Functional nitric oxide conjugate systems state/restored heart thiols of rats in modeling isadrine-pituitrin’s myocardial infarction using metabolite-tropic cardioprotector “Angiolin”

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INTRODUCTION

According to modern researches, endothelial dysfunction (ED) is one of the primary pathogenetic elements of cardiovascular diseases (myocardial infarction [MI], ischemic heart diseases, cerebral ischemic stroke, atherosclerosis, arterial hypertension, pulmonary hypertension, heart failure, dilated cardiomyopathy) as well as obesity, hyperlipidemia, diabetes and hyperhomocysteinemia. The main mechanism underlying ED is the decrease of nitric oxide (NO) formation and bioavailability at simultaneous level increase of superoxide anion and of potent vasoconstrictor’s production, and the decrease of energy products in endotheliocytes. Consequently,
ED is presented by imbalance between the mediators, which naturally provide all optimal endothelium dependent processes. The essential clinical and experimental aim is to search capabilities for task-oriented correction of ED, but at the same time, there are no medications for specific correction.\textsuperscript{1,3,6,8} Such metabolite-tropic medications as mildronate, trimetazidine, piracetam, and mexidol are used in the complex therapy for pharmacorerection of energy dysmetabolism, oxidative stress, hyperlipidemia, NOS in hypertensive disease, ischemic heart disease, chronic heart failure, cerebrovascular pathology.\textsuperscript{1,3,6-10,15} However, the lack of true endothelial protective properties and low antioxidative and the anti-ischemic activity in these medications leads to find more active drugs.\textsuperscript{8} The researches in the field exploring endothelial protective solutions indicate promising use of medications containing lysine.\textsuperscript{7} The influence of L-lysine on neurotransmitter systems and proinflammatory cytokines is showed. The substituted 1,2,4-triazolil-5-carbothioic acids with metabolite-tropic and antioxidant action are also noteworthy.\textsuperscript{5,6} All abovementioned served as ground for establishing at SPA “Farmatron” (President - Professor I.A. Mazur) a brand new metabolite-tropic cardioprotector of original structure - (S)-2,6-diaminohexanoic acid 3-methyl-1,2,4-triazolil-5-thioacetate entitled “Angiolin” which shows anti-ischemic, endothelial protective, antioxidant properties.\textsuperscript{5} Taking into account the molecular and biochemical mechanisms of myocardial ischemic injury and pharmacological properties of “Angiolin,” the aim of this work is to study the effect of “Angiolin” on thiol-disulfide system’s indicators and NO in experimental MI conditions.

METHODS

Experiments were performed on 40 Wistar rats weighing 190-210 g, received from the nursery of the Institute of Pharmacology and Toxicology, AMS of Ukraine. All manipulations were carried out according to the position on the use of animals in biomedical experiments (Strasbourg, 1986, as amended in 1998). The work received the approval of Ethic Committee of Zaporozhye State Medical University (excerption from Protocol No. 2, 09 April, 2014). MI was modeled by phased administration of isadrine and pituitrin according to the following scheme: pituitrin - 0.5 U/kg - abdominally, after 20 mins isadrine - 100 mg/kg - subcutaneously, every 6 hrs isadrine injection was repeated and after 24 hrs both agents were administrated in the same doses.\textsuperscript{11} We used pituitrin injection of AB “Endokrininiai” (Lithuania) production and isadrine of “Sigma” (USA). The investigated medication “Angiolin” was 3-fold administered within 24 hrs, combining the formation of MI abdominally 30 mins before the pituitrin and isadrine injection in dose of 100 mg/kg.\textsuperscript{7}

