Intravenous administration of the natriuretic peptide urodilatin at low doses during coronary reperfusion limits infarct size in anesthetized pigs

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Abstract

Objective: It has been shown that cGMP content is reduced in post-ischemic myocardium, and that stimulation of cGMP synthesis prevents cardiomyocyte hypercontracture and cell death in vitro. This study was aimed at determining whether administration of the natriuretic peptide urodilatin (URO) at the time of reperfusion could limit myocardial cell death secondary to transient coronary occlusion.

Methods: The relation between cGMP content in reperfused myocardium and the extent of cell death was investigated in isolated rat hearts (n=62) receiving different URO concentrations during initial reperfusion. The dose of intravenous URO necessary to obtain the targeted increase in cGMP in reperfused myocardium was investigated in ten pigs submitted to transient coronary occlusion (CO), and the effect of two selected doses of URO on infarct size was investigated in 22 pigs.

Results: cGMP was severely reduced in post-ischemic rat hearts. Addition of 0.01 μM URO during the first 15 min of reperfusion had no effect on myocardial cGMP content, functional recovery or LDH release in hearts submitted to 40 or 60 min of ischemia. At 0.05 μM, URO increased myocardial cGMP to 111% of values in normoxic hearts, improved functional recovery (P<0.01) and reduced peak LDH released by 40% (P<0.02). The beneficial effect of urodilatin was abolished by ANP receptor inhibition. At 1 μM, URO increased cGMP in reperfused myocardium to 363% of normoxic controls and had no beneficial effect. In pigs allocated to 47 min of CO and 5 min of reperfusion, cGMP was markedly reduced in reperfused myocardium. Intravenous URO at 10 ng/kg per min during the first 25 min of reperfusion normalized myocardial cGMP after 5 min of reflow (95% of control myocardium), and reduced infarct size by 40% (P=0.04). At 50 ng/kg per min, urodilatin increased myocardial cGMP in reperfused myocardium to 335% of control myocardium and failed to significantly reduce infarct size (46 vs. 66%, P=0.125). None of these doses had detectable hemodynamic effects.

Conclusions: Intravenous low-dose URO at the time of reperfusion normalizes myocardial cGMP and limits necrosis. Large doses of URO increasing myocardial cGMP well over normal values may lack this beneficial effect.

Keywords: Ischemia; Natriuretic peptide; Necrosis; Reperfusion; Second messengers

1. Introduction

During myocardial reperfusion, excessive contractile activation resulting from restoration of energy supply in the presence of abnormally high cytosolic Ca$^{2+}$ may result in disruption of cell architecture (hypercontracture), sarcolemmal rupture and cell death [1]. Hypercontracture causes an abrupt and extreme reduction of cell length during the initial minutes of reperfusion [2], and results in a characteristic ‘histological pattern of contraction band necrosis’ [3], the most prominent type of necrosis observed after early reperfusion [4–6]. If hypercontracture is prevented by transiently blocking contractility during the initial minutes of reperfusion, while cardiomyocytes recover normal Ca$^{2+}$ homeostasis, contraction band necrosis and infarct size can be limited [6,7]. However, complete contractile blockade requires regional, intracoronary administration of the blocker, and BDM, the only effective
blocker of actin-myosin cycling so far available, has many toxic effects.

We have previously shown that cGMP is reduced in myocardial cells after prolonged ischemia [8], and that stimulation of cGMP synthesis at the time of re-energization has an inhibitory contractile effect in reperfused myocardium, being able to prevent re-energization induced hypercontracture in isolated cardiomyocytes [9], isolated hearts [8], and in situ hearts [10]. Cyclic GMP can be synthesized by either soluble or membrane-bound guanylyl cyclase (sGC and mGC, respectively) [11]. sGC activity can be stimulated by increasing NO availability either directly, by NO donors, or indirectly, by increasing the availability of l-arginine, the substrate for NO synthesis. The effect of administration of NO donors during reperfusion is controversial, probably due to potential harmful free radical effects of NO [12,13], interaction with the anion superoxide [14,15], and difficult control of the actual amounts of NO released. l-Arginine supplementation has been shown to protect against reperfusion-induced hypercontracture by a cGMP dependent mechanism, and has consistently been found effective in limiting infarct size in the in situ heart [10], but has to be administered before ischemia [16].

