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THIENOPYRIDINES: EFFECTS ON CULTURED ENDOTHELIAL CELLS

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In cultured endothelial cells harvested from human umbilical vein (HUVEC) or bovine aorta (BAEC) the 30 min incubation with calcium ionophore A 23187 (1 μM) or ticlopidine (100 μM) caused an increase in nitrite generation in HUVEC from basal 227 ± 37 to 372 ± 60 or to 325 ± 33 pmoles per 10^6 cells, respectively, and in BAEC from basal 182 ± 17 to 378 ± 18 or to 423 ± 66 pmoles per 10^6 cells ($n = 6$), respectively. Calcium ionophore A 23187 (1 μM) or ticlopidine (100 μM) next to 30 min incubation with BAEC increased release of 6-keto-PGF $_{1\alpha}$ from basal level of 9.4 ± 1.8 to 96.2 ± 5.1 or to 99.5 ± 10.2 pmoles per 10^6 cells, respectively. The pretreatment with aspirin (300 μM) cut down this rise to 4.2 ± 0.1 pmoles per 10^6 cells ($n = 8$). Basal cytoplasmic calcium levels, $[\text{Ca}^{2+}]_i$, in immortalised HUVEC cell line — ECV304, HUVEC and BAEC were 47.7 ± 3.3 nM ($n = 53$), 68.3 ± 5.0 nM ($n = 30$) and 53.1 ± 3.0 nM ($n = 15$), respectively. In these cultured endothelial cells calcium ionophore A 23187 (0.1 μM) produced net maximum rise in $[\text{Ca}^{2+}]_i$ by 157 ± 27 nM ($n = 16$) [ECV304], by 107 ± 58 nM ($n = 4$) [HUVEC], and by 231.0 ± 41.3 nM ($n = 8$) [BAEC], respectively, while ticlopidine (30 μM) produced net maximum rise in $[\text{Ca}^{2+}]_i$ by 30.0 ± 3.2 nM ($n = 9$) [ECV304], 48.8 ± 15.6 nM ($n = 4$) [HUVEC] and 28.4 ± 5.4 nM ($n = 8$) [BAEC], respectively. Effect of ticlopidine on $[\text{Ca}^{2+}]_i$ was not only weaker than that of calcium A 23187 but also its maximum appeared after a lag period that was 2–3 times longer than that for A23187. In ECV304 clopidogrel at concentrations of 10, 30 and 100 μM produced maximum increment of $[\text{Ca}^{2+}]_i$ by 16.5 ± 3.8 nM ($n = 7$), 47.0 ± 6.9 nM ($n = 8$) and 67.2 ± 8.3 nM ($n = 8$), respectively. Incubation of BAEC with A23187 (1 μM), ticlopidine or clopidogrel (100 μM) for 2 h did not influence viability of cultured endothelial cells. We claim that thienopyridines, independently of their delayed anti-platelet properties *ex vivo* do release NO and PGI $_2$ from cultured endothelial cells *in vitro*. The above endothelial action of thienopyridines might be mediated by a rise in $[\text{Ca}^{2+}]_i$, however, this possibility has not been proved.

Key words: *thienopyridines, cultured endothelial cells, nitric oxide, prostacyclin*

INTRODUCTION

Thienopyridines encompass ticlopidine, i.e. [5-(2-chlorophenyl)methyl]-4,5,6,7-tetrahydro-thieno[3,2c]pyridine hydrochloride (1) and its optically active congener clopidogrel (2). These anti-platelet drugs successfully compete

