Annexin A6 is highly abundant in monocytes of obese and type 2 diabetic individuals and is downregulated by adiponectin *in vitro*

Fabian Stögbauer¹, Johanna Weigert¹,
Markus Neumeier¹, Josef Wanninger¹,
Daniela Sporrer¹, Markus Weber¹,
Andreas Schäffler¹, Carlos Enrich²,
Peta Wood³, Thomas Grewal³,
Charalampos Aslanidis⁴ and Christa Buechler^{1,5}

¹Department of Internal Medicine I
Regensburg University Hospital
Regensburg, Germany

²Departament de Biologia Cel·lular
Facultat de Medicina
Universitat de Barcelona, Spain

³Faculty of Pharmacy
Department of Pharmaceutical Chemistry
University of Sydney
Sydney, Australia

⁴Institute of Clinical Chemistry and Laboratory Medicine
Regensburg University Hospital
Regensburg, Germany

⁵Corresponding author: Tel, 49-941-944-7147;
E-mail, christa.buechler@klinik.uni-regensburg.de
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Abbreviations: AMPK, AMP-activated protein kinase; PPAR α , peroxisome proliferator-activated receptor α

Abstract

Adiponectin stimulates cholesterol efflux in macrophages and low adiponectin may in part contribute to disturbed reverse cholesterol transport in type 2 diabetes. Monocytes express high levels of annexin A6 that could inhibit cholesterol efflux and it was investigated whether the atheroprotective effects of adiponectin are accompanied by changes in annexin A6 levels. Adiponectin reduces annexin A6 protein whereas mRNA levels are not affected. Adiponectin-mediated activation of peroxisome proliferator-activated receptor α (PPAR α) and AMP-activated protein kinase (AMPK) does not account for reduced annexin A6 expression. Further, fatty acids and lipopolysaccharide that are elevated in obesity do not influence annex-

in A6 protein levels. Annexin A6 in monocytes from overweight probands or type 2 diabetic patients is significantly elevated compared to monocytes of normal-weight controls. Monocytic annexin A6 positively correlates with body mass index and negatively with systemic adiponectin of the blood donors. Therefore, the current study demonstrates that adiponectin reduces annexin A6 in monocytes and thereby may enhance cholesterol efflux. In agreement with these *in vitro* finding an increase of monocytic annexin A6 in type 2 diabetes monocytes was observed.

Keywords: adiponectin; annexin A6; diabetes mellitus, Type 2; monocytes; obesity

Introduction

The initial step in reverse cholesterol transport involves the removal and transfer of excess cholesterol from peripheral cells onto cholesterol acceptors, such as HDL or apolipoprotein A-I (Cavelier *et al.*, 2006). This is followed by the transport of cholesterol-enriched HDL to the liver for biliary excretion (Cavelier *et al.*, 2006; Schmitz *et al.*, 2008). Plasma levels of HDL or apolipoprotein A-I are inversely correlated with the risk of atherosclerosis (Cavelier *et al.*, 2006). In patients with the metabolic syndrome or type 2 diabetes mellitus low systemic HDL levels contribute to the higher risk and incidence of atherosclerotic disease (Bonora, 2006; Feig *et al.*, 2008). These findings correlate with a 30% reduction in cholesterol efflux of monocyte-derived macrophages from patients with type 2 diabetes, indicating that initial steps in cholesterol export in diabetic patients are impaired (Mauldin *et al.*, 2008). The molecular events in lipid efflux from monocytes are not fully understood, but recent studies point at the ATP-binding cassette transporters ABCA1 and ABCG1 as the main surface proteins that mediate cholesterol efflux to apolipoprotein A-I or HDL, respectively (Bodzioch *et al.*, 1999; Klucken *et al.*, 2000; Cavelier *et al.*, 2006). Lack of ABCA1 function in Tangier disease correlates with an increased risk of atherosclerotic disease (Bodzioch *et al.*, 1999; Cavelier *et al.*, 2006), suggesting that aberrant expression of ABCA1 and/or ABCG1 might also play a role in

type 2 diabetes or the metabolic syndrome. Indeed, while ABCA1 expression in diabetic monocytes was similar to control cells, ABCG1 expression was significantly reduced (Zhou *et al.*, 2008; Mauldin *et al.*, 2008).

