Endothelial ICAM-1 Protein Induction Is Regulated by Cytosolic Phospholipase A2α via Both NF-κB and CREB Transcription Factors

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Endothelial ICAM-1 Protein Induction Is Regulated by Cytosolic Phospholipase A2α via Both NF-κB and CREB Transcription Factors

Nurit Hadad,* Liron Tuval,* Vered Elgazar-Carmom,* Ron Levy,† and Rachel Levy*†

The regulated expression of ICAM-1 plays an important role in inflammatory processes and immune responses. The present study aimed to determine the in vivo involvement of cytosolic phospholipase A2α (cPLA2α) in ICAM-1 overexpression during inflammation and to elucidate the cPLA2α-specific role in signal events leading to ICAM-1 upregulation in endothelial cells. cPLA2α and ICAM-1 upregulation were detected in inflamed paws of mice with collagen-induced arthritis and in peripididymal adipose tissue of mice fed a high-fat diet. Intravenous injection of 2 mg/kg oligonucleotide antisense against cPLA2α (AS) that reduced cPLA2α upregulation also decreased ICAM-1 overexpression, suggesting a key role of cPLA2α in ICAM-1 upregulation during inflammation. Preincubation of endothelial ECV-304 cells that express ICAM-1 and of HUVEC that express ICAM-1 and VCAM-1 with 1 μM AS prevented cPLA2α and the adhesion molecule upregulation induced by TNF-α and inhibited their adherence to phagocyte-like-PLB cells. Whereas AS did not inhibit NADPH oxidase 4-NADPH oxidase activity, inhibition of oxidase activity attenuated cPLA2α activation, suggesting that NADPH oxidase acts upstream to cPLA2α. Attenuating cPLA2α activation by AS or diphenylene iodonium prevented the induction of cyclooxygenase-2 and the production of PGE2 that were essential for ICAM-1 upregulation. Inhibition of cPLA2α activity by AS inhibited the phosphorylation of both p65 NF-κB on Ser536 and protein kinase A-dependent CREB. To our knowledge, our results are the first to show that CREB activation is involved in ICAM-1 upregulation and suggest that cPLA2α activated by NADPH oxidase is required for sequential phosphorylation of NF-κB by an undefined kinase and CREB activation by PGE2-mediated protein kinase A. The Journal of Immunology, 2011, 186: 000–000.

Vascular inflammation is a pivotal event in the pathogenesis of many human diseases, including atherosclerosis, hypertension, restenosis, septic shock, autoimmune diseases, and ischemia/reperfusion damage (1, 2). In health, the endothelial cell surface of the vascular lumen is a relatively nonadhesive and nonthrombogenic conduit for the cellular and macromolecular constituents of the blood. Under inflammatory conditions, interactions between endothelial cells and blood constituents or extracellular matrix occurred by the production of adhesion molecules and their shedding onto the endothelial and leukocyte surfaces (3). ICAM-1 is a cell-surface glycoprotein member of the Ig superfamily (4). As the counterreceptor for the leukocyte β2 integrins, ICAM-1 plays a central role in inflammatory and immune responses. Although ICAM-1 is constitutively expressed, its upregulation on cytokine-activated vascular endothelial cells controls the targeted transmigration of leukocytes into specific areas of inflammation (5). Induction of ICAM-1 gene transcription by TNF-α has previously been shown to be dependent on NF-κB activation and its binding to ICAM-1 promoter (5, 6). The VCAM-1 that mediates the adhesion to monocytes and lymphocytes is also an inducible cell-surface glycoprotein on several cell types and implicated in a number of inflammatory responses. Similar to ICAM-1, induction of VCAM-1 by TNF-α has been shown to be dependent on NF-κB activation (5).

The phospholipase A2 (PLA2) superfamily consists of a broad range of enzymes that are defined by their ability to specifically catalyze the hydrolysis of sn-2 ester bond of glycerolphospholipids (7, 8). Considerable attention is focused on cytosolic PLA2α (cPLA2α), which exhibits a specific preference for arachidonic acid (AA), the precursor for generation of biologically active eicosanoids (9), including PGE2. PGE2 plays an important role in the signal transduction cascade events during induction of gene transcription (10, 11). Our recent study (12) demonstrated that there is a significant elevation in cPLA2α expression and its metabolites in the inflamed site in two mouse models of inflammation: collagen-induced arthritis (CIA) and thioglicollate-induced peritonitis. In vivo inhibition of elevated cPLA2α protein expression by i.v. administration of oligonucleotide antisense resulted in a dramatic reduction of the inflammatory process and a significant reduction of recruited neutrophils to the site of inflammation in both mouse models of inflammation.

The present study aimed to determine the in vivo involvement of cPLA2α in ICAM-1 upregulation in mouse models of inflammation and to determine its specific role in signaling events leading to induction of ICAM-1 protein expression in endothelial cells. In addition, the role of cPLA2α in VCAM-1 upregulation was determined.
Materials and Methods

Antisense oligonucleotides against cPLAα

Antisense oligonucleotides against cPLAα were engineered using the computer-based approach RNADraw V1.1 (Mazura Multimedia) and used as described in our previous study (12). A combination of three oligonucleotide antisenses (TCAAAGGCTTACATCAACA AAAAACATTCTCGATTAGG, and GCTGTCAAGGGGTTGTAG) and their corresponding senses with phosphorothioate modifications on the last three bases at both 5’ and 3’ ends was used. This antisense combination was found to be much more efficient than each individual and of other antisense combinations studied (not shown). The specificity to cPLAα was analyzed by the Basic Local Alignment Search Tool search program.

