The Antihypertensive Drug Nifedipine Modulates the Metabolism of Chondrocytes and Human Bone Marrow-Derived Mesenchymal Stem Cells

Ilona Uzieliene  
State Research Institute Centre for Innovative Medicine

Eiva Bemotiene  
State Research Institute Centre for Innovative Medicine

Greta Urbonaite  
State Research Institute Centre for Innovative Medicine

Jaroslav Denkovskij  
State Research Institute Centre for Innovative Medicine

Edvardas Bagdonas  
State Research Institute Centre for Innovative Medicine

Zygmunt Mackiewicz  
State Research Institute Centre for Innovative Medicine

Narunas Porvaneckas  
State Research Institute Centre for Innovative Medicine

Giedrius Kvederas  
State Research Institute Centre for Innovative Medicine

Ali Mobasheri  
State Research Institute Centre for Innovative Medicine  https://orcid.org/0000-0001-6261-1286

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Abstract

Aging is associated with the development of various chronic diseases, in which both hypertension and osteoarthritis (OA) are dominant. Currently, there is no effective treatment for OA, whereas hypertension is often treated using L-type voltage-operated calcium channel (VOCC) blocking drugs, nifedipine being among the most classical ones. Although nifedipine together with other VOCC inhibitors plays an important role in people wellbeing, there are unresolved questions on its possible effect on cartilage tissue homeostasis and the development of OA. Due to that, the aim of this study was to analyse the effects of nifedipine on metabolic processes in human chondrocytes and bone marrow mesenchymal stem cells (BMMSCs). To analyze whether those events were mediated specifically through VOCC, agonist BayK8644 was used. Our results demonstrate that nifedipine downregulated chondrocyte proliferation rate as well as mitochondrial respiration and ATP production (Agilent Seahorse) in both cell types. Analysis of cartilage explant histological sections by electron microscopy also suggested that part of mitochondria lose their activity in response to nifedipine. However, switch of energetic metabolic pathway towards glycolytic was observed only in chondrocytes. Stimulation with either nifedipine or BayK8644 resulted in elevated production of collagen type II and proteoglycans in micromass cultures under chondrogenic condition, although the effects of VOCC inhibitor Bay8466 were less expressed. Nitric oxide (NO) activity, as measured by flow cytometry, was upregulated by nifedipine in BMMSCs and particularly chondrocytes, suggesting that NO at least in part may account for the effects of nifedipine on metabolism in both tested cell types.

Taken together, we conclude that antihypertensive drug nifedipine inhibits mitochondrial respiration in both chondrocytes and BMMSCs and that these effects may be associated with increased NO accumulation and pro-inflammatory activity. Glycolytic capacity was enhanced only in chondrocytes, suggesting that these cells have the capacity to switch from oxidative phosphorylation to glycolysis and alter their metabolic activity in response to VOCC inhibition. Finally, nifedipine stimulated production of collagen type II and proteoglycans in both cell types, implying its potentially beneficial anabolic effects on articular cartilage. These results highlight a potential link between consumption of antihypertensive drugs and cartilage health.

Introduction

There is emerging evidence that cardiovascular disease (CVD) is a co-morbidity in many osteoarthritis (OA) patients and is mechanistically linked to the progression of OA (Rahman et al. 2013; Wang et al. 2016; Kuusalo et al., n.d.). Arrhythmia, hypertension and cardiac ischemia are most prevalent in elderly and obese individuals, with limited physical activity and in many cases, with hormonal imbalance and metabolic disorders (Chadha 2016; Berenbaum and Courties 2017).

