

Impact of O-GlcNAc on cardioprotection by remote ischaemic preconditioning in non-diabetic and diabetic patients

Rebekka V. Jensen^{1,2*}, Natasha E. Zachara³, Per H. Nielsen⁴, Hans Henrik Kimose⁴, Steen B. Kristiansen^{1,2}, and Hans Erik Bøtker^{1,2}

¹Department of Cardiology, Aarhus University Hospital, Skejby, Brendstrupgaardsvej 100, Aarhus N DK-8200, Denmark; ²Institute of Clinical Medicine, Aarhus University, Skejby, Brendstrupgaardsvej 100, Aarhus N DK-8200, Denmark; ³Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 N. Wolfe Street, Baltimore, MD 21205-2185, USA; and ⁴Department of Thoracic Surgery, Aarhus University Hospital, Skejby, Brendstrupgaardsvej 100, Aarhus N DK-8200, Denmark

Received 9 April 2012; revised 18 October 2012; accepted 6 November 2012; online publish-ahead-of-print 1 December 2012

Time for primary review: 33 days

Aims

Post-translational modification of proteins by O-linked β -N-acetylglucosamine (O-GlcNAc) is cardioprotective but its role in cardioprotection by remote ischaemic preconditioning (rIPC) and the reduced efficacy of rIPC in type 2 diabetes mellitus is unknown. In this study we achieved mechanistic insight into the remote stimulus mediating and the target organ response eliciting the cardioprotective effect by rIPC in non-diabetic and diabetic myocardium and the influence of O-GlcNAcylation.

Methods and results

The cardioprotective capacity and the influence on myocardial O-GlcNAc levels of plasma dialysate from eight healthy volunteers and eight type 2 diabetic patients drawn before and after subjection to an rIPC stimulus were tested on human isolated atrial trabeculae subjected to ischaemia/reperfusion injury. Dialysate from healthy volunteers exposed to rIPC improved post-ischaemic haemodynamic recovery (40 ± 6 vs. $16 \pm 2\%$; $P < 0.01$) and increased myocardial O-GlcNAc levels. Similar observations were made with dialysate from diabetic patients before exposure to rIPC (43 ± 3 vs. $16 \pm 2\%$; $P < 0.001$) but no additional cardioprotection or further increase in O-GlcNAc levels was achieved by perfusion with dialysate after exposure to rIPC (44 ± 4 and 42 ± 5 vs. $43 \pm 3\%$; $P = 0.7$). The glutamine:fructose-6-phosphate amidotransferase (GFAT) inhibitor azaserine abolished the cardioprotective effects and the increment in myocardial O-GlcNAc levels afforded by plasma from diabetic patients and healthy volunteers treated with rIPC.

Conclusions

rIPC and diabetes mellitus *per se* influence myocardial O-GlcNAc levels through circulating humoral factors. O-GlcNAc signalling participates in mediating rIPC-induced cardioprotection and maintaining a state of inherent chronic activation of cardioprotection in diabetic myocardium, restricting it from further protection by rIPC.

Keywords

Diabetes mellitus type 2 • Ischaemia • Remote ischaemic preconditioning • Reperfusion injury • O-linked β -N-acetylglucosamine (O-GlcNAc)

1. Introduction

In addition to the accumulation of risk factors and more extensive vascular disease, a mechanism underlying the impaired prognosis and increased mortality in type 2 diabetes mellitus (DM) after acute myocardial infarction (AMI)¹ may be increased susceptibility to ischaemia/reperfusion (IR) injury and reduced capacity for activation of endogenous cardioprotection. Activation may be achieved by

conditioning strategies among which remote ischaemic preconditioning (rIPC)—a cardioprotective mechanism where repetitive sublethal episodes of ischaemia induce resistance to myocardial IR injury^{2,3}—is most clinically applicable in AMI. However, cardioprotection by rIPC may be attenuated in diabetic myocardium.^{4–6} Conversely, diabetic animals may develop smaller infarct size after IR injury than non-diabetic animals,^{7,8} suggesting that diabetes *per se* activates basal innate metabolic cardioprotection without the ability to achieve further protection.

* Corresponding author. Tel: +45 78 45 20 29; fax: +45 78 45 22 60. Email: rrebekka.vibjerg@ki.au.dk

O-linked β -N-acetylglucosamine (O-GlcNAc) glycosylation is a novel post-translational modification of nuclear, cytoplasmic, and mitochondrial proteins that is sensitive to extracellular glucose concentrations.⁹ Elevated levels of O-GlcNAc have been associated with mediation of insulin resistance,^{10,11} atherosclerosis,¹² and cardiac dysfunction.^{13,14} O-GlcNAc levels are dynamically elevated in response to various stressors¹⁵ including local ischaemic preconditioning in non-diabetic myocardium. Notably, elevation of O-GlcNAc levels is cardioprotective, which may result from glycosylation of proteins such as voltage-dependent anion channel of the mitochondria (VDAC) and subsequent suppression of the mitochondrial permeability transition pore (mPTP).¹⁶ Exposure to high glucose and elevated expression of the O-GlcNAc transferase (OGT) increases mitochondrial protein O-GlcNAcylation and contributes to impaired mitochondrial function by compromising complex I, III, and IV activity and lower mitochondrial calcium and cellular ATP content.¹⁷ These are not only well-known abnormalities of the type 2 diabetic heart,¹⁸ but also components of the mechanisms underlying ischaemic preconditioning.^{19,20} We hypothesized that as a consequence of the possible inherent basal resistance to IR in type 2 diabetes, diabetes activates basal endogenous resistance to IR injury through up-regulation of O-GlcNAc levels and that cardioprotection by rIPC relies on O-GlcNAcylation, which cannot be further augmented because of its basal activation in diabetic patients.

rIPC liberates a dialyzable substance into the blood allowing protection to be transferred from a remotely preconditioned human donor to isolated perfused hearts.²¹ Using a dialysate of human plasma and isolated human atrial trabeculae and crossing between non-diabetic and diabetic donors and recipients, we were able to distinguish between non-diabetic and diabetic humoral responses of the effector organ and between non-diabetic and diabetic cellular effects in the target organ. The aim of this study was to achieve mechanistic insight into the remote stimulus mediating and the target organ response eliciting the cardioprotective effect by rIPC in non-diabetic and diabetic myocardium and the influence of O-GlcNAcylation.

2. Methods

A detailed description of the methods can be found in the Supplementary material online.

2.1 Remote ischaemic preconditioning protocol and preparation of dialysate

We recruited eight healthy volunteers and eight type 2 diabetic patients between 50 and 75 years of age. All the subjects underwent an oral glucose tolerance test, a physical examination, and an oral interview with a physician. Healthy volunteers with a fasting venous plasma glucose of ≥ 6.1 mmol/L or a venous plasma glucose of ≥ 7.8 mmol/L 2 h after oral glucose administration or with any sign of ischaemic heart disease or acute illness were excluded. All the type 2 diabetic patients had a fasting venous plasma glucose of ≥ 7.0 mmol/L and a 2 h venous plasma glucose of ≥ 11.1 mmol/L and when recruited for the study, HbA1c was between 6 and 9 mmol/L. Diabetic patients with any sign of ischaemic heart disease or acute illness were excluded from the study. Because of the influence of alcohol, caffeine, physical exercise, and antidiabetic medication on cardioprotection, all the subjects were instructed not to drink alcohol or caffeinated beverages and not to do physical exercise 5 days prior to examination.^{22–24} Diabetic patients were instructed not to take their oral antidiabetic drugs 4 days prior to examination and all other medicine including insulin on the day of examination.^{25,26} The investigation

conforms to the principles of the Declaration of Helsinki and the study protocol was approved by the regional Ethics Committee. Informed consent was obtained by the primary investigator.