In the isolated, crystalloid-perfused rat heart submitted to transient zero flow ischemia, stimulation of mGC with urodilatin, an ANP related peptide, at the time of reperfusion induces a rapid increase in cGMP in reperfused myocardium, and limits hypercontracture and cell death [8]. However, it is not known whether this approach is feasible during coronary reperfusion in vivo. To investigate this, the doses required to induce a sufficiently rapid and intense increase in cGMP concentration in reperfused myocardium have to be determined. Administration of the largest tolerated doses may not be an adequate approach, since it has been suggested that a too intense stimulation of cGMP synthesis could be detrimental to reperfused myocardium, and may result in enhanced apoptotic cell death [17].

The purpose of this study was to determine whether, and at what doses, intravenous administration of urodilatin at the time of coronary reperfusion can protect myocardium against cell death. The relation between cGMP concentration in reperfused myocardium and the extent of myocardial cell death was investigated in isolated rat hearts receiving different urodilatin concentrations at the onset of reperfusion. The doses of intravenous urodilatin necessary to obtain the targeted increase in cGMP in reperfused myocardium were investigated in a series of pigs submitted to transient coronary occlusion, and the effect of two selected doses on infarct size was investigated in a larger series of experiments in this model. Finally, an additional series of experiments in the isolated rat heart was used to confirm that the beneficial effect of urodilatin was mediated by stimulation of ANP receptors. Urodilatin is normally present in urine but not in blood, and possesses a pharmacokinetic profile more favorable than ANP, with a longer plasma half life [18,19], and it has been safely administered to patients [20].

2. Methods

Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and all experimental procedures were approved by the Research Commission of the Hospital General Vall d’Hebron.

2.1. Studies in the isolated rat heart

Hearts from male Sprague-Dawley rats, weighing 300–350 g, were perfused with a modified Krebs-Henseleit bicarbonate buffer (KHB) at 37°C using a Langendorff apparatus, at a constant pressure of 60 mmHg and LV pressure was monitored through the use of a water-filled latex balloon as previously described [8]. LV developed pressure was calculated as the difference between peak systolic and end-diastolic values. A total of four sets of experiments were performed. In the first set of experiments (n=20), hearts were subjected to 40 min of ischemia and reperfused for 60 min, and were allocated to receive during the first 15 min of reperfusion urodilatin at the concentration of 0 (control group), 0.01, 0.05 and 1 μmol/l. This series was used to assess the effect of different urodilatin concentrations on functional recovery. This effect cannot be assessed in the series submitted to 60 min of ischemia (see below) since after this duration, there is virtually no recovery. In the second set of experiments (n=16), the hearts were allocated to the same groups of treatment but reperfusion was reduced to 10 min, and myocardial cyclic GMP content was determined by radioimmunoassay using acetylated [3H]cGMP, as previously described [21]. In the third set of experiments (n=22), ischemia was extended to 60 min, and lactate dehydrogenase (LDH) activity was spectrophotometrically measured in the coronary effluent as previously described [16]. The 60-min series was necessary to assess the reduction of LDH and necrosis by urodilatin, that were minimal in the 40-min series. Finally, another group of rat hearts was submitted to 60 min of ischemia followed by 30 min of reperfusion, and were allocated (n=4) to receive either placebo or urodilatin 0.05 μM plus the ANP receptor antagonist isatin [22] during the first 15 min of reperfusion.

2.2. Transient coronary occlusion in the in situ pig heart

A total of 22 Large White pigs (34.7±1 kg) were premedicated with 10 mg/kg azaperone i.m., anesthetized...
with thiopental 30 mg/kg i.v., intubated and mechanically ventilated with room air. Thiopental was used to maintain deep anesthesia. A midline sternotomy was performed and the left anterior descending coronary artery (LAD) was dissected free at its midpoint. Two pairs of ultrasonic crystals inserted into the inner third of the left ventricular wall were used to monitor myocardial segment length in the LAD and circumflex territory [6]. End-diastolic (EDSL) and end-systolic segment length (ESSL) were defined as the distance between crystals at end-diastole and end-systole, respectively. Systolic shortening was calculated as the difference between EDSL and ESSL divided by EDSL and expressed as %. A pressure transducer catheter (Mikro-tip, Millar Instruments, TX, USA) was advanced into the left ventricle, and a transit time flow probe (T-106, Transonic Systems, NY, USA) was placed around the LAD to monitor coronary blood flow. A thermometer placed in the esophagus was used to monitor temperature.

