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The Dietary Flavonoid Phloretin Modulates Ca^{2+}-activated K^+ Channels Resulting in an Increase of Endothelial Nitric Oxide Production

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Abstract: The aim of this study was to investigate the effects of phloretin on the Ca^{2+}-activated K^+ channel with large conductance (BK_{Ca}) and to assess a possible contribution of the BK_{Ca} to phloretin-induced changes of endothelial proliferation and Nitric Oxide (NO) production. In this study the patch-clamp technique was used to perform single channel recordings of the BK_{Ca} in cultured endothelial cells derived from Human Umbilical Cord Veins (HUVEC). BK_{Ca} open state probability (NPo) was significantly increased after 5 min of continuous perfusion with 80 μmol L^{-1} phloretin (14 fold increase at a pipette potential of 100 mV; n=31, $p<0.05$). NPo was not increased by phloretin if cells were simultaneously incubated with the highly selective BK_{Ca}-inhibitor iberiotoxin (100 nmol L^{-1}). The production of NO was examined using a cGMP-radio-immuno-assay. Phloretin caused a significant increase of acetylcholine-induced cGMP-levels from 303.1±31.1 fmol/well (acetylcholine; 1 μmol L^{-1}) to 568.4±76.1 fmol/well (acetylcholine + phloretin; 80 μmol L^{-1}), that was significantly reduced if iberiotoxin (100 nmol L^{-1}) was added (n=9; $p<0.01$). Endothelial proliferation was significantly reduced by phloretin (-70.6%, n=12, $p<0.01$, cell counts; -86.5%, n=20, $p<0.01$, [^{3}H]-thymidine-incorporation). This effect of phloretin was independent of BK_{Ca}-activity, because it was not changed by the addition of iberiotoxin. In conclusion the results of this study demonstrated, that phloretin activates the endothelial BK_{Ca}, which causes an increase of cGMP-levels. Furthermore, the proliferation of HUVEC is decreased by phloretin. The described findings might help to understand why flavanoids like phloretin have beneficial effects in the protection against atherosclerosis.

Key words: K^+ channels, nitric oxide, phloretin, endothelial cell, proliferation

INTRODUCTION

Flavonoids such as the dihydrochalcone phloretin are polyphenolic compounds that occur ubiquitously in food of plant origin. They may have beneficial effects because of their antioxidant properties and their inhibitory role in various stages of tumor development in animal studies. Especially the peel of apples is known to contain several quercetin and phloretin glycosides[1,2]. Phlortin is present as an phloretin-2'β-D-glucoside and 2'β-D-xylosyl-(1-6.)-β-D-glucoside in concentrations between 0.2 and 0.5 mg g^{-1} of apple peel[3]. As a phytoestrogen phloretin is known to relax rabbit coronary arteries by a calcium antagonising mechanism[4].

In addition, phloretin has been shown to open calcium-activated potassium channels of large conductance (BK_{Ca}) in cloned oocytes which expressed the BK_{Ca}, as well as in myelinated nerve fibers[5]. Under physiological conditions activation of the BK_{Ca} causes a membrane hyperpolarization in endothelial cells, thereby increasing the driving force for transmembrane Ca^{2+}-influx[6,7]. For this reason modulators of the BK_{Ca} have a strong influence on the regulation of intracellular signaling cascades in endothelial cells[8]. As an example activation of this ion channel has been demonstrated to induce endothelial proliferation caused by bFGF, oxidized low density lipoprotein, or lysophosphatidylcholine[9-11]. According to the response to injury hypothesis of Russel Ross endothelial dysfunction is an early stage of atherogenesis[12]. In addition intima angiogenesis contributes to the progression and destabilization of atherosclerotic plaques[13-15]. Besides endothelial cell migration, the proliferation of endothelial cells is a prerequisite for angiogenesis. Since these two factors–endothelial proliferation on the one hand and decreased vasomotor functions on the other hand–play such a crucial role in atherogenesis, the purpose of this study was to investigate the effect of the dietary

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flavonoid phloretin on endothelial proliferation and NO-homeostasis. Since phloretin is known to be an opener of the BK_{Ca} we were further interested whether this is the case in endothelial cells as well and to assess a possible contribution of BK_{Ca}-modulation in the effects of phloretin on the above mentioned cellular functions.