with aspirin in prevention or treatment of myocardial infarction and other diseases associated with atherothrombosis (3, 4) as well as in therapy of diabetic vascular complications (5). Beneficial clinical actions of thienopyridines are usually linked with their *ex vivo* but not *in vitro* ability to block a subtype of low-affinity platelet ADP receptor. This subtype of ADP receptor is sensitive to 2-MeS-ADP-induced activation of Gi protein followed by inhibition of adenylate cyclase, fall in cyclic-AMP, mobilisation of $[Ca^{2+}]_i$ from internal stores, activation of glycoprotein GPIIb/IIIa receptors and formation of stable platelet macroaggregates (6, 7). Thienopyridines do not affect calcium influx and platelet shape change which seem to rely on activation of high-affinity ADP receptor (8). Unlike immediate anti-platelet effect of aspirin that is easily detected both *ex vivo* and *in vitro*, fine interaction between thienopyridines and platelets does occur exclusively *ex vivo*, and only after a considerable lag period following ingestion of the drug. An idea of *in vivo* biotransformation of ticlopidine or clopidogrel to active and unstable metabolites seemed obvious and sound (6) but it was hardly evidenced experimentally, although inactive metabolites of ticlopidine (1) and clopidogrel (2) were characterised in a vast number. Previously, we reported that in humans, cats and rats ticlopidine had manifested fibrinolytic or thrombolytic properties (9). We claimed that this action of ticlopidine was not depending on its anti-platelet properties, but it rather came as a result of an unknown pharmacological interaction of ticlopidine with vascular endothelial cells (9, 10). Presently, we broaden the scope of investigated thienopyridines by adding clopidogrel to ticlopidine and looking directly to their *in vitro* effects on cultured endothelial cells.

MATERIALS AND METHODS

Cell cultures

Endothelial cells were isolated from bovine aorta (BAEC) or from human umbilical veins (HUVEC). In addition, spontaneously transformed; HUVEC, cell line; ECV304 (11) was obtained from ATCC (Rockville, Maryland, USA). Primary HUVEC were obtained from human umbilical cord veins by treatment with 0.1% collagenase type I dissolved in D-PBS without Ca^{2+} and Mg^{2+} , according to Jaffe *et al.* (12). BAEC were obtained from bovine thoracic aortae after treatment with 0.05% (w/v) trypsin and 0.02% (w/v) EDTA (13). HUVEC and BAEC were pooled and seeded in T25 flasks (NUNC, Brand Products, Denmark) which were precoated with 1% gelatine. After 1 h of incubation at 37°C in CO₂ non-adherent cells were removed. HUVEC were grown in Endothelial-SFM System Medium that was supplemented with penicillin G sodium salt (100 units ml⁻¹), streptomycin sulphate (100 µg ml⁻¹), amphotericin B (0.25 µg ml⁻¹) and heparin (20 units ml⁻¹). BAEC and ECV304 cell line were cultured in OPTI-MEM I that was supplemented with foetal bovine serum (4% v/v) and antibiotics as above. All types of cell cultures were incubated at 37°C in humidified air containing 5% CO₂. The culture media were replaced three times a week.

Upon reaching confluence, cultured endothelial cells were passaged (trypsin/EDTA at a ratio of 0.05%/0.02% w/v for 1min at 37°C), and reseeded into T75 culture flasks. Primary cultures of endothelial cells usually reached confluence between 4 to 6 days. Cells between 2nd and 4th passage were used for experiments. Then endothelial cells were identified, firstly, by their typical cobblestone morphology when viewed under the Axiovert 25 Inverted Microscope (Carl Zeiss Jena GmbH, Germany), and secondly, by a positive staining for factor VIII-related antigen.

Endothelial nitrite and 6-keto-PGF_{1α} production

Nitrite production was measured in supernatant from HUVEC and BAEC cultures. These were exposed to A 23187 (1 μM) or TP (100 μM). Endothelial cells were cultured in 3 ml of appropriate medium that was instilled into a well of the six-well gelatine-coated plates. After reaching confluence the culture medium was replaced with hepes buffered solution (HBS) that contained 5 mM glucose. Following 90 min of preincubation of the confluent cells in 3 ml of HBS with 5mM glucose, the stimulants were added, and in the culture medium nitrite was assayed before and after 30 min of incubation at 37°C by the Griess reaction (14). Briefly, 500 μl of 1% sulfanilamide in 5% phosphoric acid followed by the same volume of 0.1% N-(1-naphtyl)ethylenediamine dihydrochloride were added to 900 μl of the corresponding sample. After 10 min of incubation at 22°C the absorbance was read at 550 nm using Beckman DU 640 BV diode array spectrophotometer. A change in nitrite concentrations during 30 min of incubation with a stimulant or without it (control sample) was calculated and expressed in pmoles per 10⁶ cells. In a similar way 6-keto-prostaglandin F_{1α} (6-keto-PGF_{1α}) was assayed using the enzyme immuno-assay kit (Cayman Chemical Co., Ann Arbor, MI) and the results were expressed in pmoles per 10⁶ cells.

