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Transgenic model for the discovery of novel human secretory non-pancreatic phospholipase A_2 inhibitors

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Abstract

Transgenic mice were created which overexpress human secretory non-pancreatic phospholipase A_2 ($sPLA_2$) pansomatically as a potential disease and drug-testing model. The mice were produced using a DNA construct in which the inducible mouse metallothionein gene promoter drives expression of a human $sPLA_2$ minigene. High levels of $sPLA_2$ were detected in several tissues by immunofluorescence localization. Expression in the testes caused hypospermia and male infertility. Circulating catalytically active $sPLA_2$ could be induced to levels observed in patients undergoing a systemic inflammatory response but had no detectable effect on the mice. Therefore, these results suggest that $sPLA_2$ hyperphospholipasemia alone may have only limited pathophysiological consequences. We further show that 3-[3-acetamide-1-benzyl-2-ethylindolyl-5-oxy]propane phosphonic acid (LY311727), a potent new inhibitor of phospholipase A_2 catalysis developed by our group, dramatically suppresses the circulating enzyme activity in these animals whereas 3-[3-acetamide-1-benzyl-2-propylindolyl-5-oxy]propane phosphonic acid (LY314024), a substantially less potent LY311727 analog, is without effect. These later results thus motivate the further development of this compound as a potential new therapeutic agent and valuable research tool.

Keywords: Phospholipase A_2; Inflammation; Transgenic mouse; Phospholipase inhibitor

1. Introduction

Phospholipase A_2 (PLA_2) enzymes play a key role in the generation of proinflammatory mediators. PLA_2s hydrolyze the sn-2 acyl ester bond of glycerophospholipids releasing free fatty acids and lysophospholipid. Further metabolism of arachidonic acid, released when present at the sn-2 position, liberates the proinflammatory eicosanoids in mammalian cells (Irvine, 1982; Dennis, 1987). Lysophospholipids released by PLA_2 are also damaging to cells (Stafford and Dennis, 1988) and further metabolism of lysophosphatidylcholine generates the proinflammatory mediator platelet activating factor (Hanahan, 1986).

Both intracellular and extracellular PLA_2s have been identified and classified into distinct groups based on their structural homology and enzymatic properties (Davidson and Dennis, 1990; Bonventre, 1992). Secretory non-pancreatic phospholipase A_2 ($sPLA_2$) is a 14 kD protein which belongs to the extracellular group II enzymes. $sPLA_2$ was originally isolated from arthritic joint synovial fluid (Kramer et al., 1989; Stoner et al., 1991; Vadas et al., 1993) and has since been detected in a large number of cell types including platelets (Kramer et al., 1989), leukocytes (Marshall and Roshak, 1993), chondrocytes (Lyons-Giordano et al., 1989; Nevalainen and Haapanen, 1993), mesangial cells (Pfeilschifter et al., 1993), hepatoma cells (Crowl et al., 1991), smooth muscle (Nakano et al., 1990; Vadas et al., 1993) and paneth cells (Nevalainen and Haapanen, 1993). $sPLA_2$ is also present at low levels in normal human serum (Nevalainen et al., 1992; Vadas et al., 1992; Rintala and Nevalainen, 1993) and is abundant in seminal and lacrimal gland fluid (Takayama et al., 1991; Nevalainen et al., 1993b, 1994). The normal function(s) of $sPLA_2$ is uncertain, but it is thought to act as an antimi-

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crobial agent (Wright et al., 1990; Nevalainen et al., 1994; Harwig et al., 1995).

Numerous studies have implicated $sPLA_2$ as an important proinflammatory enzyme and thus a potential therapeutic target. High local and/or systemic levels of $sPLA_2$ have been demonstrated in a number of inflammatory conditions including arthritis (Pruzanski et al., 1994), pancreatitis (Grönroos and Nevalainen, 1992; Nevalainen et al., 1993a), inflammatory bowel disease (Minami et al., 1994), SIRS (Fink, 1993; Vadas et al., 1993) and septic shock (Vadas et al., 1988; Green et al., 1991). This disease-associated $sPLA_2$ hyperphospholipasemia appears to be driven by inflammatory mediators which have been shown to stimulate expression of $sPLA_2$ in many cell types/tissues (Lyons-Giordano et al., 1989; Nakano et al., 1990; Crowl et al., 1991; Pfeilschifter et al., 1993; Vadas et al., 1993). A causal role for $sPLA_2$ is further suggested by evidence that the levels of $sPLA_2$ in the circulation correlate with disease severity in several of these clinical settings (Vadas et al., 1988; Pruzanski et al., 1994).

The catalytic activity of $sPLA_2$ together with its expression profile in the above settings is highly suggestive of a role in inflammation. However, efforts to directly demonstrate such a role experimentally have been somewhat controversial. Early studies supporting a pathophysiological role of $sPLA_2$ were based on experiments utilizing highly toxic venom-derived phospholipases as $sPLA_2$ surrogates. As it is now clear that the biological actions of these enzymes differ from those of mammalian $sPLA_2$, the relevance of such studies to the pathobiology of $sPLA_2$ has been seriously questioned (Vadas et al., 1993). However, studies employing mammalian $sPLA_2$ purified from cellular and biological fluid sources also suggest an etiopathic role for $sPLA_2$. Rat platelet $sPLA_2$ has been reported to enhance the release of prostaglandins from calcium ionophore (A23187)-activated HL-60 granulocytes (Hara et al., 1991), tumor necrosis factor-stimulated endothelial cells (Murakami et al., 1993b) and antigen-primed mast cells (Murakami et al., 1991) and to exacerbate adjuvant-induced arthritis in the rat following intradermal injection of the enzyme (Murakami et al., 1990). $sPLA_2$ extracted from human synovial fluid induced edema after injection into the mouse foot pad (Vishwanath et al., 1988) and induced inflammatory changes when injected into the knee joint of the rat (Vadas et al., 1989). $sPLA_2$ isolated from endotoxin shock rabbits when reintroduced into naive rabbits induced a shock-like hypotension which was abolished when the catalytic activity of the enzyme was inactivated with *p*-bromophenacyl bromide (Vadas and Hay, 1983).