Adiponectin is an atheroprotective adipokine whose circulating levels are reduced in patients with type 2 diabetes and/or atherosclerosis (Arita *et al.*, 1999). Some of its atheroprotective features appear to affect cholesterol homeostasis in peripheral cells, as adiponectin reduces lipid accumulation and stimulates reverse cholesterol transport in macrophages (Tsubakio-Yamamoto *et al.*, 2008; Tian *et al.*, 2009). Most interestingly, adiponectin strongly induces ABCA1 expression in monocytes, indicating that higher ABCA1 levels are atheroprotective and contribute to enhance cholesterol removal from cells (Tsubakio-Yamamoto *et al.*, 2008). In support of this hypothesis, lentiviral overexpression of adiponectin in macrophages reduces lipid accumulation and enhances cholesterol efflux to apolipoprotein A-I (Tian *et al.*, 2009). In contrast,

adiponectin-deficient mice show a significant reduction in ABCA1 and ABCG1 expression which correlates with a decreased cholesterol efflux to apolipoprotein A-I and to a minor extent to HDL, respectively (Tsubakio-Yamamoto *et al.*, 2008).

We have recently identified that elevated expression levels of annexin A6, a member of a family of Ca^{2+} -dependent binding proteins, perturb the intracellular distribution of cholesterol, leading to strongly reduced cholesterol levels at the plasma membrane (Cubells *et al.*, 2007). Consequently, cholesterol efflux to cyclodextrin and physiological cholesterol acceptors like HDL is reduced in annexin A6 overexpressing cells (Cubells *et al.*, 2007). Given that significant amounts of annexin A6 are found in monocytes and macrophages (Hoyal *et al.*, 1996; Probst-Cousin *et al.*, 2004) in the current study it was analysed whether annexin A6 levels are altered in monocytes isolated of patients with type 2 diabetes compared to controls. Furthermore the influence of recombinant adiponectin on annexin A6 abundance in monocytes was investigated. We

Figure 1. Adiponectin, but not cholesterol depletion, downregulates annexin A6 expression. (A) Annexin A6 protein levels in CHO wildtype (wt) cells, in CHO cells overexpressing annexin A6 (CHOanx6), in HeLa cells, in primary monocytes and the monocytic cell lines U937 and THP-1. (B) Annexin A6 (Anx A6) and GAPDH in monocytes of 3 blood donors cultivated with or without 10 $\mu\text{g/ml}$ adiponectin (Apm) for 24 h. (C) Annexin A6 (Anx A6) and GAPDH in monocytes cultivated with 2.5, 5.0, 7.5 or 10.0 $\mu\text{g/ml}$ adiponectin for 24 h. (D) Annexin A6 and GAPDH in monocytes cultivated with 10 $\mu\text{g/ml}$ adiponectin or without adiponectin for 12 h, 18 h, 24 h or 30 h. (E) Annexin A6 in monocytes cultivated with adiponectin, the p38 MAPK inhibitor SB 203580 or both for 24 h. (F) Annexin A6 in monocytes incubated with lovastatin or β -cyclodextrin for 16 h.

identified that annexin A6 levels are significantly elevated in monocytes from overweight probands or type 2 diabetic patients and that adiponectin reduces annexin A6 protein in monocytes. The potential role of annexin A6 as a downstream target of adiponectin to mediate its antiatheroprotective effects is discussed.

Results

Most cell types express annexin A6, and similar to HeLa cells and the previously described CHOanx6 cell line (Cubells *et al.*, 2008), blood-derived macrophages, the human monocytic cell lines U937 and THP1 express significant amounts of annexin A6 (~ 3 –5 pg/cell; Figure 1A). Elevated annexin A6 levels disturb cellular cholesterol efflux (Cubells *et al.*, 2007) and the effect of the atheroprotective adipokine adiponectin (Goldstein *et al.*, 2004) on annexin A6 levels were analysed.

Immunoblot was performed with monocytes isolated of four different normal-weight donors and incubated with 10 $\mu\text{g/ml}$ adiponectin for 24 h, and annexin A6 protein levels were found to be reduced (Figure 1B). Quantification of the immunoblots of 4 independent experiments revealed that annexin A6 protein expression was reduced to $79.0 \pm 10.2\%$ by adiponectin ($P = 0.012$) whereas annexin A6 mRNA was not altered (data not shown). A significant and similar reduction of annexin A6 was detected in monocytes of three different donors treated with 5.0, 7.5 or 10.0 $\mu\text{g/ml}$ adiponectin whereas 2.5 $\mu\text{g/ml}$ had no effect (Figure 1C).

