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## B-type natriuretic peptide expression and cardioprotection is regulated by Akt dependent signaling at early reperfusion

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## ABSTRACT

Exogenously administered B-type natriuretic peptide (BNP) has been shown to offer cardioprotection through activation of particulate guanylyl cyclase (pGC), protein kinase G (PKG) and  $K_{ATP}$  channel opening. The current study explores if cardioprotection afforded by short intermittent BNP administration involves PI3K/Akt/p70s6k dependent signaling, and whether this signaling pathway may participate in regulation of BNP mRNA expression at early reperfusion. Isolated Langendorff perfused rat hearts were subjected to 30 min of regional ischemia and 120 min of reperfusion (IR). Applying intermittent  $3 \times 30$  s infusion of BNP peptide in a postconditioning like manner (BNP<sub>Post</sub>) reduced infarct size by >50% compared to controls (BNP<sub>Post</sub>  $17 \pm 2\%$  vs. control  $42 \pm 4\%$ ,  $p < 0.001$ ). Co-treatment with inhibitors of the PI3K/Akt/p70s6k pathway (wortmannin, SH-6 and rapamycin) completely abolished the infarct-limiting effect of BNP postconditioning (BNP<sub>Post</sub> + Wi  $36 \pm 5\%$ , BNP<sub>Post</sub> + SH-6  $41 \pm 4\%$ , BNP<sub>Post</sub> + Rap  $37 \pm 6\%$  vs. BNP<sub>Post</sub>  $17 \pm 2\%$ ,  $p < 0.001$ ). Inhibition of natriuretic peptide receptors (NPR) by isatin also abrogated BNP<sub>Post</sub> cardioprotection (BNP<sub>Post</sub> + isatin  $46 \pm 2\%$  vs. BNP<sub>Post</sub>  $17 \pm 2\%$ ,  $p < 0.001$ ). BNP<sub>Post</sub> also significantly phosphorylated Akt and p70s6k at early reperfusion, and Akt phosphorylation was inhibited by SH-6 and isatin. Myocardial BNP mRNA levels in the area at risk (AA) were significantly elevated at early reperfusion as compared to the non-ischemic area (ANA) (Ctr<sub>(AA)</sub>  $2.7 \pm 0.5$  vs. Ctr<sub>(ANA)</sub>  $1.2 \pm 0.2$ ,  $p < 0.05$ ) and the ischemic control tissue (Ctr<sub>(AA)</sub>  $2.7 \pm 0.5$  vs. ischemia  $1.0 \pm 0.1$ ,  $p < 0.05$ ). Additional experiments also revealed a significant higher BNP mRNA level in ischemic postconditioned (IPost) hearts as compared to ischemic controls (IPost  $6.7 \pm 1.3$  vs. ischemia  $1.0 \pm 0.2$ ,  $p < 0.05$ ), but showed no difference from controls run in parallel (Ctr  $5.4 \pm 0.8$ ). Akt inhibition by SH-6 completely abrogated this elevation (IPost  $6.7 \pm 1.3$  vs. IPost + SH-6  $1.8 \pm 0.7$ ,  $p < 0.05$ ) (Ctr  $5.4 \pm 0.8$  vs. SH-6  $1.5 \pm 0.9$ ,  $p < 0.05$ ). In conclusion, Akt dependent signaling is involved in mediating the cardioprotection afforded by intermittent BNP infusion at early reperfusion, and may also participate in regulation of reperfusion induced BNP expression.

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### Introduction

The polypeptide hormone brain natriuretic peptide (BNP) is part of the cardiac natriuretic peptide (NP) family, comprising atrial (ANP), BNP and C-type natriuretic peptide (CNP) [1–6]. Until recently, BNP release was solely believed to be caused by ventricular wall distension, but accumulating evidence support ischemia [7] and hypoxia [8] to be independent triggers of BNP release. Furthermore, exogenous administered BNP can reduce injuries associated with ischemia reperfusion in the isolated heart [9–13].

This BNP mediated cardioprotection appears to be mediated via the natriuretic peptide receptor type A (NPR-A) [14], activating a cGMP-dependent protein kinase G (PKG) [15] that further modulates opening of mitochondrial and sarcolemmal  $K_{ATP}$  channels (m $K_{ATP}$  and s $K_{ATP}$ ) and endogenous nitric oxide synthase (eNOS) [9]. However, activation of other signaling pathways also appears pivotal in NP induced cardioprotection, as ANP administration just prior to reperfusion limited infarct size in rabbit hearts, a protection that was lost when inhibiting either NPR-A, PI3K (phosphatidylinositol 3-kinase), ERK (extracellular signal-regulated kinase), or m $K_{ATP}$  channels [16]. PI3K is part of the cytoprotective reperfusion injury salvage kinases signaling pathway (RISK) that downstream phosphorylates Akt and p70S6k [17]. Currently, however, it is unknown whether exogenously administered BNP may activate Akt dependent RISK signaling in the isolated rat heart at reperfusion.

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BNP is considered to be an immediate-early gene that has proven to be both rapidly synthesized and released (within 1 h) in response to ischemia *in vivo* [18] and after reoxygenation in the *ex vivo* perfused rat heart [8,11]. Recently, it has also been suggested that ischemia-reperfusion (IR) may lead to increased myocardial BNP mRNA independently of changes in ventricular function [19]. Scientific evidence also suggests that natriuretic peptides (NPs) may serve as autacoid mediators of ischemic postconditioning (IPost), since the natriuretic peptide receptor inhibitor isatin abolished the IPost induced protection [9]. It is well known that IPost mediates its cardioprotective effect via Akt-dependent RISK signaling. Akt participates in gene transcription regulation [20–23] and activated nucleus localized Akt has been shown to enhance ANP expression [24]. The relationship between myocardial reperfusion and BNP synthesis has yet to be fully established, and it is unknown whether Akt may participate in regulation of myocardial BNP expression level at early reperfusion.

