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ACTIVATION OF PHOSPHOLIPASE A_2 BY ENDOTHELIN IN CULTURED VASCULAR SMOOTH MUSCLE CELLS

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SUMMARY. The ability of endothelin to promote phospholipid hydrolysis has been studied in myo-[2-^{3}H]-inositol-, [^{3}H]-arachidonic acid- or methyl-[^{3}H]choline chloride-prelabelled cultured vascular smooth muscle cells (VSMC) from rat and bovine thoracic aortae and human omental vessels. The biochemical responses to endothelin were comparable between the different VSMC isolates. Endothelin promoted the accumulation of glycerolphospho[^{3}H]inositol and concomitant loss of [^{3}H]-inositol label from phosphatidylinositol. Exposure of [^{3}H]choline-labelled VSMC to endothelin resulted in a loss of radioactivity from phosphatidylcholine that was inversely parallelled by an increase in water-soluble [^{3}H]-choline metabolites. In [^{3}H]-arachidonic acid ([^{3}H]-AA)-labelled VSMC, endothelin induced extracellular release of [^{3}H]-AA which derived from both phosphatidylcholine and phosphatidylinositol. Half-maximally effective concentrations of endothelin for all these responses were $\sim$ 2-7 nM and did not vary between VSMC types. Endothelin-induced release of [^{3}H]-AA into VSMC medium-overlay was inhibited by quinacrine and nordihydroguaiaretic acid but not by neomycin or indomethacin. The data herein implicate activation of phospholipase A_2 by endothelin with subsequent metabolism of arachidonic acid via the lipoxygenase pathway.

INTRODUCTION. The potent and characteristically protracted vasoconstriction promoted by endothelin, an endothelial-derived peptide, in various isolated blood vessels is dependent on the presence of extracellular Ca^{2+} (1). In fura-2 loaded cultured vascular smooth muscle cells (VSMC) endothelin was shown to induce an initial transient- followed by a sustained - increase in intracellular Ca^{2+} (2). Since only the latter was inhibited by EGTA or Ca^{2+}-antagonist (2) mobiliza-

Abbreviations: VSCM, vascular smooth muscle cells; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; ED_{50}, half-maximally effective dose.

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tion of Ca^{2+} from intracellular stores is probably a primary response to endothelin. Additionally, endothelin-induced vasoconstriction in isolated blood vessels was reported to be reversed by H-7, a protein kinase C inhibitor (3). These data suggest that endothelin might promote phosphoinositide breakdown to produce the metabolites inositol trisphosphate and diacylglycerol which function as intracellular second messengers to mobilize intracellular Ca^{2+} stores and activate protein kinase C, respectively (4,5). Indeed we have demonstrated (6) that such rapid phospholipase C-mediated hydrolysis of phosphoinositides does occur in VSMC exposed to endothelin. Subsequent to inositol trisphosphate production and mobilization of Ca^{2+} a secondary Ca^{2+}-dependent breakdown of inositol phospholipids may occur (7). The phospholipase C hydrolysing phosphatidylinositol requires Ca^{2+} whereas those clearing polyphosphoinositides are active even in the presence of EGTA (8). Phospholipase A_2 which deacylates phosphatidylinositol to produce lysophosphatidylinositol and arachidonic acid is also Ca^{2+}-dependent (9,10). Given that endothelin elicits protracted increases in both intracellular Ca^{2+} (2) and inositol trisphosphate (6) such secondary Ca^{2+}-dependent phospholipid hydrolysis may be an important pathway through which endothelin may operate.

We report here that, in VSMC from human, rat and bovine vessels, endothelin induces phospholipase A_2-mediated degradation of phosphatidylinositol and phosphatidylcholine.