There were four groups of animals: (1) intact (10 rats); (2) control - untreated with MI (10 rats); (3) animals with MI treated with “Angiolin” (10 rats); (4) animals with MI treated with the reference medication mildronate - standard of cardioprotection’s metabolite-tropic (10 rats). The animals were taken from the experiment under anesthesia using thiopental (40 mg/kg) 60 mins after the last isadrine injection. Heart was quickly picked up and fixed in liquid nitrogen. The mitochondrial and cytosolic fractions were isolated by differential centrifugation on refrigerating centrifuge Sigma 3-30k (Germany).\textsuperscript{11} The activity of NO systems/restored thiols was estimated by the activity of NO-synthase (NOS), the content of nitrotyrosine, nitrite, arginine, methionine, cysteine, the level of total SH-groups and the activity of glutathione reductase (GR). The stable NO metabolites were determined in the cytosolic fraction of rat heart homogenate using BCM Diagnostics kits at 540 nm detection. The total NOS activity was determined in the cytosolic fraction of rat heart homogenate using NOSDetect<sup>TM</sup> Assay Kit kits, Strategene production, containing NOS inhibitors - N-nitro-L-arginine. Nitrotyrosine content was determined in the cytosolic fraction of rat heart homogenate by enzyme-linked immunosorbent assay (ELISA) (Nitrotyrosine ELISA KIT, Bycoyl biotech). The L-arginine, methionine, cysteine content in homogenates of rat’s myocardium was determined by infrared FOSS NIRSystems analyzer, model 5000. The activity of GR was determined in the cytosolic fraction of rat heart homogenate using BCM Diagnostics kits at 340 nm detection. The total content of SH-groups was determined spectrophotometrically by reaction with 5,5-dithio-bis -7 - nitrobenzoic acid at 412 nm detection.\textsuperscript{4} The protein was determined by the Lowry method. The spectrophotometer Libra S70 PC (Biochrom Ltd. production, United Kingdom) was used in the work.

The heart was fixed for 24 hrs in Carnoy’s fluid for histological studies and then, according to the standard scheme, was poured in blocks by paraplast ×100, which were prepared as serial frontal 14-micron histological sections in the apex of the heart.\textsuperscript{4}

The intensity of inducible NOS (iNOS) and endothelial NOS (eNOS) expression was determined by isolation of heart histological sections from paraplast and rehydration, thrice outwashing for 5 mins with phosphate buffer (pH=7.4) and incubation for 30 mins with 2N hydrochloric acid (T=37°C). Then sections were washed twice with phosphate buffer (pH=7.4) for 5 mins, twice with borate buffer by Holmes (pH=8.4) for 5 mins and 4 times with phosphate buffer (pH=7.4) for 5 mins, then within 30 mins were incubated with 0.1% trypsin solution in phosphate buffer (T=37°C). After incubation, the sections were washed 4 times for 5 mins with phosphate buffer (pH=7.4) and then within 24 hrs were incubated in a humid chamber (T=4-6°C) with a primary polyclonal rabbit’s antibodies IgG (1:500) eNOS (R-20 # SC-648) Santa Cruz Biotechnology, Inc., (USA) production. After incubation, the sections were washed 4 times for 5 mins with phosphate buffer (pH=7.4). Then, within 1 hr (t=37°C) sections were incubated with secondary goat antibodies to
mouse IgG fragment conjugated to a fluorescent coloring agent (FITC) of the Sigma-Aldrich company (Cat. No. F 2266). The iNOS expression was also determined by outwashing of sections after incubation 4 times with a phosphate buffer (pH=7.4) and then by incubating of sections within 24 hrs in a humid chamber (T=4-6°C) with a primary polyclonal iNOS (C -20 # SC-654 FITC) antibodies, conjugated with a fluorescent coloring agent (FITC) Santa Cruz Biotechnology, Inc. (USA) production. At final 4 times washing with phosphate buffer (pH=7.4), sections were embedded in a mixture of glycerol-phosphate buffer (9:1). The intensity of NOS isoforms expression was determined on fluorescent microscope Axioskop (Ziess, Germany) by density of eNOS-positive cells in the sections through the COHU - 4922 (USA) camcorder and the results were introduced into a digital image analysis system VIDAS - 386 (Kontron Elektronik, Germany).

Statistical data manipulation was performed using the standard analysis package of computer program “STATISTICA® for Windows 6.0” (StatSoft Inc., No. AXXR 712 D 8 33214 FAN 5). Data are presented as the sample mean±standard error of the sample mean. Verification of normality was performed using the Shapiro-Wilk test. Accuracy of differences between experimental groups E Memo was assessed using t - Student’s t-test and Fisher’s exact test.