The objectives of this study were to: (1) study if intermittent exogenous BNP administration may mimic IPost induced cardioprotection, (2) investigate if this cardioprotection is mediated via Akt and NPR dependent signaling, (3) explore how early BNP mRNA expression alters at reperfusion after an acute myocardial ischemic event, and (4) delineate whether Akt dependent signaling may regulate reperfusion induced BNP expression.

## Materials and methods

### Langendorff perfusion

All experiments were approved by the Norwegian State Commission for Laboratory Animals, and carried out in accordance with the European Communities Council Directive (2010/63/EU). Female Wistar rats ( $n = 114$ , 200–300 g) were heparinized (200 IU) and anesthetized with sodium pentobarbital (50 mg/kg) *ip*. After excision, the heart was immediately placed in Krebs Henseleit buffer (KHB, 4 °C), and rapidly mounted in Langendorff perfusion modus at constant pressure (80 mmHg). The KH-buffer contained: NaCl (118 mM), NaHCO<sub>3</sub> (25 mM), KCl (4.7 mM), MgSO<sub>4</sub> × 7 H<sub>2</sub>O (1.22 mM), KH<sub>2</sub>PO<sub>4</sub> (1.21 mM), D-glucose (11 mM), CaCl<sub>2</sub> × 2 H<sub>2</sub>O (1.8 mM) (pH 7.4, oxygenated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>). Coronary flow (CF in ml/min) was measured by timed collection of coronary effluent, and a thermo-probe placed in the pulmonary artery monitored the cardiac temperature (kept at ~37 °C). A 3-0 silk suture was passed around the main branch of the left coronary artery, and threaded through a small vinyl tube to form a snare. Regional ischemia (RI) was achieved by pulling the snare, and confirmed by a substantial fall in left ventricular developed pressure (LVDP) and CF. Reperfusion was achieved by releasing the snare.

### Measurement of ischemic risk zone and infarct size

The silk suture was securely tightened at the end of each experiment and a 0.2% (w/v) Evans Blue suspension infused to demarcate the risk zone (Duke Scientific Corp., Palo Alto, CA, USA). Thereafter, the hearts were frozen (–20 °C) and later cut into 2-mm thick slices from apex to basis of the heart, and stained with 1% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) at 37 °C for 20 min, before fixation in 4% formalin to enhance the contrast of the stain. The area of the left ventricle, the infarcted area (TTC negative) and the risk zone (not blue) were determined using a computerized planimetry program (Planimetry<sup>+</sup> v2.0; ENK, Norway) and infarct size expressed as the infarct/risk ratio (%). The coefficient of variation (CV) for the area at risk/left ventricle volume ratio (AAR/LV) were 28%.

### Experimental protocol for the *ex vivo* rat heart

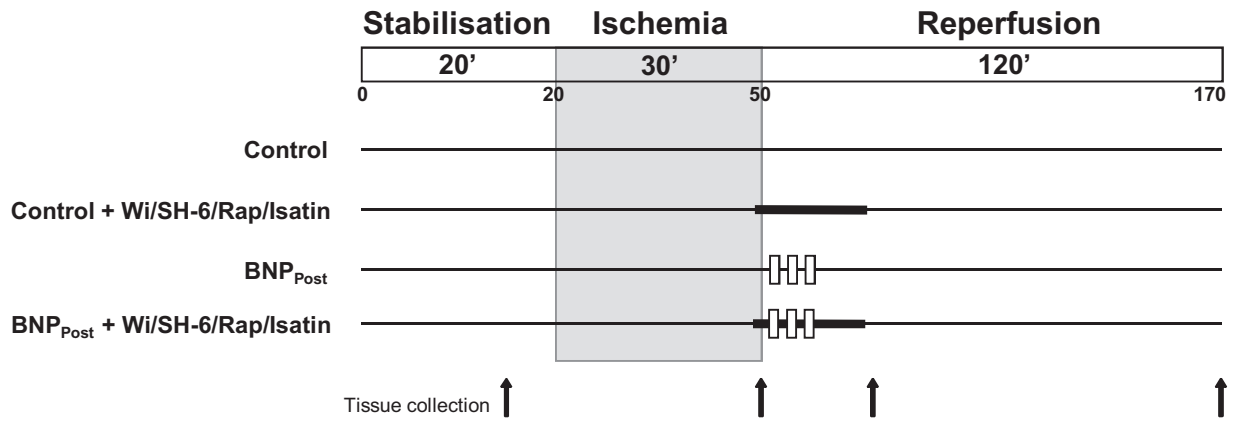
Three sets of perfusion experiments were performed: (1) infarct size determination after intermittent BNP infusion, (2) heart perfusions for tissue isolation to determine the effect of BNP treatment on phosphorylation of Akt and p70s6k and (3) heart perfusions for tissue isolation for investigation of BNP mRNA expression in the left ventricle (LV). Infarct size determination: all hearts were stabilized for 20 min before being subjected to 30 min regional ischemia and 120 min reperfusion (Fig. 1). Rat BNP (10 nM) (Sigma–Aldrich) [11] was administered in a postconditioning like manner for 3 × 30 s at immediate ischemia-reperfusion (BNP<sub>Post</sub>). To investigate the role of pro-survival RISK signaling in BNP<sub>Post</sub> induced cardioprotection, the following inhibitors ± BNP treatment were administered at ischemic reperfusion: PI3K inhibitor wortmannin (Wi; 1 μM) [25], Akt inhibitor SH-6 (SH-6; 10 μM) [26] and the mTOR/p70s6k inhibitor rapamycin (Rap; 1 nM) [25] (all from Calbiochem). The effect of inhibiting the natriuretic peptide receptor (NPR) with isatin (100 μM) [9] (Sigma) during BNP postconditioning was also investigated. Tissue isolation for immunoblotting: tissue for determination of the phosphorylation status of Akt and p70s6k in the LV were isolated at baseline and at 15 min of reperfusion. Tissue isolation for BNP mRNA expression analysis: tissue samples for mRNA analysis were collected at end ischemia, at 15 and 120 min into the ischemic-reperfusion period. Furthermore, in additional experiments, tissue was isolated from hearts exposed to ischemic postconditioning (IPost; 3 × 30 s of global ischemia) and parallel run controls at early reperfusion ± the Akt inhibitor SH-6.

