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Biochemical and Biophysical Research Communications 349 (2006) 400–407
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Resistin worsens cardiac ischaemia-reperfusion injury

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Received 10 August 2006
Available online 18 August 2006

Abstract

We provide the first report of direct effects of resistin upon haemodynamic and neurohumoral parameters in isolated perfused rat heart preparations. Pre-conditioning with 1 nmol L^{-1} recombinant human resistin prior to ischaemia significantly impaired contractile recovery during reperfusion, compared with vehicle-infused hearts ($P < 0.05$, $n = 12$). This was accompanied by a significant increase in both A-type and B-type natriuretic peptides ($P < 0.05$, $n = 12$ both ANP and BNP vs vehicle), creatine kinase, and tumour necrosis factor-α (TNF-α) release in resistin-infused hearts. Resistin had no significant effect on myocardial glucose uptake. Co-infusion of resistin with Bay 11 7082 (an NF-κB inhibitor) improved contractile recovery following ischaemia and reduced both natriuretic peptide and creatine kinase release. This is the first evidence indicating resistin impairs cardiac recovery following ischaemia, stimulates cardiac TNF-α secretion, and modulates reperfusion release of natriuretic peptides and biochemical markers of myocardial damage. A TNF-α signalling related mechanism is suggested as one component underlying these effects.

Keywords: Ischaemia; Resistin; Isolated heart; TNF-α; NF-κB

Resistin, a 92 amino acid protein primarily secreted from adipose tissue in rodents [1] and macrophages in humans [2], is the primary member of a complex family of proteins known as resistin-like molecules or RELMs, which includes RELM α, RELM β, and RELM γ [3,4]. This protein family is distinguished by the presence of a unique pattern of 11 cysteine residues and the fact that the proteins naturally form complex multimeric structures [1,4]. Resistin was initially suggested to be an important link between obesity and diabetes, as lean, healthy mice injected with resistin became insulin resistant and gained weight [5,6]. Obese humans are reported to have increased plasma levels of resistin compared with lean healthy, controls [7]. In vitro studies have indicated that resistin may play a role in adipocyte differentiation, glucose metabolism, and inflammation [8,9]. Clinical studies suggest a role in foetal development [10,11], metabolic syndrome [12,13], atherosclerotic plaque formation [14], and even Cushing's syndrome [15], although whether serum resistin levels are directly related to body mass index (BMI) or insulin sensitivity in humans is still largely unresolved [7,16]. *In vitro,* recombinant resistin has been shown to up-regulate pro-inflammatory cytokines and adhesion molecules on human endothelial cells [17], while serum levels in humans have been correlated with TNF-α, interleukin-6 (IL-6), and C-reactive protein (CRP) levels [17–19] and more recently coronary artery calcification [14,20]. Given the high cardiovascular risk associated with obesity and the metabolic syndrome, resistin may play a role in the progression from vascular inflammation to endothelial dysfunction and the eventual development of overt cardiovascular disease. Here, we provide the first evidence of direct actions of resistin on the isolated perfused rat heart and its potential to stimulate a cardiac pro-inflammatory cascade via an NF-κB pathway.

Materials and methods

Chemicals. Recombinant human resistin was purchased from Biovendor Medical Laboratory Medicine (Czech Republic) and dissolved in

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doi:10.1016/j.bbrc.2006.08.052

perfusion buffer immediately before infusion. Purity of the protein was >95% (SDS–PAGE analysis) according to the manufacturer. Bay 11 7082, which irreversibly inhibits cytokine-inducible IκBα phosphorylation without inhibiting constitutive phosphorylation, was purchased from Biomol Research Laboratories Inc. (USA). Purity of 99% was reported by the manufacturer. All concentrations expressed are final concentrations given dilution in the perfusion system.

