

Inhibition of smooth muscle cell proliferation by adiponectin requires proteolytic conversion to its globular form

Melissa Fuerst^{1,3}, Carla G Taylor^{1,2,3}, Brenda Wright³, Leslee Tworek³ and Peter Zahradka^{1,2,3}

Departments of ¹Human Nutritional Sciences and ²Physiology, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2

³Molecular Physiology Laboratory, Canadian Centre for Agri-food Research in Health and Medicine, St Boniface Hospital Research Centre, 351 Tache Avenue, Winnipeg, Manitoba, Canada R2H 2A6

(Correspondence should be addressed to P Zahradka at Molecular Physiology Laboratory, Canadian Centre for Agri-food Research in Health and Medicine, St Boniface Hospital Research Centre; Email: peterz@sbr.ca)

Abstract

Accelerated atherosclerosis is the primary cardiovascular manifestation of diabetes and correlates inversely with levels of circulating adiponectin, an anti-atherosclerotic adipokine that declines in diabetes. We therefore initiated a study to examine the mechanisms by which adiponectin, a hormone released from adipose tissue, influences the proliferation of vascular smooth muscle cells (SMCs). Addition of adiponectin to quiescent porcine coronary artery SMCs increased both protein and DNA synthesis and concurrently activated ERK1/2 and Akt. By contrast, globular adiponectin, a truncated form of this protein, exhibited anti-mitogenic properties as indicated by the inhibition of protein and DNA synthesis in SMCs stimulated with platelet-derived growth factor (PDGF). Whereas globular adiponectin did not stimulate

growth-related signal transduction pathways, it was able to block the PDGF-dependent phosphorylation of eukaryotic elongation factor 2 kinase, a regulator of protein synthesis. Proteolysis of adiponectin with trypsin, which produces globular adiponectin, reversed the growth-stimulating actions of the undigested protein. As the existence of globular adiponectin remains controversial, western blotting was used to establish its presence in rat serum. We found that globular adiponectin was detectable in rat serum, but this result was not obtained with all antibodies. The contrasting properties of adiponectin and its globular form with respect to SMC proliferation suggest that protection against atherosclerosis may therefore be mediated, in part, by the level of globular adiponectin.

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Introduction

Adipokines are hormones that are secreted by adipose tissue. While several adipokines regulate appetite and energy expenditure (Trayhurn & Bing 2006), the most abundant protein produced by adipocytes is adiponectin, a protein first identified by Scherer *et al.* (1995), which is inversely correlated with atherosclerosis (Lam & Xu 2005). Adiponectin is a 244 amino acid (30 kDa) protein also known as adipocyte complement-related protein or Acrp. This protein contains two domains (collagen-like region, complement C1q region) that show homology with the collagen superfamily, complement factors, and tumor necrosis factor- α (TNF- α ; Kadowaki & Yamauchi 2005). The collagen-like region promotes the formation of distinct 180 and 360 kDa multimers (Waki *et al.* 2003). Also low levels (~5% of total adiponectin) of an 18 kDa truncated form containing the complement C1q-like region, typically referred to as globular adiponectin, have been detected *in vivo* (Fruebis *et al.* 2001).

A causal link between adiponectin and vascular disease was first reported by Okamoto *et al.* (2002). In their study,

adenovirus was used to express adiponectin at high levels in *ApoE*^{-/-} mice, and this treatment led to a decline in the number and size of atherosclerotic lesions in the aorta. These results, which have been subsequently confirmed by others (Yamauchi *et al.* 2003a,b, Li *et al.* 2007), may explain the abundant epidemiological data that show atherosclerotic disease is elevated under conditions where circulating adiponectin levels (Scherer *et al.* 1995) are significantly reduced, such as obesity and diabetes (Weyer *et al.* 2001). Furthermore, an elegant study by Clasen *et al.* (2005) has established a link between pharmacological agents capable of limiting progression of atherosclerotic disease (e.g. ACE inhibitors and PPAR agonists) and increased serum adiponectin. These data indicate that an intervention capable of elevating adiponectin levels will likely benefit cardiovascular health.

The evidence clearly supports a role for adiponectin in maintaining the health of cardiovascular tissues (Do *et al.* 2006, DeClercq *et al.* 2008). On the other hand, whether adiponectin is responsible for all the beneficial effects ascribed to this protein has not been elucidated. Fruebis *et al.* (2001) hypothesized that adiponectin is an inactive

precursor and that globular adiponectin is the functionally relevant form of this hormone. This view, however, is not supported by evidence showing both forms of adiponectin bind and activate the adiponectin receptors AdipoR1 and AdipoR2 (reviewed in Kadowaki & Yamauchi (2005)). As well, adiponectin and globular adiponectin independently elicit distinct biological responses in various tissues. For instance, both adiponectin and globular adiponectin inhibit atherosclerotic lesion formation in *ApoE*^{-/-} mice (Okamoto *et al.* 2002, Yamauchi *et al.* 2003a,b) and activate AMP-dependent protein kinase (AMPK) in the cells of responsive tissues (Yamauchi *et al.* 2002). On the other hand, globular adiponectin activates fatty acid oxidation in skeletal muscle but not liver, whereas adiponectin stimulates fatty acid oxidation in both tissues (Yamauchi *et al.* 2003a,b). Furthermore, the small quantity of globular adiponectin found in serum has made resolution of this issue difficult. It is important to note that there have been few direct comparisons between adiponectin and globular adiponectin under identical conditions. We therefore undertook to evaluate the effects of adiponectin and globular adiponectin on smooth muscle cell (SMC) proliferation. Our results indicate that their actions on SMCs are distinct. Furthermore, we establish by western blotting that globular adiponectin is present in normal rat serum, but its detection is dependent on the antibody being used.

