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PRODUCTION OF PROSTACYCLIN AND PROSTAGLANDIN E_2 IN RESTING AND IL-1β-STIMULATED A549, HUVEC AND HYBRID EA.HY 926 CELLS

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Production of arachidonic acid (AA) metabolites - prostacyclin (PGI_2) in large vessels and prostaglandin E_2 (PGE_2) in microcirculation is intrinsically involved in maintenance of vascular wall homeostasis. EA.hy 926 is a hybrid cell line, is derived by fusion of HUVEC with A549 cells. The aim of this study was to examine the production of prostacyclin and PGE_2 in resting and IL-1β-stimulated EA.ha 926 cells, in comparison with its progenitor cells. Non-stimulated EA.hy 926 cells has been found to produce much lower amounts of prostacyclin than resting HUVEC. Resting hybrid cells produced more PGE_2 than prostacyclin, despite they expressed high levels of COX-1 and PGI_2 synthase. On the contrary to HUVEC and A549, EA.hy 926 cells did not respond to IL-1β with COX-2 induction and increase of prostaglandin production, however they did it in response to lysophosphatidylcholine (LPC). The characteristics of EA.hy 926 cells in terms of the pattern of prostanoid formation could facilitate studies on endothelial metabolism and role of these important lipid mediators.

Key words: *endothelium, prostacyclin, prostaglandin E_2, interleukin-1, EA.hy 926 cells*

INTRODUCTION

Healthy endothelium, by virtue of its endocrine action is intrinsically involved in maintenance of vascular wall homeostasis (1). Besides nitric oxide (NO) release, production of arachidonic acid (AA) metabolites - prostacyclin (PGI_2) in large vessels and prostaglandin E_2 (PGE_2) in microcirculation - represents key

marker of endothelial integrity (2-4). Inflammatory activation of endothelial cells and subsequently, dysfunction of release in endothelial mediators, has been shown to play the role in the development of atherosclerosis, hypertension, heart failure and diabetes (5).

Cultured endothelial cells of human origin are widely used as a tools for *in vitro* studies of endothelial formation and release of NO and prostaglandins (6-8). However, both primary endothelial cells, like human umbilical vein endothelial cells (HUVEC) and immortalized cell lines are not devoid of some disadvantages (9). This is why, there were several attempts to establish permanent cell lines of human endothelial cells showing as much as possible the characteristics of the primary cells (9). EA.hy 926 cell line is a hybrid derived by fusion of HUVEC with the human epithelial cell line A549 (10). EA.hy 926 cells have been characterized regarding morphology and expression of endothelial-specific markers (11;12) as well as proved to be useful in research (13-15).

It has been previously shown that EA.hy 926 cells are capable to produce and release PGI_2 in basal conditions and after short stimulation with thrombin (16). However, neither relative production of PGI_2 and PGE_2 nor the effects of pro-inflammatory stimulation on prostanoid synthesis by this cell line have been investigated.

The aim of this study was to examine the production of prostacyclin and PGE_2 in resting and IL-1β–stimulated EA.hy 926 cells, in comparison with its progenitor cells, HUVEC and A549.

MATERIALS AND METHODS

Cell culture

A549 cells originally derived by Lieber et al. (17) from a human lung carcinoma were obtained from Department of Clinical Immunology of Institute of Pediatrics of Jagiellonian University Medical College in Krakow. Cells were grown in OPTI-MEM I culture medium (Invitrogen Gibco, USA) supplemented with 4% FBS (Invitrogen Gibco, USA).

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh human umbilical veins using the method of Jaffe et al. (18). HUVEC cultures were grown to confluence on 1% (w/v) gelatin-coated flasks in OPTI-MEM I culture medium supplemented with 4% FBS and heparin (20 units/ml). Cells were used up to and including passage 2.