MATERIALS AND METHODS

Materials. Tissue culture material and chemicals were from Gibco AG, Switzerland with the exception of fetal calf serum (Fakola AG, Switzerland). All radioisotopes were from Amersham; myo-[2-^{3}H]inositol (16 Ci/mmol), [methyl-^{3}H]choline chloride (80 Ci/mmol) and [5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid (195 Ci/mmol). Porcine endothelin was purchased from Peptide Institute Inc., Japan. All other chemicals and reagents of analytical grade were commercially obtained.
Cell Culture. Procedures for the isolation, culture and characterization of vascular smooth muscle cells (VSMC) from human omental vessels, bovine and rat thoracic aortae were as previously described (11-13). VSMC between passages 4 and 13 were used at confluency and after being rendered quiescent by culture for 48 hrs in serum-free medium (where 0.1% (w/v) bovine serum albumin was substituted for fetal calf serum). Cell numbers were determined after trypsin dissociation.
Phospholipid Metabolism. VSMC were prelabelled either with myo-[^{3}H]-inositol (5 µCi/ml for 48 hrs under serum- and inositol-free conditions), with [^{3}H]-arachidonic acid (VSMC at quiescence, 1 µCi/ml for 3 hrs under serum-free conditions), or with [^{3}H]choline chloride (VSMC at quiescence, 1 µCi/ml for 4 hrs under serum-free conditions). [^{3}H]-inositol prelabelled cells were used for investigating breakdown of phosphoinositol lipids and accumulation of inositol phosphates in response to endothelin. After washing VSMC were pre-incubated for 30 min in isotonic phosphate buffered saline containing 20 mM TES/HEPES (pH 7.3) and 30 mM LiCl prior to exposure to endothelin. Reactions

were terminated following aspiration of buffer and addition of $CHCl_3$:MeOH:HCl (1:2:0.05 v/v). Dowex-1-X4 anion exchange columns were used for separating extracted inositol phosphates and phosphoinositol lipids (after deacylation) (14,15). [^{3}H]-arachidonic acid prelabelled cells were washed and pre-incubated for 10 min in minimal essential medium containing 0.1% (w/v) bovine serum albumin (MEM/BSA) before exposure to endothelin. Extracellular release of [^{3}H]-arachidonic acid was assessed by liquid scintillation spectrophotometric quantitation of radioactivity in 250 µl aliquots of medium overlay (16). Lipids were extracted after aspiration of remaining medium and addition of $CHCl_3$:MeOH (1:2 v/v) (15). Lipids were resolved on heat-activated Silica Gel 60 plates using the solvent $CHCl_3$:MeOH:CH_3COOH:H_2O (100:30:50:4, v/v); phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylethanolamine (PE) migrated with Rf values of 0.27, 0.51, 0.59 and 0.73, respectively. Standards were co-chromatographed and after visualization by iodine-staining the appropriate areas were scraped into vials, followed by addition of toluene (1 ml, 1 hr at room temperature) and quantitation of radioactivity by liquid scintillation spectrophotometry. [^{3}H]-choline chloride prelabelled cells were washed and incubated in MEM/BSA for 10 min prior to addition of endothelin. Reactions were terminated by aspiration of medium and additions of $CHCl_3$:MeOH (1:2 v/v). Following extraction, radioactivity in 1.0 ml of the aqueous phase was quantitated by liquid scintillation counting to determine [^{3}H]choline metabolites (17). Lipids in the organic phase were resolved on Silica Gel 60 plates and radioactivity in the area corresponding to PC determined using procedures given above. All experiments were performed using triplicate wells for each determination, and where appropriate Student's t-test for unpaired data was applied to determine statistical significance.

RESULTS

When myo-[^{3}H]inositol-prelabelled VSMC were exposed to endothelin glycerophospho[^{3}H]inositol levels increased in the aqueous phase of acidified chloroform/methanol extracts. This time-dependent accumulation was comparable between human, rat and bovine VSMC (Fig. 1B), and was parallelled by loss of [^{3}H]-inositol from phosphatidylinositol (Fig. 1A). Half-maximally effective endothelin concentrations (ED_{50}) promoting these responses were comparable ($\sim$ 3-6 nM; determined after 2 min endothelin (10^{-10}-10^{-9} M) exposure) and also did not vary significantly between the different VSMC isolates (dose profiles not shown).