MATERIALS AND METHODS

Isolation and culture of HUVEC: Endothelial cells were isolated from human umbilical cord veins by a collagenase digestion procedure and grown in culture as described before[16]. Briefly, human umbilical cord veins were intraluminally incubated with collagenase D (Boehringer, Mannheim, Germany) and the endothelial cell-containing suspension was purified. Cells were seeded on plastic culture dishes and grown in endothelial basal medium (EBM; Promo Cell, Heidelberg, Germany) with addition of 10% fetal calf serum (FCS; PAA, Linz, Austria). Culture medium was changed every 48 h. Cells were passaged at approximately 90% of confluency and used for experiments from passage 4 to 10. Cultures were maintained at 37°C in a fully humidified atmosphere of 5% CO_2.

Electrophysiological recordings: Single-channel membrane currents were measured by means of the patch-clamp technique in cell-attached and cell-free patches[17]. Patch pipettes of borosilikat glass (Hilgenberg, Malsfeld, Germany) were drawn with a three-step puller (Zeitz, Augsburg, Germany) and firepolished to a final resistance of 6-9 MΩ, when filled with pipett solution. For the recording, a L/M-PC patch clamp amplifier (List, Darmstadt, Germany) was used. Data were low-pass filtered at 1 kHz (6-pole Bessel filter) and digitalized (sample rate: 10 kHz) using a Digidata 12000 A (Axon Instruments, Foster City, CA, USA) A/D converter and captured on 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, USA) Open-state probability (NPo) was calculated from the ratio between the channel open time and the total time. In case of phloretin (80 μmol L^{-1}; Sigma, Deisenhofen, Germany) application intermittent recordings of BK_{Ca} were made up to 30 min. In some experiments the highly selective BK_{Ca}-inhibitor iberiotoxin (IBX; 100 nmol L^{-1}; Sigma, Deisenhofen, Germany) was added to the pipette-solution to confirm that IBX completely blocks phloretin-induced BK_{Ca}-activity.

Solutions: For electrophysiological studies, HUVEC were maintained in an extracellular (bath) solution containing (in mmol L^{-1}): NaCl 140; KCl 5; MgCl 0.5; d-glucose 5.5; HEPES 10 ; $CaCl_2$ 1.5 ; pH 7.3 (with NaOH). The standard pipett solution contained (mmol L^{-1}) K^+-aspartat 110, KCl 30 ; HEPES 5 ; $MgCl_2$ 1 ; EGTA 0,1 : ph 7.3 (with KOH). All experiments were conducted at room temperature (20-22°C).

Cell proliferation: For cell counts HUVEC of confluent primary cultures from passages 4 to 8 were trypsinized (0,5%, w/v, trypsin and 5 mmol L^{-1} EDTA containing Ca^{2+}-free solution; Sigma, Deisenhofen, Germany) and seeded in 24 well plates at the density of 6000 cells per well. On the first day (day 0) cells were incubated in above mentioned basal medium. After 24 h (day 1) medium was exchanged to serum-free basal medium to synchronize the cells in the G1 phase of the cell cycle. On the next day HUVEC were stimulated with phloretin (80 μmol L^{-1}) and/or IBX (100 nmol L^{-1}) and/or L-NMMA (300 μmol L^{-1}; Calbiochem, Bad Soden, Germany) in basal medium containing 2% FCS. Cells counts were carried out on day 4. For counting, cells were detached by trypsinizing them and samples of the mixed cell suspension were transferred to a Neubaur chamber. For further analysis, the mean values of four counts were used. To further confirm the results of the cell counts endothelial proliferation was analysed on DNA-level by measurements of [^{3}H]-thymidine-incorporation. After serum depletion for 24 h, cells were incubated with the various supplements as described in the cell count procedure and loaded with [^{3}H]-thymidine-containing medium (Amersham, Freiburg, Germany) at a concentration of 0.1 μl mL^{-1}, equivalent to 0.1 μCi mL^{-1}. To stop incorporation HUVEC were exposed to 10% trichloracetic acid (TCA, Sigma, Deisenhofen, Germany) at 4°C for 12 h. Thereafter cell lysis was performed with NaOH containing 10% SDS (Sigma, Deisenhofen, Germany) for 90 min at 37°C in CO_2 free atmosphere. The resulting lysates were transferred to szintivials and the amount of incorporated [^{3}H]-thymidine was determined using a beta-counter (Canberra Packard, Dreieich, Germany). Because the final activity of tritium-labeled thymidine is strongly influenced by the time passed after the labeling, it is not useful to calculate thymidine incorporation on the basis of absolute counts. Therefore, we defined the activity of control cells as 100% and the activity of cells treated otherwise was set in relation to the activity of the control cells.

