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Intracrine signaling of phospholipid mediators, PAF and LPA, via cognate nuclear G protein-coupled receptors

Review Article

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Abbreviations: cyclooxygenase-2, (COX-2); cytosolic phospholipase A_2, ($cPLA_2$); endothelial nitric oxide synthase, (eNOS); epidermal growth factor, (EGF); G protein-coupled receptors, (GPCRs); calcium independent phospholipase A_2, ($iPLA_2$); inducible NOS, (iNOS); lysophosphatidic acid, (LPA); mitogen-activated protein kinases, (MAPK); nuclear factor-kappa B, (NF-kB); nuclear localization signals, (NLS); platelet-activating factor, (PAF); secreted phospholipase A_2, ($sPLA_2$)

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Summary

Platelet-activating factor (PAF) and lysophosphatidic acid (LPA) are ubiquitous lipid mediators that play important roles in inflammation, cardiovascular homeostasis and immunity and are also known to modulate gene expression of specific pro-inflammatory genes. The mechanism of action of these phospholipids is thought to be primarily dependent on their specific plasma membrane receptors belonging to the superfamily of G protein-coupled receptors (GPCR). However, increasing evidence suggest the existence of a functional intracellular GPCR population. It has been suggested that immediate effects are mediated by cell surface receptors whereas long-term responses are mediated by intracellular receptors. PAF and LPA_1 receptors localize at the cell nucleus of cerebral microvascular endothelial cells of newborn pig, rat hepatocytes and cells overexpressing each receptor, and stimulation of isolated nuclei reveal biological functions, including transcriptional regulation of major genes, namely cylooxygenase-2 and inducible nitric oxide synthase. This mini review focuses on the nuclear localization and signaling of GPCRs recognizing PAF and LPA phospholipids as ligands. Theories on how nuclear PAF and LPA_1 receptors activate gene transcription and nuclear localization pathways are discussed. Intracrine signaling for lipid mediators uncover novel pathways to elicit their effects; moreover, intracellular GPCRs constitute a distinctive mode of action for gene regulation.

I. Introduction

G protein-coupled receptors (GPCRs) are part of a superfamily of receptors composed of seven transmembrane spanning domains. Although GPCRs are traditionally expressed at the plasma membrane level of cells, there is growing evidence supporting the perinuclear/nuclear localization of GPCRs for peptide ligands (angiotensin II, endothelin, somatostatin, substance P, parathyroid hormone and neurotensin) upon extracellular stimulation (Bkaily et al, 2000; Gobeil et al, 2003b). Recently, constitutive nuclear localization (independent of agonist stimulation) in human brain and in overexpressing cells of other GPCRs for peptidic ligands (apelin, angiotensin II and bradykinin) has been reported (Lee et al, 2004); a possible role for specific nuclear localization signals (NLS) has been proposed for this localization. We have also demonstrated compelling evidence for the existence of functional nuclear GPCRs of lipidic ligands, namely for prostaglandin E_2 receptors (EP_1, $EP_{3\alpha}$ and EP_4) in porcine cerebral microvascular endothelial cells, *in situ* in pig brain endothelial cells and in rat liver; the results were further corroborated in overexpression systems of these receptors in Swiss 3T3 cells and human embryonic kidney 293 cells (Bhattacharya et al, 1998; 1999). Of importance, the nuclear EP_3 receptors were found to regulate expression of the major constitutive gene for endothelial nitric oxide synthase (*eNOS*) (Gobeil et al, 2002), while surface receptors exerted distinct acute vasomotor functions; these

findings uncovered biological significance for nuclear GPCRs. Despite the growing evidence that establishes GPCRs at the cell nucleus, nuclear receptor activity has only been described for only a few, namely angiotensin II (Eggena et al, 1993), endothelin (Bkaily et al, 2000) and prostaglandin E_2 receptors (Bhattacharya et al, 1998; Gobeil et al, 2002). This mini review presents new evidence for functional nuclear GPCRs, particularly those recognizing phospholipids as ligands. Actions of nuclear platelet activating factor (PAF) and lysophosphatidic acid (LPA) in relation to gene regulation of pro-inflammatory genes, notably cyclooxygenase-2 (*COX-2*) and inducible NOS (*iNOS*), and possible mechanisms of nuclear receptor localization will be discussed.

