Lysophosphatidylcholine-induced modulation of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels contributes to ROS-dependent proliferation of cultured human endothelial cells

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Abstract

Proliferation of endothelial cells plays a crucial role in the process of atherosclerotic plaque destabilization. The major component of oxidized low-density lipoprotein lysophosphatidylcholine (LPC) has been shown to promote endothelial proliferation by increasing the production of reactive oxygen species (ROS). Since K\textsuperscript{+} channels are known to control the cell cycle, we investigated the role of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (BK\textsubscript{Ca}) in the regulation of LPC-induced endothelial proliferation and ROS generation. A significant increase of cell growth induced by LPC (20 µmol/l; cell counts (CCs): +87%, thymidin incorporation: +89%; \( n = 12, P < 0.01 \)) was observed, which was inhibited by the BK\textsubscript{Ca} inhibitor iberiotoxin (IBX; 100 nmol/l), by the NAD(P)H-oxidase inhibitor diphenyleneiodonium (5 µmol/l) and by transfection with antisense (AS) oligonucleotides against NAD(P)H oxidase, whereas \( \text{N}^\text{G}-\text{monomethyl-L-arginine (L-NMMA)} \) further increased LPC-induced cell growth. Using the patch-clamp technique a significant increase of BK\textsubscript{Ca} open-state probability (control: 0.004 ± 0.002; LPC: 0.104 ± 0.035; \( n = 21, P < 0.05 \)) by LPC was observed. Using dichlorofluorescein fluorescence microscopy a significant increase of ROS induced by LPC was reported, that was blocked by IBX and Ca\textsuperscript{2+} antagonists. Intracellular Ca\textsuperscript{2+} measurements revealed a capacitative Ca\textsuperscript{2+} influx caused by LPC. Bioactivity of nitric oxide (NO) was measured using a \([\text{3H}]\)cGMP radioimmunoassay. LPC significantly decreased acetylcholine-induced NO synthesis. LPC significantly increased cGMP levels in endothelial cells transfected with AS, which was blocked by IBX. In conclusion, our results demonstrate that LPC activates BK\textsubscript{Ca} thereby increasing ROS production which induces endothelial proliferation. In addition LPC-induced BK\textsubscript{Ca}-activation contributes to increased cGMP levels, if ROS production is prevented by AS.

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1. Introduction

Atherosclerosis can be seen as a chronic inflammatory disease, in which increased cellular turnover on the one hand and apoptotic/necrotic cell death on the other hand takes place within the vascular wall [1–3]. Besides hypertension, diabetes mellitus, and nicotine abuse, low-density lipoproteins (LDLs) play a major role in the pathogenesis of atherosclerosis. The oxidative modification of LDL, during which lysophosphatidylcholine (LPC) accumulates within the LDL particle, seems to be a key event in this process [4]. It has been shown in an animal-atherosclerosis model, that endothelium-dependent vasorelaxation is impaired, if endothelium-derived nitric oxide (NO) or its biological activity is decreased [5,6]. The vascular production of reactive oxygen species (ROS) is dramatically increased in atherosclerotic arteries, which is of great importance since superoxide is known to inactivate NO in a chemical reaction during which peroxynitrite is formed [7]. Therefore, oxidative inactivation of NO is regarded as an important cause of its decreased biological activity [8].

Another well recognized feature in atherosclerotic lesions is intimal angiogenesis. Angiogenesis is implicated in the development of atherosclerosis and associated clinical syndromes in the coronary circulation [9,10]. It has been suggested that the vascular wall of these new blood vessels is weak and therefore subject to rupture. Intramural hemorrhage from these microvessels may precipitate the clinical complications of atherosclerosis with sudden expansion and...
rupture of the plaque in association with arterial dissection, ulceration, or thrombosis [11].

It has previously been shown that oxidized LDL (oxLDL) and LPC induce endothelial proliferation by increasing the superoxide production of the NAD(P)H oxidase [12]. Recently, we have reported that modulation of Ca\(^{2+}\)-activated K\(^+\) channels (BK\(_{Ca}\)) is involved in oxLDL-induced endothelial proliferation and NO production [13]. BK\(_{Ca}\) have been shown to regulate the membrane potential and thereby influence intracellular Ca\(^{2+}\) levels. Endothelial cell functions are strongly influenced by changes of the intracellular Ca\(^{2+}\) homeostasis [14]. In endothelial cells, which do not express voltage-gated Ca\(^{2+}\) channels [15] changes of intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\)) are mainly due to Ca\(^{2+}\) release from internal stores or through transmembrane Ca\(^{2+}\) influx, which depends on membrane hyperpolarization [14].