The pathogenesis of OA and cardiovascular disorders might involve a common but neglected link, an altered Ca2+ signalling, which may contribute to the development of comorbidities in the same individual.
Furthermore, modulators of ion channels, used for the treatment of cardiovascular diseases, particularly 1,4-Dihydropyridines (1,4-DHP), may also affect the structure and function of articular cartilage. Nifedipine is one of the most commonly prescribed drugs for treating hypertension. It specifically blocks L-type voltage-operated calcium channels (VOCCs) in the vascular system and therefore, lowers high blood pressure (Rufus, Robert, and William 1983; Uzieliene 2018). Recently, it has been shown that various 1,4-DHP calcium antagonists stimulate NO release (Salameh et al. 1996; Berkers et al. 2001; Ding and Vaziri 2015). The majority of described NO effects are related to pro-inflammatory processes or mitochondrial dysfunction. Increased NO generation promotes OA through inhibition of chondrocytes cytoskeletal actin polymerization and beta-1 integrin-dependent signalling, decreased expression of IL-1β receptor antagonist and TGFβ, suppression of chondrocyte collagen and proteoglycan synthesis, activation of metalloproteinases, enhanced production of ROS by mitochondria, which is associated with NO and with some reactive nitrogen species, such as peroxynitrite and chondrocyte apoptosis (Garcia 1999; Khan et al. 2017; Species et al. 2018; Lotz 1999). Since both hypertension and OA are most prevalent in the older population with overweight and metabolic disorders, the use of these antihypertensive drugs may have additional effects on articular cartilage. The phenomenon of aberrant metabolism in OA cartilage, altered metabolic pathways, and mediators have been highlighted as potential therapeutic targets (Mobasheri et al. 2017).

However, it is challenging to predict the overall effects of ion channel modulators and their effects on the progression of OA. Several studies suggest that calcium channel antagonists (especially L-type Ca2+ channel inhibitors) may have a beneficial effect on OA by attenuating its progression (Daniilidis et al. 2015; Takamatsu et al. 2014). In this case, blockage of those channels by cardiovascular drugs may result not only in an improved heart rate and blood pressure but also in chondroprotection and attenuated OA development. As referred above, NO appears seems to have both beneficial and detrimental effects on cell death or survival outcome and may appear a potential downstream mediator of nifedipine activity may mediate or at least contribute to the above-mentioned calcium channel independent effects.

Therefore, we developed a novel hypothesis that VOCC inhibitors used to treat hypertension may, in parallel, modulate intracellular [Ca2+] levels in chondrocytes, leading to metabolic responses, which in turn affect production of extracellular matrix in articular cartilage. Therefore, the aim of this study was to analyze the metabolic and functional responses to antihypertensive VOCC inhibitor nifedipine, including mitochondrial respiration, glycolysis, proliferation, NO activity and extracellular matrix production in bone marrow mesenchymal stem cells (BMMSCs) and chondrocytes. We were also seeking to analyse whether those events were mediated specifically through VOCC, therefore as an opposite control we have used L-type VOCCs agonist – BayK8644, which prolongs Ca2+ channel opened/active state during membrane depolarization (Uzieliene Ilona, Bernotas Paulius, Mobasher Ali 2018). These data could broaden our understanding on the effects of VOCC inhibitors used for treatment of hypertension and give some mechanistic insight on the development and progression of OA, potentially offering new targets for promoting cartilage health and protection.
1 Materials And Methods

1.1. Cell isolation and culture

Human articular cartilage samples were obtained from Vilnius University Hospital Santaros Klinikos as postoperative tissues during articular surgery from patients with OA (n = 5). Cartilage was dissected from anatomical locations with morphologically similar lesions. The excised pieces of cartilage were further chopped into the small explants of 1–3 mm on each side and weighted. After weighing, cartilage explants were incubated overnight in low glucose (1 g/L) DMEM medium (Merck Millipore) without FBS at 37°C and 5% CO₂. The next day, cartilage explants were washed with PBS and incubated 1h in pronase solution (26,5U/ml) (Roche diagnostics: 18572723) at 37°C and 5% CO₂ under conditions of constant shaking. Then, cartilage explants were washed twice with PBS, chopped into smaller pieces and transferred into a new 50 ml tube for the following chondrocytes isolation with type II collagenase. 10 ml of type II collagenase solution (545U/ml) (Biochrom AG: C2–22) were prepared for each 1 g of cartilage sample. Cartilage pieces were incubated at 37°C and 5% CO₂ for 3-4 h under conditions of constant shaking. After incubation, the digested solution was filtered through cell strainers of 100 µm and 70 µm. The enzymatic activity of collagenase was stopped by adding a double volume of complete medium – DMEM (1 g/L glucose), supplemented with 10% FBS (Merck Millipore), 1% penicillin/streptomycin (Gibco, Life Technologies). Cell filtrate was centrifuged for 5 min. at 400 g, supernatant discarded and cell pellet resuspended in complete medium. Collected chondrocytes were expanded in tissue culture flasks (Gibco, Life Technologies) with complete medium, and cultured in 37°C incubator with 5% CO₂. Medium was changed twice a week. After reaching the confluence (~80%) cells were detached using trypsin-EDTA 0.25% solution (Gibco, Life Technologies), counted (CASY, Omni Life Science) and sub-cultured. Human BMMSCs were isolated from bone marrow tissues remaining after surgical procedures from five donors (50-60 years age), according to the established protocols by Centre for Innovative Medicine (IMC). BMMSCs were cultured under the same conditions as chondrocytes – in complete DMEM medium, but with addition of 1 ng/ml FGF. All the procedures made with human tissues within this study were approved by Bioethics Committee, permission No. 158200-14-741. All experiments were performed using chondrocytes and BMMSCs at passages (P) P2 to P3.

