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Identification of the phospholipase A_2 isoforms that contribute to arachidonic acid release in hypoxic endothelial cells: limits of phospholipase A_2 inhibitors

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Abstract

Changes in endothelium functions during ischemia are thought to be of importance in numerous pathological conditions, with, for instance, an increase in the release of inflammatory mediators like prostaglandins. Here, we showed that hypoxia increases phospholipase A_2 (PLA_2) activity in human umbilical vein endothelial cells. Both basal PLA_2 activity and PG synthesis are sensitive to BEL and AACOCF3, respectively, inhibitors of calcium-independent PLA_2 ($iPLA_2$) and cytosolic PLA_2 ($cPLA_2$), while OPC, an inhibitor of soluble PLA_2 ($sPLA_2$) only inhibited the hypoxia-induced AA release and $PGF_{2\alpha}$ synthesis. Hypoxia does not alter expression of $iPLA_2$, $sPLA_2$ and $cPLA_2$ and cycloheximide did not inhibit PLA_2 activation, indicating that hypoxia-induced increase in PLA_2 activity is due to activation rather than induction. However, mRNA levels for $sPLA_2$ displayed a 2-fold increase after 2 hr incubation under hypoxia. BAPTA, an intracellular calcium chelator, partially inhibited the AA release in normoxia and in hypoxia. Direct assays of specific PLA_2 activity showed an increase in $sPLA_2$ activity but not in $cPLA_2$ activity after 2 hr hypoxia. Taken together, these results indicate that the hypoxia-induced increase in PLA_2 activity is mostly due to the activation of $sPLA_2$.

Keywords: Endothelial cells; Hypoxia; Phospholipase A_2; Prostaglandins; PLA_2 inhibitors

1. Introduction

In the vasculature, the synthesis of prostaglandins (PGs) is important in maintaining normal hemostasis and vascular tone. The best-known endothelium-derived inhibitor of platelet aggregation and vasodilator is PGI_2 (prostacyclin), which is the major arachidonic acid (AA) metabolite produced by most endothelial cells. Endothelial functions, including PG release, are regulated not only by humoral factors such as growth factors or cytokines, but also by physico-chemical factors such as mechanical forces including shear stress or variations in oxygen tension. Changes in endothelium functions during ischemia are thought to be of importance in numerous pathological conditions including myocardial or cerebral infarction, thrombosis and venous insufficiency.

Decreased oxygen tension (hypoxia) has been shown to activate endothelial cells leading to increased PG [1–3] and platelet-activating factor (PAF) synthesis [4,5]. This activation results in an increased adhesiveness for neutrophils [5–7] and thus to a pro-inflammatory situation. Hypoxia also modulates gene expression through the activation of transcription factors such as hypoxia-inducible factor-1 (HIF-1) for genes involved in the adaptation of cells to hypoxic conditions [8,9] or nuclear factor-κB (NF-κB) for pro-inflammatory genes like interleukin-8 (IL-8), IL-6 or cyclooxygenase-2 (COX-2) [10].

Phospholipases A_2 (PLA_2) build up a class of enzymes which catalyse the release of AA and other unsaturated

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Abbreviations: AA, arachidonic acid; AACOCF3, arachidonyl trifluoromethyl ketone; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; COX, cyclooxygenase; bFGF, basic fibroblast growth factor; BEL, bromoenol lactone; HBSS, Hank's balanced salt solution; HUVEC, human umbilical vein endothelial cells; IL-1, interleukin-1; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; OPC, oleyloxyethyl phosphorylcholine; PAF, platelet-activating factor; c, i, $sPLA_2$, cytosolic, calcium-independent, soluble phospholipase A_2; PG, prostaglandin; RT-PCR, reverse transcription-polymerase chain reaction.

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fatty acids by hydrolysing phospholipids on the *sn*-2 position [11,12]. In endothelial cells, the availability of unesterified AA is the rate-limiting step in the formation of PGs [13,14]. Several structurally and probably functionally different isoforms of PLA_2 have been defined [15,16]. At least two distinct forms of PLA_2 are present in most human cells (see [17] for review). A family of low molecular mass (14 kDa) enzymes [18], dependent on high calcium concentrations (of the mM order) has been termed secretory PLA_2 ($sPLA_2$, type IIA). It is released at inflammatory sites and is partly homologous to the mammalian pancreatic type I PLA_2 since it lacks the pancreatic loop. A second form $cPLA_2$ (type IV) has been identified initially from monocytic cells [19] and platelets and cloned from U937 cells [20,21] $cPLA_2$ is characterised by a high molecular mass (85–100 kDa) and a selectivity for arachidonate in the *sn*-2 position of phospholipids. It is activated by low concentrations (μM) of calcium and by phosphorylation by the p42-p44 MAP kinase and translocates from the cytosol to intracellular membranes where several eicosanoid generating enzymes co-localise (COX-1, COX-2, 5-lipoxygenase) [22,23]. Recently, a third class of $iPLA_2$, has been described. The $iPLA_2$ class is extremely heterogeneous, but one of the best characterised is the cytosolic $iPLA_2$, type VI, first described in macrophages [24] and cloned from Chinese hamster ovary cells [25]. It has a molecular mass of 80–85 kDa.

Despite the multiple roles of PGs in the vasculature, the regulation of their synthesis by hypoxia in endothelial cells is not well understood. Endothelial cells express several PLA_2 isoforms, the respective functions of which remain controversial. Conflicting results about basal phospholipid remodelling and stimulus-initiated AA release are emerging in the literature. The aim of this study was to identify which PLA_2 isoform(s) is (are) involved in the hypoxia-induced increase in AA release and to investigate whether this increase occurs through activation or induction of this (these) isoform(s). To address this issue, we tested the effects of different PLA_2 inhibitors on PLA_2 activity and $PGF_{2\alpha}$ synthesis, studied the expression of three different PLA_2 isoforms and measured $sPLA_2$ and $cPLA_2$ activity.