2.2.1. Protocol

Instrumentation was followed by a 30-min waiting time after which the LAD was occluded during 47 min followed by reperfusion. The experiments were divided in two series. In a first series of experiments ten animals were used to measure the effect of urodilatin on myocardial cGMP content in reperfused and control myocardium. These animals were allocated to receive the placebo solution, urodilatin at a dose of 10 ng/kg per min or urodilatin at a dose of 50 ng/kg per min during 10 min, starting 5 min before coronary reperfusion. The apical portions of the ventricles (including the area at risk) were sectioned at 5 min of reperfusion with a dermatome blade and rapidly immersed in liquid nitrogen. The whole procedure lasted less than 5 s. In the second series of experiments, 22 animals had reperfusion time prolonged for 2 h, and the effect on infarct size of two doses of urodilatin was analyzed. A total of ten animals were randomly allocated to receive urodilatin at a dose of 10 ng/kg per min or placebo during 30 min, starting 5 min before reperfusion, and 12 animals were allocated at random to receive either urodilatin at dose of 50 ng/kg per min or placebo. The drug was infused into a femoral vein (Harvard Syringe Infusion Pump 22, Harvard Apparatus, South Nathick, MA, USA).

2.2.2. Plasma urodilatin and cGMP concentrations

Venous blood samples (5 ml) for urodilatin and cGMP determination were withdrawn at 15 min before occlusion; at 10, 20, 30, 42, 44 and 47 min of coronary occlusion; and at 5, 15, 25, 27, 30, 35, 45, 60, 90 and 120 min of reperfusion. The samples were placed in an ice-filled tray and plasma was obtained in the following 30 min. Plasma samples were stored at −20°C until processed. Plasma urodilatin levels were measured by radioimmunoassay using a specific antibody against the NH₂ terminal part of urodilatin [20]. Plasma cGMP concentration was measured by radioimmunoassay using acetylated [³H]cGMP as previously described [21].

2.2.3. Myocardial cGMP content

In order to measure myocardial cGMP concentration, frozen myocardial fragments were obtained from reperfused and control myocardium, pulverized under liquid nitrogen, and homogenized using cold trichloroacetic acid at 7.5% (w/v). Cyclic GMP concentration was measured in the homogenates as previously described [8].

2.2.4. Area at risk and infarct size

After 2 h of reperfusion the LAD was re-occluded and 5 ml of 10% fluorescein was injected into the left atrium. The heart was excised, cooled at 4°C, and cut into 5–7-mm slices perpendicular to its long axis. After being weighed in a precision balance (Precisa 180 A, Zürich, Switzerland) the slices were illuminated from the basal side with ultraviolet light to outline the area at risk, and digital images were obtained (Olympus Digital Camera C-1400L, Olympus Optical, Tokyo, Japan). The slices were then incubated at 37°C for 10 min in 1% triphenyltetrazolium chloride (TTC) buffered at pH 7.4 and imaged again under white light with a reference scale. The area at risk and the area of necrosis were measured in the digitized images using commercially available software (MicroImage, Olympus Optical, Hamburg, Germany). The masses of myocardium at risk and of the necrotic myocardium were calculated from these measurements and from the weight of the slices as previously described [6].

2.3. Statistical analysis

Investigators blind to treatment allocation performed all measurements. Statistical analysis was performed using commercially available software (SPSS for Windows 8.0). The homogeneity between groups was tested by ANOVA test for independent samples. Changes in segment length and physiologic parameters were studied by means of the MANOVA test. Post-hoc analysis was performed by means of the Scheffe test. A critical P-value of 0.05 was used for all tests. All values are expressed as mean±S.E.M.