Mouse model of CIA

The mouse model of CIA was induced by immunizing susceptible animals (DBA/1 mice) with bovine type II collagen (CII) as described (12) using bovine CII prepared at the Kennedy Institute of Rheumatology or purchased from Sigma-Aldrich (St. Louis, MO). All mice were maintained in a specific pathogen-free environment and were fed standard mouse chow and water. Bovine CII was dissolved overnight at 4°C in 10 mM acetic acid at a concentration of 4 mg/ml and emulsified with an equal volume of CFA, which was prepared by dissolving H37Ra Mycobacterium tuberculosis (Difco Laboratories, Detroit, MI) in IFA (Sigma-Aldrich) at a final concentration of 4 mg/ml. CIA was induced by intradermal injection of 100 μl emulsion containing 200 μg CII to 8-wk-old mice at the base of the tail and boosted on day 21 with CII emulsion in IFA. The severity of arthritis was assessed as described in detail in our previous study (12).

Mouse model of obesity

Male C57BL/6j mice (The Jackson Laboratory, Bar Harbor, ME) at 6 wk of age were fed either a low-fat diet (LFD; 6% calories from fat; Harlan Teklad 2018sc) or a high-fat diet (HFD; 60% calories from fat; research diets and 3x

Cell preparation and cell culture

Primary HUVEC were obtained from ScienCell Research Laboratories or were isolated from freshly obtained human umbilical cord by using the method described by Jaffe et al. (15). Briefly, the umbilical vein was rinsed twice with PBS containing 100 U/ml penicillin/streptomycin and 0.2% collagenase I (Worthington Biochemical) and incubated for 15 min at 37°C. After harvesting, endothelial cells were placed in 75 cm² tissue culture flasks and grown in endothelial cell medium (ScienCell Research Laboratories). HUVECs used in the experiments were between their third and sixth passages. The phenotype of the endothelial cell was confirmed by performing immunofluorescence analysis using mAb of von Willebrand factor (BD Biosciences, San Jose, CA). The study was approved by the Helsinki Committee of the University of Helsinki (IL-35-2006 and IL-08-01-2009).

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Primary HUVEC were obtained from ScienCell Research Laboratories or were isolated from freshly obtained human umbilical cord by using the method described by Jaffe et al. (15). Briefly, the umbilical vein was rinsed twice with PBS containing 100 U/ml penicillin/streptomycin and 0.2% collagenase I (Worthington Biochemical) and incubated for 15 min at 37°C. After harvesting, endothelial cells were placed in 75 cm² tissue culture flasks and grown in endothelial cell medium (ScienCell Research Laboratories). HUVECs used in the experiments were between their third and sixth passages. The phenotype of the endothelial cell was confirmed by performing immunofluorescence analysis using mAb of von Willebrand factor (BD Biosciences, San Jose, CA). The study was approved by the Helsinki Committee of the University of Helsinki (IL-35-2006 and IL-08-01-2009).

Immunohistochemistry

The expression of cPLAα and of ICAM-1 was detected as previously described (12, 17). Nonspecific reactivity was inhibited by incubation of deparaffinized tissue sections mounted on slides for 30 min in a solution of 4% normal horse serum (avidin–biotin VECTA-STAIN Kit Elite PK 6105; Vector Laboratories, Burlingame CA). The sections were then incubated with 1/1000 dilution of a mouse mAb against mouse cPLAα (Santa Cruz Biotechnology, Santa Cruz, CA) or Ab against ICAM-1 (R&D Systems, Minneapolis, MN) in PBS for 2 h at room temperature. Sections were incubated with secondary Abs (1/200 dilution), biotinylated goat anti-mouse IgG or rabbit anti-rat IgG, respectively, followed by avidin–biotin complex/HRP and diaminobenzidine for developing brown staining. Slides were counterstained in hematoxylin. For every staining procedure, a negative control was prepared without the primary Ab.