In this study, we investigated whether LPC modulates endothelial BK\(_{Ca}\) thereby influencing the intracellular Ca\(^{2+}\) homeostasis, and whether this ion channel is involved in the LPC-induced changes of ROS generation, cell proliferation, and NO bioactivity.

2. Materials and methods

2.1. Isolation and culture of HUVEC

Endothelial cells were isolated from human umbilical cord veins (HUVEC) by collagenase digestion procedure as described previously [13]. The endothelial cell basal medium (EBM) was enriched with 10% fetal calf serum (FCS) and the following substances: 0.4% ECGS/H, epidermal growth factor 0.1 ng/ml, hydrocortisone 1 µg/ml, and gentamicin 50 µg/ml. The culture medium was changed every 48 h. All experiments were carried out using endothelial cells from subcultures four to eight.

2.2. Electrophysiological recordings

Single-channel membrane currents were measured by means of the patch-clamp technique in the cell-attached configuration [16]. Patch pipettes of borosilicate glass (Hilgenberg, Malsfeld, Germany) with a final resistance of 5–8 MΩ when filled with pipette solution were employed. For the recording, an L/M-PC patch-clamp amplifier (List, Darmstadt, Germany) was used. The data obtained was low-pass filtered at 1 kHz (6-pole Bessel filter), digitalized (sample rate; 10 kHz) using a Digidata 1200A (Axon Instruments, Foster City, CA, USA) 4/D converter, and captured on the hard disk of an IBM-compatible personal computer. Analysis of the unitary currents was performed with pClamp 6.0.3. software (Axon Instruments, Foster City, CA, USA). Open-state probability (NPo) was calculated from the ratio between channel open time and total recording time. The mean amplitude of the unitary currents was obtained for individual patches by fitting simple Gaussian distributions to the all-points histogram.

Interruption recordings of BK\(_{Ca}\) were made up to 30 min after the application of LPC (20 µmol/l). Following the application of 100 nmol/l iberiotoxin (IBX) to the pipette solution, records were obtained after 1 min of exposure to IBX.

2.3. ROS measurements

Cellular ROS generation was analyzed using the fluorescence dye dichlorofluorescein (DCF). HUVEC were loaded with culture medium containing 5 µmol/l DCF. After an incubation period of 10–15 min, medium was exchanged with HEPES buffer composed of (in mmol/l) HEPES 25, NaCl 125, CaCl\(_2\) 1.0, KCl 2.6, MgCl\(_2\) 1.2, KH\(_2\)PO\(_4\) 1.2 at pH 7.4. Variations of the following supplements were added to the HEPES buffer: LPC (20 µmol/l), IBX (100 nmol/l); diphenyleneiodonium (DPI; 5 µmol/l); N\(^\ominus\)-monomethyl-L-arginine (L-NAME; 300 µmol/l); 2-aminoethoxydiphenyl borate (2-ABP; 100 µmol/l); dibutyrylcAMP (0.1 mol/l); 1,2-bis(o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetra acetoxyethylmethyl-ester (BAPTA; 10 µmol/l); angiotensin II (AT; 100 nmol/l). In some experiments the CaCl\(_2\) concentration of the HEPES buffer was reduced to 0.3 mmol/l. Coverslips were mounted into an incubation chamber adapted to the fluorescence microscope (IX 70, Olympus; Hamburg, Germany). Fluorescence intensities for the excitation wavelength of 475 nm were acquired after an incubation period of 15 min. Emitted light was detected at 512 nm. Changes of ROS formation in endothelial cells were analyzed with the TILL Photonics system ( Martinsried, Germany). Data were expressed as mean values of DCF fluorescence.

2.4. Measurements of intracellular \(\text{Ca}^{2+}\)

\([\text{Ca}^{2+}]_i\) were determined using the fluorescent Ca\(^{2+}\) indicator fura-2-acetoxymethyl ester (AM) (Fura-2). HUVEC were loaded with 2.5 µmol/l Fura-2. After an incubation period of 45 min, extracellular Fura-2 was removed and the medium was exchanged with the above-mentioned HEPES buffer. The excitation wavelength alternated between 340 and 380 nm. Emitted light was detected at 510 nm. Changes of \([\text{Ca}^{2+}]_i\) in endothelial cells were analyzed as described above. Data were expressed as ratio values of Fura-2 fluorescence.