Hybridoma EA.hy 926 cell line, formed by the fusion of HUVEC with the human lung carcinoma cell line, A549 (12), was kindly provided by Dr C-J. Edgell (Department of Pathology University of North Carolina, Chapel Hill, NC). EA.hy 926 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma Chemical Co.,USA) supplemented with HAT Media Supplement (100 μM hypoxanthine, 0,4 μM aminopterin, 16 μM thymidine) (Sigma Chemicals, USA) and 10% FBS.

Cell culture media were supplemented with penicillin G sodium sulphate (100 units/ml), streptomycin sulphate (100 μg/ml), and amphotericin B (0.25 μg/ml). Cells were grown in humidified atmosphere of 5% CO_2/ 95% air at 37°C and were sub-cultured every 3-4 days using 0.05% trypsin/ 0.02% EDTA.

Protocol of experiments

The cells ($3x10^4$) were seeded on wells of 96-well plates containing 200 µl of medium. The cells were serum starved overnight (relevant medium containing one-tenth of the normal FBS concentration). In the day of experiment, fresh starving medium was given and after 2 hours of equilibration, the cells were treated up to 24 hours with IL-1β (1 ng/ml), L-α-lysophosphatidylcholine (LPC, final concentrations 30 and 100 µM, given from ultrasound sonificated stock solution prepared in 1:1 v/v methanol:chloroform mixture) (Sigma Chemicals Co., USA).

For RT-PCR and Western blot measurements the cells were seeded on wells of six-well plates ($1x10^6$), serum-starved and treated as above.

The viability of cells were routinely tested by trypan blue staining; cytotoxic effect of used compounds were tested with MTT reduction assay as described previously (19;20).

Measurements of 6-keto $PGF_{1\alpha}$ and PGE_2

Supernatants of cell cultures were collected into Eppendorf's tubes, centrifuged to remove cellular debris and stored at -70° C.

Stable metabolite of prostacyclin, 6-keto$PGF_{1\alpha}$, and PGE_2 levels were measured with use of EIA kits from R&D Systems, Inc. (USA), and Cayman Chemicals (USA), respectively. All results were expressed in ng per 10^6 cells.

RT-PCR

Expression of mRNA for COX-1, and COX-2 was evaluated by semi-quantitative RT-PCR, as described previously (21) after cell homogenization and extraction of total RNA with the TRIzol® Reagent (Invitrogen, USA). Total RNA (1 µg) from each sample was reverse-transcribed to complementary DNA (cDNA) using oligo$(dT)_{12-18}$ primer and MMLV reverse transcriptase (Fermentas, USA). The final RT reaction volume was 20 µl. The reaction was performed in a thermal cycler Biometra at 42°C during 2 hrs. After completion of first-strand cDNA synthesis, the reaction was stopped by heat inactivation (5 min, 99°C). The COX-1, COX-2 and β-actin cDNA fragments (722, 305, and 308 base pair long, respectively) were amplified using specific primer pairs for COX-1: 5'- gct ggg agt ctt tct cca acg tga g and 5'- ggc aat gcg gtt gcg gta ttg gaa c-3' (22), for COX-2: 5'- ttc aaa tga gat tgt ggg aaa att gct and 5'- aga tca tct ctg cct gag tat ctt t-3' (22), and for β-actin: 5'-agc ggg aaa tcg tgc gtg-3' and 5'- cag ggt aca tgg tgg tgc c-3' (23). The polymerase chain reactions were performed with 1 µl RT product (cDNA) in a 25- µl reaction volume containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 1 U HotStarTaq DNA Polymerase (Qiagen, USA) and 1 µM of each primer. After an initial enzyme activation step for 15 min at 95°C, PCR was carried out (1 min at 94°C, 30 s at 59°C and 30 s at 72°C), followed by a 10-min extension at 72°C. For each primer pair, control experiments were performed to determine the range of cycles in which a given amount of cDNA would be amplified in a linear fashion.

