Effect of monomeric adiponectin on cardiac function and perfusion in anesthetized pig

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Abstract

Adiponectin, the most abundant adipokine released by adipose tissue, appears to play an important role in the regulation of vascular endothelial and cardiac function. To date, however, the physiological effects of human monomeric adiponectin on the coronary vasculature and myocardial systo-diastolic function, as well as on parasympathetic/sympathetic involvement and nitric oxide (NO) release, have not yet been investigated. Thus, we planned to determine the primary *in vivo* effects of human monomeric adiponectin on coronary blood flow and cardiac contractility/relaxation and the related role of autonomic nervous system, adiponectin receptors, and NO. In 30 anesthetized pigs, human monomeric adiponectin was infused into the left anterior descending coronary artery at constant heart rate and arterial blood pressure, and the effects on coronary blood flow, left ventricular systo-diastolic function, myocardial oxygen metabolism, and NO release were examined. The mechanisms of the observed hemodynamic responses were also analyzed by repeating the highest dose of human monomeric adiponectin infusion after autonomic nervous system and NO blockade, and after specific adiponectin 1 receptor antagonist administration. Intracoronary human monomeric adiponectin caused dose-related increases of coronary blood flow and cardiac function. Those effects were accompanied by increased coronary NO release and coronary adiponectin levels. Moreover, the vascular effects of the peptide were prevented by blockade of β_2-adrenoceptors and NO synthase, whereas all effects of human monomeric adiponectin were prevented by adiponectin 1 receptor inhibitor. In conclusion, human monomeric adiponectin primarily increased coronary blood flow and cardiac systo-diastolic function through the involvement of specific receptors, β_2-adrenoceptors, and NO release.

Key Words
- adipokine
- autonomic nervous system
- heart function
- nitric oxide

Journal of Endocrinology (2014) **222**, 137–149

Introduction

Both animal and clinical investigations have suggested that inflammation and dysfunction of adipose tissue could be involved in the onset of cardiovascular disease (Mazurek *et al.* 2003, Xu *et al.* 2010). Hence, peptides released by pericardial adipose tissue have been found to play a significant role in conditions such as the metabolic

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DOI: 10.1530/JOE-14-0170

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syndrome, with the adipose tissue surrounding the heart being clinically associated with coronary artery disease (Ouwens *et al.* 2010). In this respect, it is suggested that adipokines produced by adipose tissue would act as local 'vasocrine' agents in influencing a wide spectrum of hemodynamic, metabolic, and immunologic factors, including vascular reactivity, endothelial dysfunction, and vascular remodeling (Eringa *et al.* 2012, Gu & Xu 2013).

Adipokines are involved in a 'good–bad', yin–yang homeostatic balance whereby there are substantial benefits: cardioprotection, promoting endothelial function, angiogenesis, and reducing hypertension, atherosclerosis, and inflammation (Mattu & Randeva 2013). Adiponectin, the most abundant protein secreted by the adipose tissue (Arita *et al.* 1999) and circulating in human plasma as multimeric forms (Liu & Liu 2012), has been associated with endothelial improvement and vascular protection (Beltowski *et al.* 2008, Zhu *et al.* 2008) through the activation of an endothelial isoform of nitric oxide (eNOS)-related signaling, with anti-inflammatory (Ouchi & Walsh 2007) and antiatherogenic properties (Barseghian *et al.* 2011). It is also of interest that numerous epidemiological studies have correlated decreased adiponectin levels with an increased risk of cardiovascular disease in obesity and diabetes (Kumada *et al.* 2003, Pischon *et al.* 2004, Frystyk *et al.* 2007) and with coronary artery disease (Hara *et al.* 2007). As such, adiponectin deficiency has been reported to be predictive of future adverse cardiac events (Kojima *et al.* 2006) and has been associated with increased oxidative stress and inferior recovery in cardiac function (Shibata *et al.* 2008).

Moreover, adiponectin has been found to stimulate the production of NO in vascular endothelial cells (Chen *et al.* 2003), and hypoadiponectinemia has been associated with an impaired endothelium-dependent vasodilation (Tan *et al.* 2004), which is a predictor of coronary events. However, little is known about the physiological role of monomeric adiponectin in the *in vivo* acute modulation of cardiac perfusion and function, as well as any related involvement of the autonomic nervous system. There is very little information available regarding this issue, only in the form of hypotensive action elicited by adiponectin in anesthetized rats, which has been found to be partly related to changes in sympathetic nerve activity (Tanida *et al.* 2007).

Thus, the present study was planned in controlled experiments performed in anesthetized pigs to investigate the primary *in vivo* effects of human monomeric adiponectin on cardiac contractility and coronary blood flow and the mechanistic involvement of autonomic nervous system, subtype 1 of adiponectin receptors (AdipoR1) and of NO.

Materials and methods

The experiments were carried out in accordance with UK legal requirements, the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines, the national guidelines (DLGS 27/01/1992, license no. 116), and with the approval of the Local Ethical Committee.

Animal instrumentation and measurements

The experiments were carried out in 30 domestic pigs, weighing 64–73 kg, supplied by an accredited dealer (Azienda Invernizzi, Olengo, Novara, Italy). After being fasted overnight, the pigs were anesthetized with i.m. ketamine (20 mg/kg, Parke-Davis, Detroit, MI, USA), followed after about 15 min by i.v. sodium pentobarbitone (15 mg/kg, Siegfried, Zofingen, Switzerland), and artificially ventilated with oxygen-enriched air using a respiratory pump (Harvard 613; Harvard Apparatus, South Natick, MA, USA). Anesthesia was maintained throughout the experiments by a continuous i.v. infusion of sodium pentobarbitone (7 mg/kg per h) and assessed as reported previously (Linden & Mary 1983). Pressures in the ascending aorta and right atrium were recorded via catheters connected to pressure transducers (Statham P23 XL; Gould, Valley View, OH, USA) inserted, respectively, into the right femoral artery and right external jugular vein. The chest was opened in the left fourth intercostal space, the pericardium was cut, and an ultrasound flowmeter probe (model 420; Transonic Systems, Ithaca, NY, USA) was positioned around the proximal part of the left anterior descending (LAD) coronary artery to record coronary blood flow. Left ventricular pressure was measured using a catheter connected to a pressure transducer (Gould) inserted through the left atrium. To pace the heart, electrodes were sewn on the left atrial appendage and connected to a stimulator (model S8800; Grass Instruments, Quincy, MA, USA), which delivered pulses of 3–5 V for 2-ms duration at the required frequency. To assess regional contractile function, pairs of 2 mm ultrasonic segment length microtransducer crystals (Sonometrics, London, ON, Canada) were implanted in the left anterior ventricular wall in the distribution area of the LAD, about 10 mm apart and parallel to the direction of the fibers, so that the segmental shortening (SS) was in line with the orientation

