Deterioration in Hemodynamics Reaction, Baroreflex Sensitivity, Sympathetic Nerve Activity and Redox State of Thoracic Aorta in the Experimental Model of Nitrate Tolerance and Its Pharmacological Correction

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Abstract

Continuous treatment with organic nitrates causes nitrate tolerance and provides evidence for a relationship between mitochondrial complex 1 activity and mitochondrial aldehyde dehydrogenase-2 (ALDH-2) with disturbances of the hemodynamics reaction during nitroglycerin (NTG) tolerance (NTGT). The purpose of this study was the evaluation of efficacy of original oxidized form NAD-containing drug, NADCIN®, on hemodynamic reactions, baroreflex sensitivity (BRS) and reflex control of splanchnic sympathetic nerve activity (SSNA), level of redox-potential, activity of ALDH-2 and superoxide anion generation in aortic tissue in rat model of NTGT. Five groups (7 - 9 each) of male Wistar rats, including control, acute i.v. NTG (150 mcg/kg) administration, NTG tolerance NTGT treatment with NADCIN® 8 mg/kg and methylene blue (MB, 2.5 mg/kg) were used. NTGT in rats was accompanied with the greatly attenuation of hemodynamics reaction, BRS, the decreasing of the ability to reflex control of SSNA without pronounce overexpression of endothelin-1 in vessels (aorta). In NTGT rats i.v. NTG along induced less hypotensive reactions and alterations in

heart period vs single NTG treated group, more expressively decreased BRS (~34%) and reflex control of SSNA (~18%). NADCIN® significantly inhibits tolerance-inducing properties of the prolonged nitroglycerin infusion (max decrease of blood pressure response to nitroglycerin injection, % of normal controls: NTGT 51.2%, NADCIN® 91.6%, MB 55.8%). NADCIN® in NTGT rats after NTG i.v. administration increased reduced BRS (+37.8%, p < 0.05), reflex control of SSNA (+29.4%, p < 0.05) and reversed the decreasing of NAD/NADH ratio, ALDH-2 activity and decreasing in superoxide generation in thoracic aortic tissue. Thus, course treatment with NADCIN® of NTGT rats restores hemodynamics changes, BRS and SSNA throughout the increasing of redox-potential NAD/NADH and cessates the NTGT developing.

Keywords
Experimental Model of Nitroglycerin Tolerance, Baroreflex Sensitivity, Aldehyde Dehydrogenase, Redox-Potential, Splanchnic Sympathetic Nerve Activity

1. Introduction

Organic nitrates and nitroglycerin (gyceryl trinitrate, GTN) in particularly, has long been one of the key medicines for cardiovascular diseases including coronary artery disease, acute myocardial infarction and congestive heart failure for more than 100 years. The anti-ischemic effect of NTG is believed to be based on the drug-induced decrease in preload and afterload, improvement of coronary collateral flow, dilatation of stenotic coronary arteries, and the inhibition of platelet aggregation. GTN, along with other lower potency nitrates induces coronary vasodilatation throughout to its bioconversion into relaxant agent nitric oxide (NO) [1]. Propose mechanisms include neurohormonal counterregulatory mechanisms to maintain blood pressure [2], increased production of superoxide anions [3]-[5] and oxidative stress as a result, reduced biotransformation of NTG to NO, and alterations in cyclic GMP metabolism [6], endothelial dysfunction, inhibition of nitroglycerin metabolizing enzyme, changes in GNT-signaling or endogenous active substances, and so on [7]. However, long-term administration of nitroglycerin results in progressive loss of vascular sensitivity to nitrate in rodents and humans, but diminished bioactivation of GTN by deterioration in ALDH2 activity appears to be the most plausible cause [8]-[10], and that this reaction is accelerated by an allosteric action of NAD⁺ [11]. The dysfunction of Complex I (presumably through the generation of NAD⁺ deficit and/or altered NADH/NAD⁺ ratio) exerts an inhibitory effect on ALDH independently of any reactive oxygen species (ROS) participation. It is thus likely that prolonged exposure to GTN tolerance manifested as increased ROS generation accompanied by alterations in NAD⁺ availability and/or altered NADH/NAD⁺ ratio, might provoke conformational (and other) changes in ALDH-2 undermining its activity [11] [12]. In according with this, the aim of the study was to investigated the ability of original reduced form NAD-containing drug, NADCIN® [13], on the hemodynamic reactions, baroreflex sensitivity, reflex control of sympathetic nerve activity and level of redox-potential, NAD/NADH and NADP/NADPH and ALDH activity in thoracic aorta tissue in nitroglycerin-induced tolerant rats.