PCR products were separated on the ethidium bromide-stained gels (2% agarose) and bands were analyzed with freeware Scion image (Scion Corporation, USA).

Immunoblotting

Expression of COX-1, COX-2, mPGE-s, and PGI-s proteins was evaluated by Western blot. Cells were lysed in PBS containing 1% Triton X-100, 0.1% SDS, 1 mM PMSF, 100 µM leupeptin, 50 µM pepstatin A. Protein concentrations of lysates were determined using Bradford method. Samples, containing equal amounts of total protein were mixed with gel loading buffer (50 mM Tris, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2 mg/ml bromophenol blue) in a ratio 4:1 (v/v) and boiled for

4 min. Samples (30-50 μg of protein) were separated on SDS-polyacrylamide gels (7,5 – 15 %) (Mini Protean II, Bio Rad, USA) using Laemmli buffer system and proteins were semidry transferred to nitrocelulose membranes (Amersham Biosciences, USA). Membranes were blocked overnight in 4°C with 5% (w/v) non-fat dried milk in TTBS and incubated 3 hrs in room temperature with specific primary antibodies (1:1000, COX-1, COX-2, mPGE-s and PGI-s, all from Cayman Chemical, USA), then for 1 hour with HRP-conjugated secondary antibodies (Amersham Biosciences, USA). Bands were developed with use of ECL-system reagents (Amersham Biosciences, USA). Rainbow markers (Amersham Biosciences, USA) were used for molecular weight determinations. Protein bands were scanned and analyzed with freeware Scion image (Scion Corporation, USA).

Statistical analysis

All values in the figures and text are expressed as mean ± s.e. of *n* observations. A one way analysis of variance (ANOVA) followed, if appropriate, by a Bonferroni`s test for multiple comparisons was used to compare means between the groups. A *P* value less than 0,05 was considered to be statistically significant

RESULTS

All three kinds of cells accumulated easily detectable amounts of 6-keto $PGF_{1\alpha}$ and PGE_2 in culture media (*Fig. 1*). Interestingly, resting HUVEC, but not A549 and EA.hy 926 cells showed continuous release of both prostaglandins, as evidenced by gradual accumulation of 6-keto $PGF_{1\alpha}$ and PGE_2 in culture medium (*Fig. 1*). Non-stimulated A549 and EA.hy 926 cells were found to produce more PGE_2 than prostacyclin (*Tab. 1*). In contrast, HUVEC produced much more prostacyclin than PGE_2 (*Tab. 1*).

Both A549 and HUVEC responded to stimulation with IL-1β with significant increase of prostaglandin release (*Fig. 1*). Stimulation with IL-1β shifted the relative production of prostaglandins even more towards PGE_2 in A549 cells and towards prostacyclin in HUVEC (*Tab. 1*).

Interestingly, in contrast to A549 and HUVEC, stimulation with IL-1β did not influence production of prostacyclin and PGE_2 by EA.hy 926 cells (*Fig. 1*).

COX-1 protein was easy detectable in non-stimulated A549, HUVEC and EA.hy 926 cells (*Fig. 2*); treatment with IL-1β did not influenced significantly

Table 1. 6-keto $PGF_{1\alpha}$ / PGE_2 production (ng/10^6 cells) ratios in supernatants of cell cultures. Samples were taken simultaneously from non-stimulated (control) cells and cells treated with IL-1β (1 ng/ml) at indicated time points.

