DIFFERENT EFFECT OF FELODIPINE AND AMLODIPINE ON NADPH OXIDASE EXPRESSION AND FUNCTION IN HUMAN SMOOTH MUSCLE CELLS

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Summary

The aims of the study were: to evaluate the anti-oxidant potential of calcium channel blockers felodipine (Fel) and amlodipine (Aml); to estimate their effect on ROS production and on NADPH oxidase (NADPHox) expression and function in human aortic smooth muscle cells (SMCs) stimulated with angiotensin II. Human SMCs were stimulated 24h with 1µM Ang II in the presence or absence of 10 µM drugs. Intracellular ROS production was detected by spectrofluorimetry using 5 µM dichlorofluoresceine (DCF). The lucigenine-enhanced chemiluminiscence assay was used to determine NADPH oxidase activity. Quantification of protein and gene expression of NOX1 was done by Western blot and real-time PCR. By comparison to the control level, stimulation of SMCs with Ang II induced a significant increase of: intracellular ROS production; NADPHox activity; NOX1 mRNA expression and NOX1 protein level. Treatment with calcium channel blockers determined a significant decrease of: ROS production (Fel 80% vs. Aml 30%); NADPHox activity (Fel 50% vs. Aml 20%); NOX1 mRNA expression (Fel 50% vs. Aml20%); NOX1 proteic level (Fel 80% vs. Aml 5%). Felodipine and amlodipine have different anti-oxidant capacity. The unlike effects of the two compounds of the same class of L-type calcium channel blockers suggests distinct mechanisms of action and prefigurate a new therapeutic strategy for oxidative stress reduction in patients with arterial hypertension.

Key words: calcium channel blockers, oxidative stress, NADPH oxidase

Introduction

In the last decade many studies have demonstrated the importance of reactive oxygen species (ROS) production by NADPH oxidase in angiotensin II (Ang II) signaling, as well as in the development of different cardiovascular high risk states (hypertension, hypercholesterolemia, diabetes) in which this vasoactive molecule is highly implicated (Garrido, 2009; Selemidis et al., 2008). Angiotensin II stimulates NADPHox-derived ROS production in smooth muscle cells (SMCs) by activation of various intracellular pathways such as calcium channels (Guzik et al., 2008; Hool, 2008; Espinosa et al., 2009).Clinical studies suggests that calcium channel antagonists may be beneficial in reducing myocardial injury associated with oxidative stress (Perez-Ryes et al., 2009; Yao et al., 2008; Song et al., 2008; Yamagisi et al., 2008; Ackerman et al., 2008). In this context we studied the anti-oxidant potential of two calcium channel blockers, felodipine (Fel) and amlodipine (Aml), and estimated their effect on ROS production, and on NOX1-NADPH oxidase subunit expression and function in human SMC stimulated with angiotensin II.

Material and methods

Cell culture

Aortic SMCs were obtained by explantation from the media of human foetal thoracic aorta and grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS)
(v/v) supplemented with non-essential amino acids and antibiotics. At confluence, SMCs were starved for 24 hrs by incubation with DMEM in the presence of 0.2% FCS. The cells were exposed for 24 hrs to 1 µM Ang II in the presence or absence of 10 µM drugs (Fel/ Aml). Cultured cells were used between the ninth and twelfth passages.

**Quantification of reactive oxygen species (ROS)**

The generation of ROS in SMCs was measured by 2,7-dichlorofluorescein (DCF) fluorescence technique (Li et al., 2002). Briefly, cultured cells were scrapped and resuspended in HEPES-buffered saline solution, pH 7.4, containing (in mM): NaCl, 145; KCl, 5; CaCl2, 1.8; MgCl2, 1; Na2HPO4, 1; glucose, 5; HEPES, 25. After loading with 5 µmol/l DCF for 30 min at 37°C, the cells were distributed at 10⁴ cells/well into a 96-well microplate reader (Tecan, Austria). DCF fluorescence emission was detected at 535 nm with an excitation wavelength of 485 nm. The ROS production was expressed as arbitrary units.

**Determination of NADPH oxidase activity**

Quiescent SMCs were exposed for 24 hrs to 1 µM Ang II in the presence or absence of 10 µM drugs. The NADPH activity was determined in cell homogenates by lucigenin-enhanced chemiluminescence assay (Ungvari et al., 2003) using a low concentration of lucigenin (5 µmol/L) to minimize artifactual O₂⁻ production due to redox cycling (Skatchkov et al., 1999). The reaction mixture comprised 50 mM PBS, 1 mM EGTA, pH 7.0, 5 µmol/L lucigenin and 100 µmol/L NADPH. The reaction started by addition of cell homogenate (100-150 µg of protein) to the mixture and the light emission was recorded every second for 15 min in a luminometer (Berthold, Germany). The activity was expressed as arbitrary units.

