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Myocardial Concentrations of Inositol and cAMP in Intact Failed Rats

S. Paterna¹, P. Di Pasquale², A. Antona³, L. Bellanca³, O. Barberi¹, G. Licata⁴

There is a paucity of data on the way (cAMP, inositol), qualitatively or quantitatively, altered in heart failure (HF). These studies were performed separately on animal models, but no study checked cAMP and inositol changes in the same animal model simultaneously. In the present study, an experimental model was used that allowed us to check both simultaneously in the same failed intact animals. Rats weighing 180–240 g and aged 8–12 weeks, underwent uninephrectomy (41 rats survived). After a 10-day observation period, DOCA was injected at 30 mg/kg s.c. once a week for 2, 3 and 5 weeks, during which period 1% NaCl solution was given as drinking water. The blood pressure (BP) increased after 2 weeks of the treatment and uninephrectomy. After 2 weeks 8 rats were injected i.v. every 12 h with 25 micro C/kg (in 0.5 ml) of myo-3H-inositol. After 3 and 5 weeks another 12 and 14 rats underwent the same experiment. Samples of myocardial tissues were obtained from the anterior wall. The tissue samples were used to check the value of cellular cAMP and inositol from the same heart to check for the first time simultaneously cAMP and inositol levels in hearts from intact failed rats during different steps of HF. In addition, samples of normal myocardial tissues were obtained from 7 (controls) untreated and not nephrectomized rats. After 2, 3 and 5 weeks of treatment, all rats showed different but progressive overt signs of HF. The values of cAMP and inositol showed a not significant increase in cellular cAMP concentrations and a not significant change in DPM inositol levels in comparison with those from control tissues, while the percent values showed a significant reduction, p < 0.001. The value of DPM GPI and Inos 1P showed a significant difference as well as the percent value of inositol, GPI, inositol metabolites (1P, 2P, 3P). The inositol metabolites levels showed an initial significant reduction, except for the Inos3P and a subsequent increase in the rats treated for 3 and 5 weeks. This experimental method allowed us to study for the first time the concentrations of inositol metabolites and cAMP cellular concentrations simultaneously and in different stages of HF in intact animals. It appears that the changes of inositol metabolites happened in the first weeks after treatment, and during the subsequent weeks the values showed a light gradual recovery. The most important result of our study was the feasibility of the experimental model and the changes in inositol metabolites and cAMP concentrations during the progression of HF. J Clin Basic Cardiol 2002; 5: 247–51.

Key words: cAMP, heart failure, inositol, intact animals.

Several reports have demonstrated that the density of ventricular myocardial beta-adrenoceptors (BAR) is reduced in animal models and in patients with congestive HF, regardless of aetiology [1]. This reduction in BAR density is associated with a reduced sensitivity of myocardial contractile responses to beta-adrenergic stimulation [1]. In addition, it has been suggested that there is a reduction in basal cAMP in the failing heart, and that this is due to deficiencies in production separate from BAR [2]. The biochemical changes of chronically overloaded cardiac cells are not as well defined. Changes in composition of the hypertrophied and dilated myocardial cell may consist of alterations of contractile proteins by modification of gene expression probably triggered by stretching of the myocytes. Regulating structures like local hormone systems or receptors, G proteins, or ion channels and pumps may be altered, as well as calcium handling by the sarcoplasmic reticulum, coupling of energy flux between the myofibrils and mitochondria. The result is a myocyte that generates force and relaxes more slowly. Signal transduction pathways between membrane proteins that catalyze the formation of intracellular signals, such as cyclic nucleotides, inositol phosphates, or diacylglycerol or that determine the concentration of the cytosolic free Ca²⁺ are altered [3–5]. Stretch of myocardial cells generates the same intracellular signals, cAMP, inositol phosphates, and diacylglycerol [5, 6]. In addition, other authors showed an alteration of inositol trisphosphate [7–10]. When a suitable agonist binds to the alpha-1-adrenoceptor in the myocardium activate catalytic moiety of phospholipase C, situated on the inside of cell membrane phospholipid bilayer. The activated enzyme breaks down inositol lipid in the plasma membrane, particularly phoshatidylinositol 4,5 biphosphate (IP2), with production of second messenger, inositol 1,4,5 trisphosphate (IP3) and diacylglycerol (DAG) There is paucity of data on the way (cAMP, inositol), qualitatively or quantitatively, altered in heart failure (HF) [6, 11, 12]. These studies were performed separately on animals models, but no study checked cAMP and inositol concentration changes in the same animal model simultaneously. In the present, we used an experimental model that allowed us to check simultaneously and in the same animal [13] the values of cAMP and inositol in failed intact animals.

