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RESEARCH PAPER

Secretory PLA_2 inhibitor indoxam suppresses LDL modification and associated inflammatory responses in TNFα-stimulated human endothelial cells

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Background and purpose: Secretory phospholipase A_2 ($sPLA_2$) is implicated in atherosclerosis, although the effects of specific $sPLA_2$ inhibitors have not been studied. We investigated the effects of the indole analogue indoxam on low-density lipoprotein (LDL) modification by $sPLA_2$ enzymes of different types and on the associated inflammatory responses in human umbilical vein endothelial cells (HUVEC).
Experimental approach: LDL modification was assessed by measuring the contents of two major molecular species of lysophosphatidylcholine (LPC) using electrospray ionization-liquid chromatography/mass spectrometry. The proinflammatory activity of the modified LDL was evaluated by determining monocyte chemoattractant protein-1 (MCP-1) mRNA expression and transcriptional factor nuclear factor-kappaB (NF-κB) activity in HUVEC.
Key results: Indoxam dose-dependently inhibited palmitoyl- and stearoyl-LPC production in LDL incubated with snake venom $sPLA_2$ (IC_{50} 1.2 μM for palmitoyl-LPC, 0.8 μM for stearoyl-LPC). MCP-1 mRNA expression and NF-κB activity were enhanced by venom $sPLA_2$-treated LDL, which was completely suppressed by indoxam but not by thioetheramide-PC, a competitive $sPLA_2$ inhibitor. Indoxam also suppressed LPC production in LDL treated with human synovial type IIA $sPLA_2$. Tumour necrosis factor α (TNFα) increased type V sPLA2 expression in HUVEC. Indoxam dose-dependently suppressed LPC production in native and glycoxidized LDL treated with TNFα-stimulated HUVEC. Indoxam suppressed MCP-1 mRNA expression and NF-κB activity in TNFα-stimulated HUVEC incubated with native or glycoxidized LDL.
Conclusions and implications: Indoxam prevented $sPLA_2$-induced LPC production in native and glycoxidized LDL as well as LDL-induced inflammatory activity in HUVEC. Our results suggest that indoxam may be a potentially useful anti-atherogenic agent.
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Keywords: secretory phospholipase A_2; secretory phospholipase A_2 inhibitor; indoxam; lysophosphatidylcholine; atherosclerosis; monocyte chemoattractant protein-1; nuclear factor-κB; human umbilical vein endothelial cells; low-density lipoprotein; tumour-necrosis factor α

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cell; LDL, low-density lipoprotein; LPC, lysophosphatidylcholine; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor-kappa B; PC, phosphatidylcholine; $sPLA_2$, secretory PLA_2; TNFα, tumour-necrosis factor α

Introduction

Phospholipase A_2 (PLA_2) catalyses the hydrolysis of the *sn*-2 ester bond of phospholipids to produce free fatty acids and lysophospholipid. PLA_2 belongs to a large superfamily that can be divided into four categories: secretory PLA_2 ($sPLA_2$), cytosolic PLA_2, Ca^{2+}-independent PLA_2 and lipoprotein-associated PLA_2 (platelet-activating factor acetylhydrolase) (Six and Dennis, 2000; Kudo and Murakami, 2002). $sPLA_2$ is a small disulphide-rich molecule requiring millimolar Ca^{2+} concentrations for its activity. There are a large number of isoenzymes, currently 10 in humans, with various reported activities such as production of lipid mediators contributing to inflammation or tumorigenesis, fertilization, bacterial defence and phospholipid digestion in the gastrointestinal tract (Kudo and Murakami, 2002; Murakami and Kudo,

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2004). These enzymes are associated with a number of diseases, including acute pancreatitis, sepsis, rheumatoid arthritis, inflammatory bowel disease, bronchial asthma and adult respiratory distress syndrome, in which $sPLA_2$ might act to degrade pulmonary surfactant phospholipids.

Secretory PLA_2 has also been implicated in atherosclerosis (Hurt-Camejo *et al*., 2001; Murakami and Kudo, 2003), based on immunohistochemical staining for $sPLA_2$ type IIA, V and X in atherosclerotic tissue (Hurt-Camejo *et al*., 1997; Hanasaki *et al*., 2002; Rosengren *et al*., 2006). The effects of $sPLA_2$ enzymes in the development of atherosclerosis are mainly exerted through their actions on low-density lipoprotein (LDL). First, phosphatidylcholine (PC) is the most abundant phospholipid present in LDL particles, and hydrolysis of PC by $sPLA_2$ yields lysophosphatidylcholine (LPC), which has proatherogenic and proinflammatory effects on arterial wall cells, including the upregulation of adhesive molecules, monocyte chemoattractant protein-1 (MCP-1), growth factors, cell proliferation, cell migration, apoptosis, activation of protein kinase C and inhibition of endothelium-dependent relaxation (Hurt-Camejo *et al*., 2001; Zalewski and Macphee, 2005). Second, LDL modification by $sPLA_2$ exposes more arginine- and lysine-rich segments that bind strongly to extracellular matrix proteoglycans, resulting in increased retention of LDL particles. These are taken up by macrophages, leading to the formation of foam cells (Camejo *et al*., 1998). Third, $sPLA_2$ substantially reduces the PC content in the surface monolayer of LDL, resulting in smaller and denser LDL particles that are more susceptible to lipid peroxidation (Neuzil *et al*., 1998). In fact, transgenic mice expressing human type IIA $sPLA_2$ developed accelerated atherosclerosis with altered lipoprotein levels (Ivandic *et al*., 1999), and LDL receptor-deficient mice overexpressing human type IIA $sPLA_2$ in macrophages had large atherosclerotic lesions when placed on high-fat diet (Webb *et al*., 2003).

In light of the potentially causal role of $sPLA_2$ in lipoprotein modification, its inhibition may be beneficial in the prevention and treatment of atherosclerosis; however, the effects of specific $sPLA_2$ inhibitors on lipoprotein remain unknown. Here, we studied the inhibitory effects of indoxam, a 1-oxamoylindolizine derivative acting as a specific site-directed $sPLA_2$ inhibitor (Hagishita *et al*., 1996), on LPC production in LDL induced by venom $sPLA_2$, synovial fluid type IIA $sPLA_2$ and type V $sPLA_2$ in activated cultured human umbilical vein endothelial cells (HUVECs). We also investigated the effect of indoxam on $sPLA_2$-mediated inflammatory activity of native or glycoxidized LDL by assessing the activity of nuclear factor-kappa B (NF-κB) and MCP-1 mRNA expression in HUVECs. The results showed that indoxam prevented LDL modification and LDL-associated proinflammatory activity elicited by $sPLA_2$.

