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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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Abbreviations

ACh, Acetylcholine

AdipoR, adiponectin receptor

AMPK, 5' adenosine monophosphate-activated protein kinase

CAMKII, Ca²⁺ 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, N ω -nitro-L-arginine methyl ester

MAPK, mitogen-activated protein kinase

NCX, Na⁺/Ca²⁺ 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 Ca²⁺ ATPase

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 (PAE) in normal/high glucose conditions and the related mechanisms.

Main methods: PAE were treated with monomeric adiponectin alone or in presence of intracellular kinases blocker, AdipoR1 and Ca^{2+} -ATPase pump inhibitors. The role of $\text{Na}^+/\text{Ca}^{2+}$ 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 $[\text{Ca}^{2+}]_c$. 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 $\text{Na}^+/\text{Ca}^{2+}$ 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.

Keywords

Adipokine; AdipoR1; calcium transients; nitric oxide

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 type2 diabetes [3-5].

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 signaling 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.

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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. PAE 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% CO₂ in incubator. PAE (1.5 x 10⁶ cells/ml) were plated into a 96-well plate (1 x 10⁴ cells/well) with DMEM 10% FBS supplemented with L-glutamine, penicillin-streptomycin, HEPES overnight (100µl/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 NO²⁻ and NO³⁻. [20].

Cells plated in 96-well plates in starvation medium were treated for 60 s, 120 s, 180 s, 240 s, 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 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 N ω -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 ($[\text{Ca}^{2+}]_c$) measurement

The coverslips were washed twice with sterile PBS 1X and starved with DMEM 0% FBS for 4 hours. 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 PAE 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 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 PAE 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, PAE were incubated in zero Na^+ PSS [N-methyl-d-glucamine (NMDG) 126 mM, KCl 1.5 mM, $MgCl_2$ 1.2 mM, HEPES 10 mM, d-glucose 10 mM, and $CaCl_2$ mM, Sigma], as previously performed [25, 26]. Moreover, in some experiments, monomeric adiponectin was administered in zero Na^+ PSS in presence of PMCA inhibitor, carboxyeosin diacetate (25 μ M; Invitrogen, San Giuliano Milanese, Italy).

3. 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 Inc., Cary NC, USA). Data were checked for normality before statistical analysis. Quantification of $[Ca^{2+}]_c$ was conventionally obtained by measuring the Fura-2/AM fluorescence in Ca^{2+} free (0.1 M EGTA) and Ca^{2+} saturated conditions by the equation $[Ca^{2+}]_c = Kd [(RR_{min})/(R_{max}-R)]$. The fluorescence intensities obtained were corrected for cell autofluorescence at the wavelengths employed. All the results obtained were examined through one-way ANOVA followed by Bonferroni *post hoc* tests, which were used to examine changes in NO and Ca^{2+} caused by various agents among the groups. The non-parametric Mann Whitney U test for unpaired data was used to compare percentage responses. A simple-regression analysis was performed to examine the correlation between the concentration of monomeric adiponectin administered and the observed NO and $[Ca^{2+}]_c$ effects in the concentration-response study. All data are presented as means \pm SD of five independent experiments for each experimental protocol. A value of $p < 0.05$ was considered statistically significant.

4. Results

4.1. Dose-response effects of monomeric adiponectin on NO release in PAE in normal and high glucose conditions

As illustrated in Fig.1A, in the time-course study performed in normal glucose condition, monomeric adiponectin (0.3 ng, 3 ng, 30 ng, 100 ng) increased NO release in a dose-dependent way ($p < 0.05$; R: 0.81, 0.78, 0.71, 0.82). Also in PAE cultured in high glucose condition, monomeric adiponectin (0.3 ng, 3 ng, 30 ng, 100 ng) was able to increase NO production although to a lower extent relative to what was observed in normal condition. Moreover, a steady-state was reached (Fig. 1B). It is to note that the presence of endothelial dysfunction in high glucose conditions was confirmed by the results obtained by using acetylcholine, which was able to cause NO release of about 12% only. Since in the normal glucose condition, the highest effects of monomeric adiponectin were obtained at 30 ng, we have chosen that dose for all next experiments performed about NO release.

4.2. Mechanisms involved in NO release caused by monomeric adiponectin in PAE in normal glucose condition

The effects of various inhibitors alone on NO release in both normal and high glucose conditions are shown in Fig. 1 of Supplemental material. In normal glucose condition, treatment with inhibitors abolished the effects of adiponectin on NO release in PAE (Fig. 2A). Also in PAE cultured in high glucose the effects of monomeric adiponectin were abolished by H89, KN93, GTX89956-PEP and wortmannin, while SB203580 and UO126 were only able to reduce them (Fig. 2B).

4.3. Dose-response effects of monomeric adiponectin on $[Ca^{2+}]_c$ in PAE in normal and high glucose condition

In PAE cultured in normal glucose medium, monomeric adiponectin (0.3 ng, 3 ng, 30 ng, 100 ng), caused a dose-related and transient $[Ca^{2+}]_c$ increase ($p < 0.05$; Fig. 3, Table 1). Those results were linearly correlated to the dose of adiponectin administered (R: 0.74, 0.89, 0.87, 0.53). The highest effect was virtually obtained with 30 ng adiponectin for 60 s stimulation, and this

concentration was maintained for all successive experiments. In contrast, the effects of monomeric adiponectin in high glucose condition were lower and not transient (Fig. 4; Table 1).

4.3.1 Analysis of Ca^{2+} pool mobilized by monomeric adiponectin in PAE and mechanisms involved in normal glucose condition

As reported in Table 2, the effects of monomeric adiponectin on $[\text{Ca}^{2+}]_c$ were practically abolished in PAE cultured in Ca^{2+} -free medium. The noncompetitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), thapsigargin (1 μM), caused a persistent increase of $[\text{Ca}^{2+}]_c$, which at 1 min from the start of administration amounted to 132.5 ± 2.2 nM from control values of 100.4 ± 3.3 nM (Fig. 5A). The addition of 30 ng monomeric adiponectin in co-stimulation with thapsigargin markedly changed the kinetics of cytosolic Ca^{2+} fluctuations promoted by thapsigargin. Hence, at 1 min and 5 min from the beginning of thapsigargin administration, $[\text{Ca}^{2+}]_c$ amounted to 126.8 ± 2.6 nM and 122.6 ± 3 nM (Fig. 5B), respectively. Those values were lower than the ones found with thapsigargin alone ($p < 0.05$). These results demonstrated that monomeric adiponectin caused an increase of $[\text{Ca}^{2+}]_c$ by promoting Ca^{2+} mobilization from a pool mainly of extracellular origin and by modulating “capacitative Ca^{2+} entry”.

In order to achieve more details on the nature of the intracellular store affected by monomeric adiponectin, experiments were performed using extracellular ATP (10 μM) and thrombin (0.5 U/ml), which mobilize Ca^{2+} from an IP_3 -sensitive pool [25, 26]. Those agents were administrated either before or after adiponectin. As reported in Fig. 5C and D, the pool mobilized by monomeric adiponectin was partly similar to the one affected by ATP.