Materials and Methods

Materials

Nunc tissue culture plates were obtained from VWR (Mississauga, ON, Canada), and culture media and fetal bovine serum were from Invitrogen. Radioisotopes were purchased from Perkin–Elmer. Platelet-derived growth factor (PDGF)–BB was from Peprotech (Dollard des Ormeaux, QC, Canada), while recombinant (bacterially expressed) adiponectin and globular adiponectin were purchased from Alexis Biochemicals (Farmingdale, NY, USA). Acetylated trypsin and trypsin inhibitor were from New England Biolabs (Pickering, ON, Canada) and Sigma–Aldrich respectively. The ProteoExtract Albumin/IgG Removal kit was obtained from Calbiochem/EMD Millipore (Mississauga, ON, Canada). General laboratory chemicals were supplied by Sigma–Aldrich and Fisher Scientific.

Cell culture

Primary cultures of porcine coronary artery SMCs were generated from the left anterior descending coronary artery by an explant method, and quiescent cells were prepared as described previously (Saward & Zahradka 1997b) by placement into serum-free supplemented medium for 5 days.

DNA synthesis assay

Quiescent cells were prepared in 24-well dishes and stimulated by direct addition of the indicated compounds without replacing the media. When inhibitors were used, they were added 60 min before addition of 0.1 µg/ml PDGF. DNA synthesis was measured by incubating the cells with 1 µCi [³H]thymidine, added 24 h after mitogen stimulation, for 48 h as described previously (Saward & Zahradka 1997a).

Protein synthesis assay

Quiescent SMCs, prepared as described for the DNA synthesis assay, were placed into leucine-free media for 24 h. The cells were subsequently treated with inhibitors (if applicable) for 60 min before stimulation with 0.1 µg/ml PDGF. After 10 min, 10 µCi [³H]leucine was added to each well. The cells were incubated for 5 h, rinsed with PBS, and placed into 1 ml cold 10% trichloroacetic acid (TCA) for 15 min. The cells were rinsed twice with cold 10% TCA and subsequently lysed with 0.5 M NaOH/0.1% Triton X-100. The solution was transferred into scintillation vials containing EcoLume (GE Healthcare, Mississauga, ON, Canada) and the radioactivity quantified with a Beckman LS6500 Multi-Purpose Scintillation Counter.

Protease digestion

Adiponectin (1 µg) was incubated with acetylated trypsin (0.2 µg) for 24 h at 37 °C in 50 mM Tris–HCl pH 8.0/20 mM CaCl₂ (total reaction volume = 20 µl). The reaction was terminated by addition of trypsin inhibitor (0.85 µg). Appropriate amounts of the digestion product were used directly without further manipulation. Control reactions included i) adiponectin or globular adiponectin incubated in the absence of trypsin and ii) trypsin incubated without adiponectin.

Western blotting

Extracts prepared by direct addition of 150 µl 2× SDS/gel loading buffer to cells in 12-well culture dishes were loaded onto 7.5% polyacrylamide gels after boiling for 2 min (Yau *et al.* 2003). Following electrophoresis, the proteins were transferred to PVDF membrane and probed with primary antibodies to eukaryotic elongation factor 2 kinase (eEF2K), phospho-eEF2K, phosphorylated extracellular-regulated kinase (ERK1/2), phospho-Akt, phospho-AMPK, phospho-IκB kinase (IKK), phospho-NF-κB p65 (all from Cell Signaling, Pickering, ON, Canada), adiponectin (Calbiochem), and globular adiponectin (Phoenix). Band intensities were quantified as previously described (Yau *et al.* 2003). For western blotting of serum proteins from Sprague Dawley rats, abundant proteins were removed with the ProteoExtract Albumin/IgG Removal Kit before electrophoresis of 5 µg protein. Antibodies tested on the rat serum included anti-adiponectin, C1Q, and collagen domain containing

polyclonal antibody (Lifespan Biosciences, Seattle, WA, USA), anti-adiponectin/Acrp30 monoclonal antibody (R&D Systems), and anti-gAcrp30/adipolean polyclonal antibody (Peprotech).

Adiponectin oligomer formation

Adiponectin (1 µg) and globular adiponectin (0.5 µg) were mixed with 10 µl buffer consisting of 62.5 mM Tris-HCl pH 6.8 and 20% glycerol and left at ambient temperature for 10 min. Bromophenol blue was added to a final concentration of 0.005% and the samples were loaded onto a 5% polyacrylamide gel that lacked SDS. After the dye had reached the bottom of the gel, the proteins were visualized with Coomassie blue stain (Zahradka & Ebisuzaki 1984) and images were captured with a digital camera.

RT-PCR amplification

RT-PCR was conducted as described previously (Noto *et al.* 2006) with 1 µg total RNA isolated from SMCs with TRIzol (Invitrogen). Oligonucleotide primers for GAPDH (sense: 5'-CGGAGTG AACGGATTTGGCCGCAT-3', antisense: 5'-AGCCTTCTCCATGGTCGTGAAG-3'), AdipoR1 (sense: 5'-CTCTCCATCGTCTGTGTCCTGG-3', antisense: 5'-CCCATCTGGCCCCACGGTGGTGG-3'), and AdipoR2 (sense: 5'-TGAAGACAACGACTACCTGCT-3', antisense: 5'-CACCACCTTCTCCTGAAGGGG-3') were designed based on *Sus scrofa* sequences available in Genbank (AF017079, NM_001007193, and NM_001007192 respectively).

Statistical analysis

All data are presented as mean \pm S.E.M. Student's *t*-tests were conducted among means of data to detect significant differences. Differences were accepted as significant at $P < 0.05$.

