Insulin and GSK3β-inhibition abrogates the infarct sparing-effect of ischemic postconditioning in *ex vivo* rat hearts

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ABSTRACT

Objectives

Pharmacological treatment of reperfusion injury using insulin and GSK3β inhibition has been shown to be cardioprotective, however, their interaction with the endogenous cardioprotective strategy, ischemic postconditioning, is not known.

Design

Langendorff perfused *ex vivo* rat hearts were subjected to 30 min of regional ischemia and 120 min of reperfusion. For the first 15 min of reperfusion hearts received either vehicle (Ctr), insulin (Ins) or a GSK3β inhibitor (SB415286; SB41), with or without interruption of ischemic postconditioning (IPost; 3x30s of global ischemia). In addition, the combination of insulin and SB41 for 15 min was assessed.

Results

Insulin, SB41 or IPost significantly reduced infarct size versus vehicle treated controls (IPost 33.5±3.3%, Ins 33.5±3.4%, SB41 30.5±3.0% vs. Ctr 54.7±6.8%, $p<0.01$). Combining insulin and SB415286 did not confer additional cardioprotection compared to the treatments given alone (SB41 + Ins 26.7±3.5%, ns). Conversely, combining either of the pharmacological reperfusion treatments with IPost completely abrogated the cardioprotection afforded by the treatments separately (Ins + IPost 59.5±3.4% vs. Ins 33.5±3.4% and SB41 + IPost 50.2±6.6% vs. SB41 30.5±3.0%, both $p<0.01$), and was associated with blunted Akt, GSK3β and STAT3 phosphorylation.

Conclusion

Pharmacological reperfusion treatment with insulin and SB41 interferes with the cardioprotection afforded by ischemic postconditioning.

INTRODUCTION

New developments in clinical cardiology, such as thrombolysis and percutaneous coronary intervention (PCI), have allowed rapid restoration of blood flow to the ischemic heart (i.e. reperfusion) and have greatly improved survival of patients with acute coronary syndromes. However, despite the necessity of reperfusion to salvage the compromised myocardial tissue, reperfusion itself leads to additional myocardial injury (lethal ischemia reperfusion injury, IRI) [1]. Currently, no treatments aimed at reducing IRI have successfully translated into clinical practice, warranting further research in this area.

During the last decade, the use of pre-clinical models has substantially enhanced our understanding of the mechanisms behind IRI and several cardioprotective strategies have emerged; such as ischemic pre- and post-conditioning (IPC and IPost), whereby short alternating cycles of ischemia and reperfusion are applied immediately before or after a prolonged ischemic event, respectively. In addition, several pharmacological agents have been able to mimic this cardioprotection (e.g. insulin), and two common and important signal transduction pathways have been identified: The Reperfusion Injury Salvage Kinase (RISK) pathway [2] and the more recent Survival Activating Factor Enhancement (SAFE) pathway [3], both recruited at the time of myocardial reperfusion. The common mechanism seems to involve stimulation of G-protein coupled receptors on the cell membrane and signal transduction via PI3K-Akt and MEK1/2-Erk1/2 (RISK) and/or the innate immune system and activation of the JAK/STAT3 (SAFE). These signaling pathways then seem to converge on glycogen synthase kinase 3β (GSK3β), which gets phosphorylated, and thus inhibited. This will, via largely unknown mechanisms lead to inhibition or delayed opening of the mitochondrial permeability transition pore (mPTP), and cardioprotection [4,5].

There may, however, be differences in signaling that could allow for synergistic cardioprotective effects, as some have reported that insulin and GSK3β signals via a distinct cellular mechanism different from ischemic conditioning [5]. The present study therefore explores the potential synergistic effects of combining a tyrosine kinase receptor activator, insulin, with IPost, as well as a more distant signaling event, GSK3β inhibition, with IPost.

MATERIALS AND METHODS

Ethical Approval

88 isolated hearts were studied, and all experiments were approved by the Norwegian State Commission for Laboratory Animals, and carried out in accordance with the European Communities Council Directive of 1986 (2010/63/EU).

