Journal of Clinical & Experimental Pathology

Galenko-Iaroshevsky et al., J Clin Exp Pathol 2017, 7:3
DOI: 10.4172/2161-0681.1000310

Research Open Access

Possibility of Inhibition of TNF-α/NF-kB Signaling Pathway Activation in Myocardium and Reverse Cardiac Hemodynamics in Chronic Ischemic Heart Disease

Galenko-Iaroshevsky PA[1], **Sukoyan GV**[2*], **Ionov DI**[1], **Zelenskaya AV**[1] **and Khvitia NG**[2]

[1]*Department of Pharmacology, Kuban State of Medical University, Krasnodar, Russian Federation*

[2]*Department of Molecular and Clinical Pharmacology, International Research Centre of Introduction of New Biomedical Technology, Tbilisi, Georgia*

[*]**Correspondence author:** Galina V. Sukoyan, Department of Molecular and Clinical Pharmacology, International Centre of Introduction of New Biomedical Technology, Kairskaya str, 19, Tbilisi, 0137, Georgia, Tel: +995595418030; E-mail: galinasukoian@mail.com

Received date: June 12, 2017; **Accepted date:** June 21, 2017; **Published date:** June 23, 2017

Abstract

Introduction: Chronic ischemic heart disease (CIHD) caused by long term aortic stenosis characterizes the refractory to the conventional therapy. We investigated whether the restoration of redox-potential in myocardium prevents the progression of the symptoms of congestive heart failure (CHF) and myocardial inflammation in rabbits.

Material and Methods: 8 weeks after induction of CIHD by left descending coronary artery stenosis all animals were randomly assigned into 3 groups: control II - without therapy (infusion of 0,9% NaCl); main I - animals received 5,0 mg/kg Lisinopril and metoprolol, 1 mg/kg body weight administrated in drinking water and furosemide 1.0 mg/kg i.v. (bolus) once daily and main II received 10 mg/kg of Adenocin dissolved in water for injection i.v, once daily. The control I group included sham operated animals and infusion of 0.9% NaCl. All animals were euthanized throughout 14 days after beginning of treatment.

Results: Long-term aortic stenosis has led to a simultaneously developing of CHF, diagnosed by developing cardiac hypertrophy, increased level of brain natriuretic peptide (BNP) and myocardial oedema. Treatment with Adenocin® significantly improved cardiac hemodynamics, decreased water content in myocardium and BNP in plasma, decreased the content of proinflammatory cytokines and unlike treatment with furosemide, lisinopril and metoprolol, increased level of anti-inflammatory interleukin-10. Restoration of myocardium redox-potential and level of NAD under treatment with Adenocin® led to decreasing the activity of nuclear transcription factor kappa B (NF-kB) and production of vasoconstrictor component of endothelial system, endothelin-1 in myocardium. Potential important link between cellular metabolism (hypoxia/ischemia) and innate inflammatory system homeostasis is the level of redox-potential, NAD/NADH in myocardium.

Conclusion: Influence of first oxidized form of NAD-containing positive inotropic drug Adenocin® leads to the decreasing symptoms of CHF and occurs beneficial action on the balance between pro- and anti-inflammatory cytokines in myocardium and activity of NF-kB.

Keywords: Inflammation; Experimental chronic ischemic heart disease; Redox-potential; Cytokine; Endothelin-1; NAD-containing positive inotropic drug

Introduction

Persistent cardiac hypertrophy caused by ischemic heart damage can ultimately progress to cardiac dilatation, decreasing ejection fraction of left ventricle (LV) and leading to chronic heart failure (CHF) which manifested pathological ventricular dysfunction, oxygen deficit states with reduction of redox-potential (NAD/NADH) and follows contributed by oxygen free radicals, cytokines and adhesion molecules hyperproduction, interstitial fibrosis and gene expression disturbances [1]. The decreasing and disturbances in gene expression alterations including connexin 43 (Cx43, suggested as a NAD-transporting channel into the cell), gap junction disorganization, that could be the triggering and maintenance of arrhythmias [1,2]. On the other hand, addition of exogenous NAD was capable of maintaining intracellular levels of NAD and blocking the ischemic-reperfusion injury [3,4] agonist-induced cardiac hypertrophic response *in vitro* as well as *in vivo* [5-8]. It was suggested, that replacements therapy of NAD blocked the activation of pro-hypertrophic proteinkinase B (Akt1) signaling, and augmented the activity of anti-hypertrophic liver kinase B1-AMP-activated protein kinase (AMPK) signaling in the heart and strengthening anti-inflammatory effect of cardiac glycoside throughout the blockade of TNF-α/NF-kB signaling pathways in a sirtuin (Sirt)-dependent manner [9-14]. Intracellular NAD concentration regulates TNF-α synthesis include action at post-transcriptional step in Sirt dependent manner [9]. The endogenous myocardial TNF-α concentration is increased during aortic banding, acute myocardial ischemia-reperfusion, after myocardial infarction, coronary microembolization and is causally involved in the development of ventricular dysfunction [8-11]. In fact, the cardiac myocytes in the heart themselves produce TNF-α and content of TNF-α per gram of tissue as high either the liver or the spleen, both of which possess large macrophage populations and are major sources of TNF-α [15,16]. However, still controversial whether or not increased TNF-α in plasma or serum under left ventricle dysfunction originates from the