[^{3}H]-cGMP-radioimmunoassay: A [^{3}H]-cGMP-radioimmunoassay (cGMP-RIA; Amersham, Freiburg, Germany) was used to analyse NO production. HUVEC were stimulated with combinations of the following

substances for 30 min: 1 mmol L^{-1} L-arginine (Arg), 1 μmol L^{-1} acetylcholine (Ach), 80 μmol L^{-1} phloretin (Phl), 100 nmol L^{-1} iberiotoxin (IBX) (all substances were from Sigma, Deisenhofen, Germany). The stimulation was stopped by supplementing 98% ethanol and cells were maintained for 24 h at 4 °C. The cell lysate was centrifuged and measurement of cGMP levels of the supernatant were done using a beta-counter (Canberra-Packard, Dreieich, Germany).

Statistical analysis: Statistical analysis for repeated measurements of NPo after phloretin application was done by means of a Friedmann-test, followed by Wilcoxon-test. Data of cell proliferation and NO-measurements were determined using an ANOVA-test followed by a post hoc Tukey-Test (SPSS for windows, Release 10.0). Results are expressed as mean values±SEM.

RESULTS

Effects of phloretin on the BK_{Ca}: In order to test wether external phloretin can modulate BK_{Ca} in HUVEC recordings in cell-attached patches were carried out. For this purpose, phloretin was continuously perfused in a concentration of 80 μmol L^{-1} to the cell surface after recording of the open probability (NPo) under control conditions in a phloretin-free bath solution. Since BK_{Ca} activity was very low at low depolarizing test potentials, we carried out current measurements at a pipette potential of +80 and +100 mV. A representative recording of the BK_{Ca} under phloretin-perfusion is shown in Fig. 1A. External perfusion of phloretin resulted in a significant increase of compared to control conditions (n=31, p<0.05). The channel activity increased with the duration of phloretin-perfusion which was measured over a total time interval of 30 min. The result of the statistical analysis revealed a significant effect of phloretin on NPo regarding the recording time. Channel activity was significantly increased compared to control conditions after 5 min at 100 mV and after 10 min at +80 mV, respectively. In detail, using a test potential of +80 mV, the open state probability was increased from 0.0005±0.0023 (control) to 0.0166±0.0388 (n=31, p<0.05) after 10 min of phloretin treatment. When applying a test potential of + 100 mV, NPo was significantly increased from 0.0021±0.0009 (control) to 0.0285±0.0108 (n= 31; p<0.05) after 5 min of phloretin perfusion, (Fig. 1B).

To confirm that IBX completely blocks the phloretin-induced BK_{Ca}-activation IBX was added to the pipette solution during phloretin perfusion. Figure 2 shows a representative original recording of this experiment. First, BK_{Ca}-activity was recorded without the addition of phloretin. After 5 min the IBX-caused blockade was complete and now no BK_{Ca}-activity was recordable. The perfusion with phloretin was started, but even after 30 min there were still no openings of the

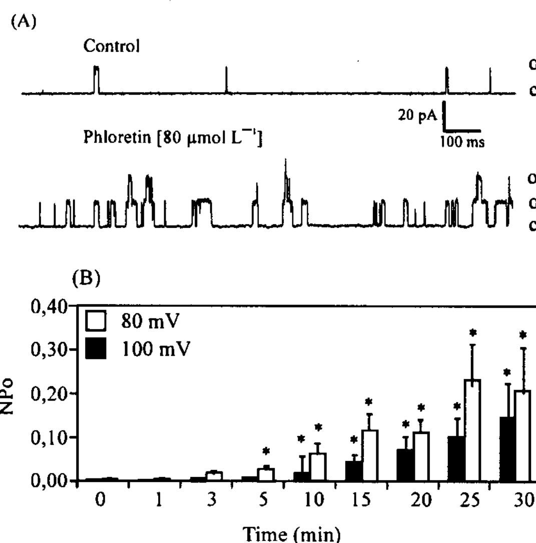

Fig. 1: Phloretin increases BK_{Ca} activity in HUVEC
(A) Original recordings of BK_{Ca} in cell-attached patches after 15 min of perfusion with phloretin (80 μmol L^{-1}) at a pipette potential of +100 mV. The closed (c) and open (o) states of the channel are indicated.
(B) Plot of the open-state probability (NPo) of BK_{Ca} as a function of time prior to (0 min/control) and during continuous application of 80 μmol L^{-1} phloretin at pipette potentials of + 80 and +100 mV as indicated by vertical bars (n=31; *p<0.05 vs. control).