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DOI: 10.1530/JOE-14-0170

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of fibers. Two additional crystals were placed at opposite ends of the left ventricular short axis to measure changes in ventricular dimension throughout cardiac cycles (Grossini *et al.* 2009, 2011*a*,*b*, 2013*a*,*b*). Arterial blood samples were used to measure pH, arterial pressure of oxygen and carbon dioxide (PO_2 and PCO_2) with a gas analyzer (Radiometer ABL505, Copenhagen, Denmark) and the hematocrit. Sampling of blood from the LAD and the anterior interventricular vein allowed the measurement of coronary arterial and venous PO_2 and arteriovenous oxygen content. Coronary venous blood samples were also taken for NO detection by Griess Reagent System (Promega) and for adiponectin confirmation by western blot analysis. The acid–base status of the animals was kept within normal limits as reported previously (Linden & Mary 1983). Infusions into the LAD were performed using a catheter connected to a butterfly needle inserted into the coronary artery distal to the flowmeter probe. To prevent changes of arterial blood pressure, a pressurized reservoir connected to the arterial system was used as described previously (Grossini *et al.* 2009, 2011*a*,*b*, 2013*a*,*b*). Coagulation of the blood was avoided by i.v. injection of heparin (Parke-Davis; initial doses of 500 IU/kg and subsequent doses of 50 IU/kg for every 30 min). The rectal temperature of the pigs was monitored and kept between 38 and 40 °C using an electric pad. Hemodynamic variables were monitored and recorded together with heart rate and the maximum and minimum rate of change of left ventricular systolic pressure ($\pm dP/dt_{max}$) using a micro1401 A/D converter (Cambridge Electronic Design, CED, Cambridge, UK) displayed on a personal computer and processed by using Spike2 Software (CED).

The dP/dt_{max} was used to define the timing of the cardiac cycle for segment length measurements with ultrasonic crystals. End-diastolic length was measured at the onset of the rapid increase in dP/dt_{max}, and end-systolic length was measured at peak negative dP/dt_{max}. End-diastolic and end-systolic ventricular volumes were obtained from data of end-diastolic and end-systolic lengths through the specific software (Sonometrics). Percentage of SS (%SS) was calculated using the following formula: %SS = (end-diastolic length − end-systolic length) × 100/end-diastolic length. The data from the sonomicrometer crystals were digitally processed by specific hardware and software (Sonometrics). Cardiac output was derived using the Sonosoft System from data recorded by piezoelectric crystals. To calculate coronary vascular resistance, the difference between mean aortic blood pressure and mean left ventricular pressure during diastole was considered as the coronary pressure gradient. Coronary vascular resistance was calculated as the ratio between this pressure gradient and mean diastolic coronary blood flow during the steady state. At the end of the experiment, each animal was killed by an i.v. injection of 90 mg/kg pentobarbitone sodium.

Western immunoblot (WB)

Coronary plasma samples, prepared by centrifugation at 1650 ***g*** for 15 min at 4 °C, were size-fractionated on 10% SDS–PAGE under reducing conditions and electro-transferred to immunoblot PVDF membranes (Bio-Rad). Membranes were incubated with monoclonal anti-adiponectin (Adipogen, Inc., Incheon, Korea) and detected

Table 1 Changes in hemodynamic variables caused by intracoronary infusion of 30 pg, 300 pg, 3 ng, 30 ng, 300 ng, and 3 μg human monomeric adiponectin for each milliliter per minute of measured coronary blood flow in 30 pigs. Data are means ± S.D.

Data	Control	30 pg	300 pg	3 ng	30 ng	300 ng	3 μg
$+dP/dt_{max}$	2055 ± 127	2213 ± 130*	2373 ± 141*,†	2617 ± 151*,‡	2665 ± 155*,§	2671 ± 159*,¶	2674 ± 159*,a
$-dP/dt_{max}$	−1865 ± 119	−1957 ± 125*,‖	−2059 ± 129*,†	−2176 ± 131*,‡	−2235 ± 128*,§	−2269 ± 135*,¶	−2278 ± 134*,a
CBF	61.4 ± 7.5	67.3 ± 8.2*	71.6 ± 8.8*,†	76 ± 8.7*,‡	76.7 ± 8.9*,§	76.8 ± 8.6*	77 ± 8.6*
CVR	1.89 ± 0.42	1.78 ± 0.4*	1.68 ± 0.4*,†	1.55 ± 0.38*,‡	1.52 ± 0.39*,‖	1.52 ± 0.38*	1.51 ± 0.38*
CO	5580 ± 468	5904 ± 520*	6242 ± 567*,†	6449 ± 545*,‡	6518 ± 594*,§	6520 ± 598*	6522 ± 597*
%SS	14.5 ± 0.7	15.4 ± 0.7*	16.8 ± 0.7*,†	18.6 ± 0.38*,‡	18.84 ± 0.38*,§	18.87 ± 0.38*,¶	18.89 ± 0.38*,a
NO	0.99 ± 0.07	1.073 ± 0.08*	1.25 ± 0.08*,†	1.58 ± 0.11*,‡	1.6 ± 0.1*,§	1.6 ± 0.1*	1.6 ± 0.1*
PO_2vc	19.9 ± 1	20.4 ± 0.9*	20.8 ± 0.9*,†	21.2 ± 0.9*,‡	21.4 ± 1*,§	21.5 ± 1*	21.51 ± 0.9*
$(AV)O_2$	11.8 ± 0.47	10.7 ± 0.49*	10.1 ± 0.54*,†	9.57 ± 0.49*,‡	9.5 ± 0.48*,‖	9.48 ± 0.47*	9.47 ± 0.49*
mVO_2	7.25 ± 0.95	7.26 ± 0.96	7.25 ± 1	7.29 ± 1	7.29 ± 1	7.3 ± 0.97	7.3 ± 1

$+dP/dt_{max}$, maximum rate of change of left ventricular systolic pressure (mmHg/s); $-dP/dt_{max}$, minimum rate of change of left ventricular systolic pressure (mmHg/s); CBF, mean coronary blood flow (ml/min); CVR, coronary vascular resistances (mmHg/ml per min); CO, cardiac output (ml/min); %SS, percentage of segmental shortening; NO, nitric oxide (μmol/1.5 μg protein); PO_2vc, coronary sinus partial pressure of oxygen (mmHg); $(AV)O_2$, coronary arteriovenous oxygen content (ml O_2/100 ml); mVO_2, myocardial oxygen consumption (ml O_2/min per 100 g). *$P<0.0001$ vs control (taken before adiponectin administration at constant HR and ABP); †$P<0.0001$ vs 30 pg adiponectin; ‡$P<0.0001$ vs 300 pg adiponectin; §$P<0.0001$ vs 3 ng adiponectin; ‖$P=0.01$ vs 3 ng adiponectin; ¶$P=0.01$ vs 30 ng adiponectin; and a$P=0.01$ vs 300 ng adiponectin.