2.5. \[^{3}H\]-cGMP radioimmunoassay

A \[^{3}H\]-cGMP radioimmunoassay (cGMP RIA) was used to analyze NO bioactivity. HUVEC were seeded at a density of 5000 cells/cm\(^2\). After 48 h, cells were stimulated for 30 min by replacing the culture medium with bath solution supplemented with the following substances: 1 mmol/l arginin (Arg; Sigma, Deisenhofen, Germany), 1 µmol/l acetylcholine (ACH; Sigma, Deisenhofen, Germany), 20 µmol/l LPC, and 100 nmol/l IBX. In some experiments LPC or IBX was applied without adding ACH. Stimulation was stopped by adding 98% ethanol. The cell lysate was centrifuged, and...
measurements of cGMP levels of the supernatant were performed using a cGMP RIA.

2.6. Cell proliferation

Proliferation of HUVEC was measured by means of CCs with the help of a Neubauer chamber and by using a \(^{[3]}\text{H}\)-thymidine incorporation (TI) assay. HUVEC of confluent cultures were seeded at a density of 10 000 cells/well. Cells were kept in serum-free medium for 24 h. Afterwards cells were stimulated for 4 h in the above described HEPES buffer, with the addition of the following substances: LPC, 20 µmol/l; IBX, 100 µmol/l; DPI, 5 µmol/l; allopurinol, 500 µmol/l; aspirin, 100 µmol/l; and L-NMMA 300 µmol/l. The Medium was exchanged to basal medium containing 2% FCS. CCs were carried out after 48 h. For \(^{[3]}\text{H}\)-TI assay cells were serum starved for 24 h. Thereafter HUVEC were stimulated with the same supplements used in the CC procedure before adding 0.2 µl/ml \(^{[3]}\text{H}\)-thymidine (0.25 MBq) to the medium. After 20 h \(^{[3]}\text{H}\)-thymidine was added to each well. Four hours later cells were washed with ice-cold PBS three times, fixed with 100% cold methanol for 15 min at 4 °C, washed with water three times, and lysed with 200 µl of 0.1 N NaOH for 30 min at room temperature. The lysate was then transferred to scinti-vials, and the content of \(^{[3]}\text{H}\)-thymidine was measured using a β-counter (Canberra-Packard, Dreieich, Germany). We defined the activity of control cells as 100%, and the activity of treated cells was set in relation to the activity of the control cells.

2.7. Oligonucleotide transfection

In some experiments cells were transfected with antisense (AS) or nonsense (NS) oligonucleotides directed against the p22-phox subunit of the NAD(P)H oxidase, as described recently [13].

2.8. Chemicals and solutions

LPC, DPI, IBX, Allopurinol, aspirin, TCA, NaOH, SDS, ACh, l-arginine, AT (all Sigma, Deisenhofen, Germany); L-NMMA, 2-ABP, BAPTA, AS and NS (Calbiochem, Bad Sooeden, Germany); DCF, Fura-2 (Molecular Probes, Leiden, Netherlands); \(^{[3]}\text{H}\)-thymidine and \(^{[3]}\text{H}\)-cGMP RIA kit (Amersham, Freiburg, Germany); EBM and cell culture supplements (Promo Cell, Heidelberg, Germany); FCS (PAA, Linz, Austria); ethanol and methanol (Riedel-de Haen, Seelze, Germany).

2.9. Statistical analysis

Statistical significance for repeated measurements of NPo after LPC application was determined using a Friedman test (\(P < 0.05\); SPSS for Windows; release 10.0) followed by multiple comparisons (Nemenyi test). Data obtained from proliferation studies, ROS- and \(\text{Ca}^{2+}\) measurements, and cGMP RIA were analyzed by means of ANOVA (SPSS for Windows; release 10.0) followed by post hoc Tukey test. Results are expressed as mean ± S.E.

3. Results

3.1. LPC activates BK\(_{\text{Ca}}\) in human endothelial cells

BK\(_{\text{Ca}}\) has been identified in HUVEC extensively in our laboratory previously [13,17]. It has been shown to be voltage dependent with a single-channel slope conductance of 170.3 ± 2.1 pS (\(n = 7\)) being well within the range of BK\(_{\text{Ca}}\)’s characteristic conductance of 150–250 pS. Furthermore, the highly specific BK\(_{\text{Ca}}\) blocker IBX (100 nmol/l) was seen to cause a complete block of unitary outward currents observed in cultured human endothelial cells, establishing that these unitary outward currents are indeed carried through BK\(_{\text{Ca}}\). These findings are consistent with the description of BK\(_{\text{Ca}}\) in other endothelial cells [18].