Methods

Cell culture

Human umbilical vein endothelial cells were isolated from umbilical cord veins using 0.25% trypsin (Difco Laboratories, Detroit, MI, USA) as described by Jaffe *et al*. (1973). HUVECs were grown in M199 medium supplemented with 10% foetal calf serum (FCS; Gibco BRL, Life Technologies, Rockville, MD, USA), 100 μg ml^{-1} heparin (Sigma Chemical Co., St Louis, MO, USA), 20 μg ml^{-1} endothelial cell growth supplement (Upstate Biotechnology, New York, NY, USA) and 0.33 mg ml^{-1} piperacillin sodium (Sankyo Co., Tokyo, Japan) in a humidified atmosphere containing 5% CO_2. Cells prior to the sixth passage were used. These cells were confirmed to incorporate Dil-acetylated LDL (Sonoki *et al*., 2002, 2003).

Modification of LDL by $sPLA_2$

Native LDL was isolated from human plasma using density-gradient ultracentrifugation (Vieira *et al*., 1996). Glycoxidized LDL was prepared by incubating an aliquot of native LDL with 200 mM glucose at 37 °C for 3 days and then with 1 μM $CuSO_4$ at 37 °C for 12 h after three dialyses with 4 l of phosphate-buffered saline. Native or glycoxidized LDL was dialysed against 0.15 M NaCl and 0.26 mM EDTA, pH 7.4, sterilized with a 0.45-μm-pore-size filter and then stored at 4 °C under N_2 gas until use. The protein concentration of each LDL preparation was determined by the Coomassie brilliant blue method using bovine serum albumin as a standard (Nacalai Tesque Inc., Kyoto, Japan), and LDL concentrations were adjusted to 1 mg ml^{-1}. For LDL modification by snake venom or human synovial type IIA $sPLA_2$, native LDL was concentrated and dialysed against a 1000-fold volume of 0.05 M HEPES buffer containing 1 M NaCl and 1 M NaOH, pH 7.4, by centrifugation through an Ultrafree-15 centrifugal filter (Millipore Co., Bedford, MA, USA) three times. LDL was incubated at 37 °C for 2 h with snake venom $sPLA_2$ (*Crotalus adamanteus*; Worthington Biochemical Co., Lakewood, NJ, USA) at 1 U ml^{-1} or human synovial fluid obtained from a patient with rheumatoid arthritis by arthrocentesis. Type IIA $sPLA_2$ concentration in the synovial fluid was measured by ELISA (Cayman Chemical Co., Ann Arbor, MI, USA).

Indoxam ((3-(biphenyl-2-yl)methyl-2-ethyl-1-oxamoyl-1-indolizine-8-yloxy)-acetic acid, molecular weight 456.503, kindly supplied by Shionogi Co., Osaka, Japan) was dissolved in dimethyl sulphoxide as a stock solution and added to the LDL at various concentrations. Another $sPLA_2$ inhibitor, thioetheramide-PC (Cayman Chemical Co.), was diluted with ethanol and added to the LDL at various concentrations. The LDL was concentrated and dialysed three times against a 1000-fold volume of 0.15 M NaCl and 0.26 mM EDTA, pH 7.4, using an Ultrafree-15 centrifugal filter for elimination of $sPLA_2$, indoxam and thioeheramide-PC and then stored at 4 °C under N_2 gas until use for the measurement of LPC content or incubation in HUVECs. For modification of native or glycoxidized LDL by HUVECs, 100 μg ml^{-1} of native or glycoxidized LDL was incubated in 6 ml of the medium for 2 h with HUVECs partly stimulated before 3 days with 100 ng ml^{-1} tumour-necrosis factor (TNFα) for 4 h. In some experiments, cells were preincubated with indoxam at various concentrations 1 h before the incubation. LDL was isolated from the medium using density-gradient ultracentrifugation prior to assaying the LPC content.

Electrophoretic mobility of modified LDL

Electrophoresis of modified LDL with or without 100 μM of indoxam or 500 μM of thioetheramide-PC was carried out using a commercial kit (Titan gel lipoproteins; Helena Laboratories, Saitama, Japan). In brief, 1 μl of each modified LDL was loaded on an agarose gel sheet and electrophoresed in barbital buffer at a voltage of 100 V for 30 min. The gel sheet was dried, stained with 0.04% fat red 7B in methanol and the distance from the applied line was measured as the electrophoretic mobility.

Measurement of LPC content in LDL by electrospray ionization-liquid chromatography/mass spectrometry

Measurement of LPC was carried out as described previously (Sonoki *et al*. 2002). Briefly, lipids were extracted from 100 μl of LDL supplemented with 500 ng of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (Avanti Polar-Lipids Inc., Alabaster, AL, USA) as an internal standard. Phospholipids were separated from the extracted lipids by the method of Kaluzny *et al*. (1985) using aminopropyl solid-phase extraction chromatography (BAKERBOND spe Columns; J.T. Baker Inc., Phillipsburg, NJ, USA). The phospholipids were then introduced into an electrospray mass spectrometer (LCQ, ThermoQuest, Tokyo, Japan) via high-performance liquid chromatography (LC-10; Shimazu, Kyoto, Japan). Reverse-phase high-performance liquid chromatography was performed by injecting 20 μl of isolated phospholipids in methanol into an STR-ODS analytical microcolumn (150×2.0 mm, 5 μM; Shimazu) at a flow rate of 0.3 ml min^{-1} and elution with a mobile solvent of methanol/acetonitrile/deionized water (84:14:2 v/v/v). The mass spectrometer was operated in positive mode employing the 'full-scan' function set from *m*/*z* 100 to 1000. Quantitative analysis of phospholipids was performed essentially as described by Han *et al*. (1996), based on their ion intensity relative to the internal standard. A liquid chromatography/mass spectrometry chromatogram of LDL showed the peaks of palmitoyl-, stearoyl-LPC and 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine as an internal standard, with retention times of 2.03 min, 2.50 min and 5.54 min, respectively. The electrospray ionization mass spectrum showed that palmitoyl- and stearoyl-LPC had four masses, that is, *m*/*z* 496.5, 497.5, 518.4 and 519.4 for palmitoyl-LPC; *m*/*z* 524.6, 525.5, 546.5 and 547.6 for stearoyl-LPC, respectively. The coefficient of variation for the electrospray ionization-liquid chromatography/mass spectrometry assay was 4.8%, and the standard-curve experiments showed that measurement by this method was linear over a wide range (0.1–5.0 ng μl^{-1}). LPC contents were normalized to LDL protein concentration or HUVEC cell number in each dish.