VSMC prelabelled with [^{3}H]-arachidonic acid released label into the extracellular space following exposure to endothelin in a manner that was both time- (Fig. 2) and dose-dependent ($ED_{50} \sim$ 4-7 nM for human, rat and bovine VSMC; assessed following 2 min treatment with 10^{-10}-10^{-6} M endothelin; dose-profiles not presented). This [^{3}H]-arachidonic acid-labelled material appeared to derive from phosphatidylcholine and phosphatidylinositol (Table 1). Furthermore, endothelin-induced release of [^{3}H]-arachidonic acid into VSMC medium overlay could be inhibited in the presence of (and with 30 min pre-incubation)

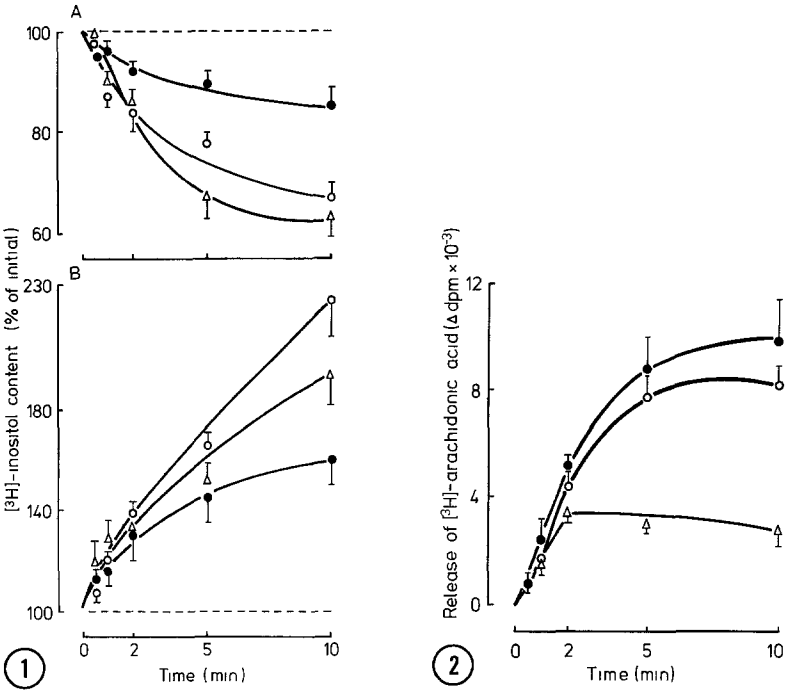

Figure 1: Endothelin promotes degradation of phosphatidylinositol and accumulation of glycerophosphoinositol. Human (○), rat (●) and bovine (△) VSMC were prelabelled with myo-[^{3}H]-inositol and incubated with 10^{-7} M endothelin for the indicated times. Glycerophospho[^{3}H]inositol (Panel B) was estimated after anion-exchange chromatography of the aqueous phase of cell extracts and represents the fraction eluted with 60 mM ammonium formate (14). Phosphatidyl[^{3}H]-inositol was determined after chromatographic separation of deacylated phospholipid extracts and represents the fraction eluted with 5 mM sodium tetraborate/0.1 M ammonium formate (14). Results (mean±SD) express the percentages of [^{3}H]-inositol content relative to those present (100%) at time zero. Absolute initial values for [^{3}H]-content in glycerophosphoinositol and phosphatidylinositol were (dpm per 1.5-2.0x10^6 cells): human VSMC (n=3, 8821±889 and 98826±7841; rat VSMC (n=3), 4964±500 and 69930±3006; bovine VSMC (n=3), 2731±163 and 49554±3430. The dashed lines indicate that neither parameter altered significantly (± 5%) for VSMC incubated for the same periods without endothelin.