1.2. Cartilage explant isolation and preparation for transmission electron microscopy study

Samples of cartilage tissue were dissected from the locations with morphologically similar lesions. Biopsy needles (3 mm, Integra Miltex, LOT: 33-32) were used to extract explant. Explants were weighed and put into 6-well plate, 100 mg of explants/well. Explants were separated into 2 groups – control group (chondrogenic medium only) and nifedipine exposure group (chondrogenic medium and nifedipine), and cultured for 7 days. Medium was changed at day 3 and 5. After 7 days explants were prepared for electron microscopy analysis.
1.3. Transmission electron microscopy study of cartilage explants

For transmission electron microscopy analysis the *ex vivo* cultivated cartilage explants derived from smooth either eroded articular surface were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer for 3 h, then with 1% OsO<sub>4</sub> in the same buffer for 1 h, alcohol dehydrated, and embedded in araldite. Ultrathin sections were prepared on a Leica EM UC6 ultratome and stained with uranyl acetate and lead citrate. The observations of ultrathin sections were carried out with Philips/FEI (Morgagni) transmission electron microscope at magnification 3000x -10000x, and photos made using the Gatan digital camera.

1.4. Proliferation assay

Chondrocytes and BMMSCs were seeded into 12-well plates (SPL, Life Sciences) at density of 20 000 cells/well in a complete medium. The next day, cells were divided into 3 treatment groups: control (with DMSO (10 µM), which is a solvent for nifedipine and BayK8644), nifedipine (10 µM) (Sigma-Aldrich) and BayK8644 (10 µM) (Sigma-Aldrich). Cell proliferation was determined at days 1, 3, 5, 8 and 12 with cell counting kit – 8 (CCK-8) (Dojindo) according to manufacturer's instructions. The medium was collected to 96 well plate (Orange Scientific) and absorbance at 450 nm was quantified with SpectraMaxvi3 spectrophotometer (Molecular Devices).

1.5. Metabolic analysis

Cellular metabolism was measured using Agilent Seahorse xFe24 metabolism analyzer and Mito-stress test kit (103010-100, Agilent Seahorse, Santa Clara, CA, USA). The cells were seeded into Seahorse 24-well plates at a density 30 000 cells/well. The next day cells were divided into treatment groups: (1) control, which was cultivated in complete medium; (2) instant nifedipine, where nifedipine (10 µM) was added only during measurement; (3) nifedipine (10 µM) and BayK8644 (10 µM) for long term (24 h.) incubation, and (4) nifedipine (10 µM) for 24 h and additional nifedipine (10 µM) added during measurement, for long and instant effect of the drug. Each group was measured in triplicates. After the treatment, complete cell medium was switched to Seahorse XF base medium (102353-100, Agilent Technologies) supplemented with 10 mM glucose, 2 mM GlutaMAX (Gibco) and 1 mM sodium pyruvate, and further incubated in CO<sub>2</sub>-free incubator for 1 hour. The measurement was completed according to Agilent recommendations, by adding oligomycin, FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) and antimycin A/rotenone, which were prepared in Seahorse XF assay medium (102353-100, Agilent Technologies) with final concentration of 1 µM, 0.5 µM and 1 µM. For glycolysis test, Glucose stress-test kit was used (103020-100, Agilent Technologies). The cells were performed the same way, only using Seahorse medium supplemented with 1 mM GlutaMAX (Gibco). During measurement, glucose, oligomycin and 2-deoxy-glucose (2-DG) were added with a final concentration of 100 mM, 10 µM and 500 mM respectively. After the measurement, the protein analysis was performed using Lowry method and the results were normalized to the amount of protein.
1.6. Intracellular calcium study