PAF and LPA are potent pro-inflammatory phospholipid mediators. Their biosynthetic cascade stems from cell membranes through the action of cytosolic phospholipase A_2 ($cPLA_2$) enzyme which upon stimulation releases arachidonic acid and immediate precursors for PAF or LPA production. Both of these phospholipids exert numerous physiological and pathological effects by mediating responses in the cardiovascular and immune systems, and by regulating cellular processes such as gene expression, secretion, and cell motility and death (Goetzl and An, 1998; Ishii and Shimizu, 2000). In order to exert these responses, PAF and LPA signal through distinct GPCRs. To date humans have been found to express a single PAF receptor (Ishii and Shimizu, 2000) whereas three receptors for LPA have been genetically identified and designated as LPA_1, LPA_2 and LPA_3 (Chun et al, 2002). PAF and LPA receptors are distributed on numerous cells and most notably on endothelium (Montrucchio et al, 2000; Fukushima et al, 2001). Depending on the cell type, PAF and LPA receptors may couple to different G proteins. PAF receptors have been reported to couple to G_q, G_s and $G_{i/o}$ proteins (Agrawal et al, 1992; Lin and Rui, 1994; Shi et al, 1996; Marrache et al, 2002) and similarly, LPA_1 and LPA_2 receptors have been shown to interact with G_q, $G_{i/o}$ and G_{12} proteins whereas LPA_3 receptor combines with $G_{i/o}$ and G_q proteins (Fukushima et al, 2001). Although many responses induced by extracellular PAF or LPA can result from their interaction with plasma membrane GPCRs, they cannot be fully explained by exclusive cell surface signaling. In the following sections we will elaborate on an intracrine mode of action for PAF and LPA.

II. Evidence for intracrine actions

The majority (80%) of newly generated PAF from endothelium are retained within the cells. Along these lines, enzymes that participate in the synthesis of PAF localize at the nuclear envelope (Schievella et al, 1995; Baker and Chang, 1996). Similarly, LPA biosynthetic and degradative pathways have also been detected at the nuclear membrane and/or within the nucleus of cells (D'Santos et al, 1998; Baker and Chang, 1999, 2000; Kim et al, 2002). The biogenesis of PAF and LPA rely on the PLA_2 enzymes that exist as calcium dependent ($cPLA_2$), secreted ($sPLA_2$) and calcium independent ($iPLA_2$) isoforms (Dennis, 1994). The occurrence of these phospholipases at the nuclear envelope favours the possibility of their nuclear actions. PLA_2 enzymes that translocate to the nucleus can act on nuclear membranes to release fatty acids (Peters-Golden et al, 1996) and in turn contribute to PAF and LPA synthesis. Data supporting the latter has recently been reported (Marrache et al, 2002). Freshly isolated nuclei from pig cerebrovascular endothelium were found to generate PAF upon $cPLA_2$ stimulation; a similar observation was made for nuclear prostaglandin E_2 production in the presence of its precursor arachidonic acid (Gobeil et al, 2002). Accordingly, since PAF and LPA can generate their own intracellular formation one could infer that these lipids could potentially activate their cognate intracellular binding sites.

Pharmacological evidence for intracellular binding sites for PAF has been presented (Marcheselli et al, 1990; Bazan, 1998; Liu et al, 2001). Separate functions for intracellular and cell surface receptors have been postulated using agents that can putatively distinguish these receptors (Bazan, 1998; Liu et al, 2001); specifically immediate (paracrine) effects have been proposed to be mediated by cell surface receptors whereas regulation of specific genes (intracrine effects) may be dependent upon intracellular receptors. This notion of functional nuclear receptors is further reinforced by the presence of signaling effectors at the nucleus. These include G proteins, ion channels, phospholipases A_2, C and D, adenylyl cyclase, nitric oxide synthase, mitogen-activated protein kinases (MAPK) and nuclear factor-kappa B (NF-κB) (Gobeil et al, 2003b). However definitive evidence for the presence of nuclear phospholipid receptors and the mechanisms by which they can elicit gene expression has only been disclosed (see below).

III. Nuclear localization of GPCRS for phospholipid ligands

A. Platelet-activating factor receptors

Using a multidisciplinary approach we have detected the presence of PAF receptors that co-localize both at the nuclear envelope and inside the nucleus of newborn pig brain microvascular endothelial cells; this was confirmed in stable transfection studies of Chinese hamster ovary cells that overexpress the wild type cDNA PAF receptor (Marrache et al, 2002). Nuclear PAF receptors were identified by radioligand binding studies and by confocal microscopy (using specific rabbit anti-PAF receptor antibodies) on isolated nuclei of endothelial cells. *In vivo* experiments on porcine brain vasculature analyzed by immunogold electron microscopy revealed receptor expression both at the nuclear envelope of endothelial cells and neurons as well as at the nuclear matrix mostly confined to euchromatin structures (**Figure 1A and B**). These results clarify intracellular PAF binding sites previously detected by pharmacological means (Marcheselli et al, 1992; Bazan, 1998) and/or immunofluorescence (Ihida et al, 1999); but in the latter reports intracellular PAF binding sites were mostly confined to the endosomes and thought to be destined for receptor recycling or degradation following internalization. However, receptor internalization has now