2. Materials and Methods
2.1. Experimental Design

Experiments were carried out in 76 male Wistar rats weighing 250 - 300 g. Animals received humane care in compliance with “Guide for the Care and Use of Laboratory animals” (National Institutes of Health publication 86-23, Revised 1996) and was performed with approval of the local Interinstitutional (International Scientific Centre of Introduction of New Biomedical Technology, Department of Pharmacology, Faculty of Medicine, I.Javakhishvili Tbilisi State University and Department of Medical Pharmacology and Pharmacotherapy, Tbilisi State Medical University, Tbilisi) Animal Care and Use Committee. Nitroglycerin-induced tolerance was re-
produced by treatment with NTG (10 mg/kg, s.c.) three times a day for 8 days and was confirmed by a reduction in hypotensive responses to intravenous NTG [14]. All animals were randomized into the five groups: Control group—s.c. injection of 0.1 ml normal saline 3 times daily for 8 days; II—were given 0.1 ml 0.9% NaCl in the same frequency and period of administration and then NTG (in form of NITRO 5 mg/ml², Orion Corporation, Finland, 150 mcg/kg) i.v. injection; III—received NTG (10 mg/kg s.c) three times a day for 8 days and then NTG (150 mcg/kg) i.v. injection; IV—received NTG (10 mg/kg s.c.) three times a day for 8 days and NADCIN®, 8.0 mg/kg of body weight resolved in 0.1 ml of water for injection i.v. from the 4th days of NTG treatment and once daily 20 min after the NTG (150 mcg/kg) i.v. injection; V—were given s.c. injection of 0.1 ml 0.9% NaCl three times a day for 8 days and then received MB—2.5 mg/kg i.v. 20 minute before i.v. injection of NTG (150 mcg/kg). After 8 days the rats were anaesthetized with pentobarbital (60 mg/kg intraperitoneally).

2.2. Hemodynamics, Baroreflex Sensitivity and Splanchnic Sympathetic Nerve Activity Study

Polyethylene catheter was placed into the right femoral artery and connected to blood pressure transducer for measuring blood pressure (BP) with electromanometer and heart period (HP) with cardiotachometer. Intravenous injections were given via catheter inserted into the rigor femoral vein. Baroreflex sensitivity (BRS) was defined by measuring HP in response to rises BP 30 - 50 mm Hg above control after i.v. injection of phenylephrine (3 - 10 mcg/kg). The slope of relationship between BP and HP was used as an index of BRS according to [15]. Splanchnic sympathetic nerve activity (SSNA) was recorded according to [16]. A thin bipolar silver electrode was placed around a branch of the splanchnic nerve and isolated carefully with silicone rubber. Nerve activity was recorded via a cable connected to an adaptor. The nerve signal was amplified and rectified and the mean nerve activity was displayed on a polygraph. BRS was assessed with respect to control of SSNA as percentage of nerve inhibition from control per mmHg of BP rise (% mmHg) and with respect to control of heart rate (HR) as HR decrease per mmHg of BP rise (beats min⁻¹ mmHg⁻¹).