3. Results

3.1. Studies in the isolated rat heart

3.1.1. Effects of urodilatin on myocardial function

After 40 min of ischemia, LV end-diastolic pressure and heart rate were identical in all groups of treatment, but LV developed pressure during reperfusion was significantly higher in hearts receiving 0.05 μM urodilatin during the first 15 min of reperfusion (P=0.01, Fig. 1). In hearts submitted to 60 min of ischemia, there was virtually no
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Fig. 2. LDH release during reperfusion in isolated rat hearts. The horizontal dashed bar indicates the time during which the treatment solution was added to the reperfusion buffer. *P<0.05 compared to control.

recovery of contractile function after ischemia in any of the groups.

3.1.2. LDH release

In hearts subjected to 60 min of ischemia, reperfusion was followed by a marked LDH release with a peak at 3 min of reperfusion. The addition of 0.05 µM urodilatin during the first 15 min of reperfusion significantly reduced LDH release (47.4±4.7 U/gdw per 30 min, vs. 69.6±4.5 U/gdw per 30 min in placebo group, P<0.01) (Fig. 2), whereas no protective effect was observed with 0.01 and 1 µM urodilatin. No LDH release was found in the hearts submitted to 40 min of ischemia. The beneficial effects of 0.05 µM urodilatin on LDH release were abolished by the simultaneous administration of the ANP receptor inhibitor isatin (56.5±1.1 U/gdw per 30 min in hearts receiving urodilatin+isatin vs. 64.1±5.9 U/gdw per 30 min in the corresponding placebo group, P=NS).

3.1.3. Myocardial cyclic GMP content

Myocardial cyclic GMP content in hearts perfused under normoxic conditions for 50 min was 36.3±4.7 fmol/mg of protein. In hearts subjected to 40 min of ischemia and 10 min of reperfusion, and receiving 0 (placebo) or 0.01 µM of urodilatin, myocardial cGMP content was significantly reduced in reperfused myocardium, as compared to normoxic control myocardium (Fig. 3). Cyclic GMP content in hearts allocated to receive urodilatin 0.05 µM was similar to that observed in control hearts during reperfus-

Fig. 1. Effect of administration of different concentrations of urodilatin during initial reperfusion on left ventricle developed pressure (LVdevP, upper panel) and left ventricle end-diastolic pressure (LVEDP, lower panel) in isolated rat hearts. The horizontal dashed bar indicates the time during which the treatment solution was added to the reperfusion buffer. *P<0.05 compared to control.

Fig. 3. cGMP content in control and reperfused myocardium of isolated rat hearts. *P<0.05 compared to control myocardium. Error bars indicate S.E.M.

3.2. Studies in the in situ pig heart

3.2.1. Myocardial cGMP content

In animals allocated to 5 min of reperfusion and not receiving urodilatin, myocardial cGMP content was significantly reduced in reperfused as compared to control myocardium (Fig. 4). In animals receiving urodilatin at 10 ng/kg per min, myocardial levels of cGMP in the risk area were nearly identical to those found in control myocardium (Fig. 4). In the urodilatin 50-ng/kg per min group, myocardial cGMP content in the risk area was much
higher than in control myocardium of the placebo group (372±100 vs. 111±34 fmol/mg of protein, P=0.06).

3.2.2. Infarct size studies
As there were no differences in any of the investigated variables between placebo groups of the two doses of urodilatin investigated, control animals were grouped into one single placebo group. Results are thus referred to three groups: placebo, urodilatin 10 ng/kg per min and urodilatin 50 ng/kg.

3.2.3. Blood chemistry and hematological determinations
There were no significant changes in hematocrit, platelet count, glucose, potassium, sodium or urea plasma concentrations throughout the experiment in any of the groups of treatment.

3.2.4. Plasma levels of urodilatin
Intravenous urodilatin infusion was followed by a dose-dependent increase in plasma urodilatin concentration in both treatment groups (Fig. 5).

3.2.5. Hemodynamics and coronary blood flow
Heart rate and mean aortic pressure were similar in all groups throughout the experiment, with a progressive increase with respect to basal values during the occlusion period and the first 15 min of reperfusion. After that, heart rate and mean artery pressure remained stable (Table 1). Changes in LV pressure during ischemia-reperfusion were not different between groups with a significant increase in the occlusion period (16.1±1 mmHg at 30 min of occlusion) and a marked increase in early reperfusion (29.9±3 mmHg at 5 min of reperfusion). Thereafter, LV end-diastolic pressure showed a progressive reduction towards normal values (17.2±2 mmHg at the end of the experiment). There were no differences between groups in coronary blood flow during reperfusion (Table 1).