Isolated heart preparation. Male Sprague–Dawley rats ($n = 60$, 250–400 g) were obtained from the animal housing facilities at the Christchurch School of Medicine and Health Sciences, Christchurch, New Zealand. Rats underwent a modified isolated heart procedure similar to that described previously [21]. Briefly, rats were anaesthetised with sodium pentobarbitone (50 mg/kg intraperitoneally), decapitated, and the chest quickly opened. The heart was cannulated above the aortic valve and perfused with oxygenated (95% O_2–5% CO_2) Krebs–Hanseleit buffer (final concentrations (mmol L^{-1}); NaCl (123), $NaHCO_3$ (22), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4 \cdot 7H_2O$ (1.1), $CaCl_2 \cdot 2H_2O$ (1.5), and glucose (11.0), final pH 7.40) at 37 °C, in a retrograde fashion (Langendorff). A constant flow rate of 12 ml/min was maintained with a peristaltic pump in a non-recirculating setup (Gilson minipuls, model MP-2). Perfusion pressure (PP), which reflects coronary resistance, was measured by using a pressure transducer (ADInstruments, Australia) situated on a side arm of the aortic cannula above the aortic root. The left atrium was removed and a fluid-filled, latex balloon connected to a pressure transducer (ADInstruments, Australia) inserted into the left ventricle. This allowed continuous measurement of heart rate (HR), developed pressure (DP), and the maximal and minimal derivatives of the left ventricular pressure ($+dP/dt_{max}$). All experiments and study protocols were approved by the University of Otago Animal Ethics Committee, and conformed to the NIH guidelines on the use of animals in laboratory research (NIH publication No. 85–23, revised 1996).

Ischaemia-reperfusion protocol. All preparations used in ischaemia-reperfusion experiments were paced to 350 beats per minute (bpm) with a stimulator (Digitimer Ltd., England) using a bipolar electrode placed on the right atrium (9 V, 1 ms). Following cannulation and initial setup, hearts were left to stabilise for 40 min. After this time, hearts were pre-conditioned for 30 min in a random protocol with either vehicle (control) or vehicle containing either 1 nmol L^{-1} recombinant human resistin alone, 100 nmol L^{-1} Bay 11 7082 alone, or 1 nmol L^{-1} resistin + Bay 11 7082. After 30 min pre-conditioning, hearts were exposed to 35 min zero-flow, global ischaemia followed by reperfusion with vehicle for 2 h. Hearts were not paced during ischaemia. For the experimental protocol, see Fig. 1A.

Measurement of natriuretic peptides and creatine kinase. Timed collections of perfusate were obtained for the measurement of ANP, BNP, and creatine kinase (CK) (Fig. 1A). Perfusate underwent SepPak extraction for ANP and BNP analysis. Immunoreactive ANP and BNP concentrations were measured as previously described [22]. CK activity was measured on an Abbot Aeroset (Canterbury Health Labs, Christchurch, New Zealand).

Myocardial glucose uptake. Isolated rat heart preparations were prepared as above. However, perfusion buffer was constantly recirculated throughout the experiment. Hearts were perfused with either vehicle or vehicle containing 1 nmol L^{-1} recombinant human resistin for 30 min followed by a further 30 min of vehicle only infusion. Collections for glucose measurements were taken at time points indicated in Fig. 1B. Perfusate was sampled from an inflowing pump connected to the main reservoir (G_{in}) and from an outlet immediately downstream from the heart (G_{out}). Following each experiment, hearts were blotted to remove excess moisture and weighed.

Glucose activity assay was performed by Canterbury Health Labs (Christchurch, New Zealand) according to manufacturers' protocols (Abbot, Illinois, USA). Myocardial glucose uptake was calculated as follows:

Glucose uptake (μg min mg^{-1}) = $[G_{in} - G_{out}] \times$ (flow rate (ml min^{-1})/tissue weight (mg)).

Measurement of rat TNF-α. Coronary effluent for TNF-α analysis was collected during 30-min resistin infusion (Fig. 1B). TNF-α concentration was determined using a commercially available ELISA (R&D Systems Ltd USA) with the following modifications. Prior to assay, samples were extracted on C18 Sep Pak columns and concentrated to a final dilution of 0.2. In the assay itself, samples were not diluted. All other steps were carried out as per manufacturer's protocols. The limit of detection of the ELISA was less than 5 pg/mL, intra-assay coefficient of variation (CV) 5.1% and inter-assay CV 9.7%, respectively. The antibodies in the ELISA recognise both recombinant and natural rat TNF-α and have no detectable cross-reactivity to mouse TNF-α or other cytokines in rat or human serum.

Statistical analysis. Data are reported as means ± SEM. Multiple group comparisons were made by one-way or repeated measures ANOVA as appropriate followed by the post hoc test for least significant differences. For comparisons between 2 groups, Student's t test was used. Significance was set at $P < 0.05$. All statistical analyses were performed using SPSS statistics software (version 13.0).