Immunofluorescence analysis of CD11b, ICAM-1, and VCAM-1

The surface expression of CD11b, ICAM-1 (CD54), and VCAM-1 (CD106) were determined as described before (19) by mixing 5 × 10⁵ cells with 10 μg FITC- conjugated mouse monoclonal anti-human–CD11b (Biolegend, San Diego, CA), FITC- conjugated mouse monoclonal anti-human–ICAM-
FIGURE 1. In vivo regulation of ICAM-1 induction by cPLA₂α in inflammatory sites. A. A mouse model of CIA. A representative immunoblot analysis out of cPLA₂α, ICAM-1, and the corresponding β-actin protein expression in paw lysates of healthy controls (H), inflamed paws of CIA mice (CIA), and paws from CIA mice i.v. daily treated with 2 mg/kg AS (CIA+AS) after development of paw inflammation. There was no difference between nontreated CIA mice and CIA mice daily treated with 2 mg/kg sense that showed identical results), and on HFD daily treated with 2 mg/kg AS. The intensity of each cPLA₂α or ICAM-1 band was divided by the intensity of each β-actin band after quantitation by densitometry, and expressed in the bar graph as arbitrary units. *Significant differences in comparison with noninflamed paw or paw from CIA mice after AS treatment, p < 0.001. B. CIA-1 immunohistochemistry of synovial sections to demonstrate the localization of increased ICAM-1 expression in mice described in A. Negative control was performed on sections from CIA mice. Original magnification ×200; ×1000 (inset). Representative results out of 10 mice in each group are shown in A and B. C. A mouse model of obesity. A representative immunoblot analysis of cPLA₂α, ICAM-1, and the corresponding β-actin protein expression in periepididymal fat tissue lysates from mice at day 3 on LFD, on HFD (nontreated or treated with 2 mg/kg sense that showed identical results), and on HFD daily treated with 2 mg/kg AS. The intensity of the cPLA₂α or ICAM-1 bands was determined as in A. *Significant differences in comparison with LFD or HFD after AS treatment, p < 0.001. D. cPLA₂α (upper panel) and ICAM-1 (lower panel) immunohistochemistry of periepididymal fat tissue sections to demonstrate the localization of increased cPLA₂α and ICAM-1 protein expression in mice described in C. Negative control was performed on sections from mice on an HFD. Original magnification ×400. Representative results out of eight mice in each group are shown in C and D.
and cPLA2α proteins was assessed in paws of healthy DBA mice (clinical score of 0 and paw thickness of 2.4 mm) in inflamed paws of DBA mice with CIA (clinical score of 3 and paw thickness of 4.2 mm) and in paws of DBA mice significantly recovered from inflammation by daily i.v. AS injection (2 mg/kg) for 7 d (clinical score of 1 and paw thickness of 3.1 mm). As shown in Fig. 1A, immunoblotting of total paw lysates showed upregulation of both cPLA2α and ICAM-1 proteins in the inflamed paws compared with a tissue of healthy control preparation. Inhibition of cPLA2α overexpression by i.v. AS injection caused a significant reduction in ICAM-1 protein expression, whereas injection of corresponding sense oligonucleotides had no effect on either cPLA2α or ICAM-1 overexpression, indicating that cPLA2α has a major role in upregulating ICAM-1 protein induction during inflammation. The role of cPLA2α in ICAM-1 upregulation was shown by immunohistochemistry staining of ICAM-1 in the mouse joints (Fig. 1B). A significant ICAM-1–positive staining is demonstrated in the vascular endothelial cells in the inflamed joint of mice with CIA compared with joints of healthy control mice. The antisense treatment that caused a significant reduction in cPLA2α upregulation in the inflamed joints of CIA mice (12) caused a significant reduction in ICAM-1–positive staining in the vascular endothelial cells. Of note, the inflammatory cells present in the inflamed joint sections of CIA mice did not express high levels of ICAM-1 and thus probably did not have a significant contribution to the elevated ICAM-1 protein expression detected in these joints by immunoblot (Fig. 1A).

The role of cPLA2α on the upregulation of ICAM-1 protein expression was further supported in a mouse model of obesity. In the recent years, obesity is increasingly accepted as a condition characterized by low-grade chronic inflammation (22). Systemically, this is evidenced by elevated levels of various inflammatory markers, including C-reactive protein, TNF-α, and IL-6, and by an activated state of circulating leukocytes (23–25). Peripheridymal adipose tissue has become recognized as an important target of inflammatory processes. We recently demonstrated (14) in C57BL/6J mice that early (3 and 7 d) after initiating obesity by an HFD, neutrophils transiently infiltrated the peripheridymal adipose tissue. Thus, we first studied whether there is cPLA2α and ICAM-1 overexpression in peripheridymal fat tissue in mice on HFD and then determined the role of cPLA2α in upregulation of ICAM-1 in this tissue by i.v. injection of AS. Immunoblot analysis, presented in Fig. 1C, shows that the protein expression of cPLA2α and ICAM-1 were elevated in peripheridymal adipose tissue of mice lysates kept for 3 d on an HFD compared with mice fed a normal LFD for 3 d. Intravenous injection of AS (2 mg/kg) 1 d prior and every day during the 3 d of HFD resulted in prevention of both cPLA2α and ICAM-1 overexpression in the peripheridymal adipose tissue lysates. The immunohistochemistry staining of cPLA2α and ICAM-1 (Fig. 1D) showed that the positive staining was detected mainly in the endothelial vascular cells derived from fat tissue of HFD mice. The expression of cPLA2α and ICAM-1 protein was much higher in fat tissue of HFD mice compared with that of mice fed a normal diet. AS treatment that inhibited cPLA2α upregulation in fat tissue of mice maintained on an HFD also inhibited ICAM-1 upregulation. Collectively, these results further support the suggestion that cPLA2α upregulates endothelial ICAM-1 protein expression.