3.2.6. Regional wall function
Changes in EDSL and systolic shortening during ischemia and reperfusion were similar in the three groups of treatment (Table 1). Coronary occlusion induced a rapid and marked increase in EDSL and abolition of systolic shortening identical in all groups of treatment. Reperfusion induced an immediate reduction of EDSL at 5 min (91±3% of basal value) that was followed by a trend towards normalization during the rest of reperfusion, without differences between groups. There was no significant recovery of contractile function during reperfusion in any of the groups.

Changes in the amplitude of segment length change observed during coronary occlusion were similar in all groups. In all animals, the amplitude of segment length change remained stable during the first minutes of ischemia and then showed a progressive reduction reflecting the development of rigor contracture [23]. This reduction started 18.8±1.2 min after coronary occlusion, without differences between groups (P=0.941). At 40 min of coronary occlusion, the amplitude of segment length was 50.1±7.4% of that measured after 5 min of ischemia, without differences between groups.

Segment length measurements in the circumflex territory were nearly identical in all groups. End-diastolic length was 101.6±0.8% of initial length after 5 min of coronary occlusion, 101.5±0.7% after 48 min, and 99.6±0.9 after 2 h of reperfusion. Systolic shortening was also similar in
Table 1
Heart rate, mean artery pressure, EDSL (end-diastolic segment length in the area at risk) and systolic shortening in the area at risk throughout the experiment

<table>
<thead>
<tr>
<th>Variable</th>
<th>Basal</th>
<th>Ischemia</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15 min</td>
<td>45 min</td>
</tr>
<tr>
<td>Heart rate (beats per minute)</td>
<td>86±5</td>
<td>94±6</td>
<td>103±7</td>
</tr>
<tr>
<td></td>
<td>URO 10</td>
<td>89±4</td>
<td>92±2</td>
</tr>
<tr>
<td></td>
<td>URO 50</td>
<td>79±6</td>
<td>87±7</td>
</tr>
<tr>
<td>Mean artery pressure (mmHg)</td>
<td>104±5</td>
<td>104±6</td>
<td>107±6</td>
</tr>
<tr>
<td></td>
<td>URO 10</td>
<td>99±9</td>
<td>99±9</td>
</tr>
<tr>
<td></td>
<td>URO 50</td>
<td>86±5</td>
<td>85±6</td>
</tr>
<tr>
<td>EDSL (% of basal value)</td>
<td>100</td>
<td>111±2</td>
<td>108±3</td>
</tr>
<tr>
<td></td>
<td>URO 10</td>
<td>100</td>
<td>114±3</td>
</tr>
<tr>
<td></td>
<td>URO 50</td>
<td>100</td>
<td>113±1</td>
</tr>
<tr>
<td>Systolic shortening (% of basal value)</td>
<td>100</td>
<td>-2±1</td>
<td>-1±2</td>
</tr>
<tr>
<td></td>
<td>URO 10</td>
<td>-1±2</td>
<td>-3±4</td>
</tr>
<tr>
<td></td>
<td>URO 50</td>
<td>-2±1</td>
<td>5±5</td>
</tr>
<tr>
<td>Coronary blood flow (% of basal value)</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>URO 10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>URO 50</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*There were no significant differences between groups.

the different groups of treatment: 102.9±6.2% of initial value after 5 min of coronary occlusion, 99.5±4.7% after 48 min, and 87±8.0% at the end of the experiment (P=NS).

3.2.7. Arrhythmias

All animals presented ventricular ectopic beats during the coronary occlusion period and runs of idioventricular accelerated rhythm during early reperfusion. A total of four animals (one in the placebo group, one receiving urodilatin 10 ng/kg per min and two receiving urodilatin 50 ng/kg per min) presented ventricular fibrillation during the occlusion period at a mean time of 27±5 min. In the first minute of reperfusion four animals developed ventricular fibrillation (two in the placebo, one in the urodilatin 10 ng/kg per min and one in the urodilatin 50-ng/kg per min groups, respectively).