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DOI: 10.1530/JOE-14-0170

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with the appropriate HRP-conjugated secondary antibody (Chemicon Millipore, Temecula, CA, USA). Immunoreactive proteins were detected using ECL (Pierce Biotechnology, Rockford, IL, USA) with image capture performed using CCD camera linked to ChemiDoc (Bio-Rad).

Experimental protocol

The experiments were begun after at least 30 min of steady-state conditions. In the 30 pigs, the effects of various doses of human monomeric adiponectin (30 kDa; Sigma) on cardiac perfusion and function were examined by infusing either human monomeric adiponectin dissolved in saline or saline only into the LAD. Each dose of adiponectin was infused over 5 min by means of an infusion pump (model 22; Harvard Apparatus, South Natick, MA, USA), working at constant rate of 1 ml/min. The infused doses amounted to 30 pg, 300 pg, 3 ng, 30 ng, 300 ng, and 3 μg for each milliliter per minute of measured coronary blood flow.

In these animals, the heart was paced to a frequency higher, by 20 beats/min, than that observed during the steady state and the arterial system was connected to the pressurized reservoir. Moreover, coronary artery and venous plasma samples were taken for measurement of PO_2, oxygen, human monomeric adiponectin, and NO content.

Recordings taken for 10 min during the steady-state conditions before infusion of human monomeric adiponectin at constant heart rate and aortic blood pressure were used as control. Measurements of hemodynamic variables, coronary artery and coronary venous PO_2, human monomeric adiponectin, arteriovenous oxygen content, and NO were obtained during the last 30 s of each dose infusion in the steady state and compared with control values. Myocardial oxygen consumption (mVO_2; ml O_2/min per 100 g) was calculated as the product of coronary arteriovenous oxygen content and coronary blood flow. Moreover, NO content in coronary venous blood was measured, as described previously

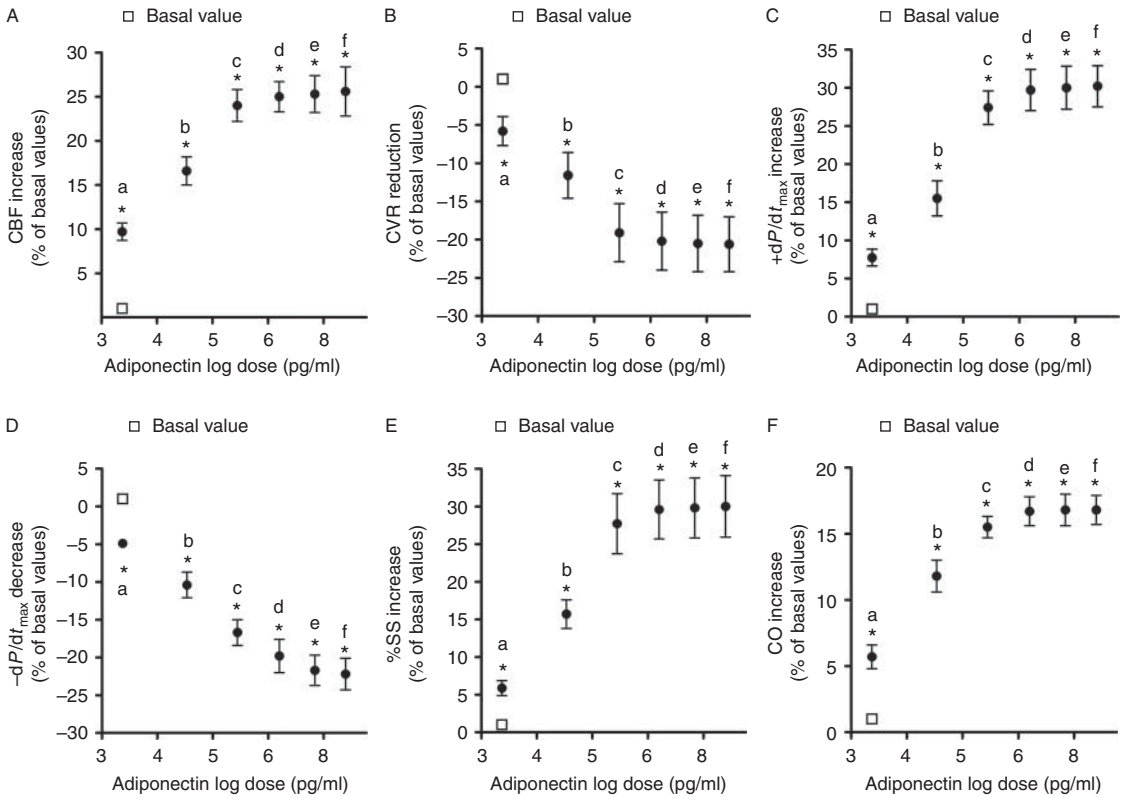

Figure 1

Effects of intracoronary infusion of human monomeric adiponectin on mean coronary blood flow (CBF; A), coronary vascular resistances (CVR; B), maximum rate of change of left systolic ventricular pressure ($+dP/dt_{max}$; C), minimum rate of change of left systolic ventricular pressure ($-dP/dt_{max}$; D), percentage of segmental shortening (%SS; E), and cardiac output (CO; F) in 30 pigs. The means of percentage changes in hemodynamic parameters obtained in the 30 pigs during the test period of measurement are plotted against the logarithm of doses of adiponectin from 30 pg to 3 μg for each milliliter per minute of measured coronary blood flow. The continuous line is the line of equality. The bars indicate S.D. *$P<0.05$ vs basal value; (b, c, d, e and f), $P<0.05$ vs (a); (c, d, e and f), $P<0.05$ vs (b); (d, e and f), $P<0.05$ vs (c); in (C), (D) and (E): (e and f), $P<0.05$ vs (d); and (f), $P<0.05$ vs (e).

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(Grossini *et al*. 2009, 2011*a*,*b*, 2013*a*,*b*), following the same time course followed for PO_2 and oxygen content measurement, and values were compared with those of control.