Results

Resistin decreases contractility following ischaemia in isolated hearts

Infusion of hearts with 1 nmol L^{-1} recombinant human resistin for 30 min had no significant effects on DP, PP or $+dP/dt_{max}$ and $-dP/dt_{min}$ (Figs. 2A–D). However, resistin pre-conditioning resulted in a significant reduction in contractile strength on reperfusion following 35 min of ischaemia. In the first 10 min of reperfusion, DP recovered to only 65.2 ± 5.8 % ($n = 12$, $P < 0.05$ vs vehicle) of pre-is-

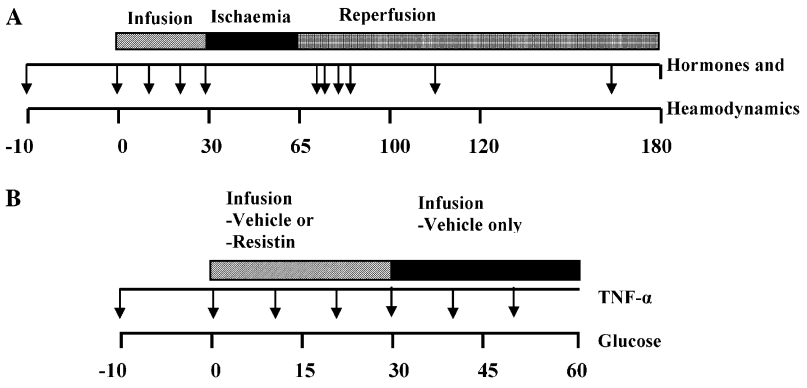

Fig. 1. (A and B). Experimental protocol for ischaemia-reperfusion experiments (A) and TNF-α and recirculating glucose experiments (B). Arrows indicate time points for collection of coronary effluent samples for hormone, CK, TNF-α, glucose, and heamodynamic measurements.