cPLA2α regulates TNF-α induction of ICAM-1 upregulation in ECV-304 cell line

To determine the role of cPLA2α in ICAM-1 expression in endothelial cells and in the adherence process to inflammatory cells, we used the ECV-304 cell line. ECV-304 is a spontaneously trans-
various cell types (10). To explore which pathway is initiated by PGE2 in the induction of ICAM-1 protein, the expression of these four EPs was examined by RT-PCR using specific primers. As demonstrated in Fig. 3B, EP3 and EP4 receptor mRNA were significantly expressed in ECV-304 cells, whereas EP1 and EP2 receptor mRNA were not detected. To study the role of PGE2 in ICAM-1 protein expression, the production of PGE2 was inhibited by a COX inhibitor, indomethacin. Addition of 30 μM indomethacin to the ECV-304 cells for 24 h caused a significant reduction in ICAM-1 expression in total cell lysates (Fig. 3C) and in ICAM-1 membrane surface expression (Fig. 3D) in the presence and absence of TNF-α. The presence of PGE2 (15 μM) significantly restored the reduced ICAM-1 protein expression in the presence of indomethacin in TNF-α-treated cells (Fig. 3C, 3D), further supporting a role of PGE2 in ICAM-1 upregulation. PGE2 by itself (in the absence of TNF-α) did not induce ICAM-1 protein expression, indicating that it is required but not sufficient for this process. The similar inhibitory effect caused by the pre-
sence of indomethacin (Fig. 3C, 3D) and of AS (Fig. 2C, 2D) on ICAM-1 expression strengthen the view that cPLA$_{2\alpha}$ is the isozyme responsible for PGE$_2$ production in these cells. It was reported that cPLA$_{2\alpha}$ regulates COX-2 expression in a mouse model of cPLA$_{2\alpha}$ knockout, though through a yet-unknown mechanism (32). Thus, the time dependency of TNF-\(\alpha\) triggered cPLA$_{2\alpha}$ activation and upregulation, and COX-2 and ICAM-1 protein induction were determined. As shown in Fig. 3E, cPLA$_{2\alpha}$ was significantly activated, as evidenced by its phosphorylation on Ser$^{505}$, detected already at the first time point assayed, 1 h, whereas a significant upregulation of cPLA$_{2\alpha}$ protein expression was detected at 24 h. The appearance of COX-2 protein at 8 h of stimulation with TNF-\(\alpha\) coincided with the time-dependent release of PGE$_2$ (Fig. 3A). As shown in Fig. 3E, ICAM-1 protein was detectable at 12 h of TNF-\(\alpha\) stimulation. The presence of AS 24 h prior to addition of TNF-\(\alpha\) significantly reduced cPLA$_{2\alpha}$ protein expression and, as a result, its phosphorylated form at all time points studied. In addition, the presence of AS (Fig. 3E) prevented COX-2 upregulation, consistent with the inhibition of PGE$_2$ secretion (Fig. 3A) and the inhibition of ICAM-1 upregulation (Fig. 2C, 2D).

Because activation of EP4 has been shown to activate protein kinase A (PKA) (10), we studied the involvement of PKA in the signaling events leading to induction of ICAM-1, using H-89, a PKA inhibitor. H-89, in the range of 2.5–20 \(\mu\)M, caused a dose-dependent inhibition of TNF-\(\alpha\)-induced ICAM-1 upregulation in total cell lysates and membrane surface expression (Fig. 4A, 4B). Furthermore, the reduced expression of ICAM-1 in the presence of indomethacin in cells treated with TNF-\(\alpha\) could be restored by addition of 10 \(\mu\)M dibutyl cAMP (dbcAMP) (Fig. 4C, 4D), a cAMP analog that directly activates PKA. As shown for PGE$_2$ (Fig. 3D), addition of dbcAMP (in the absence of TNF-\(\alpha\)) did not induce ICAM-1 protein expression, suggesting that PKA activation is required but not sufficient for this process.

**Regulation of endothelial cPLA$_{2\alpha}$ activation by NADPH oxidase**

Oxidative signals play an important role in the regulation of inflammatory gene expression in endothelial cells (33). To study whether superoxides are involved in ICAM-1 upregulation by TNF-\(\alpha\) and to determine their putative role in the signal trans-

**FIGURE 4.** Involvement of PKA in TNF-\(\alpha\) induction of ICAM-1 upregulation. \(A\) and \(B\), The dose-dependent effect of a PKA inhibitor, H-89, on ICAM-1 expression induced by TNF-\(\alpha\). A representative immunoblot analysis of ICAM-1 and of the corresponding \(\beta\)-actin protein expression in cell lysates. Quantification of ICAM-1 presented by bar graphs was determined as in Fig. 1A and expressed as arbitrary units. ICAM-1 surface expression detected by FACS analysis in Fig. 2B is representative of three different experiments. \(C\) and \(D\), The inhibited ICAM-1 protein expression and ICAM-1 cell-surface exposure in cells pretreated with 30 \(\mu\)M indomethacin and stimulated by TNF-\(\alpha\) was restored by the presence of 0.3 \(\mu\)M dbcAMP. A representative immunoblot analysis of ICAM-1 and of the corresponding \(\beta\)-actin protein expression. Quantification of ICAM-1 presented by bar graphs was determined as in Fig. 1A and expressed as arbitrary units. The results presented in \(D\) are mean \(\pm\) SEM of ICAM-1 surface expression detected by FACS analysis in three experiments. *Significant reduction by indomethacin, \(p < 0.001\); **significant ICAM-1 upregulation in the presence of TNF-\(\alpha\) and by restoration with dbcAMP in the presence of indomethacin and TNF-\(\alpha\), \(p < 0.001\).
Inhibition on p65–NF-κB-dependent activation of p65–NF-κB and CREB expression in nuclear lysates. ICAM-1 upregulation induced by TNF-α involves CREB and NF-κB activation for ICAM-1 upregulation by TNF-α (5). Because our results suggested that PGE2 stimulates NF-κB binding response element (CRE) (35). In addition, PKA can phosphorylate NF-κB in the signal events leading to ICAM-1 protein induction, the effect of DPI was studied on the time-dependent effect of TNF-α on cPLA2 activation and protein expression in relation to COX-2 and ICAM-1 upregulation. As shown in Fig. 5E, the presence of DPI, similar to the effect of AS (Fig. 3E), inhibited the phosphorylation of cPLA2 during the 24 h studied, inhibited cPLA2 activation at 24 h, and prevented the induction of COX-2 and ICAM-1 protein expression. These results support the view that the activation of NADPH oxidase is upstream to cPLA2, and its inhibition attenuates cPLA2 activity.

