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INHIBITION OF PROSTACYCLIN SYNTHESIS IN CULTURED BOVINE AORTIC ENDOTHELIAL CELLS BY VITAMIN K_1*

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Abstract—Prostacyclin (PGI_2), a potent vasodilator and inhibitor of platelet aggregation, is the predominant metabolite of arachidonic acid (AA) in endothelial cells derived from large blood vessels. Vitamin K_1 (1–100 μM) inhibited the release of PGI_2 and prostaglandin E_2 (PGE_2) by bovine aortic endothelial cells in culture, as measured by radioimmunoassay of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and PGE_2. The conversion of exogenous AA to PGI_2 was not affected by vitamin K_1 as measured by radioimmunoassay and high performance liquid chromatography of radiolabeled AA metabolites. Similarly, vitamin K_1 did not affect the conversion of prostaglandin H_2 (PGH_2) by *in vitro* enzyme systems. However, vitamin K_1 inhibited the calcium ionophore A23187-induced release of [^{3}H]AA from membrane phospholipids of bovine aortic endothelial cells. Inhibition of [^{3}H]AA release from other cells of vascular origin was also observed after exposure to vitamin K_1, but this effect was not observed in cells of non-vascular origin, including platelets. Therefore, vitamin K_1 modulates the release of AA in vascular cells and thus inhibits the capacity of blood vessels to synthesize PGI_2.

The synthesis of prostacyclin (PGI_2)‡, the potent vasodilatory and antiaggregatory eicosanoid, by cultured vascular endothelial cells was first observed by Weksler *et al.* [1]. Since this observation, cultured endothelial cells have been used extensively to investigate the regulation of PGI_2 release by endogenous compounds in vascular tissue. Much attention has focused on the inhibition of PGI_2 synthesis by lipid hydroperoxides, such as 15-hydroperoxy-arachidonic acid in microsomes from vascular tissue [2, 3] and in vascular cells [4–6], as these compounds have been associated with decreased PGI_2-synthesizing capacity in vascular disease [2, 7–9]. Vitamin K also is involved in hemostasis in that it is an essential cofactor for the formation of clotting factors II, VII, IX and X [10, 11]. The precursors of these proteins are modified via a vitamin K-dependent carboxylation of specific glutamyl residues to form the completed proteins, and evidence suggests that a hydroperoxide intermediate of vitamin K is involved in the carboxylation reaction [12]. Because of the sensitivity of PGI_2-synthase to inhibition by hydroperoxides it is possible that a hydroperoxide metabolite of vitamin K may affect PGI_2 synthesis if carboxylation of prothrombin precursor and PGI_2 synthesis occurred in proximity to each other.

We proposed in this study to investigate any possible effects of vitamin K_1 on PGI_2 production by vascular tissue, using vascular endothelial cells in culture. This is of particular interest because of the possible interaction between the extrinsic clotting mechanism and regulation of hemostasis by arachidonic acid metabolites.

EXPERIMENTAL PROCEDURES

Materials. Vitamin K_1 (phylloquinone; 2-methyl-3-phytyl-1,4-naphthoquinone) and vitamin K_3 (menadione; 2-methyl-1,4-naphthoquinone) were purchased from the Sigma Chemical Co., St. Louis, MO. [^{3}H]Arachidonic acid ([^{3}H]AA) (60–100 Ci/mmole), [^{14}C]AA (40–60 mCi/mmole), [^{3}H]6-keto-prostaglandin $F_{1\alpha}$ ([^{3}H]6-keto-$PGF_{1\alpha}$) (120–180 Ci/mmole), [^{3}H]prostaglandin E_2 ([^{3}H]PGE_2) (100–200 Ci/mmole) and Atomlight scintillation fluid were from New England Nuclear, Boston, MA.

AA was purchased from Nu-Chek Prep., Inc., Elysian, MN. 6-keto-$PGF_{1\alpha}$, PGE_2, 6-keto-$PGF_{1\alpha}$ antiserum and PGE_2 antiserum were purchased from Seragen, Inc., Boston, MA. Calcium ionophore A23187 was obtained from the Calbiochem-Behring Corp., La Jolla, CA. HPLC-grade acetonitrile and reagent grade glacial acetic acid were from Fisher Scientific, Fairlawn, NJ, and ethyl acetate was from the Baker Chemical Co., Phillipsburg, NJ. Tissue culture flasks and dishes were from Falcon, Oxnard, CA, and Costar, Cambridge, MA. Calf serum was from Hyclone Laboratories, Logan, UT, and fetal bovine serum was from Sigma. Dulbecco's modified Eagle medium (DMEM), RPMI Medium 1640 and Hanks' buffered salt solution (Ca^{2+}-, Mg^{2+}-free) (HBSS) were from the Grand Island Biological Co., Grand Island, NY. Instruments for reverse-phase high performance liquid chromatography were from Waters Associates, Milford, MA, and included model 6000A pumps, Z-module Radial Compression

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‡ Abbreviations: AA, arachidonic acid; DMEM, Dulbecco's modified Eagle medium; HBSS, Hanks' buffered salt solution; HPLC, high performance liquid chromatography; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; PGE_2, PGG_2, and PGH_2, prostaglandins E_2, G_2, and H_2; PGI_2, prostacyclin; PHS, prostaglandin H synthase; RSVM, ram seminal vesicle microsomes; and TXB_2, thromboxane B_2.

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Separation System, model 720 System Controller and Waters Intelligent Sample Processor model 710B. Separation was achieved on a Waters C_{18}, 10 μm, 5 mm i.d. Radial Pak column. A precolumn packed with Whatman (Clifton, NJ) 30–38 μm pellicular C_{18} beads was used. Radioactivity of the HPLC eluate was measured using a Radioactivity Flow Detector, Radiomatic Instruments and Chemical Co., Inc., Tampa, FL, with Hydrofluor (National Diagnostics, Somerville, NJ) as scintillation fluid.