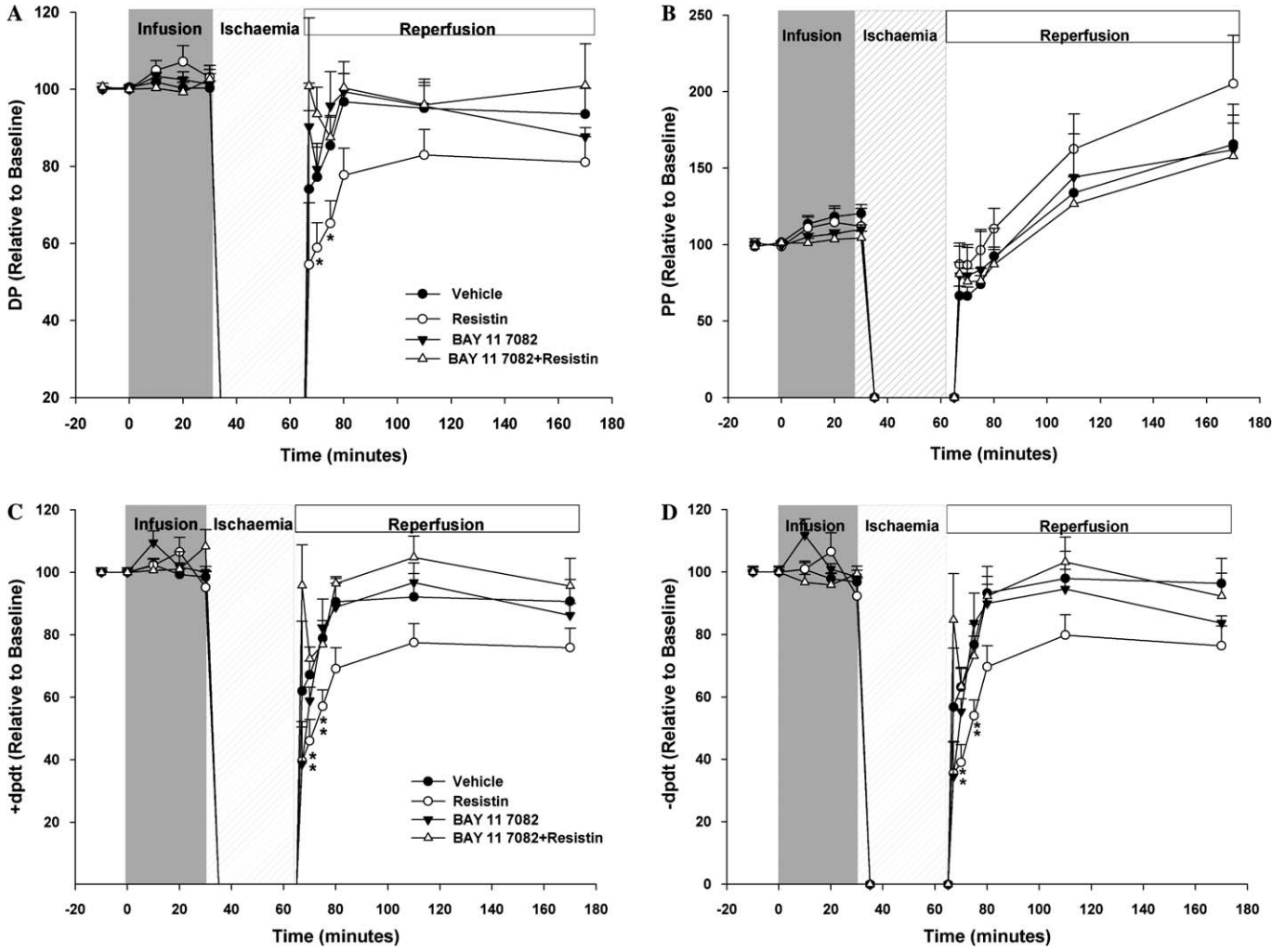

Fig. 2. (A–D) Heamodynamic changes in the isolated rat heart during ischaemia reperfusion experiments. Values are shown relative to baseline (first 10 min prior to infusion). DP (A); developed pressure, PP (B); perfusion pressure (coronary flow), $\pm dp/dt$ (C and D); positive and negative integrals of developed pressure. *Values significantly different ($P < 0.05$) from all other treatment groups. **Values significantly different ($P < 0.01$) from all other treatment groups.

chaemia DP, compared to vehicle infused hearts ($85.3 \pm 7.4\%$, $n = 12$) (Fig. 2A). This decrease in contractility was reflected in both maximal and minimal derivatives of DP (Figs. 2C and D) and was seen immediately on reperfusion, continuing for the full 2 h. Co-infusion of Bay 11 7082 significantly attenuated the contractile effects of resistin (Fig. 2A).

There was a trend in all treatment groups for coronary flow to decrease following ischaemia. Although not significantly different from any other group, the resistin group showed the greatest increase in PP of all the groups (Fig. 2B).

Resistin enhances natriuretic peptide secretion following Ischaemia

Baseline perfusate concentrations of ANP were 25 ± 3 pmol L^{-1} in resistin treated hearts and 36 ± 1 pmol L^{-1} in vehicle treated hearts (Fig. 3A), whereas baseline perfusate concentrations of BNP were 1.1 ± 0.2 pmol L^{-1} in resistin treated hearts and 1.0 ± 0.1 pmol L^{-1} in vehicle treated hearts (Fig. 3B). On reperfusion, resistin pre-conditioned hearts showed a significant increase in maximal cardiac ANP (Fig. 3A) and BNP (Fig. 3B) secretion of $380 \pm 129\%$ and $97 \pm 35\%$ ($P < 0.05$, $n = 12$), respectively, compared to baseline. In vehicle infused hearts, ANP and BNP increased by $128 \pm 43\%$ and $52 \pm 17\%$, respectively, following ischaemia. In resistin pre-conditioned hearts, ANP concentrations remained elevated throughout the 2 h of reperfusion, whereas BNP levels fell below vehicle levels by 20 min of reperfusion.

Co-infusion of resistin and Bay 11 7082 prior to ischaemia prevented the reperfusion increase in both ANP and BNP secretion seen following resistin pre-treatment. Pre-treatment with Bay 11 7082 alone did not alter ANP secretion significantly from vehicle infused hearts although Bay 11 7082 alone caused a decrease in BNP secretion in the final 10 min of pre-treatment (Fig. 3B).