Studies employing recombinant $sPLA_2$, where the purity of the enzyme is more likely assured, have also been conducted. Hence, we have shown (Snyder et al., 1993) that recombinant human $sPLA_2$ ($rhsPLA_2$) elicits the release of arachidonic acid and induces an eicosanoid-dependent contractile response in a tissue bath assay employing guinea pig lung strips. Moreover, 3-[3-acetamide-1-benzyl-2-ethylindolyl-5-oxy]propane phosphonic acid (LY-311727), a potent new $sPLA_2$ inhibitor developed by us, specifically blocks these responses demonstrating this effect is mediated through the catalytic activity of the enzyme (Schevitz et al., 1995). Bomalaski et al. (1991) found that intra-articular injection of $rhsPLA_2$ in the rabbit provoked a pathological response in the knee joint. $rhsPLA_2$ has also been shown to exacerbate polycation-induced rat paw edema (Cirino et al., 1994a) and inflammation induced by the injection of air into the subcutaneous tissue in the rat (Cirino et al., 1994b). However, no increase in eicosanoid generation was detectable in the affected tissue in this later model raising questions about the mechanism of action. Also, Morgan et al. (1993) failed to detect either the initiation or the exacerbation of pre-existing inflammation by $rhsPLA_2$ in a rat paw edema model. Hence, whereas many studies are supportive of a proinflammatory role for $sPLA_2$, others have been less corroborative.

Recent genetic studies have also shed new light on the pathobiology of $sPLA_2$. Two groups have discovered that several common mouse strains are deficient in $sPLA_2$ due to a point mutation in their $sPLA_2$ gene (Kennedy et al., 1995; MacPhee et al., 1995). The fact that these animals are viable and reproductively normal indicates that $sPLA_2$ is not essential for development. Since they appear to develop inflammation normally, this might also raise doubts about the role of $sPLA_2$ in inflammation. However, whether these mutant mice exhibit any specific pathophysiological deficits (or gains) over non-mutant animals remains to be fully explored. Moreover, as little is know about the regulation of $sPLA_2$ in inflammation in the mouse (i.e., in non-mutant mice), it is also uncertain whether a mutation in the mouse $sPLA_2$ gene should be *expected* to have any impact on inflammation in this animal species. As such, the impact of this new finding on our understanding of $sPLA_2$ pathobiology, and $sPLA_2$ hyperphospholipasemia in particular, remains to be determined.

In the present study we have superimposed an inducible human $sPLA_2$ transgene onto the $sPLA_2$-deficient C57BL/6 mouse background such that the potential pathophysiological role(s) of $sPLA_2$ could be addressed in a setting of elevated $sPLA_2$ expression. In addition, we show that this animal model provides a valuable means for testing potential $sPLA_2$ inhibitors against the most clinically relevant target, i.e., the human enzyme, in vivo.

2. Materials and methods

2.1. Transgenic mouse development

To create the $sPLA_2$ transgene, polymerase chain reaction (PCR) primers containing Bgl II linkers were used to amplify two joined segments of the human $sPLA_2$ gene

(100 bp of 5′ and 230 bp of 3′ untranslated region and poly A site inclusive; 1.9 kb of intron 4 deleted) from plasmid pHD-PLA_2 (Wery et al., 1991). This amplified Bgl II site-flanked 1508 bp region was blunt-end ligated into the Hind II site of pBluescrip KS(-) (Stratagene). The 1508 bp fragment was then excised from this plasmid with Bgl II and inserted downstream of the mouse metallothionein gene promoter in plasmid MThGH III (Palmiter et al., 1983). This was accomplished by ligation into the Bam HI site of MThGH III thereby replacing the growth hormone sequences with the $sPLA_2$ gene. For microinjection into embryos, a 3594 bp DNA fragment encompassing the metallothionein promoter-$sPLA_2$ fusion gene was excised from this plasmid (pMt-$sPLA_2$) by digestion with Eco RI and Nde I and purified free of vector sequences.

Transgenic mice were developed and identified using established methods (Fox and Solter, 1988; Hogan et al., 1986). Northern blot analyses were carried out on total RNA extracted from selected tissues as described (Chomczynski and Sacchi, 1987). Purified RNA was electrophoresed on an 18% formaldehyde/1.5% agarose gel and blotted onto nitrocellulose. Blots were probed with a ^{32}P-labeled DNA fragment (exons 3–5) generated by PCR (Schowalter and Sommer, 1989) using the Mt-$sPLA_2$ construct as template. Where $ZnSO_4$ treatment was used, the concentration of $ZnSO_4$ was gradually brought to 150 mM in the drinking water using a dosing regime of 75 mM for 1 week, 100 mM the second week and 150 mM thereafter.

Table 1
Circulating PLA_2 levels in transgenic mice

Founder mice			
Male	PLA_2 (ng/ml)	Female	PLA_2 (ng/ml)
2590	1.85	2585	7.1
2591	0.51	2589	7.5
2592	86	2608	17
2601	346.4	2619	0.77
2610	55		
2611	0.48		

Stable lineages					
Line	PLA_2 (*E. coli*) (ng/ml)	PLA_2 (ELISA) (ng/ml)	(*n*)	PLA_2^{Zn} (ELISA) (ng/ml)	(*n*)
2585	4.3±0.4	4.1±0.5	(14)	94.6±11.9	9
2589	2.2±0.4	2.8±0.6	(6)	53 ±14.7	3
2608a	1.8±0.3	1 ±0.2	(9)	172.7±10.3	60
2608b	2 ±0.8	2.3±1.0	(3)	8.8± 1.5	3
Control	0.5±0.06	ND	(5)	ND	

PLA_2 catalytic activity was measured in the plasma or serum of transgenic founders, progeny and control C57BL/6J mice. PLA_2 protein levels were also quantitated in lineage progeny for comparison with levels detected by catalytic activity (assayed in the same sample). $sPLA_2$ protein levels were also quantitated in animals treated with 150 mM $ZnSO_4$ in the drinking water for ≥1 month (values under PLA_2^{Zn}). Levels are expressed as ng/ml ± S.E.M. ND: none detected. All founders except 2591 and 2611 had elevated PLA_2 activity. Founders 2590 (infertile), 2591 and 2611 were not characterized further. Founder 2601 died prematurely of causes unrelated to the transgene. Founder 2619 did not transmit the transgene to its progeny.