The test persons were subjected to a remote ischaemic preconditioning stimulus of four times 5 min (rIPC) and four times 5 min + two times 10 min (intensified rIPC) upper arm ischaemia by inflation of a blood pressure cuff to 200 mmHg and 5 min reperfusion between inflations. Control blood samples of 120 mL were drawn from the cubital vein and collected in heparinized vials prior to the rIPC stimulus, and two rIPC blood samples were drawn after application of the two different intensities of the rIPC stimulus (Figure 1). Blood samples were centrifuged at 1520 g at 4°C for 20 min. Plasma was dialysed in 20-fold volume modified Krebs–Henseleit buffer [(pH 7.4) containing (in mmol/L) NaCl₂ (118), KCl (4.8), NaHCO₃ (27.2), MgCl₂ (1.2), KH₂PO₄ (1.0) CaCl₂ (2.0) and glucose (10)], using 12–14 kDa cut-off Spectra/Por Dialysis Membrane. Hence, six types of dialysate were prepared: (i) non-DM control dialysate; (ii) non-DM rIPC dialysate; (iii) non-DM intensified rIPC dialysate; (iv) DM control dialysate; (v) DM rIPC dialysate; and (vi) DM intensified rIPC dialysate.

2.2 Human atrial trabeculae

Atrial appendages were collected from patients undergoing elective heart surgery on extracorporeal circulation. Two study groups were included in the study by informed consent: non-diabetic patients and type 2 diabetic patients. Patients above the age of 85, patients with atrial fibrillation, and those with an ejection fraction of $<30\%$ or CKMB or Troponin T elevation within 2 weeks of surgery were excluded.

In connection with insertion of the venous cannula for extracorporeal circulation, right atrial appendages were collected. From these atrial appendages trabeculae were isolated and mounted in an organ bath with Krebs–Henseleit buffer as described in the Supplementary material online.

Force of contraction was measured continuously via a force transducer and data acquired and analysed using the Notocord Hem evolution software (Croissy sur Seine, France).

The atrial trabeculae were subjected to 75 min of stabilization, 30 min of superfusion with either control or rIPC dialysate depending on the experimental group, 90 min of simulated ischaemia, and 120 min of simulated reperfusion (Figure 1). Simulated ischaemia was induced by deoxygenating the buffer, replacing glucose and pyruvic acid with choline chloride, and increasing electrical stimulation from 1 to 3 Hz. At the end of reperfusion, trabeculae were snap-frozen in liquid nitrogen and stored at -80°C for western blotting and enzyme activity analysis for assessment of O-GlcNAc levels and formation. In three experimental groups, 80 μM azaserine, an inhibitor of glutamine:fructose-6-phosphate amidotransferase (GFAT), was added to the superfusion buffer 15 min prior to superfusion with dialysate and was present throughout the rest of the experiment.

Non-diabetic and diabetic atrial trabeculae were randomized to experimental groups listed in Figure 1.

Trabeculae that did not reach a force of contraction of 0.5 g by the end of the stabilization period were excluded. Recovery of contractile function expressed as a percentage of the baseline force of contraction is the primary endpoint. This was calculated by dividing the force of contraction reached at the end of reperfusion by the force of contraction at the end of the stabilization period for each trabecula.

2.3 O-GlcNAc analysis

Tissue samples were thawed in extraction buffer, ground with an electric grinder, sonicated for 2×10 s, and spun 18 000 g at 4°C for 30 min. Proteins were concentrated by ammonium sulfate precipitation (0–90%) on 100 μg of TCL, and samples were stored under desalting buffer for assays (below) or resuspended in $1 \times$ LDS containing dithiothreitol. Twenty micrograms of protein was loaded on two 8% PAGE gels and two 4–12% PAGE gels and western blotting was performed

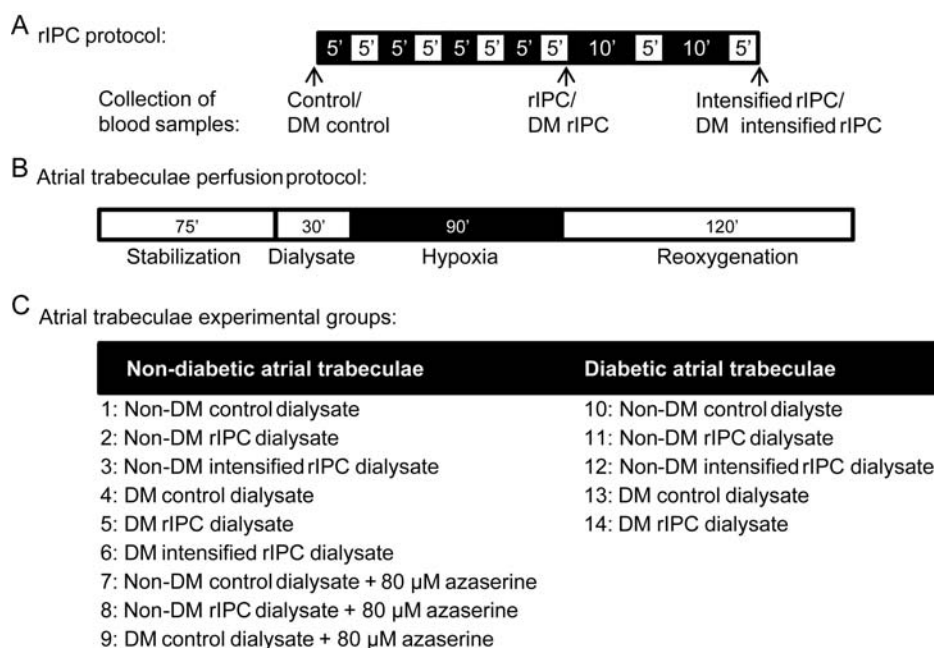


Figure 1 (A) rIPC protocol and collection of blood samples, which were prepared as plasma dialysates. (B) Experimental protocol used in the trabeculae experiments. (C) List of experimental groups.

with primary antibodies: anti-O-GlcNAc antibody (CDT 110.6), anti-O-GlcNAc antibody (CTD 110.6) + 100 mM GlcNAc (Sigma), and anti-actin antibody (Sigma). Densitometry was calculated relative to densitometry of the corresponding Actin blot.

2.4 Hexosamine biosynthetic pathway enzyme activity analysis

Proteins were concentrated by ammonium sulfate precipitation (0–90%) from 150 μ g of TCL and resuspended in Tris-HCL-buffer. O-GlcNAcase activity was estimated by measurement of the cleavage rate of a synthetic substrate *p*-nitrophenol *N*-acetylglucosamine. The activity assay was performed at 37°C for 24 h. Absorbance was measured at 400 nm. Activity is reported as picomoles of cleavage per min per mg of cell extract.