Results

Differential actions of adiponectin and globular adiponectin on SMC proliferation

DNA and protein synthesis were measured after treatment of quiescent SMCs with either 0.1 µg/ml PDGF or 10 µg/ml adiponectin. Thymidine incorporation was increased over tenfold by both treatments (Fig. 1A). Leucine incorporation was likewise elevated (PDGF ninefold and adiponectin 1.5-fold) by both agents (Fig. 1B). No additive effects were obtained if cells were treated simultaneously with both PDGF and adiponectin (Fig. 1A and B). By contrast, addition of 2 µg/ml globular adiponectin had no effect on thymidine incorporation by quiescent SMCs (Fig. 1C). However, globular adiponectin reduced thymidine incorporation in response to PDGF by 90% (Fig. 1C). These data suggest that the anti-proliferative actions of adiponectin may be mediated by globular adiponectin.

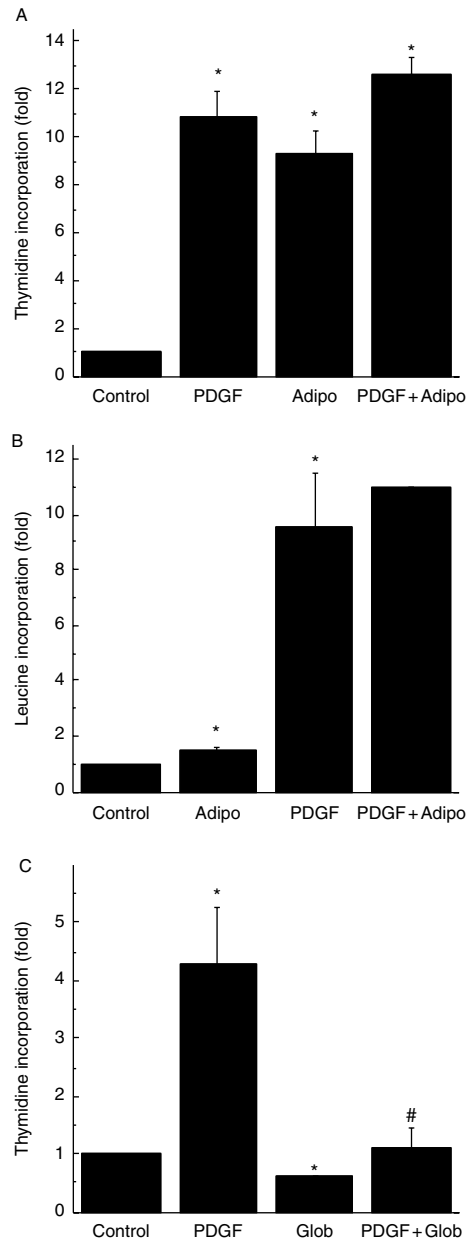
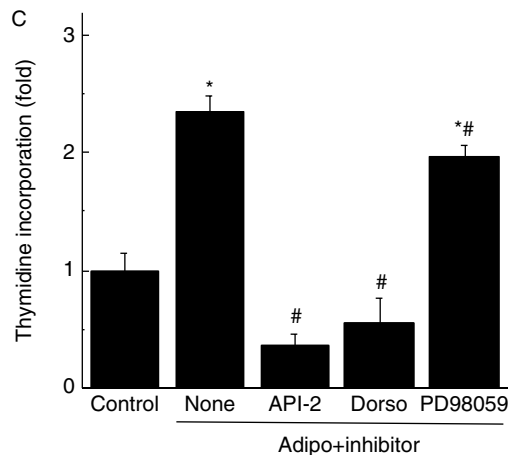
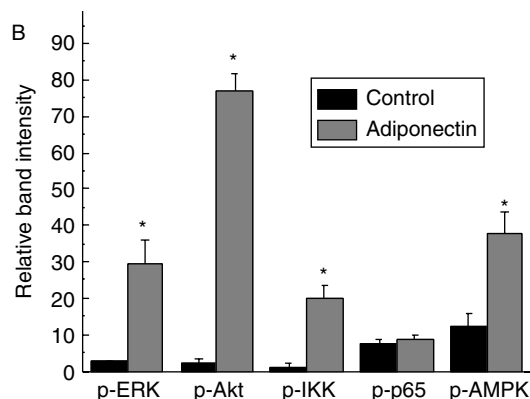
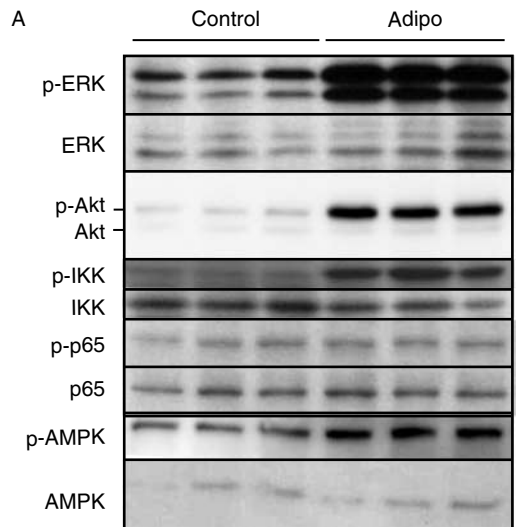


Figure 1 Smooth muscle cell proliferation in response to adiponectin and globular adiponectin. (A) Quiescent SMCs (control) were prepared in 24-well culture dishes and treated with 0.1 µg/ml PDGF, 10 µg/ml adiponectin (Adipo), or a combination of both agents. DNA synthesis was measured by incorporation of [³H]thymidine. The radiolabel was added 24 h after treatment, and cells were harvested for measurement 48 h later. (B) Protein synthesis in response to PDGF and adiponectin was measured by leucine incorporation. Treatment conditions were identical to those of panel A. Radiolabel was added immediately after treatments and cells were harvested after 5 h. (C) Quiescent cells (control) were treated with 0.1 µg/ml PDGF, 2 µg/ml globular adiponectin (Glob), or a combination of both agents and DNA synthesis was measured as described in panel A. The data are presented as mean \pm S.E.M. for $n = 3$, with the control value set to 1. *Significant at $P < 0.05$ from control; #significant at $P < 0.05$ from PDGF treatment (panel C only).

