Functionally Selective AT₁ Receptor Activation Reduces Ischemia Reperfusion Injury

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Abstract
Angiotensin II (AngII) is a key peptide in cardiovascular homeostasis and is a ligand for the Angiotensin II type 1 and 2 seven transmembrane receptors (AT₁R and AT₂R). The AT₁ receptor is a seven-transmembrane (7TM) G protein-coupled receptor (GPCR) mediating the majority of the physiological functions of AngII. The AT₁R mediates its effects through both G protein-dependent and independent signaling, which can be separated by functionally selective agonists. In the present study we investigate the effect of AngII and the β-arrestin biased agonist [SII]AngII on ischemia-reperfusion injury in rat hearts. Isolated hearts mounted in a Langendorff perfused rat heart preparations showed that preconditioning with [SII]AngII reduced the infarct size induced by global ischemia from 46±8.4% to 22±3.4%. In contrast, neither preconditioning with AngII nor postconditioning with AngII or [SII]AngII had a protective effect. Together these results demonstrate a cardioprotective effect of simultaneous blockade of G protein signaling and activation of G protein independent signaling through AT₁ receptors.

Introduction

The renin-angiotensin system (RAS) is a major endocrine regulatory system of cardiovascular physiology and its main effector peptide is angiotensin II (AngII). Most of the known physiological and pathophysiological effects of AngII are mediated by AT₁Rs. The AT₁R
is a key regulator of blood pressure and body fluid homeostasis and prolonged activation of the AT₁R can lead to severe diseases including hypertension, diabetic nephropathy and cardiac arrhythmia [1, 2]. Its importance in renal and cardiovascular pathophysiology is underscored by the widespread use of AT₁R blockers and ACE inhibitors [1, 2]. The AT₁Rs are widely distributed in all organs, including liver, adrenals, brain, lung, kidney, heart, and vasculature.

AngII binding to the AT₁R facilitates interaction with heterotrimeric Gα₁₁q, activation of PLCβ, and subsequent generation of inositol trisphosphate (IP3) and diacylglycerol (DAG). The result is an increase in cytosolic Ca²⁺ released from the sarcoplasmic reticulum, and activation of PKC [3]. The G protein component of AT₁R signaling has been associated with hypertrophy, apoptosis and fibrosis which in combination lead to deleterious cardiac remodelling and progression into cardiac failure [4-6].

Within the last decade, several studies performed in recombinant cellular systems have revealed that it is possible to separate AT₁ receptor signaling into two principle components: the G protein-dependent signaling and the G protein-independent signaling [7-9]. In this respect, β-arrestins are particularly interesting. β-arrestins bind to activated receptors and desensitize G protein signaling, promote receptor internalization, and activate distinct signal transduction cascades, which can be independent of G protein coupling [10, 11].

G protein-dependent and -independent pathways can be almost perfectly separated by pharmacological means with a modified AngII analogue, [Sar¹, Ile⁴, Ile⁸]-AngII ([SII]AngII) (unpublished data). [SII]AngII induces a receptor conformation that has limited coupling to G proteins, yet remains able to activate extracellular signal-regulated kinase 1/2 (ERK1/2) through a β-arrestin2 dependent mechanism. This means that in practice [SII]AngII will work as a functional antagonist on G protein mediated signaling while it activates G protein independent pathways [7-9, 12]. Furthermore, a recent study by Ahn et al., 2009 [13], demonstrated that β-arrestin2 can facilitate anti-apoptotic signaling through regulation of BAD phosphorylation.

The role of AngII in myocardial ischemia/reperfusion injury has been investigated in the heart [14-18]. Despite this, its involvement is still not clearly understood. The AngII concentration is reported to increase in the heart after myocardial infarction (MI) and this high AngII milieu is considered deleterious [17, 19, 20]. Several studies have demonstrated a cardioprotective effect of both pharmacological pre- and postconditioning with ACE inhibitors and AT₁ receptor blockers [21-26]. Today, one of the common clinical treatments of myocardial infarction is also based on ACE inhibitors and AT₁ receptor antagonists [27], although this acute protection is often precluded by hypotension.