2. Materials and methods

2.1. Reagents

Modified Hank's balanced salt solution (HBSS, 140 mM NaCl, 5 mM KCl, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 3 mM $Na_2HPO_4 \cdot 2H_2O$, 0.4 mM KH_2PO_4, 5.5 mM glucose, pH 7.35) containing 1 mM $CaCl_2$ (HBSS) was prepared in our laboratory. Arachidonyl trifluoromethyl ketone (AACOCF3), bromoenol lactone (BEL) and oleyloxyethyl phosphorylcholine (OPC) came from Biomol. These were dissolved at 50 mM in DMSO and stored in aliquots at −70°. 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA)-AM came from Sigma and was dissolved in DMSO at 100 mM. Anti-$cPLA_2$ (rabbit polyclonal antibody which recognises mouse, rat and human $cPLA_2$ but no other PLA_2 isoform) was purchased from Santa Cruz, anti-$sPLA_2$ (monoclonal anti-human secretory PLA_2, specific for secretory type II PLA_2 from sperm and synovial fluid) from Upstate Biotechnology and anti-$iPLA_2$ (rabbit polyclonal type VI specific antibody which recognises human, CHO cells and murine $iPLA_2$ but neither $sPLA_2$ nor $cPLA_2$) from Cayman Chemical Company. Secondary antibodies coupled to horseradish peroxidase (anti-mouse Ig from sheep or anti-rabbit Ig from donkey) came from Amersham. Human recombinant IL-1β was from R&D.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated according to Jaffe *et al.* [26]. Cords were stored at 4° just after birth in stock solution (4 mM KCl, 140 mM NaCl, 10 mM Hepes, 1 mM glucose, 100 μg/mL streptomycin, 100 U/mL penicillin and 0.25 μg/mL fungizone, pH 7.3). Cords were rinsed with 20 mL phosphate buffer saline (PBS) containing antibiotics and fungizone at concentrations aforementioned. Umbilical veins were incubated 35 min at 37° with 0.45% dispase II (Roche) in M199 (Gibco). Collected cells were then resuspended in M199 containing 20% fetal calf serum (Gibco), centrifuged for 10 min at 1000 rpm and cultured in 0.2% gelatin-coated culture dishes (Corning/Costar). The day after, cells were washed with fresh medium in order to eliminate blood cell contamination. Only primary cultures were used for these studies. Confirmation of their identity as endothelial cells was obtained by detecting factor VIII antigen assessed by immunofluorescence staining [27].

2.3. Incubation under hypoxic conditions

Cells were seeded in gelatin-coated Petri dishes (Ø = 35 mm, Falcon Plastics) for AA release, in 75 cm^2 flasks for RNA extraction and in 25 cm^2 flasks for western blotting. For incubation, cells were rinsed twice with HBSS and covered with 0.7 mL of HBSS for incubation under normoxia or hypoxia. Medium was reduced to an uniform thin layer to decrease the diffusion distances of the atmospheric gases. Hypoxia was produced with an atmosphere of 100% N_2 in an incubator gas chamber while the control cells were kept under normal atmosphere containing 20% O_2. PO_2 in the medium was 130 mmHg in normal conditions and dropped to 10 mmHg after 15 min hypoxia as described elsewhere [28]. Hypoxia never exceeded 120 min and cells retained more than 98% viability as determined by a dye exclusion method. In order to avoid the reoxygenation effect, assays were performed or cells were lysed immediately after the hypoxia exposure, i.e. in <1 min.

2.4. Arachidonic acid release assay

Fifty thousand endothelial cells were seeded in Petri dishes (Ø = 35 mm) and radiolabeled with 0.25 μCi/mL [^{3}H]-AA (specific activity = 51.3 mCi/mol, Amersham) for 18 hr in M199 containing 0.5% serum. Thereafter, the cells were washed three times with 1 mL HBSS, then 0.7 mL HBSS was added and cells were incubated under hypoxia or normoxia, in the presence or absence of the inhibitors. After incubation, the media were collected and counted in a β-scintillation liquid counter. Remaining cells were lysed with 0.5 N NaOH and radioactivity was measured [29]. The percentage of AA release was calculated as

$$\text{AA release}\,(\%) = \frac{\text{dpm in the extracellular fluid}}{\text{dpm in the cell lysate} + \text{dpm in the extracellular fluid}}$$

2.5. $sPLA_2$ and $cPLA_2$ activity assay

$sPLA_2$ and $cPLA_2$ activities were measured using kits purchased from Cayman. After incubation in normoxia or hypoxia, cells were recovered in 100 μL lysate buffer (Tris 20 mM, KCl 150 mM, EDTA 1 mM, Triton X-100 0.05%, pH 7.5, inhibitors of proteases and phosphatases) at 4°. $sPLA_2$ activity was assayed using diheptanoyl-thio-phosphatidylcholine as substrate. $cPLA_2$ activity was assayed using arachidonoyl thio-phosphatidylcholine as substrate in the presence of BEL to inhibit $iPLA_2$. Upon hydrolysis of the thio-ester bound at the *sn*-2 position, free thiols are detected using 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) at 405 nm. Activities were calculated in μmol of substrate/min using a DNTB extinction coefficient of 10.66 mM^{-1} and are reported to μg of proteins assayed by the Bradford method.

2.6. $PGF_{2\alpha}$ assay

$PGF_{2\alpha}$ released by HUVEC was measured using an immunoassay (EIA) kit (Cayman Chemical Company). The assay was performed according to the supplier protocol. Briefly, this assay is based on the competition between free $PGF_{2\alpha}$ and $PGF_{2\alpha}$ tracer ($PGF_{2\alpha}$ linked to an acetylcholinesterase molecule). Quantification of the tracer is achieved by measuring its acetylcholinesterase activity with Ellman's reagent consisting of acetylthiocholine and DTNB. Hydrolysis of acetylthiocholine produces thiocholine, which reacts with DTNB, producing 5-thio-2-nitrobenzoic acid which has a strong absorbance at 412 nm. Absorbance was then converted in $PGF_{2\alpha}$ concentration using a standard calibration curve.