Materials and Methods

We chose male wistar rats weighing 180–240 g and aged 8–12 weeks, receiving a standard regimen for small animals. All the rats underwent uninephrectomy, after ether anaesthesia, to induce hypertension to result in HF. We divided the rats in 4 groups: group 1 control (7 rats), group 2 (10 rats) receiving treatment for 2 weeks, group 3 (14 rats) receiving treatment for 3 weeks, and group 4 (17 rats) receiving treatment for 5 weeks. After a 10-day observation period (7 rats died), desoxycorticosterone acetate (DOCA, CIBA) was injected at 30 mg/kg s.c. once a week for 2 weeks (8 rats), 3 weeks (12 rats), and 5 weeks (14 rats), during which period 1% NaCl solution was given as drinking water (7 rats died before performing experiment) (Figure 1). Blood pressure (BP) was
monitored by tail-cuff plethysmography after preheating for 10-15 min at 37 °C. Systolic blood pressure (SBP) was measured twice weekly. The SBP increased after 2 weeks of the treatment and uninephrectomy (181 ± 13 mmHg), and after 3 weeks (195 ± 12 mmHg). After 5 weeks all the rats of group 4 were hypertensive (SBP 203 ± 17 mmHg). After 2 weeks group 1 (8) rats were injected intravenously every 12 h with 25μCi/kg (in 0.5 ml) of myo-3H-inositol. Treatment was given three times (every 12 h). Animals received the last injection 12 h before the experiment. Twelve hours after the last injection, the rats were given 1,000 IU of heparin i.v., and following anaesthesia with ether, the hearts were removed by thoracotomy and placed in ice-cold Krebs-Henseleit solution. Hearts were perfused in an apparatus (Radnoti-Gass technology, Monrovia, CA) by the non-recirculating Langendorff technique at 37 °C with an oxygenated (95 % O₂, 5 % CO₂) Krebs-Henseleit solution (NaCl 117 mmol/l; KCl 4.7 mmol/l; CaCl₂ 3 mmol/l; MgSO₄ 1 mmol/l; EDTA 0.5 mmol/l; Glucose 16.7 mmol/l; NaHCO₃ 24 mmol/l; pH 7.4). The temperature was constantly maintained at 37 °C, and the pressure kept at 80 mmHg. Isolated hearts were connected through an interventricular pressure transducer to a four-channel recorder Quartet (U. Basile, Varese, Italy) to ensure serial recording of heart rate (HR) and ventricular pressure. Systolic and diastolic ventricular pressures (SVP, DVP) and the intraventricular pressure time ratio (dp/dt) were measured through a small water-filled balloon connected with this apparatus. Coronary flow (CF) was also measured. All these parameters were visualized on a Philips P15 monitor and measured for the entire period of the investigation. Before the experiment started, the hearts were perfused for a 30-min period of equilibration. Thereafter, they were perfused under aerobic conditions for a 30 min period. During the course of the entire experiment, a periodic recording of haemodynamic parameters was performed (basal, at 10 min, 20 min and 30 min). At the end of the first 30 min perfusion period, samples of myocardial tissues were obtained from the anterior wall were frozen in liquid nitrogen immediately by precooled Wollenberg clamps, and stored until biochemical analysis was performed. In addition, samples of normal myocardial tissues were obtained from 7 hearts (controls) perfused under aerobic conditions after a 30 min period of equilibration. After Langendorff’s perfusion, the hearts were dissected, dried and weighed. Ventricular hypertrophy was evaluated according to Fulton criteria[14]. After three weeks group 3 rats (12 rats) underwent the same experiment (as above reported) as well as after five weeks the group 4 rats (14 rats) underwent the experiment previously described. The ratios of left ventricle to body weight (LV/BW), right ventricle to body weight (RV/BW) were determined for each heart. Left ventricular hypertrophy was presumed at an LV/BW ratio > 2–4 mg/g, together with overt signs of heart failure. The tissue samples obtained were used to check the value of cellular cAMP and inositol from the same heart, in this way we checked simultaneously cAMP and inositol cellular levels in the same myocardial tissue from intact failed animals during different steps of HF.