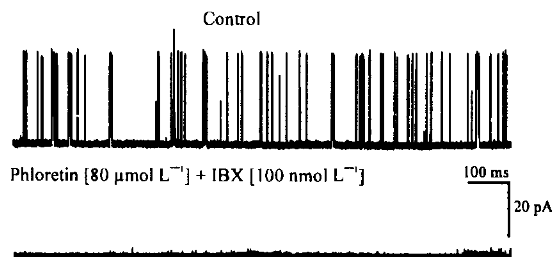

Fig. 2: Inhibition of phloretin-induced increase of BK_{Ca} activity by IBX. Original recordings of BK_{Ca} in a cell-attached patch immediately after giga-seal formation using IBX (100 nmol L^{-1}) filled pipette and 35 min later (after 30 min of continuous perfusion 80 μmol L^{-1} phloretin)

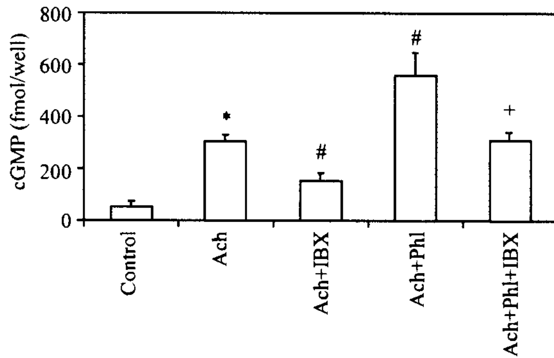

Fig. 3: Increase of acetylcholine-induced cGMP-levels by phloretin depends on BK_{Ca}-activation. Acetylcholine (Ach; 1 μmol L^{-1}) induced cGMP-levels are reduced by iberiotoxin (IBX; 100 nmol L^{-1}). Phloretin (Phl; 80 μmol L^{-1}) caused a further increase of cGMP-levels. Data represent means of cGMP-levels (in fmol/well) ± SEM (n=9; *p<0.05 vs. control; #p<0.05 vs. Ach; +p<0.05 vs. Ach+Phl)

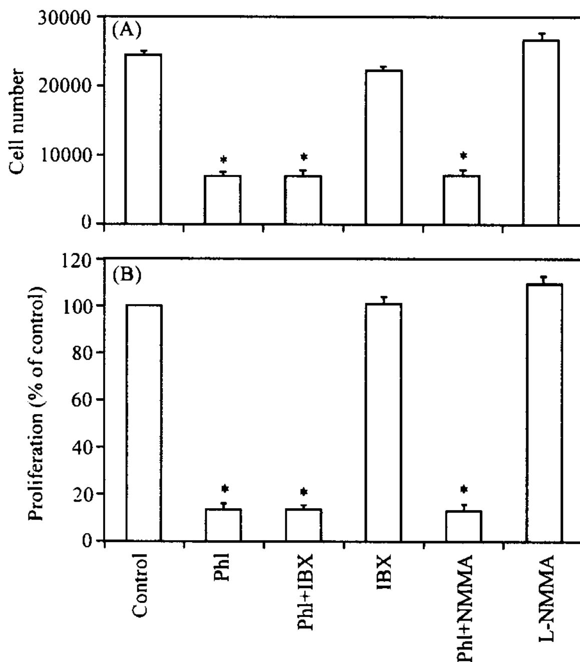

Fig. 4: Phloretin inhibits endothelial proliferation
Effect of phloretin (Phl; 80 μmol L^{-1}), iberiotoxin (IBX; 100 nmol L^{-1}) and L-NMMA (300 μmol L^{-1}) on endothelial proliferation examined by (A) cell counts (n=12) and (B) [^{3}H]-thymidine-incorporation (n=20). Data are expressed as cell numbers (A) and proliferation in % of control (B). All results are mean values ±SEM (*p<0.05 vs. control)

BK_{Ca} observed. The upper trace of Fig. 2 shows the BK_{Ca}-activity under control conditions and the lower trace shows the same cell after 30 min of phloretin perfusion.