2.3. Biochemical Markers of Functioning of Thoracic Aorta

Thoracic aorta was frozen and homogenized in liquid nitrogen, before the experiments homogenate was rapidly placed in modified Krebs’ solution [17]. The powdered tissue was dispersed in 5 vol ice-cold aqueous 30 mM potassium phosphate buffer, pH 7.5, vortexed, sonicated, and centrifuged at 10.000 g for 10 min. Isolation of mitochondria was performed using “Mitochondria isolation kit” (BioChain Institute, Inc.) according to the manufacturer’s instructions. ALDH activity in the supernatant was determined by monitoring NADH formation from NAD+. The assay mixture (0.5 mL) contained 100 mM Tris·HCl (pH 8.5), 1 mM NAD⁺, 1 mM 4-methylpyrazole, and 100 µg protein. The reaction was started by addition of 1 mM propionaldehyde to the cuvette, and absorbance changes at 340 nm as a result of NADH formation were recorded for 10 min. The mean rate of absorbance change was taken as a measure of ALDH activity (0.0125 A₃₄₀ was equivalent to 1 nmol/mg/min). The ALDH-2 inhibitor benomyl (10 - 5 mol/L) was used as a negative control. The content of pyridine nucleotide in aortic mitochondries was measured using spectrofluorimetric methods [13] [18]. The protein concentration was determined with BSA protein assay kit. Superoxide anion generation in isolated rat heart mitochondria was determined immediately following the isolation procedure. Briefly, mitochondria (0.5 mg/ml) were incubated with buffer (6 mM succinate, 70 mM sucrose, 220 mM mannitol, 2 mM, Hapes, 25 mM KH₂PO₄, 2.5 mM MgCl₂, 0.5 mM EDTA, 5 µg/ml catalase, pH 7.4) at 37°C. At the indicated time points, 40 mM acetylated cytochrome c was added and the change in absorbance at 550 nm was measured for 1 min at 37°C. Background absorbance for all groups was determined by the addition of SOD (1000 units/ml). Endothelin-1 (ET₁) in aortic homogenate concentration (pg/ml) was measures by R & D Systems for Human endothelin-1 Immunoassay (Great Britain).

2.4. Statistical Analysis.

All values are expressed as the mean ± standard error deviation of at least 6 experiments. Statistical analysis for comparison between different groups of animals was assessed by two-way unpaired Student t-test. Significance was defined as p < 0.05 or less.

Each ml of concentrate for solution preparation containing: nitroglycerin saline 10% 52.5 mg (equivalent of 5.0 mg of nitroglycerin).
3. Results

3.1. Hemodynamics, Baroreflex Sensitivity and Splanchnic Sympathetic Nerve Activity

In anaesthetized rats the baseline values of BP and HP did not show any significant differences in all groups of animals (Table 1). The study of BRS revealed great attenuation of its cardiochronotropic vagal component in rats with NTGT (0.62 ± 0.08 ms/Hg mm⁻¹) vs. control animals (0.98 ± 0.1 ms/Hg mm⁻¹, p < 0.05) that correlated with the decreasing the ability to cause bradycardia reflex (1.9 ± 0.09 beats min⁻¹ Hg mm⁻¹) and an inhibition of SSNA (Table 1). The concomitant use of NADCIIN® in NTGT rats markedly increased the reduced BRS (+39%, p < 0.001) and its ability with respect to heart rate (HR) control (+65%, p < 0.001) and inhibition of SSNA (+38.2%, p < 0.01) resulting from NTGT animals. Treatment with guanyl cyclase inhibitor, MB, similarly to NTGT, but in less degree, caused reduction in BRS (0.72 ± 0.4 ms Hg mm⁻¹) and its properties with respect to control SSNA (1.7 ± 0.1 Hg mm⁻¹) vs control group of rats. Acute single dose of NTG (150 mcg/kg i.v) administration was accompanied with significant hypotensive reactions in non-tolerance normal rats and in group treated with NADCIIN®. BRS after NTG i.v. injection was more deeply blunted in MB treated group (0.68 ± 0.15 ms Hg mm⁻¹) and especially NTGT rats (0.51 ± 0.09 ms Hg mm⁻¹) vs. group after single i.v. treatment with NTG. NADCIIN® significantly inhibits tolerance-inducing properties of the prolonged nitroglycerin infusion (BPmax, nitroglycerin response in % of normal controls: NTGT 51.2%, NADCIIN®-91.6, MB-55.8%). NADCIIN® in NTGT rats after NTG i.v. administration increased reduced BRS (+37.8%, p < 0.05), reflex control of SSNA (+29.4%, p < 0.05).