Involvement of CREB and NF-κB in the induction of ICAM-1 mediated by cPLA2α

It has been shown that PKA can phosphorylate NF-κB for its binding to cAMP response elements (CRE) (35). In addition, PKA has been reported to phosphorylate the p-65 subunit of dimeric NF-κB (36), leading to NF-κB binding response element in gene promoters. ICAM-1 gene promoters contain several NF-κB response elements (37). Moreover, it was reported that NF-κB is bound to ICAM-1 promoter and is crucial for the induction of ICAM-1 (5). Because our results suggested that PGE2 stimulates the PKA pathway, we speculated that CREB also participates in induction of ICAM-1 protein induction, we first assayed the effect of TNF-α on superoxide generation. As shown in Fig. 5A, TNF-α caused an immediate and significant release of superoxide as determined by DHE reduction, which was totally inhibited in the presence of diphenylene iodonium (DPI) (10 μM). Because our previous studies have shown that cPLA2α regulates the NAD (PH oxidase 2 (NOX2)-NADPH oxidase in phagocytic cells (12, 16), we studied whether cPLA2α regulates NOX4-NADPH oxidase present in endothelial ECV-304 cells (34). Preincubation of endothelial cells with AS that inhibited cPLA2α expression did not affect superoxide production triggered by TNF-α (Fig. 5B), indicating that NOX4-NADPH oxidase is not regulated by cPLA2α. Moreover, activation of cPLA2α determined by its phosphorylation detected as early as 10 min of exposure to TNF-α was inhibited in the presence of DPI (Fig. 5C), indicating that cPLA2α activation is regulated by NOX4-NADPH oxidase in endothelial cells. To support this view, the effect of DPI on the release of PGE2 by TNF-α was determined. As shown in Fig. 5D, DPI caused a total inhibition of the time-dependent PGE2 secretion induced by TNF-α, which was identical to that caused by inhibition of cPLA2α by means of AS (Fig. 3A). To further determine the role of superoxides in the signal events leading to ICAM-1 protein induction, the effect of DPI was studied on the time-dependent effect of TNF-α on cPLA2α activation and protein expression in relation to COX-2 and ICAM-1 upregulation. As shown in Fig. 5E, the presence of DPI, similar to the effect of AS (Fig. 3E), inhibited the phosphorylation of cPLA2α during the 24 h studied, inhibited cPLA2α upregulation at 24 h, and prevented the induction of COX-2 and ICAM-1 protein expression. These results support the view that the activation of NADPH oxidase is upstream to cPLA2α, and its inhibition attenuates cPLA2α activity.
expressed in the bar graph, and was performed as described in Fig. 1. Quantitation of four independent experiments is presented, indicating that cPLA₂ is inhibited by AS treatment (p<0.001; **significant reduction by AS treatment, p<0.0001). A detailed time-dependent phosphorylation of p65–NF-κB revealed that p65 was phosphorylated onSer(236) and on Ser(273), but not on Ser(237), and detected at 1 h and at 8–10 h, whereas phosphorylated CREB was detected at 10 h only (Fig. 6A). To study the role of cPLA₂ and PKA on these phosphorylation events, the cells were preincubated with 1 μM AS or 10 μM PKA inhibitor (H-89) before addition of 10 ng/ml TNF-α for 1 or 10 h (Fig. 6B). Preincubation with AS inhibited the presence of phospho-p65 NF-κB at both 1 and 10 h and of phospho-CREB detected at 10 h, indicating that cPLA₂ is involved in both the early and late phosphorylation events of p65 and in the phosphorylation of CREB. The presence of H-89 inhibited the phosphorylation of CREB, as expected, and the phosphorylation of p65 at 10 h but not at 1 h, suggesting that the late phosphorylation of p65 may be mediated also by a PKA-dependent process. Inhibition of NADPH oxidase activity by either DPI or apocynin prevented p65 phosphorylation at 1 h and 10 h and CREB phosphorylation at 10 h (Fig. 6C), similar to the effect of the AS (Fig. 6B). To study which event of NF-κB activation is involved in the signal transduction of ICAM-1 induction (the early at 1 h or the late at 10 h), the NADPH oxidase inhibitor was added at 2 h of activation with TNF-α after termination of the first event of p65 phosphorylation (Fig. 6A). As shown in Fig. 6D, inhibition of the second event of NF-κB by DPI did not affect ICAM-1 expression or COX-2 upregulation, indicating that only the first event related to p65–NF-κB activation is involved in the signaling of ICAM-1 expression but not a p65 phosphorylation event at 10 h. Interestingly, although inhibition of the first event of p65 phosphorylation by DPI (Fig. 6C) inhibited COX-2 upregulation, CREB phosphorylation, and ICAM-1 expression, addition of DPI at 2 h of stimulation, after termination, the first event of phosphorylation caused inhibition of the second event of p65 phosphorylation but had no effect on CREB phosphorylation (Fig. 6D), suggesting that the early p65 phosphorylation is required for COX-2 upregulation and detected by FACs analysis from three independent experiments (D) in cells treated as in A. Significance as in B, E. The role of endothelial cPLA₂ on adherence to phagocytes. Neutrophil- or monocyte-like PLB cells were stimulated with PMA (50 ng/ml) for 5 min prior to their addition to HUVEC cells untreated or pretreated with AS or correspondence sense (SE) in the presence and absence of TNF-α for 24 h as in A. Adherence was determined as described in the Materials and Methods and expressed as the percent of differentiated PLB cells that adhered to HUVEC (% Adhesion). The results are the mean ± SEM from four independent experiments. The presence of TNF-α increased the adherence in each treatment (p<0.05). The adherence was undetectable in the absence of TNF-α. F. Time-dependent activation of cPLA₂ and induction of cPLA₂, COX-2, ICAM-1, and VCAM-1 protein expression—all were inhibited by the pretreatment with 0.1 μM AS for 24 h before addition of TNF-α. Shown are representative immunoblots out of three independent experiments that gave similar results. cPLA₂ activation as detected by its phosphorylation on Ser(205) was determined by dividing the intensity of each phospho-cPLA₂ band by the intensity of each corresponding cPLA₂ band after quantitation by densitometry. Quantitation of COX-2, ICAM-1, and VCAM-1 presented in bar graphs was determined as in Fig. 1. (A)
CREB phosphorylation occurring at 10 h of TNF-α stimulation and required for ICAM-1 upregulation.

