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Significance of endothelial prostacyclin and nitric oxide in peripheral and pulmonary circulation

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SUMMARY

Background: *Vasoprotective function of endothelial cells is associated, among others, with biosynthesis and release of nitric oxide (NO), prostacyclin (PGI_2), prostaglandin E_2 (PGE_2), carbon monoxide (CO) and plasminogen activator (t-PA). These endothelial mediators calm down activated platelets and leukocytes, prevent the occurrence of parietal thrombotic events, promote thrombolysis, maintain tissue perfusion and protect vascular wall against acute damage and against chronic remodeling. Endothelial dysfunction in patients suffering from atherosclerosis or diabetes type 2 is associated not only with suppression in release of the above mediators but also with deleterious discharge of prostaglandin endoperoxides (PGH_2, PGG_2), superoxide anion (O_2^-), peroxynitrite ($ONOO^-$), and plasminogen activator inhibitor (PAI-1). We looked for mechanisms of protective endothelial function, with a special respect to the differences between peripheral and pulmonary circulation.*

Methods: *Cultured endothelial cells of bovine aorta (BAEC) were used to study physiological and pharmacological mechanisms of increasing free cytoplasmic calcium $[Ca^{2+}]i$. A porphyrinic sensor quantified the release of NO from BAEC. In cultured human umbilical vein endothelial cells (HUVEC) we looked for induction by bradykinin (Bk) of mRNAs for a number of enzymes. In blood perfused rat lungs we studied protective role of NO against injury inferred by lipopolysaccharide on pulmonary microcirculation that was accomplished by thromboxane A_2 (TXA_2), platelet activating factor (PAF), cysteinyl-leukotrienes (cyst-LTs) and the complement system. In vivo we analyzed the influence of Bk, perindopril and quinapril ('tissue type' angiotensin converting enzyme inhibitors, ACE-Is) on endothelial function in entire circulation of anaesthetized rats using a thrombolytic bioassay and EIA for 6-keto-$PGF_{1\alpha}$ and t-PA antigen.*

Results: *In BAEC Bk via kinin B_2 receptors raised in a concentration-dependent manner (1 pM – 10 nM) free cytoplasmic calcium ions $[Ca^{2+}]i$, that triggered the release of NO from BAEC. Calcium ionophore (A23187, 1–100 nM) as well as receptor agonists such as adenosine diphosphate (ADP, 10 nM – 1 μM), adrenaline (Adr, 1–10 μM) or acetylcholine (Ach, 10–100 μM) produced a similar rise in endothelial $[Ca^{2+}]i$ as did Bk at a nanomolar concentration. 'Tissue type' ACE-Is, e.g. quinapril or perindopril acted through accumulation of endogenous Bk. However, the potency of ACE-I to change endothelial function is by several orders of magnitude lower than that for exogenous Bk. In vivo the major difference between thrombolytic actions by quinapril or perindopril on one hand, and by exogenous Bk on the other was longevity of thrombolysis by ACE I and a distinct hypotensive action of exogenous Bk. Still, the long-lasting isolated thrombolytic effect of ACE I was mediated entirely by endogenous Bk as evidenced by the preventive action of icatibant, a kinin B2 receptor antagonist. Moreover, in vivo the immediate thrombolysis by ACE-I was mediated by PGI2 rather than by NO or t-PA, as shown by pharmacological analysis, and by direct blood assays of 6-keto-$PGF_{1\alpha}$ and t-PA antigen. Bradykinin as a mediator of pleiotropic endothelial action of several cardiovascular drugs (e.g. ACE-I) may complete its mission not only through B_2 receptor and $[Ca^{2+}]i$ - mediated release of PGI_2 or NO. Here, we describe a new route of the Bk action. Bk mediated induction of the $[Ca^{2+}]i$-independent, so called 'inducible', endothelial isoenzymes required for generation of CO, PGI_2 and PGE_2. After 4 hours of incubation of HUVEC with Bk (10 nM) it induced mRNAs for haemooxygenase 1 (HO-1), cyclooxygenase 2 (COX-2), prostaglandin E synthase (PGE-S) whereas mRNA for nitric oxide synthase 2 (NOS-2) was weakly affected. We proved also*

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that unlike in peripheral circulation, in pulmonary circulation only NO but not PGI_2 would play a protective role. In the blood-perfused lung, endotoxaemia liberates lipids, such as TXA_2, PAF and cyst-LTs. These toxic lipids along with the activated complement mediate pulmonary damage. Pulmonary endothelial nitric oxide is the only local protector against lung injury evoked by the phagocytised bacterial lipopolysaccharide.

Summary: *Summing up, in peripheral circulation endogenous Bk is the most efficient activator of protective endothelial function. For instance, thrombolytic action of 'tissue type' ACE-I depends on the Bk-released PGI2. Acting as an agonist of endothelial B2 kinin receptors Bk rises $[Ca^{2+}]i$ with a subsequent activation of constitutive COX 1 and NOS-3. This is followed by an immediate release of PGI_2 and NO. Moreover, acting as 'microcytokine' Bk induces mRNAs for HO-1, COX-2 and PGE S, the isoenzymes responsible for a delayed endothelial biosynthesis of CO, PGI_2 and PGE2. Activation of HO-1, apart from the CO generation may also lead to a deficiency in intracellular haeme required as a coenzyme for both COX and NOS. In peripheral circulation Bk-triggered production of PGI_2 seems to play a major role in defending endothelium against thrombosis. On the contrary, in pulmonary circulation NO seems to be the major endothelial defender against bacterial aggression coming from blood.*

BACKGROUND

As early as at the time of discovery of prostacyclin (PGI_2) [1,2] we realised that its generation in the conductance arteries might constitute a major protective mechanism against thrombosis and atherosclerosis by preventing deleterious invasion of activated platelets onto vascular wall. Moreover, we proposed that a selective inhibition of PGI_2 synthase by lipid peroxides would break protective function of endothelial barrier and invite atherosclerosis [2]. We also described a transcellular mechanism by which cytoprotective PGI_2 was biosynthesised in vascular wall when platelets fed it with the common substrate (PGH_2) for PGI_2/TXA_2 synthases [3]. A similar design of transcellular biosynthesis occurs during interaction of activated leukocytes with endothelial cells, but then pro-inflammatory cysteinyl-leukotrienes (cyst-LTs) are formed [4–6]. The significance of microcirculation in inflammatory response cannot be overestimated; in this system activation of leukocytes in the blood stream plays a crucial role. Cyclic 3',5'-AMP disarms leukocytes, however, their adenylate cyclase is insensitive to activation by PGI_2; instead it is activated by PGE_2 [4]. This is why the prevalence of generation of prostaglandin E_2 (PGE_2) over PGI_2 in microcirculation [7] makes perfect sense, especially since inducible PGE_2 synthase (PGE-S) was identified [8]. Garret Fitzgerald et al. [9] not long ago challenged the dogma that endothelial constitutive cyclooxygenase 1 (COX-1) would be exclusively responsible for making PGI_2. According to them inducible COX-2 is also likely to abide endothelial cells. Knowledge of the eicosanoid system in vascular wall is far from complete. The cytochrome P 450\NADPH dependent pathway generates 20-hydroxyeicosatetraenoic acid (20-HETE) [10] and a number of isomeric epoxyeicosatrienoic acids (EETs) with opposite effects on vascular tone. One of EETs is likely to be a vasorelaxant 'endothelium-derived hyperpolarizing factor' (EDHF) [11–14]. The family of endothelial mediators still enlarged after the discovery of endothelium-derived relaxing factor (EDRF) [15], that was soon identified as nitric oxide (NO) [16], with a vasorelaxant action mediated by cyclic 3', 5'-GMP [17]. An abbreviation EDRF(NO) is sometimes used to distinguish NO made by endothelial constitutive NO synthase (NOS 3) from NO generated by neuronal NOS-1 or by inducible NOS-2. In the body EDRF(NO) exists as a lipophylic free radical NO^- that is inactivated by superoxide anion (O_2^-) close to endothelial surface [18]. This reaction may lead to formation of inactive nitrate or to generation of the highly destructive ($ONOO^-$) [19]. Deficiency of the substrate (L arginine) or a NOS cofactor (tetrahydrobiopterin) may push NOS-3 to generate O_2^- instead of, or along with NO^- [19–21]. In consequence, the formation of $ONOO^-$ is likely to occur, especially because O_2^- reacts more avidly with NO^- than with its own scavenging enzyme - superoxide dysmutase [19]. Endothelial cells release PGI_2 and NO in a coupled manner [18], however, interactions between the systems synthesising PGI_2 and NO remain unclear. Both inhibition [22] and activation [23-25] of COX by NO were reported. Freshly, Volker Ulrich (personal communication) gave a thorough insight into that problem. It seems that peroxynitrite is a highly selective and potent inhibitor of PGI_2 synthase with EC50=50 nM. This inhibitory potency of $ONOO^-$ for PGI_2 synthase is at least 200-fold stronger than analogous inhibitory action of lipid peroxides [2]; moreover, $ONOO^-$ is much weaker inhibitor of other enzymes within the prostanoid network. In

this paper we present our data and our opinion on pathophysiological and pharmacological significance of the coupled endothelial release of PGI_2 and NO in peripheral and pulmonary circulation.