Measurement of MCP-1 mRNA expression by northern blot analysis

Briefly, total RNA was isolated from HUVECs with TRIzol (Invitrogen, San Diego, CA, USA). Total RNA (15 μg) was separated by electrophoresis and transferred to Hybond-N + nylon membranes (Amersham Biosciences, Buckinghamshire, UK). Hybridization was performed for 1 h at 65 °C in QuikHyb hybridization solution (Stratagene, La Jolla, CA, USA) with a human MCP-1 cDNA (R&D Systems Inc., Minneapolis, MN, USA) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Oncogene Research Products, Cambridge, MA, USA) that had been labelled with [γ-^{32}P] ATP (Amersham Biosciences) using the T4 polynucleotide kinase (Promega Co., Madison, WI, USA). Autoradiography and quantitative analysis were performed using a Bio-Imaging Analyzer (Fuji Photo Film Co., Kanagawa, Japan), and MCP-1 mRNA density was normalized against GAPDH density.

Measurement of NF-κB activity by electrophoretic mobility shift assay

Nuclear factor-kappa B activity was measured by electrophoretic mobility shift assay, as described previously (Sonoki *et al*., 2002). Briefly, 25 μl of 10% nonidet P-40 (Sigma) was added to HUVECs suspended in 0.4 ml buffer. The mixture was centrifuged and the nuclear pellet was suspended in 50 μl of ice-cold buffer. The suspension was sonicated and shaken at 4 °C for 15 min. The supernatant of nuclear extracts was centrifuged at 13 000 *g* in 4 °C for 5 min, and stored at −80 °C until use after determination of protein concentrations. Nuclear protein (4 μg) was incubated in 10 μl of binding buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% glycerol and 0.05 mg ml^{-1} poly(dI-dC) (Pharmacia Biotech Inc., Piscataway, NJ, USA) for 10 min at room temperature. Next, 1 μl of ^{32}P-labelled NF-κB oligonucleotide probe (Promega) was added, and the reaction mixture was incubated for 20 min at room temperature. For competition assays, 100-fold concentrations of unlabelled oligonucleotides were added to the nuclear proteins. For supershift experiments, antibodies against p50 and p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added to the nuclear proteins and incubated for 10 min at room temperature. All samples were loaded onto 6% polyacrylamide gels and electrophoresed. Gels were dried and autoradiographed on a Bio-Imaging Analyzer (Fuji Photo Film Co.). The density of NF-κB bands was expressed relative to that in the basal state.

Real-time PCR analysis of type IIA and V $sPLA_2$ mRNA expression in HUVECs

Confluent HUVECs in 6-cm gelatin-coated dish were stimulated with 100 ng ml^{-1} of TNFα (kindly supplied by Dainippon Pharma Co., Osaka, Japan) in M199 medium containing 2% FCS for 4 h, and then total RNA was extracted using TRIzol (Invitrogen). Total RNA (500 ng) was reverse-transcribed using ExScript reverse transcriptase (ExScript RT reagent Kit; Takara Bio Inc., Shiga, Japan) in the presence of random hexamers at 42 °C for 15 min. Real-time PCR was performed starting with 100 ng cDNA and 0.2 μM concentration of both sense and antisense oligonucleotides (type IIA $sPLA_2$: sense 5′-GAAGTTGAGACCACCCAGCAGAG-3′, antisense 5′-AGCCATAACTGAGTGCGGCTTC-3′; type V $sPLA_2$: sense 5′-CAAACTACGGCTTCTACGGCTGTTA-3′, antisense 5′-CCACGCGAATCTGTATTTGTAGGAC-3′; designed and produced by Takara Bio Inc., in a final volume 20 μl using

the SYBR Premix Ex Taq; Takara Bio Inc.). Fluorescence was monitored and analysed in a LightCycler (Roche Diagnostics, Basel, Switzerland). Reaction conditions were as follows: denature at 95 °C for 5 s, anneal at 55 °C for 10 s, extend at 72 °C for 10 s for 50 cycles. Analysis of GAPDH mRNA was performed in parallel to normalize gene expression (sense 5′-GCACCGTCAAGGCTGAGAAC-3′, antisense 5′-ATGGTGGTGAAGACGCCAGT-3′). Results are expressed as $2^{(Ct\ GAPDH-Ct\ type\ IIA\ or\ V\ sPLA2)}$, where Ct corresponds to the number of cycles needed to generate a fluorescent signal above a predefined threshold.

Western blot analysis of type V sPLA$_2$ protein in HUVECs

Confluent HUVECs in 10-cm gelatin-coated dishes were stimulated with 100 ng ml^{-1} of TNFα in M199 medium containing 2% FCS for 4 h. Three days later, cells were collected in a 1.5-ml tube. The mixture was centrifuged and the pellet was suspended in 50 μl of ice-cold lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride and 0.1% nonidet P-40). The extracts (20 μg) were separated on 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels and transferred to Immobilon-P polyvinylidene fluoride membrane (Millipore Co.). Membranes were blocked in 0.05% Tween-20 Tris-buffered saline (1 M Tris-HCl, pH 8.0, 5 M NaCl and 0.05% Tween-20) with 5% nonfat dry milk for 1 h at room temperature with gentle agitation. Membranes were then incubated overnight at 4 °C in 0.05% Tween-20 Tris-buffered saline containing mouse monoclonal anti-human type V sPLA$_2$ antibody (dilution 1:1000, Cayman Chemical Co.) or anti-human GAPDH antibody (dilution 1:10000, Santa Cruz Biotechnology). Membranes were washed three times with 0.05% Tween-20 Tris-buffered saline and incubated with anti-mouse IgG, peroxidase-linked antibodies (dilution 1:5000 for type V sPLA$_2$, dilution 1:10000 for GAPDH, Amersham Biosciences) for 1 h at room temperature with gentle agitation. Immunoreactive proteins were detected by ECL Plus Chemiluminescence (Amersham Biosciences) and band density analysed using NIH Image analysis software (version 1.55). Type V sPLA$_2$ density was normalized against GAPDH density and expressed relative to that in the basal state.

Statistical analysis

Data are presented as mean ± s.e.m. Statistical analysis was performed by the unpaired Student's *t*-test for comparisons between two groups and by one-way ANOVA followed by Fisher's *post hoc* test for comparisons of multiple groups. A *P*-value of less than 0.05 was considered statistically significant.