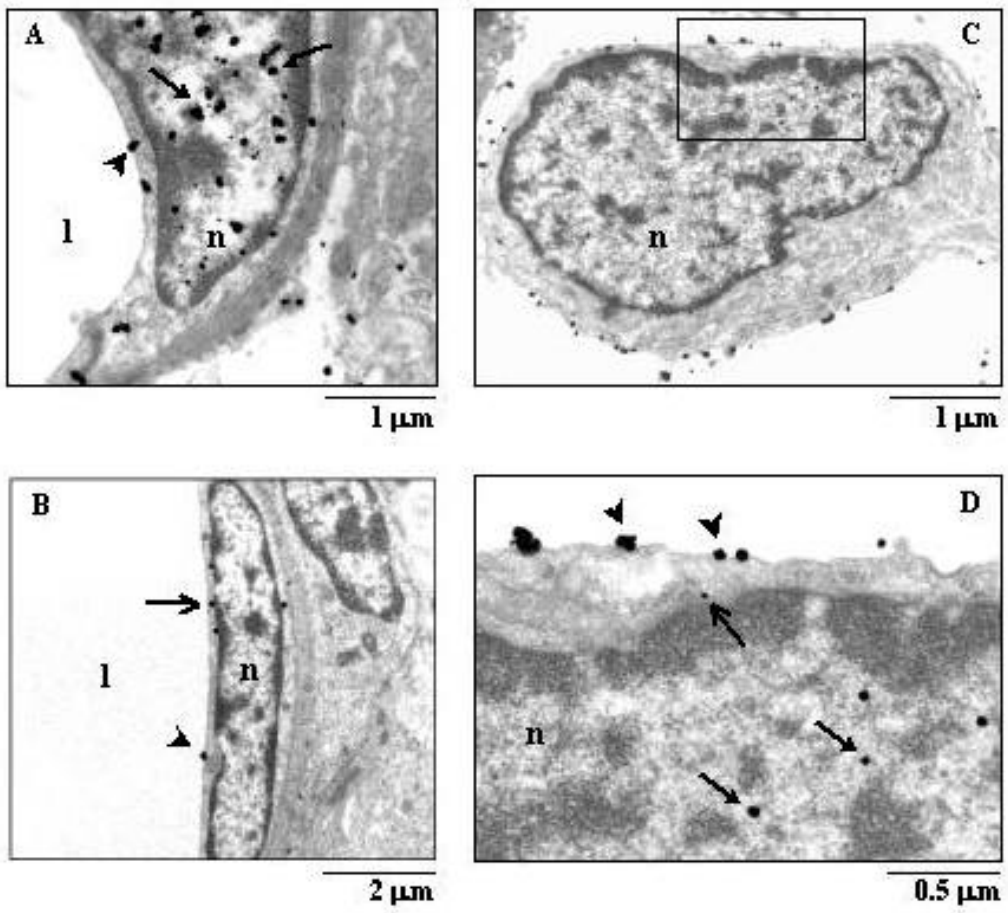

Figure 1. *In situ* localization of nuclear receptors for PAF and LPA determined by immunogold electron microscopy. **A**) In pig brain endothelial cells, PAF receptors localize at the plasma membrane (arrowhead), in the nucleus (closed arrows) and B) at the perinuclear region (open arrows). **C**) Distribution of LPA_1 receptors in rat liver nucleus of endothelial cells. **D**) Higher magnification of panel C displays LPA_1 receptor expression on plasma membrane (arrowheads), at the perinuclear region (open arrow) and in the nuclear matrix (closed arrows). l: lumen of blood vessel; n: nucleus.

been shown to exhibit a broader role by recruiting cell sorting and intracellular signaling proteins (Ferguson, 2001), consistent with our findings.

The presence of PAF receptors in different compartments of endothelial cells raises the question of receptor similarities. Western blot data indicated similar immunoreactivities and molecular masses of ~48 kDa for receptors at the plasma membrane and (isolated) nuclei, corresponding to the glycosylation form of the receptor (Garcia Rodriguez et al, 1995). Hence PAF receptors at the nucleus are intact entities similar to those at the plasma membrane.

B. Lysophosphatidic acid receptors

Specific nuclear LPA_1 receptors were revealed in unstimulated porcine cerebral microvascular endothelial cells, in HTC4 rat hepatoma cells stably transfected with the LPA_1 receptor and in rat liver using a number of complementary approaches including radioligand binding, electron and cryomicroscopy, cell fractionation and immunoblotting with three distinct antibodies against the LPA_1 receptor (Gobeil et al, 2003a). Similar to PAF receptors, these different techniques unveiled perinuclear and intranuclear localization of the LPA_1 receptor (**Figure 1C and 1D**). Western blot analyses disclosed a prominent band at 49 kDa in agreement with Zheng *et al, (*2001) in both rat liver nuclear and plasma membrane fractions.