Prostaglandin H_2 (PGH_2) was prepared from [^{14}C]AA according to the method of Crutchley *et al.* [13] using ram seminal vesicle microsomes and phenol as a reducing substrate. Ram seminal vesicles were obtained from Dr. L. Marnett, Wayne State University, Detroit, MI.

Cells. Bovine aortic endothelial cells were obtained from Dr. D. Gospodarowicz, University of California Medical Center, San Francisco, CA. The cells were identified as endothelial cells by immunofluorescence microscopy using antihemophilic factor (Factor VIII) antigen as a marker [14]. Porcine aortic endothelial cells were obtained from Dr. A. R. Whorton, Duke University Medical Center, Durham, NC. Identification of porcine endothelial cells was done by specific immunofluorescent demonstration of angiotensin-converting enzyme, α_2-macroglobulin and Factor VIII [15]. Cells were grown at 37° in 75 cm^2 tissue culture flasks under 5% CO_2 in air in DMEM with 10% calf serum for bovine cells and 10% fetal bovine serum for porcine cells. Endothelial cells from both species were subcultured at a split ratio of 1:4–1:6 and plated in 24 mm diameter wells of Costar trays and used just before confluency (5×10^5 cells/well).

Human umbilical vein endothelial and bovine aortic smooth muscle cells were obtained from Mr. M. Madden, Division of Pulmonary Medicine, University of North Carolina, Chapel Hill, NC, and were grown in DMEM containing 10% fetal bovine serum. Human endothelial cell cultures were prepared according to the method of Gimbrone [16] and were identified by immunofluorescent demonstration of Factor VIII antigen. Smooth muscle cells were prepared according to the method of Friedman *et al.* [17] and were identified by their light and electron microscopic appearance. All endothelial cells in culture were polygonal in shape and demonstrated contact inhibition.

Rat alveolar macrophages were prepared according to the method of Kouzan *et al.* [18]. They were cultured in 35 mm diameter tissue culture dishes (2×10^6 cells/dish) under 5% CO_2 in air in RPMI medium 1640 without serum.

Canine leukocytes were prepared according to the method of Böyum [19] and incubated in Ca^{2+}- and Mg^{2+}-free Krebs–Ringer buffer (2×10^7 cells/2 ml).

The experiments with human platelets were performed by Dr. J. Jakubowski, Veterans Administration Medical Center, Boston, MA. Platelets were prepared from donor blood, gel filtered and suspended in Tris buffer, pH 7, at 37°.

[^{3}H]AA labeling of cells. Bovine aortic endothelial cell monolayers were incubated for 16 hr in growth medium containing [^{3}H]AA (0.25 μCi per ml in 24 mm diameter wells or 10 μCi per 5 ml in 75 cm^2 flasks) to incorporate labeled AA into the cellular phospholipid fraction. Incorporation of [^{3}H]AA into phospholipid was confirmed by thin-layer chromatographic analysis of extracted phospholipids performed according to the method of Flower and Blackwell [20].

Porcine aortic and human umbilical vein endothelial cells and bovine aortic smooth muscle cells were incubated for 16 hr in growth medium containing 0.25 μCi [^{3}H]AA per ml in 24 mm diameter wells. Rat alveolar macrophages were incubated for 3.5 hr in RPMI medium containing 5 μCi [^{3}H]AA per 2 ml in 35 mm diameter dishes. Suspensions of human platelets (2×10^8 platelets/ml) were incubated in plastic tubes in Tris buffer containing 0.4 μCi [^{3}H]AA per ml for 30 min. Suspensions of canine leukocytes (10^7 cells/ml) were incubated in glass tubes in Krebs–Ringer buffer containing 2.5 μCi [^{3}H]AA per ml for 1 hr.

Incubation of labeled cells. Labeled cell monolayers and suspensions were washed twice with HBSS, to remove unincorporated [^{3}H]AA and serum, and preincubated in serum-free medium containing vitamin K_1 or vehicle. The preincubation medium was removed and replaced with serum-free medium containing vitamin K_1 and either A23187 or thrombin, in the case of platelets, to stimulate phospholipase-induced release of endogenous AA. Endothelial and smooth muscle cell monolayers were incubated with 5 μM A23187 for 10 min, rat macrophage monolayers with 20 μM A23187 for 20 min, canine leukocytes with 1 μM A23187 for 5 min, and platelets with 1 unit human α thrombin/ml for 5 min. At the termination of the experiment the medium was separated for counting of radioactivity or extraction of ^{3}H-labeled metabolites for HPLC.

Incubation of unlabeled cells. Monolayers of bovine endothelial cells were washed twice with HBSS and preincubated in serum-free DMEM containing vitamin K_1 or vehicle at 37° for 30 min. The preincubation medium was replaced with serum-free medium containing vitamin K_1 and A23187 (5 μM). After a 10-min incubation the supernatant fraction was removed, centrifuged at 2000 *g* for 10 min, and stored frozen at −15° for subsequent radioimmunoassay of prostanoids.

Incubation of cells with labeled precursor. Unlabeled bovine endothelial cell monolayers in 75 cm^2 plastic tissue culture flasks were washed twice with HBSS and preincubated in serum-free medium containing vitamin K_1 or vehicle at 37° for 30 min. The medium was removed and monolayers were incubated with serum-free medium containing vitamin K_1, [^{3}H]AA (10 μCi per flask) and A23187 (5 μM) for 15 min. At the termination of the experiment the supernatant fraction was removed and the prostanoids were extracted for HPLC.

