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Gene expression profiling of calcium-channel antagonists in the heart of hypertensive and normotensive rats reveals class specific effects

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ABSTRACT

Calcium channel blockers (CCB) differ in their effects on the cardiovascular system with diltiazem being less negatively ionotrop as compared to verapamil. Diltiazem is mainly used to treat supraventricular tachycardia, vasospastic angina and the Raynaud's syndrome. Little is known about the molecular effects of benzothiazepins on cardiac gene expression. We therefore investigated the effects of diltiazem on cardiac gene expression in normotensive and hypertensive rats with left ventricular hypertrophy and compared the results with our previous findings on verapamil and nifedipine. Spontaneously hypertensive (SHR) and normotensive Sprague Dawley (SD) rats were treated with 15 mg/kg diltiazem b.i.d. for 3 days. Total RNA was isolated from surgically removed hearts and the gene expression of ion channels, ion transporters and their associated partners, calcium handling proteins as well as stress and cellular differentiation markers was investigated by RT-PCR. Subsequently, hierarchical gene cluster analysis was performed to decode treatment effects of different classes of CCBs. CCB treatment of normotensive and hypertensive rats revealed class specific effects with diltiazem specifically repressing cardiac genes pertinent for ion homeostasis and excitation-contraction coupling in normotensive but not hypertensive rats. Conversely, verapamil and nifedipine caused predominantly repression of genes to affect ion homeostasis and contractile dysfunction in spontaneously hypertensive rats; nonetheless, genes coding for calcium-handling proteins were up-regulated. Unlike diltiazem treatment of normotensive rats with verapamil and/or nifedipine did not influence cardiac gene expression. The effects of diltiazem on cardiac gene expression provide a molecular rationale for its use in the treatment of vasospastic angina.

1. Introduction

Cardiovascular diseases such as coronary artery disease, heart failure or stroke are the leading cause of death in Western civilization, and the majority of patients with severe arterial hypertension develop in the long term atrial fibrillation with risk of heart failure or stroke. An early treatment of arterial hypertension is therefore essential to prevent disease progression.

According to the recommendations of the European Society of Cardiology and the American Heart Association ACE-inhibitors or angiotensin-blockers are considered to be first-line treatment for elevated blood pressure [1,2]. However, in most cases a stand-alone therapy is insufficient. Therefore, a combination of different antihypertensive drugs is frequently prescribed. Depending on various pathophysiological conditions and comorbidities calcium-channel antagonists, beta-blockers or diuretics are additionally prescribed to achieve targeted blood pressure values. Moreover, there has been great interest in modulating calcium homeostasis since calcium plays a major role in the excitation-contraction coupling of the heart and programed cell death [3–5]. Calcium channel blockers have thus been used successfully to treat literally millions of patients with hypertension. They encompass three principal classes, namely the dihydropyridines, the phenylalkylamines and the benzothiazepines and are referred to as the nifedipine-, verapamil- and diltiazem-type drugs. Although blockage of the L-type calcium channel is common to all of them, they display different effects on the cardiovascular system that offers opportunities for their selective use. For instance, dihydropyridines, such as nifedipine, preferentially block calcium channels in vascular smooth muscle cells and are mainly used in the treatment of hypertension. Phenylalkylamines, like verapamil, have cardiodepressive properties in the first line and slow the depolarization phase as well as the conduction velocity by affecting slow response myocardial tissue, e.g. sinoatrial (SA) and atrioventricular (AV) node. Consequently, phenylalkylamines are frequently used for the management of supraventricular tachycardias and for control of the ventricular rate in atrial fibrillation and atrial flutter. The

Abbreviations: AV, atrioventricular; CCB, Calcium channel blockers; Na^+-K^+-ATPase, sodium-potassium-exchanger; NCX-1, sodium-calcium-exchanger; PMCA1, plasma membrane Ca^{2+}-ATPase isoform 1; RyR, ryanodine receptor isoform; SA, sinoatrial; SERCA2b, sarcoplasmic reticulum ATPase isoform 2b.

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benzothiazepines, as represented by diltiazem, not only slow the SA node like phenylalkylamines but also reduce the peripheral resistance like dihydropyridines. Diltiazem is an effective medication in angina and exerts both cardio-depressant and vasodilatory effects. With diltiazem though, the increase in heart rate is rarely seen. Similarly, the pro-arrhythmic side effects of verapamil are less noticeable [6]. Therefore, diltiazem appeared to be a promising drug and was long thought to be the safest of all calcium channel antagonists. However, the therapeutic benefit of diltiazem is less distinctive when compared to drugs like ACE-inhibitors and beta-blockers that proved to be superior over diltiazem in terms of efficacy [7]. Nowadays, diltiazem is used to treat vasospastic angina, Raynaud's syndrome and is occasionally used in supraventricular tachycardia but there is also evidence for diltiazem to possibly play a role in the early treatment of hypertrophic cardiomyopathy [8,9]. Surprisingly and despite its use for decades, little is known about the molecular effects on the regulation of cardiac genes coding for other ion channels, ion transporters and calcium handling proteins. Specifically, we reported in the past significant changes in the expression of various cardiac specific genes upon treatment with nifedipine and verapamil in animals with systemic hypertension and observed repression of various genes pertinent for ion homeostasis and excitation-contraction coupling [10,11]. We now extend our investigation to benzothiazepines, the third class of calcium channel antagonists to gain further insight into transcript regulation of calcium channel antagonists to better understand the molecular effects of different classes of L-type calcium channel blockers in hypertension. The genes analysed in our study play a decisive role in either excitation-contraction coupling or in balancing the ion homeostasis of the myocardium and the present study documents fundamental differences in the mode of action of diltiazem when compared with other calcium channel antagonists.

