Adiponectin protects against myocardial ischaemia-reperfusion injury via AMP-activated protein kinase, Akt, and nitric oxide

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1. Introduction

Obesity and type 2 diabetes mellitus are increasing worldwide and are associated with increased risk for cardiovascular disease. Adiponectin is an adipocyte-specific protein playing a key role in the regulation of glucose and lipid metabolism. Decreased plasma concentration of adiponectin is related to obesity, insulin resistance, type 2 diabetes mellitus, and coronary artery disease.1–8 Adiponectin circulates in plasma as a trimer (low molecular weight), a hexamer (medium molecular weight), and a multimeric form (high molecular weight) that is supposed to be the most biologically active form.9 Adiponectin exerts beneficial vascular and anti-inflammatory actions. Thus, adiponectin reduces expression of endothelial cell adhesion molecules, suppresses endothelial cell apoptosis, enhances production of nitric oxide (NO) in endothelial cells and reduces cytokine production from macrophages.9,10 Accordingly, endothelium-dependent vasodilation is reduced in adiponectin-knockout mice and in patients with hypoadiponectinaemia.11,12

Based on its anti-inflammatory actions and beneficial vascular effects, adiponectin has been implicated in the regulation of myocardial ischaemia-reperfusion injury. Exogenous adiponectin protects against apoptosis/necrosis during ischaemia and reperfusion in adiponectin-knockout mice.13 The mechanism behind these effects is suggested to be related to AMP-activated protein kinase (AMPK), cyclooxygenase (COX)-2, and tumour necrosis factor (TNF)-alpha,13 as well as inhibition of the inducible isoform of NO synthase (iNOS).14 Interestingly, AMPK is of importance for phosphorylation of the endothelial isoform of NOS (eNOS) on Ser1177.15 This may imply that adiponectin following activation of AMPK downstream may phosphorylate eNOS during myocardial ischaemia-reperfusion.8 NO

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produced from eNOS exerts important biological effects such as vasodilatation, inhibition of superoxide accumulation, attenuation of platelet adhesion and aggregation, regulation of interaction between leukocytes and endothelium, and modulation of cardiac myocyte function. NO also reduces myocardial oxygen consumption. Accordingly, NO produced from eNOS is an important mediator of protection against myocardial ischaemia and reperfusion. Administration of l-arginine, the substrate for NO production, or NO donors protect from ischaemia-reperfusion injury. Extensive research has been performed on the protective effect of NO, including the use of NO donors, such as nitroglycerin and arginine esters, which increase NO production. These findings suggest that NO may be a key molecule in the regulation of cardiovascular function.

In both settings, NO is produced from the enzyme nitric oxide synthase (NOS), which is activated by the release of Ca2+ from the sarcoplasmic reticulum. The synthesis and release of NO are tightly regulated by the concentration of Ca2+, the availability of the substrate L-arginine, and the activity of the enzyme. NO is rapidly converted to nitrite and nitrate, which are stable and therefore more useful for quantitative analysis.

2. Methods

All parts of the investigations were approved by the regional ethics committee for animal research and conform with the Guide for the care and use of laboratory animals published by the US National Institute of Health (NIH publication No 85-23, revised 1985).