heart itself or it is of peripheral origin secondary to gastrointestinal congestion. In this study, we investigate the dependence between changes in redox-potential, NAD/NADH and TNF-α/NF-kB signaling pathways on experimental cardiac hypertrophy and inflammatory response in myocardium, which is regarded as a risk factor of irreversible form of CHF, and efficacy of various therapeutic action to reverse this deterioration in CHF caused by long-term aortic stenosis in rabbits.

Material and Methods

All animal experiments and procedures received institutional approval and were conducted in conformity with the "Guiding Principles in the Care and Use of Animals" of the American Physiology Society and the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication No. 85-23, revised, 1985.

All animals were secured under specific pathogen free conditions according to the Federation of European Laboratory Animal Science Associations guidelines in humidity- and temperature-controlled environment, with a 12 h light and 12 h dark cycle. Chinchilla rabbits (2.5-3.0 kg) had free access to food and water ad libitum. After 7 days of adaptation, all animals randomized into two groups: control and main. The rabbits were kept in a daylit environment, in specially designed housed (animal room) at a mean temperature 20°C, humidity 40-70%, lighting 12 h per day for at least 1 week before the experiments. Animals were fed commercial laboratory rabbit food pellet and allowed drink tap water ad libitum before the experiments. Three month after induced cardiac hypertrophy by left descending coronary artery stenosis (banding (ligation) up to one third of the original size under sterile conditions [3,4]) the symptoms of CHF has been registered (average heart mass (g) to the body mass increased from the 2.22 ± 0.24 to 4.05 ± 0.24 (p>0,001) in the CHF groups and did not changed in the control (sham operation group 2.32 ± 0.19, NS). Average survival of animals in the interval 2-7 days was about 78% (54 from 69 animals in the experiments) in the operation group and 100% in the sham operated group.

After 8 weeks all experimental animals were randomized into three group: control III (n=14) were treated with either vehicle (normal saline), main group I, in which animals received traditional therapy of CHF, containing ACE inhibitors, Lisinopril, 5 mg/kg body weight/day administered in drinking water, β1-selective adrenoceptor antagonist, Metoprolol, 1 mg/kg and diuretics, Furosemide, 1.0 mg/kg intravenously (bolus), once daily during 14 days (n=16) and main group II in which animals received cardiotonic NAD-containing drug Adenocin®, 20 mg/kg solved in 1 ml of water for injection intravenously, once daily (n=15) for 14 days. The ventricles were trimmed of atria and visible blood vessels. The heart weight (HW) and left ventricular weight (LVW), LV free wall thickness and interventricular septal thickness, the ratio of HW (HW/BW) and LVW to body weight (LVW/BW) were obtained and weighing.

Brain natriuretic peptide (BNP) as a marker of CHF development, was determinate using MyBioSource ELISA assay. Measurement of NAD and NADH, tumor necrosis factor alfa (TNF-α), IL 1β, IL-6 and IL10 and Endothelin-1 (ET1) in tissue of left ventricle ELISA kits (R&D Systems) as described below [2,3,6,17,18]. The level of NF-kB (p65) was measured in nuclear extracts of cardiomyocytes and p50DNA binding activity was measured using ELISA with 10 g/L of nuclear extract with the Trans-AM kit (Extract Motif, Carlsbad, CA).

Statistical Analysis

Statistical significance was performed with SPASS 23 statistical package (SPSS Inc). Results as mean ± standard deviation (SD). A p value of less than 0.05 was considered as statistically significant.