In order to test whether external LPC can modulate BK\(_{\text{Ca}}\), we performed single-channel recordings in cell-attached patches. After a control measurement in a LPC-free bath solution repetitive recordings of BK\(_{\text{Ca}}\) were performed during continuous perfusion with 20 µmol/l LPC. Since BK\(_{\text{Ca}}\) activity was very low at low depolarizing test potentials, we only studied the channel behavior at pipette potentials of +80 and +100 mV. A representative recording of BK\(_{\text{Ca}}\) activity after LPC perfusion is shown in Fig. 1A. The perfusion with LPC resulted in an increase of the single-channel open probability (NPo), which was significant after 15 min and lasted the whole recording time of 30 min. In detail, using a pipette potential of +80 mV the open-state probability was significantly increased from 0.001 ± 0.001 at control conditions to 0.052 ± 0.021 (\(n = 21\); \(P < 0.05\)) after 15 min of LPC treatment. When applying test potentials of +100 mV, NPo was significantly increased from 0.004 ± 0.002 (control) to 0.104 ± 0.035 after 15 min of LPC perfusion. These data are summarized in Fig. 1B. To verify that the highly selective BK\(_{\text{Ca}}\) blocker IBX is sufficient to block LPC-induced BK\(_{\text{Ca}}\) activation, we added 100 nmol/l IBX to the pipette solution and carried out cell-attached measurements of BK\(_{\text{Ca}}\). Within 2 min after giga-seal formation, we observed a total block of initial BK\(_{\text{Ca}}\) openings demonstrating the existence of BK\(_{\text{Ca}}\) in the patch. The following application of LPC did not alter the blockade of BK\(_{\text{Ca}}\) by IBX as demonstrated in Fig. 1A.

3.2. LPC-induced ROS generation can be modulated by BK\(_{\text{Ca}}\)

In order to analyze LPC-induced ROS generation DCF fluorescence measurements were conducted. Compared to the control group application of LPC (20 µmol/l) caused a significant increase of DCF fluorescence intensity (+190%), which was completely blocked by IBX (100 nmol/l) treat-
ment \( n = 30; P < 0.05 \), indicating that BKCa-activation plays a role in LPC-induced ROS formation. Since activation of BKCa contributes to changes of the intracellular Ca\(^{2+}\) homeostasis we examined whether LPC-induced radical production depends on Ca\(^{2+}\). Incubation of HUVEC with the intracellular Ca\(^{2+}\)-chelator BAPTA (10 µmol/l) completely abolished the effect of LPC. The increase of intracellular Ca\(^{2+}\) necessary for LPC-dependent ROS formation was due to transmembrane Ca\(^{2+}\) influx, because the effect of LPC was inhibited by 2-ABP (100 µmol/l), which blocks capacitative Ca\(^{2+}\) entry, or by reducing the CaCl\(_2\) concentration of the bath solution to 0.3 mmol/l \( n = 30; P < 0.05 \). These findings are demonstrated in Fig. 2A.

3.3. Influence of LPC on the intracellular Ca\(^{2+}\) homeostasis

To investigate whether LPC-induced ROS is generated by NAD(P)H-oxidase cells were transfected with AS or NS oligonucleotides directed against the p22-phox subunit of the NAD(P)H oxidase. AS transfection as well as administration of DPI (5 µmol/l) prevented LPC-mediated radical production \( n = 30; P < 0.05 \). To exclude that LPC-dependent ROS formation is caused by the endothelial NO synthase (eNOS) the NO inhibitor L-NMMA (300 µmol/l) was applied. LPC-induced DCF fluorescence intensity was not significantly changed by L-NMMA. The results are summarized in Fig. 2B.