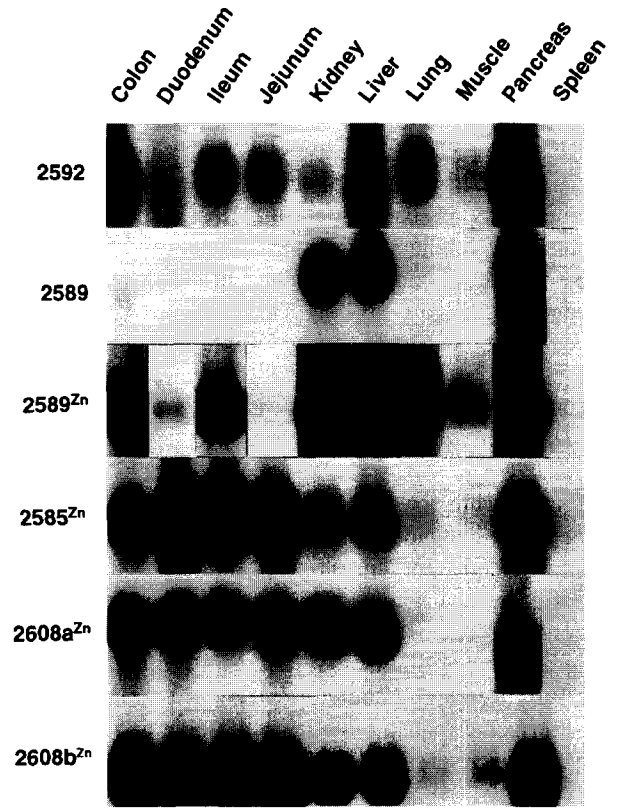

Fig. 1. Northern Blot analyses. Varying levels of the expected 1 kb transcript were detected in all tissues from founder animal 2592 and representative $ZnSO_4$-treated (Zn) or non-treated F1 progeny from the transgenic lines indicated.

2.2. PLA_2 catalytic and ELISA assays

PLA_2 catalytic activity was assayed in plasma or serum prepared from retro-orbital blood by an *E. coli* membrane assay essentially as described (Kramer et al., 1989). The amount of enzyme present was calculated from a standard curve generated using purified recombinant human $sPLA_2$ (Lilly Research Laboratories) in the assay and is expressed as ng/ml ± S.E.M. PLA_2 protein levels were quantitated by an ELISA capture assay essentially as described (Smith et al., 1992) utilizing a mouse monoclonal anti-human $sPLA_2$ antibody D3 (Lilly Research Laboratories) as the capture antibody and a second horse radish peroxidase-conjugated monoclonal anti-human $sPLA_2$ antibody (antibody C2; Lilly Research Laboratories) as the detection reagent. The amount of enzyme present was calculated from a standard curve generated using purified recombinant human $sPLA_2$ in the assay and is expressed as ng/ml ± S.E.M.

2.3. Drug testing

Mice were bled retro-orbitally prior to drug or vehicle administration and 30 min, 2 and 4 h thereafter. Com-

pounds were administered as an intravenous bolus via the tail vein in a volume of 0.15 ml (vehicle: 5% dimethylsulfoxide, 5% ethanol and 30% polyethylene glycol 300 in H_2O). Three to six mice were used for each dose. For these studies, PLA_2 catalytic activity in the serum was assayed with a modified phosphatidylcholine/deoxycholine mixed micelle assay (Schadlich et al., 1987) utilizing 3 mM sodium deoxycholate and 1 mM 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine. Values graphed represent the percentage change (±S.E.M.) in PLA_2 activity from the time zero (pre-treated) serum sample collected for each mouse.

2.4. Histology and immunofluorescence

Tissues were fixed in formalin, paraffin embedded and stained with hematoxylin (H) and eosin (E) using conventional methods for histological analyses. Indirect immunofluoresence was carried out on 6 μm frozen tissue sections as previously described (Fox and Solter, 1988) using a rabbit anti-human $sPLA_2$ antiserum (1 : 800 dilution, Lilly Research Laboratories). No cross-reactivity with non-transgenic mouse tissues was observed with this antibody.

3. Results

Transgenic mice were generated by microinjection of a hybrid mouse metallothionein promoter-human secretory PLA_2 minigene (Mt-$sPLA_2$) into C57BL/6J strain embryos (Table 1) which are devoid of endogenous $sPLA_2$ due to a mutation in their own $sPLA_2$ gene (Kennedy et al., 1995; MacPhee et al., 1995). Six male and four female transgenic founder mice were identified by PCR and Southern blot analyses. Eight of these animals had elevated PLA_2 activity in their blood as determined using an *E. coli* membrane assay (Table 1) and were therefore bred. Progeny could not be generated from transgene-expressing male founders due to fertility problems (see below), however, progeny were obtained from three female founders, 2585, 2589 and 2608. Southern analyses (not shown) of the offspring of founder 2608 revealed two transgene integrations which segregated in the germline generating genotypes 2608[a] and 2608[b]. Hence, four stable lineages, 2585, 2589, 2608[a] and 2608[b], were established from three female founders.

Extensive Northern blot and immunofluoresence analyses of transgene expression were carried out on tissues from animals in each stable line and several founders that

Table 2
Immunofluoresence localization of transgenic $sPLA_2$

Tissue	Cell type [a]	Founders		Stable lines [b]			
		2592	2610	2585	2589	2608a	2608b
Liver	Hepatocytes	++	+	±	+	+	+
	Sinusoids	++	+	±	+	+	+
Kidney	Proximal tubule	±	−	+	+	+	+
	Glomeruli	++	+	−	−	−	−
	Interstitium	+	+	−	−	−	−
Lung	Bronchial epithelium	+	−	−	−	−	NT
	Lamina propria	+	+	±	−	±	NT
CNS	White matter	+++	+++	−	−	−	−
	Gray matter	±	±	±	−	+	±
Peripheral nerve axons		++	++	+	±	+	±
GI	Gastric mucosa	+++	+	+	++	+	±
	Intestinal mucosa	++	−	+++	+	++	++
	Colonic mucosa	±	−	++	+	+	±
Skeletal muscle		−	−	−	−	−	±
Splenic reticulum		±	±	±	±	±	−
Pancreas	Acini	++++	++++	++++	++++	++++	+++
	Islets	±	±	−	−	±	−
Skin	Dermis and adnexa	+	±	−	±	−	−
Chondrocytes		++++	++++	++++	++++	++++	+++
Adipose (white and brown)		−	−	−	−	−	−
Salivary glands	Serous acini	+++	++	+	+	±	−
Prostate	Epithelium	+++	++++	++++	+++	+++	++
Testes	Interstitium	±	+	−	−	−	−
	Seminiferous tubules	++++	+++	++	++	++	++
Epididymal epithelium		+++	++	++	±	+	++
Bone marrow		−	−	−	−	−	−
Red and white blood cells		−	−	−	−	−	−

(−) Negative; (±) weakly positive; (+) positive; (++) moderately strong; (+++) strong; (++++) very strong. [a] Immunofluoresence was performed as described in Methods. Cell types normally found within a tissue which are not listed were negative. [b] 150 mM $ZnSO_4$ treated for ≥ 1 month.

were killed (Fig. 1 and Table 2). $ZnSO_4$ was added to the drinking water of some stable lineage mice to boost transgene expression (Palmiter and Brinster, 1986) and maximize the opportunity for pathology to develop. Expression of the predicted one kb transgene mRNA transcript was widespread by Northern analyses in the tissues of all transgenic mice and was induced by $ZnSO_4$ (Fig. 1). Immunofluoresence analyses demonstrated that $sPLA_2$ protein was also widely expressed and revealed a cell-type-specific pattern of expression (Table 2 and Fig. 2).