Results

Cardiac hypertrophy and congestive heart failure development

Body weight was not significantly different between animals in control and main groups. The LVH was established by increases in HW by 29%, LV weight by 24%, HW/bodyweight (BW) by 28%, LVW/BW ratio by 39%, LV free wall thickness by 22% and interventricular septal thickness by 18% in comparison with healthy control I (sham-operated group) (Table 1).

Characteristics/Group	Control 1	CHF		
		Control 2 (CHF)	+ Traditional Therapy	+Adenocin®
Body weight, kg	3.24 ± 0.19	2.79 ± 0.17*	2.90 ± 0.15	3.12 ± 0.13#
HW, g	7.0 ± 0.8	8.9 ± 0.5**	7.7 ± 0.7	7,1 ± 0.4#
HW/BW x 10^{-3}	2.16 ± 0.13	2.95 ± 0.18**	2.63 ± 0.12**	2.26 ± 0.10##xx
LVW, g	4.2 ± 0.2	5.0 ± 0.2**	4.85 ± 0.12	4.30 ± 0.13##x
LVW/BW x 10^{-3}	1.3 ± 0.2	1.8 ± 0.3*	1,7 ± 0.2*	1,4 ± 0.2#
Left ventricle hypertrophy, %	100	136 ± 8***	129 ± 7***	106 ± 8##xx
Thickness of LVFW, mm	4.28 ± 0.18	5.21 ± 0.21**	4.98 ± 0.16**	4.32 ± 0.13##xx
Thickness of IVS, mm	3.88 ± 0.15	4.57 ± 0.18**	4.67 ± 0.12**	3.97 ± 0.18##xx

Table 1: Cardiac remodeling in rabbits under chronic ischemic heart disease.

Myocardial water content increased in control II up to 8.12 ± 11 mg/g (7.84 ± 12 mg/g tissue, p<0.01). Thus, with the increased in concentration of BNP up to 3.2 ± 4 pmol/L (in control 1.0 ± 4 pmol/L, p<0.001) obtained results strongly indicated that ischemic genesis congestive heart failure reproduced.

Relationship between redox-potential and cytokine profile, disturbances and efficacy of therapy

Intracardiac level of TNF-α, IL-1β and IL-6 was increased at long-term aortic stenosis in 3, 0, 4, 3 and 5, 9 fold while the level of anti-inflammatory IL-10 significantly did not changes (Table 2).

Parameters/Group	Control I	CHF		
		Control II	+Traditional Therapy	+Adenocin®
IL-1ß, pg/mg protein	3.4 ± 0.8	24.5 ± 1.8***	20.3 ± 1.7***#	5.8 ± 1.4##xx
IL-6, pg/mg protein	6.5 ± 1.6	38.2 ± 1.9***	21.0 ± 1.9***#	7.9 ± 0.9##x
IL-10, pg/mg protein	2.8 ± 0.9	2.9 ± 0.5**	2.7 ± 0.4**	5.2 ± 0.6##xx
TNF-α, pg/mg protein	0.7 ± 0.3	9.5 ± 0.8***	8.0 ± 0.9**	1.8 ± 0.7##xx
(IL-1ß+IL-6+ TNF- α): IL- 10	3.8 ± 0.6	24.9 ± 1.6***	18.3 ± 1.5***#	3.0 ± 0.9###xxx
NF-kB (p65) activity at 450 nm (od)	0,10 ± 0,02	0,24 ± 0,04***	0,20 ± 0,03***	0,12 ± 0,03###xxx
Endothelin-1, pg/mg protein	2.4 ± 0.6	3.9 ± 0.4***	3.4 ± 0.65**	2.1 ± 0.6##xx
NAD, μMol/mg protein	7.3 ± 0.6	5.4 ± 0.6***	5.3 ± 0.4***	6.9 ± 0.7##xx
NADH, μMol/mg protein	7.1 ± 0.7	6.8 ± 0.6	7.2 ± 0.7	7.1 ± 0.8
NAD^+-, Mol/mg protein	7.3 ± 0.6	5.4 ± 0.6***	5.3 ± 0.4***	6.9 ± 0.7##xx

Table 2: Changes in cytokines profile, NF-kB and redox-potential in left ventricle myocardium of rabbits with chronic ischemic heart disease.