O-GlcNAc transferase activity was estimated as the rate of [3 H]-UDP-*N*-acetyl-D-glucosamine (American Radiolabeled Chemicals, Inc., St Louis, MO, USA) transfer to an acceptor peptide, casein kinase II peptide. Reactions were performed at room temperature for 4 h and stopped by adding 50 mM formic acid. The reactions were purified over C_{18} cartridge (Phenomenex), eluted with methanol into scintillation fluid, and counted.

2.5 Statistical analysis

One-way ANOVA and Kruskal–Wallis tests with pairwise comparison by Bonferroni and Dunns *post hoc* tests when appropriate were used to assess differences between groups. Mann–Whitney and unpaired *t*-test were used to assess differences between DM control and DM control + azaserine. Data are presented as mean \pm SEM, unless otherwise specified. A two-tailed *P*-value of <0.05 was considered statistically significant.

3. Results

3.1 Baseline characteristics

The characteristics of the test persons from whom dialysate was achieved and test persons from whom atrial trabeculae were obtained

are shown in Table 1. All non-diabetic patients had fasting blood glucose <6.7 mmol/L, which was significantly lower than in diabetic patients.

3.2 Haemodynamic recovery of human atrial trabeculae

In the atrial trabeculae from non-diabetic patients, dialysate from non-diabetic volunteers undergoing rIPC significantly improved haemodynamic recovery compared with dialysate from non-diabetic without rIPC (control) (40 ± 6 vs. $16 \pm 2\%$; $P < 0.01$; $n = 7$ –8/group) (Figure 2A). No additional protection was observed by the intensified rIPC stimulus ($39 \pm 3\%$). Control dialysate from diabetic patients improved haemodynamic recovery compared with control dialysate from non-diabetic patients (43 ± 3 vs. $16 \pm 2\%$; $P < 0.001$; $n = 7$ –8/group) (Figure 2A). No further protection was acquired by rIPC or intensified rIPC dialysate from diabetic patients compared with control dialysate from diabetic patients (44 ± 4 and 42 ± 5 vs. $43 \pm 3\%$; $P = 0.7$; $n = 8$ /group). Azaserine abolished the cardioprotective effect of both rIPC dialysate and diabetic control dialysate while having no effect on non-diabetic control dialysate ($n = 4$ –5/group) (Figure 4A and B).

In the atrial trabeculae from diabetic patients, we observed an improved recovery when perfused with non-diabetic and diabetic control dialysate compared with the non-diabetic atrial trabeculae (34 ± 4 and 35 ± 5 vs. $6 \pm 2\%$; $P < 0.05$ for both comparisons). No additional protection was acquired by rIPC or perfusion with control dialysate from diabetic patients in diabetic trabeculae (Figure 2B).

Haemodynamic data presented in gram can be found in the Supplementary material online.

Table 1 Baseline characteristics of test persons

	Dialysate test persons		Atrial trabeculae patients	
	Non-diabetic (n = 8)	Diabetic (n = 8)	Non-diabetic (n = 25)	Diabetic (n = 14)
Age, year (mean ± SD)	66 ± 6	63 ± 6	65 ± 12	70 ± 5
Male gender, n (%)	5 (60)	6 (80)	22 (88)	11 (79)
IHD, n (%)	0 (0)	0 (0)	19 (76)	13 (93)
EF, % (mean ± SD)	NA	NA	56 ± 7	53 ± 10
Total cholesterol, mmol/L (mean ± SD)	5.2 ± 0.8	4.1 ± 0.6**	4.2 ± 0.9	3.9 ± 0.6
LDL, mmol/L (mean ± SD)	3.6 ± 0.6	2.0 ± 0.4**	2.3 ± 0.8	2.1 ± 0.4
HDL, mmol/L (mean ± SD)	1.2 ± 0.2	1.5 ± 0.5	1.3 ± 0.3	1.2 ± 0.4
Triglyceride, mmol/L (mean ± SD)	1.1 ± 0.4	1.2 ± 0.6	1.4 ± 0.5	1.3 ± 0.4
Statin therapy, n (%)	0 (0)	6 (75)**	20 (80)	13 (93)
Antihypertensive therapy, n (%)	0 (0)	5 (63)*	21 (84)	14 (100)
Fasting blood glucose, mmol/L (mean ± SD)	5.3 ± 0.9	9.4 ± 2.8**	5.7 ± 0.4	7.4 ± 1.4****
HbA1c, mmol/L (mean ± SD)		0.077 ± 0.01		0.066 ± 0.01
Years with DM, year (mean ± SD)		9.9 ± 5.8		10.4 ± 6.0
<10 years with DM, n (%)		3 (37)		6 (46)
≥10 years with DM, n (%)		5 (63)		7 (54)
Metformine therapy, n (%)		4 (50)		10 (71)
Insulin therapy, n (%)		6 (75)		6 (43)
Neuropathy, n (%)		5 (63)		1 (7)
Retinopathy, n (%)		2 (25)		1 (7)

IHD, ischaemic heart disease; EF, ejection fraction; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

**** $P < 0.0001$ compared with respective non-diabetic subjects.

Mean force of contraction at baseline was 0.7 g (SEM ± 0.03 g). We found no difference in force of contraction at baseline between groups ($P = 0.6$).

3.3 Effect of remote ischaemic preconditioning on O-GlcNAc, OGT, and O-GlcNAcase levels

O-GlcNAc levels in the experimental groups are shown in Figures 2C and D, 3, and 4C, and D. In non-diabetic atrial tissue rIPC dialysate from non-diabetic volunteers significantly augmented O-GlcNAc levels compared with control dialysate from non-diabetic volunteers (1.5 ± 0.15 vs. 1.0 ± 0.06 , $P < 0.05$). Control and rIPC dialysate from diabetic patients significantly increased O-GlcNAc levels in non-diabetic atrial tissue compared with control dialysate from non-diabetic patients ($P < 0.05$ for both comparisons). Diabetic rIPC dialysate did not augment O-GlcNAc levels further compared with diabetic control dialysate ($P = 0.35$).

O-GlcNAc levels were increased in diabetic atrial tissue treated with control dialysate compared with non-diabetic atrial tissue treated with non-diabetic control dialysate ($P = 0.02$).

Azaserine blocked the increase in O-GlcNAc in non-diabetic atrial tissue treated with non-diabetic rIPC dialysate (Figures 4C and 5A). Azaserine also blocked the increase in O-GlcNAc in non-diabetic atrial tissue treated with diabetic control dialysate although not statistically significantly (Figures 4D and 5B).

3.4 O-GlcNAc transferase and O-GlcNAcase activity

OGT activity was increased by non-diabetic rIPC dialysate in non-diabetic atrial tissue (Figure 2E). The effect was abolished by azaserine (Figure 4E). No other dialysates affected OGT activity compared with non-diabetic control dialysate (Figures 2E and F, 4E and F).

Reduced O-GlcNAcase activity was seen in non-diabetic atrial tissue treated with non-diabetic rIPC dialysate, diabetic control, or diabetic rIPC dialysate compared with non-diabetic control dialysate (Figure 2G). O-GlcNAcase activity was also significantly reduced in all groups of diabetic atrial trabeculae compared with non-diabetic tissue treated with non-diabetic control dialysate ($P < 0.01$ for all comparisons) (Figure 2H). There was no additional reduction in O-GlcNAcase activity when comparing control dialysate with rIPC dialysate treatment in diabetic atrial tissue.