2.1 Animal preparation

Male Sprague-Dawley rats (weight 250–350 g, B&K Universal AB, Sollentuna, Sweden) were heparinized and anaesthetized with a mixture of alfasuninum, pentylidone, and midazolam (2.5, 0.08, and 1.25 mg/kg, respectively, i.m.; Janssen, Beerse, Belgium and Hoffmann-La Roche, Basel, Switzerland). The hearts were excised, the ascending aorta was cannulated, and immediately retrogradely perfused with non-circulating modified Krebs-Henseleit solution containing (in millimolar) NaCl 118, KCl 4.7, CaCl2 1.5, KH2PO4 1.2, MgSO4 1.2, NaHCO3 25.2, and glucose 11.1. The perfusion pressure was kept constant at 90 cm H2O. The perfusate was bubbled with 95% O2 and 5% CO2 and kept at 37°C. The hearts were randomized to four treatments (vehicle (n = 7 and 11 in protocol 1 and 2, respectively), human globular adiponectin (AstraZeneca; 3 μg/mL; n = 7 and 9), NO-synthase inhibitor N-nitro-l-arginine (l-NNA, Sigma, Stockholm, Sweden, 100 μM; n = 7 and 9) or combination of l-NNA and adiponectin (adiponectin + l-NNA; n = 7 and 9). In protocol 1, haemodynamic parameters were determined at pre-ischaemia and every 5 min during reperfusion. In protocol 2, CF and infarct size were determined.

2.3 Determination of area at risk and infarct size

At the end of the reperfusion, the hearts of protocol 2 were perfused with 2% Evans blue to demarcate the area at risk. After dismounting the heart, the LV was cut perpendicular to the base-apex axis into six slices that were scanned from both sides for planimetry of the area at risk. They were then transferred to 0.8% triphenyl tetrazolium chloride at 37°C for 12 min and scanned for the determination of viable myocardium. The area at risk and viable myocardium were calculated by counting pixels with Adobe Photoshop 7.0.

2.4 Immunoblotting of endothelial nitric oxide-synthase, AMP-activated protein kinase, and Akt

2.4.1 Tissue processing

The myocardium from hearts included in protocol 1 were used for protein analysis of phosphorylated and total eNOS, AMPK, and Akt. Additional groups of hearts given vehicle (n = 7) or adiponectin (n = 7) as in protocol 1 were collected immediately following 30 min of ischaemia for analysis of AMPK and Akt. Frozen hearts were homogenized by microdismembrator (Polytron PT1200, Kine-matica AG, Switzerland) in 400 μl ice-cold lysis buffer containing 20 mM Tris (pH 7.8), 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1% Triton X-100, 10% (w/v) glycerol, 10 mM NaF, 1 mM ethylenediamine-nitratraacetic acid, 5 mM Na-pyrophosphate, 0.5 mM Na3VO4, 1 μg/mL leupeptin, 0.2 mM phenylmethyl sulfonyl fluoride, 1 μg/mL aprotinin, 1 mM diethiothreitol, 1 mM benzamidine. Homogenates were solubilized by rotation for 30 min at 4°C and subjected to centrifugation (12 000 g for 15 min at 4°C). Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA).

2.4.2 Western blot analysis

Expression of total and phosphorylated eNOS, AMPK, and Akt were determined. An aliquot of tissue lysate (50 μg protein) was mixed in Laemmli sample buffer containing β-mercaptoethanol. Proteins were separated by 10% (eNOS) or 7.5% (AMPK and Akt) SDS-PAGE, transferred to nitrocellulose membrane (eNOS), and polyclonal antibodies against Ser1177 (1:1 000, BD Biosciences PharMingen, USA), AMPK Thr172, or Akt Ser473 (1:1 000, Cell Signaling Technology, Beverly, MA, USA). After washing in TBST, the membranes were incubated overnight at 4°C with phospho-specific antibodies against Ser1177 (1:1 000, BD Biosciences PharMingen, USA), AMPK Thr172, or Akt Ser473 (1:1 000, Cell Signaling Technology, Beverly, MA, USA).
Ser\textsuperscript{177} (1:10 000, BD Biosciences Pharmingen), and anti-rabbit IgG for AMPK Thr\textsuperscript{172} and Akt Ser\textsuperscript{473} (1:25 000, Bio-Rad, Hercules, CA, USA) for 1 h at room temperature, followed by additional washing. Proteins were visualized by enhanced chemiluminescence (ECL plus; Amersham, Arlington Heights, IL, USA) and quantified using densitometry and Molecular Analyst Software (Bio-Rad, Richmond, CA, USA). After immunoblotting with phosho-specific antibodies, membranes were stripped as described and Immunoblotted for either total eNOS (1:1000, Affinity BioReagents, USA), AMPK (1:1000, Update/Chemicon, Sweden), or Akt proteins (1:1000, Cell Signaling Technology).