![Fig. 1. Effect of LPC on Ca\(^{2+}\)-activated K\(^+\) channels in HUVEC. (A) Original recordings of BKCa in cell-attached patches before and after 15 min LPC (20 µmol/l) superfusion, with or without the application of IBX (100 nmol/l) at a pipette potential of +100 mV. The closed (c) and open (o) states of the channel are indicated. (B) Plot of the open-state probability (N\(P_0\)) as a function of time prior to (0 min; control) and during continuous bath application of 20 µmol/l LPC as indicated by the vertical bars (pipette potentials: +80 mV (black column) and +100 mV; \(n = 21; * P < 0.05 \) vs. control).](image1)

![Fig. 2. LPC-induced ROS generation depends on BKCa. ROS production was detected by DCF fluorescence. HUVEC were incubated for 15 min with combinations of the following substances: (A) LPC (20 µmol/l), IBX (100 nmol/l), BAPTA (10 µmol/l), and 2-ABP (100 µmol/l); (B) LPC (20 µmol/l), DPI (5 µmol/l), and L-NMMA (300 mmol/l). Additionally cells were transfected with AS or NS oligonucleotides against NAD(P)H oxidase (B) and in some experiments (A) extracellular Ca\(^{2+}\) was reduced (–Ca\(^{2+}\)). Values represent DCF fluorescence intensity ± S.E. \( n = 30; * P < 0.05 \) vs. control; \( P < 0.05 \) vs. LPC.](image2)

An additional set of experiments was performed to proof that IBX does not unspecifically inhibit ROS formation in HUVEC. ROS production stimulated with AT (100 nmol/l), which was not blocked by IBX (AT: 3959.8 ± 274.71; AT + IBX: 3872.74 ± 203.5; \(n = 30; P = n.s.\)).
3.4. LPC increases NO production via BK Ca if NAD(P)H oxidase is inactive

To study the effects of LPC on ACh-induced NO bioactivity HUVEC were stimulated with 1 mmol/l L-arginine, 1 µmol/l ACh, and 20 µmol/l LPC. ACh-induced cGMP levels were significantly decreased by LPC (–88%; n = 10; P < 0.01; Fig. 3A). Interestingly basal cGMP levels were significantly reduced by LPC: control (without ACh) 66.02 ± 16.41, LPC (without ACh) 35.71 ± 10.26 (n = 10; P < 0.05). To assess the role of endothelial BKCa within this process 100 nmol/l IBX was applied. The addition of IBX revealed no significant changes compared to the stimulation with LPC alone (n = 10; n.s.). Additionally IBX had no effect on basal cGMP synthesis (control (without ACh): 66.02 ± 16.41; IBX (without ACh): 65.26 ± 9.71; n = 10; n.s.). We transfected HUVEC with AS and NS oligonucleotides against the NAD(P)H-oxidase subunit p22-phox (AS and NS, respectively) to study the effect of LPC on NO production without the interaction of NAD(P)H oxidase. After HUVEC were successfully transfected, [3H]-cGMP RIA were performed under the same conditions as described above. The cGMP levels in AS-transfected cells were significantly increased when 20 µmol/l LPC was supplemented (Fig. 3B). Interestingly, this effect was completely abolished by the addition of 100 nmol/l IBX, demonstrating that LPC-induced activation of BKCa increases endothelial NO synthesis if NAD(P)H-dependent ROS production is prevented. In NS-transfected cells stimulation with LPC did not result in an increase of cGMP levels. The data are presented in Fig. 3C.

3.5. LPC-induced proliferation of HUVEC depends on ROS, eNOS, and BK Ca

Incubation of cells with LPC increased HUVEC proliferation in a concentration dependent manner (5–50 µmol/l). A maximum effect was achieved at a concentration of 20 µmol/l (data not shown). CCs were increased from 20 458 ± 1176 cells/well under control condition to 38 333 ± 2125 cells/well after LPC application. [3H]-TI was increased by 189% after addition of LPC to the culture medium. Interestingly, the co-incubation of cells with LPC and the highly selective BKCa inhibitor IBX (100 nmol/l) resulted in a significant decrease of LPC-induced endothelial proliferation by 68% (CC) and 59% (TI), respectively. Incubation with IBX alone did not result in a decrease of CC or TI indicating that there is no direct cytotoxic effect of IBX on HUVEC.