METHODS

Assay of cytosolic free calcium $[Ca^{2+}]i$ in cultured endothelial cells of bovine aorta (BAEC)

Endothelial cells were harvested from bovine thoracic aorta (BAEC) as described previously [26]. Cells from 2nd passage were used for experiments. Their homogenity was identified by their typical cobblestone morphology under the Axiovert 25 Inverted Microscope (Carl Zeiss Jena GmBH, Germany).

Cytosolic calcium ion $[Ca^{2+}]i$ assay was accomplished by the method of Grynkiewicz et al. [27], namely BAEC (0.5×10^6 cells ml^{-1})were loaded with fura-2 by incubation for 60 min at 25°C with this fluorescent dye in its membrane permeant form, i.e. acetoxymethyl ester (fura-2AM) in presence of bovine serum albumin. Then the extracellular dye was removed by centrifugation and the cells were resuspended in HBS containing glucose (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. Calibration was completed using 0.2% Triton X-100 for R_{max} and 5 mM EGTA for R_{min}. Eventually, levels of cytosolic calcium $[Ca^{2+}]i$ were calculated according to the equation of Grynkiewicz et al. [27].

Endothelial release of nitric oxide as measured by the Maliński's electrode

Nitric oxide (NO) was measured using a porphyrinic microsensor. This was prepared by cyclic voltametric scanning that deposited a film of polymeric porphyrin (Ni(II) tetrakis(3-methoxy-4-hydroxyphenyl)porphyrin) on a thermally sharpened carbon fiber electrode, then coated with nafion as described previously [28]. The microsensor's working electrode was placed close (20 ± 5 μm) to the surface of confluent monolayer of BAEC along with a platinum wire counterelectrode and a saturated calomel reference electrode. Voltametric analyser (PAR model 264A) with the current-sensitive preamplifier (PAR model 181) were used for amperometric measurements at a potential of 0.68 V. NO measurements are standardised using aqueous NO standard prepared as described by Jia and Furchgott [29]. Nitric oxide was measured over a single BAEC in Hank's balance solution at 37°C. The response time was 0.1 ms and the detection limit 10^{-15} M of NO. Bradykinin was used at a concentration from $2x10^{-15}$ to $1x10^{-12}$ M.

Influence of bradykinin on HO-1, COX-2, COX-1, PGE-S and NOS 2 mRNA expression in HUVEC

Endothelial cells were harvested from human umbilical vein (HUVEC) according to Jaffe et al. [30] and cultured as described previously [26]. HUVEC were used for experiments upon reaching 90% confluency in the third passage, which was carried out in 6-well plates ($1x10^6$ cells, NUNC, Brand Products, Denmark). HUVEC homogenity was identified by their typical cobblestone morphology under the Axiovert 25 Inverted Microscope (Carl Zeiss Jena GmBH, Germany). Then after 24 hours of HUVEC incubation in OPTI-MEM I without fetal bovine serum, cells were stimulated with Bk (10 nM) for 4 hours and total RNA was isolated by the guanidinum isothocyanate method as described by Chomczynski and Sacchi [31] using TRIZOL®Reagent (Gibco BRL, UK).

Reverse transcription (RT) of total RNA (1 μg) were perfomed with oligo $(dT)_{12-18}$ primer and M MLV reverse transcriptase (Gibco BRL, UK) for 2 hours at 42°C. Then, cDNA (1 μl) was amplified with HotStar Taq Polimerase (QIAGEN, USA) for both quantitative and qualitative RT-PCR. Specific mRNAs for HO-1, COX 2, COX 1 and NOS 2 were quantified by Gene Specific Relative RT PCR Kit (Ambion, USA) using multiplex RT-PCR protocol with endogenous 18S rRNA standard and Competimer® technology. PCR reactions were set up with GeneAmp 9600 machine (Perkin-Elmer, USA) of the following profile: 95°C – 15 min and 35 cycles: 94°C – 30 s, 59°C – 30 s, 72°C – 30 s followed by 72°C – 10 min elongation and then samples were placed on ice. Equal volumes of each sample (5 μL) were run on 3% agarose containing ethidium bromide and images were captured electronically with DC40 digital camera (Kodak, USA) and the bands were quantified using image analysis software (NIH Image, USA). Results were expressed as ratio of pixel density units for specific mRNA to internal standard (18S rRNA). For position and size of observed specific bands, marker M1 (pUC19/MspI, DNA-Gdańsk II, Poland) was run in parallel. For qualitative RT-PCR for PGE S following specific primers were used: primer 1

(sense) 5′-ATGCCTGCCCACAGCCTG-3′ (40nM) and primer 2 (antisense): 5′-TCACAGGTG-GCGGGCCGC-3′. Oligonucleotide primers were constructed by Jakobson et al [8], but trunctated for cloning restriction sites. Primer sets for NOS 2 and internal standard (18S rRNA) was obtained commercially (Ambion, USA).

Blood assays of 6-keto-$PGF_{1\alpha}$ and t-PA antigen

Blood samples (500 μl) were collected into Eppendorff tubes with indomethacin to yield its final concentration of 10 μM, and then stored at –70°C not longer than for a week. 6-Keto- $PGF_{1\alpha}$ was assayed using the enzyme immunoassay kit (Cayman Chemical Co, Ann Arbor, MI) and t-PA antigen was assayed using the enzyme immunoassay kit (Biopool TintElize t-PA antigen, Umea, Sweden). All results were expressed in ng ml^{-1}.

Thrombolytic assay for activation of entire endothelial system in rats in vivo

In vivo model for studying of thrombolysis in cats [32] was adopted to rats [33,34]. Briefly, male Wistar rats body weight 300–350 g were anaesthetised (thiopental 30 mg kg^{-1} i.p.) and unfractionated heparin at a dose of 800 units kg^{-1} i.v. was administered. Extracorporal circulation was established between left carotid artery and left jugular vein, and a collagen strip from rabbit tendon of Achilles was superfused with arterial blood at a rate of 1.5 ml min^{-1}. Its weight was continuously monitored by an auxotonic Harvard transducer. Because of deposition of thrombi [32–35] the strip gained in weight by 80–120 mg during the first 20 min of superfusion and stayed at that level during next 3–5 hours of the experiment. Mean arterial blood pressure (BP) was monitored from right carotid artery by a Harvard pressure transducer, and right femoral vein was prepared for drug administration. In the above system dispersion of thrombi used to occur next to intravenous administration of PGI_2 or iloprost at doses of 0.1–1.0 μg kg^{-1} (without concomitant fall in BP) and next to glyceryl trinitrate at doses of 30–100 μg kg^{-1} or metacholine hydrochloride (10 μg kg^{-1}) or kallikrein (100 units kg^{-1}) (with accompanying fall in BP) whereas aspirin at doses of 5–50 mg kg^{-1} did not evoke thrombolysis. Streptokinase (3–30 megaunits kg^{-1}) produced biphasic thrombogenic / thrombolytic response [36].

Blood-perfused rat endotoxaemic lungs

Lungs were isolated from Wistar rats weighing 200–250g (Lod: WIST BR from Animal laboratory of Polish Mother's Memorial Research Institute Hospital in Łódź, Poland) and mounted in an isolated rat lung set-up (Hugo Sachs Elektronik) as described previously [37]. Briefly, anaesthetised rats (thiopentone 120 mg kg^{-1}, i.p.), after laparatomy, were exsanguinated by incision of left renal artery and lungs were exposed via medial sternotomy. The pulmonary artery and left atrium were cannulated via right and left atrium, respectively. Immediately after cannulation lungs were dissected from the thorax and mounted in the water-jacketed (37°C), air-tight glass chamber (HSE), and ventilated with negative pressures. The end-expiratory pressure in the chamber was set to be –2 cm H_2O and inspiratory pressure was adjusted between -6 to –10 cm H_2O to yield the initial tidal volume (TV) of about 2.0 ml. Lungs were perfused with various fluids using a peristaltic pump (ISM 834, HSE) at constant flow of about 16 ml min^{-1}. Following parameters were measured: arterial and venous pulmonary pressures (PAP, PVP) tidal volume (TV) and the weight of lungs according to the Uhlig's method [38]. The following fluids were used to perfuse the lung preparation: rat full blood (FB), rat platelet rich plasma (PRP), rat platelet poor plasma (PPP) or Krebs-Henseleit buffer (KH) obtained as described previously [37].