4. Discussion

The results of this study indicate that the mechanism underlying the cardioprotective effect of rIPC involves post-translational modification of myocardial proteins by O-GlcNAc, which is mediated by a circulating humoral factor independent of extracellular glucose concentration. Our results also demonstrate that type 2 diabetes *per se* reduces susceptibility to IR through a mechanism involving a humoral mediator that inherently up-regulates O-GlcNAcylation and restricts the potential for further protection from rIPC.

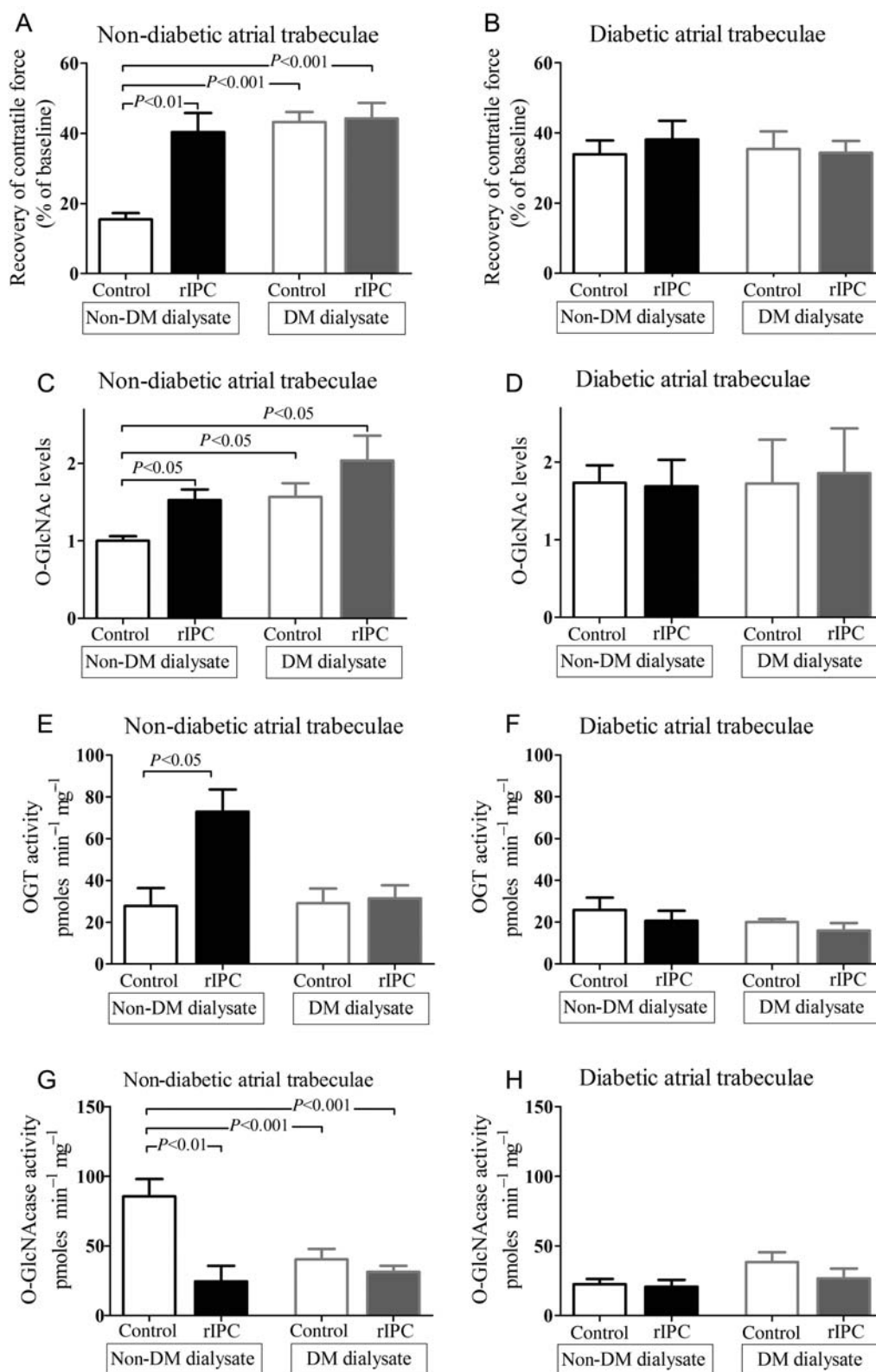


Figure 2 (A and B) Recovery of contractile force in atrial trabeculae from non-diabetic (A) and diabetic (B) patients. (A) No difference between diabetic control and diabetic rIPC dialysate. (B) No difference between any groups. (C and D) O-GlcNAc levels in atrial trabeculae from non-diabetic (C) and diabetic (D) patients. (C) No difference between diabetic control and diabetic rIPC dialysate. (D) No difference between any groups. (E and F) OGT activity in atrial trabeculae from non-diabetic (E) and diabetic (F) patients. (E) No difference between diabetic control and diabetic rIPC dialysate. (F) No difference between any groups. (G and H) O-GlcNAcase activity in atrial trabeculae from non-diabetic and diabetic patients. (G) No difference between diabetic control and diabetic rIPC dialysate. (H) No difference between any groups. Data are mean \pm SEM.

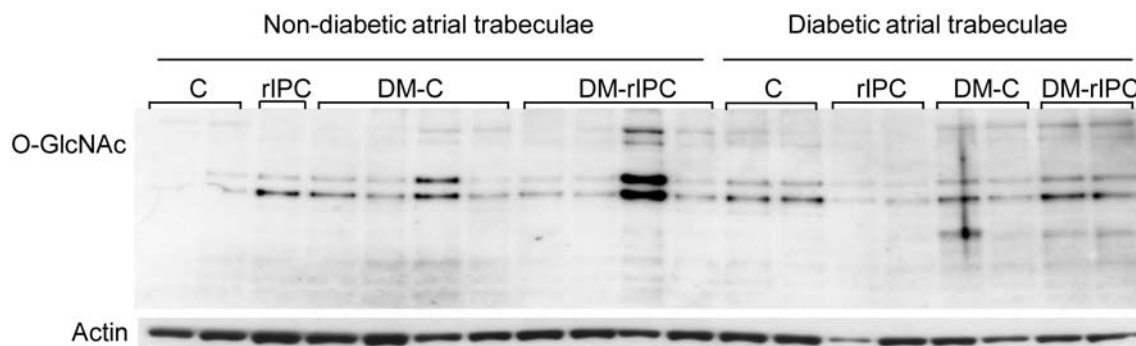


Figure 3 Representative O-GlcNAc (CTD110.6) and actin immunoblots of non-diabetic and diabetic atrial trabeculae. Note that the intensity of the O-GlcNAc bands of rIPC in diabetic atrial tissue is higher than control when corrected to actin, which reflects less protein in those lanes.

In this study we extended our previous model of ‘cross-species’ transfer of cardioprotective factors released by rIPC from a human-to-rabbit model²¹ to a human-to-human model. Using isolated human atrial trabeculae and crossing dialysate between effector and target organs from different individuals, we took advantage of the possibility of differentiating the mechanisms behind non-diabetic and diabetic humoral responses of the effector organ and the mechanisms behind non-diabetic and diabetic cellular effects in the target organ.

Similar to local ischaemic preconditioning,^{5,27} rIPC did not confer improved haemodynamic recovery by transfer of dialysate from either non-diabetic or diabetic donors. In contrast to an intensified local ischaemic preconditioning stimulus, which may elicit cardioprotection,⁵ we did not find a similar improvement by an intensified rIPC stimulus. This may be due to differences in the inherent mechanisms of local preconditioning and rIPC²⁸ and it cannot be ruled out that our intensified rIPC stimulus was insufficient.