GPCRs undergo post-translational modifications by addition of oligosaccharide moieties for proper folding and expression of the receptor. The presence of putative glycosylation consensus sequences residing in rat and human LPA_1 receptor was confirmed by electromobility shift of LPA_1 receptor protein to the predicted theoretical molecular mass (38 kDa) following treatments of a deglycosylation agent *N*-glycosidase F or by a glycosylation inhibitor tunicamycin (Gobeil et al, 2003a); the latter also did not seem to affect localization.

C. Patterns of nuclear GPCR distribution

Even though an increasing number of GPCRs localize at the nucleus this pattern of localization is not universal. For instance, it is noteworthy that β_3-adrenergic receptors are only expressed at the cell surface of cells and do not distribute intracellularly (Jockers et al, 1996). The intranuclear receptor expression pattern seen for PAF and LPA_1 receptors can also not be generalized for other nuclear GPCRs; specifically, GPCRs for angiotensin II, somatostatin, substance P, neurotensin, and prostaglandin

E_2 distribute at the nuclear envelope but negligibly within the nucleus (Gobeil et al, 2003b). The presence of perinuclear GPCRs is conceivable given that the outer nuclear membrane is contiguous with the endoplasmic reticulum (ER) (a site of protein synthesis). On the other hand, mechanisms to explain intranuclear localization are still debatable. One possibility is that since the GPCRs co-localize with euchromatin structures as reported by high resolution immunogold electron microscopy (**Figure 1**) (Marrache et al, 2002; Gobeil et al, 2003a), the receptors may contribute to gene regulation as euchromatin represents the active sites of gene transcription. This paradigm has been elegantly suggested for other types of transmembrane receptors, notably of epidermal growth factor (EGF) (Lin et al, 2001). Nonetheless, given the molecular nature of these receptors and GPCRs which contain highly hydrophobic regions, the possibility that intranuclear membrane structures exist cannot be excluded (Fricker et al, 1997; Isaac et al, 2001).

Generally, the nucleus is free of membranes other than the double (outer and inner) nuclear membrane, which makes up the nuclear envelope. Interestingly, using electron microscopy Fricker et al, (1997) detected in nucleoplasm of mammalian cells long, dynamic, branching membrane channels derived from the ER as deep, narrow invaginations of both membranes of the nuclear envelope. In addition, a cytoplasmic core and nuclear pore complexes in the channel walls suggesting nucleo-cytoplasmic transport was also identified. This *bona fide* ER in the nucleus of cells seems to be induced by a nonribosomal, nucleolar and coiled protein termed Nopp140 (nucleolar chaperone) and is contiguous with the inner nuclear membrane containing a mixture of smooth and rough ER proteins (Isaac et al, 2001). Moreover, protein translation directly within the cell nucleus (including in isolated nuclei devoid of cytoplasmic ribosomes) has recently been demonstrated (Iborra et al, 2001) and consists approximately 10-15% of total cellular protein production. Taken together, one could speculate that the existence of a nuclear ER network combined with the ability to synthesize protein provides a possible explanation for the intranuclear localization of GPCRs for PAF and LPA_1 (as well as other transmembrane receptors).

IV. Nuclear signaling pathways

It is becoming evident that the nuclear envelope plays a major role in signaling cascades. Nuclear membranes and nucleoplasm contain a number of signaling factors possibly involved in both PAF and LPA_1 receptor signal transduction pathways. For instance, nuclear localization has been identified for G proteins ($G_{i/o}$, $G_{s\alpha}$) (Takei et al, 1994; Saffitz et al, 1994; Balboa and Insel, 1995), calcium channels and pumps (IP_3, IP_4 and ryanodine receptors, Ca^{2+}-ATPase pump, Na^+/Ca^{2+} exchanger) (Malviya et al, 1990; Gerasimenko et al, 1996; Xie et al, 2002), ion channels (K^+, Na^+, Cl^-, Ca^{2+}, Zn^+, K^+/H^+ exchanger, Na^+/K^+-pump) (Mazzanti et al, 1990; Maruyama et al, 1995; Szweczyk, 1998; Masuda et al, 1998; Garner, 2002), phospholipases A_2, D and C (Divecha and Irvine, 1995; D'Santos et al, 1998; Cocco et al, 1999), adenylyl cyclase (Yamamoto et al, 1998), protein kinase C (Buchner, 1995), MAPK (Erk1, Erk2, Erk3) (Cheng et al, 1996; Kim and Kahn, 1997; Thomson et al, 1999) and IκB and NF-κB (Sachdev et al, 1998; Karin and Ben-Nariah, 2000). The autonomous nature of the nuclear signaling network brings a new dimension to cellular signaling, somewhat independent from plasma membrane/cytosolic events. Relevant components of the nuclear lipid metabolism are summarized in **Table 1**.