All lungs preparations were allowed to equilibrate for the first 15 min until baseline PAP, PVP, TV and weight became stable. LPS was injected 45 minutes after the beginning of the experiment. L-NAME was administered 15 min prior to LPS. Camonagrel (300 μM), WEB 2170 (100 μM), MK 571 (100 μM), anti-CD62P (1 μg ml^{-1}) sCR1 (100 μg ml^{-1}) were added 30 min prior to injection of LPS.

Data were expressed as means±SEM of changes in TV (ΔTV), PAP (ΔPAP), PVP (ΔPVP), or weight (Δweight) from values 30 min before LPS injection.

Reagents

Bradykinin (Bk), adrenaline hydrochloride, adenosine diphosphate (ADP), acetylocholine hydrobromide, indomethacin, N^G nitro-L-arginine methyl ester (L NAME), calcium inophore A23187, Fura 2AM, lipopolysaccharide from Escherichia coli (LPS, B: 0127) were purchased from Simga-Aldrich (USA). Platelet activating factor (PAF) receptor

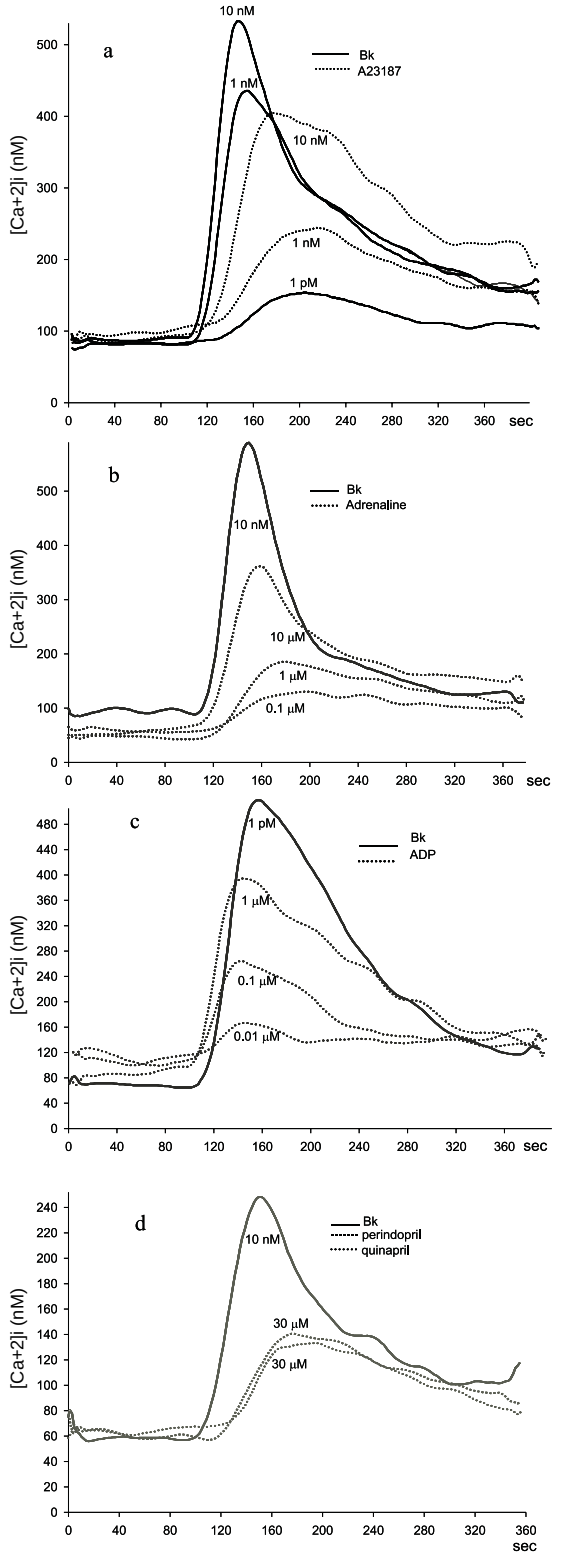

Figure 1. Increase in intracellular free calcium levels $[Ca^{+2}]i$ in cultured bovine endothelial cells (BAEC, 2nd passage) by bradykinin (Bk, 10 nM and 1 pM, solid lines), calcium ionophore (A23187, 10nM, 1 nM, dotted lines, [a]), adrenaline (10 μM, 1 μM and 0.1 μM, dotted lines, [b]), adenosine diphosphate (ADP, 1 μM, 0.1 μM and 0.01 μM, dotted lines, [c]) and angiotensin converting enzyme inhibitors (ACE-I, perindopril (30 μM), quinapril (30 μM), dotted lines, [d]).

antagonist WEB2170 was obtained from Boehringer-Ingelheim (Germany), thromboxane A_2 synthase inhibitor camonagrel from Ferreir (Spain), cysteinyl-leukotriene receptor antogonist (MK571) from Biomol (USA), anti-selectin P policlonal antibody (anti-CD62P) from Pharmingen (Germany) and alternative and classic pathway complement inhibitor (sCR1) from T CELL Boston (USA). Angiotensin converting enzyme inhibitors (ACE I); quinapril, quinaprilat were gifts from Pfizer Poland and perindopril, perindoprilat from Servier Poland. Bradykin B_2 receptor antagonist icatibant (HOE 140) was a gift from Hoechst Marion Roussel (Germany).

Statistical analysis

Arithmetical means are given with s.e. mean (SEM). Differences between groups were assessed with unpaired two-tailed t test with Welch's correction or paired Student's t-test. P less than 0.01 was assumed to denote a significant difference. In isolated lung experiments difference between groups were assessed by one-way ANOVA followed by post hoc Fischer test. P less than 0.05 was considered as statistically significant.

RESULTS

Effect of bradykinin on cytosolic free calcium $[Ca^{2+}]i$ and NO release in BAEC

Despite standardized conditions (see Methods), $[Ca^{2+}]i$ in resting or stimulated cells varied between experiments. Basal $[Ca^{2+}]i$ were 50–150 nM (the most often reading was 100 nM). After instillation of the standard Bk concentration (10 nM) the rise in $[Ca^{2+}]i$ was 4 to 8 fold. Calcium ionophore (A23187) produced a concentration dependent (1–100 nM) rise in $[Ca^{+2}]i$. Similar rise in $[Ca^{+2}]i$ was obtained by the following agonists of endothelial receptors: bradykinin (1 pM – 10 nM), adenosine diphosphate (10 nM – 1 μM), adrenaline (1–10 μM), acetylcholine (10–100 μM), as well as by ACE inhibitors perindopril and quinapril (10–30 μM) (Fig. 1). Effect of Bk (10 nM) on $[Ca^{+2}]i$ was effectively blocked by icatibant (30 nM), a B_2 kinin receptor antagonist (Fig. 2).

Sensitivity to Bk varied between batches of BAEC. In the most sensitive batch of BAEC a rise in $[Ca^{2+}]i$ occurred after instillation of Bk at a concentration as low as 1 fM (Fig. 3, left panel). Basal NO release by BAEC was 12 nM. Bk in a concentration-dependent manner (2 f M- 10 f M) stimulated within one second NO release from BAEC. Bk at higher concentrations of 10 fM to 1 pM kept NO release at the level of 535±25 nM NO (Fig. 3, right panel).

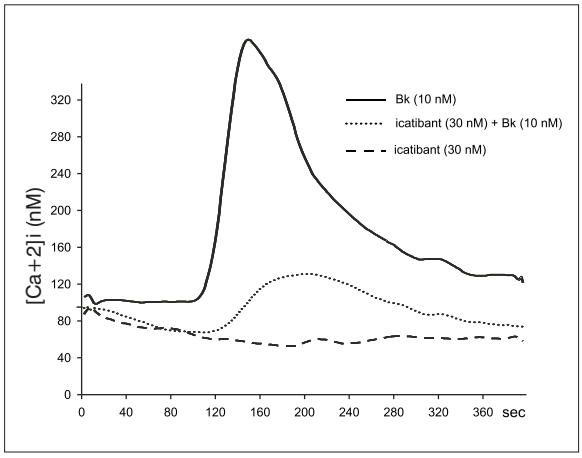

Figure 2. Inhibition by icatibant (30nM, dotted line) of increase in $[Ca^{+2}]i$ by Bk (10nM, solid line) in BAEC. Dashed line shows the icatibant (30 nM) had no effect of its own on $[Ca^{+2}]i$.