Assay for effects of vitamin K_1 on cyclooxygenase and hydroperoxidase activities of prostaglandin H synthase (PHS). Microsomes were prepared from ram seminal vesicles, a rich source of PHS, as previously described [21]. PHS was solubilized from ram seminal vesicle microsomes (RSVM) with 1.0% Tween 20 according to the method of Miyamoto *et al.* [22] and stored in 0.1 M phosphate buffer, pH 7.8, at a concentration of 5.5 mg/ml.

The effects of vitamin K_1 on the cyclooxygenase and hydroperoxidase activities of PHS were studied by monitoring oxygen incorporation into AA with a Clark oxygen electrode at 37° as described previously [21]. The effects of vitamin K_1 on cyclooxygenase activity were determined by monitoring oxygen incorporation into AA during prostaglandin G_2 (PGG_2) biosynthesis. Incubation mixtures contained 50 mM phosphate buffer, pH 7.8, 0.5 mg protein, 100 μM AA, vitamin K_1 at indicated concentrations, and water to make a total volume of 2 ml. Oxygen uptake was recorded on a Fisher Recordall Series 5000 chart recorder and rates were compared to controls to determine the effect of vitamin K_1 on oxygen uptake.

To determine if vitamin K_1 could serve as a reducing cofactor for the hydroperoxidase activity of PHS, the effect of vitamin K_1 on oxygen uptake during the hydroperoxidase-dependent co-oxidation of phenylbutazone was measured. Compounds which serve as reducing cofactors for the hydroperoxidase activity of PHS can competitively inhibit incorporation of oxygen into phenylbutazone [23]. Incubation mixtures contained buffer, RSVM protein and water as described above, 500 μM phenylbutazone and vitamin K_1 at the indicated concentrations. Oxygen uptake was initiated by the addition of 100 μM H_2O_2 and monitored as described above. The incubation was also done in the absence of phenylbutazone to investigate whether vitamin K_1 itself accepts O_2.

Incubation of RSVM with [^{14}C]PGH_2. Incubation mixtures contained 50 mM Tris buffer, pH 7.5, 1.0 mg RSVM, 0.1 μCi [^{14}C]PGH_2, vitamin K_1 at indicated concentrations, and water to make a total of 2 ml. The incubation was terminated, after 5 min at 37°, by acidification to pH 3.5 with formic acid. Prostanoid metabolites were extracted as described below.

Extraction, high performance liquid chromatography (HPLC), and radioactivity determination. Release of radioactivity into the incubation medium was measured by counting an aliquot of incubation medium directly in Atomlight scintillation fluid.

Labeled metabolites of [^{3}H]AA and [^{14}C]PGH_2 were extracted by acidifying the incubation medium to pH 3.5 with 1 M formic acid and extracting twice with 3 vol. of ethyl acetate. The organic phase was dried under vacuum and samples were reconstituted in 24% acetonitrile in water for HPLC. Recovery of internal standards during sample preparation was greater than 90%.

Separation of prostanoids by reverse-phase HPLC was achieved by isocratic elution with 24% acetonitrile in water (pH 3.5) at a flow rate of 3 ml/min [24]. Samples and standards were injected in a volume of 250 μl, and the column was eluted for 60 min with 24% acetonitrile in water. These conditions gave very good resolution of 6-keto-$PGF_{1\alpha}$, thromboxane B_2 (TXB_2), $PGF_{2\alpha}$ and PGE_2. AA and less polar metabolites were eluted in a single peak with 100% acetonitrile after the initial 60 min isocratic step.

Radioimmunoassay of prostanoids. 6-Keto-$PGF_{1\alpha}$ and PGE_2 were quantitated by radioimmunoassay using a procedure described by Seragen, Inc., Boston, MA. Briefly, this involved adding a 0.1 ml aliquot of the cell incubation medium to polypropylene tubes containing the appropriate antibody and ^{3}H-labeled prostanoid in Tris–gelatin buffer. After incubating overnight at 4°, the unbound prostanoid was precipitated by treatment with dextran-coated charcoal and centrifugation at 4°. The percentage of bound prostanoid was determined by liquid scintillation counting of the supernatant fraction and compared with known standards. Each sample was assayed in duplicate. Samples assayed at several dilutions exhibited parallelism with authentic standards.

Statistical analysis. Statistical significance for paired data was determined by Student's *t*-test. Statistical significance for dose responses to vitamin K_1 was determined by one-way analysis of variance using Williams' test for multiple comparison of data. A P value of < 0.02 was selected to denote statistical significance between groups.

RESULTS

Effect of vitamin K_1 on prostanoid synthesis by bovine aortic endothelial cells measured by radioimmunoassay. Cultured bovine aortic endothelial cells produce significant amounts of PGI_1 and PGE_2