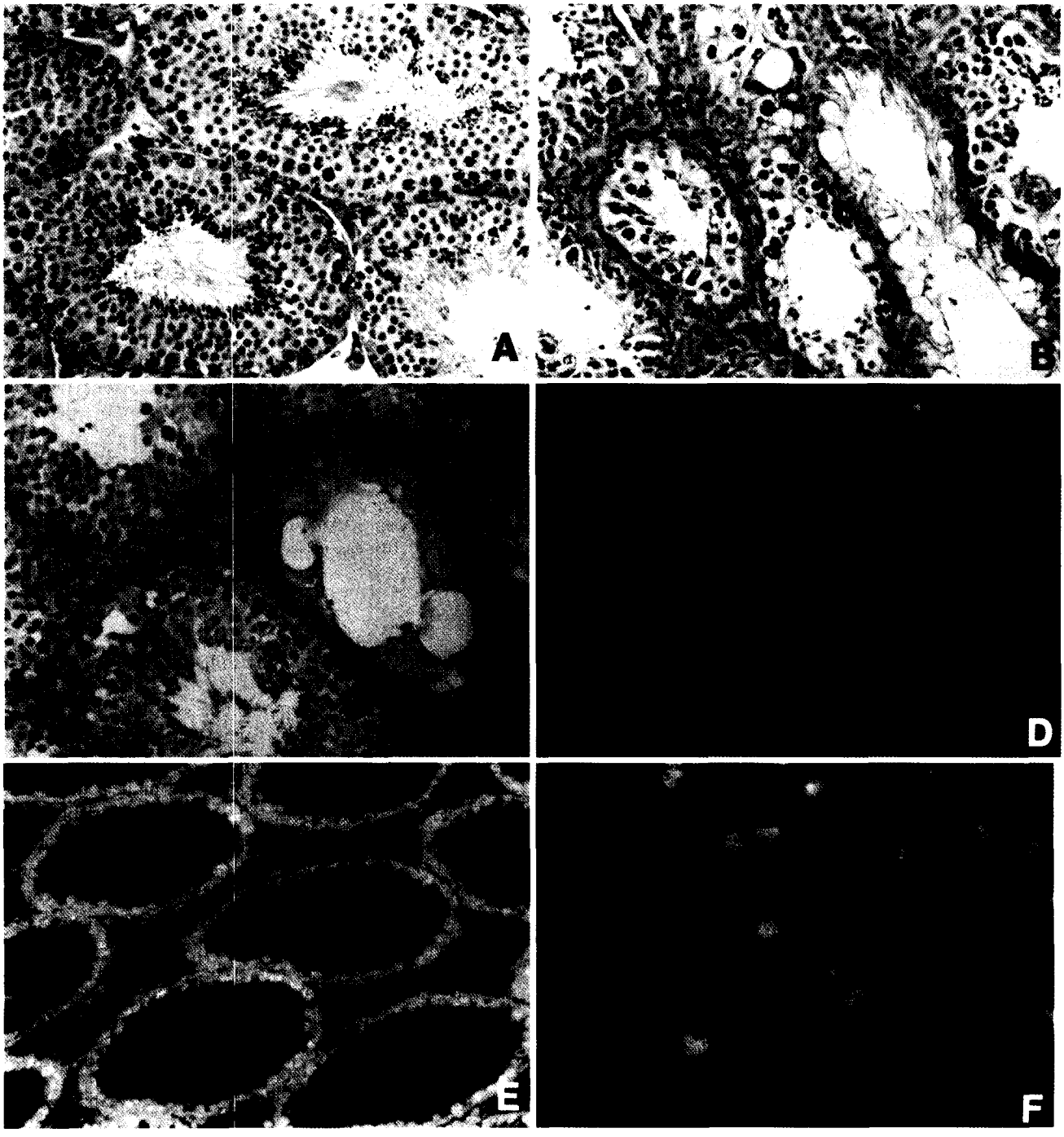

Fig. 2. Histopathology and immunofluoresence localization of human $sPLA_2$ in the testis. (A) H & E stained section of non-transgenic mouse testis: normal morphology and spermatogenesis (×50). (B) H & E stained section of founder 2592 testis: seminiferous tubules are severly hypospermatogenic and are lined largely by sertoli cells (×50). Identical changes were seen in the testes of founder 2610 (not shown). (C) H & E stained section of line 2585 progeny testis: spermatogenic epithelium is intact, though many tubules are hypospermatogenic; some tubules are depleted of spermatogenic cells (×50). (D) Immunofluoresence on non-transgenic testis: no reactivity with anti-hu-$sPLA_2$ antiserum is present (×50). (E) Immunofluoresence on 2592 testis: strong reactivity is seen along the seminiferous epithelium (×50). (F) Immunofluoresence on 2585 progeny testis: moderate focal reactivity is present within the seminiferous epithelium (×50).

No endogenous mouse mRNAs or proteins cross reacted in any tissues with the probes and conditions used here. Basal PLA_2 catalytic activity was elevated in the blood of all stable lineage transgenics and was greatly induced by $ZnSO_4$ treatment (Table 1). The inducibility varied between the lines (range of 4 to > 170-fold) and was greatest in line 2608[a]. Circulating enzyme activity closely paralleled the levels of $sPLA_2$ protein detected by ELISA assay indicating that the catalytic activity of the circulating $sPLA_2$ was not being suppressed by host factors (Table 1).

With the exception of two founder animals, all of the transgenic mice have remained healthy for a period exceeding one year. Founders 2592 and 2610 became ill at 6 and 11 months of age, respectively, with symptoms of anorexia, lethargy, mild tremors and ataxia. However, while histological examination revealed testicular abnormalities in these mice (see below), neither animal exhibited classical systemic or local inflammatory lesions nor was any other life-threatening pathology detected. Notably, relatively high levels of $sPLA_2$ were present in the CNS white matter, peripheral axons and kidneys of these founders (Table 2) and may have provoked a metabolic or other undetected pathophysiological derangement. Histopathological assessments performed on transgenic mice from each of the stable lineages also did not reveal any local/systemic inflammatory changes or other significant pathology outside the testes (see below) in any mice.

