

# Effect of Calcium Channel Blockers on Endothelin-1 Production by HDL in Cultured Human Renal Proximal Tubular Cells

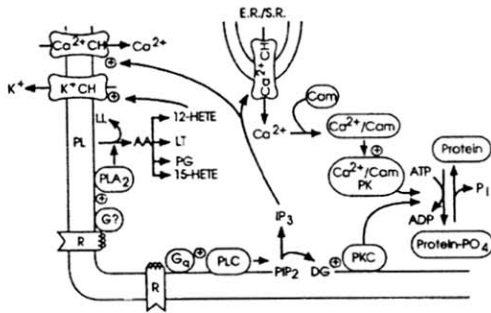
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## BACKGROUND

Human high density lipoprotein (HDL) has been known to stimulate endothelin-1 (ET-1) production in cultured human renal proximal tubular cells<sup>1)</sup>.

Endothelin-1 was produced through the phospholipase C (PLC)/Ca/protein kinase C (PKC) pathway in cultured rat mesangial cell<sup>2)</sup>.



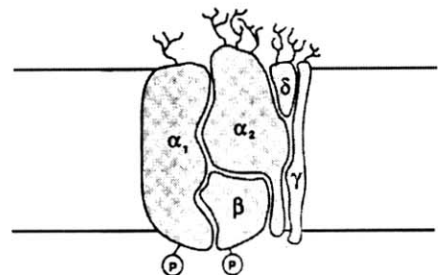
AIM

This study was designed to evaluate whether HDL-induced ET-1 production was affected by Ca channel blockers in cultured human renal proximal tubular cells (PTC).

### Classification of Selective Calcium Channel Blockers

Chemical Type	Chemical Names
Phenylalkylamines	verapamil
Benzothiazepines	diltiazem
1, 4-Dihydropyridines	nifedipine, nicardipine isradipine, felodipine amlodipine

out (closed state) : nifedipine



in (open state): verapamil  
diltiazem

## METHODS

- Human PTC were obtained from human nephrectomized tissues and cultured in six different media :  
 serum free DMEM/F12 medium  
 serum free DMEM/F12 medium + 200 μg/ml of HDL  
 serum free DMEM/F12 medium + 200 μg/ml of HDL + 0.1 volume% of 100% ethanol  
 serum free DMEM/F12 medium + 200 μg/ml of HDL + 0.1 volume% of 100% ethanol + each 10M of diltiazem, nifedipine, verapamil
- After 24 hours of exposure, ET-1 in the supernatant was measured by radioimmunoassay and marked as pg ET-1/mg cell protein/24 hour.

## 1. Proximal tubular cell culture

### 1) Media

#### (1) Materials

- ① RPMI-1640 with 25 mM HEPES + Glutamine
- ② 1% ITS (5  $\mu\text{g/ml}$  Insulin, 5  $\mu\text{g/ml}$  transferrin and 5 ng/ml sodium selenite)
- ③ 5% FCS
- ④ 0.05% Benzyl penicillin (100 U/ml) [stock 200,000 U/ml]
- ⑤ 0.1% Streptomycin sulphate (100  $\mu\text{g/ml}$ ) [stock 1 mg/ml]
- ⑥ 0.125% Amphotericin B (2.5  $\mu\text{g/ml}$ ) [stock 2 mg/ml]
- ⑦ 0.01% Hydrocortisone 21 hemisuccinate ( $2 \times 10^{-1}$  M) [stock 2 mM 10 mg/100 ml]
- ⑧ 0.005%  $T_3$  ( $5 \times 10^{-8}$  M) [stock  $1 \times 10^{-3}$  M]
- ⑨ 0.2% EGF (10 ng/ml) [stock 5  $\mu\text{g/ml}$ ]
- ⑩ 1  $\mu\text{l}$  Linoleic acid (40  $\mu\text{g/l}$ ) [stock 20 mg/ml]