When ATP was given after monomeric adiponectin, $[\text{Ca}^{2+}]_c$ amounted to 123.9 ± 2.9 nM from control values of 107 ± 2 nM. When ATP was given before adiponectin, $[\text{Ca}^{2+}]_c$ amounted to 130 ± 1.31 nM from control values of 107 ± 2.1 nM. Also the effects of monomeric adiponectin on $[\text{Ca}^{2+}]_c$ differed in relation to order of administration. When adiponectin was given before ATP, $[\text{Ca}^{2+}]_c$ amounted to 114.5 ± 2.9 nM from control values of 106.2 ± 2.1 nM. When adiponectin was given after ATP, $[\text{Ca}^{2+}]_c$ amounted to 111.2 ± 1.3 nM from control values of 105 ± 1.5 nM.

Different results were obtained with thrombin. Hence, the increase of $[\text{Ca}^{2+}]_c$ caused by monomeric adiponectin and thrombin did not significantly differ irrespective of the sequence of addition (Fig. 5E and F and Table 2).

The fact that the response of PAE to monomeric adiponectin and to an agent acting through IP_3 generation, like ATP, differs in relation to the sequence of addition, suggests that monomeric

adiponectin would act through an IP3-related signalling, which is however somehow different from that affected by another IP3-dependent Ca^{2+} mobilizing agent, like thrombin.

As shown in Fig. 6A-C, the effects of monomeric adiponectin on $[\text{Ca}^{2+}]_c$ in PAE cultured in normal glucose condition, were almost abolished by KN93, H89 and GTX88956-PEP, which demonstrated the involvement of PKA, CaMKII and AdipoR1 pathways.

As the NCX and PMCA have been shown to contribute to lower $[\text{Ca}^{2+}]_c$ in vascular endothelial cells [7, 25-27] experiments were performed to abolish their contribution to Ca^{2+} extrusion. In PAE cultured in Na^+ free medium (Na^+PSS) and normal glucose condition with or without PMCA blocker, the effects of monomeric adiponectin were markedly changed, being the decay phase mainly affected. As shown in Fig. 6D and E and reported in Table 3 the increase of $[\text{Ca}^{2+}]_c$ was lower and had not returned to control values at 5 min from the start adiponectin administration. In PAE treated with carboxyeosin acetate, a plateau in the effects of adiponectin was reached.

4.4.2 Analysis of Ca^{2+} pool mobilized by monomeric adiponectin in PAE in high glucose condition. Role of "Capacitative Calcium entry", NCX and PMCA and AdipoR1

The effects of monomeric adiponectin on $[\text{Ca}^{2+}]_c$ cultured in Na^+PSS with or without carboxyeosine acetate were not different from those previously observed during the grading study (Fig. 7A and B; Table 3).

As shown in Fig. 7C and D, in presence of EGTA, monomeric adiponectin failed to affect Ca^{2+} movements in PAE cultured in high glucose condition. Moreover, the response of PAE to thapsigargin was changed by monomeric adiponectin. At 1 min, the increase of $[\text{Ca}^{2+}]_c$ caused by thapsigargin alone reached a plateau value of 122 ± 3.7 nM from control values of 99.6 ± 2.8 nM (Fig. 7C). When monomeric adiponectin was given with thapsigargin, at 1 min from the beginning of thapsigargin administration, $[\text{Ca}^{2+}]_c$ amounted to 110.4 ± 2.4 nM from control values of 97.8 ± 3.2 nM. At 5 min from the start thapsigargin, the $[\text{Ca}^{2+}]_c$ was almost returned to basal values (Fig. 7D).

These results demonstrated that in high glucose conditions the Ca^{2+} pool mobilized by monomeric adiponectin was of extracellular origin and highly independent from the thapsigargin-sensitive one.

5. Discussion

The results of this study have shown for the first time in PAE that monomeric adiponectin increases NO release in both normal and high glucose conditions to a different extent and through a different modulation of Ca^{2+} movements. A role for intracellular signalling involving kinases leading to NOS phosphorylation and AdipoR1 has been highlighted.

All previous studies have been mainly focused on vascular effects of the principal isoforms adiponectin [1]. Those findings have generally shown protective effects on endothelial function, which were achieved through the enhancement of NO release and the involvement of PKA, PI3K and eNOS [2]. Also in oxidative stress conditions caused by high glucose medium, adiponectin was found to exert protection through elevated eNOS-related NO production and Akt and AMPK activation [28]. Those results could account for cardiovascular protection exerted by adiponectins in insulin resistance and diabetic conditions [10-12]. Relevant to this issue are the findings that endothelial dysfunction is present in a number of cardiovascular conditions such as diabetes, hypercholesterolemia and hypertension and seems to be an important feature in the pathogenesis of the atherosclerotic disease process [29]. Endothelial dysfunction could be assumed to be attributable to a nitroso-redox imbalance which is characterized by decreased endothelium-dependent vasorelaxation and changes in coagulation and inflammatory response. Decreased NO bioavailability appears to be central to the pathogenesis of this condition [30].

The results obtained in the present study have shown for the first time that monomeric adiponectin can increase NO release in PAE by affecting Ca^{2+} movements although in a different way depending on normal and high glucose conditions. The doses of monomeric adiponectin used in PAE were similar to those shown in anesthetized pigs to increase coronary blood flow and cardiac function [31].

While in PAE cultured in normal glucose medium the effects of monomeric adiponectin were linearly related to the infused dose and did not reach a steady state, in high glucose medium the response of PAE was lower and reached a plateau. It is to note that findings obtained in normal glucose conditions are in agreement with observations from anesthetized pigs in which the coronary effects caused by monomeric adiponectin were linearly related to coronary NO release [17]. Also in this study the Griess system was used for NO detection, as previously performed in same or similar cellular model [31-34].

The analysis of intracellular pathways involved in the effects of monomeric adiponectin in PAE in normal and high glucose condition has revealed a role of cAMP/PKA, CAMKII, ERK1/2,

p38MAPK, Akt and NOS. Hence, all effects of monomeric adiponectin on NO release were abolished or reduced by specific blockers of above kinases [31-34].

In addition, GTX88956-PEP, the AdipoR1 blocker, was able to inhibit the effects of monomeric adiponectin. AdipoR1 belongs to the family of adiponectin receptors, AdipoRs [35], which includes two seven transmembrane domain receptors, AdipoR1 and AdipoR2 [36]. We have chosen AdipoR1 in our study because in contrast to AdipoR2 its expression is ubiquitous and has been reported to be highly present in pig tissues [37]. Moreover, also in anesthetized pigs the cardiac and vascular effects of monomeric adiponectin were related to AdipoR1, being abolished by GTX88956-PEP [27].

Thus, at the basis of the effects of monomeric adiponectin on NO release there would be the activation of an intracellular signalling leading to Akt, ERK1/2, p38MAPK activation and NOS phosphorylation, downstream AdipoR1 and cAMP. Our findings are in agreement with previous ones about the role of those kinases and pathways in the effects of adiponectins on NO and add more information about their involvement in mediating the effects of various isoforms of peptide [38]. Hence, while Akt and AdipoR1 would play a role in eliciting the response of cells to almost all adiponectin isoforms, p38MAPK would be only involved as an intracellular mediator of the effects of the globular and monomeric ones [38].

Also the results obtained in high glucose condition are in agreement with previous observations found in human umbilical vein endothelial cells, where globular adiponectin was able to prevent the reduction of NO release caused by intermittent or constant high glucose [39].