Langendorff perfusion procedure

Male Wistar rats (Taconic Denmark) fed a standard diet were heparinized (200IU) and anesthetized using pentobarbital (50mg/kg i.p). Hearts were rapidly excised and immediately immersed in ice-cold Krebs-Henseleit buffer (118mM NaCl, 25mM $NaHCO_3$, 11mM d-Glucose, 4.7mM KCl, 1.22mM $MgSO_4*7H_2O$, 1.21mM KH_2PO_4, 1.84mM $CaCl_2*2H_2O$; pH 7.4). Within 1 minute hearts were mounted onto the Langendorff perfusion system and retrogradely perfused via the aorta with oxygenated Krebs-Henseleit buffer (95%O2 / 5%CO2, 37°C) at constant pressure (80mmHg). A silk suture was placed around the left anterior descending (LAD) coronary artery, while a water-filled latex balloon connected to a pressure transducer was placed into the left ventricle via the left atrium, and the diastolic pressure set to 5-10 mmHg. This measured left ventricular developed pressure (LVDP) and heart rate (HR), the product of which yields the rate pressure product (RPP). To monitor temperature, a thermo-probe was placed in the pulmonary artery through a small incision. Coronary flow (CF) was measured by timed collection of effluents. Regional ischemia (RI) was induced by tightening the silk suture around LAD and fastened using a pipette locking mechanism, and reperfusion achieved by loosening the suture.

Experimental protocol

All hearts were subjected to 20 min of stabilization followed by 30 min of regional ischemia (RI) and 120 min of reperfusion (Fig. 1). Hearts were randomized to receive either vehicle (Ctr), insulin (Ins [0.3 mU/ml]; Novo Nordisk A/S, Bagsværd, Denmark) or the GSK3β inhibitor SB415286 (SB41 [3μM]; Tocris Bioscience, UK) for 15 min at the onset of reperfusion, with or without interruption by 3x30s global ischemia (IPost). In addition, the combination of insulin and GSK3β inhibition for 15 min at reperfusion was evaluated. Finally, a parallel set of hearts underwent the same protocols, but were harvested at 15 min reperfusion. The atria and right ventricle were

removed and the area at risk from the left ventricle was snap frozen in liquid nitrogen for protein determination by western blotting (WB, n=3-5 for each group).

Measurement of ischemic risk zone and infarct size

At the end of the perfusion protocol LAD was re-occluded by securely tightening the silk suture, followed by infusion of a Evans Blue suspension (0.2% (w/v)) to demarcate the risk zone (Duke Scientific Corp., Palo Alto, CA, USA). Hearts were frozen (-20ºC) before being sectioned into 2-mm thick parallel slices from apex to the atrioventricular groove. Thereafter, the slices were stained for 15 min in 1% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) at 37 °C, followed by fixation in 4% formalin to enhance the stain contrast. Using a computerized planimetry program (Planimetry^{+} v2.0; ENK, Norway), the area of the left ventricle (LV), risk zone (AAR) and infarcted area were determined and multiplied by slice thickness to yield estimated volumes. Infarct size (IS) is expressed as the infarct volume/risk volume ratio (%). There were no significant differences between the different treatment groups in the relative volume of the area at risk (AAR/LV) (Table 1). A significant reduction in LVDP, CF and RPP after 5 min of regional ischemia confirmed that all groups obtained similar and expected degrees of ischemia relative to baseline (Table 2). Furthermore, there were no differences between groups with regards to recovery of LVDP, CF, HR or RPP during reperfusion (Table 2). The latter may be due to persistent stunning [6].

Immunoblot analysis

Myocardial phosphorylation of Akt at Ser^{473}, STAT-3 at Ser^{727} and GSK3β at Ser^{9} (Cell Signaling Technology, USA) in the area at risk were analysed by SDS-PAGE electrophoresis and WB analysis as previously described [7]. The tissue was homogenized in lysis buffer (20mM Tris-Hcl, 330mM Sucrose, 2mM EDTA and a protease inhibitor cocktail tablet (Roche Diagnostic)) and protein concentration was determined using a Bradford protein assay (Thermo Scientific). 40 µg of protein per sample were separated on 8-16% polyacrylamide-SDS gels (Thermo Scientific) and electrophoretically transferred onto PVDF (polyvinyl difluoride) membranes (Thermo Scientific). After transfer, membranes were activated in methanol, blocked in dry-milk, probed with primary antibody over night at 4°C followed by secondary antibody for one hour at room temperature. Western blots were developed by using an enhanced chemiluminescence detection system (Thermo Scientific) and band density imaged using Image J Software.