Adiponectin and globular adiponectin activate different intracellular signaling pathways

Quiescent SMCs were treated with 10 µg/ml adiponectin for 10 min and the cells were subsequently harvested for western blot analysis. Increased phosphorylation of the



proliferation-associated kinases ERK1/2, Akt, and IKK were observed (Fig. 2A and B). Interestingly, phosphorylation of the NF-κB p65 was unchanged under conditions where phosphorylation of IKK was increased. As reported previously (Yamauchi *et al.* 2002), adiponectin stimulated phosphorylation of AMPK.

To investigate the contribution of these signaling pathways to the mitogenic actions of adiponectin, we examined cell proliferation in response to adiponectin in the presence of inhibitors of ERK1/2 (PD98059), Akt (API-2), and AMPK (dorsomorphin). As was seen in Fig. 1, treatment of quiescent SMCs with 10 µg/ml adiponectin significantly increased thymidine incorporation; however, both API-2 and dorsomorphin blocked the actions of adiponectin (Fig. 2C). These data suggest that both Akt and AMPK are required for SMC proliferation in response to adiponectin, while ERK1/2 is not.

Treatment of SMCs with a mitogen, 0.1 µg/ml PDGF, increased the phosphorylation of ERK1/2 and Akt (Fig. 3). PDGF treatment also elevated phosphorylation of eEF2K, a mediator of protein synthesis. While no change in the phosphorylation state of ERK1/2 or Akt was detected after addition of 2 µg/ml globular adiponectin, it was observed that PDGF-dependent eEF2K phosphorylation was blocked in the presence of globular adiponectin (Fig. 3). By contrast, addition of adiponectin in conjunction with PDGF had no additional effects beyond those seen with PDGF alone (data not shown), which concur with the results previously seen with DNA and protein synthesis (Fig. 1A and B).

Activation of AMPK does not inhibit DNA synthesis

Phosphorylation of AMPK in response to adiponectin (Fig. 2), aminoimidazole carboxamide ribonucleotide (AICAR; Fig. 4A), a known activator of AMPK (Corton *et al.* 1995), and globular adiponectin (Fig. 4A) was expected based on published data. By contrast, AMPK phosphorylation in cells treated with PDGF was not anticipated (Fig. 4A).

Figure 2 Stimulation of intracellular signaling by adiponectin. Quiescent SMCs (control) were treated with 2 µg/ml adiponectin (Adipo) for 15 min. Cell extracts were subsequently analyzed by western blotting for phosphorylation of ERK1/2, Akt, IKK, NF-κB p65, and AMPK. GAPDH was used as the loading control. Panel A shows a representative blot (repeated three times) with triplicate samples from one experiment. (B) The band intensity of the phosphorylated protein was quantified by scanning densitometry and plotted relative to the intensity of the unphosphorylated protein (B). The data are presented as mean ± S.E.M., with the lowest value set to 1. *Significant at $P < 0.05$ from control. (C) Quiescent cells (control) were treated with 10 µg/ml Adipo in the absence (none) or presence of various inhibitors (0.1 µM API-2, 1 µM dorsomorphin (DORSO), and 10 µM PD98059). Inhibitors were added 15 min before stimulation with adiponectin. DNA synthesis was measured as described in Fig. 1. The data are presented as mean ± S.E.M. for $n = 3$, with the control value set to 1. *Significant at $P < 0.05$ from control; #significant at $P < 0.05$ from adiponectin treatment with no inhibitors.

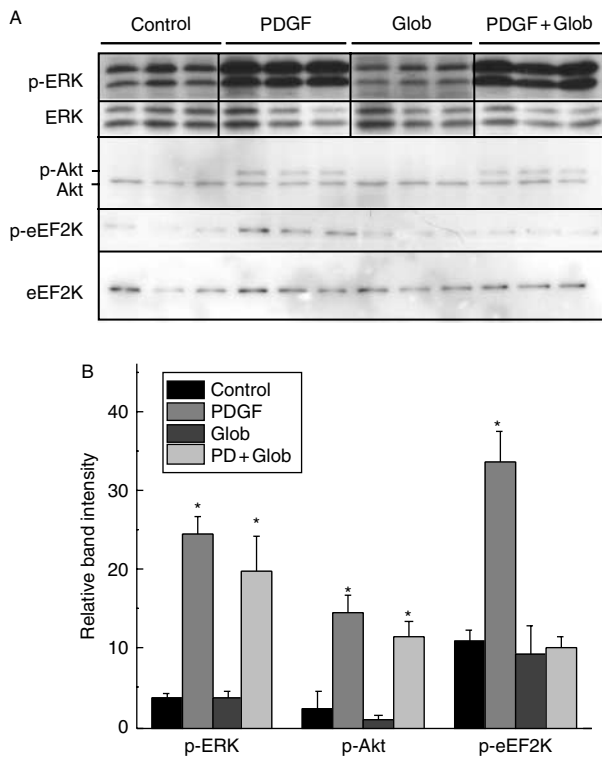


Figure 3 Effect of PDGF and globular adiponectin on intracellular signaling intermediates. Quiescent SMCs (control) were prepared in 24-well culture dishes and treated with 0.1 µg/ml PDGF, 10 µg/ml globular adiponectin (Glob), or a combination of both agents for 15 min. Cell extracts were subsequently analyzed by western blotting for phosphorylation of eEF2K, ERK1/2, Akt, and IKK. Equivalent sample loading was verified with non-phosphorylated eEF2K. Panel A shows triplicate samples from experiments that were replicated three times. Vertical lines separate groups that were taken from the same gel but rearranged to match the headings. In panel B, the band intensities of each phosphorylated protein relative to the respective unphosphorylated protein are presented as mean ± s.e.m., with the lowest value set to 1. *Significant at $P < 0.05$ from control.