### Immunoblot analysis

Myocardial Akt (Phospho-Akt, Ser<sup>473</sup>) and p70s6 kinase phosphorylation (Phospho-p70s6k, Thr<sup>389</sup>) in the area at risk was determined by SDS-PAGE electrophoresis (all antibodies from Cell Signaling Technology, USA), and phosphorylation was expressed as the ratio between phosphorylated and total protein levels. Hearts perfused with BNP for 10 min (BNP<sub>B</sub>) or KH-buffer for 10 min served as baseline controls (Ctr<sub>B</sub>). Tissue was collected at 15 min of ischemia-reperfusion, snap frozen in liquid nitrogen and stored at –80 °C. Homogenization, protein quantification, sample preparation (40 μg/lane) and electrophoresis were performed as previously described [26]. Ponceau S staining (Sigma, St. Louis, USA) confirmed successful transfer and GAPDH confirm equal loading (Santa Cruz Biotechnology, CA, USA). Densitometric analysis was performed using Quantity One software (Bio-Rad, CA, USA).

### Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Area at risk (AA, ischemic tissue) and area non at risk (ANA, non-ischemic tissue) were collected immediately after identification of area at risk by Evans Blue staining and stored in RNAlater (Ambion) according to the manufacturer's protocol. The cardiac tissue was homogenized in TRIzol (Invitrogen, 1 ml/100 mg tissue) in a Precellys 24 homogenizer (Bertin Technologies) using ceramic beads CK28 (Bertin Technologies; two times at 6000 rpm for 15 s separated with 30 s). After treatment with Recombinant DNase I (Takara) and ammonium acetate precipitation (Ambion), total RNA was further purified using RNeasy Mini Kit (Qiagen). First strand cDNA was synthesized from 3 μg total RNA in a 40 μL reaction primed with 600 ng oligo (dT)<sub>12–18</sub>; (Invitrogen) using 2 μL AffinityScript (Stratagene). A standard curve with 0.1–10 μg total RNA was made to control for reverse transcription and PCR quantification. Reactions without reverse transcriptase enzyme run in parallel were negative when tested for presence of genomic DNA. The synthesized cDNA was diluted by adding 160 μL RNase free



**Fig. 1.** Experimental protocol. Isolated female rat hearts were mounted and retrogradely perfused with Krebs Heinsleit Buffer (KHB, solid lines) in a Langendorff perfusion setup. All hearts were subjected to 20 min stabilization, 30 min regional ischemia (RI) and 120 min reperfusion. Solid lines = buffer perfusion; BNP<sub>Post</sub> = BNP postconditioning, 3 × 30 s of exogenous BNP infusion (10 nM). The PI3K inhibitor wortmannin (Wi, 1 μM), the Akt-inhibitor SH-6 (10 μM), the mTOR/p70s6k inhibitor rapamycin (Rap, 1 nM), and the natriuretic peptide receptor-inhibitor isatin (100 μM) were administered for 15 min starting 1 min prior to reperfusion. Tissue isolation for immunoblotting: tissue samples were collected at stabilization and at 15 min into the ischemic-reperfusion period. Tissue isolation for BNP mRNA expression analysis: tissue samples for mRNA analysis were collected at end ischemia, at 15 and 120 min into the ischemic-reperfusion period (indicated by arrows).

water. BNP was quantified using Messagreen (Eurogentech) with 2 μl cDNA as template with 300 nM of each primer pair for the natriuretic peptide precursor B (preproBNP, Nppb) [27] or GAPDH [28]. The qPCR was performed on a HTS 7900 (Applied Biosystem) with initial denaturation at 95 °C for 10 min and the PCR cycled at 95 °C for 1 s and 60 °C for 30 s (45 cycles) followed by a melting point analysis confirming the primer specificity by detection of one PCR product only. Amplification efficiency for each qPCR reaction was calculated from the slope of the standard curve [efficiency =  $10^{(-1/\text{slope})} - 1$ ] and the standard curve method was used to calculate individual level for each mRNA analysed. The level of Nppb mRNA was analysed and expressed relative to the house-keeping gene GAPDH. The mean of ischemic tissue prior at start of reperfusion were assigned the value 1 to simplify comparison of mRNA.

