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Monomeric adiponectin modulates nitric oxide release and calcium movements in porcine aortic endothelial cells in normal/high glucose conditions

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ABSTRACT

Aims: Perivascular adipose tissue can be involved in the process of cardiovascular pathology through the release of adipokines, namely adiponectins. Monomeric adiponectin has been shown to increase coronary blood flow in anesthetized pigs through increased nitric oxide (NO) release and the involvement of adiponectin receptor 1 (AdipoR1). The present study was therefore planned to examine the effects of monomeric adiponectin on NO release and Ca^{2+} transients in porcine aortic endothelial cells (PAEs) in normal/high glucose conditions and the related mechanisms.

Main methods: PAEs were treated with monomeric adiponectin alone or in the presence of intracellular kinases blocker, AdipoR1 and Ca^{2+}-ATPase pump inhibitors. The role of Na^+/Ca^{2+} exchanger was examined in experiments performed in zero Na^+ medium. NO release and intracellular Ca^{2+} were measured through specific probes.

Key findings: In PAE cultured in normal glucose conditions, monomeric adiponectin elevated NO production and $[Ca^{2+}]c$. Similar effects were observed in high glucose conditions, although the response was lower and not transient. The Ca^{2+} mobilized by monomeric adiponectin originated from an intracellular pool thapsigargin- and ATP-sensitive and from the extracellular space. Moreover, the effects of monomeric adiponectin were prevented by kinase blockers and AdipoR1 inhibitor. Finally, in normal glucose condition, a role for Na^+/Ca^{2+} exchanger and Ca^{2+}-ATPase pump in restoring Ca^{2+} was found.

Significance: Our results add new information about the control of endothelial function elicited by monomeric adiponectin, which would be achieved by modulation of NO release and Ca^{2+} transients. A signalling related to Akt, ERK1/2 and p38MAPK downstream AdipoR1 would be involved.

1. Introduction

Adiponectin, a protein which in humans is encoded by the ADIPOQ gene, is the most abundant adipokine produced and secreted by perivascular adipose tissue (PVAT).

Primarily three isoforms have been detected in plasma: a low molecular weight trimer (LMW), a medium molecular weight hexamer (MMW) and a high molecular weight form (HMW). In addition, fragments from adiponectin proteolysis, including the globular domains, could also be found in the plasma [1]. Of these forms, the HMW has been shown to be the most active one and the most clinically relevant in terms of protective effects against vascular diseases and metabolic syndrome [2]. Hence, plasma adiponectin levels have been found to be decreased in obesity, insulin resistance, and type 2 diabetes [3–5].

Abbreviations: ACh, acetylcholine; AdipoR, adiponectin receptor; AMPK, 5′ adenosine monophosphate-activated protein kinase; CAMKII, Ca^{2+} calmodulin kinase II; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol tetraacetic acid; eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular-signal-regulated kinases; FURA-2/AM, Fura-2/acetoxymethyl ester; L-NAME, Nω-nitro-L-arginine methyl ester; MAPK, mitogen-activated protein kinase; NCX, Na^+/Ca^{2+} exchanger; NO, nitric oxide; NOS, nitric oxide synthase; PAE, porcine aortic endothelial cells; PI3K, phosphatidylinositol 3′-kinase; PKA, protein kinase A; PMCA, plasma membrane calcium ATPase; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase.

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In addition to improving insulin sensitivity, oxidative stress and inflammation [6], *in vitro* studies have shown that globular adiponectin can exert protection against endothelial dysfunction through a cAMP/protein kinase A (PKA)-dependent signalling [7–9]. Moreover, globular adiponectin has been recognized to affect angiogenesis and endothelial function through the activation of an endothelial nitric oxide synthase (eNOS)-related signalling pathway [10–12]. In endothelial cells, globular adiponectin has also been found to increase nitric oxide (NO) production by the involvement of its specific receptors, adiponectin receptor (AdipoR), and the phosphatidylinositol 3′-kinase (PI3K) activation [13,14].

Changes of NO release have been shown to be involved in endothelial dysfunction such as that caused by high-glucose condition. Published *in vitro* data have shown that globular adiponectin could attenuate high glucose-induced oxidative stress in human umbilical vein endothelial cells by increasing NO secretion and phosphorylation of Akt, 5′ adenosine monophosphate-activated protein kinase (AMPK), and eNOS [15]. Similar results were obtained in human mesangial cells [16].

Among various adiponectin isoforms, the monomeric one has been shown to exert cardioprotective effects. Hence, in anesthetized pigs, human monomeric adiponectin was able to cause a dose-related increase of coronary blood flow through augmented coronary NO release and the involvement of the subtype 1 of AdipoR (AdipoR1) [17]. Since eNOS is a Ca^{2+} dependent enzyme, it could be hypothesized that changes in Ca^{2+} handling could be involved in those effects [18]. Regarding this issue it is notable that in C2C12 myocytes adiponectin was found to increase the intracellular Ca^{2+} concentration by acting on a pool of extracellular origin and through the involvement of AdipoR1 [19].

On the ground of the above issues, the present study was planned to examine the effects of monomeric adiponectin on NO release in normal and high glucose conditions and the mechanisms involved in porcine aortic endothelial cells (PAE). In particular, we focused on cAMP/PKA, Ca^{2+} calmodulin kinase II (CaMKII) pathways and extracellular-signal-regulated kinases (ERK1/2), Akt and p38 mitogen-activated protein kinase (MAPK) involvement. The effects of monomeric adiponectin on Ca^{2+} movements have also been examined.