AMPK activation has been reported to block DNA synthesis (Igata *et al.* 2005), a response that is inconsistent with the actions of a potent mitogen-like PDGF (Fig. 1A). Also, these results do not agree with the growth stimulatory actions of adiponectin (Fig. 1A). Consequently, the presence of phosphorylated AMPK in mitogen-treated cells suggests that AMPK does not inhibit cell proliferation. To confirm this interpretation, we compared the actions of adiponectin and AICAR on PDGF-stimulated thymidine incorporation. Treatment with PDGF increased DNA synthesis by quiescent SMCs almost 2.5-fold (Fig. 4B). The presence of adiponectin did not significantly interfere with the stimulation elicited by PDGF. Finally, AICAR was unable to block the increase in thymidine incorporation elicited by PDGF. Confirmation that AICAR specifically targets AMPK is reported elsewhere (Mohankumar *et al.* 2012). These results indicate that activation of AMPK by adiponectin and globular adiponectin

is not an indication of an anti-proliferative activity. To determine whether AMPK was required for proliferation in response to PDGF, quiescent SMCs were treated with PDGF in the presence of the AMPK inhibitor dorsomorphin. Under these conditions, induction of thymidine incorporation by PDGF was reduced to below basal levels (Fig. 4C), although there was no change in cell number or appearance (data not shown). Also, as dorsomorphin has been shown to affect SMAD activation (Yu *et al.* 2008), we examined whether PDGF stimulates SMAD phosphorylation. The finding that SMAD activation does not occur with PDGF treatment (data not shown) confirms that the effects of dorsomorphin seen in this study are mediated via AMPK. These results thus indicate that activation of AMPK appears to be a requisite for SMC proliferation following stimulation with a mitogen.

The AdipoR1 receptor mediates AMPK activation by adiponectin (Kadowaki & Yamauchi 2005). We therefore employed RT-PCR to determine whether this receptor was expressed by the SMCs used in these experiments. As shown in Fig. 4D, amplification products specific for both AdipoR1 and AdipoR2 were detectable. These data establish that AMPK activation via AdipoR1 is a reasonable expectation.

Tryptic cleavage of adiponectin alters its ability to stimulate cell proliferation

The adiponectin and globular adiponectin employed in our study had molecular masses of 30 and 18 kDa (Fig. 5A), which agree with the values for the native proteins (Fruebis *et al.* 2001). Furthermore, both proteins are capable of forming oligomeric complexes of two to six polypeptides (Fig. 5B), as previously reported (Tsao *et al.* 2002). Although the enzyme responsible for cleavage of adiponectin has not been identified, both trypsin and leukocyte elastase have been shown to generate *in vitro* a truncated form of adiponectin that consists of the globular domain (Fruebis *et al.* 2001, Waki *et al.* 2005). This protein fragment not only has a molecular mass similar to globular adiponectin, but also exhibits the biological activity associated with this protein (Fruebis *et al.* 2001, Waki *et al.* 2005). Consequently, we elected to use trypsin digestion to determine the functional relationship between the intact adiponectin and the truncated globular form.

Conditions appropriate for trypsin digestion of adiponectin were identified, and western blotting showed that the primary product was a polypeptide of ~18 kDa. Although some undigested 30 kDa adiponectin remained, as well as an intermediate 25 kDa form, more than 90% of the input was cleaved. Not only did the product co-migrate with globular adiponectin, it was also recognized immunologically as globular adiponectin (Fig. 5C). To assess the effect of cleavage on adiponectin's biological activity, thymidine incorporation was monitored in quiescent SMCs treated with adiponectin and the product of adiponectin cleaved with trypsin (Fig. 5D).

It was observed that the increased DNA synthesis obtained with adiponectin was significantly reduced following cleavage of adiponectin with trypsin. The effect of trypsin and trypsin inhibitor, the latter being used to terminate the reaction,