#### Statistical analysis

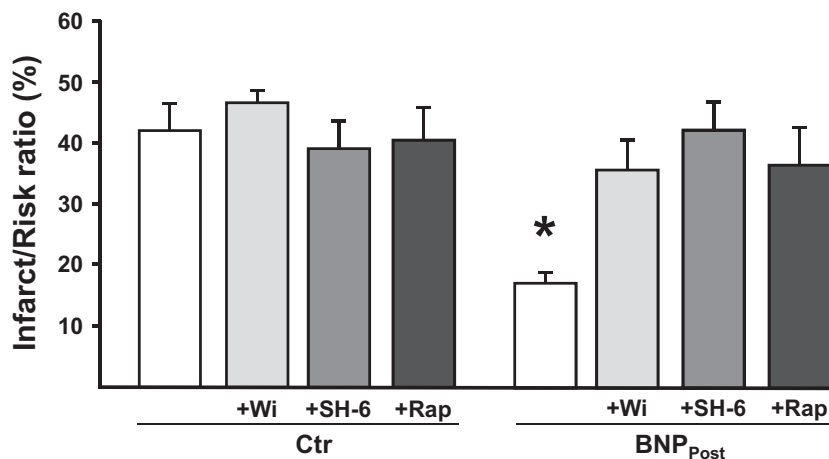
Values are presented as mean ± standard error of the mean (S.E.M.). Infarct size, AAR/LV, LVDP, HR, CF and SDS-PAGE electrophoresis results were tested for group differences by one way analysis of variance (ANOVA) combined with the Bonferroni post

hoc test. The BNP mRNA data were tested for group differences by applying a Students *t*-test. A value of  $p < 0.05$  was considered statistically significant.

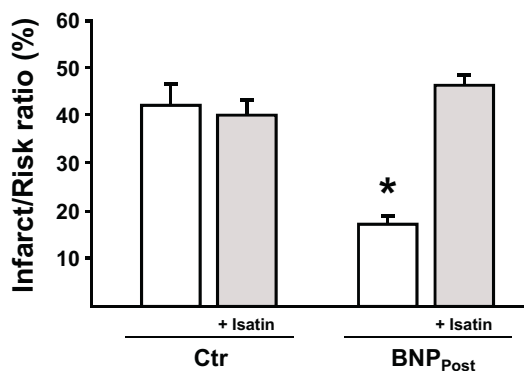
#### Results

##### *BNP postconditioning limits infarct size via PI3K/Akt/p70s6k dependent signaling and natriuretic peptide receptor inhibition*

Intermittent administration of BNP for 3 × 30 s at the immediate onset of ischemic-reperfusion (BNP<sub>Post</sub>), to mimic ischemic postconditioning, resulted in a >50% reduction in infarct size as compared to the control group (BNP<sub>Post</sub> 17 ± 2% vs. control 42 ± 4%,  $p < 0.001$ ) (Fig. 2). In order to determine the involvement of PI3K/Akt/p70s6k dependent signaling afforded by BNP postconditioning, three inhibitors of this pathway were utilized (experimental protocol, Fig. 1). The PI3K-inhibitor Wi (1 μM), Akt inhibitor SH-6 (10 μM) and the mTor/p70s6k-inhibitor Rap (1 nM) all completely abolished the effect of BNP postconditioning (BNP<sub>Post</sub> + Wi 36 ± 5%, BNP<sub>Post</sub> + SH-6 41 ± 4%, BNP<sub>Post</sub> + Rap 37 ± 6% vs. BNP<sub>Post</sub> 17 ± 2%,  $p < 0.001$ ) (Fig. 2). Inhibitor administration alone had no effect on infarct size (Wi 47 ± 2%, SH-6 39 ± 4%, Rap



**Fig. 2.** Administration of BNP as a mimetic of postconditioning limits infarct size via PI3K/Akt/p70s6k dependent signaling. Co-administration of inhibitors of RISK dependent signaling such as the PI3K inhibitor Wortmannin (Wi, 1 μM), the Akt inhibitor SH-6 (10 μM) and the mTOR/p70s6k inhibitor Rapamycin (Rap, 1 nM) abrogated the infarct size sparing effect of BNP<sub>Post</sub>. Infarct size is expressed as a percentage of the area at risk. Bars represent means ± S.E.M.  $N \geq 7$  in each group. \* $P < 0.001$  vs. Ctr group.



**Fig. 3.** Administration of BNP as a mimetic of postconditioning limits infarct size via NPR. Blocking the natriuretic peptide receptor (NPR) with isatin (100  $\mu$ M) revoked the infarct-limiting effect of BNP<sub>Post</sub>. Infarct size is expressed as percentage of the area at risk. Bars represent means  $\pm$  S.E.M.  $N \geq 7$  in each group. \* $P < 0.001$  vs. Ctr group.

**Table 1**  
Ratio of area at risk (AAR) and left ventricle (LV) volumes.

Group	Inhibitor	AAR/LV (%)
Ctr	No inhib	41.2 $\pm$ 3.4
	Wi	43.7 $\pm$ 5.1
	SH-6	48.6 $\pm$ 6.2
	Rap	40.7 $\pm$ 5.8
	Isatin	41.3 $\pm$ 2.6
BNP <sub>Post</sub>	No inhib	46.0 $\pm$ 3.8
	Wi	35.4 $\pm$ 4.3
	SH-6	45.2 $\pm$ 6.4
	Rap	37.1 $\pm$ 1.9
	Isatin	50.2 $\pm$ 3.7

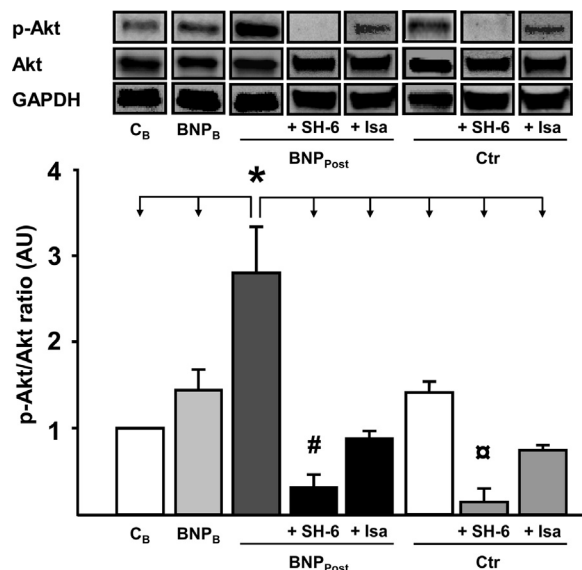
Values are mean  $\pm$  S.E.M.