In the pigs, the role of muscarinic cholinoceptors and α- and β-adrenoceptors was examined throughout by repeating 3 μg for each milliliter per minute of measured coronary blood human monomeric adiponectin administration after the i.v. administration of muscarinic cholinoceptors blocker, atropine sulfate (0.5 mg/kg, Sigma, $n=5$), α-adrenoceptor blocker, phentolamine (1 mg/kg, Sigma, $n=5$), β_1-adrenoceptor blocker, atenolol (1 mg/kg, Sigma, $n=5$), and β_2-adrenoceptor blocker, butoxamine (2.5 mg/kg, Sigma, $n=5$). The involvement of NOS and AdipoR1 was examined, respectively, by repeating human monomeric adiponectin infusion after intracoronary administration of the NOS inhibitor *N*ω-nitro-L-arginine methyl ester (L-NAME; 2 mg for each milliliter per minute of measured coronary blood flow, Sigma, $n=5$), and 3 μg for each milliliter per minute of measured coronary blood GTX89569-PEP (GeneTex, Irvine, CA, USA, $n=5$). The effect of blocking agents and human monomeric adiponectin on NO release, coronary artery and coronary venous PO_2, and myocardial oxygen consumption was examined by repeating the measurements after each blocker or adiponectin infusion in the steady state and comparing the values with those taken immediately beforehand.

All drugs were given without pacing the heart or controlling aortic pressure to assess their effects on baseline hemodynamic variables in the steady state. Thereafter, heart rate and aortic blood pressure were kept constant, and measured hemodynamic variables were taken as 'control'. In all subsequent experiments, the effects of human monomeric adiponectin in the presence of blocking agents were examined while preventing changes in heart rate and aortic blood pressure. Changes of hemodynamic variables caused by various agents were compared with control values.

Statistical analyses

All data were recorded using the Institution's database. Statistical analysis was performed by using STATVIEW, version 5.0.1 for Microsoft Windows (SAS Institute, Cary, NC, USA). Data were checked for normality before statistical analysis. Student's paired *t*-test was used to examine changes of measured variables within each animal before and after any treatment. One-way ANOVA followed by Bonferroni's *post-hoc* test was used to compare differences among groups of animals. Nonparametric Wilcoxon's signed-rank test was used to compare significance of percent changes before and after blockers. Pearson's correlation coefficient was used for linear regression analysis in the dose-dependent studies. All data are presented as means ± S.D. A value of $P<0.05$ was considered statistically significant.

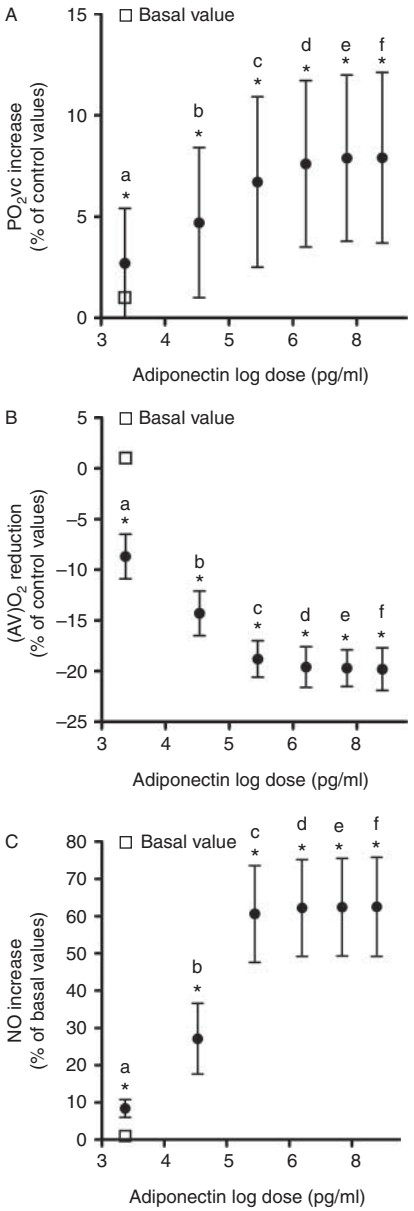

Figure 2
Effects of intracoronary infusion of human monomeric adiponectin on coronary venous PO_2 (pO_2vc; A), coronary arteriovenous oxygen content ($(AV)O_2$; B), and coronary nitric oxide (NO; C) in 30 pigs. The means of percentage changes in hemodynamic parameters obtained in the 30 pigs during the test period of measurement are plotted against the logarithm of doses of adiponectin from 30 pg to 3 μg for each milliliter per minute of measured coronary blood flow. The continuous line is the line of equality. The bars indicate S.D. *$P<0.05$ vs basal value; (b, c, d, e and f), $P<0.05$ vs (a); (c, d, e and f), $P<0.05$ vs (b); and (d, e and f), $P<0.05$ vs (c).

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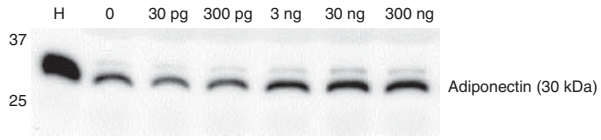

Figure 3
Western blot analysis of coronary plasma human monomeric adiponectin during various doses of human monomeric adiponectin infusion in one of the 30 pigs. H, human and 0, before human monomeric adiponectin infusion.

Results

Dose-related effects of intracoronary human monomeric adiponectin on hemodynamic variables, NO release, and myocardial oxygen metabolism

In all pigs, recordings commenced ~5 h after induction of anesthesia. The mean pH, PO_2, and PCO_2 of arterial blood were 7.39±0.01 (7.39–7.43), 119±11.5 (100–138) mmHg, and 40±1 (39–42) mmHg and the hematocrit was 39.1±1% (39–41).

The heart rate, aortic blood pressure, right atrial pressure, and left ventricular end-diastolic pressure taken while preventing changes in heart rate and aortic blood pressure in the 30 pigs, respectively, amounted to 96.8±4.7 beats/min, 100.28±8.9, 2.5±0.3, and 4.7±0.7 mmHg. These values were taken as 'control'.

Intracoronary infusion of human monomeric adiponectin caused a dose-related increase in mean coronary blood flow, $+dP/dt_{max}$, cardiac output, and %SS and an improvement of $-dP/dt_{max}$ (Table 1 and Fig. 1; Pearson's correlation coefficients: 0.87, 0.88, 0.86, 0.87, and −0.95). The increase in coronary blood flow was accompanied by a decrease of coronary vascular resistances and by an increase of NO release (Table 1, Figs 1 and 2; Pearson's correlation coefficients: −0.88 and 0.85). Moreover, a dose-related increase of coronary venous PO_2 and a reduction of arteriovenous oxygen content were found (Pearson's correlation coefficients: 0.82 and −0.86), in the absence of significant changes in myocardial oxygen consumption (Table 1 and Fig. 2).