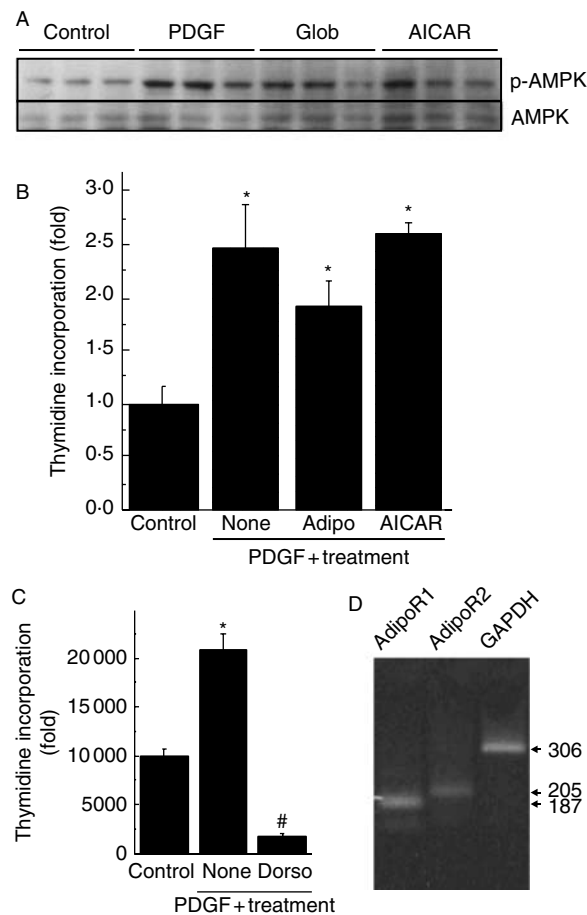


Figure 4 Modulation of AMPK phosphorylation state by PDGF, adiponectin, and globular adiponectin. (A) Phosphorylation of AMPK in response to treatment with 0.1 μ g/ml PDGF, 10 μ g/ml globular adiponectin (Glob), and 10 mM AICAR (positive control) for 15 min was measured by western blotting. The representative blot shows triplicate samples from one experiment; the experiment was replicated three times. GAPDH was used as the loading control. (B) Thymidine incorporation by PDGF-treated SMCs was measured in the presence of either 10 μ g/ml adiponectin (Adipo) or 10 μ M AICAR according to the standard protocol described in Materials and Methods section. The data are presented as mean \pm S.E.M. for $n=3$, with the value for the control set to 1. *Significantly different at $P<0.05$ relative to untreated control. (C) Thymidine incorporation by SMCs stimulated with 0.1 μ g/ml PDGF, in the absence or presence of 1 μ M dorsomorphin (Dorso) as described for panel B. The data are presented as mean \pm S.E.M. for $n=3$, with the value for the control set to 1. *Significantly different at $P<0.05$ relative to untreated control. #Significantly different at $P<0.05$ relative to PDGF-treated cells. (D) Total RNA extracted from quiescent SMCs was amplified with primers specific for the adiponectin receptors AdipoR1 and AdipoR2 or for the house-keeping gene *Gapdh*. The figure shows the positions of the amplified bands after agarose gel electrophoresis.

was determined in a complete reaction (adiponectin+trypsin) to which trypsin inhibitor was added before commencing the incubation (digestion control). Inhibition of trypsin activity prevented the decrease in DNA synthesis that was obtained with the cleaved adiponectin. Of note, the reduced activity of digested adiponectin was not significantly different from that obtained with globular adiponectin. These results suggest that conversion of adiponectin to globular adiponectin alters its ability to affect SMC proliferation.

Globular adiponectin is present in rat serum

Although numerous studies have shown that globular adiponectin is biologically active, as we have in this investigation, whether this protein fragment exists in the circulation remains contentious. To address this issue, we used western blotting to determine whether globular adiponectin could be detected in rat serum, which was used because the antibodies tested were raised against rat protein. The native 30 kDa protein was detected by three different antibodies (Fig. 6A), although a strong signal was only obtained with an antibody from R&D Systems. Depleting the serum of the most abundant proteins (albumins and immunoglobulins) improved the quality of the signal with the antibody from R&D Systems (Fig. 6B), but the signal was reduced with the other antibodies. Likewise, these antibodies detected a band at 18 kDa, the expected position of globular adiponectin, although it was faint with all but the R&D Systems antibody (Fig. 6A and B). On the other hand, the antibody from R&D Systems did not recognize either recombinant adiponectin or recombinant globular adiponectin, although recombinant adiponectin was visible with the antibody from Peptidech (Fig. 6C and D). Based on these results, we conclude that globular adiponectin is present in the circulation; however, the ability to detect its presence is dependent on the properties of the antibody being employed to distinguish between the native or recombinant proteins.

Discussion

The anti-atherogenic properties attributed to adiponectin and globular adiponectin are presumed to involve, at least in part, the ability to block SMC proliferation. While the direct effects of adiponectin on SMCs have been examined (Wang *et al.* 2005), a similar investigation of globular adiponectin has yet to be reported. We therefore undertook to compare the actions of adiponectin and globular adiponectin on SMC proliferation, using DNA and protein synthesis as surrogates for this process (Louis *et al.* 2011). In contrast to expectations, our results revealed that adiponectin stimulates SMC proliferation while globular adiponectin blocks the actions of PDGF, a potent SMC mitogen (Fig. 1). These observations were supported by evidence that adiponectin triggers signaling cascades typically associated with cell proliferation, including ERK1/2 and Akt, which also respond to PDGF,

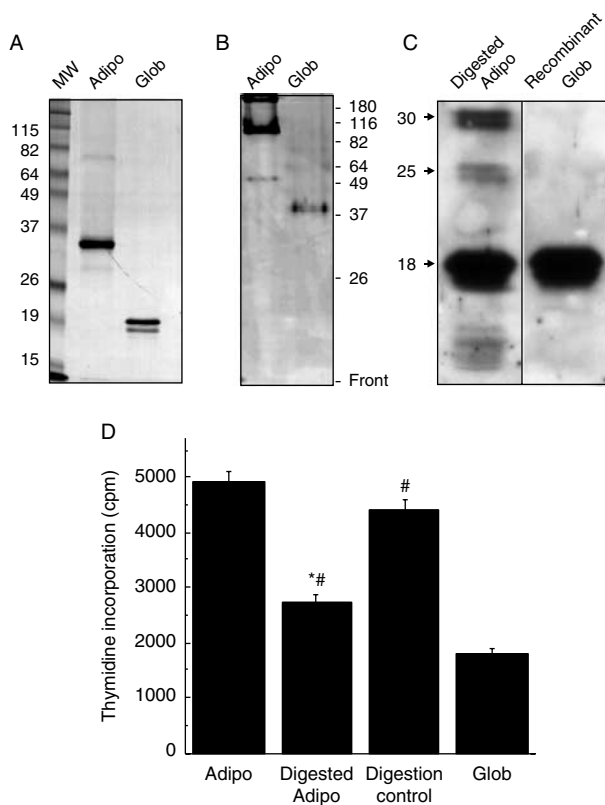


Figure 5 Effect of protease digestion on activity of adiponectin. (A) Adiponectin (Adipo) and globular adiponectin (Glob) are shown by Coomassie blue staining after SDS-PAGE. The position of the molecular mass markers run in parallel is indicated. (B) Oligomer formation by adiponectin (Adipo) and globular adiponectin (Glob) was monitored by non-denaturing PAGE as described in Materials and Methods section. The position of the molecular mass markers run in parallel is indicated. (C) SDS-PAGE of adiponectin digested with trypsin (Digested Adipo) as described in Materials and Methods section. Globular adiponectin (Glob) was loaded on the adjacent lane for comparison. The bands were detected by western blotting with a globular adiponectin-specific antibody (Peprotech). (D) Thymidine incorporation was measured after addition of 1 µg adiponectin (Adipo), 1 µg digested adiponectin (Digested Adipo), and 0.5 µg globular adiponectin (Glob) to quiescent SMCs stimulated with 0.1 µg/ml PDGF. The digested adiponectin was prepared as described in Materials and Methods section. Both adiponectin and globular adiponectin were mixed with the digestion buffer before their addition. The digestion control included trypsin inhibitor with trypsin and adiponectin during the incubation period. The raw data in c.p.m. are presented as mean ± S.E.M. for *n* = 3. *Significantly different at *P* < 0.05 relative to adiponectin (Adipo) treatment; #significantly different at *P* < 0.05 relative to globular adiponectin (Glob) treatment.