#### (2) Method

- ① DMEM:F12 1:1 with 15mM HEPES (serum free)
- ② 1% ITS (5  $\mu\text{g/ml}$  Insulin, 5  $\mu\text{g/ml}$  transferrin and 5 ng/ml sodium selenite)
- ③ 0.05% Benzyl penicillin (100 U/ml) [stock 200,000 U/ml]
- ④ 0.1% Streptomycin sulphate (100  $\mu\text{g/ml}$ ) [stock 1mg/ml]
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### 2) Preparation of proximal tubular cells

#### (1) Materials

- ① PBS
- ② Collagenase (400–600 U/ml)
- ③ Collagen coated flasks
- ④ Medium A
- ⑤ Nylon mesh or 63  $\mu\text{m}$  sieves

#### (2) Method

- ① Remove capsule from the kidney and cut the outer cortex away from the underlying medulla.
- ② Mince the cortical fragments and collagenase digest for 60–90 mins at 37°C shaking every 5 mins.
- ③ Stop digesting by adding an equal volume of serum containing medium and strain through the nylon mesh or the sieve.
- ④ Collect the filtrate and centrifuge for 5–10 mins at  $>1,000$  RPM.
- ⑤ Wash the cell pellet one or two times with PBS and plate cells into collagen coated flasks at a relatively high density (3 ml cell suspension per 25  $\text{cm}^2$  flask) and place in a humidified 37°C incubator.
- ⑥ Add a further 1ml of medium per flask after 24 h and change the medium completely 48 h later after washing off non-adherent cells and debris.
- ⑦ Change the medium completely every 1–2 days and subculture when confluent

### 3) Collagen coating tissue culture plastic ware

#### (1) Materials

- ① Vitragen or rat tail collagen
- ② 0.1 N NaOH
- ③ PBS

#### (2) Method

- ① Place 3ml of Vitragen, 6.5 ml PBS and 0.5 ml of 0.1 N NaOH in a 50 ml sterile centrifuge tube and mix by gentle inversion.
- ② Dispense 1ml of the above collagen solution per 75  $\text{cm}^2$  flask and swirl to cover the growth surface taking care not to introduce any air bubbles.
- ③ Incubate in a 37°C incubator for 1 h, remove any excess collagen and dry in a safety cabinet overnight without the caps of the flasks.
- ④ Replace caps and store.

### (3) Before using the flasks

- ① Rehydrate the collagen by adding sufficient PBS to cover the flask growth surface and removing the excess.
- ② Pre-incubate with a thin film of FCS for 1-2 h at 37°C and discard any surplus serum.

## 2. Preparative ultracentrifugation for the isolation of lipoprotein fractions

### 1) Materials

- ① 0.2 M Disodium EDTA (pH 7.4)
- ② 200 mg/ml Chloramphenicol
- ③ 0.2% Sodium Azide
- ④ 10 mg/ml Gentamicin
- ⑤ 20,000 U/ml Trasylol
- ⑥ 0.3 M Sodium Chloride
- ⑦ Sodium Bromide (NaBr)
- ⑧ Visking tubing
- ⑨ Phosphate buffered saline pH 7.4 (PBS)

### 2) Method

- ① Mix 4 ml of solution 1), 0.1 ml of 2), 1 ml of 3), 2 ml of 4), 0.125 ml of ml of 5) and 7 ml of 6) and add 1.42 ml (1.19 ml if using pen/ strep) into 30 ml universal tubes.
- ② Obtain 180 ml of blood from a healthy volunteer following an overnight fast and place 25 ml of blood/universal tube and centrifuge at 3,000 rpm for 10 minutes.
- ③ Separate the plasma and adjust density to 1.019 g/ml by using the formula given below to calculate the amount of NaBr to be added.

$$④ X = \frac{V_i (df - fi)}{1 - [V \times df]}$$

Where

X = g NaBr to be used for density adjustment  
(1.54 g/90 ml plasma for d=1.019 g/ml)

