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Polycyclic aromatic hydrocarbons present in cigarette smoke cause endothelial cell apoptosis by a phospholipase A_2-dependent mechanism

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ABSTRACT

Smoking is a major risk factor for endothelial cell injury and subsequent coronary artery disease. Epidemiological studies implicate the phospholipase A_2/arachidonic acid cascade in the mechanism by which smoking causes heart disease. However, specific components of cigarette smoke that activate this pathway have not been identified. The purpose of this study was to investigate the effects of polycyclic aromatic hydrocarbons contained in cigarette smoke on phospholipase A_2 (PLA_2) activity and apoptosis of human coronary artery endothelial cells. 1-methylanthracene (1-MA), phenanthrene (PA), and benzo(a)pyrene (B(a)P) caused significant release of ^{3}H-arachidonate from endothelial cells. 1-MA and PA, but not B(a)P, also caused significant release of ^{3}H-linoleic acid. Release of fatty acids from membrane phospholipids preceded the onset of apoptosis. ^{3}H-arachidonate release and apoptosis induced by 1-MA, B(a)P, and PA were inhibited by methylarachidonoyl-fluorophosphonate, an inhibitor of Groups IV and VI PLA_2s. Bromoenol lactone, an inhibitor of Group VI enzymes, inhibited both ^{3}H-arachidonate release and apoptosis induced by 1-MA and PA, but not B(a)P. MJ33, an inhibitor of the acidic calcium-independent PLA_2, attenuated ^{3}H-arachidonate release and apoptosis by PA, but not 1-MA or B(a)P. The presence of Groups IV and VI and the acidic $iPLA_2$ in endothelial cells was demonstrated by reverse transcriptase-polymerase chain reaction and Western analysis. These data suggest that 1-MA, B(a)P and PA induce apoptosis of endothelial cells by a mechanism that involves activation of these three distinct isoforms of PLA_2.

Key words: phospholipase A_2 • arachidonic acid • methylanthracene • benzo(a)pyrene • phenanthrene

Coronary artery disease is the leading cause of mortality in the United States, and cigarette smoking is an important risk factor for the development of this disease (1). Smoking induces many pathologic changes in the blood vessel wall, including endothelial cell injury, an event that is critical in the pathogenesis of vascular disease (2). Despite the importance of endothelial cell injury in the pathogenesis of heart disease in smokers, neither the mechanism of cell death nor the specific components of cigarette smoke responsible for this effect has been elucidated.

Apoptosis is emerging as an important mechanism of endothelial cell death during atherosclerosis (3) and has been demonstrated in atherosclerotic plaques of humans, cholesterol-fed rabbits, and hyperlipidemic knockout mice (4, 5). Although smoking is known to cause endothelial cell injury (2), the possibility that specific components in cigarette smoke promote atherosclerosis by causing endothelial cell apoptosis has not been investigated.

Polycyclic aromatic hydrocarbons (PAHs) represent one class of compounds present in cigarette smoke that is cytopathic (6). Benzo(a)pyrene is a PAH that promotes atherosclerosis in animal models (7); however, the role of endothelial cell apoptosis in the pathogenesis of this effect has not been elucidated. Methylated anthracenes and phenanthrene are present in concentrations that are 60-fold (1500 ng/cigarette) and 15-fold (362 ng/cigarette) greater than benzo(a)pyrene (25 ng/cigarette), respectively (8). Despite the prevalence of these PAHs in cigarette smoke, their ability to induce endothelial cell injury has not been investigated.

The biochemical cascade resulting from phospholipase A_2 (PLA_2) hydrolysis of arachidonic acid (AA) gives rise to more than 100 biologically active metabolites known as eicosanoids. A substantial body of evidence implicates the PLA_2/AA cascade in the acceleration of cardiovascular disease by smoking (9–12). Compared with nonsmokers, smokers preferentially metabolize AA to thrombogenic and vasoconstrictive metabolites, including thromboxane A_2 (12). Furthermore, nonsteroidal anti-inflammatory agents and diets high in omega-3 fatty acids (fish oils), both of which inhibit the AA cascade, attenuate the incidence of myocardial infarction in smokers (9–11).

The initial and rate-limiting step in this cascade is the activation of PLA_2. Components of cigarette smoke may therefore activate one or more of the several different isoforms of PLA_2 that exist. Eleven different groups of PLA_2 have been characterized that differ in substrate specificity, calcium requirements, and inhibitor profiles. These include the Group IV high-molecular-weight cytosolic PLA_2s, of which three isoforms have been described. Group IVA is arachidonoyl-selective and calcium-dependent, whereas Group IVB is arachidonoyl-nonselective and calcium-dependent. In contrast, Group IVC is an arachidonoyl-selective, calcium-independent isoform. All three isoforms are inhibited by methylarachidonoyl fluorophosphonate (MAFP; 13, 14). Low-molecular-weight, secreted, calcium-dependent enzymes have also been described (13). In addition, at least two isoforms of Group VI have been characterized, both of which are calcium-independent PLA_2s ($iPLA_2$s) and inhibited by MAFP and bromoenol lactone (BEL; 13). These enzymes differ in their substrate specificity; Group VIA is an arachidonoyl-nonselective isoform, whereas Group VIB is selective for arachidonate at the sn-2 position (15, 16). An acidic, lysosomal $iPLA_2$ ($aiPLA_2$; 17), a low-density lipoprotein-$iPLA_2$ (LDL-PLA_2; 18) and a human cytosolic, serine-dependent $iPLA_2$ (HSD-PLA_2; 19) have also been characterized.

Several of these PLA_2 isoforms have been implicated in apoptosis. Group IVA PLA_2 plays an essential role in TNF-α–mediated apoptosis (20) and group VI $iPLA_2$ is involved in the mechanism of Fas/Fas ligand-induced apoptosis (21, 22). Secreted isoforms of PLA_2 have also been implicated in apoptosis (23). Although PLA_2 isoforms play an important role in apoptosis of other cell types, their role in endothelial cell apoptosis is unknown. Moreover, it is unknown whether PAHs present in cigarette smoke are capable of activating PLA_2s and inducing apoptosis. The purpose of the present study was to investigate the effects of these compounds on endothelial cell PLA_2 activity and apoptosis. The PAHs that were studied included 1-methylanthracene, phenanthrene, and benzo(a)pyrene. The data provide evidence that these PAHs activate distinct PLA_2 isoforms in human coronary artery endothelial cells (HCAECs) that are integrally involved in apoptotic cell death. These data provide the first link between specific components of cigarette smoke and endothelial cell apoptosis, and implicate activation of PLA_2s as an important signal transduction mechanism.

MATERIALS AND METHODS

Reagents

1-methylanthracene, phenanthrene, and benzo(a)pyrene were obtained from Aldrich (Milwaukee, WI).[5,6,8,9,11,12,14,15-^{3}H] arachidonic acid (^{3}H-AA; 200–240 Ci/mmol) and [9,10,12,13-^{3}H] linoleic acid (^{3}H-LA; 60-120 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Methyl-arachidonoyl-fluorophosphonate (MAFP) and bromoenol lactone (BEL) were obtained from Biomol (Plymouth Meeting, PA). MJ33 was obtained from Calbiochem (LaJolla, CA), and 4-bromophenacyl bromide was obtained from Sigma (St. Louis, MO). Anti-PARP antibodies were obtained from Pharmingen (San Jose, CA) and Promega (Madison, WI). Cell death $^{elisa\ plus}$ apoptosis kit was obtained from Roche (Indianapolis, IN). Antibodies to Group IV PLA_2 and Group VI PLA_2 were obtained from Upstate Biotechnology (Lake Placid, NY). The polyclonal antibody to the acidic $iPLA_2$ was raised against recombinant protein (24).

Cell culture

HCAECs were obtained from Clonetics (San Diego, CA) and were maintained in microvascular endothelial cell growth media (Clonetics) containing 10% fetal bovine serum (FBS). The cells were maintained at 37°C in an atmosphere of 5% CO_2/95%O_2.