We have shown distinct G protein-coupling between plasma membrane PAF receptor (G_q) and nuclear receptor ($G_{i/o}$) (Marrache et al, 2002) suggesting different roles for each as previously proposed (Bazan, 1998; Liu et al, 2001); cell surface PAF receptors generate inositol phosphates in a phospholipase C-dependent manner while nuclear PAF receptors inhibited the activity of adenylyl cyclase through a pertussis toxin-sensitive manner. Nuclear LPA_1 receptors can elicit changes in nuclear calcium levels, which are inhibitable by pertussis toxin (Gobeil et al, 2003a), implying a $G_{i/o}$ protein-coupling. Stimulation of PAF receptors can also induce nuclear calcium transients independent of IP_3 generation. We therefore suspect that nuclear PAF and LPA_1 receptor-induced nuclear calcium transients probably involve activation of nuclear membrane ion channels through direct interaction with the βγ subunits of the $G_{i/o}$ proteins (Hughes et al, 1987; Rosenthal et al, 1988; Wickman and Clapham, 1995); interaction between nuclear K_{Ca} channels and calcium mobilization has recently been reported (Gobeil et al, 2002).

Table 1. Nuclear compartmental localization of the lipid metabolism. ud: undetermined localization

Lipid/Receptor	Nuclear Envelope	Nucleoplasm	Nuclear Matrix	Nucleolus
PGE_2 receptor	present	absent	absent	present
PAF	ud	present	ud	ud
PAF receptor	present	absent	present	ud
Phosphoinisotides	ud	present	present	ud
Cytosolic Phospholipase A_2	present	ud	ud	ud
Phospholipase C	ud	present	ud	ud
Phospholipase D	present	ud	ud	ud
LPA	ud	present	ud	ud
LPA receptor	present	absent	present	ud

V. Nuclear PAF and LPA receptors and gene transcription

A major nuclear function involves gene transcription. Nuclear calcium plays an instrumental role in many processes in the nucleus such as protein import, apoptosis and gene regulation (Malviya and Rogue, 1998) and there is evidence for PAF and LPA receptors causing pro-inflammatory gene induction (Bazan et al, 1991; 1997, 1998; Szabo et al, 1993; Bazan and Allan, 1996; Mustafa et al, 1996; Reiser et al, 1998; Palmetshofer et al, 1999). The molecular mechanisms by which GPCRs, including PAF and LPA_1 receptors, modulate gene transcription are only partially understood. Recently uncovered mechanisms to explain GPCR-mediated gene induction by extracellular ligand stimulation implicate metalloprotease-dependent MAPK activation via receptor tyrosine kinase transactivation reported for muscarinic and endothelin receptors (Pierce et al, 2001). Another mechanism documented for β_2-adrenergic receptors includes the formation of β-arrestin-c-Src complexes, which during receptor internalization can recruit other signaling molecules to phosphorylate MAPKs (Pierce et al, 2001). Alternatively, nuclear GPCRs may regulate gene transcription. The effect of nuclear PAF and LPA_1 receptors in inducing gene transcription was therefore investigated.

Using RT-PCR to amplify nuclear RNA from freshly isolated nuclei stimulated with PAF and LPA revealed induction of *COX-2* and *iNOS* gene transcription (**Figure 2A and 2B; Figure 3A and 3B**) (Marrache et al, 2002; Gobeil et al, 2003a). Differences in temporal expression for distinct genes involve different transcriptional activator interactions, which involve a sequence of signaling and nucleoplasmic sorting. Hence, this would provide an explanation for the same or different receptor to activate distinct genes. PAF and LPA induced phosphorylation of MAPKs and correspondingly, transcriptional regulation of the *COX-2* gene was found to be MAPK-dependent (**Figure 2B and Figure 3B**). PAF also induced binding of NF-κB to its consensus DNA sequence as excess unlabeled mutant competitor could not compete with the radiolabeled consensus NF-κB/DNA recognition sequence for DNA binding. Conversely, in the presence of excess unlabeled specific competitor (consensus NF-κB/DNA recognition sequence) a radioactive band could not be revealed due to competition binding (**Figure 2C**). To determine the physiological role of nuclear GPCRs on whole cells, we examined the involvement of receptor internalization, metalloprotease activation and autocrine generation of PAF or LPA in gene transcription. *COX-2* gene expression was unaltered by cells stimulated by PAF or LPA in the presence of inhibitors of metalloprotease or receptor internalization (Marrache et al, 2002; Gobeil et al, 2003). On the other hand, inhibition of the $cPLA_2$ enzyme, which is required for PAF and LPA biosynthesis prevented *COX-2* induction (**Figure 2D; Figure 3B**). Thus, extracellular PAF and LPA seem to elicit their own generation intracellularly to induce in turn pro-inflammatory gene expression. It is therefore conceivable to suggest that nuclear PAF and LPA can be locally generated to act in the vicinity of their cognate nuclear receptors to modulate gene transcription.