41  $\pm$  2% vs. control 42  $\pm$  4%, ns) (Fig. 2). In order to reveal the involvement of cGMP/PKG signaling in BNP<sub>Post</sub> induced cardioprotection, the non-specific NPR inhibitor isatin was co-administered at immediate reperfusion. Isatin did not affect infarct size under control conditions (isatin 40  $\pm$  3% vs. control 42  $\pm$  4%, ns), but completely abrogated the cardioprotection provided by BNP<sub>Post</sub> (BNP<sub>Post</sub> 17  $\pm$  2% vs. BNP<sub>Post</sub> + isatin 46  $\pm$  2%,  $p < 0.001$ ) (Fig. 3). The ischemic risk zone constituted approximately 40–50% of the left ventricular muscle volume, and there were no significant group differences in the relative sizes of the AAR (volume of area at risk/volume of left ventricle) (Table 1).

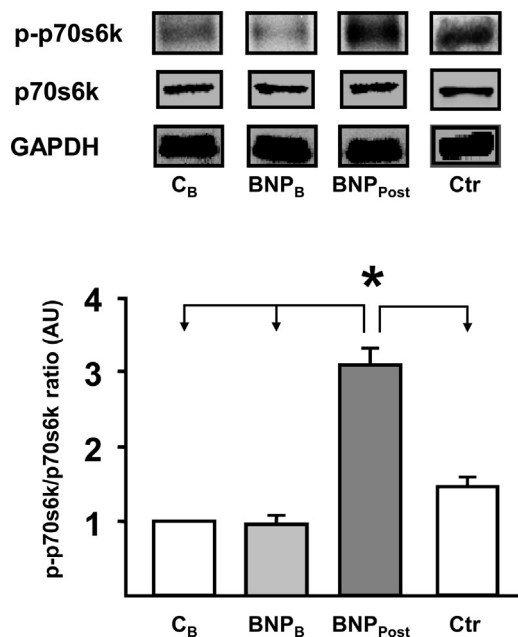
Baseline functional parameters were obtained after 18 min of stabilization. A significant reduction in LVDP and CF after 5 min of regional ischemia confirmed that all groups obtained similar and expected degrees of ischemia relative to corresponding stabilization values (Table 2). Furthermore, there was no difference between groups with regards to LVDP, CF and HR at 120 min of reperfusion.

#### BNP postconditioning phosphorylates Akt and p70s6k at early reperfusion

Administration of BNP as a mimetic of ischemic postconditioning (BNP<sub>Post</sub>) at early reperfusion resulted in a significant increase in Akt phosphorylation compared to the control group (Fig. 4). Applying the Akt inhibitor SH-6 and the NPR inhibitor isatin abrogated the BNP<sub>Post</sub> induced Akt phosphorylation (Fig. 4). The downstream Akt target p70s6k was also significantly phosphorylated after BNP postconditioning compared to the control group (Fig. 5). BNP infusion at baseline did not alter the phosphorylation status of Akt or p70s6k compared to corresponding baseline controls (Figs. 4 and 5).



**Fig. 4.** Phosphorylation status of myocardial Akt in isolated hearts exposed to BNP postconditioning. Representative immunoblots (top) and densitometric analysis (bottom) of phosphorylated Akt (Ser<sup>473</sup>) in ex vivo rat hearts demonstrates that BNP<sub>Post</sub> treatment resulted in a significant increase in Akt (Ser<sup>473</sup>) phosphorylation as compared to the control group, while the Akt inhibitor SH-6 and the NPR inhibitor isatin completely abolished it. Baseline BNP infusion (BNP<sub>B</sub>) did not significantly alter the degree of Akt phosphorylation as compared to control baseline (C<sub>B</sub>). Densitometric analysis of total and phosphorylated Akt expressed in arbitrary units (AU) where p-Akt are expressed as a ratio of total Akt with C<sub>B</sub> = 1. Bars represent means  $\pm$  S.E.M.  $N \geq 3$  in each group. \* $P < 0.001$  vs. BNP<sub>Post</sub>. # $P < 0.05$  vs. BNP<sub>B</sub>.  $\square P < 0.05$  vs. Ctr. Representative immunoblots of total and phosphorylated Akt (Ser<sup>473</sup>) (bottom). GAPDH immunoblots indicate equal loading.



**Fig. 5.** Phosphorylation status of myocardial p70s6k in ex vivo hearts exposed to BNP postconditioning. Representative immunoblots (top) and densitometric analysis (bottom) of phosphorylated p70s6k (Thr<sup>389</sup>) in ex vivo rat hearts indicate that BNP<sub>Post</sub> treatment significantly increased p70s6k phosphorylation status at Thr<sup>389</sup> as compared to the control group (Ctr). BNP infusion at baseline (BNP<sub>B</sub>) did not alter p70s6k phosphorylation as compared to control baseline (C<sub>B</sub>). Densitometric analysis of total and phosphorylated p70s6k expressed in arbitrary units (AU) where p-p70s6k are expressed as a ratio of total p70s6k with C<sub>B</sub> = 1. Bars represent means  $\pm$  S.E.M.  $N \geq 3$  in each group. \* $P < 0.001$  vs. BNP<sub>Post</sub>. GAPDH immunoblots indicate equal loading.