3.2.8. cGMP concentration

Plasma cGMP concentration showed a rapid increase after the onset of the urodilatin infusion and reached its maximal value at 30 min of reperfusion (Fig. 6). In the urodilatin 10-ng/kg per min group, plasma levels rose from 37.4±11 to 76.3±11 pmol/ml (P=0.01), whereas in the urodilatin 50-ng/kg per min group, plasma levels rose from 54.0±14.8 to 123.8±13.7 pmol/ml (P=0.008). Plasma levels of cGMP showed no significant increase after the placebo infusion (from 41.2±10 to 35.1±5.3, P=0.594).

3.2.9. Area at risk and infarct size

The area of myocardium at risk involved 10.7±0.6% of the ventricles (weight 14.2±0.9 g) without differences between groups (P=0.25). Infarct size, defined as a percent of myocardium at risk developing necrosis, was significantly smaller in animals receiving urodilatin at 10 ng/kg per min during the first 25 min of reperfusion than in those receiving placebo. In the group receiving 50 ng/kg per min of urodilatin there was a non-significant trend towards a reduced infarct size (Fig. 7).

4. Discussion

In previous studies it has been shown that stimulation of cGMP synthesis by enhancing NO availability or by
stimulation of membrane guanylate cyclase (mGC) with urodilatin limited hypercontracture and cell death during reoxygenation or reperfusion in the isolated rat heart [8,16]. The present study confirms and extends those previous observations with the following main results. (a) Myocardial cGMP concentration is severely reduced in in situ post-ischemic myocardium. (b) Stimulation of mGC with urodilatin at the time of reperfusion results in a rapid and dose-dependent increase in myocardial cGMP concentration. (c) Intravenous administration of urodilatin at low doses lacking hemodynamic effect may restore normal cGMP concentration in reperfused myocardium and limit infarct size secondary to transient coronary occlusion. (d) Higher doses of urodilatin resulting in elevation of cGMP in reperfused myocardium well above normal values attenuate or abolish this beneficial effect.

4.1. Stimulation of cGMP during myocardial ischemia-reperfusion: previous studies

There is ample and basically coherent information regarding the effect of maneuvers increasing cGMP concentration on myocardial cell death secondary to ischemia-reperfusion [12,24–28]. In most studies, l-arginine supplementation has been found to exert a protective effect against cell death secondary to transient anoxia or ischemia in different animal species [10,16,24,25]. However, most studies indicate that l-arginine has to be administered before energy deprivation in order to be effective, which limits its potential therapeutic value in the treatment of patients with acute myocardial infarction.

The effect of direct stimulation of sGC with NO donors on ischemia-reperfusion injury has been investigated in in vitro models [1,12,14,26] and in different animal species [27,28], and a large majority of these studies have demonstrated a protective effect. However, some points regarding the beneficial effect of NO donors remain obscure. First, few studies have analyzed the effect of NO donors administered at the time of reperfusion. Second, studies on the effect of inhibitors of NO synthesis on myocardial injury secondary to transient ischemia have yielded controversial results [14,26,29], and in several instances have observed a clear protective effect of these drugs [25,29]. NO can protect cells from superoxide free radical toxicity [14], but can also behave itself like a toxic radical [30] either directly, or through generation (by reacting with superoxide anion) of peroxynitrite [31]. These effects could outbalance other cGMP-mediated beneficial effects. Finally, although NO donors have been administered to patients with acute myocardial infarction receiving reperfusion therapy in two large clinical trials (ISIS-4 and GISSI-3), it is not possible to draw conclusions from these studies regarding the potential role of NO donors in the prevention of reperfusion injury [32,33]. This is mainly due to the late administration of the donors (up to 24 h after reperfusion therapy) in these trials, the high rate of cross-over between stimulation of membrane guanylate cyclase (mGC) with treatment groups, the large number of patients receiving NO donors in the control group, and the concomitant use of ACE inhibitors.

To our knowledge, the effect of ANP related peptides on myocardial reperfusion injury had not been previously investigated.