2. Materials and methods

2.1. Culture of PAE

The experiments were performed in high and normal glucose conditions. A 30 mM concentration of culture fluid containing D-glucose was applied to the hyperglycemic group. PAEs were purchased from Cell Applications, Inc. (San Diego, CA, USA) and were maintained in Dulbecco's Modified Eagle's medium (DMEM; Sigma, Milan, Italy) supplemented with 10% fetal bovine serum (FBS; Euroclone, Pero, Milan, Italy), 2 mM L-glutamine (Sigma), 1% penicillin-streptomycin (Sigma), 1% HEPES (Euroclone) at 37 °C with 5% CO_2 in incubator. PAE (1.5×10^6 cells/ml) were plated into a 96-well plate (1×10^4 cells/well) with DMEM 10% FBS supplemented with L-glutamine, penicillin-streptomycin, HEPES overnight (100 μl/well).

2.2. NO production

The NO production was measured in PAE's culture supernatants using the Griess method (Promega, Milan, Italy), which indirectly quantifies NO level, by measuring both NO^{2-} and NO^{3-}. [20].

Cells plated in 96-well plates in starvation medium were treated for 60 s, 120 s, 180 s, 240 s, and 300 s with monomeric adiponectin (0.3 ng, 3 ng, 30 ng, 100 ng; Sigma) in the concentration-related and time-course studies. In addition, in other cell samples, 600 s monomeric adiponectin (30 ng; Sigma) was given alone or in the presence of the adenylyl cyclase blocker 2′5′-dideoxyadenosine (1 μM; for 15 min; Sigma), the selective cAMP-dependent PKA inhibitor H89 (1 μM; for 15 min; Sigma), the NOS blocker Nω-nitro-L-arginine methyl ester (L-NAME; 10 mM; for 15 min; Sigma), the p38 MAPK inhibitor SB203580 (1 μM, for 30 min; Sigma), the PI3K inhibitor wortmannin (100 nM, for 30 min; Sigma), the MAPK/ERK inhibitor UO126 (10 μM, for 30 min; Sigma), the CaMKII inhibitor KN93 (100 nM, for 15 min; Sigma), the AdipoR1 blocker GTX89956-PEP (30 ng, for 15 min; GeneTex; Irvine, CA, USA). Acetylcholine chlorohydrate (10 mM, for 1 min; Sigma) was used as positive control. The agonist–antagonists and their vehicle were also tested in the basal medium without agents. H89, 2′5′-dideoxyadenosine, L-NAME, SB203580, wortmannin, UO126 and KN93 were used at similar concentrations as those that were able to prevent the effects of intermedin 1-47, human chorionic gonadotropin, urocortin II and levosimendan in endothelial cells [21–24].

At the end of the stimulations, NO production in the sample's supernatants was examined by adding an equal volume of Griess reagent following the manufacturer's instruction. At the end of incubation, the absorbance at 570 nm was measured by a spectrometer (BS1000 Spectra Count, San Jose, CA, USA) and the NO production was quantified in respect to nitrite standard curve and expressed as percentage. The values obtained corresponded to the NO (μmol) produced, after each stimulation, by samples containing 1.5 μg of proteins each.

2.3. Cytosolic calcium ($[Ca^{2+}]c$) measurement

The coverslips were washed twice with sterile PBS 1× and starved with DMEM 0% FBS for 4 h. After that they were incubated with Fura-2/acetoxymethyl ester (AM; 5 μM final concentration; Sigma) for 30–40 min in the dark in DMEM 0% FBS and without red phenol supplemented with 1% penicillin-streptomycin, 1% HEPES and 2 mM L-glutamine.

First we performed a study about dose-dependent effects of monomeric adiponectin on Ca^{2+} movements in both normal and high glucose conditions. Then, we examined the origin of the Ca^{2+} pool mobilized by monomeric adiponectin in normal glucose condition by performing the experiments in the presence or absence of ethylene glycol tetraacetic acid (EGTA, 50 mM; Sigma) or by treating PAE with agents which act through IP3 generation like adenosine triphosphate (ATP, 10 μM; Sigma) and thrombin (100 U/ml; Sigma), administrated either before or after adiponectin. These experiments were performed in order to examine if the intracellular pool affected by monomeric adiponectin was IP3-dependent. In this case different responses of PAEs to monomeric adiponectin given before/after ATP or thrombin would be expected. Moreover, some experiments were performed in PAE cultured in normal glucose condition by monomeric adiponectin administration in the absence or presence of H89 (1 μM; Sigma), GTX89956-PEP (30 ng; GeneTex) and KN93 (1 μM; Sigma). H89 and KN93 were used at similar concentrations that were able to prevent the effects of gastrin 17 and urocortin II in PAE [25,26].

In cells, the major Ca^{2+} entry pathway is the store-operated one, in which the emptying of intracellular Ca^{2+} stores activates Ca^{2+} influx ("capacitative" calcium entry). The effects of monomeric adiponectin on the "capacitative" Ca^{2+} entry through the plasma membrane Ca^{2+} channels were examined through the evaluation of the rate of Ca^{2+} overshoot in PAEs in normal and high glucose conditions. The cells on coverslips were pretreated with EGTA (50 mM) and were subsequently exposed to the Ca^{2+} ATPase inhibitor, thapsigargin (1 μM; Sigma), and monomeric adiponectin alone or in co-stimulation.

Since the return of $[Ca^{2+}]c$ to control values was shown to be related to the activation of the Na^+/Ca^{2+} exchanger (NCX) and PMCA (Plasma Membrane Calcium ATPase) in vascular endothelial cells [25,26], we performed some experiments in order to examine their role in both normal and high glucose conditions. To achieve this purpose, PAEs were incubated in zero Na^+ PSS [*N*-methyl-D-glucamine (NMDG) 126 mM, KCl 1.5 mM, MgCl2 1.2 mM, HEPES 10 mM, D-glucose 10 mM, and CaCl2 mM, Sigma], as previously performed [25,26]. Moreover, in some