Determination of ^{3}H-arachidonic acid (^{3}H-AA) or ^{3}H-linoleic acid (^{3}H-LA) released from prelabeled cells exposed to PAHs

To determine the effects of PAHs on fatty acid release, we seeded cells into six well culture plates at a density of 3×10^5 cells/well and grew them to 75% confluence. Release of ^{3}H-AA or ^{3}H-LA into the medium was determined as described previously (25). Briefly, the cells were labeled with ^{3}H-AA or ^{3}H-LA for 24 h. Release of ^{3}H-fatty acid was determined by incubating cells with compounds in Hank's Balanced Salt Solution with 0.1% albumin to inhibit reacylation

and metabolism of released AA. Therefore, the data reflect cumulative deacylation from membrane phospholipids.

When the cells were 90% confluent, concentration-dependent release of ^{3}H-AA or ^{3}H-LA was determined after exposure to PAHs for 1 h. To determine the kinetics of ^{3}H-AA release in response to PAHs, cells were treated for various times with 30 μM 1-methylanthracene, phenanthrene, benzo(a)pyrene, or vehicle. In some experiments, cells were pretreated for 30 min with PLA_2 inhibitors before stimulating with PAHs. The inhibitors included methyl arachidonoyl fluorophosphonate (MAFP; 10 μM), bromoenol lactone (BEL; 2.5 μM), MJ33 (10 μM), and 4-bromophenacyl bromide (BPB; 10 μM). At the end of the incubation period, the medium was collected into scintillation vials and radioactivity determined by scintillation counting. Radioactivity in the cellular fraction was also determined, and ^{3}H-fatty acid release was expressed as a percentage of total cellular radioactivity.

Determination of PLA_2 isoform expression by reverse transcriptase-polymerase chain reaction (RT-PCR)

We isolated total cellular RNA from HCAECs (10–20 × 10^6 cells) by using NucleoSpin nucleic acid purification kits (Clontech) according to instructions of the manufacturer. Reverse transcription of total RNA (2 μg) was performed by using the Superscript first-strand synthesis system (Invitrogen, Carlsbad, CA). We performed the reverse transcription reaction at 42˚C for 50 min and then terminated by heating at 70°C for 15 min. PCR reactions were performed in a total volume of 50 μl containing 4 μl of the reverse-transcription reaction mixture, 0.2 mM dNTPs, 0.2 μM of each primer, and 1.25 units of Taq polymerase (Invitrogen) in PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl; 1.5 mM $MgCl_2$). The conditions for PCR reactions were an initial denaturation step at 94˚C for 5 min, followed by 34 cycles of 94˚C for 30 s, 60˚C for 30 s, 72˚C for 30 s, and a final extension step at 72˚C for 10 min. As a positive control, glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was also amplified. Additional negative control reactions were performed to ensure that amplification of a gene was not generated from contaminated genomic DNA; one was performed without RNA and the other under conditions to inhibit reverse transcriptase activity. Primers used for PCR were PLA_2 group IVA (sense, 5'-CCAAAATGTCATTTATAGATCC-3'); PLA_2 group IVA (antisense, 5'-CATGAACTATGCTTTGGGTTTAC-3'); PLA_2 group IVB (sense, 5'-ACTGAGTGCCCTGCCCTCTGGTCAAG-3'); PLA_2 group IVB (antisense, 5'-TGCCCCATAAAGAACTCGGAGCCAAAGA-3'); PLA_2 group IVC (sense, 5'-AGAAAGAAGAAAAGGCGGCCGTGGAGAGAC -3'); PLA_2 group IVC (antisense, 5'-CGGCACTGAAGTCGAAGGAGAGGATGAGGT -3'); PLA_2 group VI (sense, 5'-ATGCAGTTCTTTGGCCGCCTGGTCAAT-3'); PLA_2 group VI (antisense, 5'-TGATGATCATCTCCGCACACCCCTTCTG-3'); acidic $iPLA_2$ (sense, 5'-AGAGCCCACAGAAAAGTTACCTT-3'); acidic $iPLA_2$ (antisense, 5'-CCTGCAGAGATCCAACAAGAT-3'). G3PDH primers were purchased from Clontech. The PCR products were analyzed on a 1.5% agarose gel.

Western analysis of phospholipase A_2 isoforms

Cells were washed with phosphate-buffered saline (PBS) and then lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1 mM DTT, 100 μM p-4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 μM pepstatin, and 100 μM leupeptin). The lysate was centrifuged at 10,000 g for 20 min, and the supernatant was subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were probed with antibodies against Group IV PLA_2s (Groups IVA, IVB, and IVC), Group VI $iPLA_2$ and lysosomal acidic $iPLA_2$. Bands were visualized by enhanced chemiluminescence (Amersham Pharamacia Biotech, Uppsala, Sweden).

Determination of apoptosis induced by PAHs

Apoptosis was detected by Western blotting for the cleavage of poly-ADP-polymerase (PARP), in situ terminal DNA nick-end labeling (TUNEL) and by detection of histone fragmentation.

We determined PARP cleavage after treatment with 1-methylanthracene, benzo(a)pyrene, phenanthrene, arachidonic acid, linoleic acid, or vehicle for 4 h. Hydrogen peroxide (2 mM) was used as a positive control. Crude extracts of protein were prepared by suspending in ice-cold sample buffer (62.5% Tris-HCL, pH 6.8; 6 M urea; 10% glycerol; 2% SDS, 0.003% bromophenol blue; 5% β-mercaptoethanol) containing protease inhibitors. Cells were lysed by sonication on ice for 2 ×10 s, 40% duty and lysates (20 μg of protein) subjected to 10% SDS-polyacrylamide gel electrophoresis under reducing conditions. The proteins in the gel were transferred to immobilon-P membranes via a semidry blotting apparatus, and the membrane was blocked in 10% nonfat dry milk in PBS/0.1% Tween 20 and was incubated overnight at 4°C with two different anti-PARP antibodies, one that detects both the parent protein and the cleavage products and the other that detects only the cleavage products. Visualization was enhanced by chemiluminescence (ECL).

To determine the kinetics of PAH-induced apoptosis, we performed in situ labeling of DNA fragments by use of TUNEL (Boehringer-Mannheim). ECs (2×10^5/well) were grown in tissue culture slide chambers for 24 h to 90% confluency and were then treated with 30 μM 1-methylanthracene, benzo(a)pyrene, phenanthrene, 10 μM arachidonic acid, linoleic acid, or DMSO for 1, 2 or 4 hr. At the end of the treatment period, they were washed with room temperature PBS and fixed in 4% paraformaldehyde in PBS (pH 7.4). The slides were blocked with 0.3% hydrogen peroxide in 50% methanol/50% ddH_20, washed and permeabilized with 0.1% triton X100 in 0.1% sodium citrate. After washing with PBS, 40-50 μl of a mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein labeled nucleotides (dUTPs) was added to the cells for 60 min at 37°C. The incorporation of fluorescein-conjugated dUTP into DNA was determined by incubation of slides with anti-fluorescein antibody conjugated with horse-radish peroxidase (POD), followed by incubation with DAB and counterstaining with hematoxylin. Slides were examined by light microscopy. TUNEL positive cells were identified by brown nuclear stain and altered nuclear morphology.

Apoptosis was also verified fluorimetrically by histone fragmentation by using cell death $ELISA^{plus}$. Cells seeded into sixwell plates were treated at 90% confluency with the three PAHs (30 μM), arachidonic acid (10 μM), or linoleic acid (10 μM), for 2 h. Histone fragmentation was

evaluated in cells treated with 1-methylanthracene, benzo(a)pyrene or phenanthrene in the presence and absence of the PLA_2 inhibitors listed above (BEL, MAFP, or MJ33). Cells were then washed with warm PBS, lysed, and incubated with antihistone-biotin and anti-DNA-POD at room temperature for 2 h. After being washed with incubation buffer, 100 µl of substrate solution was added to develop the color and measurements made at 405 nm by using a plate reader.