To study whether the activation of CREB in the absence of NF-κB activation is sufficient to induce ICAM-1 upregulation, PGE₂ was added to cells pretreated with the AS and stimulated with TNF-α. As shown in Fig. 6E, under these conditions, only CREB but not NF-κB was phosphorylated, but this phosphorylation was not sufficient for ICAM-1 induction.

cPLA₂α regulates TNF-α induction of ICAM-1 and VCAM-1 upregulation in HUVEC

The role of cPLA₂α in the induction of the adhesion molecules was documented also in primary endothelial cells, HUVEC, that can express both ICAM-1 and VCAM-1. As shown in Fig. 7A, preincubation with AS caused a significant reduction in cPLA₂α protein expression and prevention of cPLA₂α upregulation induced by TNF-α, whereas sense had no effect. cPLA₂α activity was elevated ~2-fold by TNF-α, and this increased activity was prevented in the presence of AS but not sense (Fig. 7B). Addition of TNF-α induced a robust upregulation of ICAM-1 (Fig. 7C, 7D) compared with that induced in the ECV-304 cell line (Fig. 2C, 2D) and of VCAM-1 proteins, whereas the presence of AS reduced their protein expression. Addition of the corresponding sense that did not inhibit cPLA₂α upregulation (Fig. 7A) had no effect on ICAM-1 or VCAM-1 protein expression (Fig. 7C, 7D). The presence of DPI caused inhibition of both ICAM-1 and VCAM-1 induction by TNF-α in HUVEC (Fig. 7C, 7D). The reduction in ICAM-1 and VCAM-1 protein expression in HUVEC in the presence of AS resulted with attenuation of the adherence to granulocyte- and monocyte-PLB cells (Fig. 7E). The time-course effect of TNF-α on cPLA₂α activation and upregulation as well as on COX-2, ICAM-1, and VCAM-1 upregulation in HUVEC cells is presented in Fig. 7F. Similar to ECV-304 cells (Fig. 3E), cPLA₂α activation determined by its phosphorylation on Ser⁵⁰⁵ was detected in HUVEC already at the first time point assayed, 1 h, whereas a significant upregulation of cPLA₂α protein expression was detected at 12 h. COX-2 protein was detected at 8 h of stimulation with TNF-α, whereas ICAM-1 and VCAM-1 proteins were detected at 10 h and 12 h, respectively. The presence of AS 24 h prior to addition of TNF-α significantly reduced cPLA₂α expression.
protein expression and, as a result, its phosphorylated form at all time points studied. The presence of AS prevented COX-2 as well as ICAM-1 and VCAM-1 upregulation.

**Phagocyte cPLA_2 and NADPH oxidase are not involved in the adherence process**

The counter partner of endothelial ICAM-1 is the phagocyte CD11b. To study the role of phagocyte cPLA_2α on the adherence process to endothelial cells, its activity was inhibited by AS. Addition of 1 μM AS at the third day of differentiation to either granulocyte- or monocyte-like cells for 24 h caused a significant inhibition in cPLA_2α expression (Fig. 8A) and sp. act. in unstimulated cells (Fig. 8B). Pretreatment with AS reduced superoxide production (Fig. 8C), but did not change the surface expression of CD11b in differentiated PLB cells before and after stimulation with PMA (Fig. 8D) nor their adherence to ECV-304 cells (Fig. 8E). These results suggest that phagocyte cPLA_2α and the NOX2-NADPH oxidase are not involved in regulation of ICAM-1 protein induction, in contrast to endothelial cPLA_2α and NOX4-NADPH oxidase that regulate ICAM-1 upregulation and thus the adherence process. The results of the current study are in accordance with our earlier study (14) that demonstrated that inhibition of NADPH oxidase in mouse neutrophils did not affect CD11b cell exposure and their adherence to adipocytes.