RESULTS

Simulation of MI leads to inhibition of the synthesis of NO, performing in terms of myocardial ischemia the role of endothelial protective and cardioprotective factor. The decrease of NOS activity by 51, 7.5%, the decrease in production of oxide-nitrate stable metabolites by 43.9% in the setting of the frank deficiency of substrate synthesis-L-arginine (reduction in the myocardium by 17.6%) indicated this conclusion (Table 1). Our data do not contradict the results of other researchers who have shown that rats with MI have the decrease of NOS in the aortic wall, the increase in iNOS activity in the heart and the decrease of eNOS activity. However, the increase of the expression of iNOS activity does not compensate total infarction NOS activity, and in the presence of L-arginine deficiency, leads to ROS synthesis enhancement.8 We have found the reduced density of eNO-positive cells in the myocardium of animals with MI and the increased density of iNO-positive cells by hystoimmunochemic procedures (Table 1). The pathogenetic link between eNOS and iNOS in myocardial ischemia lies in the fact that the expression of so-called molecules increased as a result of the eNOS inhibition and corresponded suppression of NO production by endothelium. These observations indicate that the myocardial infraction suppresses eNOS expression, which has clear cardioprotective effect and enhances the iNOS expression, which participates in the nitrosating stress program.

In parallel we recorded the abnormality of NO transport – the level of thiol-containing amino acids (cysteine by 56.5%, methionine by 24.4%) and the total number of restored thiols (38.7%) reduced (Table 2). It is well known that NO is unstable, short-lived radical and for its stabilization and subsequent transportation there are such mechanisms as the formation of thiol-containing low-molecular compounds

Table 1: Influence of “Angiolin” and reference medication on NO indicators in the myocardium in the process of experimental infraction (M±m).

<table>
<thead>
<tr>
<th>Investigated indicators</th>
<th>Intact (d=10)</th>
<th>MI (control) (d=10)</th>
<th>MI+“Angiolin” 50 mg/kg (d=10)</th>
<th>MI milodronate, 100 mg/kg (d=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOS activity (nM/mg protein/mins)</td>
<td>32.5±1.33</td>
<td>15.7±1.10</td>
<td>21.0±1.00</td>
<td>14.5±1.33</td>
</tr>
<tr>
<td>Density of eNOS-positive cells (rd)</td>
<td>482.5±17.6</td>
<td>217.5±14.8</td>
<td>280.7±20.5*</td>
<td>215.7±23.5</td>
</tr>
<tr>
<td>Density of iNOS-positive cells (rd)</td>
<td>127.6±20.1</td>
<td>185.4±20.5</td>
<td>142.1±11.0*</td>
<td>192.4±25.1*</td>
</tr>
<tr>
<td>Nitrotyrosine (nM/g tissue)</td>
<td>18.7±1.15</td>
<td>30.5±1.22</td>
<td>17.5±1.33*</td>
<td>30.7±2.12</td>
</tr>
<tr>
<td>L-arginine (mcm/kg tissue)</td>
<td>9.1±0.7</td>
<td>7.5±0.1</td>
<td>8.8±0.4</td>
<td>7.0±0.5</td>
</tr>
<tr>
<td>NO stable metabolites (NO−2) (mcm/g protein)</td>
<td>18.2±0.85</td>
<td>10.2±0.56</td>
<td>17.12±1.12*</td>
<td>10.77±0.89</td>
</tr>
</tbody>
</table>

*p<0.05 relative to the control group, †p<0.05 relative to milodronate group. NO: Nitric oxide, NOS: Nitric oxide-synthase, MI: Myocardial infarction, eNOS: Endothelial nitric oxide-synthase, iNOS: Inducible nitric oxide-synthase

Table 2: Influence of “Angiolin” and the reference medication on the indicators of restored thiols system in the myocardium in the process of experimental infraction (M±m).