Endothelial free cytoplasmic calcium levels [Ca²⁺]_i

Cytosolic calcium ion [Ca²⁺]_i assay was accomplished by the method of Grynkiewicz *et al.* 1985. BAEC, HUVEC or ECV304 were loaded with fura-2 by incubation of cultured endothelial cells with this fluorescent dye in its membrane permeant form of acetoxymethyl ester (fura—2AM) for 60 min at 25°C in presence of 0.5 mg ml⁻¹ of bovine serum albumin. Then the extracellular dye was removed by centrifugation and the cells (0.5 × 10⁶ cells ml⁻¹) were resuspended in HBS that contained glucose at 5 mM concentration. Fluorescence was measured at 37°C in a spectrofluorimeter with a dual wavelength excitation and magnetic stirring (LS 50B, Perkin-Elmer Corporation, Beaconsfield, U.K.) at 500 nm with the excitation wavelength of 340 nm and 380 nm. The calibration was completed using 0.2% Triton X-100 for R_{max} and 5 mM EGTA for R_{min}. Eventually, cytosolic calcium levels [Ca²⁺]_i were calculated according to the equation given by Grynkiewicz *et al.*, (15). [Ca²⁺]_i was continuously monitored before and for 5 min after instillation of solvent or A23187 calcium ionophore (0.1 μM) or TP (30 μM). Net effects of A23187 and TP were calculated by a computer-assisted subtraction of control [Ca²⁺]_i during a span of 5 min at the maximum increase in [Ca²⁺]_i by either drug.

Viability of BAEC or ECV304 after incubation with thienopyridines or A23187

BEAC or ECV304 (10⁵ cells per well) were seeded on 96-well plates and allowed to reach confluency. A21387 (1 μM), TP, CL, ECL (100 μM) or saline were instilled to wells. Thirty min later 20 μl of MTT (3-[4,5-dimethylthiazol-2-yl]—2,5-diphenyl-tetrazolium bromide; 5 mg ml⁻¹)

was added to each well and cells incubated for further 2 h. After removal of medium, plates were stored overnight at -70°C . Formazan was solubilized with isopropanol that contained 0.04 N HCl. A microplate ELISA reader (Biotec, USA) was used to read a difference between optical density (OD) at 562 and 630 nm ($\text{OD}_{562/630}$).

Drugs and reagents

Hydrochlorides of thienopyridines, i.e. ticlopidine (TP) and clopidogrel (CL, SR 25990C) were kindly donated by Sanofi Recherche (Toulouse, France), prostacyclin (PGI_2) by Schering Company (Berlin, Germany), lysine aspirin, calcium ionophore A 23187, fura acetoxymethyl ester (fura-2AM), bovine serum albumin and gelatine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Penicillin G and streptomycin were purchased from Polfa (Poland) and amphotericin B, collagenase type I, OPTI-MEM I from Gibco BRL, Life Technologies (UK). Sulfanilamid and N-(1-naphtyl) ethylene-diamine dihydrochloride were bought in POCH (Gliwice, Poland).