Figure 2: Endothelin causes release of [^{3}H]-arachidonic acid into VSMC medium overlay. [^{3}H]-arachidonic acid-labelled VSMC isolates from human (○), rat (●) and bovine (△) sources were incubated without or with 10^{-7} M endothelin for the indicated times. Endothelin-induced release of [^{3}H]-arachidonic acid was determined by subtracting values obtained in the absence of endothelin (which did not vary by more than 5-10% during incubation) from those obtained in the presence of the peptide. Values given are mean±SD (per 5x10^5 cells) from 4, 4 and 3 separate experiments for human, rat and bovine VSMC isolates, respectively.

the phospholipase A_2 inhibitor quinacrine (50 µM; by ~ 90-100%) or the lipoxygenase inhibitor nordihydroguaiaretic acid (50 µM; by ~ 80-90%) (data not shown). Indomethacin (50 µM) and meclofenamate (85 µM), both cyclooxygenase inhibitors, and the phospholipase C inhibitor neomycin (100 µM) were all ineffective in this regard (data not shown).

Table 1: Endothelin-induced loss of [^{3}H]-arachidonic acid from phospholipids

VSMC		[^{3}H]-arachidonic acid content (dpm x 10^{-3} per 10^6 cells)			
		Phosphatidylcholine	Phosphatidylinositol	Phosphatidylserine	Phosphatidylethanolamine
Human	-	56.6 ± 1.3	21.3 ± 1.8	1.8 ± 0.2	5.1 ± 1.0
	+	45.5 ± 2.4*	17.2 ± 0.7*	2.0 ± 0.4	4.7 ± 0.3
Rat	-	37.6 ± 2.2	17.2 ± 0.8	1.7 ± 0.4	8.8 ± 0.9
	+	27.0 ± 2.2*	15.4 ± 0.4*	2.0 ± 0.2	8.3 ± 0.8
Bovine	-	21.8 ± 0.8	9.8 ± 0.7	1.5 ± 0.1	5.8 ± 0.5
	+	18.0 ± 0.5*	7.0 ± 0.2*	1.4 ± 0.3	6.0 ± 0.3

[^{3}H]-arachidonic acid prelabelled VSMC were incubated for 5 mins without (-) or with (+) 10^{-7} M endothelin. [^{3}H] in the indicated phospholipids was quantitated after resolution of lipid extracts by thin layer chromatography. Values (mean±SD) were obtained from 3 separately performed experiments for each VSMC type. The asterisks indicate significant (p at least < 0.01) differences in [^{3}H] content of phospholipids between control and endothelin-exposed VSMC.

Confirmation that endothelin promotes phosphatidylcholine hydrolysis was obtained from experiments utilizing methyl[^{3}H]choline chloride-prelabelled VSMC in which the peptide promoted a time-dependent loss of [^{3}H]choline-label from phosphatidylcholine in lipid extracts and concomitant increase in water-soluble [^{3}H]choline products (Fig. 3). These effects were comparable between human, rat and bovine VSMC isolates. Half-maximally effective endothelin concentrations in eliciting choline accumulation and phosphatidylcholine breakdown were $\sim$2-6 nM (determined from dose (10^{-10}-10^{-6} M)-profile experiments, 3 min incubations, performed once for each type of VSMC; data not presented).

DISCUSSION

In blood vessels, the metabolism of arachidonic acid appears to be a key process in the response of the vasculature to a number of stimuli, the autocoids formed serving as important mediators of essential functions which include vascular tone and proliferation (18). Phospholipase A_2 plays an essential role in the sequence of events leading to synthesis of biologically active metabolites of arachidonic acid by liberating arachidonic acid from various membrane phospholipids such as PC, PI, PS or PE (9,10).