So far no studies have dealt with the role of functional selectivity of AT₁R in myocardial ischemia/reperfusion. Since [SII]AngII is an almost complete functional antagonist on the Gα₁₁q pathway, which is believed to cause the majority of the undesired AngII mediated effects, while preserving cardioprotective AT₁R signaling, we hypothesized that a biased agonist applied as pre- and/or postconditioning would improve myocardial survival. Therefore, the aim of this study was to analyze the functional consequences of pharmacological pre- and postconditioning with AngII and [SII]AngII in isolated perfused rat hearts subjected to ischemia/reperfusion.

Materials and Methods

Animals

Male Sprague-Dawley rats (Taconic, Denmark; body weight 409±11g, (no significant difference between groups)) were used. Prior to experiments the animals were acclimated for minimum one week while allowing free access to food and water. The study protocol was approved by the Danish Research Animal Committee, and all procedures were done in accordance with Danish National Committee for Animal Studies guidelines.
Langendorff heart preparation

Rats were anesthetized subcutaneously with a mixture of midazolam 2.5mg/kg; fluanisone 2.5 mg/kg; fentanyl citrate 0.08mg/kg followed by a subcutaneous injection with heparin (1.000 IU/kg). The rats were tracheotomized and ventilated on room air. The chest was opened and the heart was cannulated with immediate transition to retrograde coronary perfusion with a Krebs’-Henseleit (K-H) ringer that contained in mM: NaCl 119.9; KCl 3.3; NaHCO₃ 25.0; glucose 11.0; MgSO₄ 1.2; KH₂PO₄ 1.2; CaCl 1.7. The solution was warmed (37°C), and gassed with 95% O₂, 5% CO₂ to maintain pH at 7.4. The heart was removed from the animal and mounted in a Langendorff perfusion apparatus (Hugo Sachs-Harvard Apparatus GmbH, Germany). This enabled the heart to be perfused at a hydrostatic pressure of app. 60-80mmHg during the whole experiment. In order to facilitate a venous outflow, a small incision was made in the pulmonary artery.

Drugs and chemicals

AngII was used at 100nM and [SII]AngII at 18.7 µM to obtain equal near-saturating receptor occupancies [28, 29]. Drugs were prepared fresh daily by diluting the stock solution in the perfusion buffer.

Functional measurements

The Langendorff perfusion setup allowed continuous recording of left ventricular developed pressure (LVDP) (mmHg), aortic flowrate (ml/min), aortic pressure (mmHg) and heart rate (BPM) throughout the experiment. LVDP was measured through a fluid-filled latex balloon (0.06ml, Hugo Sachs-Harvard Apparatus GmbH, Germany) connected to a pressure transducer. The balloon was inserted into the left ventricle through an incision in the left atrium. The volume of the balloon was adjusted to achieve a left ventricular end-diastolic pressure (LveDP) of app. 0.5mmHg. LVDP was calculated as the difference between end-systolic and end-diastolic left ventricular pressure. All data were acquired at a sampling rate of 1 kHz by a 16-channel PowerLab system (ADInstruments, UK) and analyzed using LabChart 7 Pro Software (ADInstruments, UK).

Heart rate was measured as a peak to peak rate analysis of LVDP. Through the whole experiment the heart was submerged in the organ bath containing K-H ringer kept at 37°C. Throughout the experiment perfusion inflow temperature was measured in order to keep it constant.