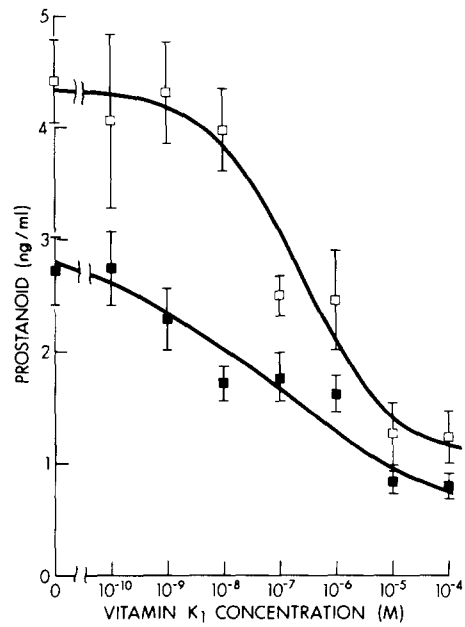

Fig. 1. Effect of vitamin K_1 on 6-keto-$PGF_{1\alpha}$ (□) and PGE_2 (■) synthesis by cultured bovine aortic endothelial cells. Monolayers were preincubated with vitamin K_1 for 30 min, followed by a 10-min incubation with A23187 (5 μM). 6-keto-$PGF_{1\alpha}$ and PGE_2 were quantitated by radioimmunoassay of the incubation medium as described in Experimental Procedures. Data represent the means ± SEM of three experiments. A P value of < 0.02 was selected to denote statistical significance between groups. Statistical significance: 6-keto-$PGF_{1\alpha}$: medium vs vitamin K_1 10^{-10}, 10^{-9}, 10^{-8} M, not significant; 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} M, $P < 0.01$; PGE_2: medium vs vitamin K_1 10^{-10}, 10^{-9} M, not significant; 10^{-8}, 10^{-7}, 10^{-6} M, $P < 0.05$ (NS); 10^{-5}, 10^{-4} M, $P < 0.02$.

when incubated *in vitro* with the calcium ionophore A23187, a known stimulus of phospholipase-induced release of endogenous AA. Pre-exposure of endothelial cells to vitamin K for 30 min significantly reduced the amount of PGI_2 released (measured as its stable hydrolysis product, 6-keto-$PGF_{1\alpha}$) and PGE_2. This effect was dependent upon the concentration of the vitamin as shown in Fig. 1 for vitamin K_1. 6-Keto-$PGF_{1\alpha}$ and PGE_2 production were equally sensitive to inhibition by vitamin K_1, suggesting that the vitamin modified production of both metabolites by a similar mechanism. The approximate IC_{50} of vitamin K_1 was $1.0–3.0 \times 10^{-7}$ M. The viability of vitamin K_1-treated endothelial cells, as judged by trypan blue dye exclusion, was comparable to that of control cells with more than 95% of cells in each group excluding the dye. The addition of vitamin K_3, either as the fat soluble form or as the sodium bisulfite salt, also inhibited the formation of PGI_2 and PGE_2 by endothelial cells. The IC_{50} was approximately 1.0×10^{-7} M. However, a biphasic effect of vitamin K_3 on PGI_2 release was observed. At concentrations above 10^{-6} M significant stimulation of PGI_2 and PGE_2 release was observed, and the vitamin appeared to be toxic to the cells. Vitamin K_3 has been shown to have toxic effects on intact cells [25]. The concentrations of vitamin K_1, which is the naturally occurring form of vitamin K, found to be effective in inhibiting prostaglandin production are well within the physiologic concentration range (see Discussion). Therefore, in all subsequent experiments vitamin K_1 was used.

Bovine endothelial cells were exposed to vitamin K_1 (10^{-6} M) for various times before addition of A23187 to the incubation medium. The inhibition of A23187-induced release of 6-keto-$PGF_{1\alpha}$ observed was dependent on the time of pre-exposure to vitamin K_1 (Fig. 2). Pretreatment of monolayers with vitamin K_1 led to a reduction in the amount of 6-keto-$PGF_{1\alpha}$ released after stimulation by A23187. This inhibition was statistically significant for pre-incubation times of 20 min or longer. However, the maximum effect was observed at 20 min with no further increase in inhibition apparent after a pre-

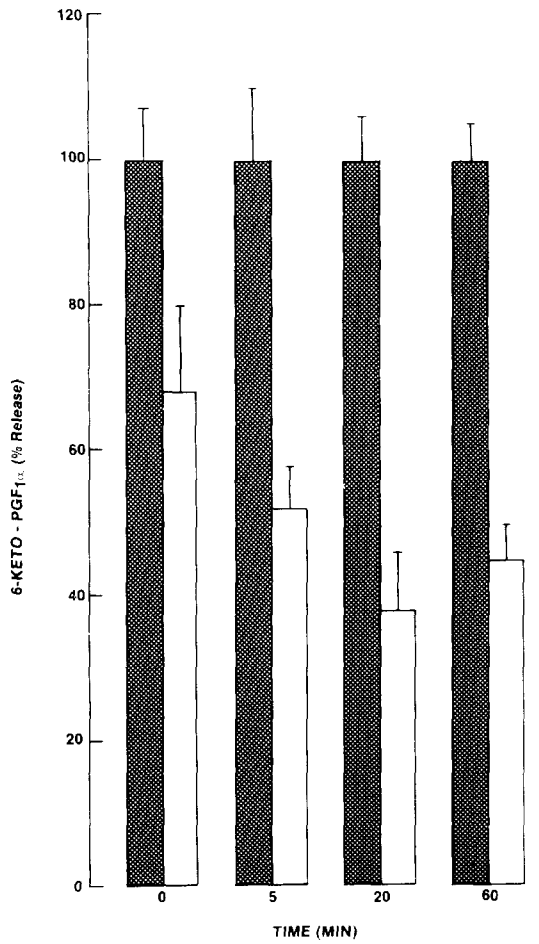

Fig. 2. Time course of the vitamin K_1 pretreatment. Bovine aortic endothelial cells were exposed for various time intervals to culture medium (shaded bars) or 10^{-6} M vitamin K_1 (open bars) before addition of A23187. 6-keto-$PGF_{1\alpha}$ was quantitated by radioimmunoassay. Control values were normalized to 100% for each pretreatment time. Zero-time control corresponded to 1.24 ± 0.22 ng/ml 6-keto-$PGF_{1\alpha}$. Statistical significance: medium vs vitamin K_1, 0 min, not significant; 5 min, $P < 0.05$ (NS); 20 min, $P < 0.02$; 60 min, $P < 0.01$.