Furthermore, the findings of an involvement of PKA, p38MAPK and Akt in mediating the effects of monomeric adiponectin on NO release in high glucose condition could assume clinical relevance; those mechanisms have been reported to play a role in inflammatory signal generation triggered by high glucose [39].

Changes of $[Ca^{2+}]_c$ levels are of primary importance in the regulation of NO production. Hence, the constitutive isoform of NOS present in endothelial cells (eNOS) is Ca^{2+} dependent [40] and [41]. For this reason we aimed to examine the effects of monomeric adiponectin on Ca^{2+} movements in PAE in both normal and high glucose conditions.

As for NO release, firstly we have performed a dose-dependent study by analyzing the effects of monomeric adiponectin on $[Ca^{2+}]_c$ in Fura 2-AM loaded PAE. Our results have shown for the first time that while in normal glucose conditions the effects of monomeric adiponectin on $[Ca^{2+}]_c$ are dose-related and transient, in high glucose condition the increase of $[Ca^{2+}]_c$ is lower and reaches a steady-state.

Then, we examined the origins of the Ca^{2+} pool mobilized by monomeric adiponectin and the mechanisms involved. In the cytosol, Ca^{2+} is maintained at a very low level and is concentrated in intracellular calcium stores such as the endoplasmic reticulum [42]. The dynamic steady state of Ca^{2+} in the cytosol is the result of the balance between active and passive fluxes through the cell membranes of various stores and is strictly regulated [43, 44]. In particular, the release from intracellular pools may occur as a consequence of IP3 generation. Also, the release of intracellular Ca^{2+} coupled to subsequent Ca^{2+} entry, known as “capacitative Ca^{2+} entry”, is a mechanism widely reported to affect Ca^{2+} homeostasis in response to various stimuli [45, 46].

Experiments performed with PAE in normal glucose condition and in presence of EGTA, ATP and thrombin have shown that the Ca^{2+} pool mobilized by monomeric adiponectin was of extracellular origin and independent from that mobilized by an agent acting through IP3 generation, like ATP [47]. Those results confirmed previous ones in C2C12 [19].

Moreover, and as observed about NO release, the effects of monomeric adiponectin on $[\text{Ca}^{2+}]_c$ were abolished by H89, KN93 and GTX88956-PEP, which showed the involvement of a signalling PKA- and CAMKII-related downstream AdipoR1.

Thus, it could be assumed that in normal glucose condition, monomeric adiponectin would cause NO release by increasing $[\text{Ca}^{2+}]_c$ through a common pathway involving AdipoR1 and intracellular signalling.

We have further examined the role of “capacitative” Ca^{2+} entry, NCX and PMCA in the effects of monomeric adiponectin in both normal and high glucose conditions. We focused on those mechanisms in an attempt to explain the observed differences in Ca^{2+} movements found in different glucose media and because those mechanisms have been reported to be variably affected in the diabetic condition [48].

When comparing the effects of thapsigargin, the non-competitive inhibitor of SERCA, on $[\text{Ca}^{2+}]_c$ in normal and high glucose conditions, it was quite clear that the “capacitative Ca^{2+} entry” would play a greater role in the effects of monomeric adiponectin in the latter condition. Hence, while in normal glucose conditions monomeric adiponectin was able to only reduce the effects of thapsigargin, in high glucose ones the response of PAE to thapsigargin was markedly changed.

The activation of the plasma membrane PMCA and of NCX would also play an important role in the restoration of basal intracellular $[\text{Ca}^{2+}]_c$ in PAE [25, 26]. In experiments performed in PAE cultured in Na^+ -free normal glucose conditions, the decay phase of the adiponectin-evoked Ca^{2+} transient was significantly longer. Moreover, in PAE cultured in Na^+ -free medium and loaded with carboxyeosin, the specific PMCA pump inhibitor, the effects of monomeric adiponectin on $[\text{Ca}^{2+}]_c$ transient reached a plateau. Thus, the results obtained showed that in normal glucose

conditions during the decay phase of the $[Ca^{2+}]_c$ transient, Ca^{2+} was extruded by both the PMCA pump and by NCX.

In contrast, the results obtained in high glucose medium showed that neither NCX nor PMCA would be involved in the effects of monomeric adiponectin. These findings could be in agreement with previous observations showing altered function of NCX and/or PMCA in diabetic conditions [48].

6. Conclusions

Our findings indicate that monomeric adiponectin increases NO release in PAE cultured in both normal and high glucose conditions through a signalling involving Akt, ERK1/2 and p38MAPK downstream AdipoR1. Those effects would be related to changes in Ca^{2+} transients, which would arise from modulation of Ca^{2+} influx from extracellular space and the release from intracellular thapsigargin-sensitive Ca^{2+} stores. Our results add new information about the control of endothelial function elicited by monomeric adiponectin in both physiological and pathological conditions. In this context, monomeric adiponectin would play a beneficial role by acting as a paracrine agent in the keeping of endothelial function and prevention of endothelial damage caused by high glucose condition. Moreover, our findings could increase the knowledge about the role played by PVAT in modulation of vascular function through the release of adipokines. Further *in vivo* experiments will be mandatory to predict clinically advantageous effects of monomeric adiponectin.

Conflict of interest statement

All the authors declare that there is no conflict of interest regarding the publication of this paper.

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Figure legends

Fig. 1. Effects of monomeric adiponectin on NO release in PAE in normal (A) and high (B) glucose conditions. In A, in the time-course study in normal glucose conditions, monomeric adiponectin (0.3 ng, 3 ng, 30 ng, 100 ng) increased NO release in a dose-dependent way. In B, in high glucose conditions the effects of monomeric adiponectin (0.3 ng, 3 ng, 30 ng, 100 ng) were lower than those found in high glucose. NO is expressed as % of basal values and corresponds to the NO (μmol) produced, after each stimulation, by samples containing 1.5 μg of proteins each. Reported data are mean \pm SD of five independent experiments.

Fig. 2. Effects of monomeric adiponectin in PAE in NO release in presence or absence of various agents. In A, and B effects of 600 s monomeric adiponectin in normal and high glucose conditions on NO release expressed as % of basal values. C= control values; A= monomeric adiponectin (30 ng); 2'5' = 2'5' dideoxyadenosine (1 μM); H89= H89 (1 μM); KN93= KN93 (1 μM); GTX89956= AdipoR1 Blocker (30 ng); Wort= wortmannin (100 nM); SB203580= SB203580 (1 μM); UO126 = UO126 (10 μM); L-NAME= L-NAME (10 mM); ACh= acetylcholine (10 mM). Reported data are mean \pm SD of five independent experiments * $p < 0.05$ vs C; # $p < 0.05$ vs A (30 ng).

Fig. 3. Dose-response effects of monomeric adiponectin on $[\text{Ca}^{2+}]_c$ in PAE in normal glucose conditions. Dose-response and time-course study and an example taken from one of five experiments are shown. Monomeric adiponectin (0.3 ng, 3 ng, 30 ng, 100 ng), caused a dose-related and transient $[\text{Ca}^{2+}]_c$ increase. The results are the mean \pm SD of 5 experiments. The abbreviations are as in previous figures.