To determine the time-dependent reduction of annexin A6 levels, monocytes of 2 different donors were treated with or without 10 $\mu\text{g/ml}$ adiponectin for 12 h, 18 h, 24 h or 30 h. Whereas annexin A6 in control treated cells was not altered, downregulation of annexin A6 was observed in the adiponectin treated cells after 24 h and 30 h (Figure 1D). Adiponectin activates the p38 MAPK (Tang *et al.*, 2007; Weigert *et al.*, 2008), and it was analysed

whether downregulation of annexin A6 depends on this kinase. Annexin A6 protein expression was similar in the monocytes incubated with serum or serum and 100 nM of the p38 MAPK inhibitor SB 203580, respectively. Adiponectin reduced annexin A6 similarly in monocytes pretreated with the inhibitor and the respective controls (Figure 1E).

Adiponectin lowers cholesterylester storage in monocytic cells (Ouchi *et al.*, 2001) but reduction of cellular cholesterol by lovastatin (5 μM) or β -cyclodextrin (1 mg/ml) did not alter annexin A6 (Figure 1F). These findings are consistent with the observation that annexin A6 levels remained unchanged in cholesterol-, LDL-loaded (Grewal *et al.*, 2000; Pons *et al.*, 2001; de Diego *et al.*, 2002; Cubells *et al.*, 2007) or acetylated LDL-loaded THP1 cells (data not shown), indicating that intracellular cholesterol levels do not alter annexin A6 protein expression. Free fatty acids are elevated in obesity (Jensen, 1997), and therefore, the effect of palmitic acid and oleic acid on annexin A6 was also investigated. Increasing concentrations of 100, 200 or 300 μM of palmitic or oleic acid did not influence annexin A6 expression. (Figure 2A).

Adiponectin and fenofibrate activate PPAR α (Neumeier *et al.*, 2006) but 0.5 μM and 1 μM fenofibrate did not reduce annexin A6 levels (Figure 2B). Similarly, IL-6 (20 ng/ml) (Figure 2B) and IL-8 (100, 200 and 300 ng/ml, data not shown) that are both induced by adiponectin (Weigert *et al.*, 2008) had no effect. Metformin and adiponectin stimulate the AMP-activated protein kinase (AMPK) (Towler *et al.*, 2007) but incubation of monocytes of 3 different donors with 3 μM , 6 μM , 100 μM and 500 μM metformin did not alter annexin A6 abundance (data not shown). In addition, the peroxisome proliferator-activated receptor γ (PPAR γ) agonist pioglitazone (12.5 μM), lipopolysaccharide (1 ng/ml) (Figure 2B) and agonists of the retinoid X receptor (RXR) and liver X receptor (LXR), 9-cis-retinoic acid (10 μM) and 25-hydroxycholesterol (10 $\mu\text{g/ml}$) were tested but did not impact significantly on annexin A6 protein levels (data not shown).

Figure 2. Fenofibrate, LPS, IL-6, and free fatty acid do not alter annexin A6 levels. (A) Annexin A6 (Anx A6) in monocytes incubated with 100, 200 or 300 μM palmitic or oleic acid for 24 h. (B) Annexin A6 (Anx A6) in monocytes incubated with fenofibrate (FF), IL-6 or LPS for 24 h.

Figure 3. Annexin A6 in primary monocytes of normal-weight and overweight controls and T2D patients. (A) Annexin A6 (Anx A6) in monocytes isolated from the blood of overweight (OW_1 to OW_3) and normal weight controls (NW_1 to NW_3). (B) Annexin A6 (Anx A6) in monocytes isolated from the blood of type 2 diabetic patients (T2D_1 to T2D_3) and normal-weight controls (NW_4 to NW_6). (C) Annexin A6 (Anx A6) in monocytes isolated from the blood of type 2 diabetic patients (T2D_4 to T2D_6) and overweight controls (OW_4 to OW_6). (D) Quantification of the immunoblots partly shown in A, B and C. (E) Correlation of monocyte annexin A6 (Anx A6) levels with the body mass index (BMI) of the respective blood donors. (F) Correlation of monocyte annexin A6 (Anx A6) with serum adiponectin (Apm) of the respective blood donors.

