

Lack of effect of dihydroergotamine on endothelial and smooth muscle cell proliferation and endothelial cell prostanoid production

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Summary. The most important effect of dihydroergotamine is vasoconstriction, but certain metabolic effects and changes in vessel prostanoid activity have also been suggested. In this study endothelial cell production of 6-keto PGF_{1α} and TxB₂ was quantitated in vitro. No evidence of altered prostanoid production was noted after incubation with dihydroergotamine (exposure ranging from 5×10^{-3} to 5×10^{-7} g/l). Similarly, no effect of dihydroergotamine on the growth rates of endothelial cells or smooth muscle cells in vitro was documented.

Key words. Dihydroergotamine; endothelial cells; smooth muscle cell; prostanoid production.

Dihydroergotamine (DHE), a dihydrogenated ergot alkaloid, has potential clinical usefulness in treating a variety of vascular diseases. Among its effects are hemodynamic alterations induced by interaction with α -adrenoreceptors. This effect of DHE can only partly be blocked by phentolamine, thus differentiating it from that of noradrenaline. The remaining tension can be blocked by inhibitors of the prostanoid system¹⁻³. In this regard, fluid in which vein strips are immersed after administering DHE contains increased activity of a prostanoid E-like substance². Similarly, increased production of prostacyclin has been reported in immersion fluid from DHE treated rabbit vessels⁴ and in human veins in vitro⁵. This effect on prostanoids could not be verified in an in vivo study on healthy volunteers⁶. Thus the question arises: does DHE cause prostanoid release? In addition DHE induces metabolic changes that theoretically could interfere with cell metabolism and cell replication⁷. This study was undertaken to ascertain whether DHE had any effect on endothelial or smooth muscle cell proliferation, or endothelial cell prostanoid synthesis in vitro.

Material and methods. Endothelial and smooth muscle cell derivation. Endothelial cells from dog external jugular veins were harvested enzymatically with trypsin and collagenase as previously described by Ford et al.⁸. A homogeneous endothelial cell culture was ascertained by morphologic study and factor VIII expression.

Smooth muscle cells were derived from canine carotid arteries. They were cleaned of adventitia and endothelium, minced, and placed into an enzyme solution of elastase type III and collagenase CLS type I. The high purity of the cell cultures was ascertained by morphological characterization, including presence of numerous myofilament bundles in the cytoplasm and vesicles near the membranes. For details of methodology see Lindblad et al.¹¹.

Endothelial and smooth muscle cell growth rate determination. Primary endothelial cell cultures, after 7–10 days growth, were trypsinized, pooled, passed and placed in serum-coated 16-mm culture wells (Costar, Cambridge, MA, USA), at a density of approximately 10,000 cells/well. The medium was medium 199, with 20% fetal bovine serum (FlowLab, Rockville, MD, USA), gentamicin (5 μ g/ml, Schering, Kenilworth, NJ, USA), and a high concentration of endothelial cell growth factor (100 μ g/ml, Seragen, Boston, MA, USA). No

heparin was added to this medium. Dihydroergotamine mesylate (DHE 45, Sandoz Inc., East Hanover, NJ, USA) was added to the medium, at concentrations of 5×10^{-3} ; 5×10^{-4} ; 5×10^{-5} ; 5×10^{-6} ; and 5×10^{-7} g/l. Medium without DHE served as a control. Sixteen wells were used for each DHE concentration studied. These DHE concentrations ranged from 1000 times higher to 10 times lower than the plasma concentration normally achieved in clinical settings. Addition of DHE did not affect the pH of the medium. The nutrient medium, with or without DHE, was changed every other day during the cultivation period.

Smooth muscle cells, after primary cultivation, were trypsinized, pooled, washed, spun, and resuspended in medium as above. Into each 16-mm tissue culture well 8000 cells were plated. Different concentrations of DHE were added giving a final concentration of DHE ranging from 5×10^{-3} to 5×10^{-7} g/l. In a control group no DHE was added. Both endothelial and smooth muscle cell cultures were maintained in a 37 °C incubator with a 100% humidified atmosphere gas consisting of 5% CO₂ and 95% air.

Initial seeding density was determined for 24 samples taken during plating. Plating efficiency was measured from 4 wells trypsinized at day 3. At the end of 7 days the remaining cells (12 wells) were trypsinized and counted using a Coulter® counter.

Endothelial cell prostanoid production. The endothelial cell culture medium was changed on day 6, and on day 7 the medium incubated for 24 h with the cells was removed and immediately frozen for later assay of prostanoid production. Prostanoid metabolites were determined after extraction of proteins and free fatty acids⁹. 6-keto PGF_{1α} was determined utilizing a radioimmunoassay kit (New England Nuclear, detection limit 3.8 pg). TxB₂ was also determined by radioimmunoassay (New England Nuclear, detection limit 0.5 pg). The assays have low cross-reactivity to related prostanoids⁹. Statistical analysis was performed with the Wilcoxon rank sum test. Mean values \pm SEM are given.