Results

Comparative effects of indoxam and thioetheramide-PC on LDL modification by venom sPLA$_2$

We first compared the inhibitory effect of indoxam on LDL modification by venom sPLA$_2$ with thioetheramide-PC, a structurally modified phospholipid that functions as a competitive, reversible inhibitor of sPLA$_2$. In electrophoresis of LDL (Figure 1a), the electrophoretic mobility was increased by venom PLA$_2$, which was prevented by 100 μM of indoxam (3.1 ± 0.3 mm in native LDL, 10.9 ± 0.8 mm in sPLA$_2$-treated LDL $P<0.001$, 2.9 ± 0.4 mm in LDL treated with sPLA$_2$ and indoxam; ns, $n=4$, respectively). However, 1 to 500 μM thioetheramide-PC did not affect the increase of the electrophoretic mobility (9.1 ± 2.2 mm in LDL treated with sPLA$_2$ and 500 μM thioetheramide-PC; ns, $n=4$). Moreover, palmitoyl-LPC content in LDL was markedly increased by venom sPLA$_2$, as we reported previously (Sonoki *et al*., 2003), which was completely suppressed by 100 μM of indoxam but only marginally by 500 μM of thioetheramide-PC (Figure 1b, $n=4$).

Next, we investigated the effects of various concentrations of indoxam on LPC production in LDL by venom sPLA$_2$ (Figure 2, $n=4$). Indoxam significantly suppressed the increases in palmitoyl-LPC (Figure 2a) and stearoyl-LPC (Figure 2b) at concentrations of ⩾1 μM. At 100 μM of indoxam, the LPC contents in LDL were not significantly different from those in native LDL. The IC_{50} of indoxam for suppressing LPC production was 1.2 μM for palmitoyl-LPC and 0.8 μM for stearoyl-LPC.

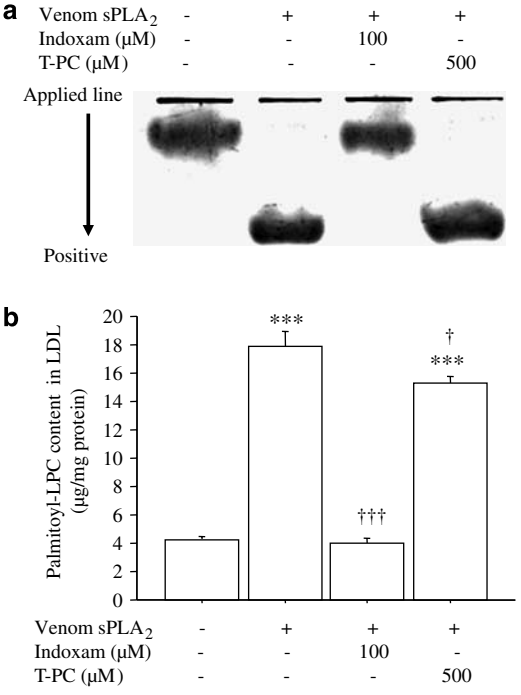

Figure 1 Comparative effects of indoxam and thioetheramide-phosphatidylcholine (PC) on modification of low-density lipoprotein (LDL) by venom secretory phospholipase A$_2$ (PLA$_2$). Native LDL was incubated with venom sPLA$_2$ with or without 100 μM indoxam or 500 μM thioetheramide-PC (T-PC) at 37 °C for 2 h. (**a**) Electrophoretic mobility: four representative sets of electrophoresis. (**b**) Palmitoyl-lysophosphatidylcholine (LPC) content in LDL measured by electrospray ionization-liquid chromatography/mass spectrometry. Data are mean ± s.e.m. of four experiments. ***$P<0.001$ vs untreated LDL, $^{\dagger}P<0.05$, $^{\dagger\dagger\dagger}P<0.001$ vs LDL modified by venom sPLA$_2$.

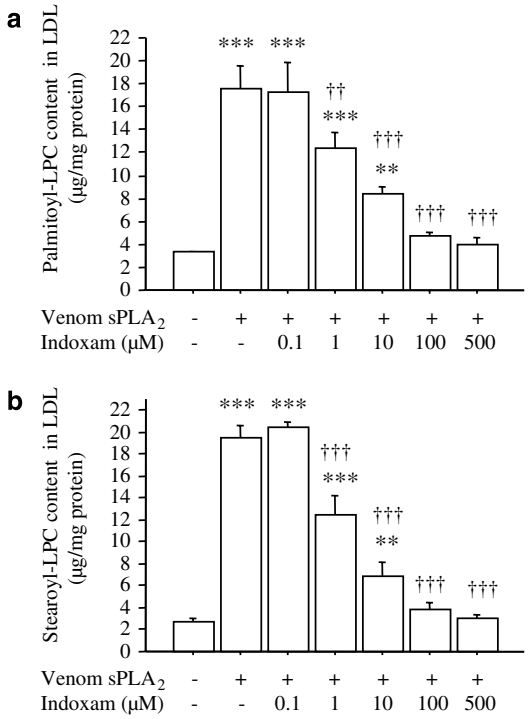

Figure 2 Effects of indoxam on palmitoyl-lysophosphatidylcholine (LPC) (**a**) and stearoyl-LPC (**b**) production in native low-density lipoprotein (LDL) by snake venom secretory phospholipase A_2 ($sPLA_2$). Native LDL was incubated with venom $sPLA_2$ in the absence or presence of indoxam at 37 °C for 2 h. Subsequently, the LPC content of the LDL was measured by electrospray ionization-liquid chromatography/mass spectrometry. Data are mean ± s.e.m. of four experiments. $^{**}P<0.01$, $^{***}P<0.001$ vs untreated LDL, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ vs LDL modified by venom $sPLA_2$.

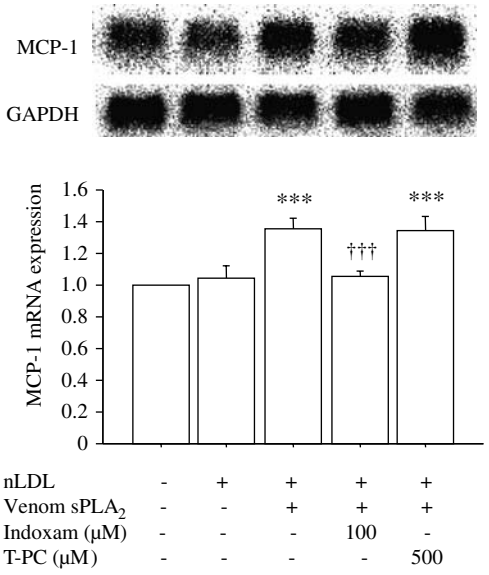

Figure 3 Effects of indoxam and thioetheramide-phosphatidylcholine (PC) on monocyte chemoattractant protein-1 (MCP-1) mRNA expression in human umbilical vein endothelial cell (HUVEC) after incubation with low-density lipoprotein (LDL) treated with snake venom secretory phospholipase A_2 ($sPLA_2$). Native low-density lipoprotein (LDL) was incubated with venom $sPLA_2$ in the absence or presence of 100 μM indoxam or 500 μM thioetheramide-PC at 37 °C for 2 h. HUVECs were incubated with 100 μg ml^{-1} of native LDL or LDL modified by venom $sPLA_2$ in the presence or absence of indoxam or thioetheramide-PC (T-PC). MCP-1 mRNA expression was measured by northern blot analysis and expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression; the basal state was set as 1.0. Data are mean ± s.e.m. of five experiments. $^{***}P<0.001$ vs HUVECs incubated with native LDL, $^{\dagger\dagger\dagger}P<0.001$ vs HUVECs incubated with LDL modified by venom $sPLA_2$.