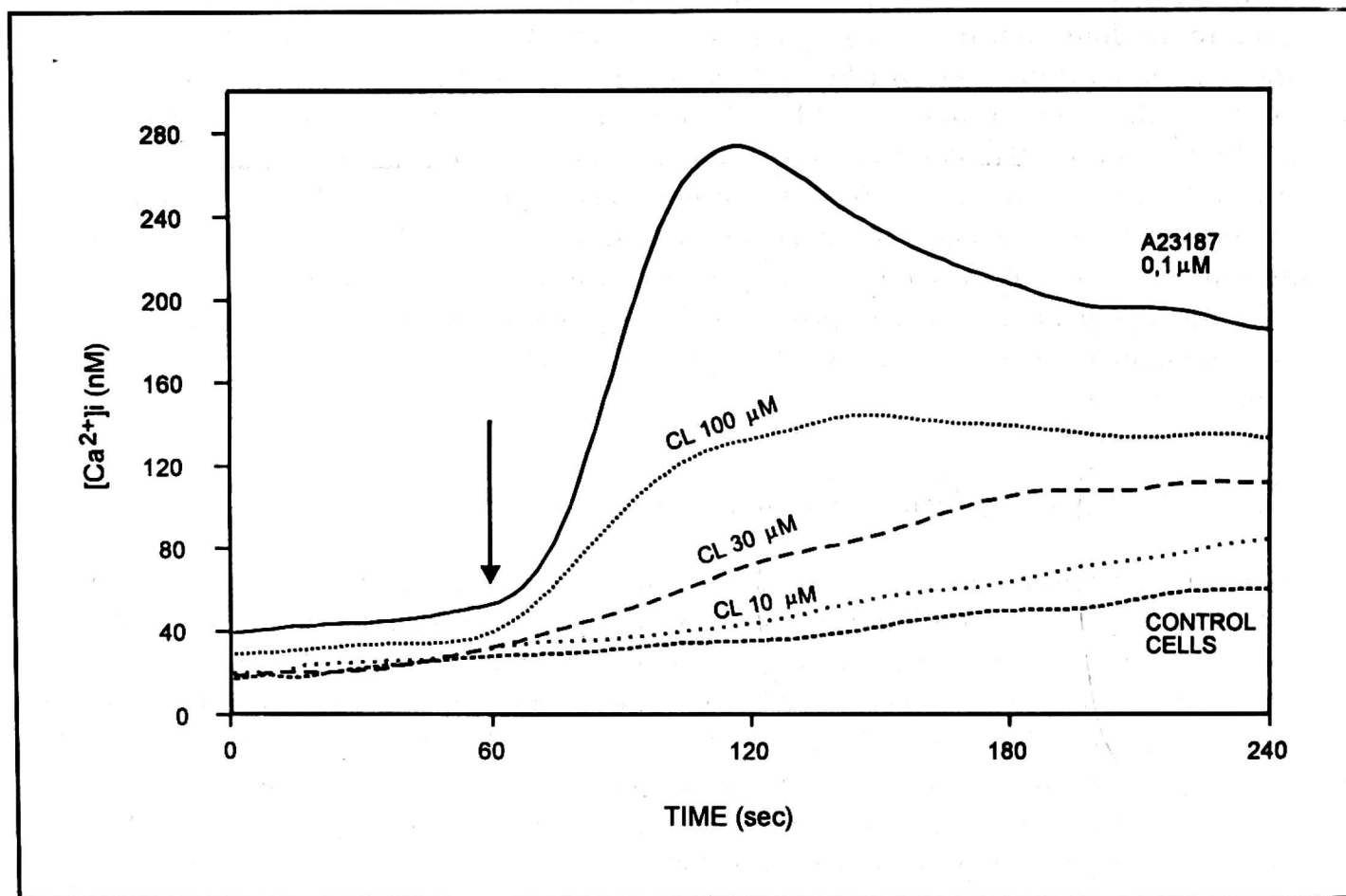


Fig. 1. Effects of calcium ionophore A 23187 (0.1 μM) and clopidogrel (CL, 10, 30 and 100 μM) on intracellular free calcium levels $[\text{Ca}^{2+}]_i$ in spontaneously transformed immortalised human umbilical vein endothelial cells (ECV304) as compared to control untreated cells.

Statistical analysis

Arithmetical means are given with s.e.mean. Differences inside group were assessed with paired Student's t test. Statistical analysis between groups was performed by one-way analysis of variance (ANOVA) followed with Scheffe's *a posteriori* test. A P value less than 0.05 was assumed to denote a significant difference.