2.5 Calculation and statistical analysis

Left ventricular-developed pressure (LVDP) is the difference between LV systolic and end-diastolic pressures. Rate pressure product (RPP) was calculated as heart rate multiplied by LVDP. The recovery of myocardial performance is expressed in percent of the pre-ischaemic value. All values are presented as mean ± SEM. Comparison between four groups were made by analysis of variance (ANOVA, area at risk, and infarct size) or two-way analysis of variance (haemodynamics) followed by Fisher’s paired least significant difference (PLSD) test. Comparison of data from western blots were analysed by Mann–Whitney U test. The significance level was set at 0.05.

3. Results

3.1 Global ischaemia-reperfusion (Protocol 1)

CF, LVDP, dP/dt, heart rate, or RPP did not differ between the groups before ischaemia (Table 1). The post-ischaemic recoveries of LVDP, dP/dt, and RPP were significantly better in the adiponectin group than in the vehicle group (P < 0.01; Figure 1A–C). Administration of L-NNA completely reversed the beneficial effect of adiponectin on LVDP, dP/dt, and RPP (Figure 1A–C). Heart rate increased in all groups during the initial phase of reperfusion but returned to pre-ischaemic levels at the end of reperfusion. There were no significant differences in heart rate between the groups. L-NNA did not affect the recovery of myocardial function per se. LVEDP during reperfusion was significantly lower in the adiponectin group (48 ± 6 mmHg at 60 min reperfusion) than in the groups given vehicle or adiponectin in combination with L-NNA (62 ± 4 mmHg and 62 ± 5 mmHg, respectively, at 60 min reperfusion; Figure 2). L-NNA slightly but significantly reduced LVEDP during reperfusion in comparison with the vehicle group (Figure 2). Recovery of CF was higher in the adiponectin group than in the vehicle group throughout the reperfusion period (Figure 1D). CF in the L-NNA group and in the group given the combination of L-NNA and adiponectin did not differ significantly from the vehicle group.

3.2. Regional ischaemia-reperfusion (Protocol 2)

The sizes of the area at risk are depicted in Figure 3A. The areas at risk in the groups receiving L-NNA and the combination of L-NNA and adiponectin were slightly but statistically larger when compared with the vehicle group. The area at risk of the adiponectin group did not differ significantly from any of the groups.

The infarct size was significantly smaller in the group receiving adiponectin (19 ± 2% of area at risk) than that of the vehicle group (41 ± 6%; Figure 3). There was no significant difference in infarct size between the vehicle group and the groups receiving L-NNA or the combination of L-NNA and adiponectin. On the other hand, the infarct size was significantly larger in the group receiving the combination of L-NNA and adiponectin than in the group receiving adiponectin only (P < 0.01, Figure 3).

CF during reperfusion was similar in the vehicle group and the adiponectin group (Figure 4). Both groups receiving L-NNA had lower CF than the vehicle and the adiponectin groups.

3.3 Immunoblotting of endothelial NO-synthase, AMP-activated protein kinase, Akt

There was no difference in the expression of total eNOS or AMPK between the vehicle and adiponectin group. On the other hand, at the end of reperfusion, phosphorylation of eNOS Ser\textsuperscript{177} and AMPK Thr\textsuperscript{172} increased significantly following the administration of adiponectin (P < 0.05, Figures 5 and 6). There was no significant difference in the expression of phosphorylated Akt Ser\textsuperscript{473} between the two groups at the end of reperfusion. In the groups collected immediately after ischaemia, there was a significant increase in phosphorylation of Akt Ser\textsuperscript{473} (Figure 7), but no change in AMPK Thr\textsuperscript{172}.