Cyclic AMP Assay
The cAMP concentrations in left ventricular tissues were determined by a commercially available radioimmunoassay kit (Amersham International, Amersham, UK). Briefly, samples of ventricular tissues were thawed and homogenized at 4 °C in 1.5 ml solution of 0.6-mol HClO₄ per litre with a Polytron PT-10 (Kinematica-Krienz, Luzern, Switzerland) set to rotate at 6 for 30 sec. The homogenate was centrifuged at 15,000 RPM (Beckman Model L8-80) for 20 min. The pellet was discarded, and the supernatant was neutralized with 250 μl of a 3.0-mol/l TRIS-BASE solution and eluted in a aluminium oxide chromatographic column [15]. The eluate was evaporated under a nitrogen stream and resuspended in 0.5 ml of 4-mmol TRIS-EDTA buffer (pH 7.5) per litre. The suspension was centrifuged at 12,000 RPM for 2 min in an Eppendorf microfuge, and cAMP was assayed in the supernatant as previously described [16].

Inositol Metabolites Assay
Frozen tissue samples from hearts were thawed, weighed, and homogenized in 2.5 ml of a Krebs-Henseleit solution at 4 °C (three bursts of 30 sec each, followed by a 30 sec intercooling period each) in a Willem Polytron PT-10 (Kinematica-Krienz, Luzern) set to rotate at 6. Samples were then added with 5 ml of cold methanol and 5 ml chloroform, vigorously vortexed and left to stand at room temperature for 1 hr [17]. After this incubation period, samples were centrifuged at 1,000 g for 10 min. Two millilitres of the upper phase of the 1,000 g supernatant were applied to columns containing 1 ml of Dowex 1 (88:formate form; Sigma) and eluted three times with 3 ml of distilled water. 5 mmol disodium tetraborate per litre / 60 mmol sodium formate per litre; 0.1 mmol formic acid per litre / 0.2 mmol ammonium formate per litre; 0.1 mmol formic acid per litre / 0.4 mmol ammonium formate per litre; and 0.1 mmol formic acid per litre / 1.0 mmol ammonium formate per litre [18, 19]. Radioactivity was determined in eluted fractions.

Statistical analysis
Data are expressed as mean ± SD. Two-way analysis of variance was used for comparison within each group with correction by Bonferroni test. A two-tailed unpaired t-test was used for comparison of means between groups. Values of p < 0.05 were considered as statistically significant.

Results
Of the 41 rat hearts studied, 7 were used as controls (and baseline values; no uninephrectomy and DOCA treatment), 8 hearts from failed rats were studied 2 weeks after

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**Figure 1. Study protocol**

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**Table 1. Study protocol**

<table>
<thead>
<tr>
<th>48 rats</th>
<th>Uninephrectomy 41 rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 rats died</td>
<td>10 days observation</td>
</tr>
<tr>
<td>34 surviving rats</td>
<td></td>
</tr>
<tr>
<td>DOCA treatment</td>
<td>1 % NaCl</td>
</tr>
<tr>
<td>After 2 weeks 3H-inositol administration</td>
<td>8 rats sacrificed</td>
</tr>
<tr>
<td>After 3 weeks 3H-inositol administration</td>
<td>12 rats sacrificed</td>
</tr>
<tr>
<td>After 5 weeks 3H-inositol administration</td>
<td>14 rats sacrificed</td>
</tr>
<tr>
<td></td>
<td>7 sacrificed untreated rats</td>
</tr>
</tbody>
</table>
uninephrectomy, DOCA treatment and 1% NaCl solution as drinking water (Figure 1), 12 hearts from failed rats were studied 3 weeks after uninephrectomy and DOCA treatment, and 14 hearts from failed rats 5 weeks after uninephrectomy and DOCA treatment. The hearts of all animals sacrificed after 2, 3 and 5 weeks of DOCA administration showed signs of increased mass. In addition, all the rats after 2, 3 and 5 weeks of treatment showed different but progressive overt signs of HF. The rats sacrificed after 5 weeks showed reduced signs of HF in comparison with rats sacrificed at 3 and 5 weeks, respectively. The rats sacrificed after 5 weeks showed the greater signs of HF. Ventricular hypertrophy was related to time (weeks) from starting treatment. The hearts studied after 2 weeks were less hypertrophized than those studied after 3 and 5 weeks. SBP rose during the 2, 3 and 5 weeks of treatment with DOCA and uninephrectomy in all animals. All the hearts either after 2, 3 and 4 weeks from treatment showed an LV/BW ratio > 2.4 mg/g. At four weeks, final SBP was 210 ± 21 mmHg. The hearts showed indices of ventricular hypertrophy in all rats sacrificed after 5 weeks after DOCA treatment and uninephrectomy: LV/BW 3.12 ± 0.17, LV/RV 5.23 ± 0.11, RV/ BV 0.59 ± 0.03. The SBP and LV mass were significantly increased in all treated animals as compared with control rats (unshown data). SVP and dp/dt ratio showed a reduction, while DVP was increased in comparison with control rats (unshown data). HR showed an increase and coronary flow did not show significant changes in comparison with controls (unshown data). Tables 1 and 2 show the values of inositol and cAMP. The values of cAMP and inositol from failed tissues showed a not significant increase of cellular cAMP concentrations and a not significant changes in DPM inositol levels in comparison with those from control tissues, while the percent value showed a significant variation, p < 0.001. The value of DPM GPI and Inos 1P showed a significant difference at variance analysis, while the percent value of inositol, GPI, Inos 1P, 2P and 3P showed significant changes to variance analysis (two ways). The values of cAMP showed a progressive increase in tissues from failed hearts according to the period of treatment (2, 3, 5 weeks). In fact, cAMP showed increase in failed hearts studied after the 2nd week, and this increase continued also in the failed hearts studied in the 3rd and 5th week even if the differences were not significant. The inositol metabolite levels showed a initial significant reduction (in failed hearts from rats treated for 2 weeks) and a subsequent increase in the hearts from treated rats for 3 and 5 weeks. The changes were significant at variance analysis. The Inos 3P showed a slight increase after 2 weeks (NS) and a reduction after 3 weeks (NS) with a subsequent increase (NS) in the 5th week. All percent values showed significant variations when analyzed by analysis of variance (two way) (Fig. 2).