3.2. Deterioration in Biochemical Markers of Redox State, Aldehyde Dehydrogenase System and Endothelial Function

Acute action of NTG did not induce significant changes in redox-potential of mitochondria of thoracic aortic tissue and did not change the rate of superoxide anion generation, with the tendency to decrease of ALDH-2 activity (Table 2). In NTGT rats after the i.v. injection of NTG the changes in BRS and SSNA was accompanied with significantly decrease of redox-potential NAD/NADH by 20% in thoracic aorta tissue (Table 2) without changes in total pyridine nucleotide pool (Table 2) and in the level of vasoconstrictor cytokine (ET-1) production in vessels. The decrease in redox-potential even without changes in the total pool of pyridine nucleotide was accompanied with increased of the rate of superoxide anion generation in 1.5 times vs NTG non tolerated group. Cellular redox status is an important regulator of energy fuel metabolism, and many enzymes are under regulation of NAD+/NADH and NADP+/NADPH ratios not only in normal condition but under various pathological states [19]-[21]. No change in NADP/NADPH redox-potential of mitochondria was observed under acute single-dose treatment with NTG in normal rats which accompanied with normal rate of superoxide generation and ALDH activity (Table 2). Under continue administration of NTG the reaction of following i/v injection of high

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control n = 12</th>
<th>C + NTG N = 10</th>
<th>NTG +NTG N = 9</th>
<th>NTG + NADCIIN® N = 9</th>
<th>NTG + MB N = 9</th>
<th>NTG + MB N = 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP, mmHg</td>
<td>126 ± 8</td>
<td>82 ± 7**</td>
<td>117 ± 6#</td>
<td>96 ± 7**</td>
<td>122 ± 5#</td>
<td>83 ± 4***</td>
</tr>
<tr>
<td>HP, ms</td>
<td>159 ± 8</td>
<td>116 ± 9***</td>
<td>148 ± 11**</td>
<td>125 ± 8**</td>
<td>160 ± 8**</td>
<td>126 ± 8***</td>
</tr>
<tr>
<td>BRS ms/ Hg mm⁻¹</td>
<td>0.98 ± 0.10</td>
<td>0.94 ± 0.1</td>
<td>0.62 ± 0.08*</td>
<td>0.51 ± 0.09**</td>
<td>0.86 ± 0.06***</td>
<td>0.82 ± 0.1***</td>
</tr>
<tr>
<td>HR beats min⁻¹ Hg mm⁻¹</td>
<td>3.6 ± 0.2</td>
<td>3.5 ± 0.2</td>
<td>1.90 ± 0.09*</td>
<td>1.7 ± 0.2**</td>
<td>2.9 ± 0.2**</td>
<td>2.8 ± 0.2**</td>
</tr>
<tr>
<td>SSNA% Hg mm⁻¹</td>
<td>2.2 ± 0.2</td>
<td>2.0 ± 0.2</td>
<td>1.64 ± 0.11*</td>
<td>1.23 ± 0.08**</td>
<td>1.94 ± 0.07</td>
<td>1.7 ± 0.2***</td>
</tr>
</tbody>
</table>

Notes: BP: blood pressure; HP: heart period; HR: heart rate (upon baroreflex activation); MB: methylene blue; n: number of animals in each group; *Comparison with control group; †Comparison with control +NTG 150 mcg group; †† With III(NTGT) group. One symbol: p < 0.05, two: p < 0.01, three: p < 0.001.
dose of NTG disturbed: occurs decreasing in NAD/NADH redox state (without significantly decrease in NADP/ NADPH potential) that leads to a increasing of generation of superoxide anion and to decrease of ALDH2 activity (Table 2) and give grounds to suggest that major source of oxidative stress in normal animals under NTG-tolerance formation is the mitochondrial complex 1 functioning disturbances [11]. Activity of mitochondrial ALDH significantly decrease (by 71%, p < 0.001 in comparison to normal animals and by 67% vs level after single i/v injection of 150 mcg/kg NTG) after the continue administration of NTG in normal animals and according with this the treatment with oxidized form NAD-containing drug, NADCIN®, is a new strategy for prevention and therapy of NTG-tolerance formation, strategy of replacement therapy.