For intracellular calcium studies chondrocytes and BMMSCs were seeded into 12-well plates at density 50 000 cells/well in a complete medium. After the cells reached confluence, the wells were subdivided into 6 different groups (2 for each treatment) and treated for 24 h with: (1) 10 µM of DMSO, (2) 10 µM of nifedipine, (3) 10 µM of BayK8644, (4) 1 ng/ml IL-1β, (5) 1 ng/ml IL-1β + 10 µM nifedipine, (6) 1 ng/ml IL-1β + 10 µM BayK8644. At the end cells were detached and stained with 1 µM of calcium specific fluorescent dye Cal-520 (Interchim, USA) for 30 min, 37°C, then measured using flow cytometer (Calibur) and the data were analyzed using FlowJo (FlowJo, USA) software.

1.7. Nitric oxide accumulation study

The intracellular nitric oxide activity was assessed in chondrocytes and BMMSCs, treated for 24 h with: (1) 10 µM of DMSO (control), (2) 10 µM of nifedipine, (3) 10 µM of BayK8644 and (5) 1 µl/ml of cigarette smoke extract (CSE) (Murty Pharmaceuticals, Lexington, USA), as a positive control. The next day cells were detached and measured for nitric oxide activity by using MUSE flow cytometer (Merck, Germany) and Muse™ Nitric Oxide Kit, (Merck, Germany), according to manufacturer’s instruction.

1.8. Chondrogenic differentiation study

Chondrogenesis was induced using standard protocol used by State Research Institute Centre for Innovative medicine. Chondrogenic medium included high glucose (4,5 g/L) DMEM medium (Merck Millipore), 1% penicillin/streptomycin, 1% insulin-transferrin-selenium (all from Gibco Life Technologies), L-proline (350nM) (Carl Roth), dexamethasone (100 nM) (Sigma Aldrich), ascorbic acid-phosphate (170 nM) (Sigma Aldrich) and TGF-β3 (10 ng/ml) (Gibco, Life Technologies). Incomplete chondrogenic medium (the same constituents without TGF-β3) was used as control.

Furthermore, each group was subdivided into 3 subgroups: (1) with addition of DMSO (10 µM), which is a solvent for nifedipine and BayK8644, (2) with nifedipine (10 µM) and (3) BayK866 (10 µM). In total, 6 subgroups of different stimulation conditions were applied for cell cultivation in pellets in 15 ml tubes (Gibco, Life Technologies) for 21 day. Extracellular matrix formation in pellets was assessed by histological methods.

1.9. Histology of chondrogenic differentiation in cell pellets

For histochemical and immunohistochemical analysis, chondrogenic differentiation pellets were fixed in 10% neutral buffered formalin and embedded into paraffin. Histological sections of 3-micrometer thickness were deparaffinised and processed for standard staining with safranin-O (Sigma-Aldrich). Immunohistochemical staining with antibodies against collagen type II (Abcam) was performed after antigen retrieval with citrate buffer pH 6.0 at +98°C for 20 min and endogenous peroxidase blocking with
0.3% hydrogen peroxide for 15 min at room temperature. ABC staining kit (Santa Cruz) and 3.3-diaminobenzidine as a chromogen were used. Stained sections were evaluated and blindly scored and independently by two histology experts.

1.10. Statistical analysis

The Student’s t-test was used to calculate statistical difference of data in proliferation, intracellular calcium, nitric oxide and metabolism assays and \( p \leq 0.05 \) was considered as statistically significant.

2 Results

2.1. Proliferation of chondrocytes and BMMSCs

To evaluate the effects of nifedipine and BayK8644 on proliferation of chondrocytes and BMMSCs, these compounds were added to cell culture medium and cell proliferation was measured during 1, 3, 5, 8 and 12 days. nifedipine significantly decreased proliferation of chondrocytes only during 12\textsuperscript{th} day of cultivation while BayK8644 did not have any significant effect (Figure 1). VOCC regulators had no significant effect on BMMSC’s proliferation (Figure 2).