	6 hours		*24 hours*	
	control	*IL-1β*	*control*	*IL-1β*
A549	0,34	0,26	0,36	0,33
HUVEC	7,58	13,03	11,32	13,58
EA.hy 926	0,71	0,52	0,51	0,71

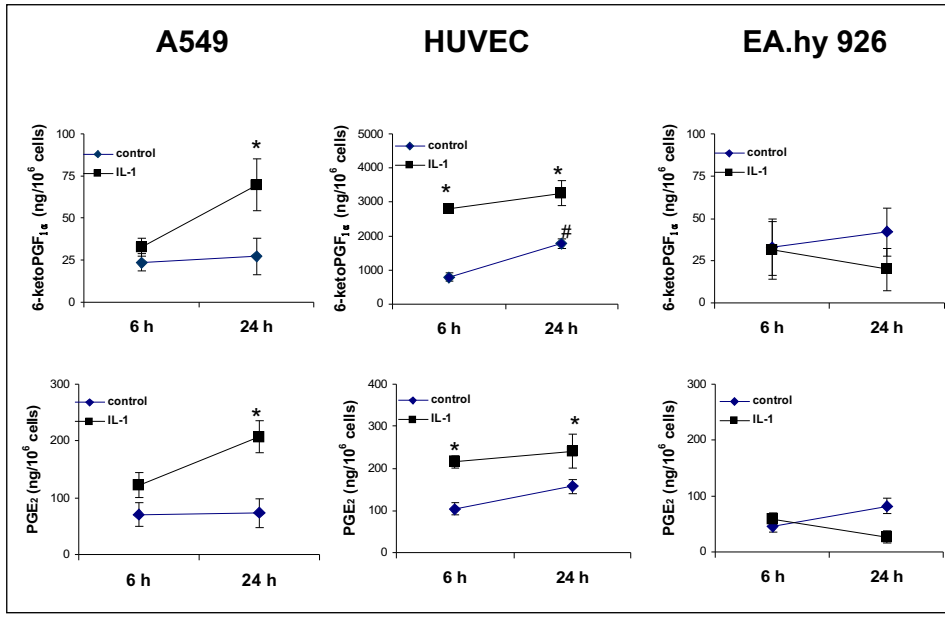

Figure 1. Time course of prostacyclin and PGE_2 production in A549, HUVEC, and EA.hy 926 cells. The cells were incubated in control medium or in the presence of IL-1β (1 ng/ ml) up to 24 hours. Data are means ± s.e. from n=4 experiments; *p< 0,05 *vs.* control; # p<0,05 *vs*. "6 h" time.

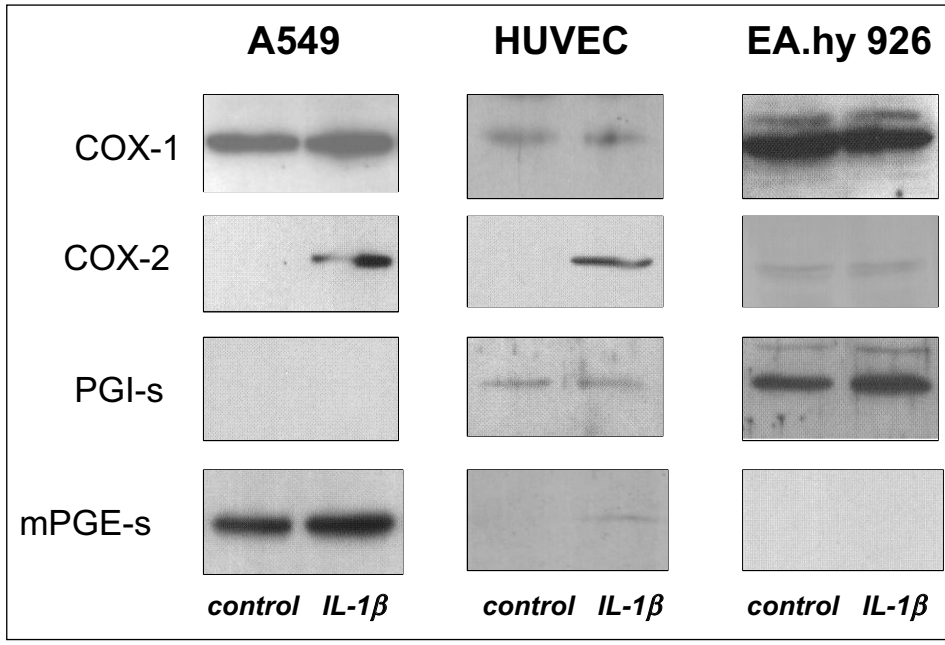

Figure 2. Expression of COX-1, COX-2, PGI-s and mPGE-s proteins in non-stimulated (control) and IL-1β–treated (1 ng/ ml, 24 hours) A549, HUVEC and EA.hy 926 cells.