2.7. Western blotting

The cells were homogenised in lysis buffer (HEPES 25 mM, NaCl 0.3 M, $MgCl_2$ 1.5 mM, β-glycerophosphate 20 mM, EDTA 2 mM, EGTA 2 mM, dithiotreitol 1 mM, Triton X-100 1%, glycerol 10%, leupeptin 10 μg/mL; aprotinin 10 μg/mL, phenylmethoxy sulfonyl fluoride 1 mM, Na_3VO_4 1 mM, pH 7.5 at 4°) and the protein concentration was determined with the Bradford protein assay (BioRad). Loading buffer was added to achieve a final concentration of Tris–HCl 62.5 mM, SDS 2%, glycerol 10% and 2-mercaptoethanol 5% before being heated to 95° for 4 min. Twenty microgram protein for each sample were used and separated by 13% SDS-PAGE. Proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore). After incubation for 1 hr at RT in PBS containing 5% milk powder, 0.1% Tween-20 and 0.001% merthiolate to reduce non-specific binding, the membranes were washed with Tris-buffered saline (TBS)-Tween-20 (Tris 25 mM, NaCl 0.5 M containing Tween-20, 0.1%, pH 7.4) containing 0.1% milk powder and incubated with primary antibodies (1:40 for $cPLA_2$, 1:200 for $sPLA_2$ and 1:250 for $iPLA_2$) for 2 hr at RT. The secondary antibodies used were anti-mouse peroxidase-conjugated Ig (1:5000) for monoclonal antibodies or anti-rabbit peroxidase-conjugated Ig (1:5000) for polyclonal antibodies for 45 min at RT. Finally, the membranes were washed in TBS-Tween-20 and revealed using enhanced chemiluminescence (Renaissance, NEN). After autoradiography, the film was scanned by an image analysis system (Visage 101, Millipore, USA) that allows quantification of the integrated optical density (IOD) corresponding to the bands.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from HUVEC was extracted using the guanidinium thiocyanate–phenol–chloroform method [30]. The RNA concentration and purity were estimated from the optical density at 260 and 280 nm (GeneQuantII, Pharmacia). Then 0.2 μg (except for $cPLA_2$: 0.05 μg was used) of total RNA from each sample was transcribed into cDNA (60 min at 60°) and amplified by polymerase chain reaction with the one-tube Access RT-PCR system (Promega) in a thermal cycler (model 2400, Perkin-Elmer). The sequences of the primers used were $cPLA_2$ sense, 5′-GAGCTGATGTTTGCAGATTGGGTTG-3′; $cPLA_2$ antisense, 5′-GTCACTCAAAGGAGACAGTGGATAAGA-3′; $sPLA_2$ sense, 5'-ATGAAGACCCTCCTACTGTTG-3′, $sPLA_2$ antisense, 5′-GCAGCAGCCTTATCACACTCAC-3′ [31]; $iPLA_2$ sense, 5′-CCGCCTGGTCAATACCTTCA-3′; $iPLA_2$ antisense, 5′-TGGAAATTCACTAGGGCGT-CAG-3′; tubulin sense 5′-GGAAGATGCTGCCAATAA-CT-3′; tubuline antisense, 5′-TGCCATAATCAACTGAC-AGG-3′. The expected size of the amplified products was 509 bp for $cPLA_2$, 284 bp for $sPLA_2$, 242 bp for $iPLA_2$ and 200 bp for tubulin. PCR was performed for 29 cycles at 60° for $cPLA_2$, for 33 cycles at 62° for $sPLA_2$, for 30 cycles at 60° for $iPLA_2$ and for 24 cycles at 60° for tubulin. The

amplification products were quantified by separation on a 5% polyacrylamide gel. The gel was then dried and autoradiographed. After autoradiography, the film was scanned by an image analysis system (Visage 101, Millipore, USA) that allows quantification of the IOD corresponding to the bands. All values were normalised to tubulin.

2.9. Electrophoretic mobility shift assay

NF-κB activity was followed by electrophoretic mobility shift assay according to Renard *et al.* [32]. After the incubations, cells were rinsed twice with cold phosphate-buffered saline before being scraped and centrifuged for 10 min at 1000 rpm. The pellet was then resuspended in a 100 μL lysis buffer (Hepes 20 mM, NaCl 0.35 M, glycerol 20%, Nonidet P-40 1%, $MgCl_2 \cdot 6H_2O$ 1 mM, EDTA 0.5 mM, EGTA 0.1 mM). After 10 min on ice, the lysate was centrifuged for 20 min at 14,000 rpm. The supernatant constitutes the total protein extract and can be kept frozen at −70°. The binding reaction occurs in a binding mixture of 20 μL containing 2 mM Hepes (pH 7.5), 5% glycerol, 75 mM KCl, 2.5 mM dithiotreitol, 2 μg polydIC, 20 μg bovine serum albumin, cell extract (25 μg protein determined according to the Bradford assay) and a ^{32}P-labelled probe (±1 ng or 20,000 cpm). The unlabeled double-stranded NF-κB probe (5′-AGTTGAGGGGACTTTCCCAGGC) was purchased from Promega, labelled with γ-^{32}P using the T4 polynucleotide kinase and purified on a Sephacryl S-200 column. After 30 min of incubation, the binding mixture was analysed on a native 4% acrylamide gel in 0.5 × TBE (Tris 0.9 M, boric acid 0.9 M, EDTA 0.02 M). After autoradiography, the film was scanned by an image analysis system (Visage 101, Millipore, USA) that allows quantification of the IOD corresponding to the shifted bands.

2.10. Colorimetric assay for NF-κB

NF-κB DNA binding activity was measured using a colorimetric assay [33] developed by Advanced Array Technology (Belgium). The assay was performed as recommended by the supplier.

2.11. Statistical analysis

Results are presented as means ± 1 SD. Statistical analyses were performed using one-way ANOVA with Scheffé's contrasts for multiple comparison.

3. Results

3.1. Effect of inhibitors on arachidonic acid release

Under hypoxic conditions, a significant increase in AA release was observed in HUVEC compared to normoxic conditions (Fig. 1). This is concomitant with an increase in the cytosolic calcium concentration [34]. A high variation in the increase in AA release induced by hypoxia is obtained between the different experiments. However, the variation is low when measurements are performed on different dishes from the same culture. This indicates that the variation observed between the experiments is not due to the assay procedure but rather to quantitative differences in the behaviour of endothelial cells originating from different donor cords. Similar variations among primary cultures from different cords have already been reported [2,35–37].