**Table 2**  
Functional parameters recorded during the experimental protocol.

	Group	18' stab	5' RI	30' rep	120' rep
LVDP (mmHg)	Ctr	137 ± 7	70 ± 7*	84 ± 6*	59 ± 6*
	Wi	134 ± 10	65 ± 7*	82 ± 6*	57 ± 5*
	SH6	141 ± 9	71 ± 9*	61 ± 2*	44 ± 5*
	Rap	126 ± 19	68 ± 7*	71 ± 3*	47 ± 5*
	Isatin	148 ± 16	78 ± 9*	86 ± 11*	62 ± 6*
	BNP <sub>Post</sub>	141 ± 10	84 ± 10*	85 ± 6*	63 ± 6*
	BNP <sub>Post</sub> + Wi	122 ± 12	85 ± 13*	85 ± 15	56 ± 15*
	BNP <sub>Post</sub> + SH6	142 ± 8	84 ± 12*	52 ± 4*#	42 ± 4*
	BNP <sub>Post</sub> + Rap	125 ± 12	87 ± 18*	90 ± 9*	61 ± 8*
	BNP <sub>Post</sub> + Isatin	135 ± 6	70 ± 12*	76 ± 6*	47 ± 7*
CF (ml/min)	Ctr	12 ± 2	7 ± 1*	10 ± 1	7 ± 1*
	Wi	10 ± 1	6 ± 1*	8 ± 2	6 ± 1*
	SH6	13 ± 1	8 ± 1*	9 ± 1*	7 ± 1*
	Rap	11 ± 1	8 ± 1*	11 ± 1	8 ± 1*
	Isatin	11 ± 1	7 ± 1*	8 ± 1*	7 ± 1*
	BNP <sub>Post</sub>	12 ± 1	6 ± 1*	9 ± 1*	7 ± 1*
	BNP <sub>Post</sub> + Wi	13 ± 1	8 ± 1*	12 ± 2	9 ± 2*
	BNP <sub>Post</sub> + SH6	12 ± 1	7 ± 1*	7 ± 1*	7 ± 2*
	BNP <sub>Post</sub> + Rap	11 ± 3	7 ± 1*	10 ± 3	8 ± 2*
	BNP <sub>Post</sub> + Isatin	12 ± 1	7 ± 1*	8 ± 2	6 ± 1*
HR (beats/min)	Ctr	259 ± 9	278 ± 12	294 ± 9	277 ± 10
	Wi	265 ± 15	261 ± 16	243 ± 18	242 ± 22
	SH6	294 ± 11	286 ± 14	253 ± 14	239 ± 17*
	Rap	274 ± 17	257 ± 21	295 ± 8	271 ± 9
	Isatin	282 ± 17	248 ± 14	248 ± 18	249 ± 17
	BNP <sub>Post</sub>	267 ± 15	228 ± 22	238 ± 21	227 ± 19
	BNP <sub>Post</sub> + Wi	285 ± 20	291 ± 24	278 ± 38	279 ± 22
	BNP <sub>Post</sub> + SH6	289 ± 5	243 ± 50	235 ± 34#	199 ± 39
	BNP <sub>Post</sub> + Rap	297 ± 27	266 ± 28	259 ± 48	275 ± 38
	BNP <sub>Post</sub> + Isatin	280 ± 18	250 ± 22	229 ± 27	231 ± 25

Values represent mean ± S.E.M.

Ctr – ischemia reperfusion control; BNP<sub>Post</sub> – BNP postconditioning; Wi – 15 min Wortmannin (1 μM) at reperfusion; SH6 – 15 min SH-6 (10 μM) at reperfusion; Rap – 15 min of rapamycin (1 nM) at reperfusion; isatin – 15 min of isatin (100 μM) at reperfusion.

\*  $P < 0.05$  vs. corresponding stab.

#  $P < 0.05$  vs. Ctr.

The level of total Akt, total p70s6k and loading control GAPDH did not alter between the groups.

#### *BNP mRNA levels at early reperfusion in the area at risk of infarction is regulated by Akt dependent signaling*

Myocardial BNP mRNA levels in area at risk (AA) were significantly elevated at early reperfusion (15 min) as compared to the non-ischemic myocardium (ANA, area not at risk) (Ctr<sub>(AA)</sub> 2.7 ± 0.5 vs. Ctr<sub>(ANA)</sub> 1.2 ± 0.2,  $p < 0.05$ ) (Fig. 6A). Furthermore, the ventricular BNP expression in AA at 15 min of reperfusion was significantly up-regulated as compared to the ischemic control (0 min reperfusion) (Ctr<sub>(AA)</sub> 2.7 ± 0.5 vs. ischemia 1.0 ± 0.1,  $p < 0.05$ ), while there were no differences between ANA and the ischemic control (Ctr<sub>(ANA)</sub> 1.2 ± 0.2 vs. ischemia 1.0 ± 0.1, ns) (Fig. 6A). The ventricular BNP expression levels in AA returned to ischemic levels after 120 min of reperfusion (Ctr<sub>(AA)</sub> 1.2 ± 0.3 vs. ischemia 1.0 ± 0.1, ns), and with no differences in expression levels between AA and ANA (Ctr<sub>(AA)</sub> 1.2 ± 0.3 vs. Ctr<sub>(ANA)</sub> 0.9 ± 0.3, ns). These results imply that the reperfusion process amplifies BNP expression in the area at risk as compared to the ischemic group and the remote non-ischemic part of the myocardium at early reperfusion.