COX-1 mRNA and protein levels in any of cell cultures (*Fig. 2, 3* and *4*). On the contrary, the levels of COX-2 protein were almost undetectable in non-stimulated cells (*Fig. 2*). Importantly, IL-1β treatment strongly induced COX-2 protein and mRNA in HUVEC and A549 cells, but not in EA.hy 926 cells (*Fig. 2, 3* and *4*).

Prostacyclin synthase (PGI-s) protein was present in non-stimulated HUVEC and EA.hy 926 cells, but almost undetectable in A549 cells (*Fig. 2*); treatment with IL-1β did not influence significantly PGI-s protein levels in any of the tested cell cultures (*Fig. 2* and *3*).

Microsomal PGE_2 synthase (mPGE-s) protein was highly expressed in non-stimulated A549 cells (*Fig. 2*); mPGE-s was not detectable in resting HUVEC and EA.hy 926 cells (*Fig. 2*). Stimulation with IL-1β significantly induced mPGE-s protein in HUVEC, but not in EA.hy 926 cells (*Fig. 2* and *3*).

Opposite to IL-1β, lysophosphatidylcholine (LPC 30, 100 μM) dose-dependently induced COX-2 protein and prostacyclin release in EA.hy 926 cell culture (*Fig. 5* and *6*). LPC-elicited increase in prostacyclin was abrogated by non-selective COX inhibitor indomethacin as well as by selective COX-2 inhibitor, rofecoxib (*Fig. 6*).

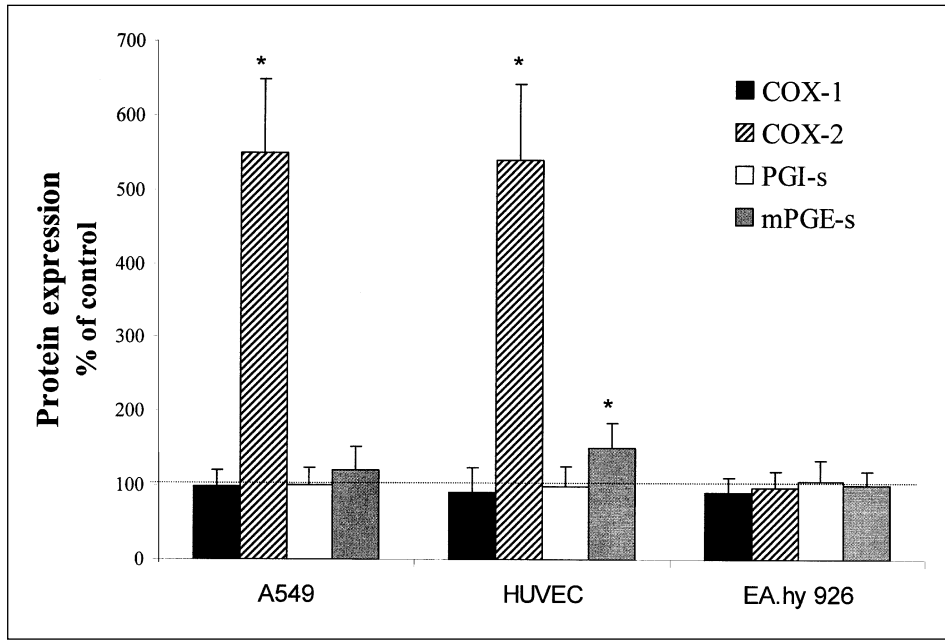

Figure 3. Densitometric analysis of changes in COX-1, COX-2, PGI-s, and mPGE-s protein levels in A549, HUVEC, and EA.hy 926 cells stimulated with IL-1β (1 ng/ml) for 24 hours. Results are normalized to β-actin and expressed as percentage of protein levels observed in control cells. Data are means ± s.e. from n=3 experiments; *p< 0,05 *vs.* control.