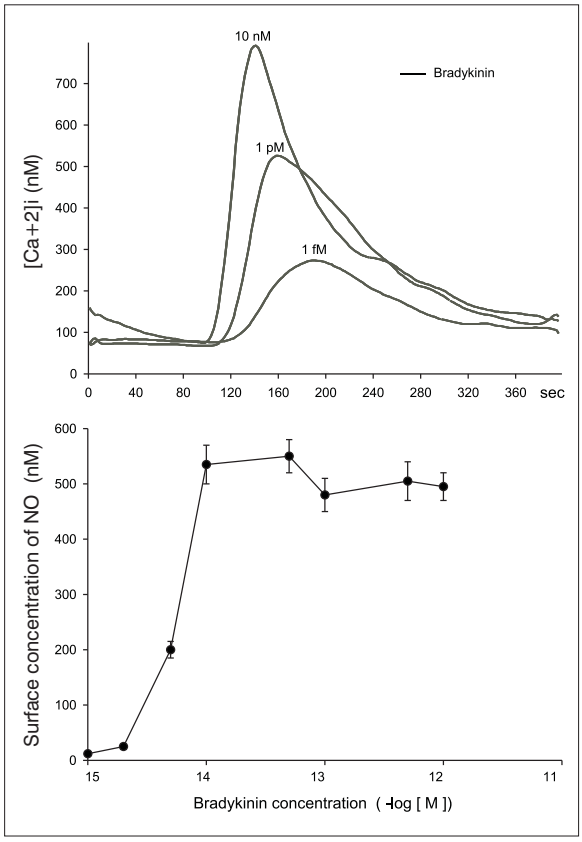

Figure 3. The effect of Bk (1 fM, 1 pM and 10 nM) on $[Ca^{+2}]i$ in BAEC (left panel) and the effect of Bk (10^{-15} – 10^{-12} M) on the surface concentration of nitric oxide in BEAC.

Effect of bradykinin on mRNAs for HO-1, COX-2, COX-1, PGE-S and NOS 2 in HUVEC

To compensate for variations in total RNA quality, initial quantification errors and random tube-to-tube variation in reverse transcription and PCR, we used multiplex RT PCR with two primer sets in one tube in a single PCR (multiplex). One set of primers was used to amplify the cDNA of interest: HO 1,

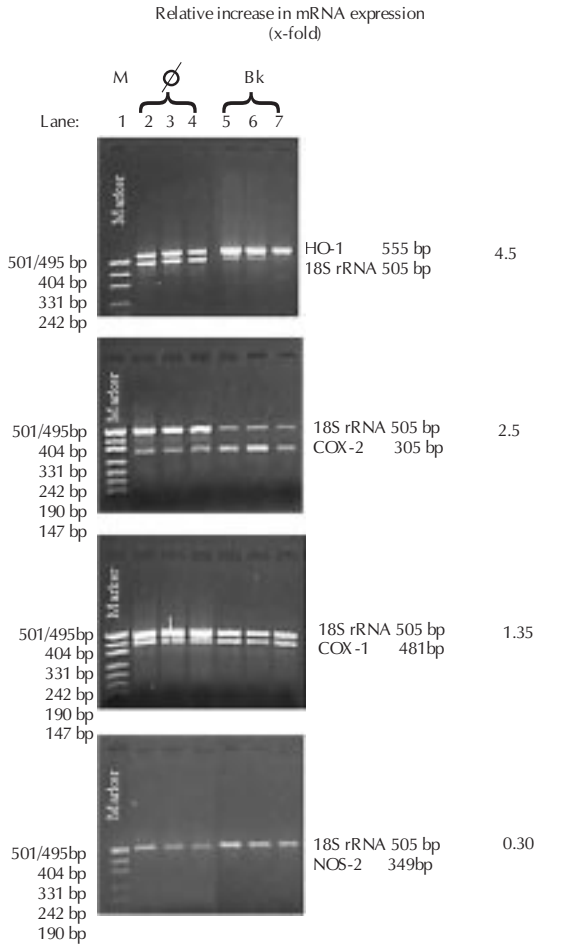

Figure 4. Multiplex RT-PCR with an endogenous standard (18S rRNA) of RNAs for HO-1, COX-2, COX-1 and NOS-2. Lane 1 displays mass standards (sizes of the DNA marker M1 are printed at left). Lanes 2, 3 and 4 contain cDNA obtained from unstimulated HUVEC, lanes 5, 6 and 7 contain cDNA from Bk (10 nM)-stimulated HUVEC for 4 hrs. cDNAs in lanes 2, 3, 4 and 5, 6, 7 were diluted 1:10, 1:20 and 1:30, respectively. The data obtained are expressed as x-fold difference in the level of specific mRNA and the level of internal standard (18S rRNA) and are shown at right.

COX 2, COX 1, and NOS 2 while second set of primers was used to amplify an invariant endogenous control 18S rRNA. Fig. 4 shows representative gels and relative RT PCR is expressed by x-fold difference in the level of specific mRNA between equivalent total RNA samples. In HUVEC incubated for 4 hours with bradykinin at a concentration of 10 nM, a significant increase in mRNAs for HO-1 (4.5-fold) and COX-2 (2.5 fold), but not for COX-1 (1.35 fold) and NOS 2 (0.3 fold) was observed. In separate RT-PCR experiments, the induction of specific PGE-S mRNA, but not NOS 2 mRNA was observed. Fig. 5 shows a gel with the band of induced PGE-S with hardly visible band of NOS 2. Total RNA content was controlled by amplifying invariant control 18S rRNA.

In vivo activation of the entire endothelial system in rats by bradykinin and by ACE I as measured by the thrombolytic assay

In rats with extracorporal circulation both perindopril and quinapril at doses 10–30 μg kg^{-1} produced a long lasting (>4 hrs) thrombolysis with no hypotensive effect. The same doses of perindoprilat and quinaprilat (active metabolites of the drugs) produced a shorter (< 2 hrs) thrombolytic effect, again with no concomitant fall in blood pressure. Exogenous Bk (1–30 μg kg^{-1}) produced a short-lasting thrombolysis and a profound arterial hypotension (Fig. 6) All of the above effects were inhibited by pretreatment with icatibant at a dose of 100 μg kg^{-1}. Thrombolysis by quinapril and perindopril was also abolished by pretreatment with indomethacin (5 mg kg^{-1}), while L NAME (5 mg kg^{-1}) only partially and temporally inhibited thrombolytic response to ACE-I (Fig. 7). Quinapril and perindopril at doses 30 μg kg^{-1} within first 15 min after intravenous injection raised blood levels of 6-keto-$PGF_{1\alpha}$ (Fig. 8). These elevated levels of PGI_2 breakdown product would be staying for next 3–4 hrs till thrombolysis lasted (Fig. 7, 8). t PA antigen plasma concentration started to rise significantly only 1 hr after administration of ACE Is and then continued to rise during the observation period (Fig. 8). As mentioned above a long-lasting thrombolytic response to quinapril (Fig. 7) was associated with a concomitant rise in blood level of 6-keto-$PGF_{1\alpha}$. Cyclooxygenase inhibition by indomethacin (5 mg kg^{-1} i.v.) was evidenced by eradicating the bio-

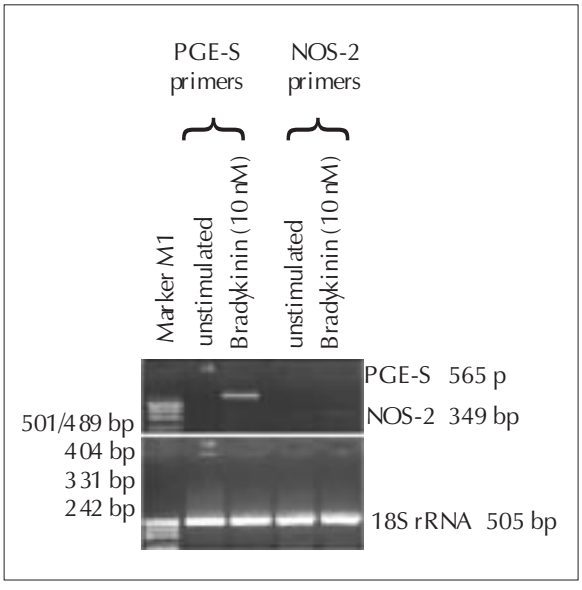

Figure 5. RT-PCR of mRNAs for PGE-S, NOS-2 (upper gel) and 18S rRNA (house keeping gene, lower gel) in unstimulated and Bk (10 nM) stimulated for 4 hours HUVEC. Positions and sizes of the DNA marker M1 are printed at left.

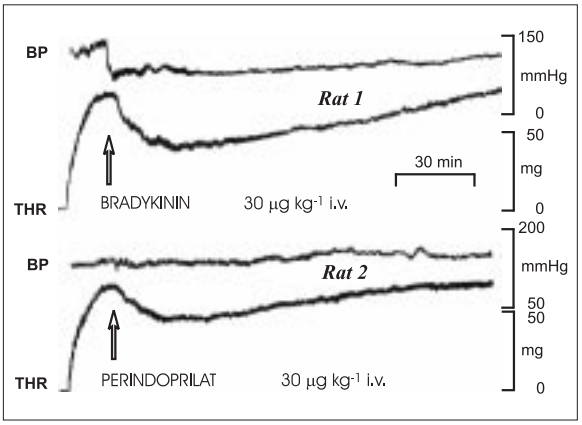

Figure 6. Effects of exogenous Bk (30 μg kg^{-1}) and endogenous Bk (perindoprilat, 30 μg kg^{-1}) on arterial blood pressure (BP) and blood-superfused thrombus weight (THR) in rats #1 and #2.