Comparative effects of indoxam and thioetheramide-PC on MCP-1 mRNA expression and NF-κB activity in HUVEC

As indoxam prevented the increase in LPC content in LDL treated with venom $sPLA_2$ (Figure 1b), we investigated its effect on MCP-1 mRNA expression in HUVECs (Figure 3, $n=5$). MCP-1 mediates the recruitment of mononuclear cells into subendothelial layers, which is an important initial step of atherogenesis. Northern blot analysis showed approximately 1.3-fold increase of MCP-1 mRNA expression in HUVECs incubated with $sPLA_2$-treated LDL compared with HUVECs in the basal state or incubated with native LDL. This enhancement of MCP-1 mRNA expression was prevented by coincubation with 100 μM indoxam but not with 500 μM thioetheramide-PC. As expression of MCP-1 mRNA is regulated by the transcription factor NF-κB, we investigated NF-κB activity in HUVECs by electrophoretic mobility shift assay (Figure 4, $n=5$). LDL treated by venom $sPLA_2$ significantly increased NF-κB activity (1.4-fold) relative to the basal state, and such activation was suppressed by coincubation with 100 μM indoxam, but not with 500 μM thioetheramide-PC.

Effect of indoxam on LPC production in LDL by human synovial type IIA $sPLA_2$

As human type IIA $sPLA_2$ was not commercially available at the time of this study, we used synovial fluid obtained from the knee joint of a patient with rheumatoid arthritis. Such fluid is known to contain large amounts of type IIA $sPLA_2$ (Kramer *et al*., 1989). When LDL was incubated with human type IIA $sPLA_2$ at 0–40 ng ml^{-1}, the palmitoyl- and stearoyl-LPC contents in LDL showed a mild dose-dependent increase, and 100 μM of indoxam significantly suppressed these increases of LPC contents in LDL treated with 40 ng ml^{-1} of synovial type IIA $sPLA_2$ (Figure 5, $n=6$).

Type V $sPLA_2$ expression in TNFα-stimulated HUVECs

Indoxam inhibits LPC production in LDL by $sPLA_2$ derived from snake venom or by type IIA $sPLA_2$ in human synovial fluid. We next used $sPLA_2$ derived directly from human endothelial cells to approach more closely to the conditions in vascular tissue: HUVECs were stimulated by TNFα for 4 h, based on the TNFα-induced $sPLA_2$ expression in other cells (Andreani *et al*., 2000). Real-time PCR revealed that TNFα stimulated type V $sPLA_2$ mRNA expression in HUVECs by 1.6-fold relative to the basal state (Figure 6a, $n=4$), whereas type IIA $sPLA_2$ mRNA was not detected ($n=4$). Western blot analysis revealed that TNFα-stimulated HUVECs expressed type V $sPLA_2$ protein at a higher level (1.5-fold) than that in unstimulated HUVECs at 3 days after the TNFα stimulation (Figure 6b, $n=6$).

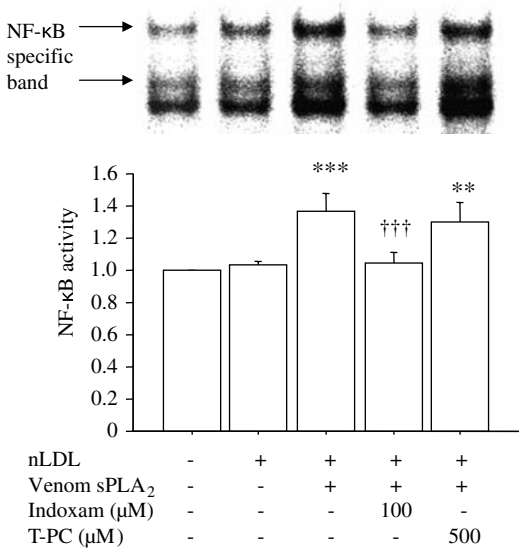

Figure 4 Effects of indoxam and thioetheramide-phosphatidylcholine (T-PC) on nuclear factor-kappa B (NF-κB) activity in human umbilical vein endothelial cell (HUVEC) after incubation with low-density lipoprotein (LDL) treated with snake venom secretory phospholipase A_2 ($sPLA_2$). Native LDL was modified by venom $sPLA_2$ in the presence or absence of 100 μM indoxam or 500 μM thioetheramide-PC at 37 °C for 2 h. HUVECs were incubated with 100 μg ml^{-1} of native LDL or LDL modified by venom $sPLA_2$ in the absence or presence of indoxam or thioetheramide-PC. NF-κB activity was measured by electrophoretic mobility shift assay and expressed relative to the basal state, set as 1.0. Data are mean ± s.e.m. of five experiments. $^{**}P<0.01$, $^{***}P<0.001$ vs HUVEC incubated with native LDL, $^{†††}P<0.001$ vs HUVEC incubated with LDL modified by venom $sPLA_2$.