Perfusion protocols

Spontaneously beating hearts were stabilized for 40 min. After that, hearts were assigned randomly into six groups, each composed by three to six hearts. Control: Hearts in the Control group (Control) (n=6) were subjected to 30 min of global ischemia followed by 120 min of reperfusion in K-H ringer. Pharmacological preconditioning: Prior to 30 min of global ischemia, hearts in the Angiotensin II preconditioning group (AngII pre) (n=6) and the [Sar¹, Ile⁴, Ile⁸]Angiotensin II preconditioning group ([SII]AngII pre)(n=5) were subjected to 15 min of AngII (100nM) or [SII]AngII (18.7 µM) perfusion, respectively. After 30 min of global ischemia the hearts underwent 120 min of reperfusion in K-H ringer. Pharmacological postconditioning: After 30 min of global ischemia, hearts in the Angiotensin II postconditioning group (AngII post) (n=6) and the [Sar¹, Ile⁴, Ile⁸]Angiotensin II postconditioning group ([SII]AngII post) (n=5) were subjected to 15 min of either AngII (100nM) or [SII]AngII (18.7 µM) perfusion followed by 105 min of reperfusion in K-H ringer. Ischemic preconditioning: Prior to 30 min of global ischemia, hearts underwent Ischemic preconditioning (IP) (n=3) consisting of two 5-min occlusion periods (global no-flow ischemia) interspersed with 5 min of reperfusion. The 30 min of global ischemia was followed by 120 min of reperfusion in K-H ringer. All protocols are summarized in Fig. 1.

Quantification of myocardial infarct size

The extent of infarcted myocardium was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining. After each experiment the heart was rapidly removed from the Langendorff perfusion apparatus, and frozen at -80°C overnight. The following day the frozen heart was cut into 2 mm-thick transverse sections, thawed, and incubated in TTC (1 % phosphate buffer pH 7.4, 37°C) for 5 min in order to determine the viable and non-viable myocardium. Immediately after staining the tissue slices were fixed in 4% formalin for 24 h. Next day the heart slices were weighed, and both sides of each slice were scanned using a flatbed scanner (HP Scanjet G4050). The area of infarct and the total area of slices were assessed by planimetry (Image-Tool 2.0 software, NIH, USA). Total infarct size determined as a percentage of total muscle mass (representing
total area, AAR) was calculated as the weighted mean of individual slice values: $(AI1/AAR1)\times(W1/Wtotal)+ (AI2/AAR2)\times(W2/Wtotal)+ (AI3/AAR3)\times(W3/Wtotal)+ (AI4/AAR4)\times(W4/Wtotal)+ (AI5/AAR5)\times(W5/Wtotal)$, where $AI_n$ is the area of infarct for the slice (n) and $W_n$ is the weight of the respective section (n) and Wtotal is the sum of all the slice weights. Quantification of infarct size was performed by an investigator who was blinded to the different experimental groups.

**Exclusion criteria**

Hearts were excluded from the study if one of the following criteria was fulfilled:

1. If the aortic wall was damaged during cannulation leading to an inability to maintain a constant aortic pressure at 60-80mmHg.
2. If hearts showed clear ischemic damage during operation or stabilization, e.g. clear white areas on the epicardium.
3. If hearts suffered from sustained irreversible arrhythmia during stabilization.
4. If left ventricular developed pressure (LVDP) was below 60mmHg during stabilization.
5. If hearts that developed ventricular fibrillation (VF) at the onset of reperfusion and did not revert spontaneously within 15 min were defibrillated by a gentle mechanical stimulation. If this procedure was unsuccessful the experiment was excluded from further analysis.

Eleven rats were excluded due to irreversible VF during reperfusion (Three [IP], three [SII]AngII post, three [SII]AngII pre and two AngII pre). One (Control) rat was excluded due to problems with the TTC-staining.

**Statistical analysis**

All data are presented as mean±SEM using GraphPad Prism software (San Diego, CA, USA). Comparison of infarct sizes as well as cardiac function and hemodynamic effects of pharmacological preconditioning with AngII and [SII]AngII was performed using one-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test. To compare the effect of treatment on cardiac function and hemodynamics during reperfusion, two-way analysis of variance followed by a Bonferroni post hoc test was performed at stabilization, and
Results