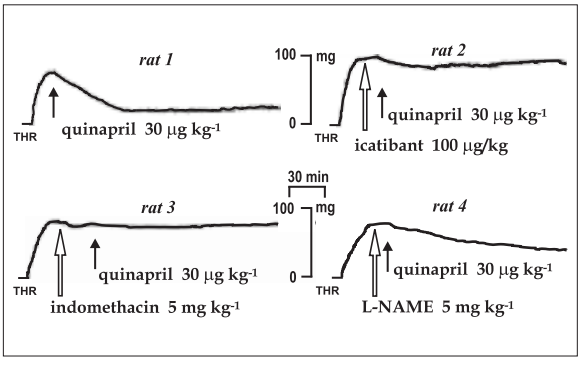

Figure 7. Pharmacological analysis of thrombolytic action (THR) of quinapril (30 μg kg^{-1}, i. v, rat #1). Effects of the pretreatment with icatibant (100 μg kg^{-1}, i. v, rat #2), indomethacin (5 mg kg^{-1}, i. v, rat#3) and L-NAME (5 mg kg^{-1}, i. v, rat#4).

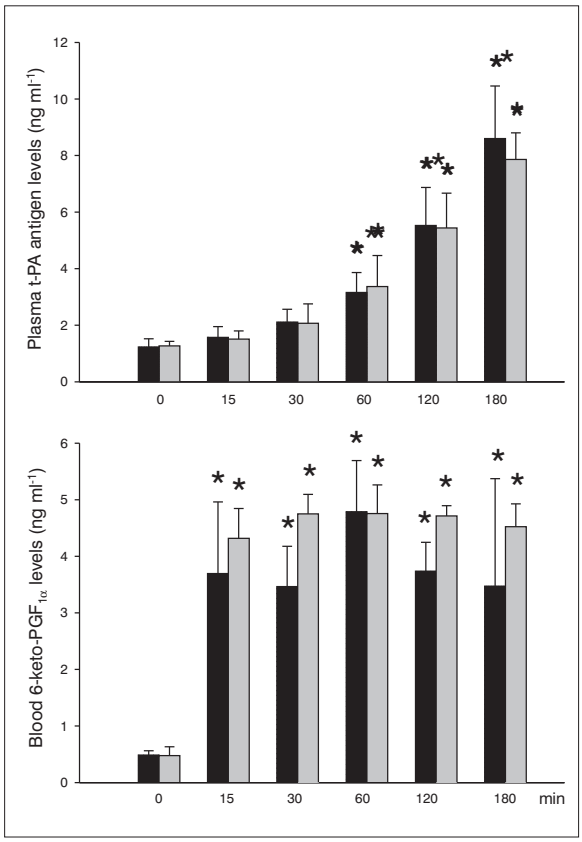

Figure 8. Time-dependent (0 – 180 min) effects of quinapril (■ 30 mg kg^{-1}, i. v.) and perindopril (■ 30 μg kg^{-1}, i. v.) in rats on plasma or blood levels of t-PA antigen (n=8) or 6-keto-$PGF_{1\alpha}$(n=6) (mean±SEM) ($p<0.01$ as compared with time 0.

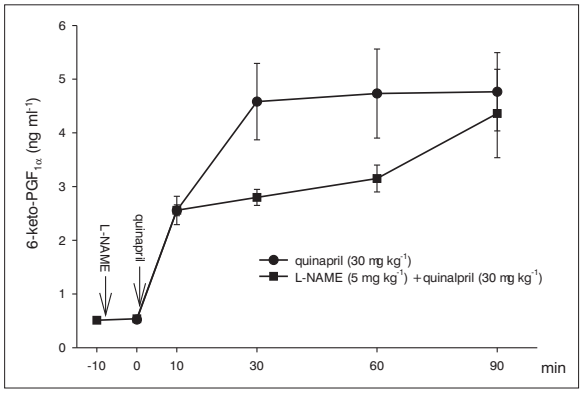

Figure 9. Time-dependent (0–90 min) changes in blood levels of 6-keto-$PGF_{1\alpha}$ in rats after intravenous administration of quinapril (30 μg kg^{-1}) administration before (●) and after (■) pretreatment with L-NAME (5 mg kg^{-1}, i. v.), (mean±SEM, n=4).

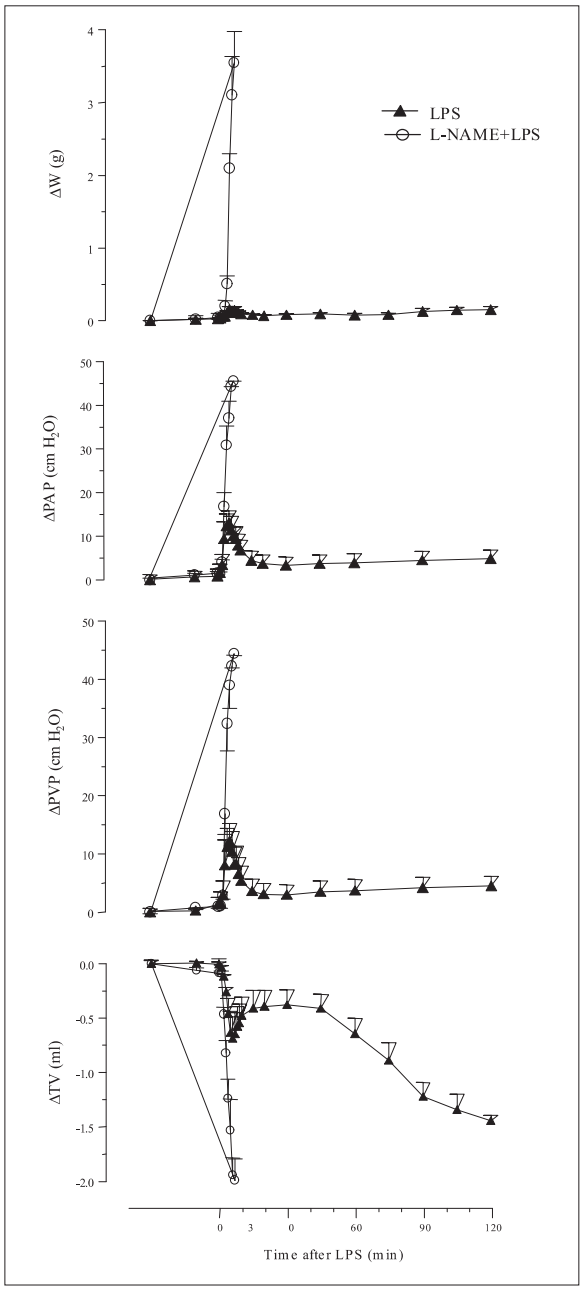

Figure 10. Blood-perfused rat lungs. Early response to lipopolysaccharide (LPS, 300 μg ml^{-1}) before (▲) and after (❍) pretreatment with (L-NAME, 300 μM). LPS produced immediate but transient constriction of pulmonary vessels (ΔPAP and ΔPVP) and a fall in tidal volume (ΔTV). Within next 2 hrs only a decrease in ΔTV was agravated. After pretreatment with L-NAME LPS produced an abrupt and irreversible rise in ΔPAP, ΔPVP and a fall in ΔTV accompanied by pulmonary oedema. Data show mean±SEM for n=6 experiments.

chemical response to quinapril. In blood no rise in the decomposition product of PGI_2 occurred (data not shown). This clear cut effect of indomethacin was coupled with loss of thrombolytic properties of quinapril (Fig. 7). Interestingly, pretreatment with L-NAME (5 mg kg^{-1}, i.v.) not only delayed thrombolysis by quinapril (Fig. 7). Absence of the endo-

genous source of NO did also hindered quinapril-induced appearance of 6-keto-$PGF_{1\alpha}$ in blood (Fig. 9).

Role of endogenous NO in the immediate response of isolated blood-perfused rat lung to lipopolysaccharide (LPS)

Injection of LPS (300 μg ml^{-1}) into the isolated blood-perfused rat lungs induced a biphasic response. An immediate transient phase of this response consisted of an increase in pulmonary arterial and venous pressures (PAP and PVP) with a simultaneous decrease in tidal volume (TV). This response was not accompanied by a significant change in weight of the lung. A delayed phase of lung response to LPS consisted of a gradual decline in TV without accompanying changes in TV, PAP PVP and weight (Fig 10). After pretreatment with L NAME (300 μM), injection of LPS (300 μg ml^{-1}) led to the immediate arrest of lung function. L-NAME + LPS - induced immediate rise in PAP and PVP as well as drop in TV were substantially augmented as compared to the response evoked by LPS alone, and were accompanied by a profound lung oedema (Fig. 10).