Our study provides further knowledge about the mechanism behind the absence of effect of rIPC in human diabetic myocardial tissue, because dialysate from diabetic patients even before exposure to rIPC improved post-ischaemic haemodynamic recovery in non-diabetic atrial trabeculae. Similarly, diabetic atrial trabeculae perfused with control dialysate enhanced post-ischaemic haemodynamic recovery compared with non-diabetic trabeculae. However, as post-ischaemic functional recovery of the chronically cardioprotected diabetic trabeculae is similar to recovery of non-diabetic trabeculae treated with rIPC or dialysate from diabetic patients, our findings suggest that a basal potential of the end effector of cardioprotection is present in diabetic hearts. However, this potential is fully utilized and not accessible for further activation. As a consequence, type 2 DM *per se* induces a state of inherent basal resistance to IR by release of a circulating humoral factor, which also restricts the potential for further protection by rIPC.

Even though resistance to IR injury may be increased in type 2 diabetes, the drawback appears to be that the potential for further organ protection by rIPC is compromised. Our results appear to provide an explanation for this by our demonstration of the involvement of O-GlcNAc in cardioprotection.

Diabetic animal models are characterized by increased myocardial O-GlcNAc level.¹⁴ Consistent with these observations, O-GlcNAc

levels were elevated in our human atrial tissue samples from diabetic patients subjected to IR injury. This increment was induced by a circulating humoral factor as demonstrated by the fact that non-diabetic atrial tissue perfused with dialysate from diabetic patients not exposed to rIPC also increased myocardial O-GlcNAc levels. Like the cardioprotective effect of dialysate from diabetic patients, the elevation of O-GlcNAc levels was blocked by azaserine, suggesting that the cardioprotective effect of diabetes is related to a similar humoral factor and mediated by O-GlcNAc. Azaserine is a commonly used GFAT inhibitor used to block an increase in O-GlcNAc levels and cardioprotection *in vitro* with the same concentration as used in our studies. However, azaserine may have other cellular actions moderating its specificity.^{29,30} Even though the reduction of O-GlcNAc levels in the diabetic control group did not reach statistical significance, we consider this limitation mainly related to the restricted number of patients.

Cardioprotection by rIPC was associated with increased myocardial O-GlcNAc levels. A mechanistic connection between cardioprotection by rIPC and myocardial O-GlcNAc levels is substantiated by our finding that the absence of the capability to further augment O-GlcNAc levels by rIPC is associated with reduced efficacy of rIPC in diabetic patients. The cardioprotective effect of dialysate from diabetic patients in non-diabetic trabeculae implies that diabetic patients are capable of triggering a state of cardioprotection.

Like the inherent diabetic cardioprotection, perfusion with the GFAT inhibitor azaserine abolished the cardioprotective effects and the elevation of O-GlcNAc levels, demonstrating that cardioprotection by rIPC is dependent on augmentation of O-GlcNAc. Similarly, Jones *et al.*¹⁶ demonstrated that ischaemic preconditioning enhances O-GlcNAc levels *in vivo* and reduces sensitivity to mPTP formation. There are several potential mechanistic links between O-GlcNAc modification and cardioprotection. Exposure of cardiomyocytes to the O-GlcNAcase inhibitor O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc) causes O-GlcNAc modification of VDAC,^{16,31} which is a central element in formation of mPTP, and make them resistant to induction of mPTP and death. Augmentation of O-GlcNAc attenuates cell injury following IR by inhibiting the opening of mPTP and reducing Ca²⁺ overload and ROS

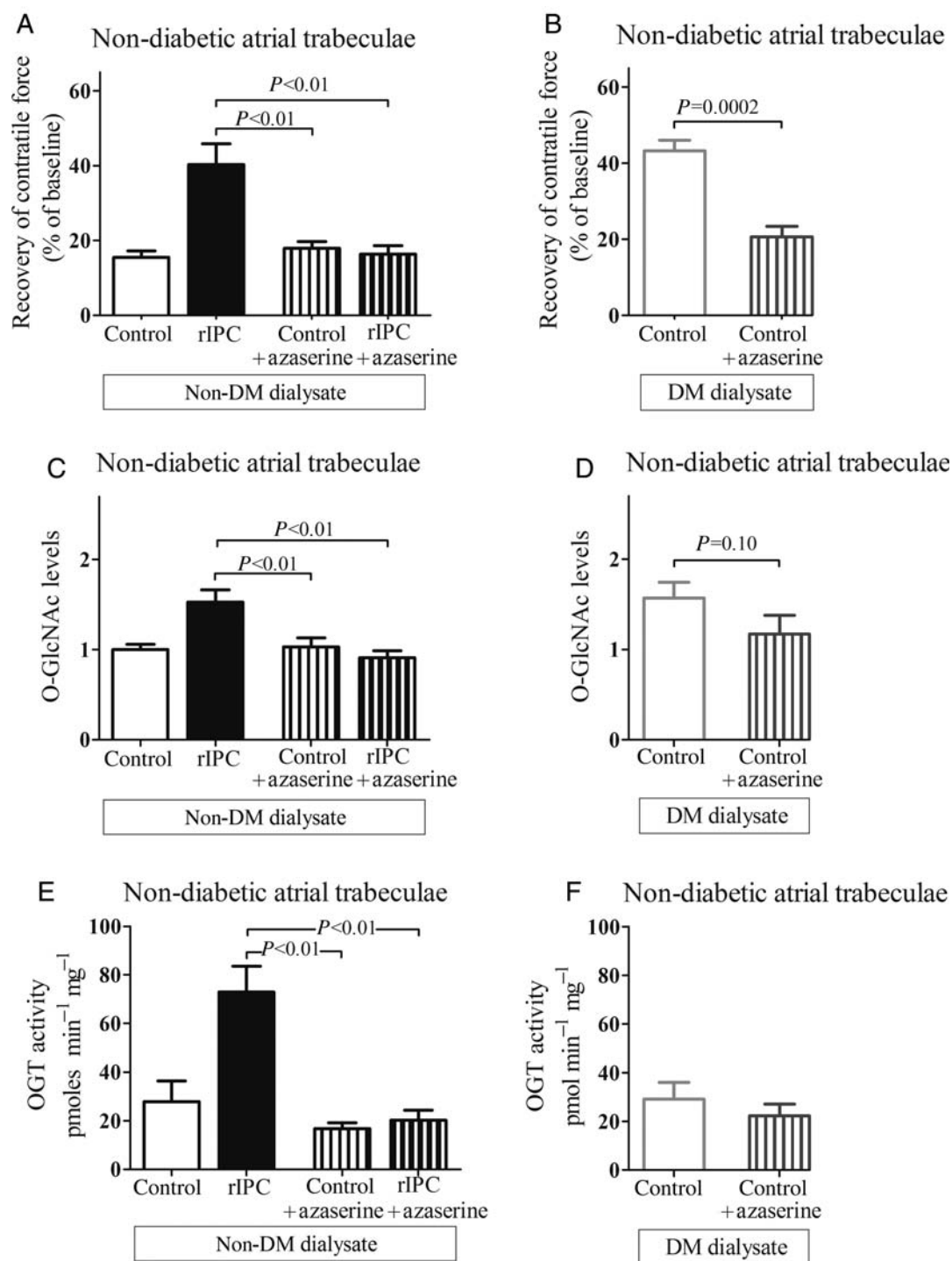
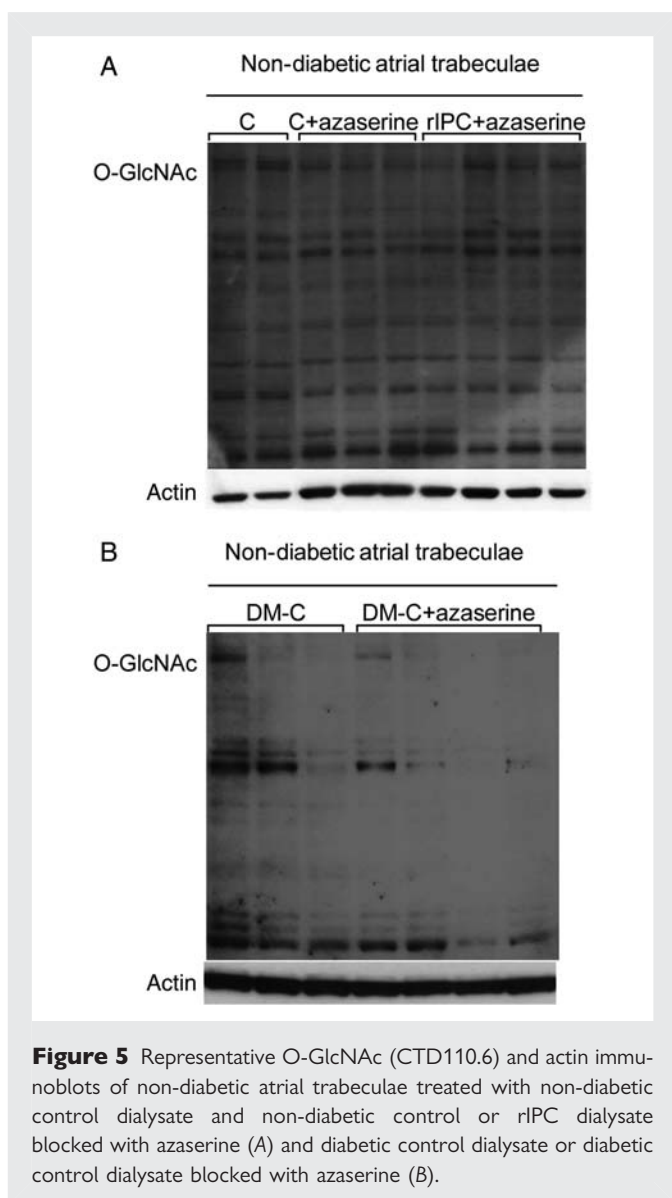