To investigate whether the reduction of annexin A6 expression by adiponectin identified *in vitro* is also relevant *in vivo*, monocytes were isolated from the blood of probands with low systemic adiponectin. Quantification of immunoblots from monocytes of 8 type 2 diabetic patients (T2D), 7 overweight donors (OW) and 9 normal-weight controls (NW) (Figure 3A to C; Table 1) revealed that annexin A6 was 1.1 ± 0.2 in T2D monocytes, 1.0 ± 0.1 in OW cells and 0.9 ± 0.1 in NW monocytes ($P = 0.002$ compared to T2D and $P = 0.005$ compared to OW) (Figure 3D). Thus annexin A6 in

primary monocytes of overweight probands and type 2 diabetics was significantly elevated when compared to monocytes of normal-weight controls. Monocyte annexin A6 positively correlated with the BMI of the blood donors ($r = 0.809$, $P < 0.0001$, Figure 3E) and negatively with their systemic adiponectin levels ($r = -0.442$, $P = 0.035$, Figure 3F). However, correlation of monocyte annexin A6 with adiponectin was lost after adjusting for BMI indicating that annexin A6 levels are not directly suppressed by adiponectin.

Table 1. Anthropometric and metabolic characteristics of the male study group.

	T2D	OW (WHR > 1)	NW (WHR < 1)	P-value
Proband (n)	n = 8	n = 7	n = 9	
Age (years)	61.9 ± 10.1	63.8 ± 10.3	61.4 ± 8.1	
BMI (kg/m ²)	32.5 ± 10.6	27.6 ± 3.2	23.8 ± 2.5	0.026 [#] , 0.015 [§]
WHR	0.99 ± 0.09	1.04 ± 0.03	0.94 ± 0.04	0.002 ^{#,§}
Adiponectin (μg/ml)	1.6 ± 0.7	3.7 ± 2.8	4.1 ± 1.9	0.002 [§]

[#]OW versus NW, [§]T2D versus NW. BMI, body mass index; NW, normal-weight control group; OW, overweight control group; T2D; type 2 diabetes; WHR, waist to hip ratio.

Discussion

In the current study it is demonstrated that adiponectin reduces annexin A6 expression in primary human monocytes. In accordance with this *in-vitro* finding elevated annexin A6 is detected in monocytes of T2D patients with low adiponectin. Monocytic annexin A6 levels strongly and positively correlate with the BMI of the blood donors indicating that overweight is associated with elevated monocytic annexin A6. Adiponectin is reduced in obesity and a modest negative correlation of monocytic annexin A6 with systemic adiponectin was identified but this association is lost after adjusting for BMI. This finding indicates that factors that are altered in obesity lead to increased annexin A6 in circulating monocytes and adiponectin has only a minor impact on monocytic annexin A6 *in vivo*. Annexin A6 is even elevated in monocytes from healthy overweight probands although systemic adiponectin levels of these donors are comparable to those observed in the normal-weight blood donors.

Circulating IL-6 and IL-8 are increased in obesity but annexin A6 expression is not altered by IL-6 or IL-8 (Devaraj *et al.*, 2000; Schober *et al.*, 2007; Weigert *et al.*, 2008). Free fatty acids and lipopolysaccharide are also elevated in overweight individuals (Jensen, 1997; Cani *et al.*, 2008) but have no effect on annexin A6. Therefore, the mechanisms that lead to elevated annexin A6 levels in obesity have yet to be identified.

Metformin and adiponectin activate the AMPK and thereby lower cellular cholesterol (Yamauchi *et al.*, 2007; Misra, 2008). Neither metformin nor pharmaceutical reduction of cellular cholesterol reduce annexin A6 indicating that stimulation of AMPK and lowering of cellular cholesterol levels do not alter annexin A6. Annexin A6 levels also remain unchanged in cholesterol-loaded monocytic cells (Grewal *et al.*, 2000; Pons *et al.*, 2001; de Diego *et al.*, 2002; Cubells *et al.*, 2007) further excluding that cellular cholesterol content regulates annexin A6. Beside AMPK, PPAR α is also activated by adiponectin (Yamauchi *et al.*, 2007) but a PPAR α antagonist (Neumeier *et al.*, 2006) does not inhibit adiponectin mediated suppression of annexin A6 (own unpublished observation). Therefore, the adiponectin-stimulated pathways that downregulate annexin A6 levels have still to be identified in further studies.

PPAR α and PPAR γ agonists like fenofibrate and pioglitazone, respectively, upregulate ABCA1 and ABCG1 expression and thereby stimulate cholesterol efflux (Schmitz *et al.*, 2005) but cellular annexin A6 is not affected by these drugs. There-

fore, reduction of annexin A6 seems to be regulated independent of ABCA1 and ABCG1 expression.