### Table 1. Changes in cAMP (fmol/mg 10) concentrations in myo-cardial tissues from failed rats according to weeks of treatment

<table>
<thead>
<tr>
<th>cAMP concentrations</th>
<th>Controls (7 hearts)</th>
<th>II Week (8 hearts after 2 weeks)</th>
<th>III Week (12 hearts after 3 weeks)</th>
<th>V Week (14 hearts after 5 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP concentrations</td>
<td>524.34 ± 284.99</td>
<td>616.95 ± 185.14</td>
<td>686.53 ± 149.88</td>
<td>745.20 ± 260.50</td>
</tr>
<tr>
<td>p = NS</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

### Table 2. Labelling of water-soluble inositol metabolites in rat heart (left ventricular), percent of radioactivity of water-soluble inositol metabolites

<table>
<thead>
<tr>
<th>Radioactivity DPM</th>
<th>Percent radioactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (7 hearts)</td>
<td>Radioactivity DPM</td>
</tr>
<tr>
<td>After 2 weeks (8 hearts)</td>
<td>After 3 weeks (12 hearts)</td>
</tr>
<tr>
<td>Inositol</td>
<td>2831.25 ± 786.92</td>
</tr>
<tr>
<td>GPI</td>
<td>*417.25 ± 37.34</td>
</tr>
<tr>
<td>Inos 1P</td>
<td>*563.75 ± 61.63</td>
</tr>
<tr>
<td>Inos 2P</td>
<td>*501.50 ± 19.84</td>
</tr>
<tr>
<td>Inos 3P</td>
<td>*529.75 ± 14.00</td>
</tr>
</tbody>
</table>

**Percent radioactivity (%)**

<table>
<thead>
<tr>
<th>Radioactivity DPM</th>
<th>Percent radioactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (7 hearts)</td>
<td>Radioactivity DPM</td>
</tr>
<tr>
<td>After 2 weeks (8 hearts)</td>
<td>After 3 weeks (12 hearts)</td>
</tr>
<tr>
<td>Inositol</td>
<td>*57.50 ± 5.48 %</td>
</tr>
<tr>
<td>GPI</td>
<td>*8.83 ± 1.97</td>
</tr>
<tr>
<td>1P</td>
<td>*11.75 ± 1.58</td>
</tr>
<tr>
<td>2P</td>
<td>*10.50 ± 1.72</td>
</tr>
<tr>
<td>3P</td>
<td>*10.98 ± 2.04</td>
</tr>
</tbody>
</table>