Thus, the integrative parameter of ratio between pro-and anti-inflammatory cytokines in myocardium decreased and innate inflammatory process activated in this model of CHF by 500%. These accompanied with the decreasing of level of NAD in LV of myocardium by 27%, the sum of NAD+NADH by 15% and redox-potential NAD/NADH by 22.5%. The increasing of TNF-α in LV myocardium was negative correlated with level of NAD (r=-0.69, p<0.01) and more strongly with redox-potential NAD/NADH (r=-0.79, p<0.001). The redox potential, but not level of NAD, strongly correlated with integrative parameters of dysbalance in innate immune system (TNF-α+IL-1β+IL-6): IL10 (r=-0.81, p<0.001). The increasing of level of proinflammatory cytokines IL-1β and IL-6 did not appeared in linear correlation with decreasing of NAD in myocardium while decreasing of level of anti-inflammatory cytokine positive correlate with level of NAD (r=-0.69, p<0.02) and redox-potential NAD/NADH (r=-0.78, p<0.001). The level of ET-1 and level of NF-kB (p65) of LV myocardium of animals with CHF increased by 63% and 140%, respectively, and its changes negative correlated with redox- potential NAD/NADH (r=-0,81, p<0,001 and r=-0,74, p<0,001, respectively).

Cardiac growth was inhibited by treatment with traditional therapy (TT, combination of metoprolol, lisinopril and furosemide) by 7% and Adenocin® by 20% (Table 1). Both ratio of whole heart and LV weight to body weight notably decreased compared with the control groups in TT groups by 9% and 13% and in Adenocin®-treated group by 27% and 24%, respectively. At the same time, Adenocin® fully deremodeling the LV free wall thickness and interventricular septal thickness. These dates and decreasing of myocardial water content and level of BNP up to control level under treatment with Adenocin® and by 24% under treatment with traditional combination suggest that Adenocin® can stopped the development of LV hypertrophy and CHF induced by pressure-overload. Early was shown, that t-treatment with Adenocin® significantly reduced LV hypertrophy and improved LV performance in patients with CHF and could inhibition of the proinflammatory activities of cytokines in circulation [17,18].

We sought to determine whether exogenous NAD in form of the first oxidized NAD-containing cardiotrophic positive inotropic drug, Adenocin®, with multiple targets of action, independent of beta-receptors blockades, inhibits the development and progression the myocardial inflammation in CHF caused by long-term aortic stenosis. The productions of IL-6 and IL-1β in the heart tissue were reduced significantly in the combination of lisinopril, metoprolol and furosemide, and TNF-α showed a similar tendency. Under treatment with Adenocin® the level of all proinflammatory cytokines markedly decreased to control level and in contrast to traditional combination accompanied with the increasing the level of anti-inflammatory component of cytokines system, IL-10 and restored the activity of NF-kB (Table 2).

As a result the ratio between pro- and antiinflammatory cytokines decreased by 87% (p<0.001) while under traditional therapy only by 35% (p<0.01). The level of vasoconstrictor, endothelin-1, did not changed in traditional-treated group and significantly decreased in LV myocardium in Adenocin®-treated rabbits. Improvement in inflammatory link of myocardium in group with Lisinopril, metaprolol and furosemide therapy did not leads to improvement in the NAD-redox-potential signaling pathways while influence of Adenocin® increased the level of NAD and restored redox-potential in LV myocardium.

Discussion

The chronic pressure-overload-induced congestive heart failure accompanies with LV hypertrophy and deterioration in TNF-α/NF-kB signaling pathway in myocardium. The decreasing of level of redox-potential NAD/NADH, content of NAD and sum of NAD+NADH in LV hypertrophy myocardium confirms that develops maladaptive hypertrophy which coupled with injury in inflammatory and endothelial systems of the myocardium. The traditional therapy with β-adrenoblockers, inhibitor of ACE and diuretics leads to decrease of proinflammatory cytokine of TNF-α and IL-1β production, but not IL-6, and protect against acute endothelial injury induced by stress-induced atherosclerosis throughout ability of metoprolol to ameliorate sympathetic nerve sprouting in rabbits after myocardial infarction and is associated in part with inhibiting NF-κB activity [19-23].