Hemodynamic parameters and coronary flow

Effects of AngII and [SII]AngII preconditioning on cardiac function. Pharmacological preconditioning with AngII and [SII]AngII on isolated perfused heart preparations was performed to investigate their potential cardioprotective effect. Protocols are shown in Fig. 1. Prior to global ischemia the changes in hemodynamic parameters due to pharmacological preconditioning was investigated. Coronary flow rate, heart rate, systolic (LVDP) and diastolic (LveDP) function were measured during the different drug preconditioning trials, where 100 nM AngII and 18.7 µM [SII]AngII were used. Values were calculated as the difference between baseline values (during stabilization) and a mean of the last 5 min of 15 min drug treatment. Values were normalized to those of control hearts, which did not receive any drug preconditioning. The hearts that received pharmacological treatment with AngII experienced a reduced coronary flow when compared to Control group (67±10% versus 100±5%, respectively, P<0.05) (Fig. 2). By contrast the hearts that received [SII]AngII did not exhibit vasoconstriction. Diastolic and systolic function as well as heart rate were similar in all groups at baseline and were not influenced significantly by drug administration in either group (Table 1 and Fig. 2).

Hemodynamic consequences of ischemia. Figure 3 shows the time course for ischemic contracture expressed as a percentage of peak contracture (AngII post: 57±6 mmHg at 18 min). 30 min of global ischemia markedly elevated left ventricular end-diastolic pressure (LveDP) in all experimental groups (Fig. 3 is divided into an A and B part for clarity). The time to maximal LveDP varied with treatment. At 16 min, the ischemic preconditioned (IP) group (20±6mmHg), the [SII]AngII pre group (35±6mmHg), the AngII pre (49±11mmHg) and the control group (51±7mmHg) reached their maximum. A significant difference in peak contracture was found between control group and IP group (P<0.05).

Table 1. Hemodynamic parameters. Baseline values represents a mean of the last 10 min of stabilization (mean ± SEM) – prior to drug treatment (preconditioning). No significant difference was found between groups (one-way ANOVA test).

| Animal weight (g) | LVDP during stabilization (mmHg) | LveDP during stabilization (mmHg) | Heart rate during stabilization (BPM) | Flow rate during stabilization (ml/min) | Aortic pressure during stabilization (mmHg) |
|-------------------|----------------------------------|----------------------------------|--------------------------------------|----------------------------------------|--------------------------------------------|
| Control (n=7)     | 408 ± 18.7                       | 105 ± 19.8                       | -4.5 ± 0.79                          | 221 ± 20.9                             | 13.1 ± 0.62                                |
| Angiotensin II post (n=6) | 425 ± 10.9                       | 84 ± 3.3                         | -2.7 ± 1.32                          | 262 ± 7.9                              | 16.3 ± 1.08                                |
| Angiotensin II pre (n=6) | 433 ± 24.5                       | 112 ± 21.5                       | 1.0 ± 1.98                           | 208 ± 8.9                              | 18.0 ± 1.32                                |
| [SII]AngII post (n=6) | 364 ± 13.7                       | 101 ± 8.1                        | -3.9 ± 1.42                          | 233 ± 16.2                             | 14.2 ± 1.39                                |
| [SII]AngII pre (n=6) | 395 ± 22.5                       | 81 ± 6.9                         | -1.2 ± 1.49                          | 210 ± 21.4                             | 16.4 ± 1.77                                |
| IP (n=3)          | 434 ± 57.9                       | 107 ± 28.9                       | -2.6 ± 3.54                          | 249 ± 14.6                             | 18.1 ± 3.24                                |