These responses were not seen in lungs perfused with plasma or Krebs-Henseleit buffer. Interestingly, during an immediate lung response to LPS and L-NAME + LPS there was a comparable pool of leukocytes (data not shown) and platelets sequestered within pulmonary circulation, the latter in response to LPS (Fig. 11).

Immediate lung responses to LPS or L-NAME+LPS were both abrogated by a complement inhibitor sCR1 (100 μg ml^{-1}). Responses induced by LPS and L-NAME+LPS were also affected by inhibitors of lipid mediators. Immediate transient lung response to LPS was inhibited by PAF receptor antagonist, WEB 2170 (100 μM), and a TXA_2 synthase inhibitor, Camonagrel (300 μM), but not by a leukotriene receptor antagonist MK 571 (300 μM), whereas immediate irreversible lung response to L-NAME+LPS was inhibited by WEB 2170 (100 μM), Camonagrel (300 μM) as well as by MK 571 (300 μM). Responses induced by L-NAME+LPS but not that induced by LPS were inhibited by antibody against selectin P, anti-CD62P (1 μg ml^{-1}) (data not shown).

At the morphological level, the immediate transient lung response induced by LPS did not leave any signs of injury in the lung histology. However, irreversible response induced by L-NAME+LPS was associated with alveolar haemorrhagic oedema and a damage of alveolar barrier as evidenced by light and electron microscopy (Fig. 12, 13). Importantly, electron microscopy also revealed that sequestration of neutrophils and platelets during an immediate phase of lung response to LPS was confined to pulmonary microcirculation and associated with internalisation of LPS by neutrophils (Fig. 14).

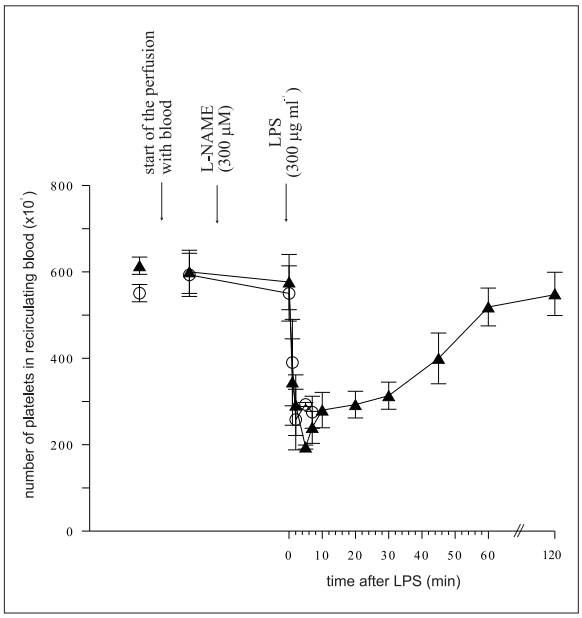

Figure 11. Pulmonary sequestration of platelets in the isolated blood-perfused rat lung, challenged with either LPS or L-NAME+LPS (see legend to Fig. 10). LPS induced an immediate retention of platelets in pulmonary circulation that lasted approximately 1 h. L-NAME+LPS produced a similar pulmonary sequestration of platelets, although it was irreversible and associated with lung injury. Data show mean±SEM for n=6 experiments.

DISCUSSION

Cultured endothelial cells from bovine aorta (BAEC) or from human umbilical vein (HUVEC) have a capacity to generate two major endothelial mediators, i.e. prostacyclin (PGI_2) and nitric oxide (NO). Constitutive enzymes responsible for that generation are dioxygenases, namely, cyclooxygenase 1 (COX-1) and nitric oxide synthase (NOS-3). Inside the cells these enzymes are activated by a rise in free cytoplasmic calcium ions $[Ca^{2+}]i$. Here we report that in BAEC calcium ionophore (A 23187, 1–100 nM), and a number of physiological receptor agonists such as bradykinin (Bk, 1 pM – 10 nM), adenosine diphosphate (ADP, 10 nM – 1 μM), adrenaline (1–10 μM) or acetylcholine

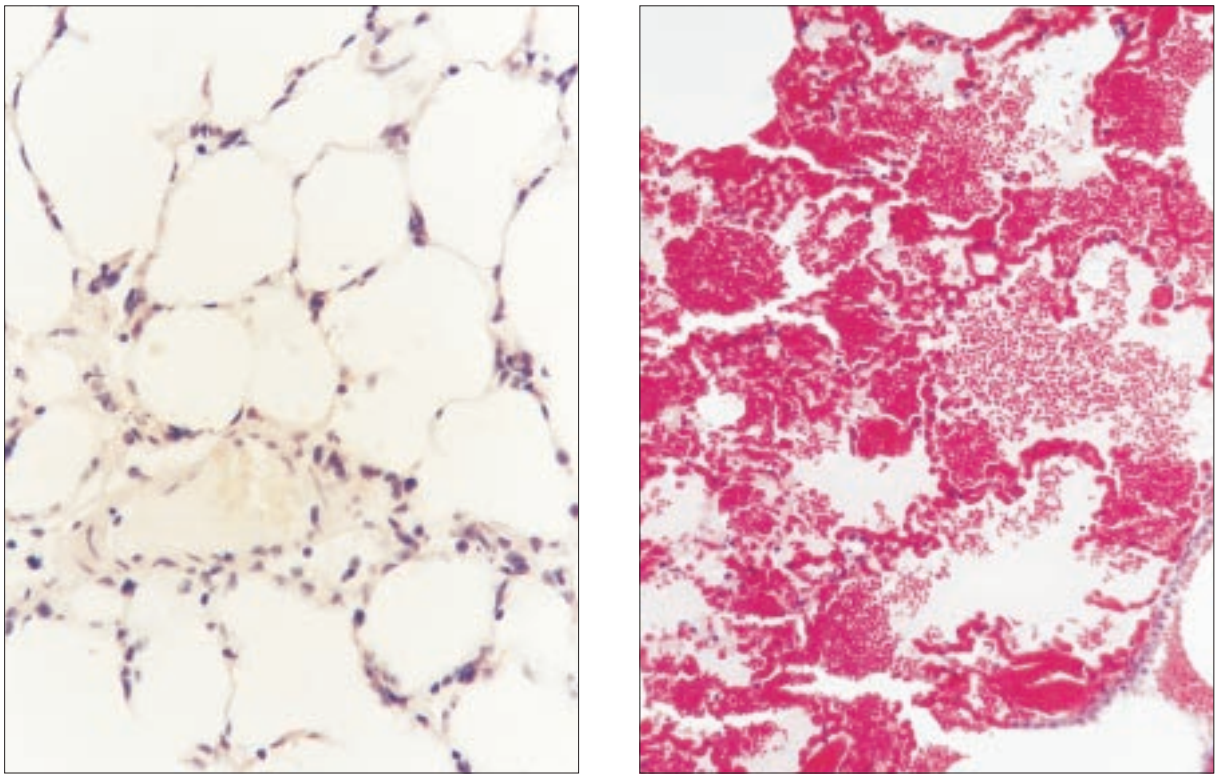

Figure 12. Light micrograph of isolated blood-perfused rat lung shortly after a challenge with LPS (upper panel) and L-NAME+LPS (lower panel). Immediate phase of lung response to LPS was not associated with changes in lung histology (upper panel) while injection of L-NAME+LPS induced massive alveolar haemorrhages (lower panel). Hematoxylin-eosin stain, Objective magnification 40x.

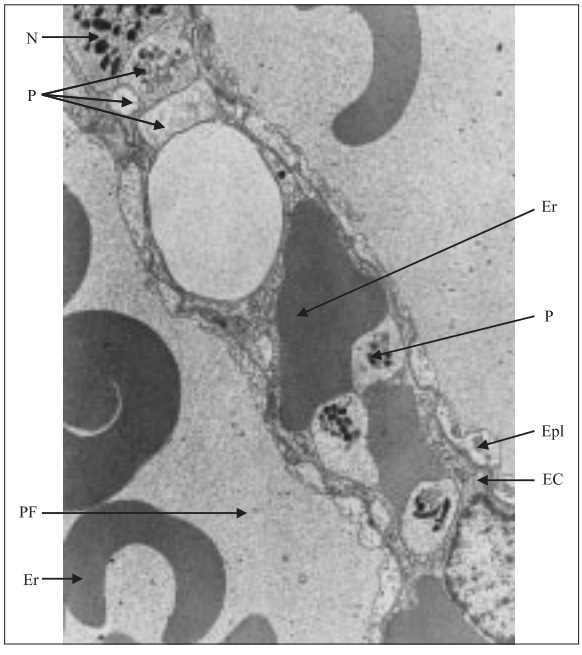

Figure 13. Electron micrograph of pulmonary capillary of isolated blood-perfused rat lung shortly after a challenge with L-NAME+LPS. Capillary endothelial cells (EC) and epithelial cell type I (EpI) are damaged as evidenced by decrease in electron density and oedema of these cells. Inside capillary vessel sequestrated platelets (P) and neutrophil (N) are seen. In alveolar space protein rich fluid (PF) and erythrocytes (Er) are present. Magnification 6000x.