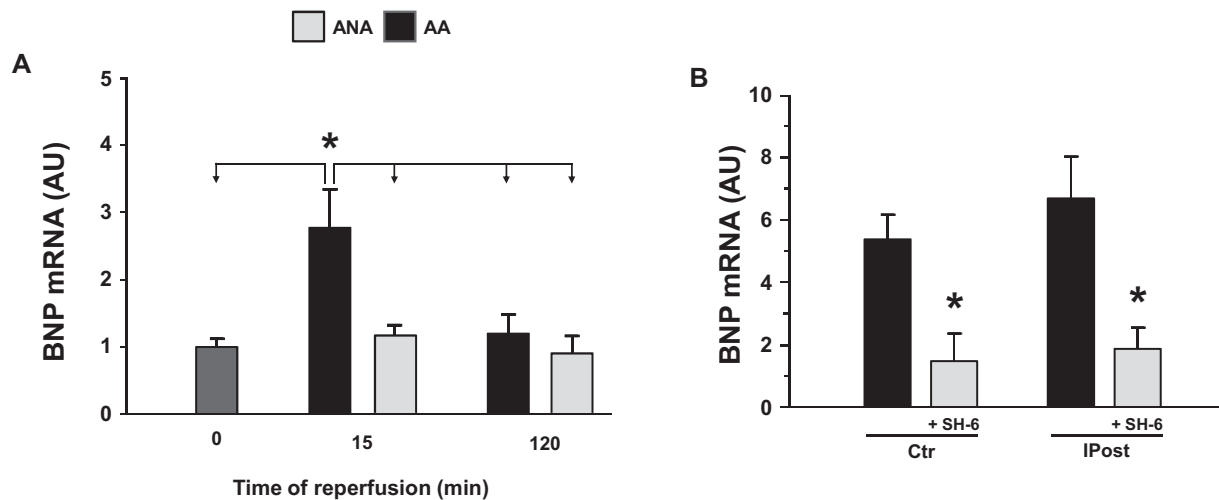
In separate experiments, we also investigated the effect of ischemic postconditioning (IPost) and Akt inhibition on BNP mRNA expression at early reperfusion in the area at risk of infarction. Myocardial BNP mRNA was significantly up-regulated in ischemic postconditioned (IPost) hearts as compared to ischemic controls at early reperfusion (IPost 6.7 ± 1.3 vs. ischemia 1.0 ± 0.2,  $p < 0.05$ ) (Fig. 6B). BNP expression in the parallel run controls were also elevated (Ctr 5.4 ± 0.8 vs. ischemia 1.0 ± 0.2,  $p < 0.05$ ). Furthermore, inhibiting Akt with SH-6 completely abrogated the elevated BNP

mRNA expression levels both in the IPost and control group (IPost 6.7 ± 1.3 vs. IPost + SH-6 1.8 ± 0.7,  $p < 0.05$ ) (Ctr 5.4 ± 0.8 vs. SH-6 1.5 ± 0.9,  $p < 0.05$ ) (Fig. 6B), indicating that Akt may modulate BNP expression levels at early reperfusion.

## Discussion

The findings can be summarized as follows: (1) when exogenous B-type natriuretic peptide (BNP) was intermittently infused for 3 × 30 s, in a postconditioning like fashion (BNP<sub>Post</sub>), it significantly reduced infarct size in the ex vivo rat heart. (2) BNP<sub>Post</sub> cardioprotection was abolished by inhibiting PI3K/Akt/p70s6k dependent signaling and by inhibition of natriuretic peptide receptors using isatin. (3) Pharmacological postconditioning with BNP peptides elevated Akt and p70s6k phosphorylation at early reperfusion. (4) The myocardial BNP mRNA levels in the area at risk (AA) were significantly elevated at early reperfusion compared to ischemic control tissue and remote ANA (area not at risk). (5) Ischemic postconditioning (IPost) did not elevate the BNP mRNA levels beyond the control group at early reperfusion. (6) Inhibition of Akt abrogated the early reperfusion induced BNP mRNA expression levels.

It has previously been shown that exogenous administration of BNP prior to, during and immediately after coronary occlusion protects the heart against ischemia reperfusion induced injury [9–13] via a cGMP/PKG driven mechanism [11], regulating the opening of intracellular K<sub>ATP</sub>-channels [9,29]. It is well known that the RISK member Akt serves as a focal point for signal transduction pathways coding for cell survival [30] and Akt mediated signaling events has been associated with activation of cGMP and PKG [31]. In the current study, we show that intermittent pharmacological manipulation of the heart for 3 × 30 s with BNP at early reperfusion, in



**Fig. 6.** Relative expression of left ventricular BNP mRNA at ischemic reperfusion. (A) BNP messenger RNA was quantified by real time quantitative qRT-PCR at various time points during reperfusion (0=end ischemia, 15 and 120 min) in the area at risk (AA, black bar) and the area not at risk (ANA, gray bar) of infarction. \* $P < 0.05$  vs.  $Ctrl_{R+15(AA)}$ . (B) In additional experiments, BNP mRNA expression analysis in the AA of hearts exposed to ischemic postconditioning (IPost;  $3 \times 30$  s of global ischemia), with or without the Akt inhibitor SH-6, was compared to parallel run control hearts. The mRNA data was normalized to GAPDH and expressed in arbitrary units (AU). Bars represent means  $\pm$  S.E.M.  $N \geq 3$  in each group. \* $P < 0.05$  vs. corresponding control group.

a postconditioning like fashion ( $BNP_{Post}$ ), reduced infarct size in isolated rat hearts. This  $BNP_{Post}$  induced cardioprotection was lost when inhibiting the classical RISK-kinases; PI3K with wortmannin, Akt with SH-6 and p70s6k with rapamycin.  $BNP_{Post}$  treatment also enhanced the phosphorylation of Akt and p70s6k. In addition, inhibition of Akt with SH-6 at reperfusion completely blunted BNP induced Akt phosphorylation. Our findings are in accordance with previous studies where BNP treatment during reperfusion have been found to activate both Akt and PKC in isolated mouse hearts [10], when administrated at hypoxia-reoxygenation to neonatal rat cardiomyocytes to prevented opening of the mitochondrial permeability pore (mPTP), partly via PI3K/Akt dependent signaling [32]. Administration of ANP infusion just prior to reperfusion also activated the RISK members PI3K and ERK in isolated perfused rabbit hearts [16].