Figure 4 (A and B) The effect of perfusion with the GFAT inhibitor azaserine on the recovery of contractile force in atrial trabeculae from non-diabetic patients, when treated with non-diabetic dialysate (A) and diabetic dialysate (B). (A) No difference in recovery between control + azaserine and rIPC + azaserine. Non-diabetic control and non-diabetic rIPC same data as in Figure 2A. (B) Diabetic control same data as in Figure 2B. (C and D) O-GlcNAc levels in atrial trabeculae from non-diabetic patients, when treated with non-diabetic dialysate (C) and diabetic dialysate (D). (C) No difference in recovery between control + azaserine and rIPC + azaserine. Non-diabetic control and non-diabetic rIPC same data as in Figure 2C. (D) Difference between diabetic control and diabetic control + azaserine not significant $P = 0.10$. Diabetic control same data as in Figure 2D. (E and F) OGT activity in atrial trabeculae from non-diabetic patients, when treated with non-diabetic dialysate (E) and diabetic dialysate (F). (E) No difference in recovery between control + azaserine and rIPC + azaserine. Non-diabetic control and non-diabetic rIPC same data as in Figure 2E. (F) No difference between diabetic control and diabetic control + azaserine. Diabetic control same data as in Figure 2F. Data are mean \pm SEM.



generation³² and by increasing mitochondrial Bcl-2 levels.³³ This anti-apoptotic protein in the outer mitochondrial membrane plays an important role in the regulation of mitochondria-mediated apoptosis and is thought to inhibit opening of mPTP by interaction with VDAC.³⁴ Prevention of mPTP opening in early reperfusion is a well-known mechanism underlying cardioprotection by local IPC,^{35,36} and may also be associated with rIPC.³⁷ In addition PUGNAc reduces IR injury by attenuating calpain-mediated proteolysis of α -fodrin and Ca^{2+} /calmodulin-dependent protein kinase II during reperfusion.³⁸

The exact nature of the circulating cardioprotective factor(s) released by rIPC remains unknown. RIPC by transient limb ischaemia is dependent on intact neural pathways and nitric oxide-sensitive nerve stimulation to release of a blood-borne, hydrophobic, and small (molecular-mass <15 kDa) circulating factor(s),³⁹ which modify mitogen-activated protein kinase Akt, ERK1/2 and GSK-3 β ,⁴⁰ redistribution of PKC ϵ in subcellular compartments⁴¹ and STAT5 signalling,⁴² and finally converge at mitochondrial level to prevent mPTP opening in early reperfusion. Several substances such as adenosine, bradykinin, opioids, and calcitonin gene-related peptide are known humoral neurotransmitters involved in cardioprotection by rIPC.

It is unlikely that these transmitters are transferred in the plasma dialysate because of their short half-life and size.^{43,44} These agents more likely stimulate neural release of other transmitters, but the precise signal transduction pathway from the rIPC stimulus to O-GlcNAcylation and prevention of mPTP opening is unknown.

O-GlcNAc modification is regulated in a glucose-dependent manner and may provide a mechanistic explanation to the finding that type 2 DM modifies signalling pathways responsible for cardioprotection through chronic metabolic influence primarily by hyperglycaemia. Indeed, a difference in plasma glucose was present between non-diabetic volunteers and diabetic patients during the rIPC stimulus and collection of blood for dialysate. However, after dialyzation glucose concentrations were identical in all dialysates, indicating that the humoral cardioprotective factor in diabetic plasma that simultaneously increases myocardial O-GlcNAc levels acutely after IR injury is not glucose. In addition to extracellular glucose concentration, O-GlcNAcylation is sensitive to circulating glutamine concentrations because glucose and an amine group from glutamine converted into glutamate enter the hexosamine biosynthesis pathway to UDP-GlcNAc. Glutamine mediates cardioprotection through O-GlcNAcylation⁴⁵ also in DM. Although cardioprotection is induced by glutamine, efficacy is dependent on circulating glucose concentrations,⁴⁶ indicating that an increase in myocardial glucose uptake by IPC⁴⁷ may boost the flux through hexosamine biosynthetic pathway to increase O-GlcNAc levels.