Statistics

Values are presented as mean ± standard error of the mean (s.e.m). Infarct size, AAR/LV and protein phosphorylation were tested for group differences by one way analysis of variance (ANOVA) combined with Fisher's post hoc test. Comparisons of LVDP, CF, HR and RPP between groups were tested with mixed ANOVA combined with Tukey's post hoc test for any significant differences. We tested stabilization vs. regional ischemia to confirm both adequate ischemia within groups and equal degree of ischemia between groups, and tested % recovery of 18 min stabilization at 5, 30 and 120 min of reperfusion to determine any differences after treatment. All statistics were performed in IBM SPSS (version 20.0.0). A value of $p<0.05$ was considered statistically significant.

RESULTS

Combination of Insulin and Ischemic Postconditioning Abrogates Cardioprotection

In this study we confirmed previous results where administration of insulin [0.3 mU/ml] for the first 15 minutes of reperfusion [8] or 3x30s ischemic postconditioning at the onset of reperfusion [12] significantly reduced infarct size compared to controls (Ins 33.5±3.4% or IPost 33.5±3.3% vs. Ctr 54.7±6.8%, $p<0.01$) (Fig. 2). Both treatments were associated with a significant increase in phosphorylated Akt, GSK3β and STAT3 vs. Ctr (Fig. 3A-C). Surprisingly, not only did we *not* get any additional reduction in infarct size when combining insulin with IPost, but the infarct sparing effect of the separate treatments was lost altogether (Ins + IPost 59.5±3.4% vs. Ctr 54.7±6.8%, ns) (Fig. 2). This was accompanied by significantly blunted levels of phosphorylated Akt, GSK3β and STAT3 (Fig. 3A-C).

Combination of GSK3β-inhibition and Ischemic Postconditioning Abrogates Cardioprotection

As crucial steps in RISK and SAFE signaling were abrogated with the combination of insulin and IPost, we wanted to bypass these kinases by targeting the putative common signaling kinase in both pathways, i.e. GSK3β. The ATP-competitive inhibitor of GSK3β, SB415286, was applied for the first 15 min of reperfusion, and it significantly reduced infarct size compared to controls (SB41 30.5±3.0% vs. Ctr 54.7±6.8%, $p<0.05$) (Fig. 2). As expected, this treatment alone had no effect on the phosphorylation of Akt, GSK3β or STAT3 (Fig. 3A-C), but when it was combined with IPost, this also abolished the infarct sparing effect (SB41 + IPost 50.2±6.6% vs. Ctr 54.7±6.8%, ns) (Fig. 2), and, even more surprisingly, significantly reduced the phosphorylation of Akt, GSK3β and STAT3 compared to IPost (Fig. 3A-C).

Combination of Insulin and GSK3β inhibition did not lead to Additional Cardioprotection

The combination of insulin and the GSK3β inhibitor SB415286 eluted directly in the drug reservoir and administered for 15 min at reperfusion, did not reduce infarct size compared to either treatment alone (Ins 33.5±3.4% or SB41 30.5±3.0% vs. Ins + SB41 26.7±3.5%, ns) (Fig. 2). The phosphorylation level of GSK3β and STAT3 was similar as insulin treatment alone (Fig. 3B and C), while the Akt phosphorylation was lower than the insulin group, but significantly higher than the SB41 group (Fig. 3A) (GSK3β phosphorylation by insulin is not affected by SB41 [25]).