(10–100 μM) may cause 4- to 8-fold increase in $[Ca^{2+}]i$. Bradykinin is known to release EDRF(NO) from BAEC in a $[Ca^{2+}]i$-dependent manner, although calcium channel antagonists do not inhibit this release [39] Much less active stimulators of $[Ca^{2+}]i$ in BAEC are 'tissue type' inhibitors of angiotensin converting enzyme (ACE-I), such as quinapril or perindopril (10–30 μM), which, in our opinion, seem to act as 'bradykinin potentiating factors' (BPF) (40), i.e. kininase II inhibitors, rather than as angiotensin I/II convertase inhibitors [41]. We believe that the bradykinin-mediated activation of endothelial protective secretory function by 'tissue ACE-I' is responsible for a substatial reduction in the death rate from myocardial infarction and ischaemic stroke in high risk patients treated with ramipril (another 'tissue ACE-I') [42].

Indeed, as early as fifteen years ago we reported that bradykinin at a concentration of 20 nM or

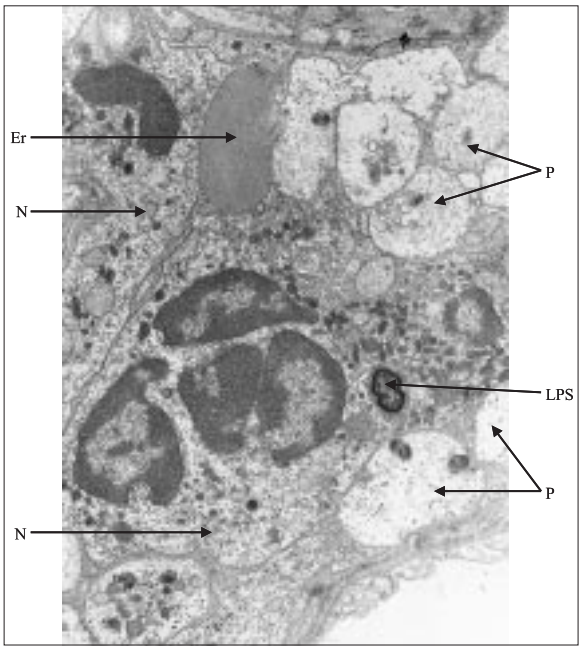

Figure 14. Electron micrograph of pulmonary microvessel of isolated blood-perfused rat lung challenged with L-NAME+LPS. Neutrophils (N), erythrocytes (Er) and aggregated platelets (P) are seen inside the lumen of pulmonary microvessel. Inside a neutrophil is visible internalised LPS with electron dense lipid A, and less osmophilic polisaccharide chains of the LPS structure. Magnification 10000x.

A23187 at a concentration of 2 mM would both result in a coupled release of EDRF(NO) and PGI_2 from cultured porcine aortic endothelial cells [43]. The order of potencies of bradykinin and A23187 to release PGI_2 and EDRF(NO) in a coupled manner from BAEC [44] was similar to that for increasing $[Ca^{2+}]i$, as reported here. No doubt, bradykinin is the most potent physiological stimulator for increasing $[Ca^{2+}]i$ in BAEC. ADP is next to it. In vivo, the coupled release of PGI_2 and EDRF(NO) by ADP is likely to occur when endogenous ADP is being released from aggregating platelets, and then ADP-triggered endothelial defence against thrombosis turns up. In vivo, the coupled release of PGI_2 and EDRF(NO) by bradykinin may be expected when this peptide is locally generated by vascular endothelium, provided that bradykinin would suc-

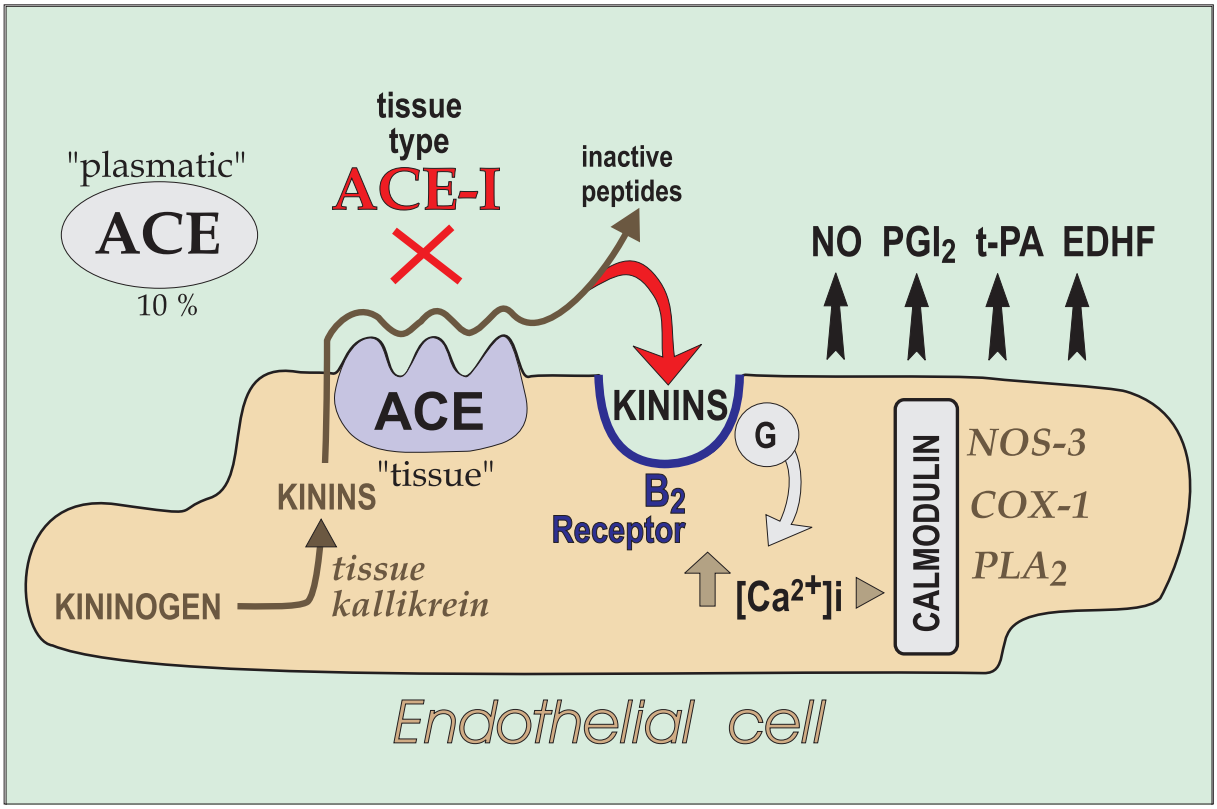

Figure 15. A scheme of inhibitory action of 'tissue type' ACE-I (e. g. quinapril or perindopril) on endothelial ACE. Inhibition of this enzyme allows endothelial kinins to escape the distruction and to activate kinin B_2 receptor. In consequence, via protein G $[Ca^{+2}]i$ is mobilized and calmodulin complex is formed. This last activates a number of enzymes such as phospholipase A_2 (PLA_2), cyclooxygenase-1 (COX-1), and NO synthase (NOS-3), which are responsible for synthesis and release of endothelial defensive mediators (eg. PGI_2, NO, EDHF).