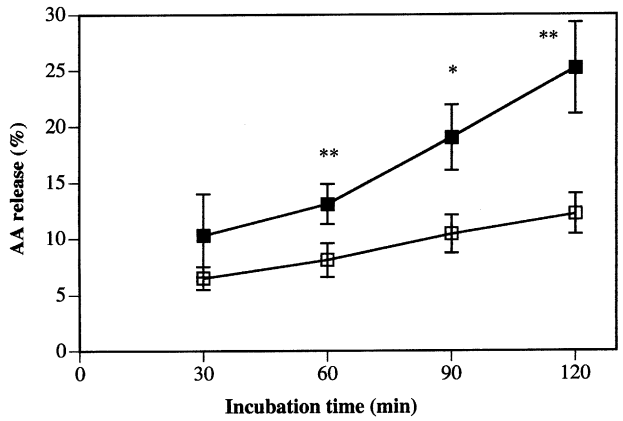

Fig. 1. Effect of hypoxia on AA release. HUVEC were incubated in normoxic (□) or hypoxic (■) conditions for different incubation times and the release of [^{3}H]-AA was measured. Results are expressed in percentage of AA release (see Section 2) as means ± 1 SD (for two experiments in triplicates, $n = 6$). (*) $P < 0.05$ or (**) $P < 0.01$ vs. normoxic control (ANOVA).

Different inhibitors have been tested to identify which PLA_2 isoform(s) is (are) involved in hypoxia. AACOCF3 specifically inhibits $cPLA_2$ and to a lesser extent $iPLA_2$ and cyclooxygenases [38]. K_i value for the inhibition by AACOCF3 for $cPLA_2$ is 5×10^{-5} mole fraction [38] while it is 0.0075 for $iPLA_2$ [39] so that AACOCF3 is 150 more potent for inhibiting $cPLA_2$ than $iPLA_2$. BEL inhibits $iPLA_2$ [39] and OPC, $sPLA_2$ [40]. BEL and AACOCF3 inhibited to some extent the basal AA release observed in normoxic conditions, with a maximal effect of 34% at 2 μM for AACOCF3 and of 43% at 5 μM for BEL (Fig. 2). The three molecules inhibited the hypoxia-induced AA release in a dose-dependent manner (Fig. 2): OPC completely inhibited the hypoxia-induced increase in AA release while BEL decreased the AA release below the level of normoxic control cells. AACOCF3 also inhibited the hypoxia-induced AA release, even taking into account that AACOCF3 also partially inhibited the basal AA release.

$cPLA_2$ and $sPLA_2$ are activated by calcium and hypoxia increases the cytosolic calcium concentration [34]. BAPTA, an intracellular calcium buffering agent, dose-dependently decreased the AA release in normoxic as well as in hypoxic conditions with a maximal inhibition at 5 μM (Fig. 3).

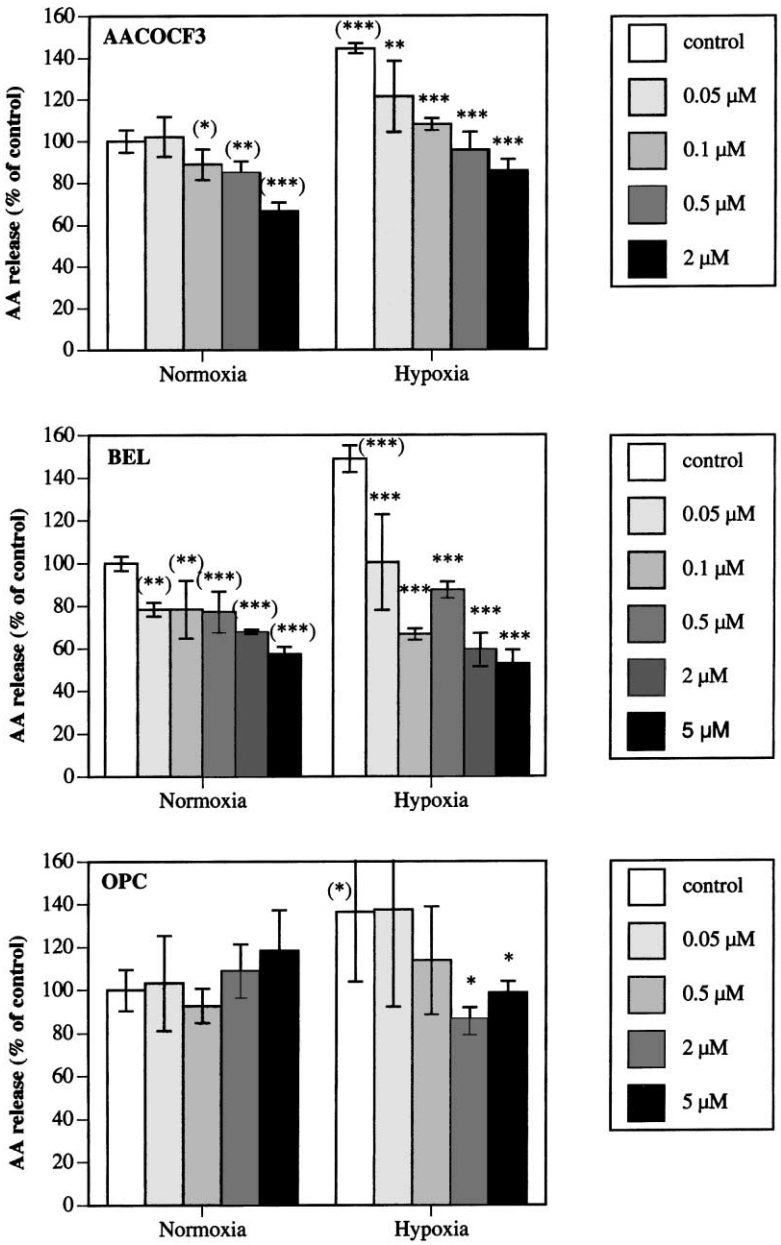

Fig. 2. Effect of PLA_2 inhibitors on basal and hypoxia-induced AA release. HUVEC were incubated 2 hr in normoxic or hypoxic conditions in the presence or absence of different concentrations of AACOCF3, BEL or OPC and the release of [^{3}H]-AA was measured. Results are expressed as percentage of normoxic controls and presented as means ± 1 SD ($n = 3$). (*) $P < 0.05$, (**) $P < 0.01$ or (***) $P < 0.001$ vs. normoxic control; (*) $P < 0.05$, (**) $P < 0.01$ or (***) $P < 0.001$ vs. hypoxic control (ANOVA with Scheffé's contrast).