2.2. Alterations in cellular metabolism as a response to nifedipine and BayK8644

Agilent Seahorse metabolism analyzer provides an informative study of cells energy metabolism. The sequential compound injection allows to measure basal cell respiration capacity, ATP production, maximal respiration rate and spare respiration capacity during mitochondrial respiration, and glycolysis, glycolytic capacity and glycolytic reserve during glycolysis.

Mitochondrial spare respiratory capacity in chondrocytes was significantly reduced by an instant treatment with nifedipine during measurement, but not by long treatment. However, ATP production was significantly downregulated by all of the treatments, especially BayK8644. Moreover, BayK8644 also significantly reduced spare respiratory capacity in chondrocytes (Figure 3).

Nifedipine long treatment significantly increased glycolysis in chondrocytes (Figure 4). Furthermore, nifedipine and BayK8644 significantly increased glycolytic reserve, which is an important parameter in cells energy metabolism, as it indicates the capability of a cell to respond to an energetic demand as well as shows how close the glycolytic function is to the cell’s theoretical maximum.

Long term (24h) incubation with nifedipine downregulated basal mitochondrial respiration in BMMSCs, while instant treatment had no significant effect (Figure 5). Pre-treatment with BayK8644 also downregulated basal respiration. nifedipine resulted in a repression of ATP production, but only a
combination of long and instant treatments reached statistical significance. None of the treatments had any significant effects on spare respiratory capacity.

Neither nifedipine, nor BayK8644 had a significant effect on glycolytic capacity or glycolytic reserve in BMMSCs (Figure 6).

2.3. Transmission electron microscopy study of cartilage explants treated with nifedipine

After a long-term incubation with nifedipine (7 days), cartilage explants were analyzed by transmission electron microscopy, as shown in Figure 7. Upon nifedipine treatment, the mitochondrial matrix became dark as compared to untreated, structurally unchanged chondrocyte in control samples.

2.4. Nifedipine treatment increased intracellular calcium levels in chondrocytes and BMMSCs

To investigate the effects of nifedipine and BayK8644 on their direct targets – VOCC, changes in intracellular calcium concentrations were studied. The cells were additionally incubated with IL-1β, which is an important cytokine in the early stages of OA, as well as IL-1β + nifedipine and IL-1β + BayK8644. As shown in Figure 8, nifedipine significantly increased intracellular calcium concentration [Ca2+] in both cell types. While IL-1β significantly decreased intracellular [Ca2+] in chondrocytes, IL-1β + nifedipine treatment increased it as compared to control. BayK8644 treatment didn’t significantly change [Ca2+] in chondrocytes, as compared to control. In BMMSCs, nifedipine, IL-1β + nifedipine and IL-1β + BayK8644 significantly increased it.

2.5. Nifedipine increased nitric oxide accumulation in chondrocytes and BMMSCs

Nitric oxide (NO) activity in chondrocytes and BMMSCs was analyzed due to its direct roles in mitochondria functions and inflammation processes. NO activity increased when cigarette smoke extract (CSE) was added. CSE was chosen as positive control since it has been known to induce NO in MSCs from previous experiments (unpublished data). Nifedipine significantly increased NO activity in chondrocytes and BMMSCs (Figure 9), as compared to control. BayK8644 had no significant effect on both cell types.

2.6. Chondrogenic differentiation of nifedipine and BayK8644 treated chondrocytes and BMMSCs
Treatment with nifedipine and BayK8644 induced chondrogenic differentiation both in chondrocytes and in BMMSCs (Figure 10). Most surprisingly, nifedipine induced strong synthesis of collagen type II and proteoglycans both in absence and in presence of TGFβ3 and in both cell types.

3 Discussion

In the present study, we were seeking to elucidate the role of antihypertensive drug nifedipine on functions and energy metabolism in chondrocytes and BMMSCs. Nifedipine is a VOCC antagonist, therefore, in order to understand if those effects were mediated through [Ca2+] channels, we have also compared its effects to those of agonist BayK8644.

First, the downregulation of proliferation was observed in both chondrocytes and BMMSCs, however only in chondrocytes it was significant. This may signify potential cytotoxic or cytostatic effects of Nifedipine. It is noteworthy that chondrogenic differentiation is also associated with cell cycle arrest (Beier et al. 1999). BayK8644 had no such an effect and even tended to stimulate proliferation in chondrocytes. This are on the contrary to the data on breast cancer cell line, where nifedipine promoted cell proliferation (Guo et al. 2014).