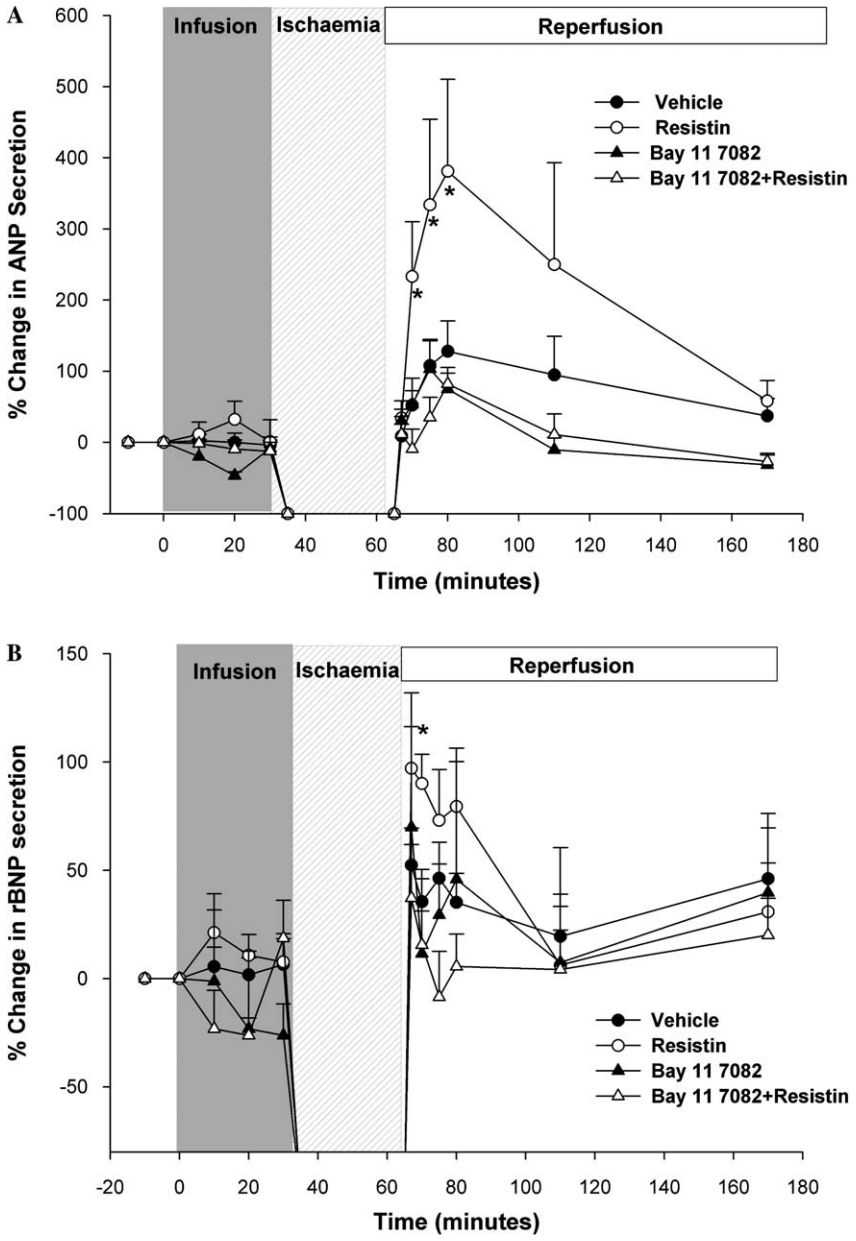

Fig. 3. (A and B). A and B type natriuretic peptide secretion during infusion and on reperfusion following ischaemia in the isolated rat heart. Values are represented as percentage change from baseline.*Values significantly different ($P < 0.05$) from all other treatment groups.

Resistin promotes myocardial creatine kinase secretion following ischaemia

Baseline CK perfusate concentration prior to ischaemia in all treatment groups was <5 U L^{-1}. Following ischaemia, there was a significant ($P < 0.05$, $n = 6$) increase in CK concentration in the coronary effluent from hearts pre-conditioned with 1 nmol L^{-1} recombinant human resistin (Fig. 4). Cumulative CK release from resistin-treated hearts and vehicle hearts was 3133 ± 835 U L^{-1} and 1463 ± 688 U L^{-1}, respectively ($P < 0.05$, Fig. 4 inset). CK secretion peaked during the first 15 min of reperfusion in all groups, and fell during late reperfusion. Co-infusion of resistin and Bay 11 7082 prior to ischaemia prevented the reperfusion increase in CK seen following resistin pre-treatment (Fig. 4). Pre-treatment with Bay 11 7082 alone did not significantly alter CK secretion from vehicle infused hearts.