Table 1. Effect of vitamin K_1 on the cyclooxygenase and hydroperoxidase activities of prostaglandin H synthase of RSVM

Cyclooxygenase activity		Hydroperoxidase activity		
Vitamin K_1 (M)	% O_2 uptake/sec	Vitamin K_1 (M)	Phenylbutazone*	% O_2 uptake/sec
0	5.55	0	+	3.09
10^{-8}	5.38	10^{-8}	+	3.17
10^{-7}	5.21	10^{-7}	+	3.11
10^{-6}	5.29	10^{-6}	+	3.09
10^{-5}	5.21	10^{-5}	+	3.78
		10^{-4}	+	3.13
		0	-	0
		10^{-6}	-	0

Cyclooxygenase and hydroperoxidase activities were measured as described in Experimental Procedures and are expressed as percent total O_2 uptake per sec. Cyclooxygenase activity was assayed by monitoring O_2 incorporation into AA during PGG_2 biosynthesis. Hydroperoxidase activity was assayed by monitoring O_2 uptake during hydroperoxidase-dependent co-oxidation of phenylbutazone.

* Phenylbutazone concentration = 500 μM.

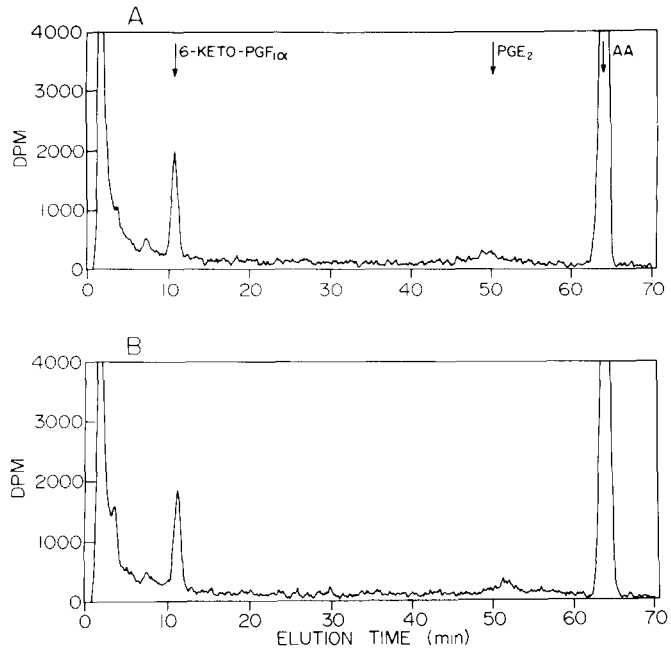

Fig. 3. Effect of vitamin K_1 on metabolism of exogenous AA by bovine aortic endothelial cells. After preincubation in the absence (A) and presence (B) of vitamin K_1 (10^{-4} M), monolayers were incubated with [^{3}H]AA and A23187 (5 μM) for 10 min. Labeled metabolites were extracted from the medium and separated by HPLC.

exposure time of 60 min. Hence, a preincubation time of 30 min was used in all subsequent experiments.

Effect of vitamin K_1 on prostaglandin H synthase and PGI_2 synthase. To investigate which enzymes of the AA metabolism cascade are involved in the inhibition of prostanoid production by vitamin K_1, the effect of vitamin K_1 on the cyclooxygenase and hydroperoxidase activities of PHS was examined. Ram seminal vesicles were used since they are a conveniently obtained and highly active source of PHS and PGI_2 synthase, the enzymes under study in vascular endothelial cells. Table 1 shows that vitamin K_1, up to a concentration of 10^{-5} M, did not affect the cyclooxygenase activity of PHS, measured as O_2 uptake into AA during PGG_2 biosynthesis. Similarly, the hydroperoxidase activity of PHS was not affected by vitamin K_1 as determined by oxygraph studies in which vitamin K_1 had no effect on hydroperoxidase-dependent co-oxidation of phenylbutazone (Table 1). Vitamin K_1 also did not act as a reducing cofactor and accept O_2 itself as there was no O_2 uptake in the absence of phenylbutazone (Table 1).

To further substantiate the lack of effect of vitamin K_1 on PHS, the effect of vitamin K_1 on the metabolism of exogenous [^{3}H]AA by bovine aortic endothelial cells was examined. Pretreatment of endothelial cells with vitamin K_1 did not inhibit PGI_2 production, as determined by HPLC separation of radiolabeled AA metabolites (Fig. 3). Indeed, the ability of endothelial cells to transform exogenous [^{3}H]AA into prostanoids was identical for control (6-keto-$PGF_{1\alpha}$, 8.83% and PGE_2, 1.75% of total

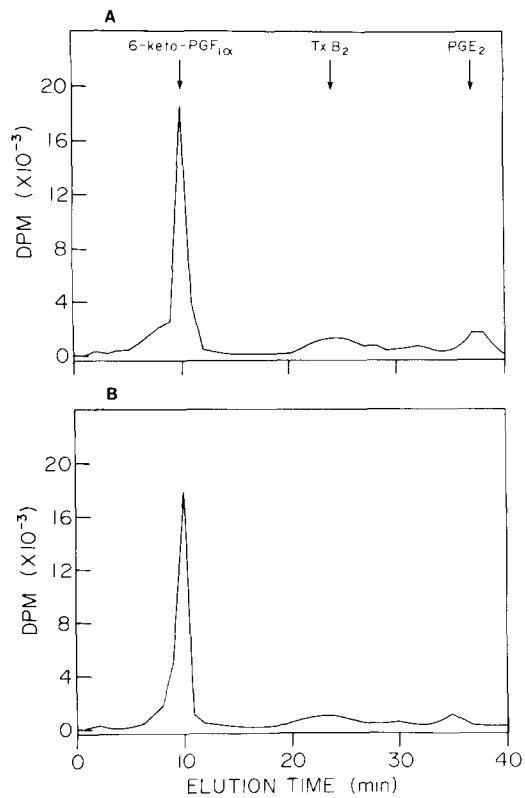

Fig. 4. Effect of vitamin K_1 on metabolism of PGH_2 by RSVM. RSVM were incubated with [^{14}H]PGH_2 in the absence (A) and presence (B) of vitamin K_1 (10^{-4} M) for 5 min. Labeled prostanoids were separated by HPLC.