3.2. Effect of inhibitors on $PGF_{2\alpha}$ synthesis

When released from phospholipids by PLA_2, AA is metabolised mainly into PGs in endothelial cells. In order to investigate which PLA_2 isoform activity is upstream of PG synthesis, the different inhibitors were tested on $PGF_{2\alpha}$ synthesis. BEL, the $iPLA_2$ inhibitor and AACOCF3, the $cPLA_2$ and $iPLA_2$ inhibitor, both inhibited the basal synthesis in normoxia of $PGF_{2\alpha}$ with 27 and 51% inhibition, respectively (Fig. 4). Interestingly, OPC, the $sPLA_2$ inhibitor, has no effect on basal $PGF_{2\alpha}$ production. As for the AA release, hypoxia led to an increased $PGF_{2\alpha}$ synthesis and all three inhibitors decreased this synthesis in hypoxic conditions (Fig. 4), but as for AA release, only OPC can be considered as an inhibitor acting on hypoxia specifically induced events, in this case, increased $PGF_{2\alpha}$ production.

3.3. Effect of hypoxia on specific PLA_2 activities

$sPLA_2$ and $cPLA_2$ activities were measured in HUVEC in hypoxia (Table 1). A significant 30% increase in $sPLA_2$ activity was observed. In contrast, $cPLA_2$ activity was highly variable but would rather decrease after 2 hr of hypoxia (Table 1). The remaining BEL-inhibitable activity

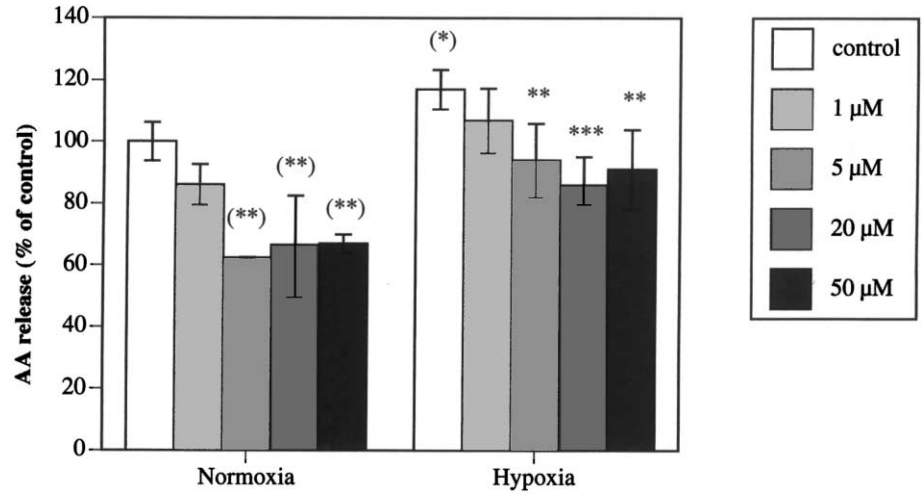

Fig. 3. Effect of BAPTA on basal and hypoxia-induced AA release. HUVEC were incubated 2 hr in normoxic or hypoxic conditions in the presence or absence of increasing concentrations of BAPTA and the release of [^{3}H]-AA was measured. Results are expressed as percentage of normoxic controls and presented as means ± 1 SD ($n = 3$). (**) $P < 0.01$ vs. normoxic control; (**) $P < 0.01$ or (***) $P < 0.001$ vs. hypoxic control (ANOVA with Scheffé's contrast).

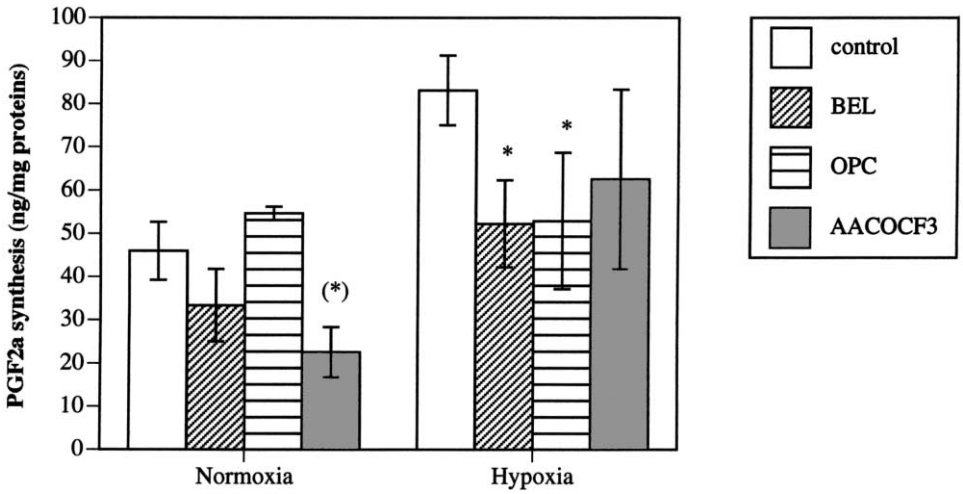

Fig. 4. Effect of PLA_2 inhibitors on $PGF_{2\alpha}$ synthesis. HUVEC were incubated 2 hr in normoxic or hypoxic conditions in the presence or absence of 2 μM AACOCF3, 0.5 μM BEL or 2 μM OPC and $PGF_{2\alpha}$ synthesis was assayed. Results are expressed in nanogram of $PGF_{2\alpha}$ per milligram proteins and presented as means ± 1 SD ($n = 3$ for normoxia and $n = 6$ for hypoxia). (*) $P < 0.05$ vs. normoxic control; (*) $P < 0.05$ vs. hypoxic control (ANOVA with Scheffé's contrast).

in the $cPLA_2$ assay, corresponding to $iPLA_2$, was very low in both conditions.

3.4. $cPLA_2$, $sPLA_2$ and $iPLA_2$ expression

Increase in PLA_2 activity during hypoxia can be the result of either activation of a protein already present in an inactive form or of induction of its expression. The hypoxia-induced increase in PLA_2 activity is insensitive to cycloheximide, a *de novo* protein synthesis inhibitor (Table 2), indicating that protein synthesis was not required. In order to confirm this result, $cPLA_2$, $sPLA_2$

Table 1
Effect of hypoxia on $sPLA_2$ and $cPLA_2$ activities[a]

	Normoxia	Hypoxia
$sPLA_2$	0.43 ± 0.05	0.55 ± 0.07*
$cPLA_2$	0.39 ± 0.19	0.18 ± 0.14

[a] HUVEC were incubated 2 hr in normoxic or hypoxic conditions in HBSS and the cells were recovered for $sPLA_2$ or $cPLA_2$ assay. Results are expressed in μmol substrate/min/μg proteins and presented as means ± 1 SD ($n = 6$ for $sPLA_2$ and $n = 5$ for $cPLA_2$).