4. Discussion

The main findings of this study is that the protective effect of adiponectin against myocardial contractile dysfunction and infarction following ischaemia and reperfusion is inhibited by pharmacological blockade of NO production, and is associated with phosphorylation of AMPK Thr\textsuperscript{172}, Akt Ser\textsuperscript{473}, and eNOS Ser\textsuperscript{177}. These observations suggest that the protection against myocardial ischaemia and reperfusion injury mediated by adiponectin is related to activation of eNOS and production of NO.

The observation that exogenous adiponectin exerts cardioprotective effects during myocardial ischaemia and reperfusion is in agreement with previous studies performed in

| Table 1 Pre-ischaemic values of the hearts in protocol 1 (global ischaemia) |
|-----------------------------|---------|----------|------------------|------------------|
|                             | Vehicle | Adiponectin | Nitro-L-arginine (L-NNA) | Adiponectin + L-NNA |
| Coronary flow (mL/min)      | 16.6 ± 0.5 | 15.5 ± 0.9 | 15.4 ± 0.7 | 15.2 ± 0.3 |
| Left ventricular-developed pressure (mmHg) | 104 ± 3 | 102 ± 2 | 101 ± 3 | 105 ± 2 |
| Maximum dP/dt (mmHg/s)      | 4992 ± 162 | 4257 ± 172 | 4851 ± 183 | 4838 ± 147 |
| Heart rate (b.p.m.)         | 330 ± 13 | 310 ± 16 | 317 ± 15 | 329 ± 9 |
| Rate pressure product       | 34316 ± 1145 | 31538 ± 1366 | 32055 ± 1392 | 34320 ± 743 |

The hearts received either vehicle, adiponectin, L-NNA, or the combination of adiponectin and L-NNA.
Adiponectin-deficient mice in vivo. Shibata et al. further demonstrated that adiponectin inhibits apoptosis by activating AMPK and suppresses the production of TNF-alpha via a COX 2-dependent mechanism. In addition, there are observations suggesting that adiponectin stimulates the production of NO in endothelial cells. Production and release of NO during ischaemia and reperfusion are of importance for mediating cardioprotection during ischaemia and reperfusion. The hypothesis that NO is involved in the cardioprotective effect mediated by adiponectin was tested by investigating both functional recovery following global ischaemia-reperfusion and infarct size following regional ischaemia-reperfusion. Administration of the NOS inhibitor L-NNA completely blocked the cardioprotective effect of adiponectin in both experimental protocols, which supports our hypothesis. Importantly, L-NNA did not affect the recovery of myocardial contractile function or infarct size per se when compared with the vehicle group, suggesting that it interfered with the protective mechanisms induced by adiponectin.

In the global ischaemia experiments, adiponectin increased CF during reperfusion in comparison with the vehicle group. This may suggest that the improvement in

Figure 1  The percent recoveries of (A) left ventricular (LV)-developed pressure, (B) first derivative of LV pressure (dP/dt), (C) rate-pressure product, and (D) coronary flow during reperfusion in rat hearts given vehicle, adiponectin, the NOS inhibitor L-NNA, or the combination of adiponectin and L-NNA. Data are presented as mean ± SEM. Significant differences from the vehicle group during the entire reperfusion period are shown (two-way analysis of variance); ***P < 0.001.

Figure 2  Left ventricular end-diastolic pressure during ischaemia and reperfusion of rat hearts given vehicle, adiponectin, the NOS inhibitor L-NNA, or the combination of adiponectin and L-NNA. Data are presented as mean ± SEM. Significant differences from the vehicle group during the entire reperfusion period are shown (two-way analysis of variance); ***P < 0.001.
myocardial function is due to increased perfusion. However, improved CF may be owing to a reduction in the area of no-reflow and thereby secondary to inhibition of the reperfusion injury. Previous observations suggest that reperfusion injury leads to decrease in myocardial perfusion, suggesting that inhibition of reperfusion injury will lead to increased flow. In the regional ischaemia experiments, adiponectin did not affect post-ischaemic CF in comparison with the vehicle group suggesting that changes in CF did not contribute to the difference in infarct size.