Water-soluble inositol metabolites in the aqueous phase were reported in Dowex-1 (formate form) columns. Radioactivity from the fraction ranged from 150 to 16,500 DPM. Results are means ± SD of 7, 8, 12, 14 hearts. GPI = glycerophosphoinositol; *p = 0.0001, *p = 0.001, **p = 0.01
Discussion
The sympathetic system is stimulated in HF and is an early and important compensatory response. The BAR is situated on the outer surface of the sarcolemma and is coupled to adenyl cyclase by the stimulatory G protein. Adenyl cyclase is the only enzyme system producing cAMP and specifically requires low concentrations of adenosine triphosphate as substrate. A basic feature of the concept of cAMP as second messenger is its rapid turnover as a result of a constant dynamic balance between its formation by adenylyl cyclase and removal by another enzyme, phosphodiesterase. In general, changes in the tissue content of cAMP can be related to the effects of catecholamines in stimulating the contractile activity of the heart. Beta-1-adrenergic stimulation is followed by an increase of tissue cAMP then the active kinase A increases, and thereafter there is increased contractility. The overall effect of beta-1-adrenergic stimulation includes the positive inotropic effect, the relaxant effect, the chronotropic effect, and the dromotropic effect. It is controversial whether B-2-adrenergic receptors are linked, as beta-1-receptors, to the adenyl cyclase-cAMP-protein kinase A signal system [20]. The beta-adrenergic cAMP system is down-regulated in severe HF. Excess circulating catecholamines are thought to be detrimental. The major changes are: defective functioning of adenyl cyclase, B1-receptor downgrading, and relative upgrading of the B2-receptors, which may be associated with an impaired contractile response [21]. In HF caused by ischemic cardiomyopathy, the alpha1-receptor system undergoes several changes, including increased receptor density and downstream changes that limit the ultimate inotropic response [22]. In the myocardium from end stage of HF studies have shown that Gi is increased [23], which may lead to inhibition of adenyl cyclase and poor formation of cAMP. Inos P3 is one of the second messengers and stimulates the release of Ca++ from sarcoplasmic reticulum, which explains why alpha-receptor stimulation causes vascular smooth muscle to contract without any entry of Ca++ from the outside. Although P3 travels to the sarcoplasmic reticulum to liberate Ca++, 1,2-diacylglycerol stays in the cell membrane, being highly lipophilic. It stimulates into activity another protein kinase (C). Besides P3, at least two other similar compounds, P4 and P5, may form in response to alpha-adrenergic stimulation. Normally, Inos P3 metabolism in intact cells was studied upon incorporation of (3H) inositol into Inos Pn. Apart from artefacts introduced by differences in extraction procedures, problems in studying Inos P3 metabolism arise from the fact that the dephosphorylation reactions are extremely rapid [24–26]. The latter problem is partially circumvented by the addition of Li+ that inhibits the Inos P3 and Inos P2-1-phosphatase and Inos P-monophosphatase activities [27].
Up to now, inositol was studied in cultured cells and isolated hearts namely to study reperfusion arrhythmias [17]. These studies have been undertaken by studying D-myo(3H)-inositol incorporation at cellular level [28]. Recent studies have additionally determined the inositol levels in myocardial tissue [29]. In these experiments, the isolated rat hearts were pre-labelled with 3H-inositol for 2 hr by a non-recirculating perfusion technique according to Langendorff [29]. Previous studies by Strosznajder [19] have suggested to us the possibility to investigate also in vivo the metabolic turnover of inositol in intact animals. In a previous experiment, we studied the inositol turnover in the heart of rabbits injected i.v. with 3H-myo-inositol to assess the differences in the distribution of phospho-inositol metabolites between control and ischaemic hearts [13]. In the present report we used the same protocol in failed rat hearts. The 3H-inositol was administered i.v. for three different times (every 12 hours) at the end of the 2nd, 3rd and 5th week of treatment (uninephrectomy and DOCA administration). Twelve hours after the last administration, we checked 3H-inositol and cAMP in myocardial tissues. We obtained different groups at different times and levels of HF. In fact, rats studied after 2 weeks of treatment were less decompensated than those studied after 3 weeks and 5 weeks. Our method allowed us to study the concentrations of inositol metabolites and cAMP cellular concentrations simultaneously and in different steps of HF in intact animals. In fact, 3H-inositol was administered before sacrificing rats and the hearts were not perfused with 3-H-inositol as reported previously. The experimental model evidences different values of cAMP and inositol metabolites, and these findings, perhaps are due to the adrenergic stimulation during the decompensated status. Inositol showed (heart rats used as control received 3H-inositol before being sacrificed) statistically significant differences in comparison with the controls, especially for percent values. It appears that the changes of inositol metabolites happened in the first weeks after treatment, and during the subsequent weeks the variations showed a light gradual recovery. We are not able to explain this later recovery of inositol metabolite levels. It is possible that the different technique may alter the inositol concentrations. Our study further evidences the low supply cost of our experimental model in comparison with in vitro assays due mainly to lower amount of labelled compound needed to undertake these studies in vivo. To our knowledge, this study is the first one that has measured the cardiac concentrations of inositol in intact failed animals. We thought that inositol determination after i.v. administration was more physiological compared to that measured by cellular cultures and perfusion of isolated failed hearts [28, 29]. It is possible that i.v. administration allows inositol to recycle binding to myocardial tissue following physiological turnover. Further studies are required to confirm our data.

References
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