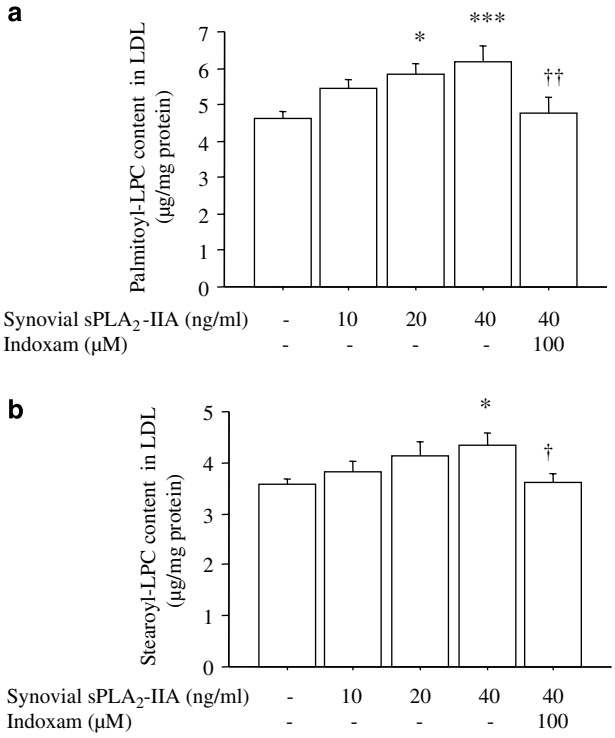

Figure 5 Effects of indoxam on palmitoyl-lysophosphatidylcholine (LPC) (**a**) and stearoyl-LPC (**b**) production in native low-density lipoprotein (LDL) by human synovial type IIA secretory phospholipase A_2 ($sPLA_2$). Native LDL was incubated with human synovial type IIA $sPLA_2$ in the absence or presence of 100 μM indoxam at 37 °C for 2 h. Subsequently, LPC content in the LDL was measured by electrospray ionization-liquid chromatography/mass spectrometry. Data are mean ± s.e.m. of six experiments. $^{*}P<0.05$, $^{***}P<0.001$ vs untreated LDL, $^{†}P<0.05$, $^{††}P<0.01$ vs LDL modified by 40 ng ml^{-1} of human synovial type IIA $sPLA_2$.

Effect of indoxam on LPC production induced by TNFα-stimulated HUVECs in native or glycoxidized LDL

Native or glycoxidized LDL was incubated with TNFα-stimulated HUVECs with or without indoxam, and then LDL was retrieved from the medium to measure LPC. Native LDL incubated with TNFα-stimulated HUVECs contained more palmitoyl- and stearoyl-LPC compared with LDL incubated with -unstimulated HUVECs (Figure 7, $n=5$). Coincubation with indoxam reduced the LPC content dose dependently, the reduction was statistically significant at 100 μM of indoxam for palmitoyl-LPC and at 200 μM of indoxam for stearoyl-LPC. Glycoxidized LDL contained higher concentrations of palmitoyl- and stearoyl-LPC compared with native LDL (palmitoyl-LPC 4.70 ± 0.24 μg per mg protein per 10^6 cells; stearoyl-LPC 4.69 ± 0.43 μg per mg protein per 10^6 cells, $n=6$, respectively), as we reported previously (Sonoki *et al*., 2002). TNFα-stimulated HUVECs further increased the LPC content in glycoxidized LDL (palmitoyl-LPC 7.63 ± 0.98 μg per mg protein per 10^6 cells; stearoyl-LPC 7.64 ± 1.09 μg per mg protein per 10^6 cells, $P<0.01$, $n=6$). Coincubation with indoxam (100 μM) did not significantly reduce palmitoyl- or stearoyl-LPC in LDL (data not shown), whereas at 200 μM, it reduced palmitoyl-LPC (5.15 ± 0.55 μg per mg protein per 10^6 cells, $P<0.05$, $n=6$), but not stearoyl-LPC (5.99 ± 0.39 μg per mg protein per 10^6 cells, ns, $n=6$).

To investigate the PLA_2 activity released into the medium from TNFα-stimulated HUVECs, we measured type IIA $sPLA_2$ in the medium using the ELISA kit ($n=10$), but it was undetectable (as expected from the results of the PCR experiments). The medium from TNFα-stimulated or unstimulated HUVECs was applied to native LDL for 2 h and then the LPC contents in the retrieved LDL were compared. There was no difference in LPC contents between LDL incubated with the medium from TNFα-stimulated and unstimulated HUVECs (data not shown). These results suggest that $sPLA_2$ was not released into the medium, but was anchored to the cell surface by heparan sulphate proteoglycan (Murakami *et al*., 1996).

Effect of indoxam on MCP-1 mRNA expression and NF-κB activity in TNFα-stimulated HUVECs incubated with native or glycoxidized LDL

Lastly, we investigated the effects of indoxam on MCP-1 mRNA expression in TNFα-stimulated HUVECs incubated with 100 μg ml^{-1} of native (Figure 8a, $n=4$) or glycoxidized LDL (Figure 8b, $n=4$). The results showed a significant increase in MCP-1 mRNA expression upon the addition of native or glycoxidized LDL compared with TNFα-stimulated HUVECs without LDL. Indoxam significantly prevented this increase at 50 μM with native LDL and at 100 μM of indoxam with glycoxidized LDL. As shown in Figure 9 ($n=6$), the

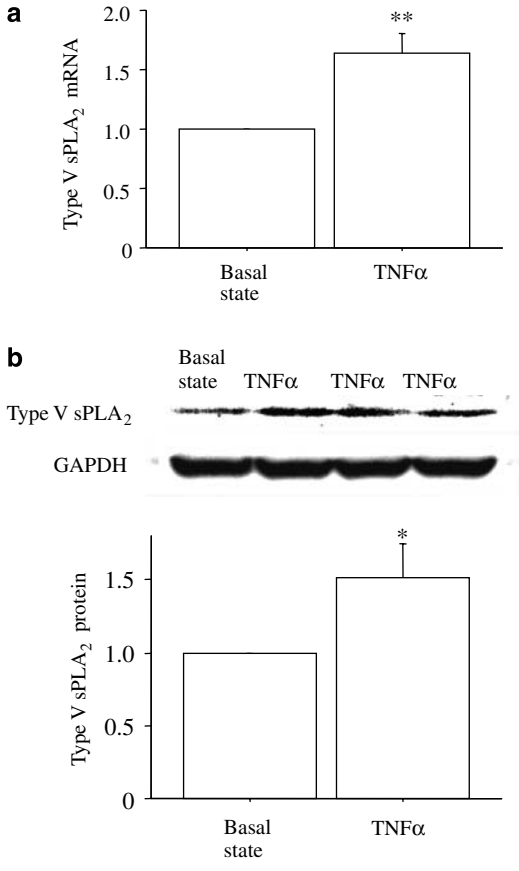

Figure 6 Type V secretory phospholipase A_2 ($sPLA_2$) mRNA expression (**a**) and protein expression (**b**) in human umbilical vein endothelial cell (HUVEC) after tumour-necrosis factor α (TNFα)-stimulation. Confluent HUVECs were stimulated with 100 ng ml^{-1} of TNFα for 4 h, and real-time PCR was performed. At 3 days after TNFα-stimulation, western blot analysis was performed and expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein expression. Data are mean ± s.e.m. of four experiments for mRNA expression and of six experiments for protein expression. *$P<0.05$, **$P<0.01$ vs basal state.