Finally, analysis by WB was able to detect a dose-dependent increase in human monomeric adiponectin from the coronary plasma taken in pigs (Fig. 3).

The effects of various doses of human monomeric adiponectin began within about 30 s after starting each dose and reached a steady state in about 2 min at each dose (Fig. 4). The effects of the last dose of adiponectin were almost abolished at 10 min after the end of administration. The intracoronary infusion of the vehicle did not affect hemodynamic variables. In addition, no changes in other hemodynamic variables were observed during adiponectin infusion.

Mechanisms of the responses

Tables 2 and 3 show the effects of the blocking agents and human monomeric adiponectin given after the blocking agents on hemodynamic variables. In Table 3, coronary venous PO_2, arteriovenous oxygen content, and myocardial oxygen consumption before and after butoxamine, L-NAME, GTX89569-PEP followed by adiponectin given in animals treated with the above agents are reported as well.

In the 15 pigs, blockade of muscarinic cholinoceptors ($n=5$), α-adrenoceptors ($n=5$), and β_1 adrenoceptors ($n=5$) did not affect either the coronary and cardiac responses or the NO release caused by the intracoronary infusion of human monomeric adiponectin (Table 2 and Fig. 5).

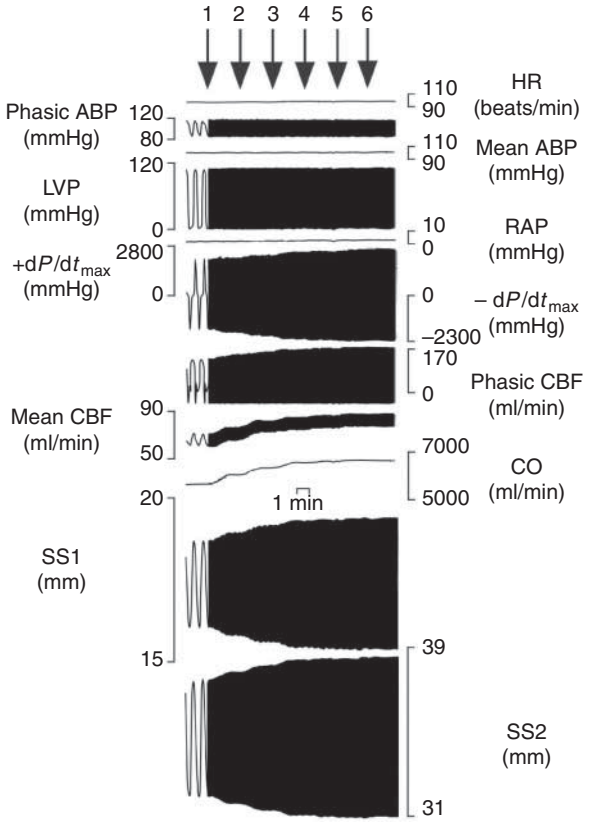

Figure 4
Example of experimental recording taken in one of the 30 pigs. From top to bottom are shown heart rate (HR), phasic and mean aortic blood pressure (ABP), left ventricular pressure (LVP), right atrial pressure (RAP), $\pm dP/dt_{max}$, phasic and mean CBF, CO, and segment length (SL1 and SL2). The other abbreviations are as in Fig. 1. The arrow indicates the start of various doses of human monomeric adiponectin infusion (1, 30 pg; 2, 300 pg; 3, 3 ng; 4, 30 ng; 5, 300 ng; and 6, 3 μg).

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Table 2 Effect of human monomeric adiponectin on hemodynamic variables after blockade of muscarinic cholinoceptors and α- and β_1-adrenoceptors at constant HR and ABP. Data are means ± S.D. Baseline, hemodynamic variables without keeping heart rate and aortic blood pressure constant. Blocking agent, effects of various agents on hemodynamic variables. Control, hemodynamic variables after blocking agents, at constant heart rate and aortic blood pressure. Adiponectin, effects of 3 μg human monomeric adiponectin given after blocking agents at heart rate and aortic blood pressure constant

Data	Baseline	Blocking agent	Control	Adiponectin
Atropine				
HR (beats/min)	75.2±4.7	85±4.4*	104.6±4.4	104.8±4.7
ABP (mmHg)	99.8±2.5	100±2.5	100.4±2.6	100.6±2.7
CBF (ml/min)	56.2±10.6	57±10.8$^{P=0.01}$	67.8±12	83.6±13.6*
$+dP/dt_{max}$ (mmHg/s)	1735±104	1968±107†	2345±119	3003±81*
$-dP/dt_{max}$ (mmHg/s)	−1927±83	−1872±83†	−1766±83	−2131±137*
CO (ml/min)	4200±369	4672±322$^{P=0.01}$	6131±368	7148±283*
%SS	14.34±1.04	14.44±0.4$^{P=0.03}$	14.94±0.38	19.1±0.5*
Phentolamine				
HR (beats/min)	74.4±2.9	84.6±3.2*	105±3.8	105.2±3.7
ABP (mmHg)	90±8.6	81.4±8.2*	82±7.8	82.2±8.5
CBF (ml/min)	48.8±4.2	47.8±4.6	58±5.5	72.2±6.5*
$+dP/dt_{max}$ (mmHg/s)	1622±36	1623±36.6	2051±50	2588±108*
$-dP/dt_{max}$ (mmHg/s)	−1946±156	−1822±156$^{P=0.002}$	−1764±153	−2068±146*
CO (ml/min)	4048±357	4062±400	5086±575	5898±615*
%SS	13.66±0.7	13.7±0.66	14.1±0.7	18.36±0.2†
Atenolol				
HR (beats/min)	78.6±7.4	69.2±7.2*	89.2±7	89.4±7.3
ABP (mmHg)	97.4±9.1	92±9.4*	92.6±9.5	93±9
CBF (ml/min)	52.4±6.7	49.2±6.7*	58.6±6.4	72±6.9*
$+dP/dt_{max}$ (mmHg/s)	1673±90	1350±107*	1688±111	2120±133*
$-dP/dt_{max}$ (mmHg/s)	−1998±98	−2068±102†	−1932±110	−2365±146*
CO (ml/min)	4258±665	3370±659*	4710±758	5400±766*
%SS	13.88±0.65	12.76±0.65*	13.24±0.6	16.9±0.5*

HR, heart rate; ABP, mean aortic blood pressure; CBF, mean coronary blood flow; $+dP/dt_{max}$, maximum rate of change of left ventricular systolic pressure; $-dP/dt_{max}$, minimum rate of change of left ventricular systolic pressure; CO, cardiac output; %SS, percentage of segmental shortening. *$P<0.0001$ vs baseline or control. $^{\dagger}P<0.002$ vs baseline or control.