Resistin enhances myocardial TNF-α release, but does not modify glucose uptake

Infusion of recombinant human resistin in isolated rat hearts caused a significant increase in perfusate TNF-α concentration, compared to baseline (Fig. 5A). Following 30 min of resistin infusion, there was a $68.5 \pm 33.7\%$ ($P < 0.05$, $n = 8$) increase in perfusate TNF-α concentration compared to vehicle infused hearts which showed an

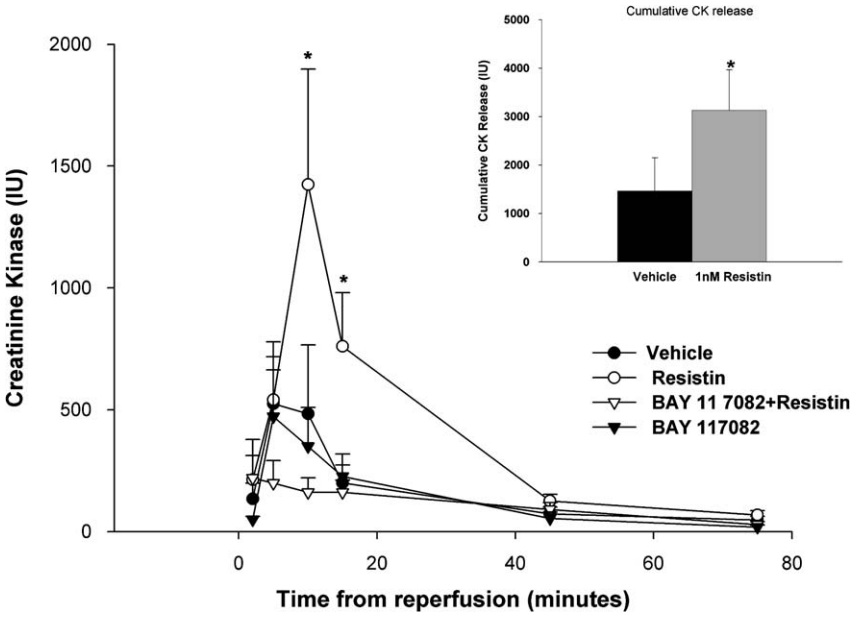

Fig. 4. Creatine kinase secretion (IU) from isolated rat heart during reperfusion. *Values significantly different ($P < 0.05$) from all other treatment groups. Inset; cumulative creatine kinase secretion from either vehicle or 1 nmol L^{-1} resistin infused hearts.

increase of 8.44 ± 10.5%. Cumulative TNF-α secretion was significantly higher in resistin infused hearts than in vehicle infused hearts (Fig. 5A, inset). Resistin (1 nmol L^{-1}) did not alter myocardial glucose uptake compared to vehicle infused hearts (Fig. 5B) during a 30-min infusion period.

Discussion

In the setting of cardiovascular disease (CVD), the role of plasma resistin is uncertain although recent studies have indicated a role in the inflammatory cascade associated with CVD [13,20,23]. To determine if resistin has direct cardiac actions, we infused recombinant human resistin in an isolated rat heart model of ischaemia-reperfusion injury. Accordingly, we provide the first documentation that resistin (1) significantly impairs cardiac ischaemia reperfusion recovery, (2) directly stimulates the release of TNF-α, (3) promotes the secretion of the cardiac markers ANP, BNP, and CK, and (4) that these actions are dependent upon an NF-κB related signalling mechanism.

Resistin impairs contractile recovery in the ischaemic heart

The depression of contractile function following ischaemia in resistin pre-conditioned hearts was reflected in both the $+dp/dt$ and $-dp/dt$, indicating resistin affects both systolic and diastolic functioning of the isolated heart. In addition to this contractile dysfunction, resistin treated hearts released high levels of natriuretic peptides upon reperfusion. Both ANP and BNP are generally considered to be negatively inotropic, although this effect depends on the vascular bed being studied [24]. This negative inotropic effect may in part account for some of the contractile dysfunction observed during reperfusion, as ANP secretion throughout reperfusion inversely mirrored developed pressure.