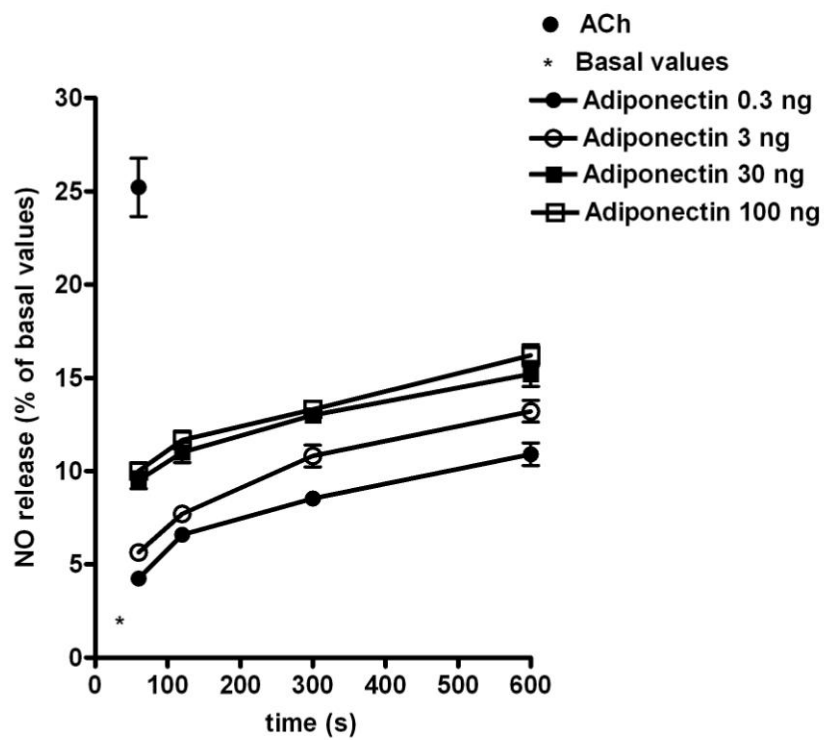
Fig. 4. Dose-response effects of monomeric adiponectin on $[\text{Ca}^{2+}]_c$ in PAE in high glucose conditions. Dose-response and time-course study and an example taken from one of five experiments are shown. Monomeric adiponectin (0.3 ng, 3 ng, 30 ng, 100 ng), caused a lower but not transient effect. The results are the mean \pm SD of 5 experiments. The abbreviations are as in previous figures.

Fig. 5. Analysis of intracellular Ca^{2+} pool mobilized by monomeric adiponectin in PAE in normal glucose conditions. In A and B, effects of monomeric adiponectin on thapsigargin-dependent Ca^{2+} pool. In C and D, effects of monomeric adiponectin on ATP-dependent Ca^{2+} pool. In E and F, effects of monomeric adiponectin on thrombin-dependent Ca^{2+} pool. An example of each experimental protocol is shown. In A, thapsigargin (SERCA inhibitor, 1 μM) alone. In B monomeric adiponectin (A 30 ng) with thapsigargin; EGTA (50 mM). In C and D, ATP was administrated either before or after monomeric adiponectin. In E and F, monomeric adiponectin was administrated before or after thrombin. The results are the mean \pm SD of 5 experiments for each experimental protocol.

Fig. 6. Effects of monomeric adiponectin in PAE in presence of various agents and role of NCX and PMCA in the effects of monomeric adiponectin in normal glucose conditions. In A-C, effects of monomeric adiponectin (A 30 ng) in presence of KN93 (1 μM), H89 (1 μM) and GTX899565-PEP (30 ng). In D and E, effects of monomeric adiponectin (A 0.3 ng and A 100 ng) on $[\text{Ca}^{2+}]_c$ in PAE incubated in Na^+PSS in the absence or presence of carboxyeosin diacetate (25 μM) are shown. Each example was taken from one of the five different experiments for each experimental protocol. Car E, carboxyeosin diacetate.

Fig. 7. Role of NCX and PMCA in the effects of monomeric adiponectin on Ca^{2+} movements and effects of monomeric adiponectin on thapsigargin-dependent Ca^{2+} pool in high glucose conditions. In A and B, effects of monomeric adiponectin (A 0.3 ng and A 100 ng) on $[\text{Ca}^{2+}]_c$ in PAE incubated in Na^+PSS in the absence or presence of carboxyeosin diacetate (25 μM) are shown. In C and D, effects of monomeric adiponectin on thapsigargin-dependent Ca^{2+} pool. An example was taken from one of the five different experiments for each experimental protocol. Car E, carboxyeosin diacetate.

A



B

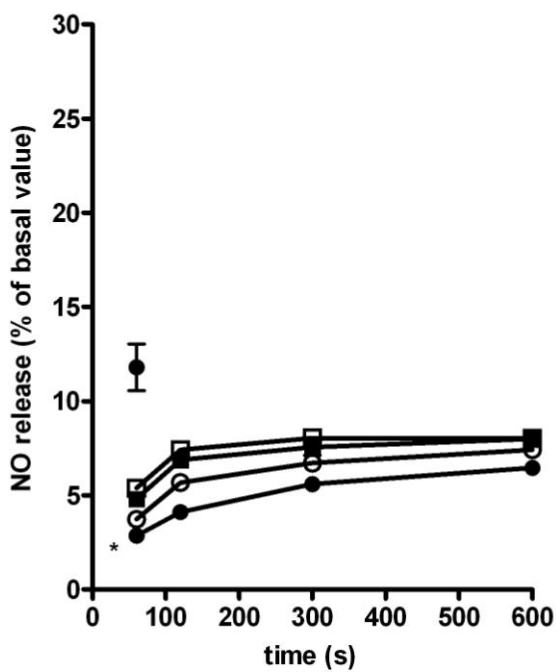
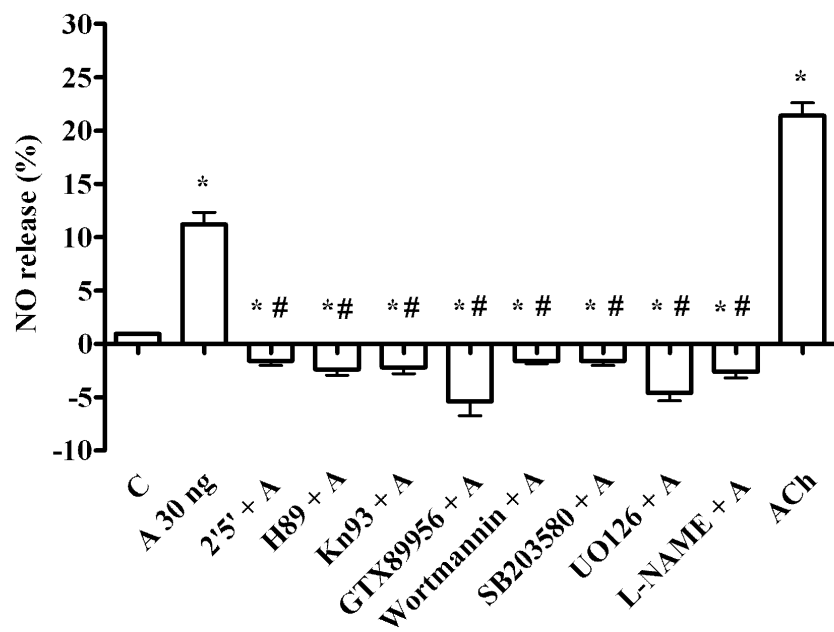