DISCUSSION

The present study verifies that insulin for 15 min at reperfusion reduces infarct size and signals via Akt, GSK3β and STAT3, as reported earlier [8-10]. Similar results were obtained using a 3x30s protocol of IPost, also in concordance with others [11]. The rational for combining these two treatments was a study in which insulin (and GSK3β inhibition), in contrast to ischemic conditioning, was demonstrated to induce cardioprotection *independently* of mitochondrial ATP-sensitive potassium (mK_{ATP}) channels and subsequent release of reactive oxygen species (ROS) [5]. Opening of mK_{ATP}-channels has been demonstrated to be a vital trigger in many cardioprotective treatments (reviewed in [12]), including IPost [13], and thus it would seem logical to combine insulin and IPost. Much to our surprise, this combined treatment completely abolished any infarct sparing effect, and event that is not straightforward to explain. However, there is some evidence to suggest that protective mitochondrial signaling requires a cyclic activation at reperfusion: Bradykinin or direct mK_{ATP} channel opening by diazoxide is ineffective when applied continuously for 3 min at reperfusion, but reduces infarct size when applied for 5x10s (same as their IPost protocol) [14]. We replicated these findings with insulin: 1 or 5 min continuous insulin treatment was ineffective, but 3x30s intermittent infusion (InsPost) reduced infarct size to a similar degree as IPost, acting via mK_{ATP} and ROS [15]. Continuous (15 min) insulin treatment also signals via mK_{ATP} channels (results not shown). Thus, the signaling pathways in insulin and IPost induced cardioprotection may be more overlapping than initially thought. One could therefore speculate whether intermittent treatment activates and opens the mK_{ATP} channel faster than shorter continuous treatment, and/or that intermittent and longer continuous insulin produces just the right type and/or amount of ROS in the right intracellular compartment to induce protection from reperfusion induced injury. However, this still does not fully explain why the combination of two, seemingly channel-activating cardioprotective treatments like IPost and continuous insulin (15min), would be detrimental. Nevertheless, our data show a clear link between the loss of protection and diminished

phosphorylation of important RISK and SAFE signaling components like Akt, GSK3β and STAT3. The lack of protection in the shorter (1 or 5 min) insulin treatment groups also corresponded with reduced Akt phosphorylation [15]. Transient small bursts of cellular ROS may play an important role in insulin mediated signaling [16], while high concentration of ROS may attenuate insulin-mediated phosphorylation of Akt and GSK3β [17]. We therefore speculate whether the combination of IPost with insulin generates a larger, and hence, adverse ROS concentration causing blunted RISK and SAFE signaling, and loss of protection.

Based on our current knowledge from the literature, it is quite difficult to conceive how the combination of IPost with either GSK3β-inhibition (or insulin) would be detrimental. Phosphorylation, and thus, inhibition of GSK3β, a central, integrative kinase downstream of both Akt and STAT3, is crucial for cardioprotection from IPost [18], IPC [19], opioids [20], erythropoietin [21] and adenosine [22]. In addition, pharmacological inhibition of GSK3β has been demonstrated to reduce infarct size when administered prior to ischemia or five min before reperfusion [20]. Our results, where the use of an ATP competitive GSK3β inhibitor, SB415286, for 15 min at reperfusion reduced the infarct size is thus in concordance with previous findings. By directly targeting GSK3β, any upstream interference should be bypassed and a potential benefit of combining GSK3β inhibition with IPost should be revealed. However, SB41 *also* reversed the infarct sparing effect of IPost when the two treatments were combined. SB41 had no impact on Akt or STAT3 phosphorylation, as expected for a downstream kinase, but it did reduce the IPost induced phosphorylation of Akt and STAT3 when the two treatments were combined. This is a surprising finding and implies that there is some sort of cross-talk between GSK3β and more proximal kinases in RISK and SAFE. Although much evidence suggests that GSK3β is one of the last steps in the signaling cascade before inhibition of mPTP, some evidence suggests that mK_{ATP} channels are downstream of GSK3β inhibition [23]. In addition, Downey and co-workers have extensively studied signaling in IPC and hypothesized that the ROS signal produced from opening of mK_{ATP} channels induce a PKC mediated increase in affinity of the adenosine A2b receptors, which in turn, is responsible for higher levels of RISK activation at reperfusion [24]. Further research is needed to establish if this link between mitochondrial signaling and upstream RISK (and SAFE) signaling pertains to IPost as well.

The combination of insulin and GSK3β inhibition did not further decrease infarct size compared to either treatment alone. Activity assays from muscle and fat cell cultures have revealed greater inhibition of GSK3β by SB41 than by insulin [25], and thus we hypothesized an even greater reduction of infarct size. When this appears not to be the case, it would seem that insulin's inactivation of GSK3β already maximally exploits the potential for reduction of infarct size.