* $P < 0.05$ vs. normoxia.

Table 2
Effect of cycloheximide on the hypoxia-induced AA release[a]

Incubation	AA release
Normoxia	100 ± 17.2
Normoxia + cycloheximide	142.0 ± 7.0
Hypoxia	149.2 ± 15.8
Hypoxia + cycloheximide	169.3 ± 16.9

[a] HUVEC were pre-incubated 4 hr in the presence or absence of 5×10^{-5} M cycloheximide in complete medium. Cells were then washed and incubated 2 hr in normoxic or hypoxic conditions in HBSS and the release of [^{3}H]-AA was assayed. Results are expressed as percentage of normoxic controls and presented as means ± 1 SD ($n = 3$).

and iPLA$_2$ expression was studied both at the mRNA and protein levels.

All three PLA$_2$ isoforms are expressed at the mRNA level in normoxic conditions, as shown by RT-PCR (Fig. 5). No increase in cPLA$_2$ and iPLA$_2$ mRNA levels (normalised to tubulin) was evidenced after 2 hr of hypoxia. On the other hand, a 80% increase of sPLA$_2$ expression was observed after 2 hr hypoxia. Longer incubation times under hypoxia cannot be tested in this model since they give rise to significant loss of viability.

Western blot analysis showed similar contents of cPLA$_2$, iPLA$_2$ and sPLA$_2$ protein in HUVEC exposed to 2 hr hypoxia in comparison to normoxia (Fig. 6). It must be noted that two bands are revealed by the Western blot analysis for cPLA$_2$, probably corresponding to the unphosphorylated and to the phosphorylated form of cPLA$_2$. However, no change in the ratio between the two forms was observed after hypoxia. This is in agreement with the observation that there is no increase in p42–p44 MAP kinase phosphorylation in hypoxia compared to normoxia (data not shown).

Since the data obtained by RT-PCR suggest that sPLA$_2$ may be induced by hypoxia, and given the fact that COX-2 is induced by hypoxia in human microcirculation endothelial cells through the activation of NF-κB [42,43], we checked whether there is an activation of the transcription factor NF-κB in the experimental

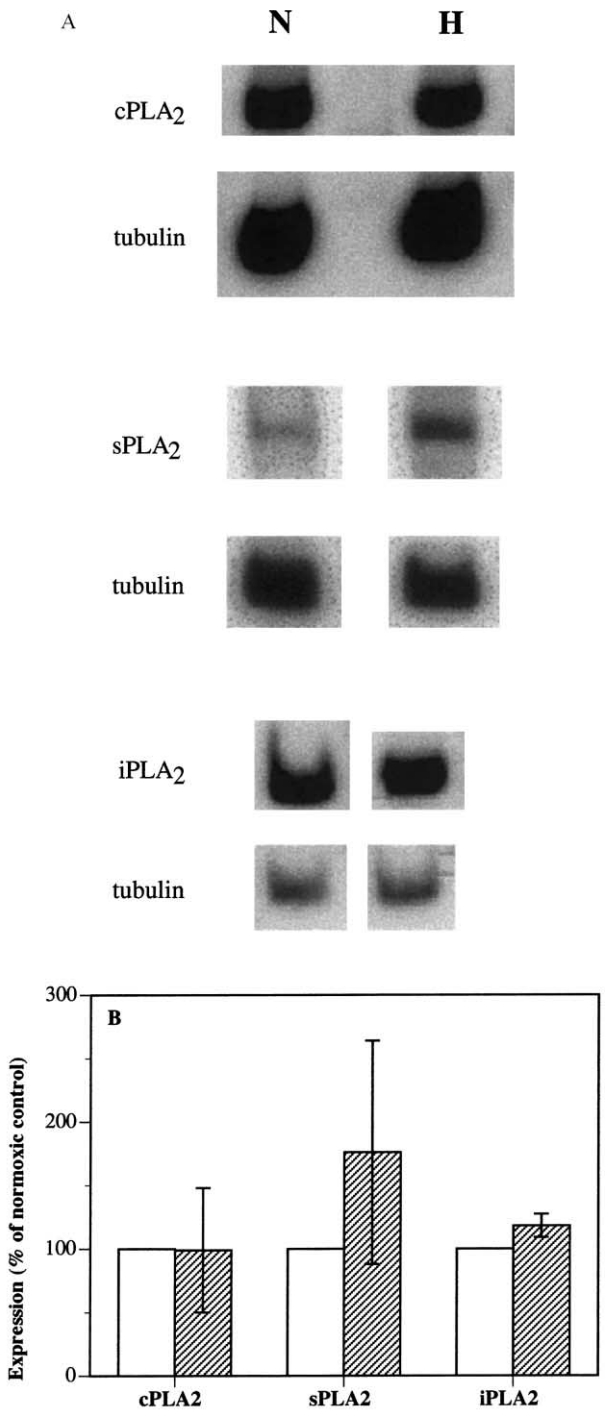

Fig. 5. Effect of hypoxia on mRNA expression of cPLA$_2$, sPLA$_2$ and iPLA$_2$ in HUVEC. Cells were incubated 2 hr in normoxic or hypoxic conditions. (A) Representative RT-PCR analysis (N: normoxia; H: hypoxia). (B) Normalised mRNA level determined by RT-PCR analysis. Values of normoxic control are taken as 100%. Results are presented as means ± 1 SD ($n = 4$).