VI. Mechanisms of GPCR nuclear localization

The data presented above unveils compelling evidence for the presence of functional perinuclear/nuclear PAF and LPA_1 receptors. But the mechanisms for this nuclear localization are not known, albeit the following possibilities can be considered. Nuclear distribution may involve nuclear NLSs that consist of short stretches of basic amino acids. These sequences are found either in the third intracellular loop or downstream of the seventh transmembrane domain within the eighth helix of the receptor as reported for other nuclear GPCRs such as the angiotensin II and apelin receptors (Lu et al, 1998; Lee et al, 2004). It is proposed that a Ran-GTP/importin pathway mediates the mechanism of NLS nuclear sequestration. Interestingly, a putative NLS ($KKFRKH^{298-303}$) exists at the C-terminal tail of the PAF receptor (Chase et al, 1993). ER retention sequences ($NLSK^{338-341}$) present on PAF receptor C-terminus (Teasdale and Jackson, 1996) may also participate in PAF receptor nuclear distribution; these sequences may also be implicated in vesicular trafficking and translocation of receptors to the nucleus as suggested for angiotensin II (Chen et al, 2000). However, deletion of the PAF receptor C-terminus does not affect its cellular localization (Marrache et al, 2002). Along these lines, the LPA_1 receptor does not contain a putative NLS sequence (Lee et al, 2004) and infers to the role of other mechanisms such as those possibly involved in nuclear import and endocytosis. In this context, LPA_1 receptors were found to partition in both clathrin and caveolar endocytotic microdomains in plasma membrane of endothelial cells but co-localized with caveolar fraction only in nuclear preparations as analyzed by co-immunoprecipitation (Gobeil et al, 2003a); clathrin pathways are usually ascribed for receptor recycling or degradation whereas caveolar pathways serve as a carrier system responsible for intracellular redistribution of various signaling components.

Another possible mechanism of nuclear localization is post-translational modifications of proteins such as glycosylation. Prostaglandin E_2 receptors (EP_1, $EP_{3\alpha}$ and EP_4) have been reported by us to localize at the nucleus (Bhattacharya et al, 1998; 1999; Gobeil et al, 2002). Non-glycosylated EP_3 receptors seem to be retained in the ER whereas glycosylated receptors distribute to the cell surface (Boer et al, 2000); this suggests a role for glycosylation in cellular compartmentalization. However Western blot analyses of PAF and LPA_1 receptors revealed identical molecular masses in plasma membrane and nuclear fractions (Marrache et al, 2002; Gobeil et al, 2003a), which could suggest comparable degree of glycosylation of these proteins in the different cell compartments. This does not however exclude the possibility of distinct glycoprotein adducts in different cellular locations. All in all, the mechanisms that determine receptor expression on plasma membrane versus nuclear membrane remain to be clarified.

VII. Conclusion

In summary, the discovery of nuclear GPCRs for PAF and LPA provides a novel mechanism of GPCR signaling – specifically a pathway operating within the nucleus rather than having a seemingly less direct and more unwieldy molecular mechanism which would transfer signal from the cell surface to the cell's interior; a schematic diagram depicting this newly described cellular process is presented in **Figure 4**. Nuclear receptor population can offer PAF and LPA geographic advantage considering the short half-life (minutes) of these phospholipids. Having PAF and LPA synthesis in close proximity of their nuclear receptors would therefore give spatial shelter from degrading enzymes that could inactivate PAF and LPA produced at a distance, and consequently yield a more efficient system. Moreover, distinct localization of GPCRs may dictate different mechanisms for the same receptor to manifest its various functions; specifically, plasma membrane receptors elicit acute responses while nuclear receptors would participate in the intracrine mode of actions of phospholipids in regulating gene transcription.

It is of interest to point out that separate localization of GPCRs has been observed in the clinical setting and may have significant implications (Shibuta et al, 2002; Spano et al, 2004). For instance, CXCR4 receptors have been found in nuclei of tumour cells but not in non-cancerous cells (Shibuta et al, 2002; Spano et al, 2004), as a means of possibly modulating cell proliferation. Intracellular compartmentalization of GPCRs exhibiting distinct functions may also have important pharmacological consequences. At present, therapies aimed towards conditions involving GPCRs do not distinguish sites (small molecules) or apply to cell surface receptors (antibodies). By elucidating functional nuclear GPCRs, site-targeted drug design will be favoured.