One apparent pathophysiological effect of the overexpressed $sPLA_2$ was male infertility. This occurred immediately after puberty in all transgenic male mice that expressed the transgene (without $ZnSO_4$ treatment) but presented differently (histologically) in the founders versus the stable lines. The seminiferous tubules of the testes were severely hypospermic in founders 2592 and 2610 (Fig. 2B). Immunofluoresence analyses revealed high levels of transgene expression in the testis/reproductive tract in both animals (Fig. 2 and Table 2). However, in the stable lineage transgenics, the amount of $sPLA_2$ expressed in the testes was much lower than in the founders (Fig. 2F) and the hypospermatogenesis was much less severe (Fig. 2C). Thus, the degree of hypospermia in the testes appeared to correlate with local levels of $sPLA_2$. Transgenic $sPLA_2$ was not detected in the female reproductive organs (not shown) and all female founders and progeny appear to reproduce and transmit the transgene normally.

An important goal and achievement of this work was to engineer a small animal to express high levels of human $sPLA_2$ in the circulation thereby modeling the situation in patients undergoing a systemic inflammatory response. While not exhibiting signs of inflammation in their current state (see below), these mice have nevertheless proven very useful for the in vivo evaluation of $sPLA_2$ inhibitors. Candidate inhibitors identified by in vitro screening by our group have been tested for their ability to reduce the serum PLA_2 activity in Mt-$sPLA_2$ transgenic mice. Results for one of our most promising inhibitors, 3-[3-acetamide-1-benzyl-2-ethylindolyl-5-oxy]propane phosphonic acid (LY311727), are shown in Fig. 3A. LY311727 significantly and dose dependently suppressed the PLA_2 activity in the serum of Mt-$sPLA_2$ transgenic mice following the intravenous (i.v.) administration of the drug. Inhibition was > 80% over the course of 4 h at the highest dose tested (30 mg/kg). To rule out the possibility that this was a non-specific effect of the compound, the inhibitory activity of LY311727 was compared with a close structural analog, 3-[3-acetamide-1-benzyl-2-propylindolyl-5-oxy]propane phosphonic acid (LY314024) (Fig. 3B), which is ~ 250-fold less active than LY311727 in in vitro biochemical assays (Schevitz et al., 1995). When compared at a dose of 10 mg/kg (Fig. 3B), LY311727 inhibited sPLA2 activity > 80% at 30 min while LY314024 was no more effective than vehicle alone (Fig. 3B).

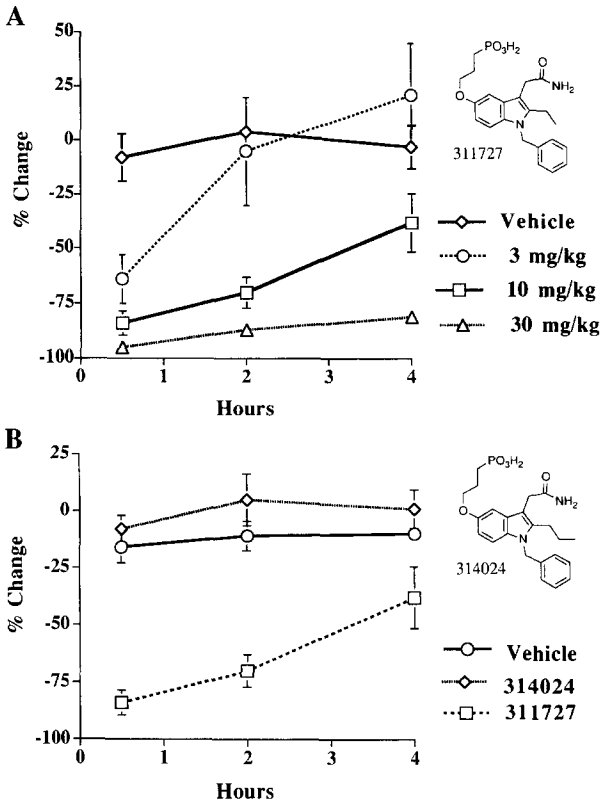

Fig. 3. Inhibition of circulating human $sPLA_2$ catalytic activity by enzyme inhibitors. (A) Mice treated with 3, 10 or 30 mg/kg of compound 311727. (B) Mice treated with 10 mg/kg of compound 311727 or 314024.

4. Discussion

A provocative question regarding $sPLA_2$ is whether this enzyme initiates, promotes or has little role in inflammation. The impact of our results on this question should be considered in light of our current understanding of $sPLA_2$ pathobiology. In the in vitro and in vivo studies

where it has been possible to elicit a reaction to $sPLA_2$, some type of prior cell/tissue stimulation or perturbation has often been required to obtain a response. Thus, enhanced prostaglandin liberation in $sPLA_2$ treated granulocytes, mast cells or endothelial cells was only observed following presensitization of the cells (see Introduction). Similarly, $sPLA_2$ *exacerbated* the foot pad edema caused by adjuvant arthritis (Murakami et al., 1990) or poly-L-arginine arthritis (Cirino et al., 1994a) in the rat but had no effect in normal rats or on carrageenan-induced edema (Murakami et al., 1990). These and other observations therefore suggest a synergistic interaction between $sPLA_2$ and other, possibly tissue/disease specific, inflammatory/pathogenic factors. Such factors may act to perturb the membrane in such a way as to expose phospholipid substrate on the cell surface (Murakami et al., 1991, 1993a; Pruzanski et al., 1993; Vadas et al., 1993). Fourcade et al. (1995) have recently shown, for example, that treatment of cells with pathogenic mediators such as lipopolysaccharide or *S. aureus* α-toxin induces the shedding of microvesicals bearing relevant phospholipid substrates for $sPLA_2$ on the outer membrane surface. In light of these findings it may be concluded that $sPLA_2$ activity acts primarily to *exacerbate* rather then initiate inflammation.

The fact that spontaneous pathology in our $sPLA_2$ transgenics was very limited i.e., confined to the testes, is consistent with the idea that $sPLA_2$ is not a major *initiator* of disease. Notably, we have observed levels of $sPLA_2$ as high as 300 ng/ml in the circulation of subsets of transgenics treated with $ZnSO_4$ (M. Song and N. Fox unpublished results). These levels are in the range observed in patients undergoing a systemic inflammatory response to sepsis (Nevalainen et al., 1992; Rintala and Nevalainen, 1993) yet do not evoke a systemic response in our mice. Unlike our transgenics, however, these patients typically suffer from concomitant clinical disorders which may predispose them to the actions of $sPLA_2$ as discussed above. Moreover, the most severely ill sepsis patients often have μg/ml levels of $sPLA_2$ in the circulation (Vadas et al., 1992). Hence, we cannot exclude the possibility that had we achieved still higher levels of $sPLA_2$ expression in these transgenic mice, we would have observed more spontaneous pathology.