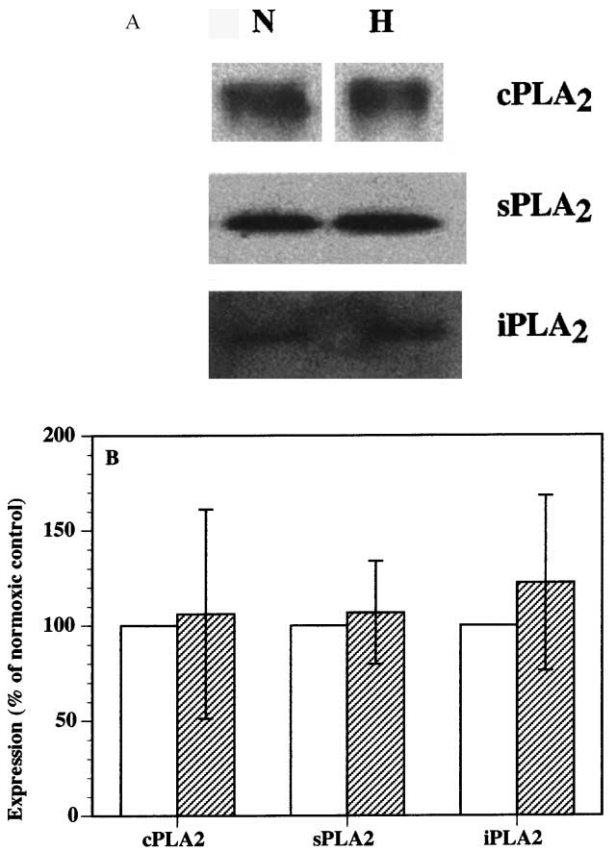

Fig. 6. Effect of hypoxia on protein expression for cPLA$_2$, sPLA$_2$ and iPLA$_2$ in HUVEC. Cells were incubated 2 hr in normoxic or hypoxic conditions. (A) Representative western blot (N: normoxia; H: hypoxia). (B) Protein expression determined by western blot analysis. Values of normoxic control are taken as 100%. Results are presented as means ± 1 SD ($n = 4$).

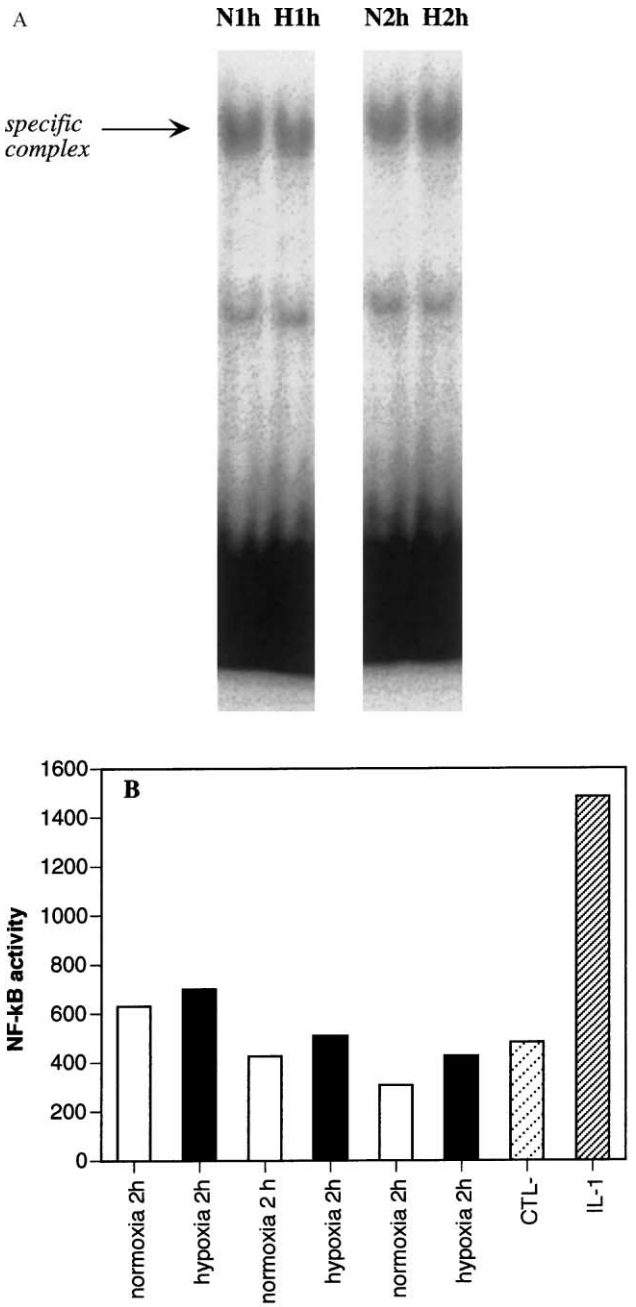

Fig. 7. Effect of hypoxia on NF-κB activation. Cells were incubated either 1 or 2 hr in normoxic or hypoxic conditions or 30 min in the presence or absence of 5 ng/mL IL-1β. (A) NF-κB DNA binding activity was assayed by EMSA (N: normoxia; H: hypoxia). (B) NF-κB DNA binding activity was assayed using a colorimetric assay in a multiwell plate (three different experiments performed on three different HUVEC strains are represented for 2 hr of hypoxia).

conditions used in this work, that if present, could be responsible for $sPLA_2$ induction. Fig. 7 shows that while IL-1β (5 ng/mL, 30 min), used as a positive control, was able to induce the activation of NF-κB, hypoxia (1 or 2 hr) did not increase the DNA-binding activity for NF-κB as measured by EMSA or using a colorimetric assay.

4. Discussion

Endothelial cells are activated by hypoxia mimicking ischemic conditions which occurs following blood flow impairment. This activation may be responsible at least in part for the inflammatory reaction taking place in ischemic organs. This activation leads to an increase in cytosolic calcium concentration [34] and in AA release. In parallel, PG and PAF synthesis is markedly stimulated leading to the recruitment of neutrophils [44] and to their adherence to the endothelial cells [5]. PLA_2 activity, because it controls the availability of AA and lysoPAF as precursors, is rate-limiting for the synthesis of PGs and PAF. Different isoforms of PLA_2 are present in mammalian cells. A better understanding of the mechanism leading to the hypoxia-induced increase in PLA_2 activity in endothelial cells would be very useful not only for a better comprehension of the pathophysiological events involved but also for developing new intervention strategies to limit the release of inflammatory mediators in ischemic organs.

Our present data show that human endothelial cells contain at least three isoforms of PLA_2: $cPLA_2$, $sPLA_2$ IIA and $iPLA_2$. The three enzymes are detected both at the mRNA and protein levels in resting normoxic cells. Expression of $cPLA_2$ and of $sPLA_2$ has already been reported in HUVEC [45]. $iPLA_2$ expression has been described in macrophages [24], neutrophils [46], mast cells [47] and myocytes [48]. This study is the first demonstration of the expression of $iPLA_2$ in HUVEC. However, Creer and McHowat [49] previously described calcium-independent PLA_2 activity in pig aorta endothelial cells.