Circulating humoral factor(s) mediating cardioprotection by rIPC and type 2 diabetes may share mechanistic elements but do not appear to be completely identical. We identified a difference in the mechanism behind elevation of O-GlcNAc level by diabetes and rIPC. Plasma from non-diabetic patients subjected to rIPC caused an increase in OGT activity and a decrease in O-GlcNAcase activity, whereas elevation in O-GlcNAc level and cardioprotection by diabetes was characterized by a reduction in O-GlcNAcase activity only. Only limited knowledge of the rearrangement in the O-GlcNAc network in human diabetes is available. In most animal studies, expression of OGT is elevated, whereas expression of O-GlcNAcase is reduced. Our results are in accordance with the finding that O-GlcNAcase expression is reduced in type 2 diabetes with progression of the disease.⁴⁸ In contrast, an acute stress signal like rIPC activates not only OGT, but also reduces O-GlcNAcase activity to increase O-GlcNAc levels in the heart.

We have previously demonstrated that diabetic peripheral neuropathy influences the release of the transferable cardioprotective factors in diabetic patients.²¹ In the study in question, five of the diabetic patients undergoing preconditioning and only one patient from whom atrial tissue was obtained had neuropathy. Despite this limitation, we were able to demonstrate that diabetes *per se* activates cardioprotection through augmentation of O-GlcNAc. In contrast to our findings in the present human-to-human transfer, our human-to-rabbit transfer only conferred cardioprotection after preconditioning and not by non-conditioned diabetic dialysate. This difference may reflect species-specific differences in the target organ.

A limitation of the study in question is that atrial trabeculae may not reflect the metabolism and physiology of ventricular myocardium. Nevertheless, important similarities between atrial and ventricular were demonstrated.^{49,50} Antidiabetic medication and caffeine have known cardioprotective effects. Diabetic patients were restricted from taking these agents prior to the rIPC stimulus and collection of blood samples and therefore did not confound the result.

In conclusion, this study demonstrates that the cardioprotective effects of rIPC are connected to augmentation of O-GlcNAc levels in humans. Furthermore, type 2 DM *per se* increases myocardial O-GlcNAc levels and induces a state of inherent chronic cardioprotection, which restricts the potential for further organ protection by rIPC in diabetic patients. The mechanisms seem to differ as rIPC increases O-GlcNAc levels by increasing OGT activity and decreasing O-GlcNAcase activity, while diabetes mellitus decreases only O-GlcNAcase activity.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

We greatly appreciate the technical assistance from Russell A. Reeves, Anja Helveg Larsen, and Casper Elkjær.

Conflict of interest: none declared.

Funding

This work was supported by Leducq (CVD 06), the Danish Research Council (11-108354), The Danish Strategic Research Council (11-1115818), American Heart Association (SD0930162N to N.E.Z.), and the National Heart Lung and Blood Institute (R21-HL-108003 and PO1HL107153 to N.E.Z.).