To further explore the effect of adiponectin on NO production, the phosphorylation and the expression of eNOS protein was investigated. Administration of adiponectin did not increase total eNOS expression, but it increased the expression of phosphorylated eNOS Ser\(^{1177}\). It was recently demonstrated that adiponectin increases eNOS phosphorylation in the mouse heart but the phosphorylation site was not specified. This is of importance since phosphorylation at different sites may result in opposite effects on enzyme activity. Phosphorylation of eNOS at residue Ser\(^{1177}\) is associated with increased enzyme activity.
and increased production of NO. We have previously demonstrated that L-NNA does not influence the phosphorylation of eNOS per se. Collectively, these observations suggest that adiponectin stimulates phosphorylation of eNOS at Ser1177 resulting in increased production of NO which mediates the cardioprotection during ischaemia and reperfusion.

To study the possible link between adiponectin and phosphorylation of eNOS, we determined the expression and phosphorylation of AMPK and Akt at different time points. AMPK is an important regulatory mechanism involved in the generation of adenosine triphosphate, fatty acid oxidation, glycolysis, increasing muscle glucose uptake, and inhibiting energy-consuming anabolic pathways. Adiponectin stimulates AMPK signalling in isolated cardiomyocytes and in the ischaemic hindlimb. Furthermore, adiponectin protects the murine heart against ischaemia and reperfusion injury via an AMPK-dependent pathway. In this study, we show that phosphorylation of the Thr172 residue of AMPK is increased by adiponectin in the myocardium 60 min following ischaemia. Since it has been demonstrated that AMPK phosphorylates eNOS Ser1177 in rat heart extracts and endothelial cells, these observations suggest that adiponectin stimulates phosphorylation of AMPK, which results in downstream phosphorylation of eNOS in the ischaemic/reperfused myocardium. The effect of adiponectin has also been suggested to involve the PI3-kinase/Akt signalling pathway. In the present study we did not detected any change in Akt at the end of reperfusion, but at the onset of reperfusion there was a significant increase in phosphorylation of Akt in the group receiving adiponectin. Taken together these observations indicate that adiponectin activates both AMPK and Akt, but at different time points during myocardial ischaemia-reperfusion.

There are limitations with the present study. The studies were performed on isolated hearts and the results need to be confirmed in vivo. On the other hand, it has been demonstrated that adiponectin reduces infarct size in adiponectin knockout mice in vivo. The present study extends earlier knowledge by demonstrating that adiponectin improves post-ischaemic myocardial function and reduces infarct size via an effect that is dependent on the availability of NO. In an earlier study it has been shown that adiponectin may be locally produced in cardiomyocytes. However, the present study indicates that local production of adiponectin does not give optimal efficacy and supplemental administration of adiponectin during ischaemia could exert additional protective effects. A further limitation is that the authors, in this presently used model, cannot determine which molecular form of adiponectin that is protective. Previous data suggest the high molecular weight fraction to be of biological importance, but this issue remains to be elucidated in ischaemia-reperfusion studies. Further, the degree of tissue penetration and thereby the target cell type for the effect of adiponectin under these experimental conditions is unknown. The molecular size of adiponectin may indicate that its effect is confined to the vasculature and the endothelial cells. An effect on the endothelial cells may be sufficient in order to release NO, which in turn may affect cells on the abluminal side of the vasculature including cardiomyocytes.

In conclusion, administration of adiponectin at the onset of ischaemia followed by reperfusion induces phosphorylation of AMPK Thr172, Akt Ser473, and eNOS Ser1177 and protects from myocardial contractile dysfunction and
infarction. Pharmacological blockade of NOS abrogated the cardioprotective effect of adiponectin. These observations suggest that the cardioprotective effect of adiponectin is mediated via NO by phosphorylation of eNOS.

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