It has already been shown that LPC induces the proliferation of HUVEC via NAD(P)H-dependent superoxide formation [12]. To determine if other oxidative systems are involved in LPC-induced O2•−-dependent endothelial proliferation, HUVEC were additionally incubated with the following substances: 5 µmol/l DPI, 100 µmol/l aspirin, 500 µmol/l allopurinol, and 300 µmol/l L-NMMA. Aspirin and allopurinol failed to prevent LPC-induced proliferation, whereas DPI significantly reduced endothelial cell growth. To further confirm this finding proliferation experiments were repeated using cells which were transfected with AS and NS. The proliferative response of AS-treated cells was significantly lower than that of AS- or untransfected HUVEC (n = 12; P < 0.05). Interestingly, L-NMMA caused a further significant increase of endothelial proliferation induced by LPC compared to stimulation with LPC alone (n = 12; P < 0.05). When HUVEC were stimulated with L-NMMA, DPI, aspirin or allopurinol without the substitution of LPC no significant changes compared to the control group were observed. In addition no changes of cell growth were seen if...
cells were transfected with AS or NS compared to non-transfected HUVEC. The data of the proliferation studies are summarized in Table 1.

### 4. Discussion

The aim of our study was to investigate the underlying mechanisms that are involved in LPC-induced ROS formation and its contribution to endothelial proliferation. During the oxidative modification of LDL, LPC accumulates in the oxLDL particle [19].

Data, recently published by our group, demonstrates that oxLDL modulates BK$_{Ca}$ in human endothelial cells [13]. Therefore, we examined the effect of LPC on BK$_{Ca}$ single-channel activity in cell-attached patches, using the patch-clamp technique. Our electrophysiological data show that the major component of oxLDL—LPC—activates BK$_{Ca}$ in endothelial cells. A modulation of K$^+$ channels by LPC has already been described in cardiomyocytes. In these cells LPC inhibits the slow component of cardiac delayed rectifier K$^+$ currents as well as the inward rectifier K$^+$ current [20–22]. The present study demonstrates for the first time an LPC-induced modulation of BK$_{Ca}$ in human endothelial cells.

In human monocye-derived macrophages ATP/UTP-induced NAD(P)H-oxidase-dependent superoxide generation is blocked by the BK$_{Ca}$ inhibitor charybdotoxin [23]. Enhanced superoxide production was observed in endothelial cells after exposure to oxLDL or LPC [12,24]. The NAD(P)H oxidase was identified as most possible source of oxLDL/LPC-dependent superoxide release. Our DCF fluorescence data are in line with the findings of Heinloth et al. [12]. We have clearly demonstrated that LPC-induced ROS generation is due to NAD(P)Hoxidase activation. Another possible signaling pathway leading to increased radical formation by LPC would have been peroxynitrite-induced uncoupling of the eNOS. Since ROS generation was not blocked by l-NMMA we can exclude that LPC-induced radicals derived from the eNOS. The highly selective BK$_{Ca}$ inhibitor IBX significantly inhibited superoxide production, therefore, our experiments suggest that endothelial NAD(P)H oxidase is regulated by BK$_{Ca}$ in a similar manner as it was shown for macrophages [23]. Unspecific side effects of IBX seem to be very unlikely, because IBX was not able to block angiotensin-induced radical production. In addition we were able to show the Ca$^{2+}$ dependency of LPC-induced radical production. Endothelial NAD(P)H-oxidase-dependent ROS formation was inhibited by decreasing the transmembrane Ca$^{2+}$ influx. This result is in line with the findings of Holland et al., who have reported that the production of ROS can be inhibited by intracellular Ca$^{2+}$ antagonists [25].

To further confirm our findings we performed measurements of [Ca$^{2+}$], LPC caused a significant increase of [Ca$^{2+}$], that was inhibited by reducing the amount of extracellular Ca$^{2+}$, or by the addition of 2-ABP, which has been reported to block capacitative Ca$^{2+}$ entry in endothelial cells [26]. These results are consistent with the data of Yokoyama et al., who have shown an LPC-induced increase of [Ca$^{2+}$], [27]. In addition we have observed that the LPC-dependent changes of the intracellular Ca$^{2+}$ homeostasis were due to BK$_{Ca}$ activation, which fits well with the finding that the Ca$^{2+}$ increase was due to extracellular Ca$^{2+}$ influx, because under physiological conditions activation of endothelial BK$_{Ca}$ hyperpolarizes the endothelial cell membrane and thereby increases the electrochemical gradient for a maintained Ca$^{2+}$ entry [14,28]. Since endothelial cells do not express voltage-gated Ca$^{2+}$ channels, this increase of intracellular Ca$^{2+}$ is of great importance for intracellular signaling cascades [29,30].