Hypoxia is able to increase the AA release of endothelial cells in a time-dependent way. This increase in AA release is probably the result of the activation of enzymes already present within the cells, since it is not prevented by cycloheximide and since the protein level of all three isoforms did not change after 2 hr of hypoxia. It must be stressed, however, that while $cPLA_2$ and $iPLA_2$ mRNA levels did not change, an increase in $sPLA_2$ mRNA level was detected. This result suggests that $sPLA_2$ would probably be induced by longer exposure times to hypoxic conditions. This isoform has indeed shown to be induced by IL-1 in osteoblasts [50], astrocytes [51] or fibroblasts [52] or by a non-steroidal anti-inflammatory drug in mesangial cells [53] and in vivo in adjuvant arthritis in rats [54].

Hypoxia induces the expression of several genes through the activation of the transcription factor HIF-1 (for a review, see [55]). However, NF-κB seems to be the transcription factor responsible for the hypoxia-induced induction of pro-inflammatory genes such as COX-2 [42,43] or IL-8 [56]. While $sPLA_2$ mRNA level seemed to increase after 2 hr of hypoxia, NF-κB was not activated. Further investigation is thus required to determine

whether another transcription factor is involved or whether mRNA stability is increased. A recent study showed that $sPLA_2$ can also be induced by interleukin-1 (IL-1). This induction is rapid, within 2 hr, and does not require NF-κB but involves the transcription factor C/EBPdelta [57].

Hypoxia increases the cytosol calcium concentration in HUVEC within the same time frame as the one observed for PLA_2 activation [34]. The intracellular calcium chelator BAPTA partially inhibited the AA release in normoxia as well as in hypoxia, indicating that calcium-dependent isoform(s) are involved. The use of the inhibitors indicates that $iPLA_2$ and $cPLA_2$ could be involved in the basal AA release, while all three inhibitors decreased the hypoxia-induced increase in AA release. However, since AACOCF3 also inhibits to a lower extent $iPLA_2$ in addition to $cPLA_2$ and BEL also inhibits other enzymes than $iPLA_2$, other more specific approaches for example using antisense oligonucleotides may be used to confirm these data. An interesting observation is that BEL, an inhibitor of $iPLA_2$, inhibits the basal AA release in normoxia and decreased the hypoxia-induced AA release below the level of normoxic control. These results suggest that this isoform may be involved in the basal AA release in resting cells. This is in agreement with observations by Balsinde *et al.* [58] showing that $iPLA_2$ plays a major role in regulating phospholipid remodelling by providing lysophospholipid acceptor molecules required for the reacylation reaction in phospholipid turnover. $cPLA_2$ is also probably involved in the basal AA release since BAPTA was able to inhibit this release by 40%. $cPLA_2$, to be fully active, requires phosphorylation and MAP kinase appears to be critical in this phosphorylation [41,59,22], as shown in endothelial cells stimulated with vascular endothelial growth factor [60], basic fibroblast growth factor (bFGF) [61] or by shear stress [62]. However, our results suggest that $cPLA_2$ is not activated by hypoxia in HUVEC.

All together, these results indicate that $sPLA_2$ is the main isoform, specifically activated by hypoxia. However, $iPLA_2$ also clearly participates to AA metabolism in hypoxic cells, as demonstrated by the effects of BEL on AA release and $PGF_{2\alpha}$ synthesis. This is also in agreement with the limited effect of BAPTA in hypoxic cells.

Histamine also triggers AA release in endothelial cells. This release is inhibited by AACOCF3 and by BAPTA, indicating that the Ca-dependent $cPLA_2$ is mainly responsible for this response [63]. Compared to hypoxia, histamine induces a much faster stimulation with the cytosolic calcium concentration rising within 30 s and AA release being maximal after 20 min, which could explain the difference in the PLA_2 isoform involved.

Balsinde and Dennis [64] showed that $cPLA_2$ and type II $sPLA_2$ were sequentially activated following stimulation of mouse macrophages by lipopolysaccharide and PAF. In the early phase, $cPLA_2$ is providing AA intracellularly, which is followed by $sPLA_2$-mediated extracellular release of AA. Strikingly, selective inhibition of $cPLA_2$ was accompanied by inhibition of subsequent $sPLA_2$-mediated delayed AA release, suggesting that a functionally active $cPLA_2$ is required for $sPLA_2$ intervention. This is consistent with our observations since in normoxic cells, $cPLA_2$ is already active: the hypoxia-induced increase in Ca^{2+} could activate $sPLA_2$ which increases AA release and PG synthesis in hypoxia.

The metabolism of AA into PGs was also investigated. COX-1, associated with the endoplasmic reticulum, is generating the basal PG synthesis, whereas inducible COX-2 mediates the cytokine-induced delayed phase of eicosanoid production [65]. $cPLA_2$ regulates the early AA metabolism in response to various stimuli which involve Ca^{2+} mobilisation and COX-1, to generate PGs. Nevertheless, $cPLA_2$ may also be involved in the delayed phase of eicosanoid generation in monocytes [66] and in synovial cells [67], together with COX-2. Moreover, recent findings have shown that $cPLA_2$ activity is required for cytokine-induced expression of $sPLA_2$ in fibroblasts through the synthesis of certain metabolites, suggesting functional segregation and crosstalk between the two isoforms [68]. Here, we showed that AACOCF3 and BEL inhibit basal PG synthesis suggesting that $cPLA_2$ and $iPLA_2$ are the major isoforms involved in this process, whereas $sPLA_2$ is specifically activated in hypoxia. This does not exclude the role of $iPLA_2$ in hypoxic cells.

These observations are in favour of a functional segregation between distinct PLA_2 isoforms in endothelial cells, as described in fibroblasts and Chinese hamster ovary cells [69].

PLA_2 is involved in the synthesis of several inflammatory mediators involved in neutrophil infiltration in ischemic organs. In generating low density lipoprotein (LDL) modification, $sPLA_2$ also plays a role in early atherogenesis [70,71]. Moreover, $sPLA_2$ is present in the intima of atherosclerotic arteries, but not in normal arteries [31]. A correlation between the expression of this enzyme and the degree of atherosclerosis in aortic samples further underlines its possible role in pathophysiological conditions affecting the endothelium [72]. $sPLA_2$ is detected in macrophages present in human atherosclerotic lesions and in vitro, $sPLA_2$ expression is increased in these cells by mildly modified LDL [73]. $sPLA_2$ overexpression also alters HDL metabolism [74,75]. The present findings on the role of $sPLA_2$ in the hypoxia-induced AA release open up new perspectives in developing intervention strategies addressed to these cardiovascular diseases.

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