Results. Endothelial cell growth (table). The mean initial seeding density for the endothelial cells was $11,206 \pm 253$ /well. Plating efficiency measured at day 3 did not differ markedly, being $15,162 \pm 765$ – $16,376 \pm 534$. This cell density increased approximately 15-fold by the 7th day being $158,300 \pm 1,315$ in control wells. No difference between the

Endothelial cell and smooth muscle cell growth*, at 7 days from an initial cell density of $11,206 \pm 253$ and 7613 ± 176 cells, respectively (n = 12, $\times \pm$ SEM)

	Dihydroergotamine concentration (g/l)					
	5×10^{-3}	5×10^{-4}	5×10^{-5}	5×10^{-6}	5×10^{-7}	None (control)
Endothelial cells	153,530 ± 1120	159,370 ± 860	151,745 ± 1710	167,594 ± 2060	166,370 ± 1625	158,300 ± 1315
Smooth muscle cells	9036 ± 389	9051 ± 288	8876 ± 421	8872 ± 332	8963 ± 312	9017 ± 246

* No statistically significant differences, comparing any concentration of DHE to controls.

groups which were exposed to different DHE levels was seen (range $151,745 \pm 1710$ – $167,594 \pm 2060$ cells/well). Examination of cells by phase contrast microscopy revealed no changes that could be ascribed to DHE.

Smooth muscle cell growth (table). Initial seeding density of smooth muscle was 7613 ± 176 cells/well. Plating efficiency measured at day 3 did not differ markedly being 5865 ± 380 – 5976 ± 412 . By day 7 after seeding, the cell count had increased, with control wells containing 9017 ± 246 cells and DHE treated wells between 8872 ± 332 and 9051 ± 288 cells. Phase contrast microscopy revealed no changes in cell configuration.

Endothelial cell prostanoid production. The 24-h incubated media had concentrations of 6-keto $\text{PGF}_{1\alpha}$ of 3380 ± 380 pg/ml in controls, and a range of 3160 ± 425 – 3370 ± 510 pg/ml in DHE wells. TxB_2 levels were 5810 ± 1020 pg/ml in controls and ranged from 4210 ± 1400 to 6960 ± 1310 pg/ml in DHE wells. No significant differences existed between control medium and that to which DHE had been added.

Discussion. In the current study we could not verify increased activity of prostanoids in vitro from cultured endothelial cells exposed to DHE. We have reported previously on the effects in vivo on healthy volunteers of indomethacin pretreatment before DHE administration. After pretreatment the same hemodynamic effects were observed as with no pretreatment; namely, increased venous flow velocity, reduced venous cross-sectional area, and reduced resting calf blood flow volume^{6,10}. These results, contrary to those of others, suggest little importance of prostanoids in the action of DHE. Measured under identical conditions, other agents show substantial variation in the prostanoid activity. Reduced release of prostanoids has been noted for acetylsalicylic acid (–55%) and other cyclooxygenase and thromboxane inhibitors, whereas dipyridamole, a phosphodiesterase inhibitor, did not alter prostanoid activity, and thrombin administration increased prostanoid activity¹¹. In addition to the direct vascular effects of DHE, metabolic alterations have also been ascribed to DHE. Included among

these are reductions in liver glucose output, inhibition of adrenaline-induced hyperglycemia, stimulation of lipolysis, inhibition of cAMP degradation, interference with phosphodiesterase and increased pyruvate/lactate ratio during relative hypoxia^{7,12}. Because of the potential effect of these changes on cell growth, we attempted to define whether there was any interaction between DHE and in vitro endothelial or smooth muscle plating efficiency and cell proliferation. Our data show that no such effect exists in this setting.

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Inhibition by tetanus and botulinum A toxin of the release of [³H]noradrenaline and [³H]GABA from rat brain homogenate

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Summary. Rat brain homogenate was preloaded with [³H]noradrenaline or [³H]GABA and stimulated with high K^+ . Tetanus toxin and botulinum A neurotoxin partially prevent the evoked [³H]noradrenaline release in the same range of toxin concentrations starting below 10^{-10} M. In contrast, release of γ -amino butyric acid (GABA) is much more sensitive to tetanus than to botulinum A toxin.

Key words. Tetanus toxin; botulinum toxin; noradrenaline; GABA; brain.

Recently it has been claimed¹ that tetanus toxin does not inhibit the evoked release of [³H]noradrenaline from rat cerebral cortex slices. The statement contradicts our positive findings, obtained using two different experimental designs, on rat brain homogenate^{2,3}. Using an improved superfusion system, we now show that both tetanus and, still better, botulinum A toxin prevent noradrenaline release. In contrast, the release of GABA, which is known as an inhibitory neurotransmitter, is inhibited preferentially by tetanus toxin. **Method.** Rat brain cortex was cut into 0.3×0.3 mm prisms with a McIlwain chopper and suspended in Hepes (10 mM,

pH 7.4) buffered ice-cold Krebs-Ringer solution (KRH). After a short trituration with an Eppendorf pipette, the suspension (10% w/v) was passed three times by hand through a Potter homogenizer with a loose-fitting pestle. After 3 min centrifugation at $4500 \times g$ and washing once, the sediment was taken up to give 2% (w/v) with respect to original weight. For studying noradrenaline release, this suspension was mixed with half its volume of KRH containing (final concentration) noradrenaline (1 mCi/l, 1 μM), iproniazid (10 μM) and ascorbic acid (20 μM). All subsequent solutions contained iproniazid and ascorbic acid. After 15 min shak-