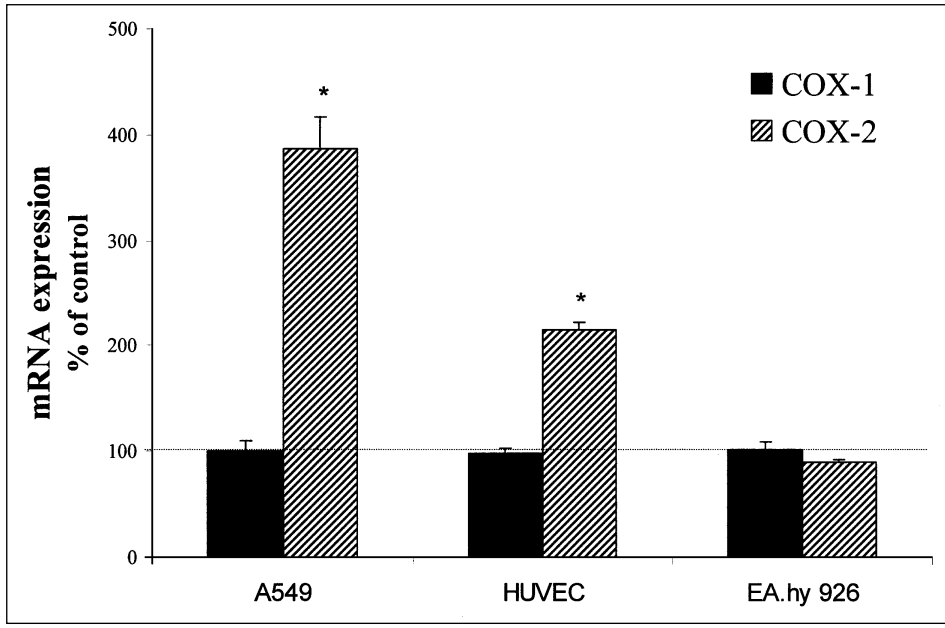

Figure 4. Densitometric analysis of COX-1 and COX-2 mRNA changes in A549, HUVEC, and EA.hy 926 cells stimulated with IL-1β (1 ng/ml) for 6 hours. Results are normalized to β-actin and expressed as percentage of mRNA levels observed in control cells. Data are means ± s.e. from n=3 experiments; *p< 0,05 *vs.* control.