Also worth noting are suggestions that some of the effects of $sPLA_2$ may not be mediated through its catalytic activity per se. $sPLA_2$ is a highly basic protein and readily associates with the cell surface via heparin sulfated proteoglycans (Murakami et al., 1989, 1993a,b; Suga et al., 1993). Hence, it has been speculated that some of $sPLA_2$'s pathophysiological properties may be due to the membrane-altering effects exhibited by such cationic proteins (Cirino et al., 1994a; Lomonte et al., 1995). A cell surface receptor for the enzyme has also been reported (Lambeau et al., 1994) and may be involved in cellular activation. Though speculative, a mode of action could be proposed wherein $sPLA_2$ amplifies its own hydrolytic effects by stimulating the transversion/transmigration of phospholipid substrate to the cell surface for catalysis through these surface interactions. In this scenario, $sPLA_2$ could act to both initiate and promote pathology as might occur in situations where the systemic or local levels of $sPLA_2$ are especially high. This could be the case, for example, in the testes of our transgenics where the severity of the morphologic changes correlated with the levels of $sPLA_2$. Hence, localized elevations of $sPLA_2$ alone may be sufficient to invoke a pathological response in some tissues.

In order to fully understand the pathobiology of $sPLA_2$ it will be important to examine the sensitivity of our $sPLA_2$ transgenics to challenge by various biological mediators such as endotoxin, tumor necrosis factor, interleukin-1 etc., which might collaborate with $sPLA_2$ in disease. Though the $sPLA_2$ transgene in these animals is driven by a foreign promoter, the expression pattern is very similar to that of the native $sPLA_2$ gene in man (see Introduction and Table 2). Basal levels of circulating $sPLA_2$ in the transgenics are very similar to those in man (Nevalainen et al., 1992; Vadas et al., 1992; Rintala and Nevalainen, 1993) and are not confounded by endogenous mouse $sPLA_2$ expression on the $sPLA_2$-deficient C57BL/6 mouse background used to make the mice (Kennedy et al., 1995; MacPhee et al., 1995). Moreover, the induciblity of the metallothionein gene promoter by both heavy metals and inflammatory mediators provides a means for mimicking the induction of the native human gene in sepsis and inflammation (Palmiter et al., 1983; Durnam et al., 1984; Palmiter and Brinster, 1986). Also noteworthy is the fact that these animals express $sPLA_2$ in tissues which may be relevant to the pathobiology of the arthridities and pancreatitis, i.e., chondrocytes and pancreatic acinar cells as well as in the gastrointestinal tract where $sPLA_2$ has been implicated as a tumor suppresser (see MacPhee et al., 1995). Hence, multiple opportunities for disease exploration exist in these animals.

The discovery by Kennedy et al. (1995) and MacPhee et al. (1995) that $sPLA_2$ is not essential for normal reproduction is intriguing in light of historical data implicating the eicosanoids and phospholipase A_2s in both normal and abnormal reproductive physiology. Seminal plasma is normally a rich source of PLA_2 enzyme activity and prostaglandins (Bergstrom et al., 1968; Kunze et al., 1974; Ronkko and Rasanen, 1992) and the group II nonpancreatic $sPLA_2$ enzyme has been specifically identified in the seminal plasma in man (Takayama et al., 1991). Abnormal levels (high or low) of testicular or seminal plasma prostaglandins and/or PLA_2 activity have been associated with impaired male reproductive functions (Tso and Lacy, 1975; Conte et al., 1985; Ronkko and Rasanen, 1992). While not essential for normal reproductive function in all species, it remains possible that $sPLA_2$ hyperphospholipasemia or hyperphospholipasemia due to other PLA_2s in the reproductive system play a role in the pathogenesis of infertility and reproductive disease. In-

deed, our results suggest that hyperphospholipasemia in the testes was the primary cause of infertility in our transgenics though abnormalities in sperm transport and/or sperm or semen physiology have not been ruled out. Hence, additional study of the $sPLA_2$-mediated infertility in Mt-$sPLA_2$ transgenics as a specific or surrogate model for other PLA_2s may shed further light on the role of the PLA_2s and eicosanoids in reproductive pathophysiology. Newly developed inhibitors of $sPLA_2$ catalysis (see below) may also be employed to establish whether the catalytic activity of $sPLA_2$ is causally linked to this infertility.

The current study additionally demonstrates the utility of genetically engineered small animals in the development of novel pharmacologic agents to treat inflammation. We have used this model to evaluate the in vivo potency of lead $sPLA_2$ inhibitors selected by broad screening and structure based drug design. These transgenics have allowed us to rapidly and easily select compounds with the greatest probability of success in vivo from lead compounds having very similar in vitro potencies. To date, these efforts have culminated in the development of LY311727, the most potent $sPLA_2$ inhibitor yet described (Schevitz et al., 1995 and present report). Our studies provide powerful in vivo results demonstrating that LY311727 inhibits $sPLA_2$ through its specific interaction with the catalytic domain of $sPLA_2$ as opposed to some non-specific effect. Future studies employing the current transgenic model and LY311727 will no doubt yield the final word on $sPLA_2$'s role in inflammation.

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References

Bomalaski, J.S., P. Lawton and J.L. Browning, 1991, Human extracellular recombinant phospholipase A_2 induces an inflammatory response in rabbit joints, J. Immunol. 146, 3904.

Bergstrom, S., L.A. Carlson and J.R. Weeks, 1968, The prostaglandins: a family of biologically active lipids, Pharmacol. Rev. 20, 1.

Bonventre, J.V., 1992, Phospholipase A_2 and signal transduction, J. Am. Soc. Nephrol. 3, 128.

Chomczynski, P. and N. Sacchi, 1987, Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162, 156.

Cirino, G., C. Cicala and L. Sorrentino, 1994a, Human recombinant platelet phospholipase A_2 exacerbates poly-L-arginine induced rat paw edema, Inflammation 18, 59.

Cirino, G., C. Cicala and L. Sorrentino, F.M. Maiello and J.L. Browning, 1994b, Recombinant secreted nonpancreatic phospholipase A_2 induces a synovitis-like inflammation in the rat air pouch, J. Rheumatol. 12, 824.

Conte, D., M. Nordio, F. Romanelli, F. Manganelli, P. Giovenco, F. Dondero and A.J. Isidori, 1985, Role of seminal prostaglandins in male fertility. II Effects of prostaglandin synthesis inhibition on spermatogenesis in man, Endocrinol. Invest. 8, 289.

Crowl, R.M., T.J. Stoller, R.R. Conroy and C.R. Stoner, 1991, Induction of phospholipase A_2 gene expression in human hepatoma cells by mediators of the acute phase response, J. Biol. Chem. 266, 2647.

Davidson, F.F. and E.A. Dennis, 1990, Evolutionary relationships and implications for the regulation of phospholipase A_2 from snake venom to human secreted forms, J. Mol. Evol. 31, 228.