**Real Time PCR**

Total cellular RNA was isolated from cultured SMCs using GenElute® Mammalian Total RNA kit (Sigma). First-strand cDNA synthesis was performed employing 1 µg of total RNA and MMLV reverse transcriptase, according to the manufacturer’s protocol (Invitrogen). Quantification of NOX1 mRNA expression was done by amplification of cDNA using an Opticon 2 DNA Engine real-time thermocycler (MJ Research) and SYBR Green I chemistry. Optimized amplification conditions were 0.2 µM of each primer, 2.5 mM MgCl₂, annealing at 58 °C and extension at 72 °C for 40 cycles. GAPDH gene was used as internal control. The relative quantification was done using the comparative C_T method and expressed as arbitrary units.

**Western-blot analysis**

Cultured cells were washed twice in ice-cold PBS before lysis in 2 x Laemmli’s electrophoresis sample buffer and boiled for 10 min. Protein concentration was quantified by the Amido Black method. Equal amounts of protein (70 µg) were run on 10 % SDS-PAGE and electrophoblated onto nitrocellulose membranes. The membranes were exposed to TBS Blotto A, and then incubated overnight at 4 °C with the primary antibodies against NOX1 (rabbit polyclonal, sc-25545) or β actin (mouse monoclonal, sc-47778), followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit IgG-HRP). The protein bands were detected using chemiluminescence substrate solution and images were taken with a gel analyzer system (ImageMaster VDS, Pharmacia Biotech). The quantification of the NOX1 protein was determined by normalization to β actin protein and expressed as arbitrary units.

**Statistical analysis**

Data were expressed as means ± SD. Statistical evaluation was done by 1-way ANOVA test; p≤0.05 was considered statistically significant.
Results and discussion

Modulation of ROS production and NADPH oxidase activity by amlodipine and felodipine

Stimulation of SMCs with Ang II (100 nM - 1 μM) resulted in a dose- and time-dependent increase of intracellular ROS production and NADPH oxidase activity. Compared to control level, treatment with 1 μM Ang II augmented significantly the ROS production (≈ 30 %) and NADPH-dependent O₂⁻ production (≈ 60 %) after 4 h of stimulation to a level that was maintained for 24 hours.

To determine whether amlodipine and felodipine contributes to the regulation of NADPH oxidase activity, SMCs were incubated for 24 hours with 1 μM Ang II in the presence of 10 μM of each drug. The results showed that treatment with calcium channel blockers determined a significant decrease of ROS production (Fel 80% vs. Aml 30%) and NADPH oxidase activity (Fel 50% vs. Aml 20%) (Figure 1).

Regulation of NOX1 gene and protein expression by amlodipine and felodipine

NOX1 gene and protein expression was evaluated by real time PCR and Western blot in SMCs exposed to 1 μM Ang II (24 h) in the presence or absence of 10 μM amlodipine or felodipine. The results showed that stimulation of SMCs with Ang II caused a significant increase in NOX1 mRNA and protein expression. The Nox1 gene expression attained a maximum level at 6 hours after stimulation (≈ 2 fold above the control level) and the level was sustained for 24 hours. NOX1 protein level was significantly augmented (≈ 35-40 % above the baseline) after 24 hours incubation with Ang II. Treatment with calcium channel blockers determined a significant decrease of NOX1 mRNA (Fel 50% vs. Aml 20%) and protein level (Fel 80% vs. Aml 5%) (Figure 2).

Conclusions

Felodipine and amlodipine have different anti-oxidant capacity. The unlike effects of the two compounds of the same class of L-type calcium channel blockers suggests distinct mechanisms of action and prefigure a new therapeutic strategy for oxidative stress reduction in patients with arterial hypertension. Therapies targeted against specific Nox isoformes to decrease ROS generation may be useful in minimizing vascular injury, and thereby in preventing or regressing target organ damage associated with hypertension.
Figure 2. Regulation of NOX1 mRNA (A) and protein expression (B) by amlodipine and felodipine in Ang II – stimulated SMCs; n=3, *p<0.05, **p<0.01, ***p<0.001.

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References