**Discussion**

The results of the current study demonstrate that cPLA_2α has a central role in ICAM-1 overexpression during inflammation both in vivo and in vitro systems. As shown in Fig. 1, ICAM-1 upregulation in the inflamed paws of CIA mice and in peri-epididymal adipose tissue in mice fed an HFD coincided with the upregulation of cPLA_2α expression, in contrast to endothelial cPLA_2α upregulation, by i.v. injection of AS against cPLA_2α, in inflamed tissues in both mouse models resulted in prevention of ICAM-1 upregulation. The immunohistochemistry analysis of the peri-epididymal adipose tissue of mice fed an HFD diet showed elevated cPLA_2α and ICAM-1 protein expression mainly on the vascular cell membranes that were significantly lower in mice treated with AS. The inhibition of the elevated ICAM-1 protein expression in the vascular endothelial cell membranes is the inflamed joints of CIA mice by the AS treatment further demonstrates the significant role of cPLA_2α in ICAM-1 upregulation during inflammation. In our previous study in DBA mice with CIA (12), we have demonstrated the i.v. administration of AS that reduced cPLA_2α protein expression in inflamed paws also inhibited the recruitment of neutrophils to the inflamed site through cPLA_2α-dependent LTB_4 production. The present study suggests that cPLA_2α may also control neutrophil recruitment by an additional mechanism (i.e., regulating ICAM-1 protein induction).

The role of cPLA_2α in the transduction events leading to induction of ICAM-1 protein expression was elucidated in the endothelial cell line, ECV-304, that expresses only this adhesion molecule and documented in HUVEC for both ICAM-1 and VCAM-1. Similar to the results in the in vivo models, AS against cPLA_2α that efficiently prevented TNF-α-induced increase in cPLA_2α expression and activity (Figs. 2A, 2B, 7A, 7B) also prevented the induction of ICAM-1 protein in ECV-304 cells (Fig. 2C, 2D) and both adhesion molecules in HUVEC (Fig. 7C, 7D). The role of cPLA_2α in VCAM-1 upregulation shown in HUVEC suggests that cPLA_2α may serve as a target not only for treatment of inflammation but also as a target for intervention in atherosclerosis.

The involvement of various PLA2s in ICAM-1 expression in vitro systems was reported in earlier studies (39, 40), although understanding the molecular events has not yet been elucidated. In addition to the reported involvement of NF-κB in TNF-α induction of ICAM-1 and VCAM-1 upregulation (5, 6), to our knowledge the results of the current study are the first to demonstrate that CREB transcription factors are also required for ICAM-1 upregulation and that both transcription factors are regulated by cPLA_2α. We report in this study that ICAM-1 gene promoter contains a single CRE binding domain, extending between 2234 and 2246 bp. Although VCAM-1 gene promoter does not contain a CRE binding domain, it was reported that inhibition of CREB signaling by overexpression of a dominant-negative form of CREB suppresses TNF-α-induced VCAM-1 in bovine aortic endothelial cells (41). The presence of the AS against cPLA_2α that inhibited cPLA_2α overexpression and activity and the induction of ICAM-1 protein upregulation in ECV-304 cells (Fig. 2A–D) also inhibited the activation of both transcription factors NF-κB and CREB, as determined by their phosphorylated forms in the nuclear fraction (Fig. 6B). As shown previ-
ously, CREB transcription factor undergoes activation by PKA-dependent phosphorylation on Ser\textsuperscript{133} (42), whereas p65 NF-kB ReLA is phosphorylated by PKA on Ser\textsuperscript{382} (42, 43) or by a redox-sensitive mechanism on Ser\textsuperscript{526} (44–46). The present study demonstrates a PKA-dependent phosphorylation of CREB on Ser\textsuperscript{133} at 10 h and two events of phosphorylation of p65 NF-kB on Ser\textsuperscript{526} at 1 h and 8–10 h of TNF-\alpha exposure, whereas phosphorylation of p65 NF-kB Ser\textsuperscript{526} was not detected under these conditions, suggesting that PKA is not involved in NF-kB activation. Only the first event of p65 NF-kB phosphorylation is required for the induction of ICAM-1, as its inhibition by addition of NADPH oxidase inhibitors (DPI or apocynin) or cPLA\textsubscript{2}\alpha inhibitor (AS) prior to exposure to TNF-\alpha prevent the induction of ICAM-1, whereas inhibition of the second phosphorylation event by addition of DPI at 2 h of stimulation with TNF-\alpha, after the termination of the first phosphorylation event, did not affect ICAM-1 expression (Fig. 6D). The sensitivity of p65 NF-kB phosphorylation on Ser\textsuperscript{526} to DPI or apocynin could suggest that activation of NF-kB is mediated by NADPH oxidase-producing superoxides as suggested previously (45). However, in the presence of AS against cPLA\textsubscript{2}\alpha, superoxides were produced normally (Fig. 5B), yet the phosphorylation of p65 NF-kB on Ser\textsuperscript{526} was inhibited (Fig. 6B), indicating that NF-kB phosphorylation is not mediated directly by superoxides. Because cPLA\textsubscript{2}\alpha was found to be activated by superoxide production as it was sensitive to DPI (Fig. 5C, 5E), it is possible that the phosphorylation of NF-kB is mediated by cPLA\textsubscript{2}\alpha and thus can be prevented either in the presence of AS or DPI, as both reduce cPLA\textsubscript{2}\alpha activity, although the kinase involved in this phosphorylation process is not yet identified. In accordance with our suggestion, it was reported (40) that LT\textsubscript{4} produced AA release by cPLA\textsubscript{2} is secreted and induces cPLA\textsubscript{2}\alpha phosphorylation through leukotriene receptors resulting in NF-kB activation via an yet unknown mechanism. Our present study suggests that the cPLA\textsubscript{2}\alpha-dependent AA metabolite that regulates ICAM-1 expression is PGE\textsubscript{2}. The presence of AS or DPI, which inhibited cPLA\textsubscript{2}\alpha upregulation and activation (Figs. 2A, 2B, 5C, 5E), inhibited the release of PGE\textsubscript{2} detected 8 h after addition of TNF-\alpha (Figs. 3A, 5D) and the induction of ICAM-1 protein expression (Figs. 2C, 2D, 5E). Likewise, the presence of a COX inhibitor, indomethacin, that inhibits PGE\textsubscript{2} secretion (Fig. 3A) inhibited ICAM-1 upregulation by TNF-\alpha (Fig. 3C, 3D). This inhibition was removed by addition of PGE\textsubscript{2} probably by activation of PKA. The suggestion that PKA pathway participates in signaling ICAM-1 induction is supported by the expression of EP\textsubscript{4} (Fig. 3B), the inhibition of ICAM-1 induction by a specific PKA inhibitor (Fig. 4A, 4B), and the restoration of the inhibited ICAM-1 protein expression in cells pretreated with indomethacin by a cAMP analog (Fig. 4C, 4D), which directly activates PKA. The participation of PKA in ICAM-1 production is in accordance with a previous study in HUVEC (47). The results of the current study shown in Fig. 6 are in agreement with the known role of CREB stimulation by PKA in cAMP-mediated activation of gene transcription (48). CREB phosphorylation on Ser\textsuperscript{133} was PKA dependent (Fig. 6B), and it was obligatory for ICAM-1 upregulation by TNF-\alpha (Fig. 4A, 4B). However, activation of CREB is not sufficient to induce ICAM-1, as PGE\textsubscript{2} could not restore the inhibited ICAM-1 upregulation caused by the presence of either AS (Fig. 6E) or DPI (not shown) that inhibited the first event of NF-kB activation. Likewise, as shown in Figs. 3C, 3D, 4C, and 4D, there was no induction of ICAM-1 by addition of PGE\textsubscript{2} or dbcAMP (both known to phosphorylate CREB) in the absence of TNF-\alpha. These results suggest that CREB phosphorylation, although required, is not sufficient for ICAM-1 induction, and the first event of NF-kB activation is also necessary. The inhibition of CREB phosphorylation in the presence of DPI or AS in cells stimulated with TNF-\alpha (Fig. 6C) raises the possibility that the first phosphorylation event of p65–NF-kB on Ser\textsuperscript{382} is required for the phosphorylation of CREB, in addition to its role in activation of ICAM-1 promoter (5, 6). Thus, it might possibly be that the activation of NF-kB at 1 h that is dependent on cPLA\textsubscript{2}\alpha, as is inhibited in the presence of AS (Fig. 3B), is also required for COX-2 induction and responsible for the elevated release of PGE\textsubscript{2} that in turn phosphorylates CREB through activation of PKA. The time-dependent formation of COX-2 and PGE\textsubscript{2} release at 8 h, CREB phosphorylation at 10 h, and ICAM-1 upregulation at 24 h of TNF-\alpha stimulation and their inhibition in the presence of DPI or AS that inhibits the first NF-kB activation support this suggestion. The mechanism by which cPLA\textsubscript{2}\alpha regulates COX-2 upregulation is not yet clear. However, it was reported (49) that COX-2 induction in smooth muscle cells is mediated by the CRE through an autocrine loop involving endogenous PGE\textsubscript{2} produced by COX-1 using AA liberated by cPLA\textsubscript{2}\alpha.