Resistin stimulates release of the pro-inflammatory cytokine TNF-α

TNF-α is a pro-inflammatory cytokine with potent negative inotropic effects, acting through the cell surface receptor TNFR. In feline cardiomyocytes, these effects are the direct result of alterations in intracellular calcium homeostasis, specifically associated with decreased peak calcium levels during systolic contraction [25]. Additionally, TNF-α stimulates the direct release of free radicals from the myocardial epithelium, which in turn is a self-amplifying process, shown to further increase TNF-α production [26]. In our study, resistin treated hearts showed elevated levels of CK on reperfusion. Creatine kinase is a sensitive indicator of myocardial damage, released following cellular necrosis. Compared with control ischaemia hearts, resistin pre-conditioned hearts generated almost three times the CK release. Our results are in agreement with previous experimental and clinical reports which have demonstrated strong post-MI correlations of circulating TNF-α, CK, and myocardial necrosis [27] and that monoclonal antibody inhibition of TNF-α activity significantly improves left ventricular peak systolic pressures, coronary flow, and oxygen consumption, and reduced CK levels on reperfusion following ischaemia [28].

Thus, our demonstration that co-infusion of Bay 11 7082 significantly attenuated both the reperfusion contractile dysfunction (both developed pressure and coronary flow) and CK increase induced by resistin pre-conditioning suggests that resistin is another potential TNF-α inducing cardiac depressant cytokine.