RESULTS

Nitrite and 6-keto-PGF_{1α} production by HUVEC and BAEC

Incubation of HUVEC or BAEC with A 23187 (1 μM) for 30 min increased generation of nitrite by endothelial cells from control values by 64% and 105%, respectively, while for TP (100 μM) a corresponding rise in nitrite was 43% and 132%, respectively. Changes in nitrite production by both A23187 and TP were statistically significant (*Table 1*). Incubation of BAEC with A23187 (1 μM) or TP (100 μM) brought about a more vigorous rise of 6-keto-PGF_{1α} production than that of nitrate. Production of 6-keto-PGF_{1α} by BAEC after their treatment with either A 23187 or TP increased more than 10-fold. This increase was not seen in the presence of aspirin (300 μM) (*Table 2*). Treatment of BAEC with aspirin (300 μM) did not change significantly their basal release of 6-keto-PGF_{1α} which was 4.2 ± 0.1 pmoles 10^6 cells⁻¹ (n = 8, p = 0.989).

Table 1. Nitrite production (pmoles 10^6 cells⁻¹) by cultured human umbilical vein endothelial cells (HUVEC) and cultured bovine aortic endothelial cells (BAEC) during 30 min of incubation with calcium ionophore (A 23187, 1 μM) or with ticlopidine (TP, 100 μM).

Type of cells	Nitrite production pmoles 10^6 cells ⁻¹		
	Basal	A23187 (1 μM)	TP (100 μM)
HUVEC	227 ± 37	372 ± 60 (p = 0.012)	325 ± 33 (p = 0.015)
BAEC	182 ± 17	378 ± 18 (p = 2.8×10^{-9})	423 ± 66 (p = 1.2×10^{-5})

Values are mean \pm s.e. mean, n = 6.
p was calculated vs. basal release.

Table 2. 6-Keto PGF_{1α} production (pmoles 10^6 cells⁻¹) by cultured bovine aortic endothelial cells (BAEC) during 30 min of incubation with calcium ionophore (A 23187, 1 μM) or ticlopidine (TP, 100 μM) in absence or presence of aspirin (ASA, 300 μM).

6-keto-PGF _{1α} production pmoles 10^6 cells ⁻¹			
Basal	A 23187 (1 μM)	TP (100 μM)	ASA (300 μM) + TP (100 μM)
9.4 ± 1.8	96.2 ± 5.1 (p = 9.2×10^{-12})	99.5 ± 10.2 (p = 3.3×10^{-12})	4.2 ± 0.1 (p = 0.989)

Values are mean \pm s.e. mean, n = 8.
p was calculated vs. basal release.

β Ca²⁺/i in cultured endothelial cells

Basal levels of $[Ca^{2+}]_i$ in ECV304, HUVEC and BAEC were 67.7 ± 3.3 nM (n = 53); 68.3 ± 5.5 nM (n = 30) and 53.1 ± 3.0 nM (n = 15), respectively. A 23187 (0.1 μ M) or TP (30 μ M) when instilled to ECV304, HUVEC or BAEC raised $[Ca^{2+}]_i$ from the above basal levels additionally by 94.1 ± 12.8 nM (n = 13) or 30.0 ± 3.2 nM (n = 9) [ECV304], by 107.0 ± 58.5 nM (n = 4) or 48.7 ± 15.6 nM (n = 4) [HUVEC], and by 231.0 ± 41.3 nM (n = 8) or 28.4 ± 5.4 nM (n = 8) [BAEC]. In all types of cultured endothelial cells the effect of TP (30 μ M) on $[Ca^{2+}]_i$ was not only weaker than that of A23187 (0.1 μ M) but also its maximum appeared later. After instillation of A23187 or TP maximum rise in $[Ca^{2+}]_i$ was achieved within following periods of time (seconds):

59 ± 4 (n = 13) or 111 ± 3 (n = 9) [ECV304], 43 ± 5 (n = 4) or 89 ± 10 (n = 4) [HUVEC], and 53 ± 3 (n = 10) or 153 ± 16 (n = 12) [BAEC]. Except for HUVEC these differences between responses to A23187 (0.1 μ M) and TP (30 μ M) were statistically significant ($P < 0.05$).