Statistical analysis

Data are expressed as mean ± SE. Data were analyzed by analysis of variance, and group means were compared by using Student-Newman-Keuls'. Appropriate transformations were performed on all data that did not follow a normal distribution (e.g., percentage of data). If transformation failed to normalize the data, nonparametric statistics (Mann-Whitney rank sum test) were used. For all studies, the criterion for statistical significance was $P<0.05$.

RESULTS

PAH-induced release of ^{3}H-arachidonic acid or ^{3}H-linoleic acid from human coronary artery endothelial cells

A minimal amount of ^{3}H-AA or ^{3}H-LA was released from unstimulated HCAECs (Fig. 1A–C). Exposure to 1-methylanthracene for 1 h caused concentration-dependent release of both ^{3}H-AA and ^{3}H-LA (Fig. 1A). In contrast, benzo(a)pyrene caused significant release of fatty acid from ^{3}H-AA-prelabeled cells with no release from cells prelabeled with ^{3}H-LA (Fig. 1B). Phenanthrene, like 1-methylanthracene, caused significant release of both ^{3}H-AA and ^{3}H-LA (Fig. 1C).

The kinetics of ^{3}H-AA release is shown in Figure 2. Significant release of ^{3}H-AA was observed within 5 min in HCAECs stimulated with phenanthrene and within 10 min in cells exposed to benzo(a)pyrene or 1-methylanthracene. No further increase in release was seen after 10 min in any treatment group.

To begin to investigate the characteristics of the PLA_2 isoforms involved in PAH-induced ^{3}H-AA release, we performed experiments in the presence and absence of the inhibitor of Groups IV and VI PLA_2s, MAFP, the inhibitor of Group VI $iPLA_2$, BEL, the inhibitor of the acidic calcium-independent PLA_2, MJ33, and the inhibitor of low-molecular-weight secreted isoforms 4-bromophenacyl bromide (BPB). As shown in Figure 3, MAFP attenuated significantly ^{3}H-AA release from HCAECs treated with all three PAHs. Preincubation with BEL significantly inhibited ^{3}H-AA release induced by 1-methylanthracene and phenanthrene but not benzo(a)pyrene; however, MJ33 inhibited only the response to phenanthrene (Fig. 3). The inhibitor of $sPLA_2$ isoforms, BPB, did not alter ^{3}H-AA release from cells treated with any of the PAHs (data not shown).

Figure 4A demonstrates the expression of five different isoforms of PLA_2 in HCAECs as determined by RT-PCR. Amplicons of the appropriate size were identified for the acidic $iPLA_2$ (677 bp), the Group VI $iPLA_2$ (817 bp), and the Groups IVA (2237 bp), IVB (801 bp), and IVC

PLA_2s (1206 bp). Western blots utilizing antibodies against Groups IV and VI and acidic $iPLA_2$ are shown in Figure 4B. At least two Group IV enzymes are expressed by HCAECs. In addition, the Group VI and the acidic $iPLA_2$ proteins are also expressed.

Induction of apoptosis by PAHs

Western analysis with two different antibodies for PARP cleavage was used as a measure of apoptosis in HCAECs after exposure to PAHs or fatty acids. One antibody recognizes both the parent protein and cleavage products (Fig. 5; top panel); and the other antibody recognizes only the cleavage product of PARP (Fig. 5; bottom panel). There was no evidence of PARP cleavage in vehicle-treated cells; however, significant amounts of the 89 kD fragment were observed in cells after exposure for 4 h to 1-methylanthracene, benzo(a)pyrene, phenanthrene, arachidonic acid, or linoleic acid.

To examine the timecourse of apoptosis, we treated ECs with 1-methylanthracene, benzo(a)pyrene, phenanthrene, arachidonic acid, or linoleic acid, and we performed TUNEL assays. No TUNEL-positive cells were seen in vehicle-treated cells (Fig. 6A**,** B; Panel A) or in cells treated with 1-methylanthracene, benzo(a)pyrene or phenanthrene at 1 h (Fig. 6A). The same results were obtained for cells exposed to arachidonic acid and linoleic acid (data not shown). However, numerous TUNEL-positive cells were seen at 2 h and thereafter in cells treated with 1-methylanthracene (Fig. 6A, B; Panel B), benzo(a)pyrene (Fig. 6A**,** B**;** Panel C), phenanthrene (Fig. 6A**,** B**;** Panel D), arachidonic acid (Fig. 6B; Panel E), and linoleic acid (Fig. 6B**;** Panel F).

Inhibition of apoptosis by PLA_2 inhibitors

To examine the role of PLA_2-mediated release of fatty acids in PAH-induced apoptosis, we evaluated histone fragmentation in HCAECs exposed to PAHs in the presence and absence of MAFP, BEL, or MJ33. MAFP attenuated the apoptotic response to 1-methylanthracene, benzo(a)pyrene, and phenanthrene at a concentration that also inhibited ^{3}H-AA release (Fig. 7). Pretreatment with BEL significantly inhibited 1-methylanthracene- and phenanthrene-induced apoptosis; however, the effect of benzo(a)pyrene was not altered. In contrast, MJ33 inhibited only the apoptotic response to phenanthrene (Fig. 7).

DISCUSSION

It is well known from epidemiologic studies that the PLA_2/AA cascade is important in the mechanism by which cigarette smoking causes heart disease (9–12); however, little work has focused on identifying specific components of cigarette smoke that are responsible for this effect. This study is the first to identify specific PAHs present in high concentrations in cigarette smoke that stimulate PLA_2-mediated release of membrane fatty acids. These results suggest that three distinct isoforms of PLA_2 are activated by PAHs with different structural features. The data indicate that 1-methylanthracene, a three-ring compound with a bay-like region, activates the Group VI $iPLA_2$; whereas benzo(a)pyrene, a five-ring compound, activates a Group IV enzyme. In contrast, phenanthrene, with a four-membered ring and a true bay region, activates both the Group VI and the acidic $iPLA_2$. Moreover, this study is the first to link exposure of endothelial

cells to cigarette smoke components with PLA_2 activation, fatty acid release, and apoptosis, events known to be important in the etiology of atherosclerosis.

1-methylanthracene, benzo(a)pyrene and phenanthrene induced concentration- and time-dependent release of ^{3}H-AA from prelabeled human coronary artery endothelial cells. Release of ^{3}H-AA was significant within 10 min in cells exposed to 1-methylanthracene or benzo(a)pyrene and within 5 min in cells treated with phenanthrene. The release of arachidonic acid preceded the onset of apoptosis that was first observed 2 h after treatment. Treatment with free arachidonic acid or its precursor, linoleic acid, also induced significant apoptosis, and this effect had a time course similar to PAH-induced apoptosis. These data are consistent with the hypothesis that 1-methylanthracene, benzo(a)pyrene, and phenanthrene may cause apoptotic death of HCAECs by a mechanism that involves phospholipase-mediated release of arachidonic acid. This conclusion is substantiated further by experiments with MAFP, BEL, and MJ33. These PLA_2 inhibitors caused significant and parallel inhibition of both ^{3}H-AA release and apoptosis in response to various PAHs.

Although the specific PLA_2 isoforms activated by PAHs were not delineated conclusively in this study, the data suggest that 1-methylanthracene, benzo(a)pyrene, and phenanthrene activate enzymes with different biochemical characteristics. Benzo(a)pyrene cleaved arachidonate, but not linoleic acid, from the sn-2 position of membrane phospholipids, which suggests that this compound activates an arachidonoyl-selective PLA_2. In contrast, 1-methylanthracene activated an enzyme that cleaved both linoleic and arachidonic acid from membrane phospholipids, but with a preference for linoleic acid. Phenanthrene also displayed a lack of substrate specificity because it caused release of both arachidonate and linoleate; however, in contrast to 1-methylanthracene, phenanthrene at low concentrations preferentially acted on phospholipids containing arachidonic acid. These results may reflect not only the substrate selectivity of the enzymes studied but also the different subcellular locations of the enzymes, because the two fatty acids may be distributed differentially among membrane phospholipid pools. In vitro assays are necessary to validate the substrate specificity of the enzymes studied.