and that inhibitors of these signal transduction pathways block the proliferative response (Fig. 2). By contrast, globular adiponectin interfered with activation of eEF2K by PDGF. As AMPK was phosphorylated in response to adiponectin, PDGF, and globular adiponectin (Fig. 4), it was difficult to conclude that AMPK was associated with SMC proliferation. However, the observation that inhibition of AMPK blocks

the mitogenic actions of PDGF and adiponectin (Figs 2C and 4C) indicates that AMPK activation is necessary for cell proliferation. Finally, proteolytic conversion of adiponectin to globular adiponectin, which we show is detectable in normal rat serum, altered the ability of this molecule to stimulate SMC proliferation (Fig. 5), thus confirming that adiponectin and globular adiponectin had opposite effects on this process.

An increase in nitric oxide (NO) production by vascular endothelial cells (Chen *et al.* 2003) is one mechanism that has been proposed to explain the inverse relationship between serum adiponectin levels and the elevated incidence of vascular disease in diabetes, metabolic syndrome, atherosclerosis, and hypertension (Giannesi *et al.* 2007, Hung *et al.* 2008). NO is a potent anti-atherogenic agent as atherosclerosis is prevalent in mice that lack NO synthase (NOS; Knowles *et al.* 2000) and NO inhibits SMC proliferation (Sato *et al.* 2000). Chen *et al.* (2003) reported that adiponectin increases endothelial NOS (eNOS) activity in endothelial cells through AMPK-dependent phosphorylation. Globular adiponectin likewise was shown to increase eNOS activity and additionally stimulate eNOS expression (Hattori *et al.* 2003). Both forms of adiponectin can thus influence vasodilation (Schmid *et al.* 2011).

Direct inhibition of vascular SMC proliferation could also explain the anti-atherosclerotic actions of adiponectin (Zhu *et al.* 2008), although promoting differentiation to the quiescent phenotype would have a similar effect (Ding *et al.* 2011). Adiponectin has been reported to inhibit SMC proliferation in response to a variety of different growth-promoting agents (Matsuda *et al.* 2002), possibly by sequestering growth factors (Wang *et al.* 2005). Matsuda *et al.* (2002) also reported that adiponectin reduced neointimal hyperplasia in response to arterial injury (restenosis), a process that is closely associated

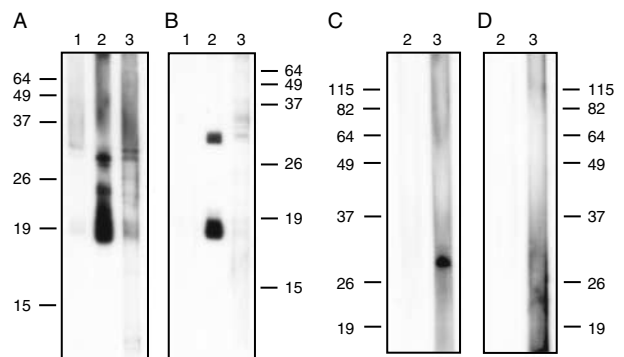


Figure 6 Antibody-dependent detection of globular adiponectin. Western blotting was used to compare various commercial antibodies for their ability to detect native and recombinant forms of adiponectin and globular adiponectin. Rat serum was tested (A) before or (B) after depletion of abundant proteins with antibodies from Lifespan Biosciences (1), R&D Systems (2), and Peprotech (3). Antibodies 2 and 3 were also tested with 50 ng of adiponectin (C) and globular adiponectin (D). Molecular mass markers were run in parallel on each gel. The results were replicated three times.

with SMC proliferation. The latter observation is supported by several studies that have linked restenosis with low circulating adiponectin levels (Kubota *et al.* 2002, Nishimura *et al.* 2006, Kitta *et al.* 2008). However, these investigations have neither tested the effect of globular adiponectin in their systems nor verified the physical characteristics of the protein. In contrast to these observations, however, Lee *et al.* (2008) reported that treatment of SMCs with adiponectin resulted in ERK1/2 activation. Furthermore, these investigators showed a similar response occurred in endothelial and HEK293 cells and that adiponectin stimulated the proliferation of HEK293 cells (Lee *et al.* 2008).

With respect to other cell types, there is no consistency regarding the effect of adiponectin on growth. For instance, adiponectin has been reported to inhibit the proliferation of hepatic stellate and breast cancer cells (Adachi & Brenner 2008, Nakayama *et al.* 2008) and promote the proliferation of epithelial, breast, and prostate cancer cells, as well as osteoblasts and hematopoietic stem cells (Luo *et al.* 2006, Ogunwobi & Beales 2006, DiMascio *et al.* 2007, Mistry *et al.* 2008, Pfeiler *et al.* 2008). Additionally, adiponectin had no effect on the proliferation of normal and transformed breast cancer cells and adenocarcinomas (Grossmann *et al.* 2008, Ogunwobi & Beales 2008). Similar results have been obtained with globular adiponectin, with i) growth of epithelial cancer cells, fibroblasts, and hematopoietic stem cells increased (Ogunwobi & Beales 2006, DiMascio *et al.* 2007, Hattori *et al.* 2007); ii) epithelial and breast cancer cells and adenocarcinomas growth inhibited (Fenton *et al.* 2008, Grossmann *et al.* 2008, Ogunwobi & Beales 2008); and iii) growth of normal breast cells and prostate cancer cells unaffected (Grossmann *et al.* 2008, Mistry *et al.* 2008). In studies that directly compared the two forms of adiponectin, both different (breast, prostate, and adenocarcinoma) and similar (epithelial and stem cells) responses have been observed.