Carvedilol, a beta-blocker and particularly α-1 receptor blockers containing an antioxidative property, inhibits T cell activation *via* downregulating NF-kB activity. Chronic administration of metoprolol during three years slows the progression of intima media thickness in humans and alters the grey scale of carotid plaques and decrease serum levels of proinflammatory cytokines [22,23]. On the chronic canine model of multivessel ischemic cardiomyopathy with left ventricular dysfunction, was shown that myocardial interstitial fluid occurred greater attenuation of leukocytosis and had higher IL-10 level with carvedilol compared to metoprolol, after 3 months of treatment resulted in better resting global and regional function as well as better regional function at stress compared to metoprolol. ACE- inhibitors, ramipril or qiunapril, as well as AT1 receptor blockage with losartan or valsartan, leads to inhibition NF-kB activity as a results of downregulation of activation of angiotensin II, the triggers for NF-kB, and thus reduces inflammation [19,20,24-26]. ACE inhibitors exert their effects by modulating the neurohumoral milieu, and may increase natriuretic peptides, bradykinin, and perhaps endothelin-1 in patients with CHF. Thus, in patients (n=107) with ischemic or dilated cardiomyopathy, New York Heart Association functional class II to III, with LV ejection fraction <40%, the ACE inhibitor lisinopril 20 mg/day tended to decrease BNP, proinflammatory cytokine IL-6 and ET-1 levels and increased anti-inflammatory cytokine IL-10.

In case of Adenocin, as NAD^+- and lower dose β-acetyldigoxin containing drug, decrease of inflammatory system activation occurs throughout direct action on the key points in immune response signaling pathways in myocardium. Level of NAD+ or more correctly redox-potential NAD/NADH, has previously been shown to regulate TNF-α synthesis and TNF-α to regulate NAD+ homoeostasis providing a link between a pro-inflammatory response and redox status. Moreover, the capacity to produce TNF-α appears to be directly correlated with intracellular NAD levels, and at the same time NAD as a cofactor of poly-(ADP-ribose) Polymerase (PARP) -1 has been shown to act as a transcriptional modulator of NF-kB [27].

Intracellular level of NAD were found to affect a relatively proximal step in the necroptotic signaling cascade initiated by TNF receptor1 by the reduced capacity of TNF to induced serine-threonine kinase 1/2 receptor interacting complexes in cell dysplaying low intracellular level of NAD. Despite the reduced number of regulatory T cells and an increased number of proinflammatory T helper 17 cells under immune homeostasis disturbances in various diseases, NAD able to promote an impressive allograft survival through a robust systemic IL-10 production independent of CD4+CD25+Foxp3 states [10]. These relationship and ability of NAD to prevent overproduction of proinflammatory cytokines strengthening by inosine which themselves prevent overproduction of proinflammatory cytokines, while it can enhance the production of the protective IL-10 *via* at least partially mediated *via* adenosine receptors and main at the posttranscriptional and did not involve interference with the activation of p38, p42/44, c-Jun N-terminal protein kinase, degradation of inhibitor of kappa B, or elevation of intracellular cAMP levels.

The posttranscriptional nature of inosine's mechanism of action can be considered as preferable to transcriptional inhibitors, because it is expected to increase the window of therapeutic opportunity, and may remain effective even in a posttreatment paradigm [28]. Additionally, cardiac glycoside, small doses of beta-acetyldigoxin in Adenocin®, included another antiinflammtory signaling pathways dependent of modulation of Na^+-K^+-ATPase which in turn regulated the inflammatory process, possibility efficacy *via* the Ca^{2+} signaling cascades or by increasing levels of endogenous brain hydrogen sulfide (H2S) [29]. Treatment with digoxin, in a mouse and human $CD4^+$ T cell culture, inhibited naïve CD4 polarization to Th17 cells and reduced IL-17A protein expression.

These effects were due to the inhibition of RORγt transcriptional activity. Furthermore, in experimental autoimmune encephalomyelitis, a multiple sclerosis model and Th17-mediated disease, treatment with digoxin, delays disease onset and reduces disease severity. IL-17 levels produced by infiltrating cells were also decreased [11,12,14]. Cardiac glycosides in lower doses were found to inhibit NF-kB signaling not by induce cellular apoptosis but through blocking TNF-α dependent binding of the TNF receptor to the TNF receptor-associated death domain in the TNF-α/NF-kB signaling pathway [20,29] and to suppress the upstream cascade of TNF-α/NF-kB signaling pathway in dose-dependent manner [11,12,14,30]. Early was shown beneficial effect of small dose of digoxin to decrease level of proinflammatory cytokines in patients with CH [1,13,17,18]. Thus, in the basis of beneficial therapeutic potential of Adenocin® leads its direct positive effect on key targets of inflammation *via* the restoration normal function of immune-metabolic-axis.

Acknowledgments

The authors express appreciation for E. Tsivtsivadze, Director of "Biotechpharm GE", Ltd Georgia, for the provision of preparation Adenocin for investigation.

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