DISCUSSION

A coupled release of PGI₂ and EDRF (NO) from cultured endothelial cells seems to be a rule (16, 17), no matter what releasers are used. Here we show that thienopyridines, i.e. ticlopidine and clopidogrel, stay in line with this rule. In this respect potency of thienopyridines at a range of concentrations of 30–100 μ M was equivalent to that of calcium ionophore A 23187 at a concentration of 1 μ M. Then pharmacologically induced release of nitrate (an index of NO generation) was increased 1.5–2 times, while the release of 6-keto-PGF_{1 α} (an index of PGI₂ generation) was increased 10 — fold. Ticlopidine or clopidogrel at a dose of 10 mg kg⁻¹ were reported to hamper vasoconstriction produced by platelet-rich plasma, serotonin or endothelins in arterial preparations excised half from rats, rabbits (18) or dogs (19) half-an-hour after intravenous administration of either drug. Interestingly, the authors speculate that thienopyridines combat vasoconstriction through their metabolites which modulate activity of an esoteric „ADP receptor on the vessel wall”. It may well be that non-metabolised molecules of thienopyridines moderate vasoconstrictor response through releasing endothelial NO, as we have shown here in cultured endothelial cells. In patients with intermittent claudication the endothelial mechanism of action of thienopyridines may contribute to their beneficial effects on blood flow when administered together with captopril (20). Also in patients with chronic heart failure enalapril reduces systemic vascular resistance more effectively when given in combination with ticlopidine rather than with aspirin (21). In this last study an additive

interaction between ticlopidine and enalapril cannot be excluded. However, the authors do not care about this contention, since as they say: „there is no pharmacological background for such interaction.” We believe that our paper provides a missing link, i.e. an evidence that thienopyridines release vasodilator and antithrombotic NO and PGI₂ from vascular endothelium. Mechanism of their release is as yet not clear. Initially, we presumed that thienopyridines would act as calcium ionophores, and a common denominator for their endothelial effects might be a rise in cytoplasmic [Ca²⁺]_i. Indeed, TP produced a rise in [Ca²⁺]_i in three types of cultured endothelial cells (ECV304, HUVEC and BAEC). However, in comparison to calcium ionophore A23187 (0.1 μM) the effective concentration of thienopyridines were rather high (30 μM), a rise in [Ca²⁺]_i was feeble and it appeared with a delay. Rising in [Ca²⁺]_i by thienopyridines is not only much weaker than that by A23187, but also its kinetics does not resemble a typical pattern of response to calcium ionophore. It may well be that a common trigger for thienopyridine-induced release of NO and PGI₂ from endothelium is different from [Ca²⁺]_i. Nonetheless, we propose that endothelial release of NO and PGI₂ by thienopyridines may add up to their direct anti-platelet effect (8). Not only mechanism of anti-platelet action of thienopyridines but also their stimulatory effect on vascular endothelium makes them different from aspirin (22). Aspirin has a potential to inhibit synthesis of prostanoids, both in platelets and in endothelial cells while thienopyridines like some inhibitors of angiotensin I/angiotensin II converting enzyme (ACE) (23) are endowed with a capacity to stimulate the secretory function of vascular endothelium including PGI₂ release. Hence, appearance of clinical compatibility between ACE inhibitors and thienopyridines in the treatment of patients with intermittent claudication (20) or with chronic heart failure (21). This does not show up when thienopyridines are replaced by aspirin. Here, we demonstrated for the first time directly, an existence of endothelium — centred mechanism of action of thienopyridines which so far were considered to be antiplatelet drugs only.

REFERENCES

1. Panak E, Maffrand JP, Picard-Fraire C, Vallee E, Blanchard J, Roncucci R. Ticlopidine: A promise for the prevention and its complications. *Haemostasis* 1983; 13, 1—54.
2. Coukell AJ, Markham A. Clopidogrel. *Drugs* 1997; 54: 745—750.
3. CAPRIE Steering Committee. A randomised, blinded trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). *Lancet* 1996; 348: 1329—1339.
4. Gent M. Benefit of clopidogrel in patients with coronary disease. *Circulation* 1997; 96: 465—467.
5. TIMAD Study Group. Ticlopidine treatment reduces the progression of non-proliferative diabetic retinopathy. *Arch Ophthalmol.* 1990; 108: 1577—1583.