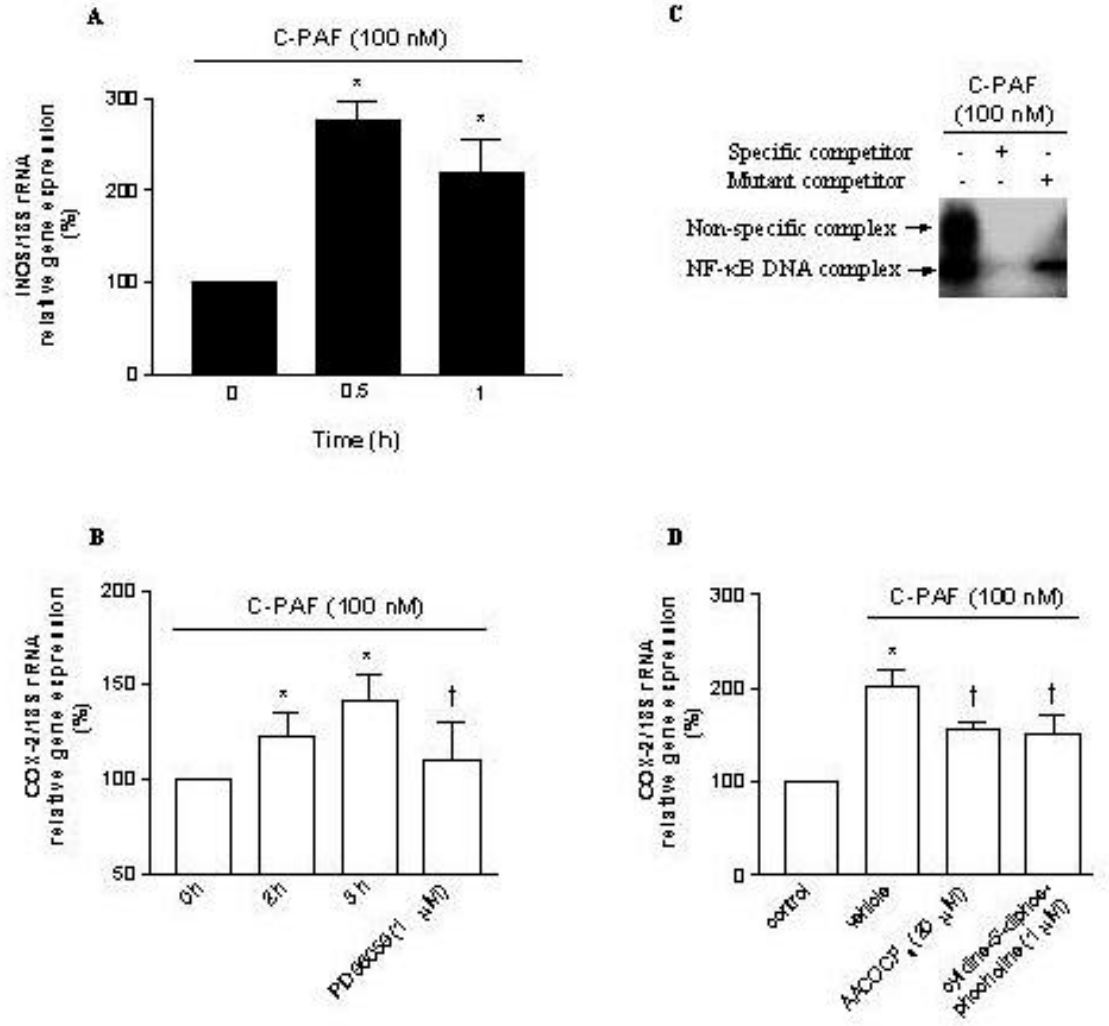

Figure 2. Pro-inflammatory gene induction by stimulation of isolated nuclei of endothelial cells with PAF (A-C), and in intact endothelial cells (D). A) Stimulation of nuclei with PAF induces *iNOS* and B) *COX-2* gene expression determined by RT-PCR. MEK inhibitor PD 98059 prevents *COX-2* gene transcription at 3 h. C) Binding of NF-κB to DNA consensus sequence reveals NF-κB-DNA binding complex after 1 h of stimulation of nuclei with PAF. D) $AACOCF_3$ and cytidine-5-diphosphocholine ($cPLA_2$ inhibitors) diminishes *COX-2* transcription (at 3 h) induced by extracellular PAF. *$p<0.05$ compared to 0 h or control; †$p<0.5$ compared to C-PAF.