2. Material and methods

2.1. Animals

All animal work followed strictly the Public Health Service (PHS) Policy on Human Care and Use of Laboratory Animals. Formal approval to carry out animal studies was granted by the institutional ethics board of the Fraunhofer Institute ITEM, Hannover, and animal procedures were approved by the Lower Saxony State Office for Consumer Protection and Food Safety (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, LAVES, Hannover), Germany, reference number 33.9-42502-04-06/1081. Male Sprague Dawley (SD) rats and male Spontaneously Hypertensive rats (SHR) were obtained from Charles River (Sulzfeld, Germany). Food and water was given ad libitum. Animals were kept in a 12 hour light/dark cycle. All animals were of the age of approximately 12 weeks with established arterial hypertension and heart to body weight was increased as previously reported [10].

2.2. Experimental design

Over a period of three days animals were treated twice a day, i.e. 12 hours interval with the calcium channel antagonist diltiazem. The medication was given i.p. at a dose of 15 mg/kg bodyweight and is based on published findings. On day three, animals were sacrificed at 2, 4, 8, 12 and 20 h after the last injection. Altogether, $n = 36$ animals were used with $n = 3$ animals per group. Control animals were treated with 0.9% saline using an identical treatment regime and were sacrificed 8 h after the last injection.

2.3. Heart explantation

Rats were anaesthetised with 1 ml of ketamine/kg/body weight and 0.5 ml of xylazine/kg body weight. In addition, 2000 international units of heparin were given i.p. prior to surgery. Under anaesthesia, the thorax was opened and blood was collected retrograde from the thoracic aorta. There was no blood left within the chambers of the heart. After removal, the heart was examined for any obvious macroscopically visible pathologic change, e.g. dilatation of the atria or abnormal coloring of the myocardium. Thereafter, afferent and efferent blood vessels were removed and the heart was rinsed with phosphate buffered saline and weighed to eventually divide the heart at the level of the AV-valves by a clean cut with a sharp razor blade. The right and left ventricle were separated and the interventricular septum remained as part of the left ventricle. The ventricles were individually frozen in liquid nitrogen to await further analysis. Tissue for RNA isolation was taken from the lateral parts of the heart, strictly resembling right and left ventricle.

2.4. RNA and cDNA

Total RNA was isolated from right and left ventricle of explanted hearts with the NucleoSpin RNA kit from Macherey&Nagel, according to the manufacturer's recommendation. The integrity of isolated RNA was verified using a 1,0% agarose gel. 2 μg total RNA was used for reverse transcription. RNA and random primer (Promega) were preheated for 10 min at 70 °C and then chilled on ice for 2 min. 5x RT-Avian myoblastosis virus (AMV) buffer (Promega), dNTP's (10 mM, Promega), RNAsin (Promega), AMV-buffer (Promega) and DEPC-H_20, were added to a final volume of 20 μl. Then, reverse transcription was carried out for 60 min at 42 °C and was stopped by heating to 95 °C for 5 min. The resulting cDNA was diluted with 80 μl DEPC and then frozen at −20 °C to await further analysis.

2.5. Primer design

Primer design was done with the program Primer 3 (http://www.genome.wi.unit.edu/cgi-bin/primer/prime3.cgi). Cross reaction of primers with the genes was excluded by comparison of the sequence of interest with a databank (Blast, US National Centre for Biotechnology Information).

2.6. Thermocycler RT-PCR

PCR reactions were done with a 20 μl reaction mixture that consisted of HotStarTaq Master Mix (Qiagen), DEPC, 1 μl of cDNA and 1,0 μM concentration of the 3′ and 5′-specific oligomers (synthesized by Invitrogen, Germany). PCR reactions were carried out with a thermal cycler (T3, Biometra, Germany) using the following conditions: 95 °C for 15 min, 94 °C for 30 s, followed by individual conditions for each gene as reported previously by us [11]. PCR reactions were done within the linear range of amplification and were separated using a 1,5% agarose gel. They were visualized by ethidiumbromide under UV transillumination. Quantification of PCR-products was done with the software of the Kodak Image Station version ID 3.5.

2.7. Hierarchical gene cluster analysis

Hierarchical gene cluster analysis was done as originally described by [12]. Gene expressions are given as ratios, where the area count of a gene of interest is divided by the area count of the housekeeping gene GAPDH. The ratios were log transformed and a hierarchical clustering algorithm produced a table of results wherein PCR experiments are grouped together based on similarities in their expression patterns and are presented graphically as coloured images. The genes are arranged as ordered by the clustering algorithm. The colour image is proportional to the ratio.

2.8. Statistical analysis

We used the General Linear Model (GLM) with the time as between-factor and the left–right ventricle as within-factor. If the time effect was