The profile of inhibitors that altered ^{3}H-AA release and apoptosis in response to the three PAHs also differed markedly, which is consistent with the conclusion that activation of three distinct enzymes was involved. MAFP, an inhibitor of Group IV and VI enzymes, attenuated significantly ^{3}H-AA release and apoptosis induced by 1-methylanthracene. Similar results were observed with BEL, a selective inhibitor of Group VI calcium-independent isoforms; however, BPB, an inhibitor of low-molecular-weight secreted PLA_2s was without effect. These data are consistent with the hypothesis that 1-methylanthracene activates a Group VI $iPLA_2$. Expression of a Group VI enzyme in human coronary artery endothelial cells was demonstrated by RT-PCR and Western blot, which further substantiated the hypothesis that 1-methylanthracene activates this enzyme. This is the first published study to demonstrate the presence of this isozyme in endothelial cells. The Group VI $iPLA_2$s, of which several different splice variants exist, (27), are normally constitutively active at very low levels and function to maintain cell membrane integrity (28). Recently, it has become evident that these enzymes also function in the process of apoptosis (21, 22).

^{3}H-arachidonic acid release and apoptosis induced by benzo(a)pyrene were inhibited by MAFP

but not by BEL, BPB, or MJ33. These data, in addition to the apparent enzyme specificity for AA, are consistent with the possibility that a Group IV PLA_2 is involved in benzo(a)pyrene-induced endothelial cell apoptosis. Several reports link Group IV PLA_2s to apoptosis in other cell types (20, 29). Hayakawa et al. (20) demonstrated that a TNF-α–resistant cell line shows reduced Group IVA expression and transfection with this enzyme renders the cells susceptible to TNF-α–mediated apoptosis. Similar results were reported by Wissing et al. (29). A study by Duan et al. (30) showed that a Group IV PLA_2 isoform was responsible for TNF-induced apoptosis of lymphocytes; however, because both PLA_2 Group IVA and PLA_2 IVC were expressed by these cells, it was not possible to discern which enzyme caused this effect. The results with RT-PCR for the Group IVA PLA_2 indicate that mRNA for this enzyme is present; however, Western analysis indicates that, under the conditions of this study, the 85 kD protein is not expressed by HCAECs. In contrast, both the RT-PCR and Western blot results suggest that Groups IVB (~110 kD; 31) and IVC (~60 kD; 32) are expressed by these cells. It is likely that Group IVC was the enzyme responsible for benzo(a)pyrene-induced apoptosis because this enzyme is arachidonoyl-selective (31) and because benzo(a)pyrene targeted an arachidonoyl-selective Group IV PLA_2.

Phenanthrene caused AA release and apoptosis under conditions that differed from both 1-methylanthracene and benzo(a)pyrene. In contrast to 1-methylanthracene and benzo(a)pyrene, phenanthrene caused release of both ^{3}H-AA and ^{3}H-LA from HCAECs that was inhibited by MAFP, BEL, and the acidic $iPLA_2$ inhibitor, MJ33. Because activity of the acidic $iPLA_2$ is not altered by BEL or MAFP (24, 25), it is likely that phenanthrene activates two different isoforms of PLA_2: the Group VI $iPLA_2$ and the acidic $iPLA_2$. RT-PCR and Western analysis confirmed the presence of this enzyme in HCAECs. A role for acidic $iPLA_2$ in apoptosis has not been reported previously; however, this enzyme is associated with lung injury that occurs during oxidative stress (34). A nonspecific effect of MJ33 cannot be ruled out because this inhibitor has been shown to also alter activity of small molecular weight secreted isoforms of PLA_2 (35); however, this is unlikely because BPB, an inhibitor of these enzymes, was without effect.

Although 1-methylanthracene and phenanthrene caused significantly more arachidonic acid release from endothelial cells than benzo(a)pyrene, the magnitude of the apoptotic response was similar for all three PAHs. There are several possible explanations for these results. It is plausible that benzo(a)pyrene causes apoptosis by mechanisms other than PLA_2 activation. Of the PAHs studied, only benzo(a)pyrene has significant affinity for the aryl hydrocarbon (Ah) receptor (36). Because Ah receptor activation can result in apoptosis (37), it is likely that more than one mechanism is involved in benzo(a)pyrene-induced apoptosis; one mechanism may require phospholipase A_2 and the other, the Ah receptor. Furthermore, the downstream events involved in apoptosis induced by the three different phospholipases may differ. Phospholipases can induce apoptosis by a direct effect on the membrane or by indirect mechanisms that involve alterations of signaling pathways by arachidonate and/or its metabolites. Previous studies suggest that $iPLA_2$ activity is important in phosphatidylserine externalization (21, 22), an early event in apoptosis that promotes the recognition and destruction of apoptotic cells by phagocytes (38). In addition, this enzyme, which is poorly linked to eicosanoid metabolism (25), results in the release of free fatty acids from membrane phospholipids. These fatty acids directly activate c-jun N-terminal kinase and p38, enzymes involved in apoptosis (40). However, the Group IV PLA_2, which is intimately associated with the arachidonic acid-metabolizing enzymes, has been

shown to induce apoptosis due to eicosanoid formation (41). Because downstream signaling mechanisms by the three different isoforms of PLA_2 may differ, it is not surprising that the magnitude of fatty acid release and the magnitude of apoptosis are not correlated in a simple and direct manner.

The ability of arachidonate and other fatty acids to induce apoptosis is well-documented (42–45). Arachidonic acid and other polyunsaturated fatty acids interact with key apoptotic pathways, including p38/cJun-N-terminal kinase (JNK; 40) and the sphingomyelinase/ceramide pathway, to regulate caspase activity (42). Thus, PAH-induced activation of the PLA_2/AA pathway may result in apoptosis by causing multiple changes that include membrane alterations (PS externalization) as well as alterations in cellular signal transduction pathways and gene expression (JNK/caspase activation/PARP cleavage). Whether the apoptotic effects of the PAHs and the free fatty acids demonstrated in this study were dependent on eicosanoid metabolites was not addressed herein. However, because the precursor to arachidonate, linoleate, was as effective as arachidonate in inducing apoptosis, it is likely that the fatty acids themselves are responsible for this effect. The role of eicoisanoids in apoptosis induced by PAHs is the subject of future studies.

The results of the present study may be significant in light of several studies that have linked cigarette smoking, PLA_2/arachidonic acid activation, and coronary artery disease. The Honolulu Heart Project initiated in 1968 followed a cohort of 8006 Japanese-American men aged 45 to 65 years of age. The results of this study demonstrated clearly that mortality and morbidity due to coronary heart disease was significantly less (50%) in heavy smokers who consumed diets high in omega-3 fatty acids (11), compounds known to inhibit the arachidonic acid cascade at several different levels (46). The same protection was not observed in non-smokers (11). This study, in addition to others (9, 10), suggests that components of cigarette smoke activate this cascade. The present study is the first to describe PLA_2 activation and subsequent endothelial cell apoptosis caused by specific components of cigarette smoke. Because endothelial cell loss/injury is known to be important in the process of coronary artery disease and atherosclerotic disease involving other arteries as well, induction of endothelial cell apoptosis by cigarette components may represent an important mechanism of smoking-induced augmentation of cardiovascular diseases. Identification of specific components of cigarette smoke that alter pathways known to be important in heart disease will likely provide means for discovering effective preventative and therapeutic strategies in smokers.