In conclusion, the current study demonstrates, as summarized in Fig. 9, that addition of TNF-\alpha to endothelial cells stimulates the production of superoxides probably by NOX4-NADPH oxidase that causes cPLA\textsubscript{2}\alpha activation detected as early as 10 min. cPLA\textsubscript{2}\alpha is involved in the first event of NF-kB activation required for ICAM-1 induction, at 1 h of TNF-\alpha stimulation. cPLA\textsubscript{2}\alpha inhibition by means of AS did not affect the normal production of superoxides by NOX4-NADPH oxidase triggered by TNF-\alpha, yet NF-kB phosphorylation and ICAM-1 upregulation were attenuated, suggesting that superoxides do not directly activate NF-kB but do so through cPLA\textsubscript{2}. cPLA\textsubscript{2}\alpha is also involved in a later stage of ICAM-1 induction, by liberating AA and by regulating the COX-2 protein induction (Fig. 3D). At 8 h of TNF-\alpha stimulation, there is a significant release of PGE\textsubscript{2} which coincided with the protein expression of COX-2 (both inhibited in the presence of AS). The activation of PKA by PGE\textsubscript{2} through EP\textsubscript{4} stimulates CREB phosphorylation on Ser\textsuperscript{133}. Phosphorylated CREB is required but is not sufficient for ICAM-1 induction. p65 NF-kB phosphorylation on Ser\textsuperscript{382} probably participates in ICAM-1 promoter activation as reported before. However, this event is not sufficient to induce ICAM-1 upregulation, as in its presence and absence of CREB activation, by a PKA inhibitor, there is no induction of ICAM-1. Thus, we suggest that two sequential cPLA\textsubscript{2}\alpha-dependent synchronized activation processes of two transcription factors NF-kB and CREB participate in upregulation of ICAM-1 expression.

Disclosures

The authors have no financial conflicts of interest.

References