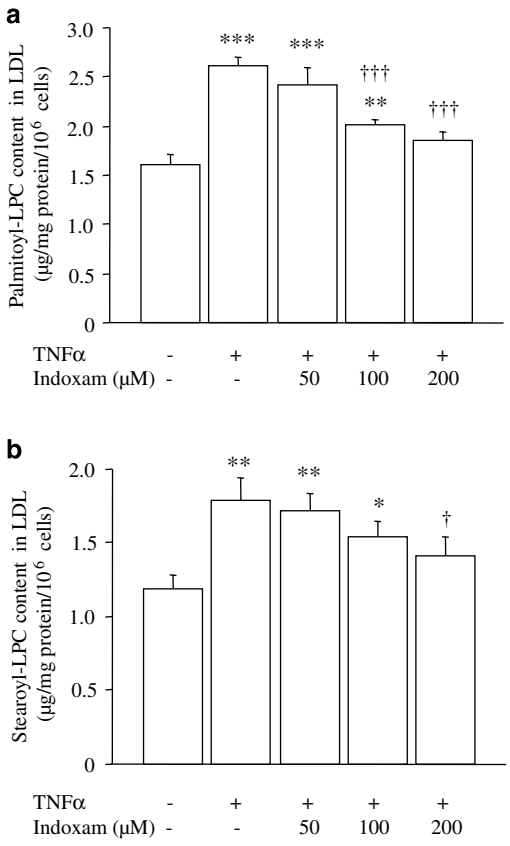

Figure 7 Effects of indoxam on palmitoyl-lysophosphatidylcholine (LPC) (**a**) and stearoyl-LPC (**b**) production in native low-density lipoprotein (LDL) by tumour-necrosis factor α (TNFα)-stimulated human umbilical vein endothelial cell (HUVEC). Native LDL was incubated for 2 h with unstimulated or TNFα-stimulated HUVEC that had been preincubated 1 h earlier with indoxam. LDL was isolated from the medium using density-gradient ultracentrifugation, and LPC content of the LDL was measured by electrospray ionization-liquid chromatography/mass spectrometry and normalized to cell number in each dish. Data are mean ± s.e.m. of five experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs native LDL incubated with unstimulated HUVEC, $^{\dagger}P<0.05$, $^{\dagger\dagger\dagger}P<0.001$ vs native LDL modified by TNFα-stimulated HUVEC.

DNA-binding activity of NF-κB was enhanced by coincubation with native or glycoxidized LDL in TNFα-stimulated HUVECs, whereas 100 μM of indoxam significantly suppressed the activation of NF-κB activity.

Discussion and conclusions

In the present study, the indole analogue indoxam, dose dependently prevented LPC production in LDL by venom $sPLA_2$, synovial fluid type IIA $sPLA_2$ and type V $sPLA_2$ in TNFα-stimulated HUVECs. Indoxam also suppressed NF-κB activity and MCP-1 mRNA expression in HUVECs incubated with $sPLA_2$-treated LDL. On the other hand, thioetheramide-PC, a competitive, reversible inhibitor of $sPLA_2$ had no effects on LPC production by venom $sPLA_2$, NF-κB activity or MCP-1 mRNA expression. Thioetheramide-PC is an analogue of PC containing a thioether at the *sn*-1 position and an amide at the *sn*-2 position and acts on lipid interfaces. Thioetheramide-PC may be intercalated into the interface and dilute the surface concentrations of the substrate (Yu *et al*., 1990). There are numerous $sPLA_2$ inhibitors, which are nonspecific or active site-directed competitive inhibitors, although no single compound is highly selective for a single $sPLA_2$ enzyme. Indoxam is known to be the most generally potent $sPLA_2$ inhibitor (Singer *et al*., 2002).

In vivo indoxam administration prevented endotoxin shock in type IIA $sPLA_2$-deficient C57BL/6J mice (Yokota *et al*., 1999) and ameliorated cerebral infarction following middle cerebral artery occlusion in rats (Yagami *et al*., 2002). The pharmacokinetic study of indoxam in mice revealed that maximal plasma concentration (*C*max) was approximately 8 μM at 15–30 min after injection of 10 mg kg^{-1} (Yokota *et al*., 1999), although a dose of 50 mg kg^{-1} was needed to prevent a rise in plasma TNFα during endotoxic shock. *In vitro* treatment with 20 μM indoxam suppressed cytokine and eicosanoid production in macrophages (Saiga *et al*., 2001; Granata *et al*., 2005) and neuronal cell death induced by type IIA $sPLA_2$ (Yagami *et al*., 2001). In the present study, the IC_{50} of indoxam to prevent palmitoyl-LPC production in LDL by

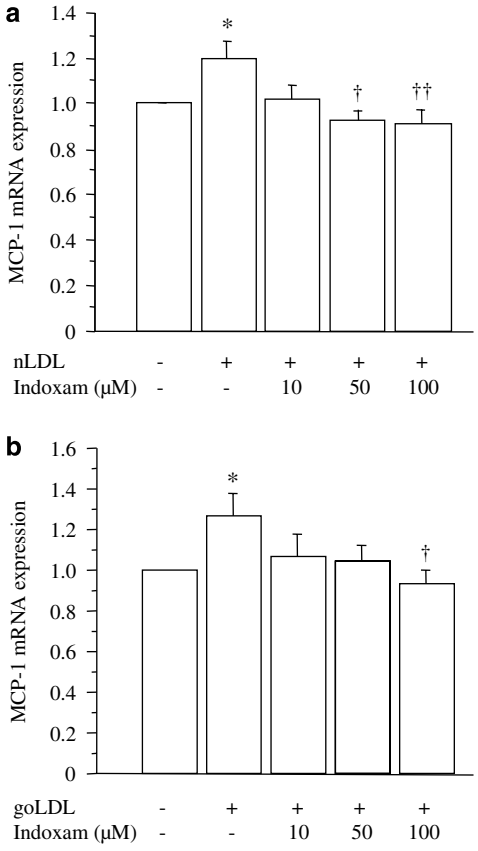

Figure 8 Effects of indoxam on monocyte chemoattractant protein-1 (MCP-1) mRNA expression after incubation with native low-density lipoprotein (LDL) (**a**) or glycoxidized LDL (**b**) in TNFα-stimulated HUVEC. Native (nLDL) or glycoxidized LDL (goLDL) was incubated with tumour-necrosis factor α (TNFα)-stimulated human umbilical vein endothelial cell (HUVEC) that had been preincubated 1 h earlier with indoxam. MCP-1 mRNA was measured by northern blot analysis and the mRNA level was corrected by that of the corresponding glyceraldehyde-3-phosphate dehydrogenase mRNA and expressed relative to the value of TNFα-stimulated HUVEC without LDL. Data are mean ± s.e.m. of four experiments. $^{*}P<0.05$ vs TNFα-stimulated HUVEC without LDL, $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs TNFα-stimulated HUVEC incubated with LDL alone.