Limitations

First, although the Langendorff perfused rat heart represents a good compromise between quantity and quality of data, it is far from the *in vivo* setting with respects to oxidative stress, neuro-hormonal influence and several other components that may potentially influence the results obtained in the present study. Second, there appear to be differences in signaling pathways between rats and other species, and specifically a recent study of remote ischemic preconditioning in humans found STAT5 to be activated [26], unlike the more extensively studied STAT3 in rodents. Third, the present study was performed on young, healthy animals, while most myocardial infarct patients have comorbidities such as diabetes and hypertension. Studies on animal models of type 2 diabetes and post-infarct remodeling has shown impaired PI3K-Akt signaling [27, 28], and thus it is uncertain if myocardial signaling at reperfusion in human patients will behave similarly.

CONCLUSION

The present study demonstrated that direct pharmacological treatment of IRI by insulin or GSK3β-inhibition somehow interferes with the infarct sparing effect of the endogenous ischemic postconditioning mechanism, and that this coincides with attenuation of RISK and SAFE signaling. This could have important implications for clinical trials with ischemic postconditioning in which combination with insulin, or other agents inducing GSK3β-inhibition, could bereave patients of this treatment's clinical benefit.

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CONFLICTS OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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

Group	N	AAR/LV (%)
Ctr	8	41.5 ± 2.5
Ins	13	42.2 ± 2.5
IPost	6	49.1 ± 3.0
Ins+IPost	8	44.5 ± 2.5
SB41	11	41.1 ± 2.8
Ins+SB41	6	32.7 ± 4.8
IPost+SB41	9	44.6 ± 5.2

Values represent mean ± SEM.

Table 2 Functional parameters recorded during the experimental protocol.

	Group	18' Stab	5' RI	5' Rep	30' Rep	120' Rep
				% recovery of 18' Stab		
LVDP mmHg	Ctr	133 ± 11	70 ± 8 #	82 ± 10	65 ± 7	41 ± 6
	Ins	161 ± 10	88 ± 7 #	80 ± 5	74 ± 4	52 ± 4
	IPost	131 ± 8	58 ± 11#	69 ± 6	68 ± 4	50 ± 5
	Ins+IPost	148 ± 16	69 ± 8 #	76 ± 8	77 ± 15	51 ± 6
	SB41	161 ± 10	84 ± 13#	60 ± 4	63 ± 5	51 ± 3
	Ins+SB41	163 ± 10	89 ± 13#	74 ± 5	69 ± 4	52 ± 5
	IPost+SB41	143 ± 5	75 ± 5 #	80 ± 7	74 ± 5	61 ± 5
CF ml min^{-1}	Ctr	11.5 ± 1.3	7.4 ± 0.9#	88 ± 8	82 ± 6	52 ± 6
	Ins	12.8 ± 0.9	7.6 ± 0.4#	99 ± 9	81 ± 7	62 ± 7
	IPost	13.1 ± 1.0	7.8 ± 1.1#	88 ± 7	74 ± 8	70 ± 7
	Ins+IPost	13.3 ± 1.6	7.6 ± 0.6#	94 ± 10	84 ± 14	61 ± 6
	SB41	13.7 ± 0.8	8.0 ± 0.7#	68 ± 7	70 ± 4	53 ± 3
	Ins+SB41	14.9 ± 0.9	9.5 ± 1.1#	76 ± 9	78 ± 5	58 ± 5
	IPost+SB41	12.6 ± 1.2	6.8 ± 0.3#	99 ± 12	88 ± 5	67 ± 4
HR beats min^{-1}	Ctr	279 ± 14	262 ± 26	97 ± 1	94 ± 1	91 ± 1
	Ins	276 ± 11	259 ± 13	84 ± 1	95 ± 3	85 ± 2
	IPost	319 ± 9	295 ± 17	95 ± 2	96 ± 3	94 ± 2
	Ins+IPost	283 ± 13	270 ± 17	92 ± 1	96 ± 1	91 ± 1
	SB41	302 ± 6	275 ± 9#	89 ± 1	100 ± 1	94 ± 1
	Ins+SB41	295 ± 12	286 ± 12	93 ± 1	103 ± 1	97 ± 1
	IPost+SB41	286 ± 12	258 ± 8	91 ± 1	104 ± 3	98 ± 2
RPP beats*mmHg	Ctr	42349 ± 6003	17863 ± 2370#	67 ± 10	60 ± 8	35 ± 8
	Ins	44577 ± 2813	22123 ± 1691#	73 ± 11	67 ± 5	48 ± 5
	IPost	41681 ± 2078	17074 ± 3227#	62 ± 7	63 ± 5	46 ± 6
	Ins+IPost	42231 ± 5196	18290 ± 2049#	62 ± 8	65 ± 13	48 ± 6
	SB41	48933 ± 3055	22797 ± 3597#	45 ± 6	61 ± 5	47 ± 3
	Ins+SB41	47066 ± 4132	23503 ± 3541#	56 ± 4	70 ± 3	50 ± 4
	IPost+SB41	41125 ± 2489	19437 ± 1507#	66 ± 6	76 ± 4	59 ± 4