These results are contradictory to a previously published study by Sohn et al., who have demonstrated that depolarization of endothelial cells resulted in an activation of NAD(P)H oxidase [31]. It seems to be very unlikely, that the two opposing mechanisms—depolarization and hyperpolarization—both result in an activation of the NAD(P)H oxidase. Nevertheless, increased [Ca$^{2+}$], result in a rise in NAD(P)H-oxidase-dependent radical formation, which is in contrast to the results of Sohn et al., as membrane depolarization is not associated with raised [Ca$^{2+}$], [14,25]. Recently published data demonstrated, that ROS are potent inhibitors of BK$_{Ca}$ in endothelial cells, which might be a negative feedback mechanism to antagonize BK$_{Ca}$-dependent activation of the NAD(P)H oxidase [32].

Cominacini et al. have demonstrated that oxLDL inactivates NO through an increased cellular production of superoxide [24]. Very recent published data by our group have demonstrated that oxLDL reduces ACh-induced CGMP levels, if NAD(P)H oxidase is not inactivated by transfection of HUVEC with AS oligonucleotides directed against the p22-phox subunit of the NAD(P)H oxidase [13].
To test whether this is also the case for LPC, cGMP levels were analyzed in cells that were transfected, AS, or NS transfected. In HUVEC that were not transfected LPC caused a significant reduction of ACh-induced cGMP levels. When AS oligonucleotides were applied, ACh-induced cGMP levels were significantly increased by LPC. This increase of cGMP levels was due to the activation of BKCa since the addition of IBX completely abolished this effect of LPC. Our results confirm the findings of Cominacini et al. and demonstrate the important role of endothelial BKCa in the modulation of the NO/cGMP pathway. It is tempting to conclude that LPC-induced BKca-activation regulates NAD(P)H oxidase and eNOS at the same time. In general, it is very likely that BKCa activation is an amplifier for eNOS and NAD(P)H oxidase and that it depends on the stimulus which enzyme is predominantly activated.

The proliferation of endothelial cells is another important feature of atherosclerotic lesions because it is an important step in angiogenesis and thereby contributes to intimal thickening [9,33,34]. The weakness of the new blood vessels within the atherosclerotic plaque contributes to intraplaque hemorrhage, which has been related to a sudden increase in plaque volume and the development of plaque instability leading to plaque rupture [9,10,34]. Our proliferation studies demonstrate a concentration dependent increase of endothelial cell growth induced by LPC with a maximum effect at a concentration of 20 µmol/l. The data presented by Heinloth et al. revealed a maximum effect of LPC at a concentration of 10 µmol/l, which might be due to different stimulation protocols [12]. Previously published data indicate growing evidence that BKCa and other K+ channels are involved in the progression of the cell cycle thereby regulating endothelial proliferation [13,17,35]. To assess the role of BKCa in LPC-induced endothelial cell proliferation, we investigated whether specific blockade of BKCa with IBX affected the proliferation of HUVEC. Our results demonstrate an important role of BKCa activation in LPC-induced endothelial cell growth. IBX at the concentration of 100 nmol/l caused a significant inhibition of LPC-mediated HUVEC proliferation. A direct cytotoxic effect of IBX was excluded, since 100 nmol/l IBX did not significantly change endothelial cell growth examined by CCs and [3H]-TI. In addition it has been shown that superoxide formation regulates the endothelial cell cycle [36]. The findings of our cell growth experiments are in line with previously published data [12]. DPI significantly decreased LPC-induced cell growth, indicating a role of NAD(P)H oxidase. This finding was further confirmed, since the effect of LPC was abolished by AS transfection. There are many enzymatic sources for superoxide generation in almost all cell types [37]. In agreement with the results of Cominacini et al. allopyrinol as inhibitor of xanthine oxidase and aspirin as blocker of cyclo-oxygenase failed to inhibit superoxide-induced endothelial proliferation caused by LPC. Interestingly, the eNOS inhibitor L-NMMA further increased LPC-induced cell growth, supporting the hypothesis that in the presence of eNOS inhibitors, less radicals are quenched by the reduced intracellular NO [24]. Additionally it was shown that NO by itself inhibits endothelial cell growth [38], which would be another possible explanation for the effect of L-NMMA on LPC-induced HUVEC proliferation.

In conclusion, the results of our study demonstrate that activation of the BKCa followed by a capacitative Ca2+ influx leads to an increase of ROS generation, that is responsible for LPC-induced reduction of NO bioavailability and increased proliferation of cultured human endothelial cells.

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