<table>
<thead>
<tr>
<th>Investigated indicators</th>
<th>Intact (d=10)</th>
<th>MI (control) (d=10)</th>
<th>MI+“Angiolin” 50 mg/kg (d=10)</th>
<th>MI milodronate, 100 mg/kg (d=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine (mcm/kg tissue)</td>
<td>4.9±0.1</td>
<td>3.7±0.03</td>
<td>5.0±0.05*</td>
<td>3.9±0.1</td>
</tr>
<tr>
<td>Cysteine (mcm/kg tissue)</td>
<td>2.3±0.03</td>
<td>1.0±0.01</td>
<td>2.7±0.01*</td>
<td>1.1±0.07</td>
</tr>
<tr>
<td>Common restored SH-groups (mcm/g tissue)</td>
<td>147.8±7.4</td>
<td>90.5±5.1</td>
<td>138.5±8.08*</td>
<td>98.7±3.1</td>
</tr>
<tr>
<td>Glutathione reductase activity (mcm/mg protein/mins)</td>
<td>18.7±0.71</td>
<td>9.6±0.31</td>
<td>27.5±0.21*</td>
<td>14.7±0.77</td>
</tr>
</tbody>
</table>

*p<0.05 relative to the control group, †p<0.05 relative to milodronate group. MI: Myocardial infarction
(glutathione, cysteine, methionine) of stable S-nitrazol’s complex. The given lack of thiol’s compounds disrupted the NO transportation because it’s attacked by reactive oxygen species - superoxyradical and hydroxylradical with the conversion of cytotoxic product - peroxynitrite. We have found the increase of peroxynitrite marker in the myocardium of experimental animals - nitrotyrosine by 63.1% in the MI modeling. Peroxynitrite plays a significant role in strengthening the ischemic lesion of cardiocytes, intensifying the reaction of oxidative and nitrosating stress. The level of thiols is settled by such enzyme as GR. The inhibition of GR activities by 48.6% is observed in acute myocardial ischemia.

DISCUSSION

The prescription of “Angiolin” to the rats with MI has no significant effect on the NOS activity, L-arginine level and iNOS-NOS-positive cells density, i.e., has no direct influence on the NO production in ischemia. However, “Angiolin” has unique properties to provide a protective effect on NO transport due to saving the restored thiols. Thus, it was found that “Angiolin” injection to animals with MI exceeds the level of restored thiol’s groups by 53.3%, and thiol-containing amino acids - methionine by 35.1%, cysteine – by 170%, both through direct antioxidant action of thiol’s groups in the molecule of the medication, and by increasing GR activity by 186%. The reference medication mildronate do not have a protective effect on the thiol-disulfide system. In addition, we assume that “Angiolin” itself may be NO transmitter, forming with it stable S-nitrosyl complexes. Thus, “Angiolin” prevents the conversion of NO under the influence of reactive oxygen species in peroxynitrite (as evidenced by the reduction in nitrotyrosine by 42% under the influence of studied medication), maintaining its endothelial protective properties that distinguishes it from mildronate which does not not show similar properties. The prescription of mildronate to the rats with MI did not have a protective effect on the NO system. The mechanism of the anti-ischemic action of mildronate in acute myocardial ischemia consists in its influence on metabolic energy chain, which provides an adequate function of cells in low oxygen. These effects are achieved by reducing of the intensity of the oxidation of fatty acids in the ischemia (energy saving) and by activation of glycolysis for energy production. Some authors suggest that false mildronate esthers are structurally analogous to acetylcholine. Consequently mildronate through the stimulation of acetylcholine receptors apparently may cause the induction of eNOS. As a result, the synthesis of NO increases. From there mildronate was selected as a reference medication. This effect of mildronate was not seen on the model of isadrine pituitrin - MI.

CONCLUSIONS

1. The prescription of “Angiolin” 2.5% solution abdominally in dose of 50 mg/kg to rats with MI resulted to the increase of NOS activity by 33.7%, of the density of eNOS-positive cells by 29%, of the level of stable NO metabolites by 70% and the reduce of nitrotyrosine by 42% and of the density of NOS-positive cells by 23.3% in heart tissue.
2. The injection of “Angiolin” to animals with MI increases the content of restored thiols by 53%, methionine by 35%, cysteine by 174% and the increase of GR activities by 186%.
3. Our results indicate that “Angiolin” normalizes the ratio of conjugated NO-thiol-disulfide systems and the fact that this medication has endothelial protective and antioxidant properties in experimental MI.
4. “Angiolin” significantly excels the efficacy of mildronate reference medication by therapeutic effect.

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Conflict of interest: None declared
Ethical approval: The study was approved by the Ethics Committee of Zaporozhye State Medical University (excretion from Protocol No. 2, 09 April, 2014)

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