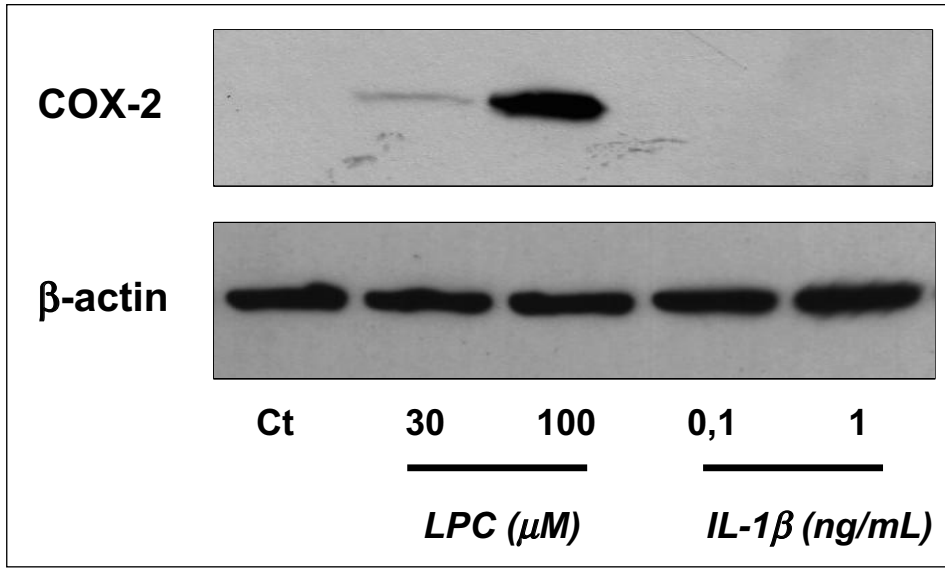

Figure 5. Expression of COX-2 protein in EA.hy 926 cells stimulated for 24 hours with L-α-lysophosphatidylcholine (LPC, 30 and 100 μM) and IL-1β (0,1 and 1 ng/ml). β-actin was used as internal control.

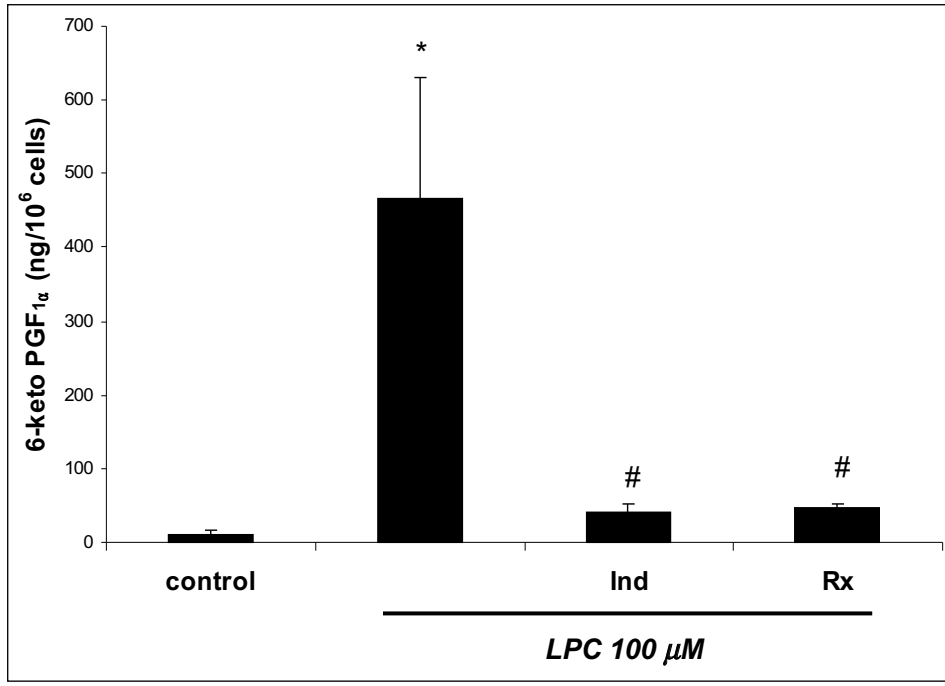

Figure 6. Prostacyclin production in EA.hy 926 cells stimulated by LPC (100 μM). The cells were pre-treated with indomethacin (Ind; 10 μM) or rofecoxib (Rx; 5 μM) prior to 24-hr stimulation with IL-1β (0,1 and 1 ng/ml). Data are means ± s.e. from n=4 experiments; *p< 0,05 *vs*. control, # p< 0,05 *vs*. LPC.

DISCUSSION

The pattern of prostanoid production in resting EA.hy 926 cells resembled the pattern observed in A549 cell culture: (i) EA.hy 926 cells accumulated similar amounts of 6-keto $PGF_{1\alpha}$ in culture medium; (ii) similarly to A549 cells, they produced more PGE_2 than prostacyclin. This similarity could be surprising, taking into granted different pattern of expression of terminal synthases (PGI-s and mPGE-s) in resting A549 and EA.hy 926 cells.