Natriuretic peptides (NPs) interact with three types of membrane natriuretic peptide receptors (NPR) A, B and C. BNP has been shown to activate both NPR-A and C [15]. The density of NPR-C in most tissue is higher than that of NPR-A/B, and NPR-C has a high affinity for BNP [33]. Several studies have reported that NPs recruit the PI3/Akt signaling pathway via NPR-C [33,34]. Thus, cGMP/PKG and RISK signaling could be the result of BNP binding to two different receptors; cGMP is produced when BNP binds NPR-A and RISK signaling activated when BNP binds the NPR-C [9,33]. In this study, the  $BNP_{Post}$  induced cardioprotection and Akt phosphorylation was completely abolished by the non-selective NP receptor inhibitor isatin. At the concentration applied in this study (100  $\mu$ M), isatin has been shown to inhibit both particulate guanylyl cyclase activation via the NPR-A receptor, and also the NPR-C receptor (reviewed in [35]). Unfortunately, other non-peptide specific antagonists of NPRs are currently not available, and we are therefore unable to conclude which NPR that conveys the RISK mediated cardioprotection by  $BNP_{Post}$ .

Acute myocardial ischemia (AMI) up-regulates BNP gene expression in cardiac ventricles [7,8], but lately it has been suggested that ischemia-reperfusion (IR) may serve as an independent trigger of increased BNP mRNA myocardial content [19]. Our results indicate that reperfusion per se stimulates BNP mRNA expression beyond the ischemia induced expression level in the area at risk (AA). No regulation was evident in the remote non-ischemic area (ANA) at the same time point, and within 120 min of reperfusion

the BNP expression levels had returned to ischemic levels, with no difference between the AA and ANA. These results are in accordance with the results of Ramos et al. [19], who found that the BNP mRNA level was increased in the IR region (AA) as compared to the non-ischemic area (ANA) of the isolated rat heart independent of changes in the ventricular function. Seen in conjunction with our study, this implies that early reperfusion induced modulation of BNP gene expression is restricted to the ischemic area, reinforcing the concept of temporal molecular changes in the IR myocardium (AA) at very early reperfusion as compared to the remote myocardium (ANA).

In addition to being a marker of AMI and heart failure, Burley et al. speculated that NPs may be autacoid mediators of ischemic postconditioning (IPost), since the nonspecific NPR-A/C inhibitor isatin abolished the IPost induced protection [9]. Our results show that IPost does not influence BNP mRNA expression levels in the area at risk beyond the control group at early reperfusion. Despite comparable mRNA expression levels in IPost and control, it may not be representative for the BNP content/activity or the amount of BNP released from the myocardium. However, Goetze et al. found that plasma BNP and proBNP concentrations most likely reflected an increase in BNP gene expression in the ischemic human left ventricle, since the concentration were closely associated with ventricular mRNA expression [36].

Accumulation of nucleus activated Akt promote cardiomyocytes survival [37] via phosphorylation of multiple protein substrates and gene transcription regulation [20–23]. Nucleus targeted Akt has also been shown to promote ANP expression via an autocrine/paracrine stimulated PI3K-dependent signaling cascade [38]. Our results indicate that inhibition of Akt using SH-6 has the ability to depress left ventricular BNP mRNA expression. This implies that Akt-dependent signaling may be involved in regulating cardiac BNP expression at early reperfusion. Further studies are warranted to fully delineate the role of Akt in IR induced regulation of cardiac BNP mRNA levels, and to further evaluate whether the shift in gene expression is transitional or if it has long-term functional implications.

Several studies have documented a correlation between the plasma BNP concentration and infarct size in patients suffering from acute myocardial infarction (AMI) [7,39,40]. Normally, plasma BNP concentrations lie between 0 and 10 pmol/L, and circulating

levels may increase several 100-folds during AMI [39]. Interestingly, Hillock et al. observed that a 60 h exogenous BNP infusion (0.01  $\mu\text{g}/\text{kg}/\text{min}$ ) induced an incremental plasma BNP concentration within the range observed in severe MI and heart failure, but without reporting any safety hazard such as symptomatic hypotension, renal function or mortality [41]. Similarly, BNP infusion (0.05  $\mu\text{g}/\text{kg}/\text{min}$ ) for 4 h in an acute AMI pig model were well tolerated post-MI, with a peak plasma concentration of 1003 pmol/L [42]. We applied short and cyclic  $3 \times 30$  s infusion of BNP (10 nM) at early reperfusion, leading to a total administration time of only 1.5 min. The applied dose is in the supra-physiological range, but only 10 times higher than the peak plasma BNP concentration measured post-MI in the study of Kousholt et al. [42]. Hence, it is clear that the optimal concentration, timing and duration of BNP infusion after MI should be addressed in future studies, acknowledging that higher BNP dosages may have several serious side effects in vivo [43].

In conclusion, BNP administered in a postconditioning like manner reduced infarct size in the ex vivo rat heart. This cardioprotection is mediated via PI3K/Akt/p70s6k dependent RISK signaling, but further investigation is needed to elucidate the specific contribution of NPR-A vs. NPR-C. The data also indicate that Akt signaling may participate in regulation of BNP mRNA expression at early reperfusion.

### Conflict of interest

The authors declare that they have no conflict of interest.

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