The present study demonstrates that endothelin stimulates phospholipase A_2-mediated deacylation of phospholipids in VSMC from human, rat and bovine vessels. This was evidenced by a time- and dose-dependent loss of [^{3}H]-arachidonic acid from PC and PI that was accompanied by secretion of [^{3}H]-arachidonic acid label into the extracellular

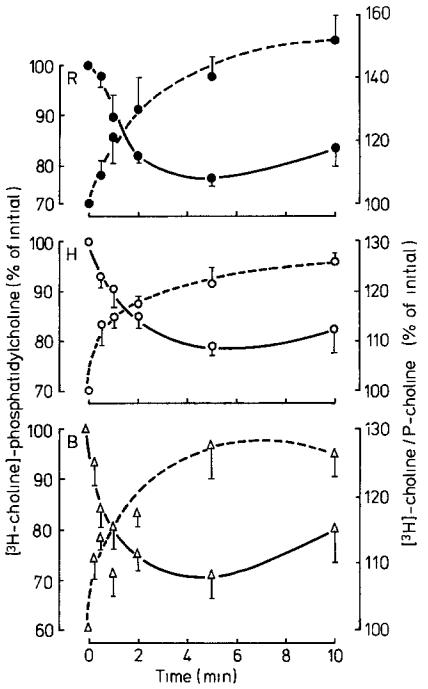

Figure 3. Endothelin-induced hydrolysis of phosphatidylcholine in VSMC. [^{3}H]-choline prelabelled VSMC from human (○, panel H), rat (●, panel R) and bovine (△, panel B) vessels were incubated for the indicated times in the presence of 10^{-7} M endothelin. [^{3}H]-choline products (determined in the aqueous phase of lipid extracts) and [^{3}H]-phosphatidylcholine (determined after thin layer chromatography) are represented by the dashed and solid lines, respectively. Values (mean±SD, n=3 for each VSMC type) represent the percentages of [^{3}H]-choline label relative to those (100%) at zero time.

space. Support for activation of this pathway by endothelin was obtained in experiments demonstrating glycerophosphoinositol accumulation. Glycerophosphoinositol is produced by phospholipase B action on lysophosphatidylinositol subsequent to generation of the latter (and arachidonic acid) by phospholipase A_2-mediated deacylation of PI (9,10). Additionally release of [^{3}H]-arachidonic acid could be inhibited by quinacrine, an inhibitor of phospholipase A_2. The alternative phospholipase C-diacylglycerol lipase pathway for generation of arachidonic acid (10) appears not to be operative in endothelin-stimulated VSMC since neomycin (0.1 mM), a phospholipase C inhibitor, did not inhibit [^{3}H]-arachidonic acid release. The lack of inhibition by neomycin also raises the possibility that activation of phospholipase A_2 by endothelin may occur through a pathway that is independently parallel rather than secondarily sequential to the phospholipase C pathway. Such independent pathways have been proposed for α_1-adrenergic receptors (16,19,20).

Experiments with methyl[^{3}H]-choline prelabelled VSMC confirmed that PC was a substrate for endothelin activated phospholipase(s). We have not investigated the nature of intracellular water-soluble [^{3}H]-choline products (choline, glycerophosphorylcholine or phosphorylcholine (19)), and thus cannot determine whether endothelin promotes phospholipase C-hydrolysis of PC (analogous to that demonstrated for PI (6)) in addition to phospholipase A_2-deacylation. Such PC hydrolysis by phospholipase C has been proposed to provide a source of diacylglycerol (either independent of or additional to that derived from PI) for activation of protein kinase C in response to certain growth factors and hormones (19-21).

We observed almost complete inhibition of [^{3}H]-arachidonic acid release by nordihydroguaiaretic acid, suggesting that such extracellular labelled material are eicosanoid metabolites derived from lipoxygenase activities. Cyclooxygenase derivatives are not indicated since endothelin-induced [^{3}H]-arachidonic acid release was relatively insensitive to both indomethacin and meclofenamate. Although Yanagisawa et al (1) have reported resistance of the endothelin-induced constrictive response to nordihydroguaiaretic acid, studies in our laboratory (Lüscher T, and Yang Z, personal communication) have yielded contrary results in that this lipoxygenase inhibitor significantly decreased the sensitivity of isolated human blood vessels to contraction by endothelin. We have yet to establish the identity of arachidonic acid metabolites released by endothelin-exposed VSMC, and to determine whether such compounds may play some autocrine role in mediating the vascular effects of endothelin.

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