after 1, 60 and 120 min of reperfusion. Each point during reperfusion represents a mean of the preceding 1 min of recording, while the point during stabilization represents a mean of the last 10 min of stabilization. P<0.05 was considered significant.
Impaired hemodynamic function after ischemia. Changes in contractile (systolic and diastolic) function after 30 min of global ischemia were investigated to measure the cardioprotective effects of the different treatments. During reperfusion, coronary flow did not resume at pre-ischemic levels, and diastolic and systolic functions were significantly altered (Fig. 4 and 5). Figure 4A and 4B show the time course for left ventricular developed pressure (LVDP) expressed as a percentage of baseline values (during stabilization) in each group. At reperfusion, hearts in all groups showed a restricted developed pressure during the
first 15 to 30 min. This was a consequence of prolonged arrhythmia occurring at the onset of reperfusion. In general, systolic function was significantly altered in all groups after 120 min of reperfusion compared to pre-ischemic levels (Fig. 4A, B). Nevertheless, the ischemically preconditioned group (IP) had less impaired developed pressure compared to the control group (at 60 min, \(P<0.01\); at 120 min, \(P<0.05\)). Relaxation was significantly impaired in all groups during reperfusion. At the onset of reperfusion (at 15 min), hearts in all groups showed an initial peak in left end diastolic pressure (LveDP), from which it decreased before leveling off during the last 60 min of reperfusion (Fig. 5A, B). Hearts exposed to IP showed a significantly lower LveDP compared to the other groups (at 60 min, \(P<0.05\)).

**Fig. 4.** Effects of different treatments on left ventricular developed pressure (LVDP). A: The effect of 100 nM AngII and 18.7 µM [SII]AngII preconditioning on LVDP (mean±SEM) during stabilization (time; 10 min prior to ischemia), ischemia (time; the last 10 min of ischemia) and reperfusion (time; every 15 th min (mean of 1 min) for 120 min). (Two-way ANOVA) *: \(P<0.05\) and **: \(P<0.01\); Control vs. IP. B: The effect of 100 nM AngII and 18.7 µM [SII]AngII postconditioning on LVDP. No significant difference between groups.

**Fig. 5.** Left ventricular end-diastolic pressure (LveDP). Effects of different treatments on LveDP expressed as a percentage of mean initial value (t=0; stabilization) in each group. A: The effect of IP, 100 nM AngII and 18.7 µM [SII]AngII preconditioning on LveDP (mean±SEM) during stabilization (time; 10 min prior to ischemia), ischemia (time; the last 10 min of ischemia) and reperfusion (time; every 15 min (mean of 1 min) for 120 min). *: \(P<0.05\); Control vs. IP (At 60 min, two-way ANOVA). B: The effect of 100 nM AngII and 18.7 µM [SII]AngII postconditioning on LveDP (mean±SEM) during stabilization, ischemia and 120 min of reperfusion. No significant difference between groups.
The extent of infarct size after ischemia-reperfusion (I/R) was determined by a 2,3,5-triphenyltetrazolium chloride (TTC) staining. In the control group, 30 min of global no-flow normothermic ischemia followed by 120 min of reperfusion resulted in an infarct size of 46±8.4% of the total area. The infarct sizes in the different groups are summarized in Figure 6 and a representative visualization of ischemic hearts is shown in Figure 7.

Protection was achieved by ischemic preconditioning (IP). Here, the infarct size was significantly reduced to 14±5.2% compared to the 46±8.4% observed in the control group (P<0.05) (Fig. 6). A significant reduction was also achieved when 18.7 µM [SII]AngII was administered for 15 min prior to global ischemia ([SII]AngII pre) (infarct size: 22±3.4% (P<0.05)). In contrast, 15 min of [SII]AngII 18.7 µM postconditioning ([SII]AngII post), administered at the onset of reperfusion, did not reduce infarct size significantly (infarct size: 48±7.3%) compared to control conditions. Similarly, 100 nM of angiotensin II (AngII) administered either 15 min prior to global ischemia (AngII pre) or during the first 15 min of reperfusion (AngII post) did not reduce infarct size significantly (36±4.1% and 45±5.3%, respectively).

**Discussion**

Ischemic preconditioning (IP) is a well established technique to induce cardioprotection [30, 31]. Consistent with previous studies, our experiments also show significant improvement of relaxation and systolic function (Fig. 4 and 5), as well as reduced myocardial infarction (Fig. 6) when short episodes of no-reflow ischemia were introduced prior to prolonged ischemia [30, 31].