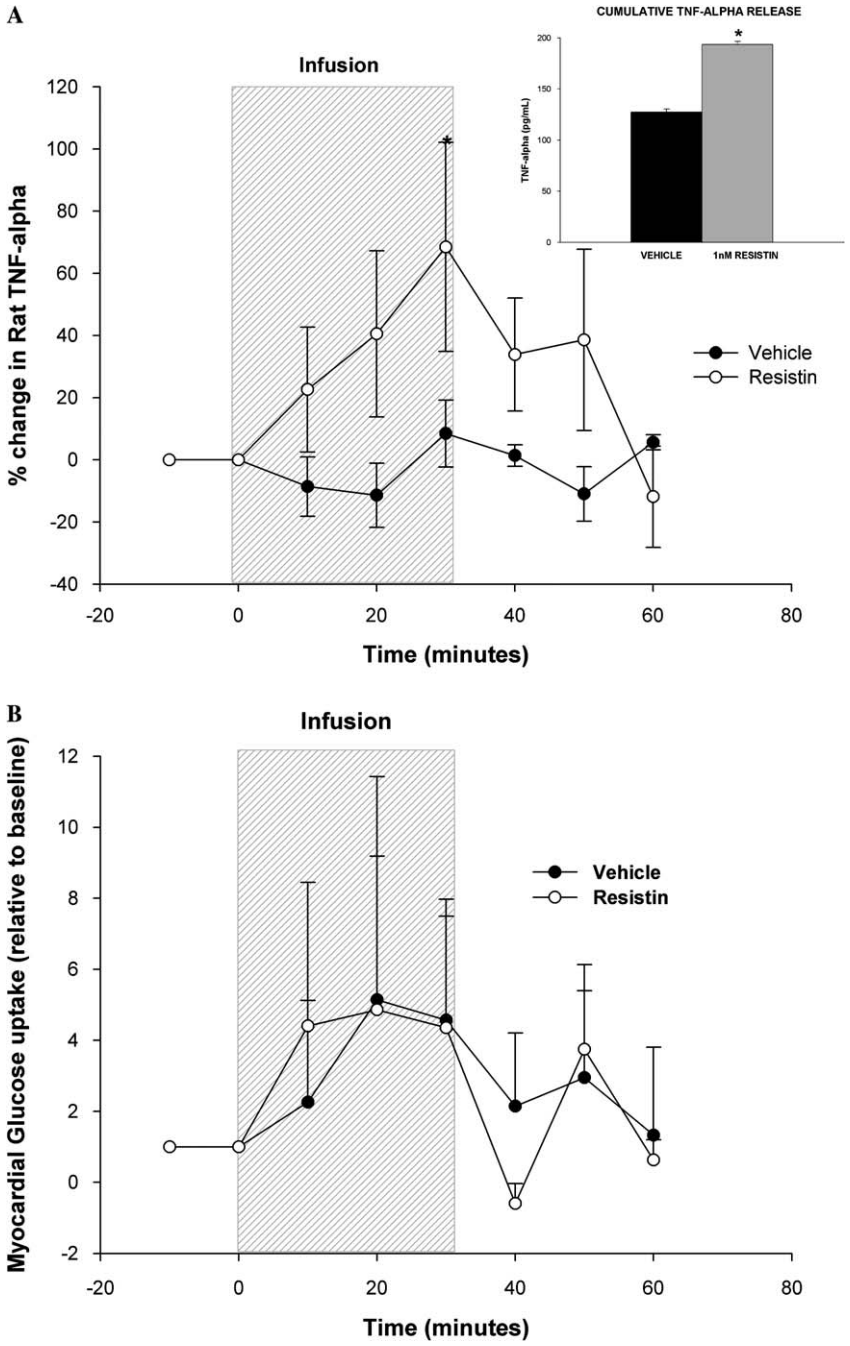

Fig. 5. (A and B). TNF-α secretion (A) and myocardial glucose uptake (B) in the isolated rat heart during 30-min infusion with either vehicle or 1 nmol L^{-1} recombinant human resistin. *Significant difference ($P < 0.05$) from baseline values.

In the present study, the ability of the IκBα inhibitor Bay 11 7082 to attenuate the actions of resistin indicates that resistin may act via the cytokine inducible NF-κB signalling pathway. The NF-κB family of proteins are heterodimers that contain DNA binding subunits known as p50 and p52 and transcriptional activating subunits known as p65 and RelB. The activation of NF-κB is associated with the induction of phosphorylation of the inhibitory IκBα subunit. Upon stimulation, this inhibitory complex is degraded to p50–p65 and p53-RelB subunits which then move into the nucleus and activate gene expression. Genes regulated by NF-κB include those coding for the interleukins, IL-2, IL-6, and IL-8, various cell adhesion molecules, and even TNF-α itself [29]. In human macrophages, recombinant human resistin has been shown to induce secretion of TNF-α and IL-12 via nuclear translocation of the NF-κB transcription factor [23].

In addition to its other actions, TNF-α stimulates endothelin-1 (ET-1) production both *in vivo* and *in vitro* [30,31] causing significant coronary constriction. These effects are reduced by the addition of ET_A receptor antagonists or TNF-α antibodies [30]. ET-1 is a potent constrictor of ves-

sels and is accumulated during myocardial ischaemia. In the present study, perfusion pressure (PP), a direct measurement of coronary constriction, was highest in the resistin pre-conditioned group and continued to increase throughout reperfusion. This trend was attenuated by co-infusion of Bay 11 7082. Although ET-1 was not measured in the coronary effluent, given the significant increase in TNF-α seen during resistin infusion, it is reasonable to suggest this increase in PP is at least partly due to cytokine stimulated ET-1 production.

Resistin does not alter cardiac glucose uptake

Treatment of normal mice with recombinant resistin impairs glucose tolerance and insulin action [1]. Consequently, there have been many reports suggesting resistin is either up regulated [32–36] or is down regulated or unchanged [16,37–39] in the insulin-resistant and diabetic state. Given this apparent discrepancy between studies, we measured myocardial glucose uptake during resistin infusion in the isolated perfused rat heart. Myocardial glucose uptake in resistin treated hearts was not significantly different from vehicle infused hearts. However, this was an acute measurement over only 1 h, and it is possible that longer-term administration of resistin (several hours) may affect *in vitro* and/or *in vivo* changes in cardiac glucose metabolism.

In conclusion, we provide the first report that recombinant human resistin significantly exacerbates cardiac ischaemia–reperfusion injury, with impaired recovery of contraction, and significant elevation of release of cardiac injury markers (creatine kinase and the natriuretic peptides). These actions are attenuated by co-infusion with a specific NF-κB antagonist, indicating that in the isolated heart, resistin can act via the TNF-α inducible NF-κB pathway. These findings indicate a potentially important role for resistin in stimulating pro-inflammatory mechanisms relevant to the evolution of both vascular disease and the post-injury response of the heart.

Acknowledgments

The authors thank the Canterbury Medical Research Foundation, the Health Research Council of New Zealand, Maurice and Phyllis Paykel Trust, and the National Heart Foundation of New Zealand for financial support of this project. AMR holds the National Heart Foundation chair of Cardiovascular studies.

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