Overexpression of annexin A6 in CHO cells disturbs cholesterol efflux (Cubells *et al.*, 2007) and elevated annexin A6 in monocytes may contribute to reduced cholesterol release that has been described in type 2 diabetic monocytes (Li *et al.*, 2008; Mauldin *et al.*, 2008). Adiponectin stimulates cholesterol release in monocytic cells and besides induction of ABC transporters (Tsubakio-Yamamoto *et al.*, 2008; Tian *et al.*, 2009) the suppression of annexin A6 may reduce foam cell formation. Therefore, the current study provides new insights into the potential mechanisms of adiponectin which may impact the development of atherosclerotic diseases.

Methods

Material

Recombinant human adiponectin was from R&D Systems (Wiesbaden-Nordenstadt, Germany), Vacutainer CPT was from Becton Dickinson (Franklin Lakes, NJ). GAPDH antibody was from New England Biolabs GmbH (Frankfurt, Germany). The annexin A6 antibody used in this study was recently described (Grewal *et al.*, 2000; de Diego *et al.*, 2002). The p38 MAPK inhibitor SB 203580, and lovastatin were from Calbiochem-Merck (Darmstadt, Germany). LightCycler FastStart DNA Master SYBR Green I was purchased from Roche (Mannheim, Germany). Metformin, AICAR, methyl- β -cyclodextrin, 9-cis-retinoic acid, 25-hydroxycholesterol, LPS (*Escherichia coli* serotype 055:B5), fenofibrate, pioglitazone, β -actin antibody, palmitic acid, and oleic acid were ordered from Sigma (Deisenhofen, Germany). Fatty acids were complexed to fatty acid-free bovine serum albumin (Roche) with a molar ratio of 1:1. Equal amounts of bovine serum albumin were added to control cells.

Subjects

The study protocol was approved by the local ethics committee and the investigation conforms with the principles outlined in the Declaration of Helsinki (1997). Each proband gave written informed consent. Monocytes were isolated from the blood of 8 male T2D patients, 7 male controls with a waist to hip ratio (WHR) above 1.0 (OW) and 9 male controls with a WHR below 1.0 (NW). Details of the study groups are given in Table 1. Monocytes isolated of healthy blood donors were used for the *in-vitro* studies.

Real-time RT-PCR

Real-time RT-PCR was performed as described elsewhere (Weigert *et al.*, 2008). Annexin A6 was amplified with AnxA6uni (5'-TGGCCTATCAGATGTGGAAC-3') and AnxA6rev (5'-CTGCGTCAGGGTTGAAGTCAT-3').

Isolation and culture of primary blood monocytes

Peripheral blood leukocytes were isolated from 30 ml of whole blood by Vacutainer CPT, and monocytes were further purified by magnetic separation with CD14 beads (Miltenyi Biotec, Bergisch Gladbach, Germany) (Stogbauer *et al.*, 2008). Serum was coagulated with Thromborel S (Roche) and CaCl_2 and was dialyzed 3-times against PBS for 2 h each. 3×10^6 monocytes were cultivated in 1 ml RPMI supplemented with 10% autologous serum for 24 h. Subsequently, the medium was replaced. Monocytes were either cultivated in RPMI supplemented with 10% autologous serum or in the identical medium supplemented with 10 $\mu\text{g/ml}$ adiponectin. Supernatants were collected 24 h later and used for ELISA.

Cell culture

The generation of CHO cells overexpressing annexin A6 (CHOanx6) is described in detail elsewhere (Grewal *et al.*, 2000). CHO cells (CHOwt) and CHOanx6 cells were grown in Ham's F12, HeLa cells in DMEM, THP-1 and U937 cells in RPMI 1640 with 10% fetal calf serum (FCS), glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 $\mu\text{g/ml}$) at 37°C and 5% CO_2 . THP1 and U937 monocytes were differentiated into macrophages with PMA (50 ng/ml) for 72 h.

SDS-PAGE and immunoblotting

3×10^6 monocytes were washed with PBS and solubilized in 50 μl RIPA buffer and 15 μg of protein was used for immunoblot as recently described (Weigert *et al.*, 2008). Quantification of the immunoblots was performed using OptiQuant software.

Statistics

Data are presented as the mean value $\pm$ standard deviation (SPSS 15.0 for Windows). Statistical differences were analyzed by two-tailed Mann-Whitney U Test or Students *t*-test for paired samples, and a value of $P < 0.05$ was regarded as statistically significant. The Pearson's correlation was calculated using the SPSS 15.0 software.

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