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Fig. 1

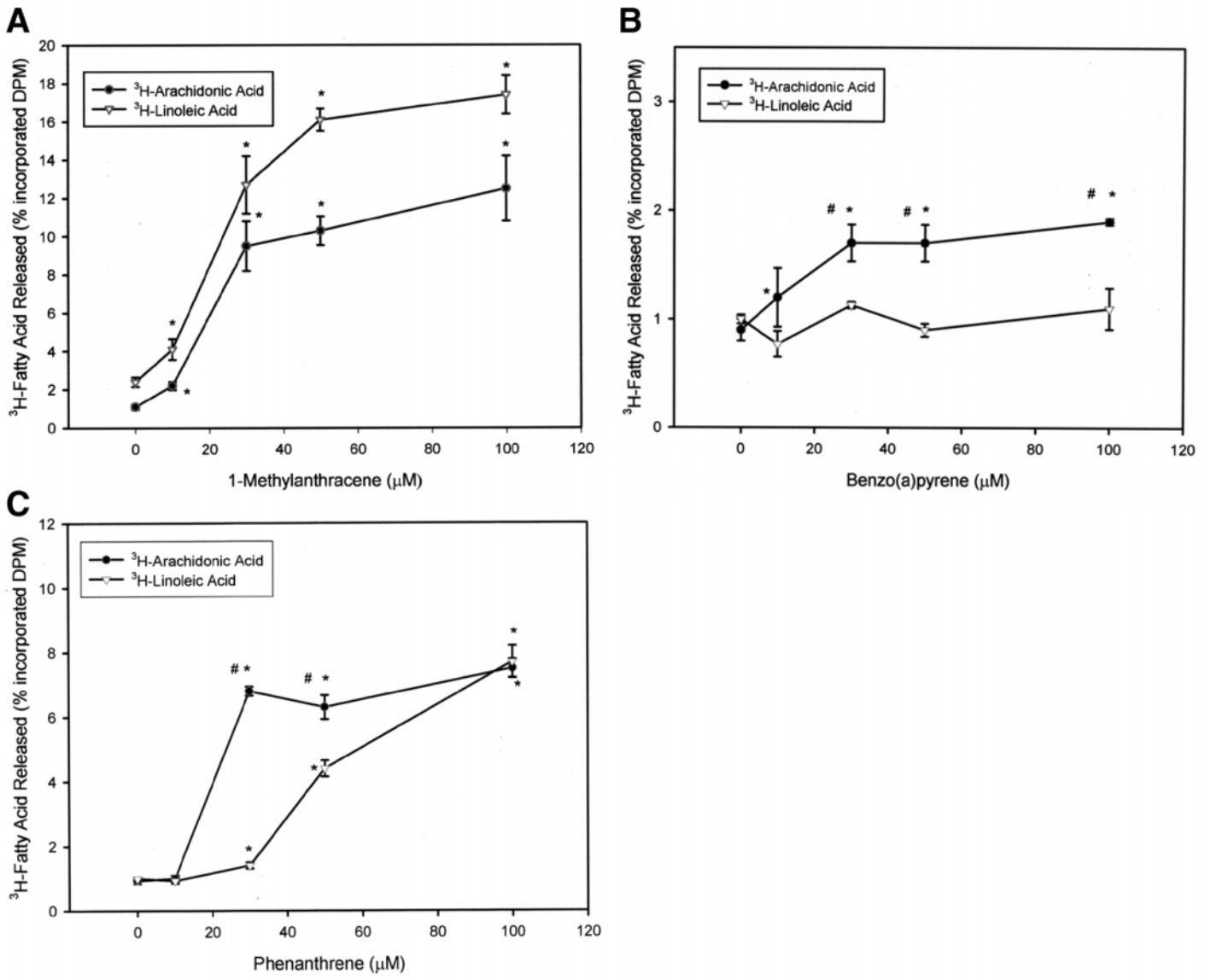

Figure 1. Concentration-dependent release of ^{3}H-fatty acid from HCAECs exposed to PAHs. Cells pre-labeled for 24 h with ^{3}H-AA or ^{3}H-LA were exposed to various concentrations of 1-methylanthracene **(A)**, benzo(a)pyrene **(B)**, or phenanthrene **(C)** for 1 h. Cumulative release of ^{3}H-fatty acid into the medium was measured as described in Materials and Methods. The data represent the results of three separate experiments performed in triplicate. (*) Significantly different from vehicle-treated control; $P<0.05$. (#) Significantly different from response obtained in ^{3}H-AA-prelabeled cells; $P<0.05$.

Fig. 2

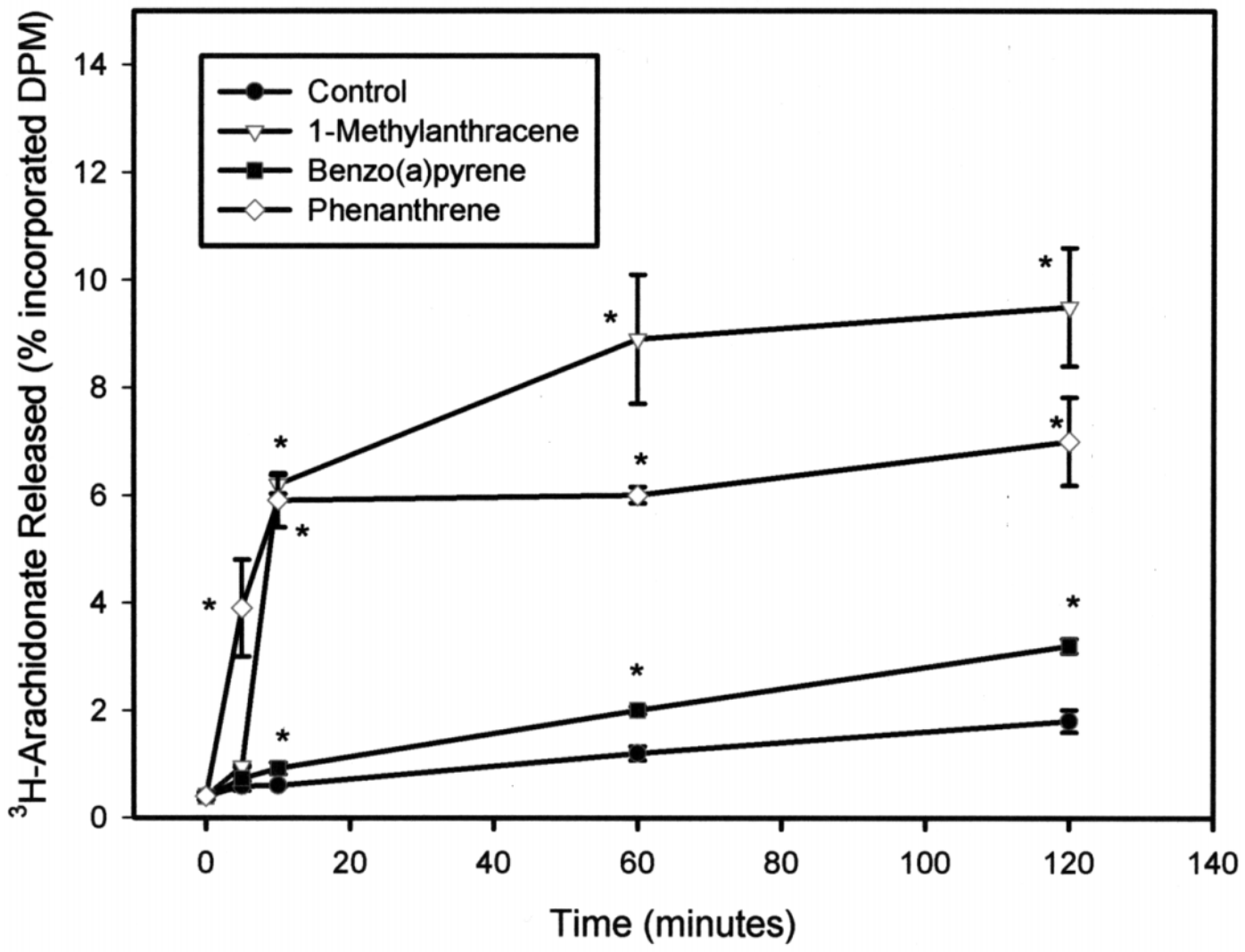

Figure 2. Time-dependent release of ^{3}H-AA from HCAECs exposed to PAHs. Cells pre-labeled with ^{3}H-AA were treated for various times with 30 μM 1-methylanthracene, benzo(a)pyrene, or phenanthrene; cumulative release of ^{3}H-AA into the medium measured as described in Materials and Methods. The data represent the results of three separate experiments performed in triplicate. (*) Significantly different from vehicle-treated control at same timepoint; $P<0.05$.