Dennis, E.A., 1987, Regulation of eicosanoid production: role of phospholipase and inhibitors, Biotechnology 5, 1294.

Durnam, D.M., J.S. Hoffman, C.J. Quaife, E.P. Benditt, H.Y. Chen, R.L. Brinster and R.D. Palmiter, 1984, Induction of mouse metallothionein-I mRNA by bacterial endotoxin is independent of metals and glucocorticoid hormones, Proc. Natl. Acad. Sci. USA 81, 1053.

Fink, M.P., 1993, Phospholipases A_2: potential mediators of the systemic inflammatory response syndrome and the multiple organ dysfunction syndrome, Crit. Care Med. 21, 957.

Fourcade, O., M.-F. Simon, C. Viodé, N. Rugani, F.L.A. Ragab, B. Fournié, L. Sarda and H. Chap, 1995, Secretory phospholipase A_2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells, Cell 80, 919.

Fox, N. and D. Solter, 1988, Expression and regulation of the pituitary- and placenta-specific human glycoprotein hormone alpha-subunit gene is restricted to the pituitary in transgenic mice, Mol. Cell. Biol. 8, 5470.

Green, J.A., G.M. Smith, R. Buchta, R. Lee, K.Y. Ho, I.A. Rajkovic and K.F. Scott, 1991, Circulating phospholipase A_2 activity associated with sepsis and septic shock is indistinguishable from that associated with rheumatoid arthritis, Inflammation 15, 355.

Grönroos, J.M. and T.J. Nevalainen, 1992, Increased concentrations of synovial-type phospholipase A_2 in serum and pulmonary and renal complications in acute pancreatitis, Digestion 52, 232.

Hanahan, D.J., 1986, Platelet activating factor: a biologically active phosphoglyceride, Ann. Rev. Biochem. 55, 483.

Hara, S., I. Kudo and K. Inoue, 1991, Augmentation of prostaglandin E_2 production by mammalian phospholipase A_2 added exogenously, J. Biochem. 110, 163.

Harwig, S.S.L., L. Tan, X.D. Qu, Y. Cho, P.B. Eisenhauer and R.I. Lehrer, 1995, Bactericidal properties of murine intestinal phospholipase A_2, J. Clin. Invest. 95, 603.

Hogan, B., F. Costantini and E. Lacy, 1986, Manipulating the Mouse Embryo: A Laboratory Manual (Cold Spring Harbor Laboratory, New York).

Irvine, R.F., 1982, How is the level of free arachidonic acid controlled in mammalian cells?, Biochem. J. 204, 3.

Kennedy, B.P., P. Payette, J. Mudgett, P. Vadas, W. Pruzanski, M. Kwan, C. Tang, D.E. Rancourt and W.A. Cromlish, 1995, A natural disruption of the secretory group II phospholipase A_2 gene in inbred mouse strains, J. Biol. Chem. 270, 22378.

Kramer, R.M., C. Hession, B. Johansen, G. Hayes, P. McGray, E.P. Chow, R. Tizard and R.B. Pepinsky, 1989, Structure and properties of a human non-pancreatic phospholipase A_2, J. Biol. Chem. 264, 5768.

Kunze, H., N. Nahas and M. Wurl, 1974, Phospholipases in human seminal plasma, Biochim. Biophys. Acta 348, 35.

Lambeau, G., P. Ancian, J. Barhanin and M. Lazdunski, 1994, Cloning and expression of a membrane receptor for secretory phospholipases A_2, J. Biol. Chem. 269, 1575.

Lomonte, B., A. Tarkowski and L.Å. Hanson, 1995, Phospholipase A_2 and inflamation, Mol. Med. Today Vol. 1, 9.

Lyons-Giordano, B., G.L. Davis, W. Galbraith, M.A. Pratta and E.C. Arner, 1989, Interleukin-1b stimulates phospholipase A_2 mRNA synthesis in rabbit articular chondrocytes, Biochem. Biophys. Res. Commun. 164, 488.

MacPhee, M., K.P. Chepenik, R.A. Liddell, K.K. Nelson, L.D. Siracusa and A.M. Buchberg, 1995, The secretory phospholipase A_2 gene is a

candidate for the Mom1 locus, a major modifier of Apc^{Min}-induced intestinal neoplasia, Cell 81, 957.

Marshall, L. and A. Roshak, 1993, Co-existence of two biochemically distinct phospholipase A_2 activities in human platelet, monocyte and neutrophil, Biochem. Cell Biol. 71, 1993.

Minami, T., H. Tojo, Y. Shinomura, Y. Matsuzawa and M. Okamoto, 1994, Increased group II phospholipase A_2 in colonic mucosa of patients with Crohn's disease and ulcerative colitis, Gut 35, 1593.

Morgan, D.W., C.M. Anderson and K.P. Meyers, 1993, Recombinant human type II phospholipase A_2 lacks edema producing activity in rat, Eur. J. Pharmacol. 235, 45.

Murakami, M., I. Kudo and K. Inoue, 1989, In vivo release and clearance of rat platelet phospholipase A_2, Biochim. Biophys. Acta 1005, 270.

Murakami, M., I. Kudo, H. Nakamura, Y. Yokoyama, H. Mori and K. Inoue, 1990, Exacerbation of rat adjuvant arthritis by intradermal injection of purified mammalian 14-kDa group II phospholipase A_2, FEBS Lett. 268, 113.

Murakami, M., I. Kudo and K. Inoue, 1991, Eicosanoid generation from antigen-primed mast cells by extracellular mammalian 14-kDa group II phospholipase A_2, FEBS Lett. 294, 247.

Murakami, M., N. Hara, I.K. and K. Inoue, 1993a, Triggering of degranulation in mast cells by exogenous type II phospholipase A_2, J. Immunol. 151, 5675.

Murakami, M., I. Kudo and K. Inoue, 1993b, Molecular nature of phospholipases A_2 involved in prostaglandin I_2 synthesis in human umbilical vein endothelial cells, J. Biol. Chem. 268, 839.

Nakano, T., O. Ohara, H. Teraoka and H. Arita, 1990, Group II phospholipase A_2 mRNA synthesis is stimulated by two distinct mechanisms in rat vascular smooth muscle cells, FEBS Lett. 261, 171.

Nevalainen, T.J. and T.J. Haapanen, 1993, Distribution of pancreatic (group I) and synovial-type (group II) phospholipases A_2 in human tissues, Inflammation 17, 453.

Nevalainen, T.J., P.T. Kortesuo, E. Rintala and F. Märki, 1992, Immunochemical detection of group I and group II phospholipases A_2 in human serum, Clin. Chem. 38, 1824.