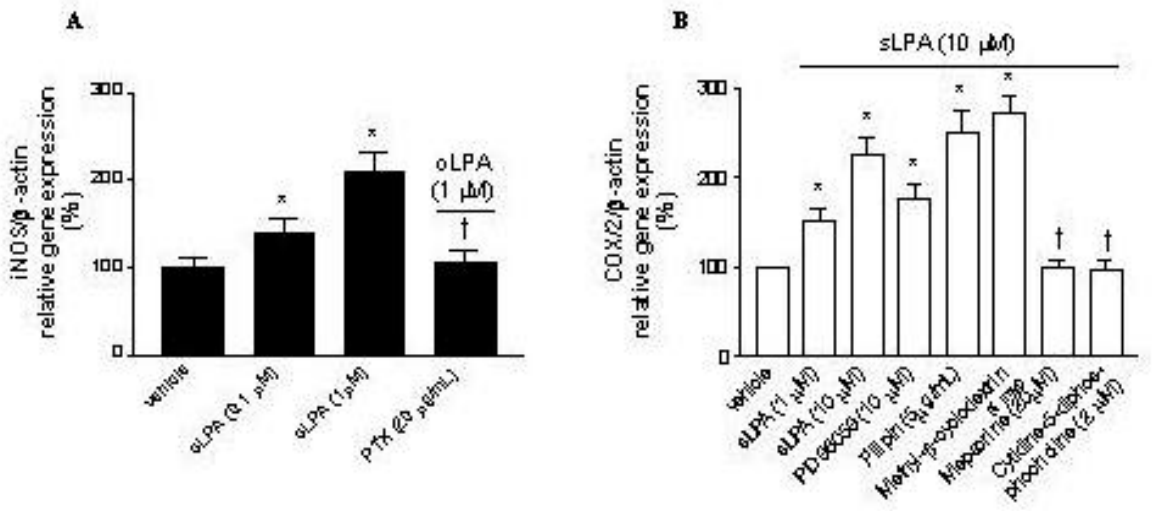

Figure 3. Pro-inflammatory gene induction by stimulation of nuclei of rat hepatocytes (A) and intact endothelial cells (B) with LPA. A) Stimulation of nuclei with LPA for 1 h induces a dose-dependent rise in *iNOS* transcription (determined by RT-PCR); the latter is PTX sensitive ($G_{i/o}$ protein-coupling). B) Stimulation of cells with LPA induces *COX-2* gene transcription. This LPA-induced *COX-2* expression was inhibited by MEK blocker PD98059 and by specific $cPLA_2$ inhibitors (mepacrine and cytidine-5-diphosphocholine), but not by caveolae-disrupting agents filipin and methyl-β-cyclodextrin. *p<0.05 compared to vehicle; †p<0.5 compared to LPA.

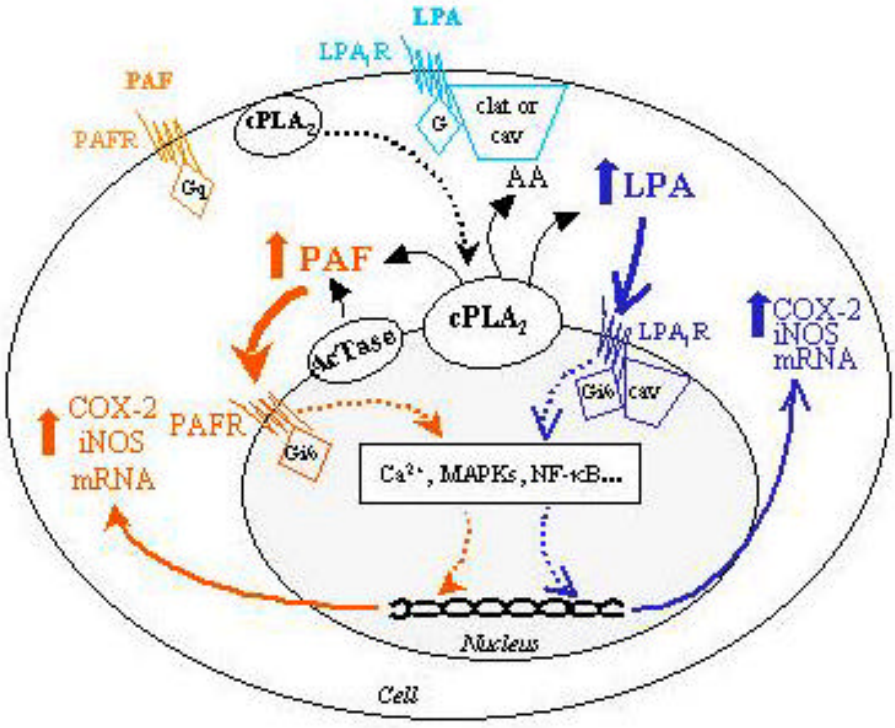

Figure 4. Proposed model depicting cellular mechanism for nuclear GPCR signaling. PAF receptors (orange) and LPA_1 receptors (blue) localize at the plasma as well as at nuclear membranes. Distinct G protein-coupling for PAF receptors is noted on plasma membrane and nuclei. The LPA_1 receptor co-localizes with different endocytotic microdomains either with clathrin and/or caveolae at the cell surface or simply with caveolae at the nucleus. Translocation of the $cPLA_2$ enzyme to the nuclear membrane favours nuclear production of PAF and LPA. The ability of PAF and LPA to generate their own intracellular production enables these mediators to act on their nearby nuclear receptors to elicit a signaling cascade involving calcium, MAPK and NF-κB, which in turn leads to gene transcription. AA: arachidonic acid; AcTase: acetyltransferase; clat: clathrin; cav: caveolae; LPA_1R: LPA_1 receptor; PAFR: PAF receptor.

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