Fig. 3

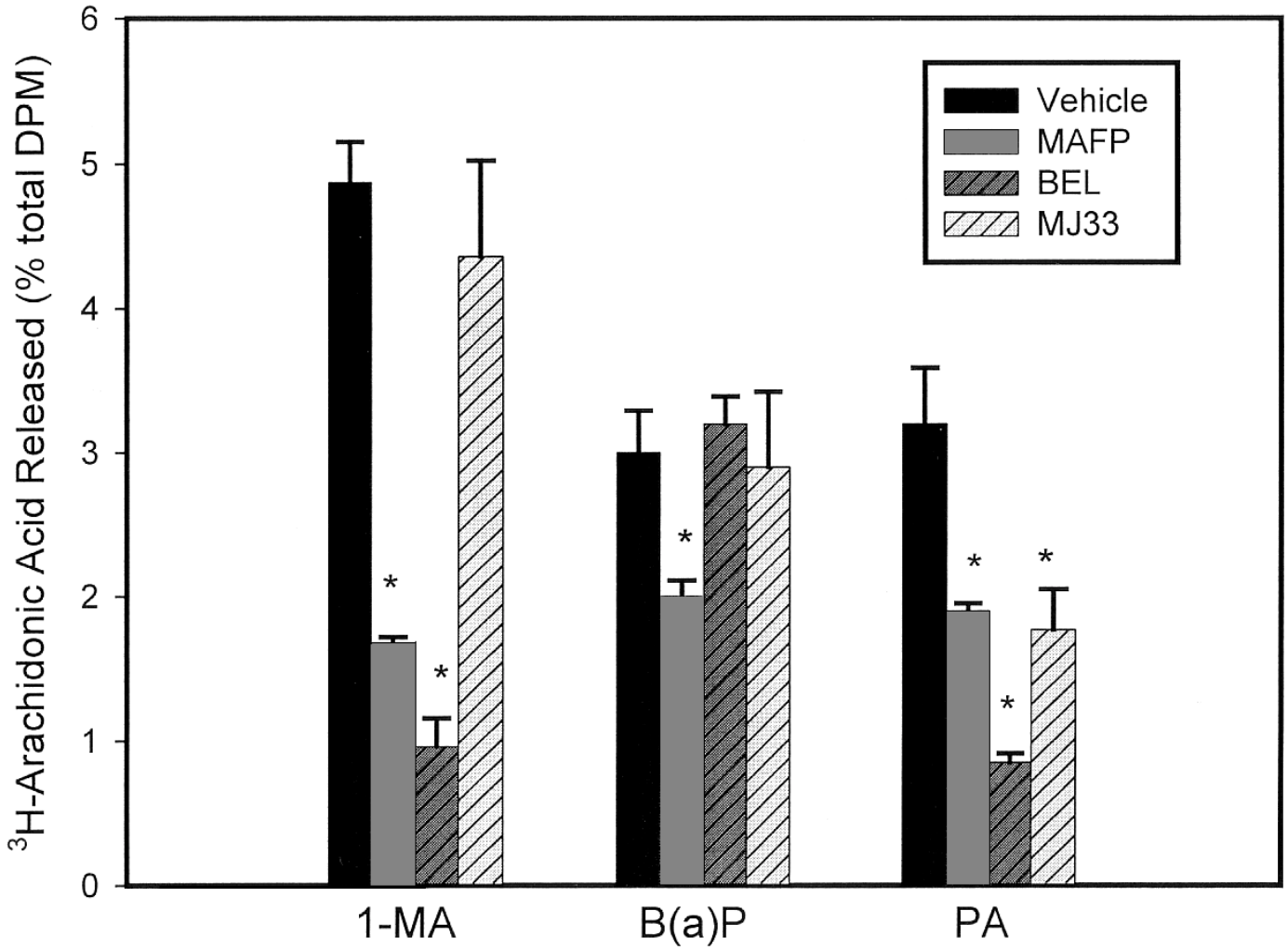

Figure 3. Attenuation of ^{3}H-AA release by PLA_2 inhibitors. Endothelial cells were pre-labeled with ^{3}H-AA as described in the legend to **Figure 1**. The cells were pretreated with 10 μM MAFP, 2.5 μM BEL, 10 μM MJ33, or the appropriate vehicle for 30 min and then exposed to vehicle, 1-methylanthracene, benzo(a)pyrene, or phenanthrene for 1 h. Release of ^{3}H-AA into the medium was measured as described in Materials and Methods. The data represent the results of three separate experiments performed in triplicate and are expressed as a percentage of total incorporated DPM. (*) Significantly different from results obtained in the absence of inhibitor; $P<0.05$.