In pigs treated with β_2-adrenoceptor blocker ($n=5$) and NOS inhibitor ($n=5$), the effects of human monomeric adiponectin on coronary blood flow and NO release were abolished in the absence of significant changes of responses of cardiac function (Table 3 and Fig. 5). In addition, changes in myocardial oxygen metabolism were not significant (Fig. 6).

It is notable that the administration of GTX89569-PEP ($n=5$) caused a reduction of basal $\pm dP/dt_{max}$ and coronary blood flow (Table 3). In addition, the AdipoR1 blocker completely prevented the effects of human monomeric adiponectin on both cardiac function and perfusion and coronary venous PO_2 and coronary arteriovenous oxygen content (Figs 5 and 6).

Discussion

This is the first study showing the direct effects of human monomeric adiponectin on myocardial perfusion and function through the involvement of AdipoR1 and β_2-adrenoceptor-related NO release in anesthetized pigs.

Adiponectin, an adipokine predominantly secreted from adipose tissue, exerts multiple protective properties against obesity, insulin resistance (Lau *et al.* 2011), and cardiovascular diseases (Hui *et al.* 2012). Adiponectin circulates in plasma as homo-oligomers, trimer, hexamer, and high-molecular-weight (HMW) forms, as well as a truncated form corresponding to the globular domain.

The results of the present study have shown for the first time that intracoronary human monomeric adiponectin infusion in anesthetized pigs acutely increased myocardial perfusion and improved systolic and diastolic ventricular function in a dose-dependent way. It is noteworthy that those results were obtained by human monomeric adiponectin infusion starting from a very low dose up to doses similar to the ones reported for circulating adiponectin, which range from 0.5 to 30 μg/ml (Lau *et al.* 2011). Moreover, the infusion of such doses of adiponectin was found to result in a dose-related increase of coronary plasma human monomeric adiponectin levels.

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Table 3 Effect of human monomeric adiponectin on hemodynamic variables after blockade of β_2-adrenoceptors, NOS, and subtype 1 adiponectin receptors at constant HR and ABP. Data are means±S.D.

Data	Baseline	Blocking agent	Control	Adiponectin
Butoxamine				
HR (beats/min)	76±7.2	70.4±7.6*	90.4±7.6	90.6±7.5
ABP (mmHg)	99±11.3	105±11.6*	106±11.5	106±11.3
CBF (ml/min)	53.2±8.7	48.6±8.5*	57.2±9.3	57±9.5
$+dP/dt_{max}$ (mmHg/s)	1703±165	1703±163	2077±209	2635±205*
$-dP/dt_{max}$ (mmHg/s)	−1922±26	−1961±23	−1872±19	−2310±20*
CO (ml/min)	4346±707	4346±659	5394±672	6300±738 $P=0.0001$
%SS	14±1.1	14±1.1	14.56±1.1	18.46±0.9*
L-NAME				
HR (beats/min)	74±1.6	69±1.6 $P=0.0002$	89±1.4	89.2±1.6
ABP (mmHg)	105±3.7	114.2±3.7*	114.6±4	114.4±4
CBF (ml/min)	52.8±3.5	52.6±5.1	63.2±5.1	63.2±5
$+dP/dt_{max}$ (mmHg/s)	1769±102	1771±98	2113±98	2693±85*
$-dP/dt_{max}$ (mmHg/s)	−2037±22	−1990±16	−1872±39	−2254±81*
CO (ml/min)	4262±383	4237±197	5554±162	6441±236*
%SS	14.58±0.3	14.62±0.3	15±0.3	18.78±0.1*
GTX89569				
HR (beats/min)	77±3.4	77±4.4	97±4.4	97.4±4.6
ABP (mmHg)	100±3.3	100±2.2	101±1.9	100.6±2
CBF (ml/min)	48.4±2.4	45.6±2.5 $P=0.001$	54.6±2.7	54.4±2.9
$+dP/dt_{max}$ (mmHg/s)	1691±78	1491±79 $P=0.0003$	1843±59	1847±55
$-dP/dt_{max}$ (mmHg/s)	−1905±185	−1652±128 $P=0.002$	−1592±131	−1598±124
CO (ml/min)	4056±169	3878±141 $P=0.001$	4807±172	4810±185
%SS	14±0.2	13.6±0.19 $P=0.002$	14±0.18	14±0.19

GTX89569, subtype 1 adiponectin receptor blocker. Layout and other abbreviations are as in Table 2. *$P<0.0001$ vs baseline or control.

Since the experiments were carried out while preventing changes in heart rate and arterial blood pressure and in the absence of changes in cardiac filling pressures, the observed responses represented the primary effects of human monomeric adiponectin on the coronary circulation and cardiac function. Furthermore, none of those responses to adiponectin could be obtained during intracoronary infusion of the vehicle alone given at the same rate as that of the peptide. In addition, the above effects were accompanied by a significant increase of coronary venous PO_2 and a decrease of arteriovenous sinus oxygen content, which could be related to the increased coronary blood flow caused by adiponectin (Tune *et al.* 2004).

It is notable that to date, knowledge of the exact role of adiponectin in cardiac function has been confusing and conflicting. While it may be accepted that adiponectin could be involved in the pathophysiology of heart disease, it is not so clear if it acts as a positive or negative modulator. On the one hand, the association of 'hyperadiponectinemia' with increased mortality risk was found to be more pronounced in patients with cardiovascular disease than in those without so that the expected cardioprotective effect fails to materialize (Hui *et al.* 2012). The observed paradoxical increase in adiponectin has been hypothesized to be either a kind of modulatory response of inflamed tissue to counter the atherosclerotic process or the consequence of 'adiponectin resistance' of tissues (Bidulescu *et al.* 2013). On the other hand, circulating adiponectin in type 2 diabetic patients has been reported to be lower in the presence of coronary artery disease, and clinical observations have demonstrated that plasma adiponectin levels obtained after myocardial infarction correlate positively with myocardial salvage index and ejection fraction recovery (Shibata *et al.* 2008). As such, reduced adiponectin production has been recognized as a risk factor of cardiovascular disease.

The results obtained in the present study are in agreement with the latter observations and with those taken in adiponectin knock-out mice and in anesthetized pigs with myocardial ischemia/reperfusion injury, in which adiponectin was able to exert protective effects on cardiac function (Shibata *et al.* 2005, Tao *et al.* 2007, Kondo *et al.* 2010). In addition, our findings on ventricular relaxation are in line with those taken in patients showing an association between low plasma adiponectin and a worsened diastolic dysfunction (Negi *et al.* 2012).