It was demonstrated that high constitutive expression of mPGE-s could be responsible for prevailing production of PGE_2 in A549 cells (24). Surprisingly, despite similar to A549 cells level of PGE_2 release, mPGE-s protein was almost undetectable in resting EA.hy 926 cells. On the contrary, resting EA.hy 926 cells showed high expression of PGI-s protein, however the prostacyclin production seemed to be much lower than in non-stimulated HUVEC.

The reasons of these discrepancies remain unclear. At least in the case of PGE_2 formation in resting cells, we cannot exclude other than mPGE-s-dependent pathways (25). Also, it should be noted that both PGI-s and microsomal PGE_2

synthase were characterized as very active enzymes (24;26), and even in the presence of their minimal amounts, production of both prostanoids in EA.hy 926 cells could be determined entirely by COX activity and/or cell-specific coupling of COX isoforms with terminal synthases (27;28).

Both, non-stimulated HUVEC and EA.hy 926 cells expressed relatively high levels of COX-1 and PGI-s proteins, however, in resting state COX-1 fuelled significantly prostacyclin production only in HUVEC.

In theory, there are several critical steps of prostaglandin synthesis pathway at which EA.hy 926 cells may differ from HUVEC. It is possible that main difference could occur at early stages of AA cascade, namely at the stage of generation of AA from membrane phospholipids by phospholipase A_2 (PLA_2) (29). Interestingly, by-passing of PLA_2 by giving exogenous AA resulted in rapid increase of prostacyclin production by resting EA.hy 926 cells (data not shown). However, the differences in PLA_2 isoform regulation between hybrid cells and HUVEC remain to be tested.

Endothelial cells *in vivo* respond to a variety stimuli (like thrombin, acetylcholine, or calcium ionophore) with transient activation of AA cascade and subsequent release of small amounts of prostacyclin (30;31). This delicate, physiological response could be disturbed in chronic cardiovascular diseases, when upon influence of various pro-inflammatory stimuli, endothelial cells undergo inflammatory change of phenotype (5;32). Analysis of action of pro-inflammatory cytokines on cultured endothelial cells is widely used in studies pertaining to molecular mechanisms of endothelial dysfunction (6;33). It has been shown that IL-1β increases prostacyclin generation by cultured endothelial cells, but its effect is delayed in onset and require induction of COX-2 (26;34;35).

In our hands, stimulation with IL-1β caused significant increase of production of prostacyclin and PGE_2 in HUVEC and A549 cells (IL-1β affected much more formation of prostacyclin in HUVEC and PGE_2 in A549 cells). On the contrary, treatment with IL-1β influenced neither formation of prostacyclin nor PGE_2 in EA.hy 926 cells. The ability of cells to respond to IL-1β with increase of prostanoid release was clearly related to COX-2 induction - treatment with IL-1β caused significant induction of COX-2 in HUVEC and A549, but not in EA.hy 926 cells. In agreement with previous reports, in our study IL-1β induced mPGE-s in HUVEC (36) and upregulated slightly mPGE-s protein in A549 cells (37).

In this study we cannot answer the question why, in contrary to HUVEC and A549 cells, hybrid cells do not induce COX-2 in response to IL-1β. Importantly, EA.hy 926 cells are able to induce COX-2 and subsequently increase production of prostacyclin in response to lysophosphatidylcholine (LPC), oxidized LDL component and cellular activator (38;39).

Because of unlimited replication potential and clonal purity EA.hy 926 cells have proved to be very convenient for *in vitro* research. Their characteristics in terms of the pattern of prostanoid formation, expression of enzymatic components of AA cascade and responsiveness to inflammatory stimuli could

facilitate studies of endothelial formation and pathophysiological role of these important lipid mediators.

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