Figure 1

A



B

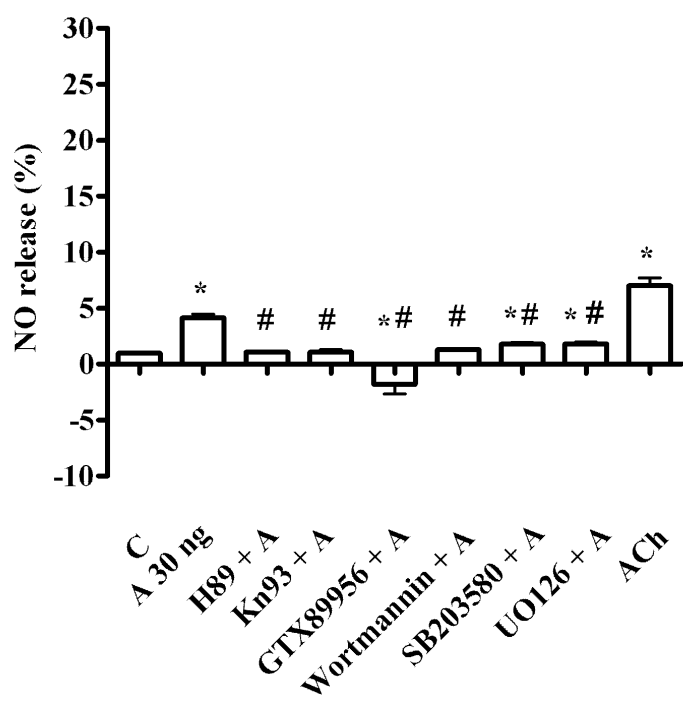


Figure 2

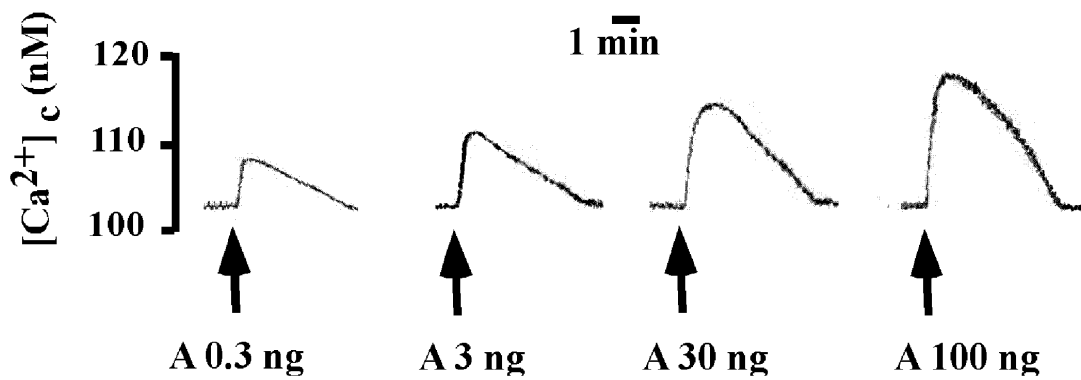
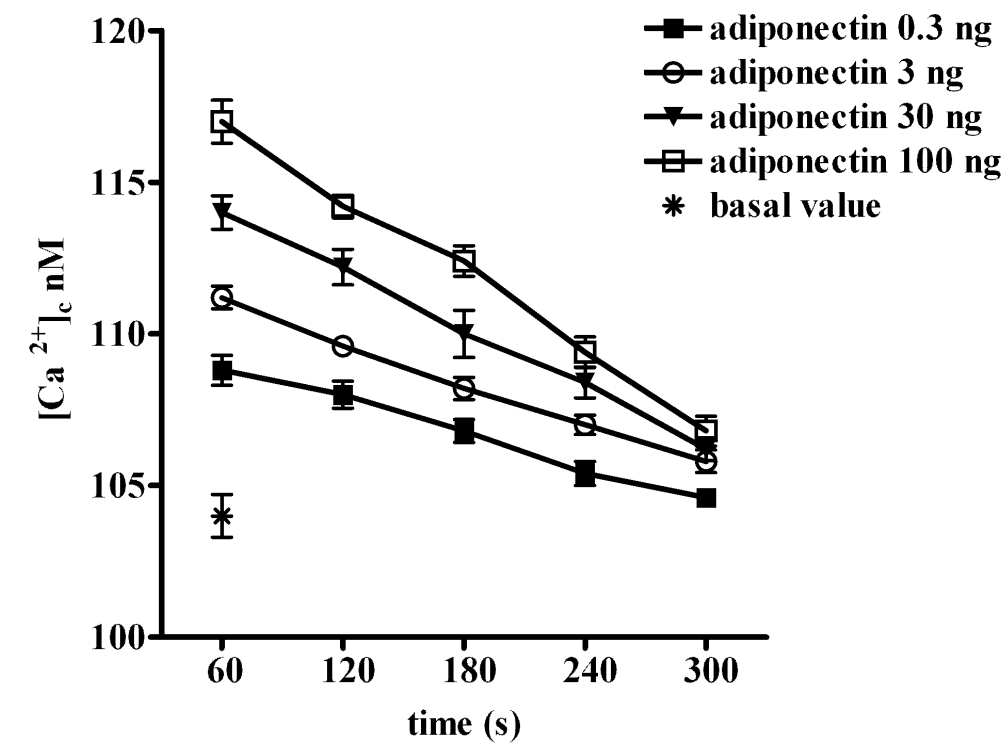