In response to nifedipine and BayK8644, changes in cell metabolism were analysed, particularly mitochondrial respiration and glycolysis, that are the main energy generating processes in cells.

In chondrocytes, application of nifedipine for either instant or long (24h) duration significantly downregulated ATP production, suggesting blockage of mitochondrial respiration. Noteworthy, both spare respiratory capacity and glycolytic capacity were significantly lower after instant nifedipine treatment as compared to the 24h application suggesting that those parameters respond immediately and then gradually are compensated. On the other hand, only long nifedipine treatment augmented glycolytic reserve, suggesting efficient switch to compensatory energetic production in chondrocytes.

BMMSCs responded differently: only long (24h) application downregulated basal respiration level and ATP production, whereas no induction of glycolysis was observed.

Altogether these data suggest that nifedipine may lead to energetic arrest in BMMSCs and chondrocytes, which could also, at least in part, account for the reduced proliferation, as was shown in the study with barbeine in HepG2, HeLa and Hepa1-6 cell lines (Yan et al. 2017). In agreement to that, the analysis of chondrocyte mitochondria by electron microscopy in cartilage explant histological sections has also suggested that part of mitochondria lose their activity in response to nifedipine.

Unexpectedly, VOCC agonist BayK8644 had similar metabolic effects to nifedipine, including induction of glycolytic reserve in chondrocytes and blockage of ATP production in both chondrocytes and BMMSC. These data imply that additional mechanisms than modulation of [Ca2+] channels may be involved in the effects of antihypertensive drug nifedipine on BMMSCs and chondrocytes.
Therefore, the roles of nifedipine and BayK8644 to their direct target changes in intracellular [Ca2+] concentration were analyzed by flow cytometry. In this study, we included the most efficient pro-inflammatory factor during OA – IL-1β (Mabey 2015). Intracellular calcium levels were not decreased, but unexpectedly increased in nifedipine, but not BayK8644 treated cells of both types. Induction of intracellular calcium intake from endoplasmic reticulum ryanodine sensitive stores by nifedipine has been previously reported (Piriz 2003), suggesting compensation from intracellular [Ca2+] stores, however such potential process is still under investigation.

To further understand the mechanism by which nifedipine blocks mitochondrial functions, the production of NO was investigated. nifedipine has been shown to increase endothelial NO bioavailability (Berkels et al. 2001), and upregulating intracellular calcium in striatal neurons (HORN et al. 2002), whereas inhibition of mitochondrial activity by NO has been demonstrated (Brown 2001). Similarly, in the present study, NO activity was stimulated by nifedipine in BMMSCs and particularly chondrocytes, suggesting that NO at least in part may account for the effects of nifedipine on metabolism in both tested cell types. On the other hand, BayK8644 had no effect on NO activity, although was the most potent blocker of ATP in chondrocytes, suggesting that different mechanisms might be implicated in its action on mitochondrial respiration.

Finally, the effects of nifedipine and BayK8644 on chondrogenesis and extracellular matrix production was assessed in chondrocytes and BMMSCs. To the best of our knowledge, we are the first to demonstrate, that stimulation with either nifedipine or BayK8644 resulted in induction of collagen type II and proteoglycan production even in the absence of TGFβ3, although the effects of VOCC inhibitor Bay8466 was less pronounced.

To the best of our knowledge, we are the first research group to demonstrate the effects of nifedipine on metabolic activity of chondrocytes and BMMSCs, and these data hold promising information for the future experiments using DHP. These results extend our understanding on chondrocyte nature and reaction responses to DHP drugs, particularly nifedipine, and might provide a promising link between hypertension treatment struggling people with OA. Nevertheless, deeper analysis of the effects of those [Ca2+] channel modulators on chondrocyte hypertrophy, inflammatory responses and modulation of catabolic enzymes involved in cartilage degradation, including matrix metalloproteinases, and ADAMTS (disintegrin and metalloproteinase with thrombospondin motifs) could better elucidate the implication of [Ca2+] blocking in OA pathogenesis in the future.