4.2. Mechanisms of action

It has been proposed that l-arginine and NO donors could effect their beneficial effects on reperfused myocardium through improvement of microvascular function and limitation of neutrophil-mediated injury [12,24]. However, this mechanism cannot explain the beneficial effect of these interventions in in vitro systems or in crystalloid perfused preparations [14,16,26]. We have proposed that the protective effect of these interventions would be mediated, at least in part, by a direct effect of cGMP on cardiomyocytes [16].

In the present study, the cause/effect relationship between cGMP increase and the beneficial effects of urodilatin was supported by the lack of effects of too low doses that failed to normalize myocardial cGMP in perfused myocardium, and by the fact that the beneficial effect of urodilatin was abolished by ANP receptor blockade.

Stimulating cGMP synthesis has been found to protect against reoxygenation induced hypercontracture in isolated cardiomyocytes and in isolated perfused rat hearts [8,9]. This effect can be mimicked by administration of the soluble cGMP analog 8-Br-cGMP [8], and can be abolished by inhibitors of cGMP synthesis [16]. Cyclic GMP has been described to have a negative inotropic effect [34]. Although this effect is small in normal myocardium under normoxic conditions, it could be larger in reperfused myocardium, in which myofilament sensitivity to Ca$^{2+}$ is already depressed (stunning). A significant negative inotropic effect of urodilatin in reperfused, but not in control myocardium, has been previously shown in
the isolated rat heart [8]. This negative inotropic effect seems to be due to a desensitizing effect of cGMP on myofilaments [34]. This desensitization effect could be mediated by cytosolic acidification, since cGMP dependent kinases have been shown to inhibit sarcolemmal Na⁺/H⁺ exchange [35]. Although some previous studies have failed to document an effect of cGMP on Ca²⁺ transients during normoxic conditions, an effect on Ca²⁺ handling during initial reperfusion has not been ruled out. Moreover, there are data suggesting that such an effect could be mediated through the actions of cGMP dependent protein kinases on the sarcolemmal Na⁺/Ca²⁺ exchanger [36]. Reverse mode Na⁺/Ca²⁺ exchange has been recently shown to have a prominent role in Ca²⁺ overload during ischemia [37] and reperfusion [38].

Previous studies on the effects of ischemia and reperfusion on myocardial cGMP content are scant and contradictory [39,40]. This and previous studies from our group demonstrate that ischemia induces a severe reduction in myocardial cGMP concentration [8,10], and that altered cGMP production due to damage of both mGC and sGC contribute to this effect in cardiomyocytes and endothelial cells [41]. This study shows that stimulation of mGC at the time of reperfusion allows a rapid normalization of myocardial cGMP concentration. The study also suggests that excessive stimulation of mGC may have harmful effects. In the present study, aimed at characterizing the effects of urodiolatin on immediate reperfusion injury, the reperfusion time was limited to 2 h, and this prevented adequate quantification of the apoptosis induced by ischemia-reperfusion. However, high cGMP concentrations have been implicated in apoptosis in endothelial cells [42] and cardiomyocytes [17]. Since very high doses of urodiolatin, lacking any beneficial effect in the intact reperfused heart, have been shown to protect against reoxygenation hypercontracture of isolated cardiomyocytes, it can be speculated that the adverse effects of these high concentrations are independent of the effects on hypercontracture. An interesting possibility is that the adverse effects take place at endothelial cells. Recent observations indicate that stimulation of mGC is particularly effective in endothelial cells as compared to cardiomyocytes, and can result in increases of several orders of magnitude in cGMP content of endothelial cells either during normoxia or during reperfusion [41]. Several-fold increases in the cGMP content of endothelial cells, accounting for less than 5% of cells in myocardial tissue [43], could have relatively little impact on myocardial cGMP content.

4.3. Therapeutic implications

The present study demonstrates that the intravenous administration of urodiolatin at the time of coronary reperfusion at low doses, lacking detectable side-effects, may effectively limit infarct size in pigs. Direct extrapolation to patients with acute myocardial infarction is not warranted due to potential interspecies differences, and to particular features of acute coronary thrombosis in the context of human coronary heart disease not adequately reproduced in the pig model used in the present study. The present results, however, open the way for clinical pilot studies.

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