Figure 3

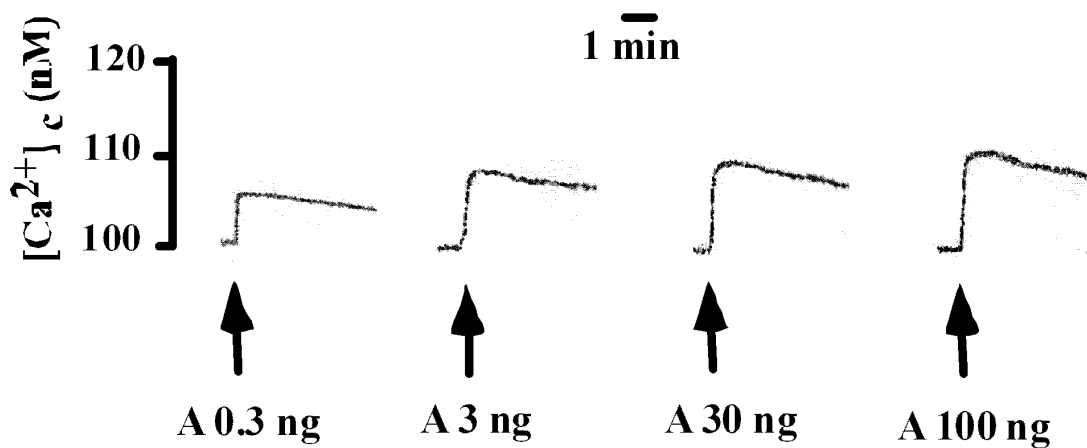
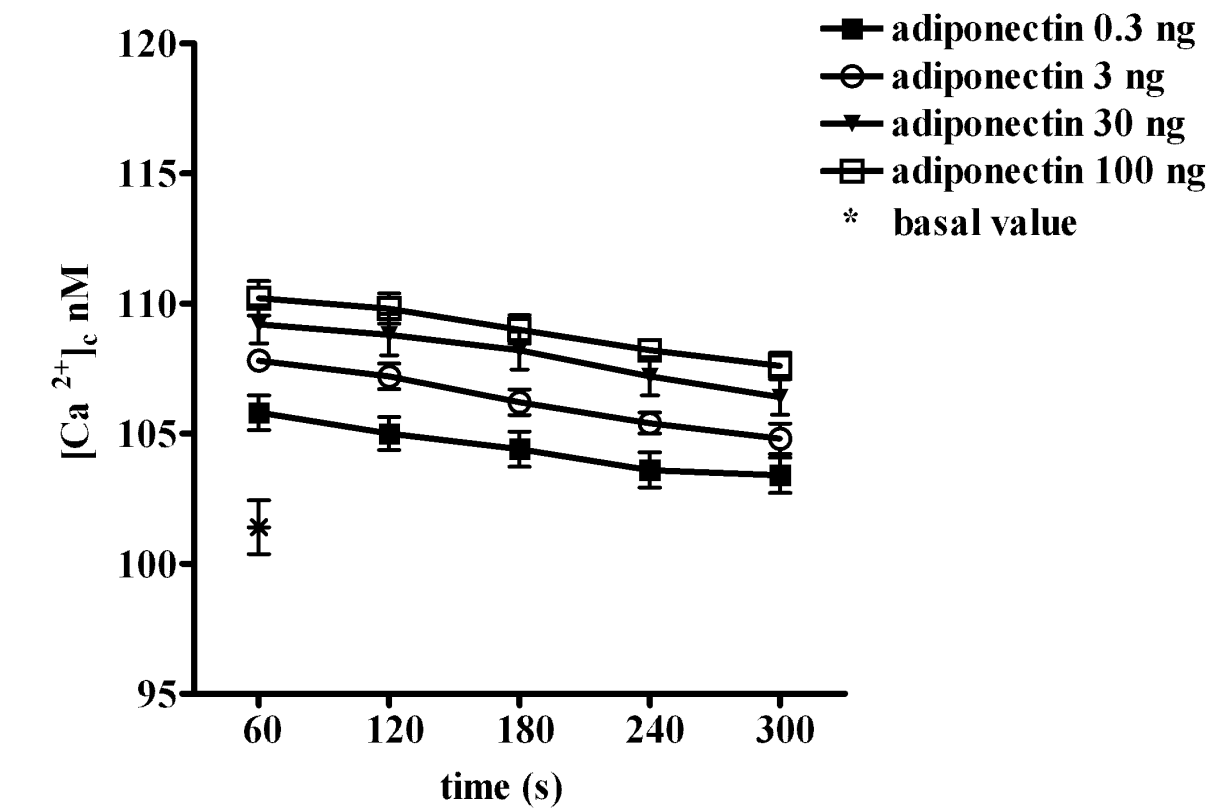