No significant changes in post-ischemic recovery of cardiac contractility were observed by pharmacological AngII treatment (Fig. 4 and 5). This was the case for both pre- and postconditioning and the current findings correlate with what previous studies have seen [17, 21]. In 2000, Zhu et al., investigated the comparative effects of pretreatment with the ACE inhibitor, captopril, and the AT1 receptor blocker, losartan, on myocardial infarct size. Sprague-Dawley rats were given either captopril or losartan 10 weeks prior to I/R studies. Myocardial I/R was then induced in a model of left anterior descending coronary artery (LAD) occlusion (17 min) and reperfusion (120 min). They found that myocardial infarct size was significantly smaller in the losartan and captopril groups than in the control group [21]. Several other experimental models have similarly demonstrated the protective effects of both ACE inhibitors and AT1 antagonists in I/R studies [22, 24, 26]. There is thus solid evidence of a deleterious effect connected with particularly G protein-dependent signaling from the AT1 in the setting of Ischemia and Reperfusion. To investigate whether selective G protein-independent pathways are more beneficial in the ischemic heart, we included
pre- and postconditioning protocols with the selective β-arrestin-biased AngII analogue [SII]AngII. In contrast to AngII, the peptide ligand [SII]AngII is able to induce a receptor active conformation with very limited ability to activate G proteins, yet capable of activating ERK 1/2 by the β-arrestin2 pathway [7-9, 32].

Pre-ischemic measurements revealed that presence of AngII in the perfusate caused vasoconstriction to decrease flow rates by 23% relative to controls. [SII]AngII treatment in contrast did not influence any of the measured hemodynamic parameters (Fig. 2). These findings support the notion that acute regulation of blood pressure by the AT$_1$R is caused by G protein stimulated Ca$^{2+}$ mobilization and is not (directly) affected by β-arrestin dependent signaling [33].

The constriction of coronary arteries seen with AngII perfusion is intuitively not desirable in the aftermath of a coronary occlusion, but did not lead to an increase in final infarct size (48±7%) (Fig. 6). Though we do not demonstrate deleterious effects of AngII in this isolated test of short-term AngII stimulation in a global ischemic model, it may be so when factors like partial reperfusion and collateral compensation are in play. Preconditioning with isolated G protein-independent AT$_1$R stimulation was evaluated with [SII]AngII, and induced a significant reduction in infarct size (22±3%) and a tendency towards superior recovery in developed pressure (LVDP and LVeDP) as compared to the control group. Similar testing of [SII]AngII in the postconditioning protocol had no significant effect on infarct size, but by contrast to AngII did not entail reduction of coronary flowrates.

These results corroborate previous reports that AT$_1$R blockers mediate cardioprotective effects through inhibition of deleterious Gprotein signaling. Futher to this notion, preservation of G protein-independent signaling (β-arrestin2-dependent signaling) has previously been indicated to induce cardioprotective signaling [13, 34, 35]. To further support an anti-apoptotic role of β-arrestin signaling, we have found [SII]AngII to inhibit caspase-3 cleavage in isolated cardio-myocytes exposed to apoptotic conditions (data not shown).

Currently Trevena Inc is developing agonists with functional selectivity for β-arrestin signaling (TRV027) for the treatment of acute myocardial infarction, and in two recent studies another potent, selective β-arrestin biased ligand of AT$_1$R (TRV120027) increased cardiac performance through a reduced mean arterial pressure and preserved cardiac stroke volume [34, 35]. However, these studies did not perform investigations in relation to I/R.

In summary, the present study establishes that pharmacological preconditioning with the functionally selective AngII analogue [SII]AngII induces cardioprotection as revealed by a significant reduction in myocardial infarct sizes after exposure to ischemic reperfusion. In contrast, pharmacological pre- and postconditioning with AngII does not alter myocardial infarct size. Together these findings strongly indicate that AT$_1$ receptor mediated β-arrestin
signaling plays an important role in I/R-induced cardioprotection and highlight the potential of targeting specific receptor functions in future drug discovery.

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