V<sub>i</sub> = The volume of solution to be density adjusted

df = Final density required

di = Initial density

V = Partial specific volume of NaBr = 0.235

- ⑤ Centrifuge for 20 h at 4°C in a Beckman ultracentrifuge at 40,000rpm using a 50.3 Ti rotor.
- ⑥ Cut tubes or aspirate VLDL and IDL using a 5 or 10 ml syringe with a 19 G needle.
- ⑦ Pool infranatant and adjust density to 1.063 g/ml by adding sodium bromide using the formula given above and centrifuge as described earlier.
- ⑧ Aspirate LDL fraction and dialyse for 24 h against phosphate buffered saline changing the dialysis medium 4 times pass through a 22 μm filter and refrigerate.
- ⑨ If the HDL fraction is needed adjust the LDL infranatant to a density 1.21 g/ml and centrifuge for a further 20 h at 40,000 rpm at 4°C.
- ⑩ Dialyse and store HDL.

## RESULTS

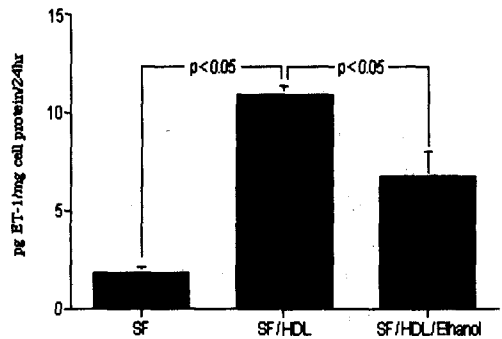
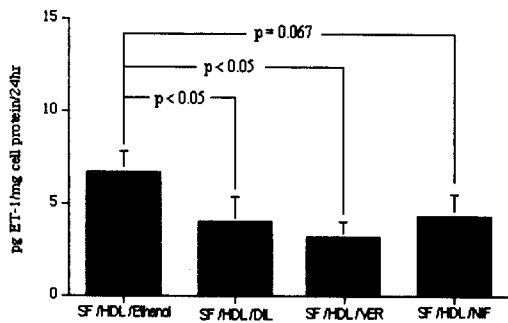


Fig. 1. Effect of HDL and ethanol on endothelin-1 production in human proximal tubular cells after 24 hr of exposure. SF/HDL, SF added 200 μg/ml of HDL; SF/HDL/Ethanol, SF added 200 μg/ml of HDL and 0.1 volume% of 100% ethanol as a control.



**Fig. 2.** Effect of  $Ca^{2+}$  channel blockers on ET-1 production by HDL in human proximal tubular cells. SF/HDL/DIL, SF added 200  $\mu$ g/ml of HDL and 10  $\mu$ M/L of diltiazem solved in 0.1 volume % of 100% ethanol; SF/HDL/VER, SF added 200  $\mu$ g/ml of HDL and 10  $\mu$ M/L of verapamil solved in 0.1 volume% of 100% ethanol; SF/HDL/NIF, SF added 200  $\mu$ g/HDL and 10  $\mu$ M/L of nifedipine solved in 0.1 volume% of 100% ethanol.

## CONCLUSION

These results suggest that the HDL-induced endothelin-1 may be produced through the phospholipase C (PLC)/Ca/protein kinase C (PKC) pathway in cultured human renal proximal tubular cells.

These results show that diltiazem and verapamil significantly, but nifedipine insignificantly decreased the HDL-induced endothelin-1 production in cultured human renal proximal tubular cells.

## REFERENCES

- 1) Ong ACM, Jowett TP, Moorhead JF, Owen JS: Human high density lipoproteins stimulate endothelin-1 release by cultured human renal proximal tubular cells. *Kidney Int* 46:1315-1321, 1994
- 2) Sakamoto H, Sasaki S, Nakamura Y, Fushimi K, Marumo F: Regulation of endothelin production in cultured rat mesangial cells. *Kidney Int* 41:350-355, 1992