4. Discussion

Experimental and clinical investigations have provided evidence that prolonged exposure to organic nitrates during cardiovascular disease induces tolerance and endothelial dysfunction [16] [22]. However there is controversial data suggesting the development of tolerance related to long-term continues nitrate therapy [1] [8] [11] [22] [23]. It was demonstrated that a nonvasodilated concentration of NTG exerts a direct myocardial anti-ischemic effect independent of its vascular actions in isolated rat hearts and conscious rabbits [21] [24]. In addition, this effect is not diminished by the development of vascular tolerance to NTG [22] [23]. In the state of nitrate tolerance well known that the enzymatic bioconversion of NTG to NO and the consequent increase is cGMP level is responsible for the vascular effects of NTG is impaired [6] [9] [25] and administration of NTG does not affect cardiac cGMP content in the rat heart in vivo [16] [25]. In other investigations long-term continuous NTG therapy has been associated with endothelium dysfunction and altered autonomic neural function, including impaired baroreflex activity and prevalence of sympathetic to parasympathetic tone in the regulation of heart rate [26] with increased sensitivity to receptor-dependent vasoconstrictors such as serotonin, phenylephrine, angiotensin II and thromboxane [16] [22]. BRS proved to be highly effective for correct identification the animals at high risk of developing ventricular fibrillation during transient ischemia. Vasodilator effects of nitrates on large arterial conductance vessels are, in general, preserved even in the presence of neurohormonal activation, while tolerance to NTG-induced changes in coronary flow is usually established [16]. These observations indicate that increased levels of vasoconstrictive agents encountered with the initiation of NTG therapy cannot override the vasodilator effects of NTG on large arterial vessels but may induce vascular constriction at the arteriolar level. Our experiments showed that in NTG tolerant rats BRS was greatly attenuated with reduction of its cardiochronotropic vagal component that correlated with decreased ability to cause reflex bradycardia.

Table 2. Influence of NADCIN® on the redox-potential, ALDH-2 activity and superoxide anion generation in aortic rings of rats with nitroglycerin tolerance.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>NTG, 150 mcg/kg</th>
<th>NTG tolerance</th>
<th>NTG tolerance + NADCIN®</th>
<th>NTG tolerance + MB</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD, nmol/mg wet tissue</td>
<td>6.75 ± 0.23</td>
<td>6.54 ± 0.37</td>
<td>6.10 ± 0.27*</td>
<td>6.80 ± 0.22*</td>
<td>6.09 ± 0.14*</td>
</tr>
<tr>
<td>NADH, nmol/mg wet tissue</td>
<td>6.28 ± 0.39</td>
<td>6.55 ± 0.27</td>
<td>7.05 ± 0.17*</td>
<td>6.25 ± 0.17*</td>
<td>6.98 ± 0.12*</td>
</tr>
<tr>
<td>Redox-potential, NAD/NADH</td>
<td>1.08 ± 0.08</td>
<td>1.0 ± 0.1</td>
<td>0.86 ± 0.06**</td>
<td>1.09 ± 0.09*</td>
<td>0.87 ± 0.07*</td>
</tr>
<tr>
<td>NADPH, nmol/mg wet tissue</td>
<td>5.70 ± 0.14</td>
<td>5.54 ± 0.17</td>
<td>5.40 ± 0.20</td>
<td>5.80 ± 0.20</td>
<td>5.39 ± 0.24</td>
</tr>
<tr>
<td>Redox-potential, NADP/NADPH</td>
<td>4.67 ± 0.33</td>
<td>4.55 ± 0.19</td>
<td>4.65 ± 0.20</td>
<td>4.65 ± 0.15</td>
<td>4.68 ± 0.22</td>
</tr>
<tr>
<td>Total pool of pyridine nucleotide</td>
<td>23.4 ± 0.5</td>
<td>23.2 ± 0.4</td>
<td>23.2 ± 0.4</td>
<td>23.5 ± 0.4</td>
<td>23.1 ± 0.3</td>
</tr>
<tr>
<td>ALDH-2, NADH nmol/mg/min</td>
<td>2.25 ± 0.38</td>
<td>1.98 ± 0.27*</td>
<td>0.65 ± 0.07***</td>
<td>2.32 ± 0.23**</td>
<td>1.75 ± 0.24*</td>
</tr>
<tr>
<td>Superoxide anion, µmol/mg protein/min</td>
<td>28 ± 4</td>
<td>30 ± 7</td>
<td>45 ± 10</td>
<td>27 ± 5</td>
<td>46 ± 6</td>
</tr>
<tr>
<td>ET-1, µg/mg protein</td>
<td>2.34 ± 0.21</td>
<td>2.14 ± 0.23</td>
<td>2.76 ± 0.21</td>
<td>2.03 ± 0.13</td>
<td>2.57 ± 0.18</td>
</tr>
</tbody>
</table>
and an inhibition of SSNA. Such alterations in this group of animals were associated with increased of the level of redox-potential, NAD/NADH, but not NADP/NADPH, that more markedly was expressed after NTG intravenous administration. Thus, even in normal animals without significant increase in vasoconstrictor and proinflammatory cytokine, ET-1 production (Table 2), in a response to continue NTG infusion occurs decreased in the redox-potential of mitochondria of thoracic aortic tissue that leads to the overproduction of superoxide anion in aortic (vessels) tissue and as a result the risk of superoxide scavenging by NO with the generation of the strong oxidant peroxynitrite (ONOO\(^-\)) increased under continue treatment with the NTG and could be one of the intimate mechanism of NTG tolerance formation. We showed that mitochondria isolated from aorta after the NTG tolerance developed in normal animals the content of ET-1 does not significantly rise (increased only for 18%, N.S.) and about 1, 6 fold greater amount of superoxide anion production compared to untreated animals.