Nevalainen, T.J., J.M. Grönroos and P.T. Kortesuo, 1993a, Pancreatic and synovial type phospholipases A_2 in serum samples from patients with severe acute pancreatitis. Gut 34, 1133.

Nevalainen, T.J., K.-M. Meri and M. Niemi, 1993b, Synovial-type (group II) phospholipase A_2 human seminal plasma, Andrologia 25, 355.

Nevalainen, T.J., H.J. Aho and H Peuravuori, 1994, Secretion of group 2 phospholipase A_2 by lacrimal glands, Invest. Ophthalmol. Visual Sci. 35, 417.

Palmiter, R.D., G. Norstedt, R.E. Gelinas, R.E. Hammer and R.L. Brinster, 1983, Metallothionein-human GH fusion genes stimulate growth of mice, Science 222, 809.

Palmiter, R.D. and R.L. Brinster, 1986, Germ-line transformation of mice, Annu. Rev. Genet. 20, 465.

Pfeilschifter, J., C. Schalkwijk, V.A. Briner and H. Van den Bosch, 1993, Cytokine-stimulated secretion of group II phospholipase A_2 by rat mesangial cells, J. Clin. Invest. 92, 2516.

Pruzanski, W., P. Vadas and J. Browning, 1993, Secretory non-pancreatic group II phospholipase A_2: role in physiologic and inflammatory processes, J. Lipid Mediat. 8, 161.

Pruzanski, W., K. Albin-Cook, R.M. Laxer, J. MacMillan, E. Stefanski, P. Vadas and E.D. Silverman, 1994, Phospholipase A_2 in juvenile rheumatoid arthritis: correlation to disease type and activity, J. Rheumatol. 21, 1951.

Rintala, E.M. and T.J. Nevalainen, 1993, Group II phospholipase A_2 in sera of febrile patients with microbiologically or clinically documented infections, Clin. Infect. Dis. 17, 864.

Ronkko, S. and M. Rasanen, 1992, Studies on phospholipase A_2 in human seminal plasma, Int. J. Biochem. 24, 987.

Schadlich, H.R., M. Buchler and H. G. Beger, 1987, Improved method for the determination of phospholipase A_2 catalytic activity concentration in human serum and ascitics, J. Clin. Chem. Clin. Biochem. 25, 505.

Schevitz, R.W., N.J. Bach, D.G. Carlson, N.Y. Chirgadze, D.K. Clawson, R.D. Dillard, S.E. Draheim, L.W. Hartley, N.D. Jones, E.D. Mihelich, J.L. Olkowski, D.W. Snyder, C. Sommers and J.-P. Wery, 1995, Structure-based design of the first potent and selective inhibitor of human non-pancreatic secretory phospholipase A_2, Nature Struct. Biol. 2, 458.

Schowalter, D.B. and S.S. Sommer, 1989, The generation of radiolabeled DNA and RNA probes with polymerase chain reaction, Anal. Biochem. 177, 90.

Smith, G.M., R.L. Ward, L. McGuigan, I.A. Rajkovic and K.F. Scott, 1992, Measurement of human phospholipase A_2 in arthritis plasma using a newly developed sandwich elisa, Br. J. Rheumatol. 31, 175.

Snyder, D.W., C.D. Sommers, J.L. Bobbitt and E.D. Mihelich, 1993, Characterization of the contractile effects of human recombinant nonpancreatic secretory phospholipase A_2 (PLA_2) and other PLA_2s on guinea pig lung pleural strips, J. Pharmacol. Exp. Ther. 266, 1147.

Stafford, R.E. and E.A. Dennis, 1988, Lysophospholipids and biosurfactants, Colloids Surf. 30, 47.

Stoner, C.R., L.M. Reik, M. Donohue, W. Levin and R.M. Crowl, 1991, Human group II phospholipase A_2. Characterization of monoclonal antibodies and immunochemical quantitation of the protein in synovial fluid, J. Immunol. Methods 145, 127.

Suga, H., M. Makoto, I. Kudo and K. Inoue, 1993, Participation in cellular prostaglandin synthesis of type-II phospholipasse A_2 secreted and anchored on cell-surface heparan sulfate proteoglycan, Eur. J. Biochem. 218, 807.

Takayama, K., H. Shuntaro, I. Kudo and K. Inoue, 1991, Detection of 14-kDa group II phospholipase A_2 in human seminal plasma, Biochem. Biophys. Res. Commun. 178, 1505.

Tso, E.C.-F. and D.J. Lacy, 1975, Effects of prostagladin $F_{2\alpha}$ on the reproductive system of the male rat, Reprod. Fert. 44, 545.

Vadas, P. and J.B. Hay, 1983, Involvement of circulating phospholipase A_2 in the pathogenesis of the hemodynamic changes in endotoxin shock, Can. J. Physiol. Pharmacol. 61, 561.

Vadas, P., W. Pruzanski and E. Stefanski, 1988, Extracellular phospholipase A_2: causative agent in circulatory collapse of septic shock?, Agents Actions 24, 320.

Vadas, P., W. Pruzanski, J. Kim and V. Fornasier, 1989, The proinflammatory effect of intra-articular injection of soluble human and venom phospholipase A_2, Am. J. Pathol. 134, 807.

Vadas, P., K. Scott, G. Smith, I. Rajkovic, E. Stefanski, B.D. Schouten, R. Singh and W. Pruzanski, 1992, Serum phospholipase A_2 enzyme activity and immunoreactivity in a prospective analysis of patients with septic shock, Life Sci. 50, 807.

Vadas, P., J. Browning, J. Edelson and W. Pruzanski, 1993, Extracellular phospholipase A_2 expression and inflammation: the relationship with associated disease states, J. Lipid Mediat. 8, 1.

Vishwanath, B.S., A.A. Fawzy and R.C. Franson, 1988, Edema-inducing activity of phospholipase A_2 purified from human synovial fluid and inhibition by aristolochic acid, Inflammation 12, 549.

Wright, G.W., C.E. Ooi, J. Weiss and P. Elsbach, 1990, Purification of a cellular (granulocyte) and an extracellular (serum) phospholipase A_2 that participate in the destruction of *Escherichia coli* in a rabbit inflammatory exudate, J. Biol. Chem. 265, 6675.

Wery, J.P., R.W. Schevitz, D.K. Clawson, J.L. Bobbitt, E.R. Dow, G. Gamboa, T. Goodson, Jr., R.B. Hermann, R.M. Kramer, D.B. McClure, E.D. Mihelich, J.E. Putnam, J.D. Sharp, D.H. Stark, C. Teater, M.W. Warrick and N.D. Jones, 1991, Structure of recombinant human rheumatoid arthritic synovial fluid phospholipase A_2 at 2.2 Å resolution, Nature 352, 79.