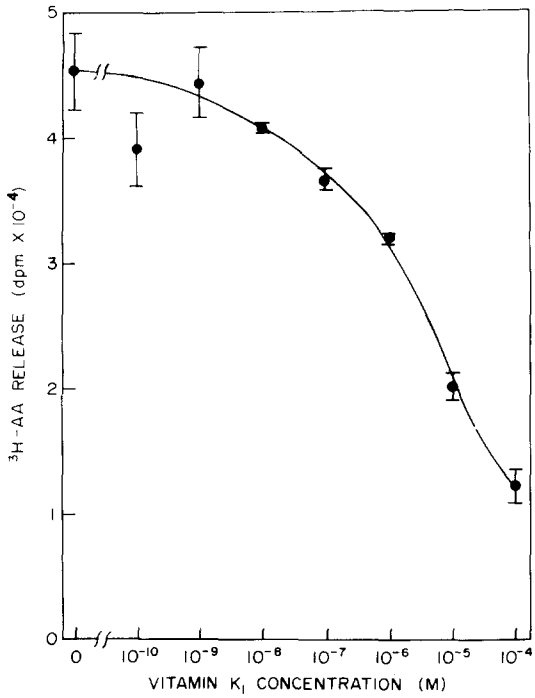

Fig. 5. Effect of vitamin K_1 on release of [^{3}H]AA from bovine aortic endothelial cells. Cellular phospholipids were labeled by incubation of monolayers for 16 hr with [^{3}H]AA. After preincubation with vitamin K_1 for 30 min, release of AA was stimulated by incubation of cells with A23187 (5 μM) for 10 min. Aliquots of incubation medium were assayed for released radioactivity. Data represents mean ± SEM of three experiments. Statistical significance: medium vs vitamin K_1 10^{-10}, 10^{-9}, 10^{-8} M, $P < 0.05$ (NS); 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} M, $P < 0.01$.

radioactivity) and vitamin K_1-treated cells (6-keto-$PGF_{1\alpha}$, 8.63% and PGE_2, 1.88%). These data indicate that vitamin K_1 does not inhibit PHS. The ratio of 6-keto-$PGF_{1\alpha}$ to PGE_2 observed in this experiment differs from that shown in Figs. 1 and 3. This difference in the ratio of metabolites between experiments is typical for cells in culture and appears to depend on cell passage number and degree of confluency.

The effect of vitamin K_1 on PGI_2 synthase was investigated by examining the metabolism of [^{14}C]PGH_2 to prostanoids by RSVM. On incubation with the microsomes, PGH_2 was metabolized to mainly PGI_2. Little, if any, other prostanoid was observed. The amounts of [^{14}C]PGI_2 measured as [^{14}C]6-keto-$PGF_{1\alpha}$, produced in the presence and absence of vitamin K_1, were identical (Fig. 4), indicating that vitamin K_1 does not inhibit PGI_2 synthase in RSVM.

Effect of vitamin K_1 on phospholipase activity. Phospholipase activity was quantitated by measuring the A23187-induced release of radioactivity from cells prelabeled with [^{3}H]AA. A23187 triggered a 5-fold increase in release of radioactivity into the medium above basal release. Vitamin K_1 caused a dose-dependent inhibition in A23187-induced release of [^{3}H]AA from bovine aortic endothelial cells (Fig. 5). Inhibition was maximal (75%) at a concentration of 10^{-4} M vitamin K_1 and paralleled the inhibition of 6-keto-$PGF_{1\alpha}$ and PGE_2 synthesis by vitamin K_1 shown in Fig. 1. In several experiments, the IC_{50} of vitamin K_1 ranged from 1.0 to 6.0×10^{-7} M. The IC_{50} for inhibition of release of [^{3}H]AA by vitamin K_1 is therefore very similar to the IC_{50} for inhibition of release of 6-keto-$PGF_{1\alpha}$ and PGE_2 by vitamin K_1. The metabolism of [^{3}H]AA

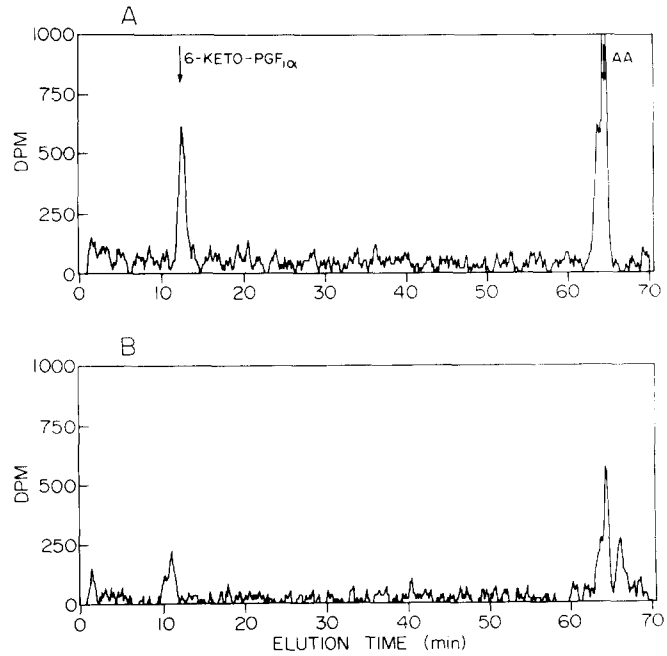

Fig. 6. Effect of vitamin K_1 on release and metabolism of [^{3}H]AA by bovine aortic endothelial cells. After preincubation for 30 min in the absence (A) and presence (B) of 10^{-4} M vitamin K_1, release of esterified [^{3}H]AA was stimulated with A23187 (5 μM) for 10 min. [^{3}H]AA and metabolites were extracted from the incubation medium and separated by HPLC.