It was demonstrated that is NTG-induced tolerance the decreased release of CGRP is associated with the decrease in aldehyde dehydrogenase (ALDH-2) activity [11] [24] [27]. NTG produced a depressor effect concomitantly with an increase in plasma CGRP concentration, which may be prevented by the pretreatment with ALDH-2 inhibitors. These results correlate with our previous investigation where CGRP receptors antagonist reduced NTG hemodynamic effects in NTG tolerant rats and increased mortality in these animals during acute myocardial infarction [3] [7] [14]. The oxidative stress concept could be compatible with the multiple different observations associated with long-term nitrate therapy [28].

5. Conclusion

NADCIN\(^R\), unlike MB, has ability to restored hemodynamic reaction and BRS in NTG tolerant rats that were associated with improved control of the SSNA throughout restoration of NAD/NADH redox-potential sate and decreased oxidative stress under NTG continue treatment. Because cellular level of NAD can modulate the SIRT1 activity (enzymatic activity of SIRT1 depends on the cellular level of NAD [11] [12]) and expression. Decreasing of the redox-potential and enhancing of oxidized state of endothelium of vessels and shear-stress deterioration may be one of the possible mechanisms by which laminar/pulsatile flow is disturbed and deteriorated vascular homeostasis. Restoring of the NAD/NADH potential with following normalization the activity SIRT1 functioning could prevent endothelium cell dysfunction [29] and counteract with the prevention of atherosclerosis development and NTG-tolerance formation. In the clinical study it was shown that NAD containing drug in patients with ischemic heart disease restored the redox-potential, decreased the superoxide production and ET\(_1\) plasma level improving clinical symptoms of ischemia-reperfusion injury in patients with chronic form [25]. Thus, the reduction of redox-potential NAD/NADH is the target-point in the development of tolerance to NTG in normal animals which accompanied by inadequate functioning of the ALDH and inducer of oxidative stress formation even without significant deterioration in endothelin-1 system functioning. Course treatment of NADCIN\(^R\), first original reduced form of NAD-containing drug, leads to complete normalization in sensitivity to NTG and to restoration of the redox-potential NAD/NADH in aortic tissue and to elimination of the overexpression of superoxide anion and to the changes in ALDH functioning.

Conflict of Interest

The authors report no conflict of interest. The authors alone are responsible for the conduct and writing of this manuscript.

Authorship Contributions

Participated in research design: Gongadze N, Sukoyan G, Kezeli T. Conducted experiments: Sukoyan G, Dolidze N, Mirziashvili M, Chipashvili M. Contributed new reagents or analytical tools: Sukoyan G, Gongadze N, Chapichadze Z. Performed data analysis: Gongadze N, Sukoyan G, Kezeli T, Dolidze N. Wrote or contributed to the writing of the manuscript: Gongadze N, Sukoyan G, Kezeli T, Dolidze N.

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