG, Marcheselli VL, Bodker M, Botkjaer A, Gotlinger K, Serhan CN, Bazan NG. A role for docosahexaenoic acid-derived neuroprotectin D1 in neural cell survival and Alzheimer disease. *J Clin Invest* 2005;115:2774–2783. [PubMed: 16151530]
- [21]. Tong N, Sanchez JF, Maggirwar SB, Ramirez SH, Guo H, Dewhurst S, Gelbard HA. Activation of glycogen synthase kinase 3 beta (GSK-3beta) by platelet activating factor mediates migration and cell death in cerebellar granule neurons. *Eur J Neurosci* 2001;13:1913–1922. [PubMed: 11403684]
- [22]. Xu Y, Tao YX. Involvement of the NMDA receptor/nitric oxide signal pathway in platelet-activating factor-induced neurotoxicity. *Neuroreport* 2004;15:263–266. [PubMed: 15076749]
- [23]. Aihara M, Ishii S, Kume K, Shimizu T. Interactions between neuron and microglia mediated by platelet-activating factor. *Genes Cells* 2000;5:397–406. [PubMed: 10886367]
- [24]. Ohmori T, Hirashima Y, Kurimoto M, Endo S, Takaku A. In vitro hypoxia of cortical and hippocampal CA1 neurons: glutamate, nitric oxide, and platelet activating factor participate in the

mechanism of selective neural death in CA1 neurons. *Brain Res* 1996;743:109–115. [PubMed: 9017237]

- [25]. Higai K, Ishihara S, Matsumoto K. NFkappaB-p65 dependent transcriptional regulation of glycosyltransferases in human colon adenocarcinoma HT-29 by stimulation with tumor necrosis factor alpha. *Biol Pharm Bull* 2006;29:2372–2377. [PubMed: 17142966]
- [26]. Yao Y, Chinnici C, Tang H, Trojanowski JQ, Lee VMY, Praticò D. Brain inflammation and oxidative stress in a transgenic mouse model of Alzheimer-like brain amyloidosis. *J Neuroinflammation* 2004;1:21–25. [PubMed: 15500684]
- [27]. Townsend KP, Pratico D. Novel therapeutic opportunities for Alzheimer's disease. *FASEB J* 2005;19:1592–1601. [PubMed: 16195368]

Fig. 1.

Experimental design. HN cells were exposed to PAF alone or to BUDeR, LAU-0901 and/or PBN 1 h prior to treatment with PAF [13,14,20]. Total RNA and proteins were isolated at 0, 1, 2, 3, 6 and 24 h and analyzed for COX-2, TNF α and β -actin mRNA, TNF α protein and PGE₂ abundance. HN cells were examined for apoptotic index using Hoescht 33258 assay at 0, 3, 6 and 24 h (asterisks) [5,20].

Fig. 2.

PAF-induced up-regulation of a rapid pro-inflammatory response in HN cells in primary culture. **(a)** Merged neuron-specific (β tubulin III, red), glial-specific (GFAP, green) and nuclear (Hoescht 33258, blue) staining of HN cells show approximate equal populations of neurons and glia in 3 w old cultures; note prominence of neuronal and glial nuclei; magnification 20X. **(b)** Relative mRNA levels were compared against the COX-2 signal at '0' time that was arbitrarily set to 1.0 (dashed horizontal line). At '0' time in 3 w old HN cell cultures TNF α mRNA was about half as abundant as that for COX-2 mRNA; at 3 h TNF α reached 80% of the COX-2 signal. N=5 for each determination at each time-point; *significance above '0' time controls for each respective transcript, $p < 0.05$.

Fig. 3.

Analysis of basal and PAF-induced COX-2, TNFα mRNA and protein, and PGE₂ in the presence and absence of BUDeR, LAU-0901, and/or PBN. **(a)** Quantification of up-regulated COX-2 and TNFα mRNA signal intensities compared to β-actin mRNA signal intensities. At 3 h fully induced COX-2 mRNA was assigned a relative signal strength of 100; at 3 h TNFα mRNA signal achieved a relative signal strength of about 80 (Fig. 2). The greatest inhibition of both COX-2 and TNFα mRNA signals were obtained using BUDeR, LAU-0901 and PBN in combination yielding a signal for COX-2 and TNFα mRNA of 25% and 13%, respectively, of non drug-treated controls (maximally repressed TNFα mRNA levels are arbitrarily marked by a dashed horizontal line). **(b)** Quantification of PGE₂ and TNFα protein levels in PAF-induced HN cell NPM in the absence and presence of BUDeR, LAU-0901 or PBN. In the presence of PAF the mean PGE₂ levels were induced at 3 h to 6-fold over untreated controls, to about 610 pg/ml NPM (lane 1). BUDeR, LAU-0901 or PBN were found to inhibit

PGE₂ abundance in a graded fashion; the most effective single inhibitor was PBN that reduced PGE₂ to about 56% of the control value. Again, BUDeR, LAU-0901, and PBN in combination repressed the maximally induced PGE₂ levels to a relative signal strength of about 100 pg/ml PGE₂ (dashed horizontal line), which is near control (basal, un-induced) PGE₂ levels. Similar reductions were observed for the pro-inflammatory cytokine TNF α [8-11]. N=5 for each determination at each time-point; significance of each drug treatment over fully induced mRNA abundance (Fig. 3a) or TNF α protein or PGE₂ (Fig. 3b) levels, * p <0.05, ** p <0.01.

Survival of HN cells after PAF and various drug treatments, based on Hoescht 33258 HN cell viability assay [5,20]. Ten microscope fields for each condition were examined, quantified electronically and compared to controls. Numbers under time (0, 3, 6 and 24 h) represent mean percentage of neurons in the HN cell cultures showing no evidence of nuclear apoptosis, a useful index of HN cell survival [20].

Table 1

PAF	treatment			time (h)			
	BUDeR	LAU-0901	PBN	0	3	6	24
-	-	-	-	100	100	100	99
+	-	-	-	100	99	54	29
+	+	-	-	100	99	72	56
+	-	+	-	100	98	73	62
+	-	-	+	100	99	79	71
+	+	+	-	100	98	81	69
+	-	+	+	100	99	83	75
+	+	-	+	100	98	88	81
+	+	+	+	100	98	96	91