6. Gachet C, Cattaneo M, Ohlmann P, Hechler B, Lecchi A, Chevalier J, Cassel D, Mannucci PM, Cazenave JP. Purinoceptors on blood platelets: further pharmacological and clinical evidence to suggest the presence of two ADP receptors. *Br J Pharmacol* 1995; 91: 434—444.
7. Humbert M, Nurden P, Bihour C, Pasquet JM, Winckler J, Heilmann E, Savi P, Herbert JM, Kunicki TJ, Nurden AT. Ultrastructural studies of platelet aggregates from human subjects receiving clopidogrel and from a patient with an inherited defect of an ADP-dependent pathway of platelet activation. *Arterioscler Thromb Vasc Biol* 1996; 16: 1532—1543.
8. Savi P, Bornia J, Salel V, Delfaud M, Herbert JM. Characterization of P2 × 1 purinoreceptors on rat platelets: effect of clopidogrel. *Br J Haematol* 1997; 98: 880—886.
9. Gryglewski RJ, Korbut R, Świąż J. Thrombolytic action of ticlopidine: possible mechanisms. *Eur. J. Pharmacol.* 1997; 308: 61—67.
10. Gryglewski RJ, Korbut R, Świąż J, Uracz W. Effect of ticlopidine on streptokinase-induced thrombolysis in rats. *Wien Klin Wochenschr* 1999; 111: 98—102.
11. Takahashi K, Sawasaki Y, Hata J, Mukai K, Goto T. Spontaneous transformation and immortalization of human endothelial cells. *In Vitro Cell Dev Biol* 1990; 26: 265—274.
12. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 1973; 52: 2745—2765.
13. Makarski JS. Stimulation of cyclic AMP production by vasoactive agents in cultured bovine aortic and pulmonary artery endothelial cells. *In Vitro* 1981; 17: 450—458.
14. Green IC, Ruiz de Luzuriaga K, Wagner DA. Nitrate biosynthesis in man. *Proc Natl Acad Sci USA* 1981; 78: 7764—7768.
15. Gryniewicz G, Poenie M, Tsien RY. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 1985; 260: 3440—3450.
16. Gryglewski RJ, Moncada S, Palmer RM Bioassay of prostacyclin and endothelium-derived relaxing factor (EDRF) from porcine aortic endothelial cells. *Br J Pharmacol* 1986; 87: 685—694.
17. de Nucci G, Gryglewski RJ, Warner TD, Vane JR. Receptor mediated release of endothelium-derived relaxing factor (EDRF) and prostacyclin from bovine aortic endothelial cells is coupled. *Proc Natl Acad Sci USA* 1988; 85: 2334—2338.
18. Yang LH, Fareed J. Vasomodulatory action of clopidogrel and ticlopidine. *Thromb Res* 1997; 86: 479—491.
19. Yang LH, Hoppensteadt D, Fareed J. Modulation of vasoconstriction by clopidogrel and ticlopidine. *Thromb Res* 1998; 92: 83—89.
20. Novo S, Abrignani MG, Pavone G, Zamuchi M, Pernice C, Geraci AM, Longo B, Caruso R, Strano A. Effect of captopril and ticlopidine, alone or in combination, in hypertensive patients with intermittent claudication. *Int Angiol* 1996; 15: 169—174.
21. Spaulding C, Charbonnier B, Cohen-Solal A, Juilliere Y, Kromer EP, Benhamda K, Cador R, Weber S. Acute hemodynamic interaction of aspirin and ticlopidine with enalapril. Results of a double-blind, randomized comparative trial. *Circulation* 1998; 98: 757—765.
22. Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action of aspirin-like drugs. *Nature* 1971; 231: 232—235.
23. Maeso R, Navarro-Cid J, Rodrigo E, Ruilope LM, Cachafeiro V, Lahera V. Effects of antihypertensive therapy on factors mediating endothelium-dependent relaxation in rats treated chronically with L-NAME. *J Hypert* 1999; 17: 221—227.

Received: September 7, 1999

Accepted: September 21, 1999

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