Fig. 4

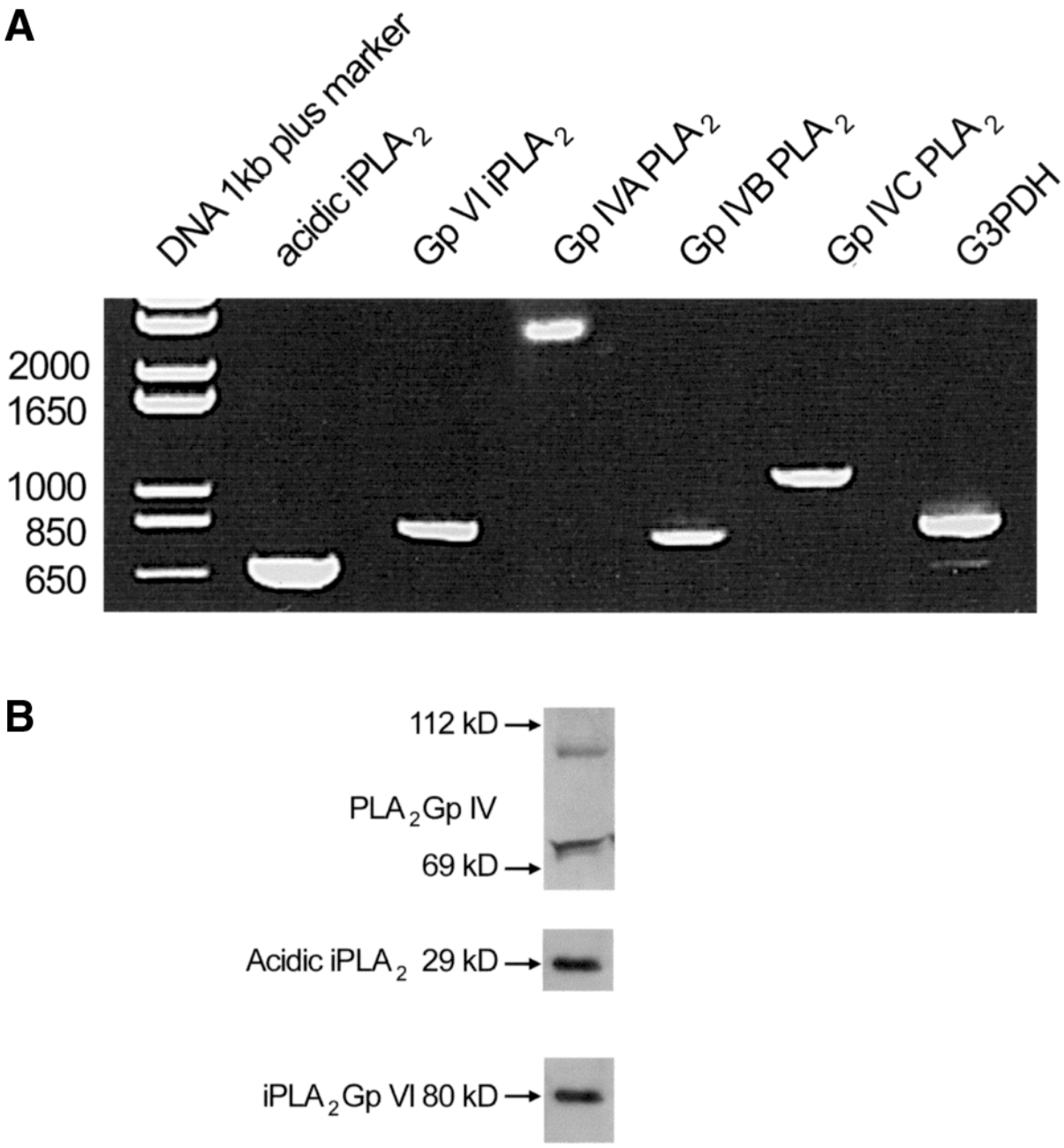

Figure 4. A) Expression of PLA_2 isoforms in human coronary artery endothelial cells. RT-PCR analysis of total RNA from human coronary artery endothelial cells by using PLA_2 primers was performed as described in Materials and Methods. A 10-μl aliquot of the PCR reaction was analyzed on a 1.5% agarose gel containing ethidium bromide. Amplification of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was performed as a control by using the same reaction conditions. DNA size markers are in bp. **B)** Western blotting for acidic, calcium-independent PLA_2 ($aiPLA_2$), Group VI, calcium-independent PLA_2 ($iPLA_2$), and Group IV PLA_2s in human coronary artery endothelial cells. Lysates were separated by SDS-PAGE, and Western blotting was performed by using antibodies to $aiPLA_2$, Group VI $iPLA_2$ and the Group IV PLA_2s, as described in Materials and Methods. Human coronary artery endothelial cells express the $aiPLA_2$, the Group VI $iPLA_2$, and at least two isoforms of the Group IV enzymes. The blots are representative of three separate experiments.

Fig. 5

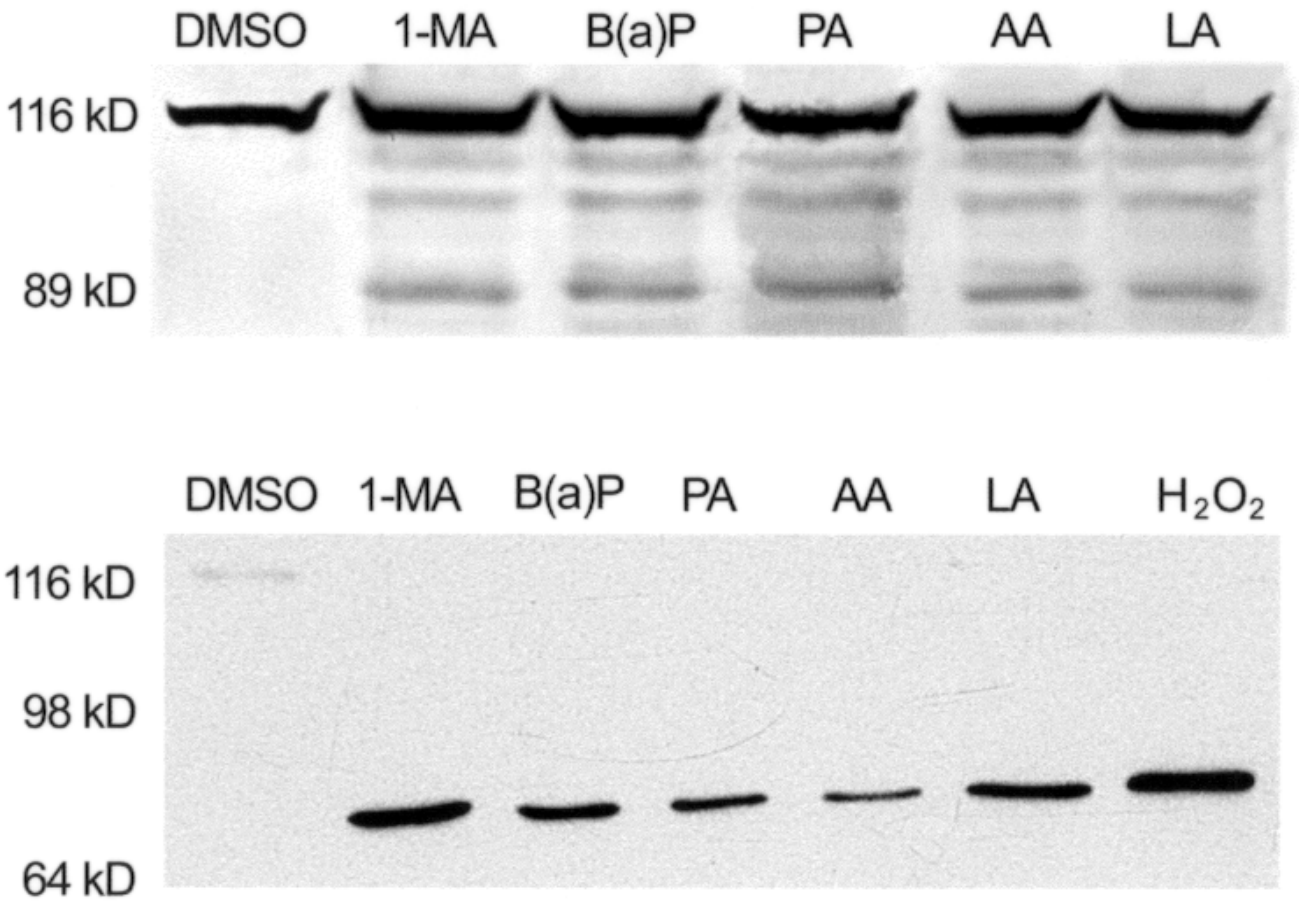

Figure 5. PAHs and fatty acids induce apoptosis of HCAECs. Cells were incubated for 4 h with vehicle, 1-methylanthracene (1-MA; 30 μM), benzo(a)pyrene (B(a)P; 30 μM) phenanthrene (PA; 30 μM) arachidonic acid (AA; 10 μM), linoleic acid (LA; 10 μM), or hydrogen peroxide (2 mM) as a positive control. Cell lysates were prepared and PARP cleavage was determined by Western blotting with two different anti-PARP antibodies as described in Materials and Methods. One antibody recognizes both the parent protein and the cleavage products (top panel); the other recognizes a cleavage product only (bottom panel). Both the parent protein and the cleaved products are present in PAH- and fatty acid-treated groups, but not in the vehicle-treated group (top panel). Only the PARP fragment was identified in lysates taken from PAH- and fatty acid-treated cells (bottom panel).