Table 2. Comparison of effect of vitamin K_1 on arachidonic acid release on various cell types

Cells	[^{3}H]AA release*		
	Control	Vitamin K_1 (10^{-4} M)	% Inhibition
Bovine aortic endothelial	45,275 ± 3,198†	12,389 ± 1,355	
	1.58 ± 0.11‡	0.43 ± 0.05	72.6§
Porcine aortic endothelial	34,412 ± 2,465	24,018 ± 1,175	
	28.23 ± 0.02	19.71 ± 0.01	30.2§
Human umbilical vein endothelial	4,886 ± 148	2,897 ± 191	40.8§
	8.1 ± 0.2	4.8 ± 0.3	
Bovine aortic smooth muscle	9,389 ± 628	4,129 ± 190	
	3.9 ± 0.3	1.7 ± 0.1	56.0§
Human platelets	62,584 ± 30,202	56,720 ± 20,654	
	12.06 ± 5.82	10.93 ± 3.98	9.4‖
Canine leukocytes	148,792 ± 11,079	120,281 ± 10,069	
	4.96 ± 0.37	4.01 ± 0.34	19.2‖
Rat alveolar macrophages	116,420 ± 9,825	95,885 ± 6,040	
	3.28 ± 0.28	2.71 ± 0.17	17.6‖

Cells were incubated with [^{3}H]AA to label the phospholipid fraction. After the prelabeling period, the cells were incubated with medium alone or medium containing vitamin K_1 and then stimulated to release AA as described in Experimental Procedures. Results shown are from a single experiment verified in at least three separate studies.

* Values are mean ± SEM, N = 3–6.

† Disintegrations per min. per incubation.

‡ Percentage of incorporated [^{3}H]AA.

§ $P < 0.01$.

‖ Not significant.

released by A23187 in bovine aortic endothelial cells was examined using HPLC of radiolabeled products (Fig. 6). Vitamin K_1 inhibited release of [^{3}H]AA from cellular phospholipid but had no effect on conversion of endogenous [^{3}H]AA to [^{3}H]6-keto-$PGF_{1\alpha}$, providing further evidence that vitamin K_1 exerted its effect on phospholipase and not on subsequent enzymes in the AA metabolic pathway.

To investigate the possibility that vitamin K_1 reduced the availability of free AA by enhancement of reesterification of AA into phospholipids by acyl transferases rather than inhibition of phospholipase activation, the effect of vitamin K_1 on uptake of [^{3}H]AA by cultured bovine aortic endothelial cells was examined. Neither the rate nor the extent of incorporation of [^{3}H]AA into the cells was affected by vitamin K_1.*

Effect of vitamin K_1 on phospholipase activity of various cell types. In view of the above observation with bovine aortic endothelial cells, the effect of vitamin K_1 on arachidonic acid release by several different cell types was investigated to determine the cell specificity of this response. The various cell preparations were incubated with [^{3}H]AA to label the phospholipid fraction and then incubated with vitamin K_1 as described in Experimental Procedures. The release of [^{3}H]AA from the cell preparations was then measured after stimulation of phospholipase activity. Phospholipase activity was inhibited by vitamin K_1 (10^{-4} M) in all of the vascular cell types studied, which were bovine and porcine aortic endothelial cells, human umbilical vein endothelial cells and bovine aortic smooth muscle cells (Table 2). The largest effect was seen on the two bovine cell cultures. No effect of vitamin K_1 on AA release could be demonstrated in human blood platelets, canine leukocytes or rat alveolar macrophages (Table 2).

* R. D. Nolan, unpublished observations.

DISCUSSION

Results of our studies indicate that the release of AA from membrane phospholipids in vascular endothelial and smooth muscle cells is inhibited by vitamin K_1, one of the more biologically active forms of vitamin K formed in animal tissues. Since the availability of precursor AA is reduced, the synthesis of eicosanoids by endothelial cells is also reduced by vitamin K_1. This is reflected in the levels of 6-keto-$PGF_{1\alpha}$ and PGE_2 measured. The inhibitory effect of vitamin K_1 is directed towards the phospholipases, the series of enzymes that catalyze the liberation of AA from esterified stores, rather than subsequent enzymes in the AA metabolic pathway. This conclusion is based on several lines of evidence: (i) vitamin K_1 inhibited the release of ^{3}H-label from cells that had been prelabeled with [^{3}H]AA, (ii) vitamin K_1 did not directly inhibit PHS or PGI_2 synthase, and (iii) inhibition of AA release paralleled the decrease in release of PGI_2 and PGE_2 by endothelium, suggesting that vitamin K_1 inhibits the rate-limiting step in the metabolic cascade common to the three compounds measured, namely hydrolysis of phospholipid by phospholipases. The possibility that the decreased release of AA observed after vitamin K_1 treatment was due to a stimulation of reesterification of AA into phospholipids was ruled out by the finding that uptake of [^{3}H]AA by endothelial cell monolayers was not affected by vitamin K_1. The mechanism by which AA is released from

phospholipids in vascular endothelial cells in response to a specific stimulus is unknown. Hong and Deykin [26] suggest that bradykinin activates a phosphatidylinositol-hydrolyzing phospholipase A_2 as well as a phospholipase C in porcine aortic endothelial cells. Whether this phospholipase A_2 cleaves AA directly from phospholipid or acts on phosphatidic acid formed by the sequential action of phospholipase C and diglyceride kinase remains to be determined. Alternatively, it has been demonstrated in human platelets that AA is cleaved from diglyceride by a diglyceride lipase following the action of phospholipase C [27, 28]. Thus, vitamin K_1 could interfere with the action of one or more of these enzymes in vascular endothelial cells.