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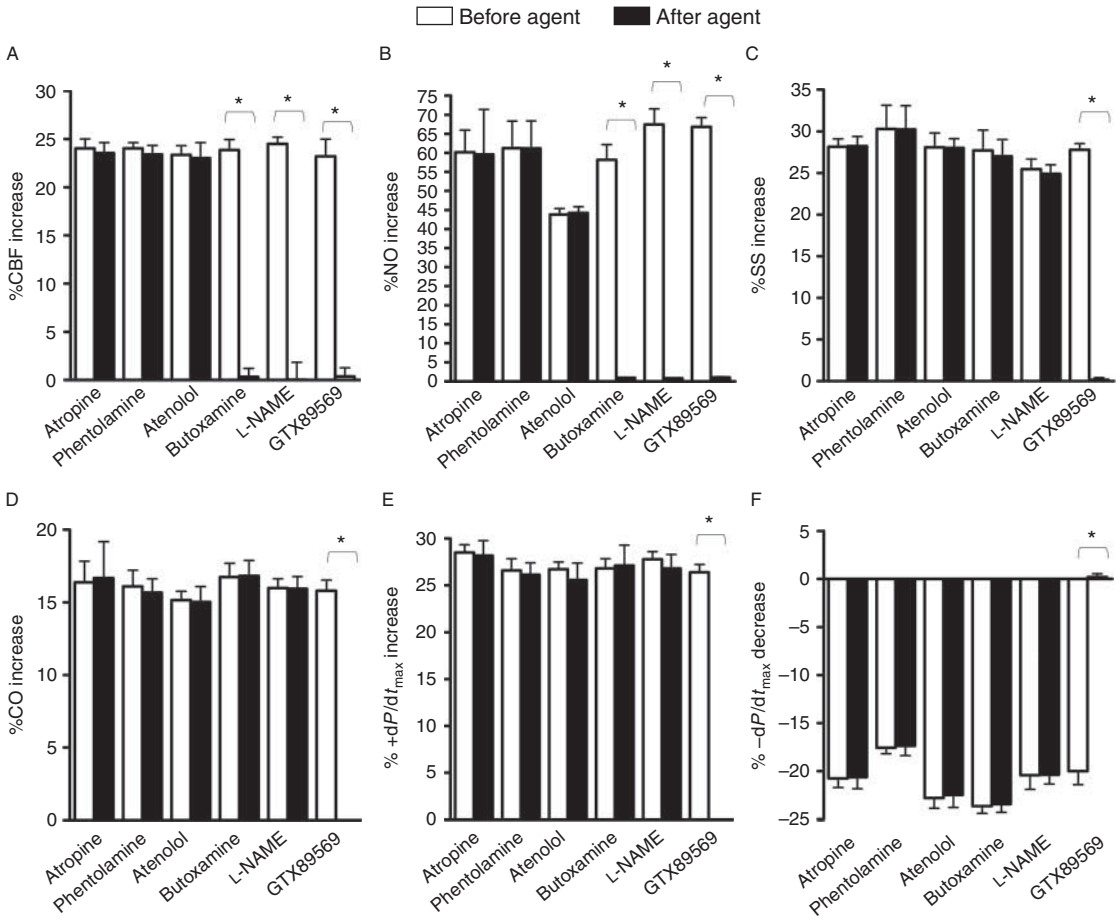

Figure 5
Effects of intracoronary infusion of human monomeric adiponectin on CBF (A), nitric oxide (NO; B), %SS (C), CO (D), $+dP/dt_{max}$ (E), $-dP/dt_{max}$ (F) before and after blockade of muscarinic cholinoreceptors ($n=5$), α-adrenoceptors ($n=5$), β_1-adrenoceptors ($n=5$), β_2-adrenoceptors ($n=5$), the NO synthase ($n=5$), and subtype 1 of adiponectin receptors ($n=5$). Adiponectin was infused before (open columns) and after (filled columns) giving atropine, phentolamine, atenolol, butoxamine, L-NAME, and GTX89569. Abbreviations are the same as previous figures. The bars indicate S.D. *$P<0.0001$.

The results obtained about $-dP/dt_{max}$ and those regarding myocardial oxygen metabolism are of particular relevance and strengthen the role of human monomeric adiponectin as a beneficial modulator of the cardiovascular system. It is to note that our findings on myocardial oxygen consumption are novel and are in disagreement with previous observations where the beneficial metabolic effects elicited by adiponectin were accompanied by an increase of mVO_2 (Fang *et al.* 2010). Differences in experimental protocol and setup could explain that discrepancy. Hence, in that study primary cardiomyocytes and isolated working perfused hearts were used, which is a quite different condition from the *in vivo* experimental setup. It is also to note that those tissues were taken from rats and mice and not from pigs. Moreover, full-length adiponectin instead of monomeric adiponectin was tested for 60 min at higher concentration (4 μg/ml) than those used in the present study. Finally, in cardiomyocytes the effects of adiponectin on metabolism and oxygen consumption were found to be related to APPL1, an adaptor protein containing a pleckstrin homology domain, a phosphotyrosine binding domain, and a leucine zipper motif, which is a direct interacting partner of both AdipoR1 and AdipoR2 (Mao *et al.* 2006). However, in addition to APPL1, several other signaling molecules, such as receptor for activated protein kinase C1 (RACK1), the regulatory subunit of protein kinase CK2 (CK2β), endoplasmic reticulum protein 46 (ERp46), and lymphotoxin-β, have been identified as interacting partners of AdipoR1 and intracellular mediators of the response to adiponectin. Thus, the discrepancy found about mVO_2 could also rely on

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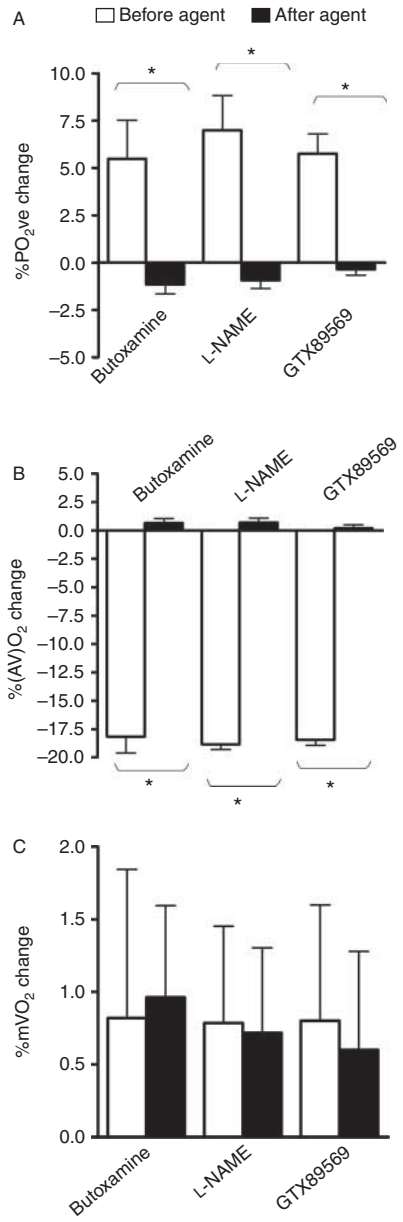