Phloretin-induced BK_{Ca} activation induces NO-production: Endothelial nitric oxide production was examined by measuring the NO-dependent second messenger cGMP using a cGMP-radio-immuno-assay. Cellular cGMP-levels were increased by the addition of 1 μmol L^{-1} acetylcholine (control: 46.4±20.5 fmol/well, acetylcholine: 303.1±31.1 fmol/well), which was significantly reduced in the presence of the BK_{Ca}-inhibitor IBX (n=9, p<0.01). Stimulation with phloretin lead to a further increase of acetylcholine-dependent cGMP levels (+87.5%), that was again significantly reduced by blockade of BK_{Ca} (n=9, p<0.01) (Fig. 3).

Inhibition of endothelial proliferation by phloretin: The proliferation of HUVEC was analysed by cell counts (cc) and [^{3}H]-thymidine-incorporation (TI). A significant reduction of endothelial proliferation was observed, if the cells were incubated with phloretin (cc: -70.6%, TI: -86.5%; n=12 and n=20, respectively, p<0.05). Interestingly this phloretin induced effect was not affected by blockade of the BK_{Ca} with IBX (100 nmol L^{-1}), or inhibition of the eNOS with L-NMMA (300 μmol L^{-1}) (Fig. 4A and B).

DISCUSSION

The aim of this study was to examine the effects of the dietary flavonoid phloretin on endothelial cell functions that are relevant in the process of atherosclerosis. The major findings were: Phloretin caused an activation of the BK_{Ca} in endothelial cells; Phloretin increased acetylcholine-dependent NO-formation involving the BK_{Ca}; and endothelial proliferation is inhibited by phloretin independent of BK_{Ca}-activity or NO-formation.

The presented electrophysiological data demonstrated for the first time that the flavonoid phloretin activates the BK_{Ca} in human endothelial cells. Activation of this ion channel has been demonstrated by other working groups in different cell types: BK_{Ca} modulation by phloretin was reported in HEK 293 cells[18], human glioma cells[19], in myelinated nerve fibres[5] and in xenopus laevis oocytes[18]. In endothelial cells, which do not express voltage gated Ca^{2+}-channels calcium influx mainly occurs through store-operated calcium channels and through non-selective cation channels[7,20,21]. This influx of extracellular calcium depends on hyper polarization, because the electrochemical driving force for calcium influx is increased by agonist-induced hyperpolarization[6,8]. The regulation of the intracellular calcium homeostasis is essential for endothelial cell

functions like NO-synthesis and proliferation, which are of great importance in atherogenesis[8,12].

The BK_{Ca} has been shown to play an important role in the regulation of NO-synthesis by increasing intracellular calcium[22-24]. A cGMP-radio immunoassay was used to investigate phloretin-induced changes of endothelial NO-production. Phloretin significantly increased endothelial cGMP-levels, which was blocked by our IBX, indicating a contribution of the BK_{Ca}. These findings are in line with data previously published by our working group. It could demonstrated, that acetylcholine-induced cGMP-levels are significantly reduced by IBX[9]. Furthermore, we have shown that stain-induced NO-release depends on BK_{Ca}-activation[25]. Up to now, there are no reports of phloretin induced changes of cellular nitric oxide formation. Figtree *et al.*[4] examined the effect of phloretin and other plant-derived flavonoids on the relaxation of isolated rabbit coronary artery rings, with the result that these substance cause a vasodilatation of the vessel segments.

As mentioned above intima angiogenesis plays a crucial role in the progression and destabilization of atherosclerotic plaques[13-15]. For this reason we studied the effects of phloretin on endothelial cell proliferation. Phloretin has been shown to have antiproliferative effects on hepatic cells and lymphocytes[26,27]. The activation of K^+ channels promotes the progression of the endothelial cell cycle clock[9,11,26,27]. In contrast to the above mentioned findings the present study demonstrates that phloretin reduces endothelial proliferation although BK_{Ca} activity is largely increased. Since this effect was not affected by IBX, it seems to be independent of BK_{Ca} activity. Another possible mechanism that could be responsible for the antiproliferative response of the endothelial cells to phloretin, results from the fact that nitric oxide donors inhibit the proliferation of human endothelial cells[28]. Since we could not observe an inhibition of the phloretin-induced reduction of HUVEC growth, we conclude that this effect is independent of nitric oxide as well.

In conclusion the present study demonstrated that phloretin induces BK_{Ca} activation in HUVEC, which results in an increase of nitric oxide production. In addition phloretin reduced endothelial proliferation independent of nitric oxide formation and BK_{Ca}-activity. The results of this study give further insight in the signalling mechanism underlying the beneficial effects of dietary flavonoids as phloretin.

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