LVDP = left ventricular developed pressure (mmHg); CF = coronary flow (ml/min); HR = heart rate (beats/min);

RPP = rate pressure product (beats/mmHg); Stab = stabilization; RI = regional ischemia; Rep = reperfusion.

Values represent mean ± SEM. # $P<0.05$ vs. corresponding 18`Stab.

FIGURE LEGENDS

Fig. 1 Experimental protocol. Stab = stabilization; **RI** = regional ischemia; Open bars = buffer perfusion; **Ctr** = ischemia–reperfusion controls; **Ins** = insulin [0.3 mU/ml] for the first 15 min of reperfusion; **IPost** = ischemic postconditioning, 3x30s of global ischemia (GI); **Ins + IPost** = insulin interrupted by ischemic postconditioning; **SB41** = GSK3β inhibitor SB415286 [3 μM] for the first 15 min of reperfusion; **Ins + SB41** = co-administration of insulin and GSK3β inhibitor for the first 15 min of reperfusion. **IPost + SB41** = SB415286 interrupted by ischemic postconditioning; Arrows indicates time points for tissue collection from a parallel set of hearts.

Fig. 2 Infarct size. Infarct size is expressed as percentage of the area at risk. SB41 [3 μM] and insulin [0.3 mU/ml] reduced infarct size with ~40% compared to controls (Ctr). The combination of insulin and SB41 did not result in any further reduction of infarct size compared to the treatments alone. IPost reduced infarct size by ~36% compared to controls. However, combining IPost with either insulin [0.3 mU/ml] or SB41 [3 μM] completely abrogated the infarct-sparing effect of IPost. Bars represent mean ± SEM. N≥6 in each group. *p<0.05 vs. control group, ¤ p<0.05 vs. IPost, $ p<0.05 vs. Ins, # p<0.05 vs. SB41.

Fig. 3 Phosphorylation status of myocardial Akt, GSK3β and STAT3. Representative immunoblots (top) and densitometric analysis (bottom) of **(A)** total and phosphorylated Akt (Ser^{473}), **(B)** total and phosphorylated GSK3β (Ser^{9}) and **(C)** total and phosphorylated STAT3 (Ser^{727}). GAPDH serves as loading control. Reperfusion treatment with either Ins, SB41 or IPost lead to increased levels of phosphorylated Akt, GSK3β and STAT3, while the combination of Ins or SB41 and IPost reduced this phosphorylation. SB41 alone had no effect on phosphorylated Akt, GSK3β or STAT3. The combination of Ins and SB41 caused a significant elevation in Akt and GSK3β phosphorylation as compared to control, while STAT3 phosphorylation was not significantly elevated as compared to control, but not different from the Ins group either. Ins_B = baseline insulin perfusion for 20 min served as positive control. Ctr = KHB perfused ischemia-reperfusion. Densitometric analysis of total and phosphorylated proteins expressed in arbitrary units (AU) where the phosphorylated proteins are expressed as a ratio of the corresponding total proteins. Bars represent means ± S.E.M. N ≥ 3 in each group. Significant differences (P < 0.05) are as denoted in the bar graphs.