Recently, Blackwell *et al.* [29] reported that several vitamin K analogs (K_3, K_4, K_7) inhibit platelet AA liberation and aggregation. The IC_{50} values for inhibition of aggregation induced by ADP, collagen, and thrombin were at least 2×10^{-5} M. However, IC_{50} values of $7–8 \times 10^{-4}$ M were observed with calcium ionophore-induced aggregation. In fact, the concentration of vitamin K_3 required to inhibit platelet aggregation and AA liberation was more than 1000-fold that for inhibition by vitamin K_3 of ionophore-induced PGI_2 release from endothelial cells in this study. We tested vitamin K_1 in concentrations as high as 10^{-4} M in platelets and did not observe an effect on AA release. Interpretation of the effects of vitamin K_3 (menadione) may be complicated because it is generally assumed that menadione must be alkylated by intestinal microorganisms or tissue alkylating enzymes to menaquinone to exert its biological effect [30].

The phospholipase-induced release of AA from vascular endothelial cells in response to specific stimuli, such as bradykinin [15], histamine, thrombin and ionophore A23187 [31], most likely involves the mobilization of Ca^{2+} within the cell. Preliminary experiments in this laboratory suggest that, in human umbilical vein endothelial cells, vitamin K_1 inhibits release of AA in response to bradykinin. Thus, it is expected that vitamin K_1 would inhibit phospholipase activity in response to stimulation by bradykinin, histamine and thrombin as well as by A23187. It is possible that the tissue-specific sensitivity to vitamin K_1 observed in this study is related to the variations in sensitivities of different phospholipases to Ca^{2+} activation reported by Michell [32] and Hong *et al.* [31]. Future studies to investigate the activities of the individual phospholipases and the Ca^{2+} dependence of these enzymes, using methods to separate the lipid species involved or using purified enzymes and substrates, are necessary to understand the action of vitamin K_1.

The results from this study indicate that vitamin K_1 interferes with the metabolism of AA in cells of the vascular system but is devoid of such activity in other cell types. This may be of physiological importance since vascular tissue is a major source of PGI_2 which is a significant contributor to the regulation of hemostasis. The effect of this reduced PGI_2 production may be especially pronounced if thromboxane A_2 production by platelets is unaffected by vitamin K_1, as these studies suggest. Vitamin K_1 may therefore contribute to the development of thrombosis by specifically lowering the capacity of vascular tissue to synthesize PGI_2 yet not affecting the capacity of the platelet to synthesize thromboxane A_2. Whether this effect of vitamin K_1 is of physiological significance remains to be determined. Certainly the normal circulating levels of the vitamin are low [33], in the order of 10^{-9} M which is much lower than the concentrations effective in inhibiting AA release in vascular cell cultures. However, the levels of circulating vitamin K_1 are elevated dramatically in cases of hyperlipidemia [33], since vitamin K_1 is transported by lipoproteins, and after pharmacological administration of vitamin K_1 for treatment of hemorrhagic disease in which doses of 25 mg are used. Plasma concentrations of vitamin K_1 reach levels of 10^{-6} M within hours of pharmacological administration [34]. Moreover, high concentrations of vitamin K_1 (10^{-4} to 10^{-5} M) are required for carboxylation of prothrombin precursor by preparations of rat liver microsomes [35]. The intracellular levels of vitamin K_1 may be high particularly in tissues where the vitamin accumulates and could account for the intracellular effects of vitamin K_1 on vascular cells. Anderson and Barnhart [36, 37] demonstrated that prothrombin is produced in the liver but other tissues such as bone [38] and kidney [39] are able to carry out vitamin K-dependent carboxylation of proteins. Vitamin K was found to be distributed throughout most tissues of the body [40, 41] including cardiovascular and other, highly vascularized tissues, but whether its distribution indicates any physiological function of the vitamin in tissues other than liver cannot be determined from those studies. However, it has been established that vitamin K analogs inhibit such neutrophil functions as phagocytosis, bactericidal activity, peroxide formation and lysosomal enzyme release [42].

The possibility that vitamin K_1 modulates the production of PGI_2 by vascular tissue *in vivo* is especially intriguing because it would implicate the regulation of the fibrin clotting cascade with the regulation of platelet aggregation and therefore represent a common regulatory step in the control of both hemostatic mechanisms. The concept of regulation of two separate aspects of blood clotting by a single agent, such as vitamin K_1, would not be unprecedented. It is well known that von Willebrand factor is necessary for platelet adhesion to blood vessel walls and also the regulation of plasma levels of factor VIII C [43], a clotting factor necessary for fibrin clot formation. As with von Willebrand factor, alterations in the plasma levels of vitamin K_1 may affect the formation of the platelet aggregate as well as the fibrin clot.

In summary, our study shows that vitamin K_1 has a potent inhibitory activity on vascular AA metabolism *in vitro*. The mechanism of action of vitamin K_1 on AA release in vascular cells and its physiological significance remain to be studied.

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