Fig. 6

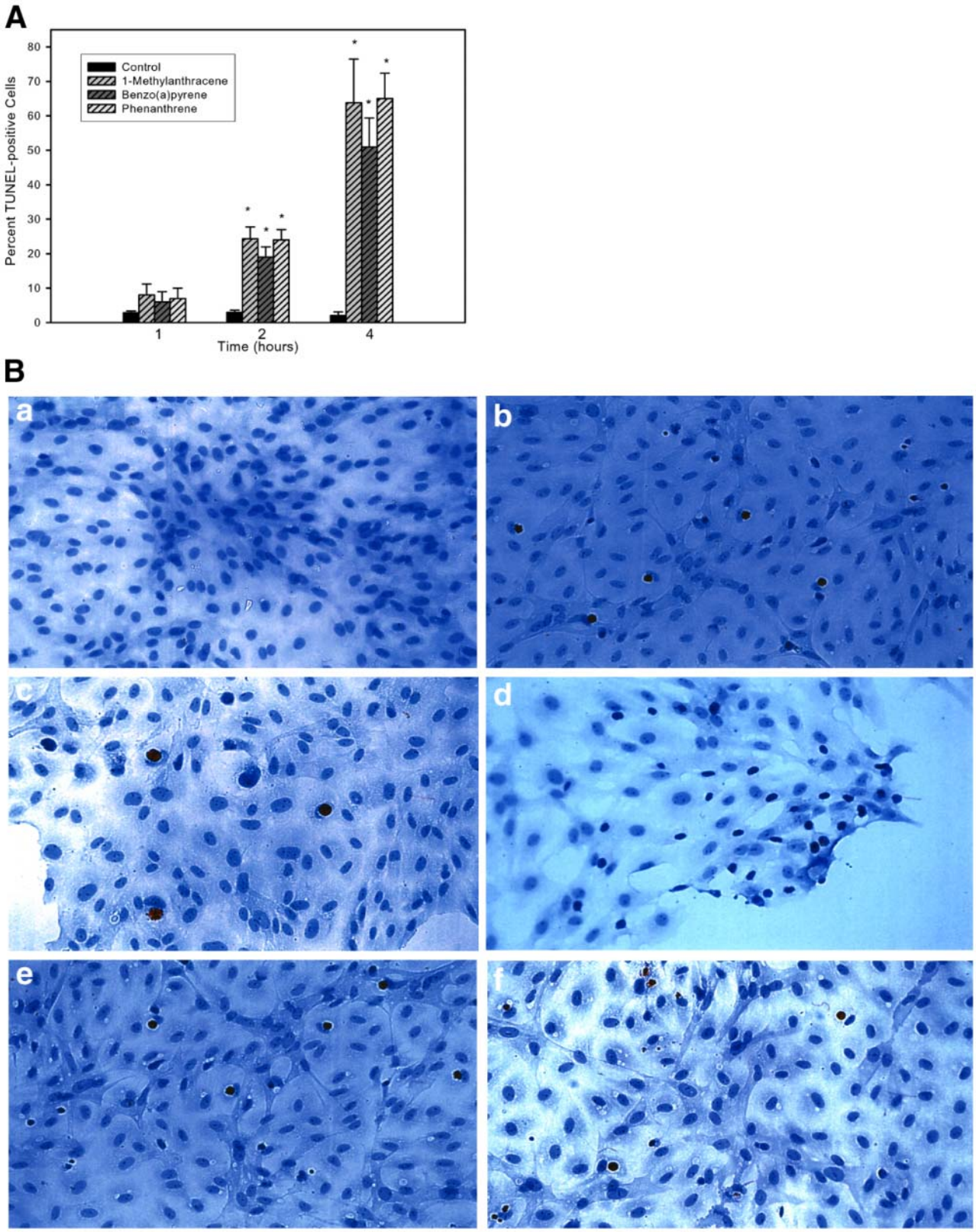

Figure 6. A) The kinetics of PAH-induced apoptosis were determined by in situ DNA end-labeling in cells treated for 1, 2, or 4 h with 1-methylanthracene (30 μM), benzo(a)pyrene (30 μM), or phenanthrene (30 μM) as described in Materials and Methods. (*) Significantly different from vehicle-treated control; $P<0.05$. **B)** Representative data from the 2-h timepoint after treatment with vehicle **(a)**, 1-methylanthracene **(b)**, benzo(a)pyrene **(c)**, phenanthrene **(d)**, arachidonic acid **(e)**, or linoleic acid **(f)**.

Fig. 7

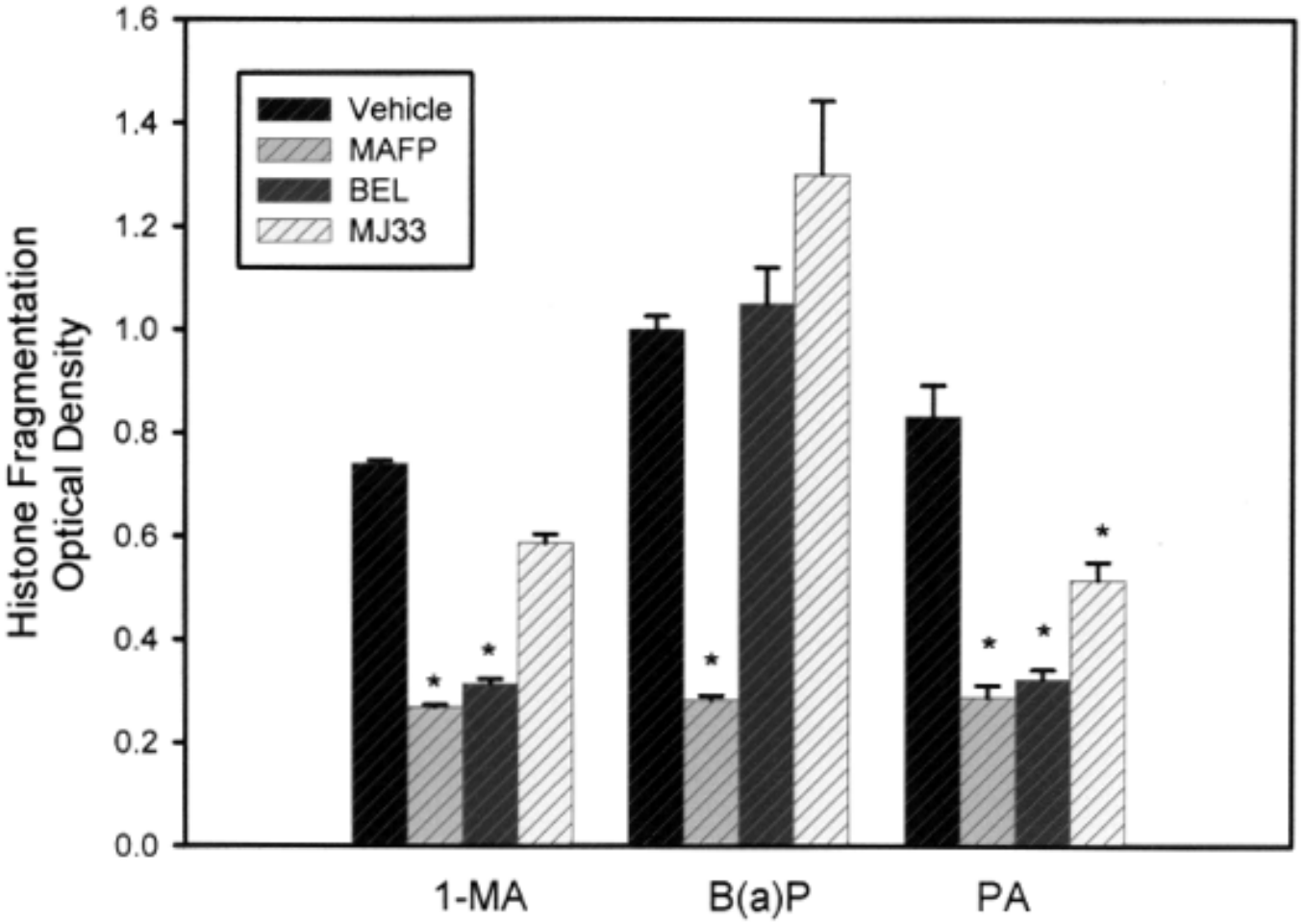

Figure 7. Inhibition of PAH-induced apoptosis of human coronary artery endothelial cells by specific PLA_2 inhibitors. Human coronary artery endothelial cells were pretreated with 10 μM methylarachidonoyl fluorophosphonate (MAFP), 2.5 μM bromoenol lactone (BEL), 10 μM MJ33, or vehicle for 30 min and then WERE treated with 30 μM 1-methylanthracene, benzo(a)pyrene, or phenanthrene for 2 h. Apoptosis was determined by histone fragmentation as described in Materials and Methods. The data represent two separate experiments run in triplicate. (*) Significantly different from respective value obtained in the absence of inhibitor.