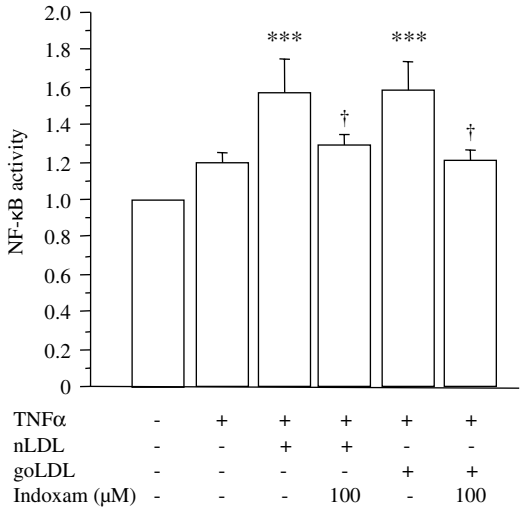

Figure 9 Effects of indoxam on nuclear factor-kappa B (NF-κB) activity after incubation with native or glycoxidized low-density lipoprotein (LDL) in tumour-necrosis factor α (TNFα)-stimulated human umbilical vein endothelial cell (HUVEC). Native (nLDL) or glycoxidized LDL (goLDL) was incubated with TNFα-stimulated HUVEC that had been preincubated 1 h earlier with indoxam. NF-κB activity was measured by electrophoretic mobility shift assay (EMSA) and the activity in the basal state was set as 1.0. Data are mean ± s.e.m. of six experiments. $^{***}P<0.001$ vs basal state, $^{\dagger}P<0.05$ vs TNFα-stimulated HUVEC incubated with native or glycoxidized LDL alone.

venom sPLA2, which exhibits a more potent hydrolysis of phospholipids than mammalian $sPLA_2$ (Verheij *et al*., 1980), was 1.2 μM. On the other hand, Degousee *et al*. (2002) reported that the IC_{50} of indoxam was 10 nM for recombinant type IIA $sPLA_2$ activity and 40 nM for recombinant type V $sPLA_2$ activity on substrates of [^{3}H]oleate-labelled membranes of *Escherichia coli*. Although the protein-binding capacity of indoxam has not been determined yet (Dr Kato, Shionogi Co., personal communication), this agent seems to be more potent in experiments using [^{3}H]oleate-labelled *E. coli* membrane as the substrate.

Type IIA $sPLA_2$ is secreted by a variety of cells including macrophages, and its overexpression in transgenic mice, and specifically in macrophages, causes a marked increase in atherosclerotic lesions (Ivandic *et al*., 1999; Webb *et al*., 2003). Moreover, increased serum type IIA $sPLA_2$ levels are a risk factor for coronary artery disease (Boekholdt *et al*., 2005). However, recent studies showed that type V $sPLA_2$ is important in enzymatic modification of LDL and foam-cell formation in the arterial wall (Wooton-Kee *et al*., 2004; Boyanovsky *et al*., 2005) and contributed to the atherosclerotic process in genetically altered mice (Bostrom *et al*., 2007). In addition, type V $sPLA_2$ is about 20–30 times more potent in phospholipid degradation than type IIA $sPLA_2$ (Gesquiere *et al*., 2002; Pruzanski *et al*., 2005). This is in agreement with our findings that synovial type IIA $sPLA_2$ only weakly degraded the PC in LDL. On the other hand, type V $sPLA_2$ preferentially hydrolyses linoleic acid and discriminated against arachidonic acid, in contrast to venom $sPLA_2$ (Gesquiere *et al*., 2002) and type IIA $sPLA_2$ (Pruzanski *et al*., 2005). Therefore, the pathological effects of type V $sPLA_2$ are less likely due to the direct stimulation of eicosanoid synthesis, but more to the generation of LPC. $sPLA_2$ is expressed in response to a variety of inflammatory cytokines, including interleukin 1β and TNFα (van der Helm *et al*., 2000; Akiba *et al*., 2001; Hurt-Camejo *et al*., 2001), and $sPLA_2$ itself directly induces the expression of chemokines and adhesion molecules in microvascular endothelium (Beck *et al*., 2003). In the present study, TNFα enhanced type V $sPLA_2$ expression in HUVECs. Recently, Rosengren *et al*. (2006) showed type V $sPLA_2$ in the endothelium of advanced atherosclerotic lesions, and reverse-transcription-PCR of cultured human arterial endothelial cells detected the mRNA expression of type V $sPLA_2$ but not type IIA $sPLA_2$. We also failed to detect type IIA $sPLA_2$ mRNA in TNFα-stimulated HUVECs. Higher concentrations of LPC were identified in glycoxidized LDL, which further increased in activated HUVECs. Indoxam dose dependently suppressed the modification of native and glycoxidized LDL by TNFα-stimulated HUVECs. Moreover, indoxam blocked the increase in MCP-1 mRNA expression and NF-κB activity in TNFα-stimulated

HUVECs incubated with native or glycoxidized LDL. However, the suppression of MCP-1 mRNA expression was observed at lower doses of indoxam without the suppression of LPC production. These results suggest that indoxam may suppress MCP-1 mRNA expression in activated HUVECs via mechanisms other than the suppression of LPC production. It was reported that types IB and IIA $sPLA_2$ exerted some of their activities independent of their catalytic activity via specific M-type receptors (Ancian *et al*., 1995; Jaulmes *et al*., 2005), and indoxam inhibited type IB and X $sPLA_2$ binding to M-type PLA_2 receptors in a dose-dependent manner (Yokota *et al*., 1999, 2000; Granata *et al*., 2005). In addition, indoxam is not membrane permeable (Mounier *et al*., 2004). Therefore, further studies are required to ascertain whether indoxam interacts directly with $sPLA_2$ receptors on endothelial cells.

In conclusion, indoxam, a $sPLA_2$ inhibitor, prevented $sPLA_2$-induced production of LPC in native and glycoxidized LDL as well as LDL-associated MCP-1 mRNA expression in HUVECs. Although specific type IIA $sPLA_2$ inhibitors, structurally related to indoxam, had been reported to have no beneficial effects in patients with rheumatoid arthritis (Bradley *et al*., 2005) or severe sepsis (Zeiher *et al*., 2005), our results suggest that $sPLA_2$ inhibitors, such as indoxam, which suppress LDL modification merit further investigation for the prevention and treatment of atherosclerotic diseases.

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Conflict of interest

The authors state no conflict of interest.

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