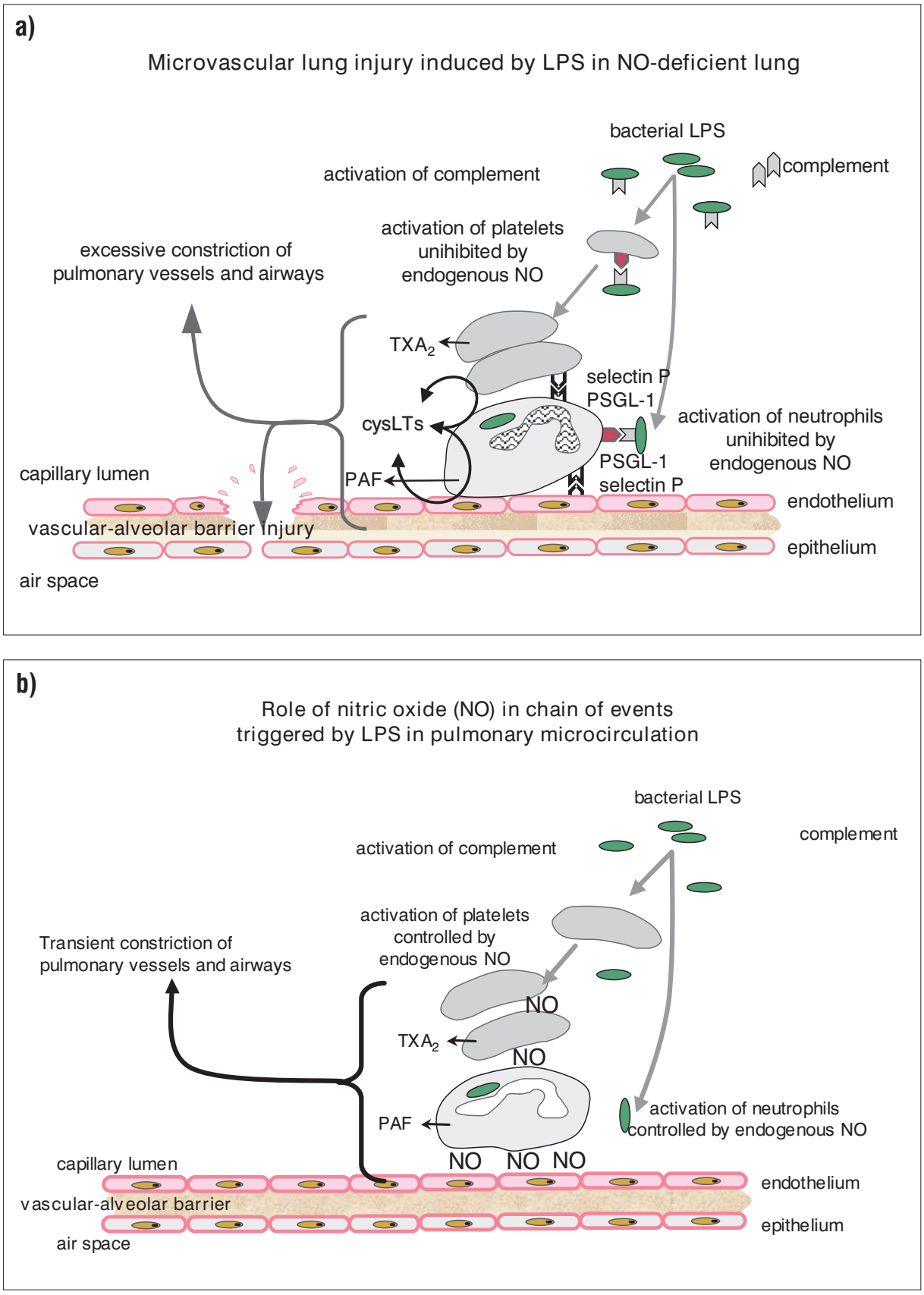

Figure 16. Role of pulmonary NO in the early response of the lung to LPS **(a)** and consequences of pharmacological removal of pulmonary NO, which are showing up as microvascular lung injury by endogenous toxins **(b)**.

ceed escaping destruction by the kininase II function of endothelial caveolar ACE (Fig. 15). Indeed, we show presently that in vivo in rats quinapril and perindopril activate the secretory endothelial function through a bradykinin mechanism. In vivo thrombolytic response has been used, and allowed to demonstrate, that quinapril or perindopril induced, bradykinin-mediated thrombolysis is mediated predominantly by PGI_2. EDRF(NO) plays only a permissive role, while t-PA may participate in a late phase of thrombolysis.

Bradykinin is a potent agonist of the receptor B_2 mediated instantaneous endothelial secretory response. However, in this paper we report that bradykinin may have yet another endothelial mechanism of action. In cultured HUVEC incubated with bradykinin at a low concentration of 10 nM for four hours we observed an induction of transcripts for three enzymes: haemooxygenase 1 (HO-1), prostaglandin E synthase (PGE-S), cyclooxygenase 2 (COX-2) and NO synthase 2 (NOS-2). The potency of induction for corresponding mRNAs decreased in order HO-1>PGE-S>COX-2>>NOS-2. Actually, the expression of NOS-2 mRNA was only slightly touched by bradykinin. We have not been as yet studying effects of bradykinin on corresponding enzymic proteins or on their activities. Would they go along with their mRNAs, then bradykinin acts as a 'microcytokine' enzymatic inducer. Induction of HO-1 is associated not only with an increased production of CO but also with an increased consumption of haeme required as a prosthetic moiety both in cyclooxygenase and in NO synthase, as well as in a number of other haemoprotein enzymes [45]. The above pattern of induction of endothelial mRNAs may suggest that the delayed microcytokine-like effect of bradykinin on activities of COX-2 and NOS-2 will be tempered by a concomitant rise in HO-1 activity, and therefore the net 'inductive' effect of bradykinin on prostanoid generation might be stronger than its effect on NO generation. This assumption requires an experimental confirmation, especially that an increased generation of CO by inducible HO-1 may assist vasodepressor function of PGI_2 or NO [46]. Not long ago, the involvement of cytosolic phosholipase A_2 in bradykinin-mediated release of PGI_2 from HUVEC was reported [47]. As it has been said PGI_2 is a major endothelial mediator involved in housekeeping function of the conductance blood vessels of peripheral circulation, while in microcirculation PGE_2 seems to play a similar role. This is why induction by bradykinin of PGE-S in endothelial cells of microcirculation would be of great significance for controlling inflammatory responses, especially those mediated by activated neutrophils whose adenylate cyclase is sensitive to stimulation by PGE_2 rather than by PGI_2.

In pulmonary circulation NO but not PGI_2 protects endotoxaemic lung from damage both in vivo [48] and in isolated blood perfused rat lung, as shown presently. In this preparation an immediate bronchoconstrictor and vasoconstrictor responses to bacterial lipopolysaccharide (LPS) are mediated by platelet activating factor (PAF) and thromboxane A_2 (TXA_2) being released from activated neutrophils and platelets, respectively. Usually these responses are transient, since LPS-induced, complement-dependent activation of blood morphological elements are tempered by endogenous NO.

The above reasoning is supported by our finding that pharmacological blockade of NOS enforced LPS to precipitate unrestricted adhesion of platelets and neutrophils to each other and to endothelial cells. These interactions mediated by the complement system and selectin P draw a scenario favourable for transcellular synthesis of cysteinyl leukotrienes (cyst-LTs) [5,6]. In absence of endogenous NO the immediate response to LPS is no more transient. Now TXA_2, PAF and cyst-LTs have their chance to act in an unrestricted way leading to the arrest of all lung functions, vascular and bronchial constriction, acute microvascular lung injury and haemorrhagic lung oedema. It is tempting to speculate that the role of pulmonary NO is to ensure the quick and safe elimination from blood of bacterial LPS internalised in neutrophils. Indeed, platelets-stimulated [49] and otherwise stimulated neutrophils are main phagocytising cells in blood [50].

It goes as no surprise that the safeguard role of NO in pulmonary endothelium in the early endotoxaemia cannot be replaced by PGI_2. It is NO but not PGI_2 that inhibits intercellular adhesion [51, 52]. Although PGI_2 is a potent inhibitor of platelet aggregation [53] it hardly influences platelet adhesion [54] and it has virtually no effect on leukocyte function [4]. Thus, pulmonary NO appears to be the major if not the only endogenous mediator that alleviates the necessary but dangerous events leading to removal of bacterial LPS from pulmonary circulation (Fig. 16).

Summing up, endothelial bradykinin seems to regulate through a calcium-dependent mechanism the activities of dioxygenases, which produce

prostacyclin and nitric oxide in the vascular endothelium. This immediate action of bradykinin is supplemented by its delayed induction of transcripts for several endothelial enzymes. Irrespectively to mechanisms of their generation prostacyclin and prostaglandin E_2 are likely to play a major protective role in peripheral circulation, whereas nitric oxide is a major defender in pulmonary circulation.

CONCLUSION

1. In peripheral circulation vasoprotective function of the endothelium is mediated predominantly by prostacyclin (PGI_2) whereas in pulmonary circulation it is mediated mainly by nitric oxide (NO).

2. In cultured endothelial cells, of all receptor agonists studied, bradykinin (Bk) proved to be the most potent stimulator to increase their free cytoplasmic calcium $[Ca^{2+}]i$.

3. Bradykinin (Bk) activates endothelial enzymes by fast rising of free cytoplasmic calcium $[Ca^{2+}]i$ or it induces transcripts of endothelial enzymes in a delayed 'microcytokine' – like manner.

Acknowledgments

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