Figure 4

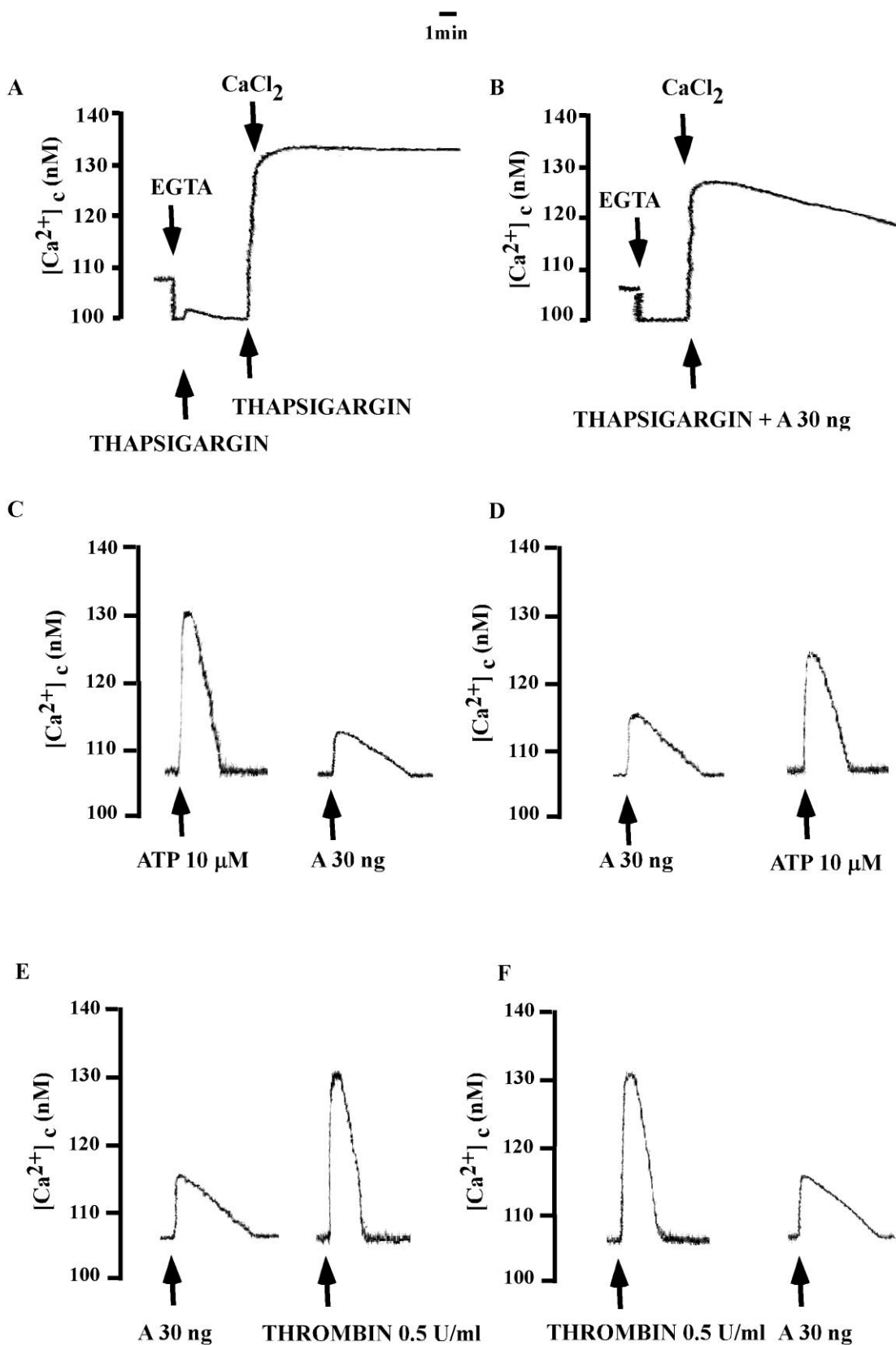


Figure 5

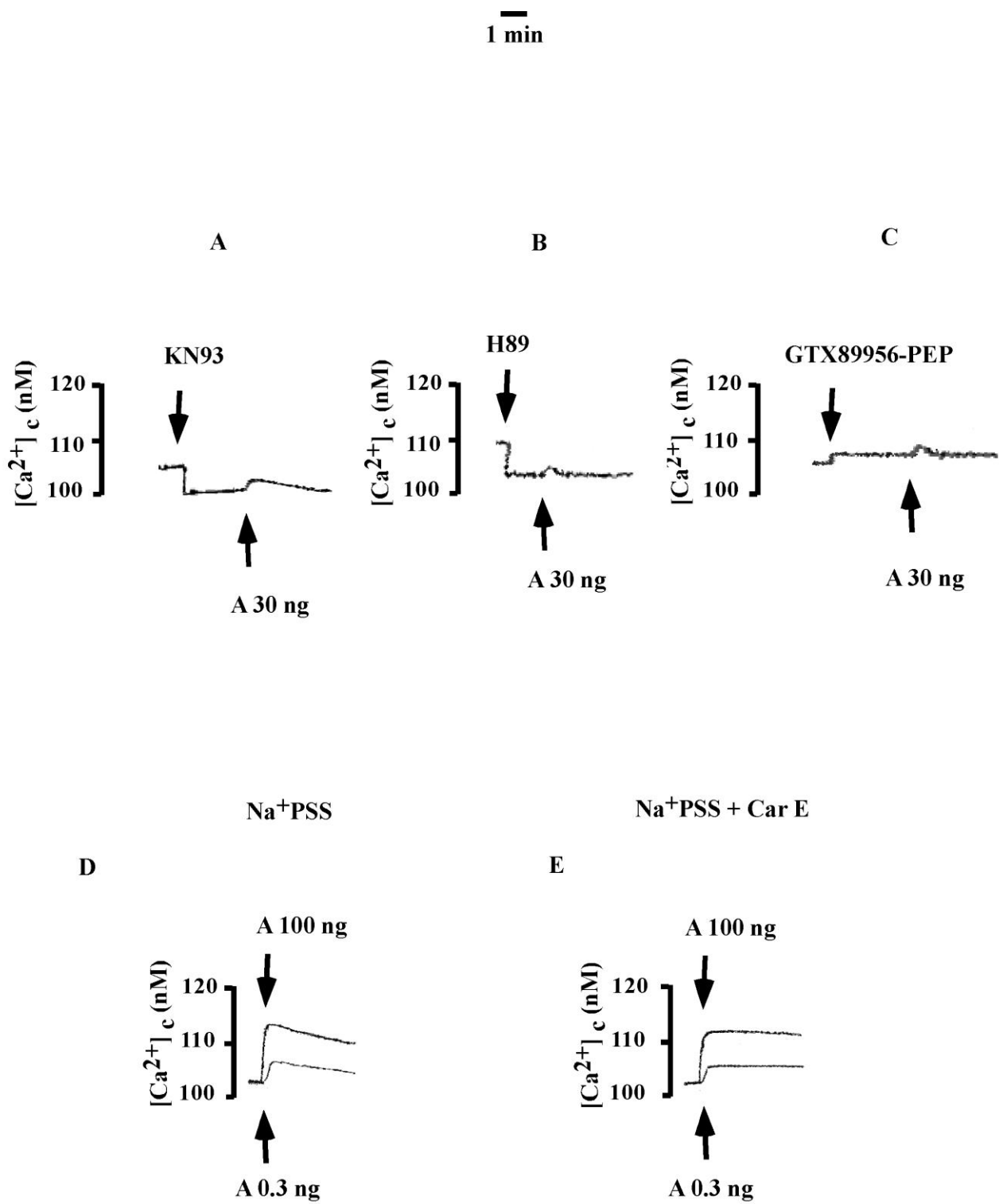


Figure 6

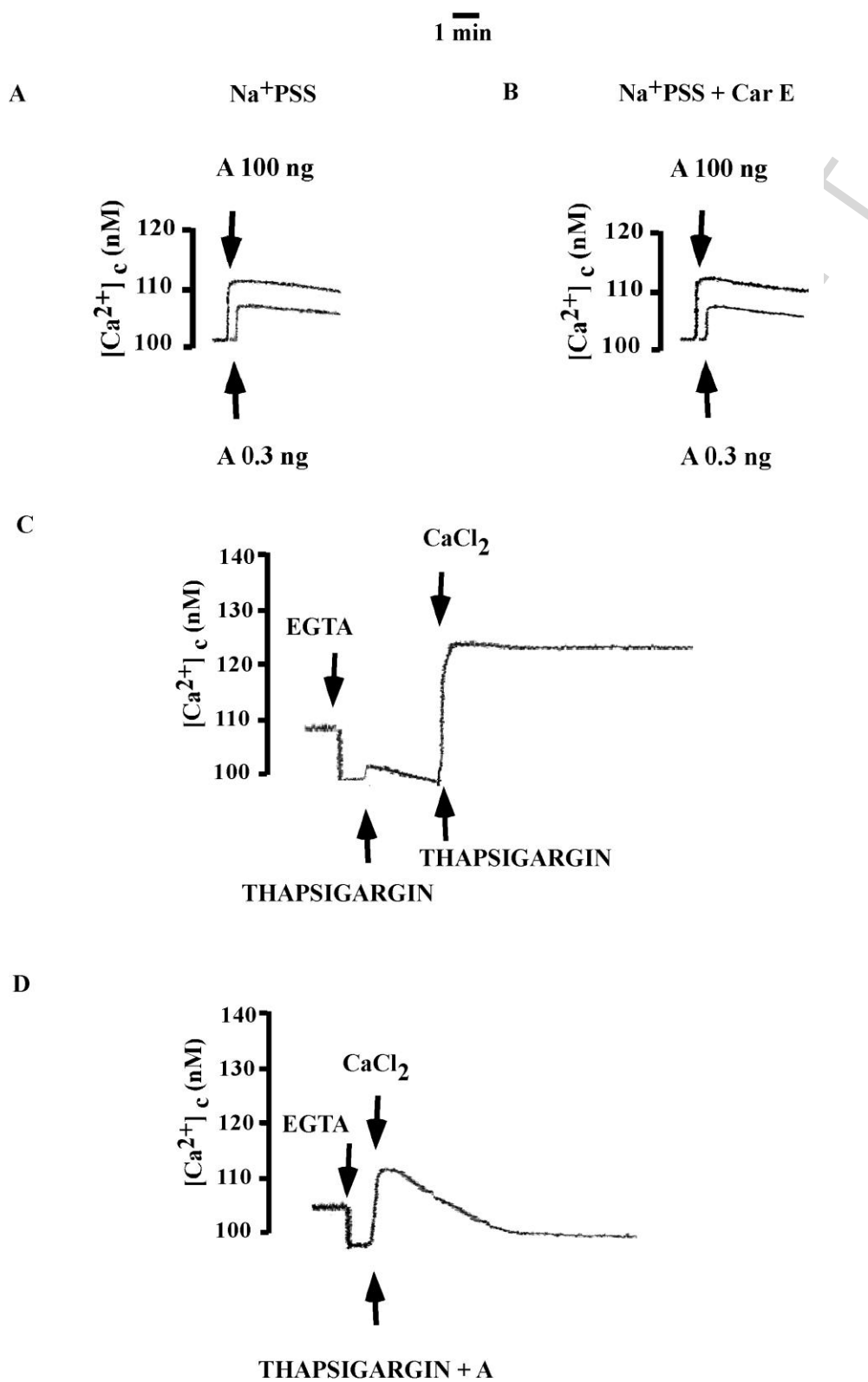
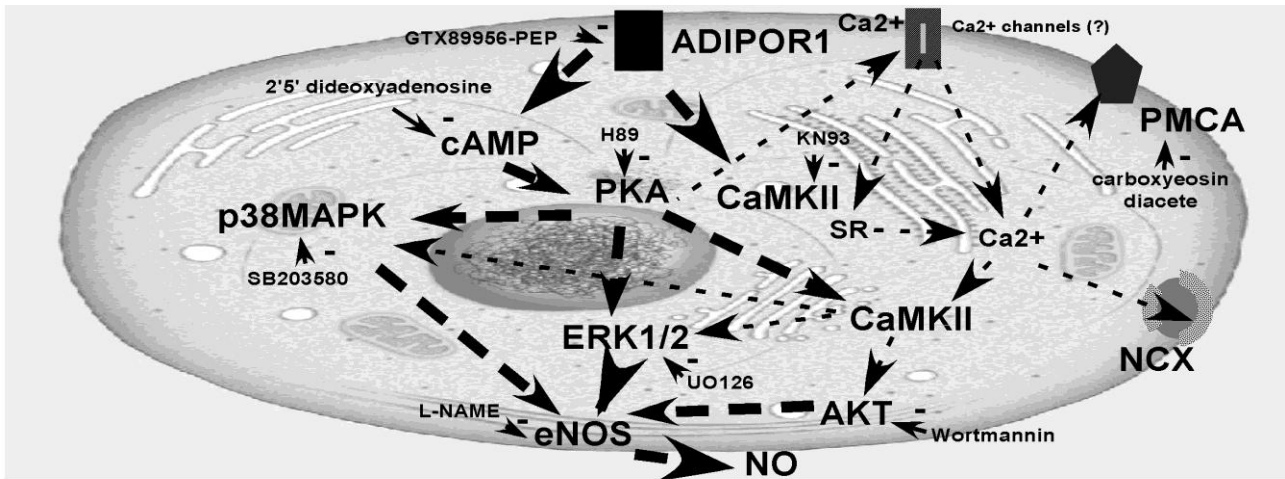