Figure 6
Effects of intracoronary infusion of human monomeric adiponectin on coronary venous PO_2 (pO_2vc; A), coronary arteriovenous oxygen content ($(AV)O_2$; B) and myocardial oxygen consumption (mVO_2; C) before and after blockade of β_2-adrenoceptors ($n=5$), the NO synthase ($n=5$), and subtype 1 of adiponectin receptors ($n=5$). Adiponectin was infused before (open columns) and after (filled columns) giving butoxamine, L-NAME, and GTX89569. Abbreviations are the same as previous figures. The bars indicate S.D. *$P<0.0001$.

different intracellular pathways activated by adiponectin in different animal species.

In the same controlled anesthetized animals, the administration of atropine, phentolamine, atenolol, butoxamine, and L-NAME, infused at the same doses as the ones previously used (Grossini *et al.* 2009, 2011*a*,*b*, 2013*a*,*b*), was applied to examine the involvement of muscarinic cholinoceptors, α-, β_1-, β_2-adrenoceptors, and NOS on the cardiovascular effects elicited by human monomeric adiponectin. Neither the blockade of muscarinic cholinoceptors nor the administration of phentolamine or atenolol affected the hemodynamic responses to adiponectin, indicating that the above effects did not involve muscarinic cholinoceptors or β_1-adrenoceptors.

In contrast, the results obtained after butoxamine and L-NAME administration showed the involvement of both β_2-adrenoceptors and NO on coronary effects elicited by local human monomeric adiponectin infusion, in that the coronary response was abolished by both agents. The idea that NO could play a role in the coronary response to adiponectin was also supported by plasma NO measurement, which was performed by the Griess method as described previously (Grossini *et al.* 2009, 2011*a*,*b*, 2013*a*,*b*). Hence, for the first time, intracoronary human monomeric adiponectin was found to increase NO release in a dose-dependent way. Taken together, these results highlight the role of human monomeric adiponectin as an agent that can cause endothelium-dependent vasodilation in the coronary vasculature and are in agreement with previous findings about the regulation of vascular tone elicited by adiponectin in various regions (Fesus *et al.* 2007, Bussey *et al.* 2011) and NO release in endothelial cells (Cheng *et al.* 2007) or ischemic–reperfused tissue (Tao *et al.* 2007). Moreover, as reported previously, the observed NO release may contribute to the cardiovascular protective effects elicited by adiponectin in physiological conditions (Ignarro 1989).

Furthermore, the present observations regarding the involvement of β_2-adrenoceptors in the coronary response to human monomeric adiponectin are new findings and confirm previous data on this issue. Hence, β_2-adrenoceptor excitation has been reported to increase cellular uptake of L-arginine and eNOS activity in human endothelial cells (Conti *et al.* 2013). Moreover, the involvement of β_2-adrenoceptor-related NO release has been found to play a role in the coronary effects of other agents intracoronary infused in the same animal model (Grossini *et al.* 2009, 2011*a*,*b*, 2013*a*,*b*). Information about the relationship between β-adrenoceptors and adiponectin has been scarce and only concerned the modulation of adiponectin gene expression in adipose tissue, which has been found to be inhibited by β-adrenergic stimulation (Fasshauer *et al.* 2001). When those observations are taken together, despite being obtained in a different context, they would support the

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existence of a type of feedback regulation between adiponectin and β-adrenoceptors. Future studies are necessary to better address this issue.

Globular and full-length adiponectin are widely reported to exert their effects through interaction with two distinct AdipoRs, AdipoR1 and AdipoR2, which are highly related and share about 80% sequence identity (Yamauchi *et al*. 2003, Buechler *et al*. 2010). In addition, T-cadherin has been found to act as a receptor for hexameric and HMW forms of adiponectin (Hug *et al*. 2004, Hui *et al*. 2012). Specifically, AdipoR1 in pig tissues has been reported to be highly expressed (Kiezun *et al*. 2013), which is the reason why we focused on this subtype of AdipoRs. In addition, only Adipor1 knockdown has been found to be able to prevent the acute effects of the globular form of adiponectin on rat cardiac metabolism and oxidation (Palanivel *et al*. 2007). In the present study, the blockade of AdipoR1 through GTX89569-PEP reduced basal values of systo-diastolic function and cardiac perfusion in the absence of changes in the heart rate and aortic blood pressure. Hence, this is the first study showing those direct hemodynamic changes in response to an AdipoR1 blocker, which revealed an important role for AdipoR1 receptors in physiological control of cardiac function and perfusion in anesthetized pigs. In addition, further speculations could be raised if considering that WB analysis was able to detect a basal release of monomeric adiponectin in coronary blood flow of pigs; that is, basal monomeric adiponectin secretion could be involved in physiological modulation of cardiac function and perfusion through interaction with AdipoR1. Data obtained from experiments performed with GTX89569-PEP, that prevented all hemodynamic effects of adiponectin, would confirm that hypothesis. The role played by the AdipoR2, as well as the cell-surface glycoprotein T-cadherin should be examined in the future.

In conclusion, the results obtained in the present study highlight beneficial acute effects elicited by local human monomeric adiponectin administration on both heart function and perfusion. These findings are of particular relevance when considering that adiponectin is the most abundantly produced adipokine by adipose tissue, which surrounds the heart and blood vessels. Thus, adiponectin plays a protective role against cardiovascular disease not only by its action as insulin-sensitizing and anti-inflammatory agent, but also by direct positive effects on cardiac systo-diastolic function and myocardial perfusion, exerted via the modulation of the β_2-adrenoceptor-dependent NO release.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This research has received generous sponsorship from Università del Piemonte Orientale 'A. Avogadro'.

Author contribution statement
The authors have participated actively in the study in terms of substantial contribution to design, analysis, and interpretation of data and manuscript drafting.

Acknowledgements
We thank the Azienda Ospedaliera Maggiore della Carità di Novara for its help.

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Received in final form 7 May 2014
Accepted 16 May 2014
Accepted Preprint published online 23 May 2014

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-14-0170

Published by Bioscientifica Ltd.