References

- Malmberg K, Yusuf S, Gerstein HC, Brown J, Zhao F, Hunt D *et al.* Impact of diabetes on long-term prognosis in patients with unstable angina and non-Q-wave myocardial infarction: results of the OASIS (Organization to Assess Strategies for Ischemic Syndromes) Registry. *Circulation* 2000;**102**:1014–1019.
- Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986;**74**:1124–1136.
- Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. *Circulation* 1993;**87**:893–899.
- Botker HE, Kharbanda R, Schmidt MR, Bottcher M, Klotz AK, Terkelsen CJ *et al.* Remote ischaemic conditioning before hospital admission, as a complement to angioplasty, and effect on myocardial salvage in patients with acute myocardial infarction: a randomised trial. *Lancet* 2010;**375**:727–734.
- Sivaraman V, Hausenloy DJ, Wynne AM, Yellon DM. Preconditioning the diabetic human myocardium. *J Cell Mol Med* 2010;**14**:1740–1746.
- Tsang A, Hausenloy DJ, Mocanu MM, Carr RD, Yellon DM. Preconditioning the diabetic heart: the importance of Akt phosphorylation. *Diabetes* 2005;**54**:2360–2364.
- Galagudza MM, Nekrasova MK, Syrenskii AV, Nifontov EM. Resistance of the myocardium to ischemia and the efficacy of ischemic preconditioning in experimental diabetes mellitus. *Neurosci Behav Physiol* 2007;**37**:489–493.
- Kristiansen SB, Lofgren B, Stottrup NB, Khatir D, Nielsen-Kudsk JE, Nielsen TT *et al.* Ischaemic preconditioning does not protect the heart in obese and lean animal models of type 2 diabetes. *Diabetologia* 2004;**47**:1716–1721.
- Robinson KA, Weinstein ML, Lindenmayer GE, Buse MG. Effects of diabetes and hyperglycemia on the hexosamine synthesis pathway in rat muscle and liver. *Diabetes* 1995;**44**:1438–1446.
- Marshall S, Bacote V, Traxinger RR. Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system. Role of hexosamine biosynthesis in the induction of insulin resistance. *J Biol Chem* 1991;**266**:4706–4712.
- Vosseller K, Wells L, Lane MD, Hart GW. Elevated nucleocytoplasmic glycosylation by O-GlcNAc results in insulin resistance associated with defects in Akt activation in 3T3-L1 adipocytes. *Proc Natl Acad Sci U S A* 2002;**99**:5313–5318.
- Federici M, Menghini R, Mauriello A, Hribal ML, Ferrelli F, Lauro D *et al.* Insulin-dependent activation of endothelial nitric oxide synthase is impaired by O-linked glycosylation modification of signaling proteins in human coronary endothelial cells. *Circulation* 2002;**106**:466–472.
- Clark RJ, McDonough PM, Swanson E, Trost SU, Suzuki M, Fukuda M *et al.* Diabetes and the accompanying hyperglycemia impairs cardiomyocyte calcium cycling through increased nuclear O-GlcNAcylation. *J Biol Chem* 2003;**278**:44230–44237.
- Fulop N, Mason MM, Dutta K, Wang P, Davidoff AJ, Marchase RB *et al.* Impact of type 2 diabetes and aging on cardiomyocyte function and O-linked N-acetylglucosamine levels in the heart. *Am J Physiol Cell Physiol* 2007;**292**:C1370–C1378.
- Zachara NE, O'Donnell N, Cheung WD, Mercer JJ, Marth JD, Hart GW. Dynamic O-GlcNAc modification of nucleocytoplasmic proteins in response to stress. A survival response of mammalian cells. *J Biol Chem* 2004;**279**:30133–30142.
- Jones SP, Zachara NE, Ngoh GA, Hill BG, Teshima Y, Bhatnagar A *et al.* Cardioprotection by N-acetylglucosamine linkage to cellular proteins. *Circulation* 2008;**117**:1172–1182.
- Hu Y, Suarez J, Fricovsky E, Wang H, Scott BT, Trauger SA *et al.* Increased enzymatic O-GlcNAcylation of mitochondrial proteins impairs mitochondrial function in cardiac myocytes exposed to high glucose. *J Biol Chem* 2009;**284**:547–555.
- Lowell BB, Shulman GI. Mitochondrial dysfunction and type 2 diabetes. *Science* 2005;**307**:384–387.
- da Silva MM, Sartori A, Belisle E, Kowaltowski AJ. Ischemic preconditioning inhibits mitochondrial respiration, increases H₂O₂ release, and enhances K⁺ transport. *Am J Physiol Heart Circ Physiol* 2003;**285**:H154–H162.
- Wojtovich AP, Brookes PS. The endogenous mitochondrial complex II inhibitor malonate regulates mitochondrial ATP-sensitive potassium channels: implications for ischemic preconditioning. *Biochim Biophys Acta* 2008;**1777**:882–889.
- Jensen RV, Stottrup NB, Kristiansen SB, Botker HE. Release of a humoral circulating cardioprotective factor by remote ischemic preconditioning is dependent on preserved neural pathways in diabetic patients. *Basic Res Cardiol* 2012;**107**:285.
- Domenech R, Macho P, Schwarze H, Sanchez G. Exercise induces early and late myocardial preconditioning in dogs. *Cardiovasc Res* 2002;**55**:561–566.
- Miyamae M, Camacho SA, Zhou HZ, Diamond I, Figueredo VM. Alcohol consumption reduces ischemia-reperfusion injury by species-specific signaling in guinea pigs and rats. *Am J Physiol* 1998;**275**:H50–H56.
- Riksen NP, Zhou Z, Oyen WJ, Jaspers R, Ramakers BP, Brouwer RM *et al.* Caffeine prevents protection in two human models of ischemic preconditioning. *J Am Coll Cardiol* 2006;**48**:700–707.
- Jonassen AK, Sack MN, Mjos OD, Yellon DM. Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. *Circ Res* 2001;**89**:1191–1198.
- Ye Y, Perez-Polo JR, Aguilar D, Birnbaum Y. The potential effects of anti-diabetic medications on myocardial ischemia-reperfusion injury. *Basic Res Cardiol* 2011;**106**:925–952.
- Ghosh S, Standen NB, Galinanes M. Failure to precondition pathological human myocardium. *J Am Coll Cardiol* 2001;**37**:711–718.
- Heinen NM, Putz VE, Gorgens JJ, Huhn R, Gruber Y, Barthuber C *et al.* Cardioprotection by remote ischemic preconditioning exhibits a signaling pattern different from local ischemic preconditioning. *Shock* 2011;**36**:45–53.
- Lyons SD, Sant ME, Christopherson RL. Cytotoxic mechanisms of glutamine antagonists in mouse L1210 leukemia. *J Biol Chem* 1990;**265**:11377–11381.
- Suzuki H, Kumagai H, Tochikura T. gamma-Glutamyltranspeptidase from *Escherichia coli* K-12: purification and properties. *J Bacteriol* 1986;**168**:1325–1331.
- Hirose K, Tsutsumi YM, Tsutsumi R, Shono M, Katayama E, Kinoshita M *et al.* Role of the O-linked beta-N-acetylglucosamine in the cardioprotection induced by isoflurane. *Anesthesiology* 2011;**115**:955–962.
- Ngoh GA, Watson LJ, Facundo HT, Jones SP. Augmented O-GlcNAc signaling attenuates oxidative stress and calcium overload in cardiomyocytes. *Amino Acids* 2011;**40**:895–911.
- Champattanachai V, Marchase RB, Chatham JC. Glucosamine protects neonatal cardiomyocytes from ischemia-reperfusion injury via increased protein O-GlcNAc and increased mitochondrial Bcl-2. *Am J Physiol Cell Physiol* 2008;**294**:C1509–C1520.
- Tsujimoto Y, Nakagawa T, Shimizu S. Mitochondrial membrane permeability transition and cell death. *Biochim Biophys Acta* 2006;**1757**:1297–1300.
- Argaud L, Gateau-Roesch O, Chababreyse L, Gomez L, Loufouat J, Thivolet-Bejui F *et al.* Preconditioning delays Ca²⁺-induced mitochondrial permeability transition. *Cardiovasc Res* 2004;**61**:115–122.
- Javadov SA, Clarke S, Das M, Griffiths EJ, Lim KH, Halestrap AP. Ischaemic preconditioning inhibits opening of mitochondrial permeability transition pores in the reperfusion rat heart. *J Physiol* 2003;**549**:513–524.
- Zhang SZ, Wang NF, Xu J, Gao Q, Lin GH, Bruce IC *et al.* Kappa-opioid receptors mediate cardioprotection by remote preconditioning. *Anesthesiology* 2006;**105**:550–556.
- Liu J, Marchase RB, Chatham JC. Increased O-GlcNAc levels during reperfusion lead to improved functional recovery and reduced calpain proteolysis. *Am J Physiol Heart Circ Physiol* 2007;**293**:H1391–H1399.
- Steensrud T, Li J, Dai X, Manliot C, Kharbanda RK, Tropak M *et al.* Pretreatment with the nitric oxide donor SNAP or nerve transection blocks humoral preconditioning by remote limb ischemia or intra-arterial adenosine. *Am J Physiol Heart Circ Physiol* 2010;**299**:H1598–H1603.
- Tamareille S, Mateus V, Ghaboura N, Jeanneteau J, Croue A, Henrion D *et al.* RISK and SAFE signaling pathway interactions in remote limb ischemic preconditioning in combination with local ischemic postconditioning. *Basic Res Cardiol* 2011;**106**:1329–1339.
- Wolfgram S, Schneider K, Heidbreder M, Nienstedt J, Dominiak P, Dendorfer A. Remote preconditioning protects the heart by activating myocardial PKCepsilon-isoform. *Cardiovasc Res* 2002;**55**:583–589.

42. Heusch G, Musiolik J, Kottenberg E, Peters J, Jakob H, Thielmann M. STAT5 activation and cardioprotection by remote ischemic preconditioning in humans: short communication. *Circ Res* 2012;**110**:111–115.
43. Dickson EW, Lorbar M, Porcaro WA, Fenton RA, Reinhardt CP, Gysembergh A et al. Rabbit heart can be 'preconditioned' via transfer of coronary effluent. *Am J Physiol* 1999;**277**:H2451–H2457.
44. Serejo FC, Rodrigues LF Jr, da Silva Tavares KC, de Carvalho AC, Nascimento JH. Cardioprotective properties of humoral factors released from rat hearts subject to ischemic preconditioning. *J Cardiovasc Pharmacol* 2007;**49**:214–220.
45. Liu J, Marchase RB, Chatham JC. Glutamine-induced protection of isolated rat heart from ischemia/reperfusion injury is mediated via the hexosamine biosynthesis pathway and increased protein O-GlcNAc levels. *J Mol Cell Cardiol* 2007;**42**:177–185.
46. Ugurlucan M, Erer D, Karatepe O, Ziyade S, Haholu A, Gungor UF et al. Glutamine enhances the heat shock protein 70 expression as a cardioprotective mechanism in left heart tissues in the presence of diabetes mellitus. *Expert Opin Ther Targets* 2010;**14**:1143–1156.
47. Tong H, Chen W, London RE, Murphy E, Steenbergen C. Preconditioning enhanced glucose uptake is mediated by p38 MAP kinase not by phosphatidylinositol 3-kinase. *J Biol Chem* 2000;**275**:11981–11986.
48. Lehman DM, Fu DJ, Freeman AB, Hunt KJ, Leach RJ, Johnson-Pais T et al. A single nucleotide polymorphism in MGEA5 encoding O-GlcNAc-selective N-acetyl-beta-D glucosaminidase is associated with type 2 diabetes in Mexican Americans. *Diabetes* 2005;**54**:1214–1221.
49. Bohm M, Pieske B, Ungerer M, Erdmann E. Characterization of A1 adenosine receptors in atrial and ventricular myocardium from diseased human hearts. *Circ Res* 1989;**65**:1201–1211.
50. Heidbuchel H, Vereecke J, Carmeliet E. Three different potassium channels in human atrium. Contribution to the basal potassium conductance. *Circ Res* 1990;**66**:1277–1286.