Are these varied results due to the different oligomeric structures formed by adiponectin and globular adiponectin, or are they due to the presence of different receptors on the cells? The issue of oligomers is an important one given the fact these exist *in vivo* (Schraw *et al.* 2008) and have been shown to affect the differentiation state of SMCs (Ding *et al.* 2011). As part of this investigation, we established that full-length adiponectin formed dimers, trimers, and structures > 180 kDa (likely hexamers) according to analysis with non-denaturing gel electrophoresis (Fig. 5B). These data resemble those reported by Palanivel *et al.* (2007) who found recombinant adiponectin produced with a mammalian expression system likewise preferentially formed high and medium size oligomers. By contrast, globular adiponectin predominantly formed dimers, with only small amounts of trimers and possibly tetramers visible. The adiponectin receptors exhibit different affinities for adiponectin and globular adiponectin (Yamauchi *et al.* 2003a,b). Specifically, it has been shown that globular adiponectin binds to both AdipoR1 and AdipoR2, while adiponectin binds more favorably to AdipoR2. However, as the porcine coronary

artery SMCs employed in this investigation express both adiponectin receptors (Fig. 5E), and both adiponectin forms elicited a biological response (Figs 1, 2 and 3), the different results seen with adiponectin and globular adiponectin are most likely not a consequence of differences in the receptors.

As AMPK phosphorylation has been linked to inhibition of SMC proliferation (Nagata *et al.* 2004, Igata *et al.* 2005), activation of AMPK would not be expected if adiponectin and globular adiponectin stimulate SMC proliferation. On the other hand, AMPK phosphorylation occurred upon treatment with PDGF, a potent mitogen (Figs 2 and 3), which suggests that AMPK activation may not be strictly associated with growth inhibition. Although these data do not agree with the concept that AMPK has anti-proliferative activity (Motoshima *et al.* 2006), two recent reports have shown that AMPK promotes the proliferation of osteoblastic cells and fibroblasts (Hattori *et al.* 2006, Kanazawa *et al.* 2008). In agreement with these findings, inhibition of AMPK blocked SMC proliferation in response to both PDGF and adiponectin, which thus implies that AMPK likely has a role in SMC proliferation. Regardless, the fact that AMPK becomes phosphorylated in SMCs treated with adiponectin and globular adiponectin suggests that this protein is required for signal transduction from AdipoR1, which both proteins stimulate, whether proliferation occurs or not. It is worth noting, however, that T cadherin has also been shown to mediate activation of AMPK in response to adiponectin (Denzel *et al.* 2010), and this protein is highly abundant in SMCs (Takeuchi *et al.* 2007).

Although numerous studies have examined the effects of globular adiponectin on cells and animals, the physiological relevance of this protein remains unresolved. We were able to detect globular adiponectin in rat serum with three different antibodies, with only the monoclonal antibody providing a strong signal (Fig. 6) and only with the native protein. Fruebis *et al.* (2001) were the first to propose that proteolytic cleavage was a factor in the regulation of adiponectin's biological function. Consequently, our demonstration that globular adiponectin can be detected in serum samples (albeit this is antibody dependent) may be considered validation of this premise. Furthermore, many different studies using globular adiponectin have been published. As described earlier, there are many examples where responses to globular adiponectin were similar to those obtained with full-length adiponectin. However, there are equally as many reports showing that cells exhibit quite distinct responses to these different forms of adiponectin. This investigation has attempted to bridge the gap by comparing the response of one cell type to both forms of adiponectin. Even so, there is essentially no information about globular adiponectin *in vivo*. The limitation for studying globular adiponectin may be attributable to a lack of resources for detection and quantification of this polypeptide. Few antibodies capable of binding globular adiponectin are available, and an ELISA that is specific for globular adiponectin does not exist. Indeed, our data suggest that the

ability to bind native vs recombinant adiponectin or globular adiponectin may also be a factor. Thus, while there is still controversy regarding the presence of globular adiponectin in human serum, it is also true that very few attempts have been made to search for it. A recent report, however, provides some intriguing information regarding the possible target for globular adiponectin. Specifically, Almer *et al.* (2011) have shown that fluorescently tagged globular adiponectin accumulates in the fibrous cap region of atherosclerotic lesions. Although these results do not provide information about the production, circulating levels, or function of globular adiponectin *in vivo*, they do strongly support the existence of a relationship between this protein and atherosclerotic disease.

It is now evident that the complement C1q region of adiponectin is capable of binding to the adiponectin receptors when separated from the rest of the adiponectin molecule (Yamauchi *et al.* 2003a,b). Whether it exists as a separate biologically active 'globular adiponectin' remains to be determined. Nevertheless, proteolytic cleavage of polypeptide hormones is an accepted mechanism of regulating activity. Both Fruebis *et al.* (2001) and Waki *et al.* (2005) have shown that proteolytic cleavage of adiponectin *in vitro* with either trypsin or leukocyte elastase respectively generates a proteolytic fragment that physically and pharmacologically resembles the globular adiponectin molecule found in human serum. While Waki *et al.* (2005) argue that leukocyte elastase is the serum factor that is responsible for globular adiponectin formation *in vivo*, it remains to be seen whether this is the case. Further study will therefore be required to establish whether globular adiponectin is biologically active at the concentration found in serum and to determine the physiological relevance of adiponectin cleavage with respect to vascular disease. Nevertheless, these results establish that globular adiponectin is a naturally occurring protein and further investigation of its physiological function *in vivo* is warranted.

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.

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