Figure 7



Graphical abstract summarizing the effects of monomeric adiponectin on NO release and Ca²⁺ movements in porcine aortic endothelial cells. ADIPOR1: subtype 1 of adiponectin receptor; cAMP: 3' 5' cyclic adenosine monophosphate; CaMKII:Ca²⁺ calmodulin kinase II; eNOS: endothelial nitric oxide synthase;ERK1/2, extracellular-signal-regulated kinases; L-NAME: Nw-nitro-L-arginine methyl ester; MAPK, mitogen-activated protein kinase; NCX:Na⁺/ Ca²⁺ exchanger; NO: nitric oxide; PKA, protein kinase A; PMCA: plasma membrane calcium ATPase; SR: sarcoplasmic reticulum

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Table 1. Effects of monomeric adiponectin on $[Ca^{2+}]_c$ in PAE

	NORMAL GLUCOSE	HIGH GLUCOSE
C	104 ± 1.6 nM	101.4 ± 2.3 nM
A 0.3 ng	108.8 ± 1 ^{* a} nM	105.8 ± 1.4 [*] nM
A 3 ng	111.2 ± 0.8 ^{*#a} nM	107.8 ± 0.8 ^{*#} nM
A 30 ng	114 ± 1.2 ^{*#†a} nM	109.2 ± 1.6 ^{*#} nM
A 100 ng	117 ± 1.5 ^{*#†a} nM	110.2 ± 1.5 ^{*#} nM

In Table, the effects on $[Ca^{2+}]_c$ caused by monomeric adiponectin at 60 s stimulation, are shown. Data are means ± DS of 5 different experiments for each experimental protocol. A= monomeric adiponectin. * p<0.05 vs C (Control); # p<0.05 vs A 0.3 ng; † p<0.05 vs A 3 ng; a p <0.05 vs high glucose.

Table 2. Effects of monomeric adiponectin on $[Ca^{2+}]_c$ in presence/absence of various agents in PAE

	NORMAL GLUCOSE
C	99.6 ± 3.3 nM
A 30 ng/ml	112.2 ± 2.2* nM
C	106.9 ± 1.7 nM
EGTA + A 30 ng/ml	102 ± 2.2* nM
C	106.2 ± 2.1 nM
A 30 ng/ml PRE THR (0.5U/ml)	115 ± 1.7* nM
C	106.3 ± 1.9 nM
A 30 ng/ml POST THR (0.5U/ml)	115.6 ± 1.4* nM
C	105.3 ± 1.2 nM
THR (0.5U/ml) POST A 30 ng/ml	130.8 ± 2.8* nM
C	105.8 ± 1.6 nM
THR (0.5U/ml) PRE A 30 ng/ml	131 ± 2.4* nM
C	105.6 ± 2.4 nM
KN93	100.2 ± 2.5* nM
KN93 + A 30 ng/ml	100.8 ± 2.6* nM
C	106.6 ± 2.4 nM
H89	101.6 ± 2* nM
H89 + A 30 ng/ml	102.2 ± 2.7* nM
C	105 ± 3.3 nM
GTX89956-PEP	106.6 ± 3.8 nM

GTX89956-PEP + A 30 ng/ml	106.4 ± 4.1 nM
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In Table, the effects on $[Ca^{2+}]_c$ caused by monomeric adiponectin at 60 s stimulation, in presence/absence of various agents are shown. Data are means ± DS of 5 different experiments for each experimental protocol. A= monomeric adiponectin, THR=Thrombin * $p < 0.05$ vs Control.

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Table 3. Effects of monomeric adiponectin on $[Ca^{2+}]_c$ in PAE cultured in normal and high glucose medium- Na^+ PSS alone or with carboxyeosin diacetate

	Na^+ PSS	Na^+ PSS + CARBOXYEOSIN DIACETATE
	NORMAL GLUCOSE	
C	103 ± 2.2 nM	102.8 ± 2.6 nM
A 0.3 ng peak	107.6 ± 3.2 ^{*†} nM	106.4 ± 2.6 [*] nM
A 0.3 ng 5 min	104 ± 2.5 ^{*#} nM	
A 100 ng peak	113.4 ± 3.2 ^{*†} nM	112.6 ± 3.8 [*] nM
A 100 ng 5 min	111.6 ± 3 ^{*#} nM	
	HIGH GLUCOSE	
C	101.9 ± 1.7 nM	101.6 ± 1.3 nM
A 0.3 ng peak	107.1 ± 1.6 ^{*†} nM	106.8 ± 1.8 [*] nM
A 0.3 ng 5 min	106.1 ± 1.7 ^{*#} nM	
A 100 ng peak	111.5 ± 1.8 ^{*†} nM	111.4 ± 1.3 [*] nM
A 100 ng 5 min	110.9 ± 1.2 ^{*#} nM	

In Table, the effects on $[Ca^{2+}]_c$ caused by monomeric adiponectin (peak and at 5 min) Na^+ PSS medium in absence or presence of carboxyeosin acetate are shown. Data are means ± DS of 5 different experiments for each experimental protocol. Layout is as in previous Tables. * $p < 0.05$ vs Control; # $p < 0.05$ vs peak; † Na^+ PSS alone $p < 0.05$ vs Na^+ PSS + carboxyeosin diacetate.