4 Conclusion

Taken together, we conclude that antihypertensive drug nifedipine inhibits mitochondrial respiration in both chondrocytes and BMMSCs and that these effects may be associated with increased NO production and pro-inflammatory activity. Glycolytic capacity was enhanced only in chondrocytes, suggesting that these cells have the capacity to switch from oxidative phosphorylation to glycolysis and alter their metabolic activity in response to VOCC inhibition. Finally, nifedipine stimulated production of collagen
type II and proteoglycans in both cell types, implying its potentially beneficial anabolic effects on articular cartilage. These results highlight a potential link between consumption of antihypertensive drugs and cartilage health.

5-8 Declarations

5 Conflict of Interest

The authors declare no conflict of interest.

6 Author Contributions

Writing-Original Draft Preparation I.U., E.Bern., G.U., E.Bagd., J.D; GK, NP patient selection and tissue sample preparation, manuscript editing; study design and supervision, E.Bern.; conceptualization and editing, A.M;

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9 References


**Figures**
**Figure 1**

Proliferation of chondrocytes after the treatment with nifedipine (10 µM) and BayK8644 (10 µM) for 12 days. CCK-8 assay, * - p<0.05.
Figure 2

Proliferation of BMMSCs after the treatment with nifedipine (10 μM) and BayK8644 (10 μM) for 12 days. CCK-8 assay.
Figure 3

Mitochondrial respiration capacity in chondrocytes. The cells were treated with nifedipine (10 µM) and BayK8644 (10 µM) for 24 hour (long t.), and additionally treated with nifedipine (10 µM) during the measurement (instant t.). Basal, spare respiratory capacity and ATP production are presented. OCR – oxygen consumption rate, * - p<0.05.
Figure 4

Glycolysis in chondrocytes. The cells were treated with nifedipine (10 µM) and BayK8644 (10 µM) for 24 hour period (long t.), and additionally treated with nifedipine (10 µM) during the measurement (instant t.). ECAR - extracellular acidification rate. * - p<0.05.
Figure 5

Figure 5. Mitochondrial respiration in BMMSCs. The cells were treated with nifedipine (10 µM) and BayK8644 (10 µM) for 24 hour period (long t.), and additionally treated with nifedipine (10 µM) during the measurement (instant t.). OCR - oxygen consumption rate. * - p<0.05.
Figure 6

Glycolysis in BMMSCs. The cells were treated with nifedipine (10 µM) and BayK8644 (10 µM) for 24 hour period (long t.), and additionally treated with nifedipine (10 µM) during the measurement (instant t.). ECAR – extracellular acidification rate.

Figure 7
Sections of cartilage explants derived from smooth-surface cartilage samples, untreated (control) and treated with nifedipine (10 µM). Control sample – unchanged chondrocyte in ECM, with normal, light mitochondria (marked by arrows). Magnification ×4k. Nifedipine -treated sample – matrix of some mitochondria is darker (marked by arrows). Magnification ×10k. ECM – extracellular matrix.

**Figure 8**

Mean fluorescence intensity (MFI) of intracellular calcium [Ca2+] dye Cal-520 in chondrocytes and BMMSCs after treatment with DMSO (10 µM) (Control), nifedipine (10 µM) (Nif), BayK8644 (10 µM) (BayK), IL-1β (1 ng/ml) (IL-1b), IL-1β (1 ng/ml) + BayK8644 (10 µM) (BayK) and IL-1β (1 ng/ml) + nifedipine (10 µM) (Nif) for 24 hours. * - p<0.05.
Figure 9
Nitric oxide (NO) activity in chondrocytes and BMMSCs. Treatment with cigarette smoke extract (CSE) (1µg/ml), DMSO (10 µM) (Control), nifedipine (10 µM) (Nif), BayK8644 (10 µM) (BayK) for 24 hours. * - p<0.05.

Figure 10
Chondrogenic differentiation of BMMSCs and chondrocytes. Cells were incubated in chondrogenic differentiation medium with and without TGFβ3. Each group was divided into: Control, treated with nifedipine (10 µM) and treated with BayK8644 (10 µM). A – Histological sections of cell pellets, stained with Safranin-O and anti-collagen type II antibodies, ×100 magnification. B – Macroscopic view of each group of cell pellets. C – Control staining for histological sections with Safranin-O and anti-collagen type II antibodies, where positive is cartilage and negative – salivary gland.