Annexin A1 attenuates microvascular complications through restoration of Akt signalling in a murine model of type 1 diabetes

Gareth S. D. Purvis 1 · Fausto Chiazza 2 · Jianmin Chen 1 · Rodrigo Azevedo-Loiola 1 · Lukas Martin 1 · Dennis H. M. Kusters 3,4 · Chris Reutelingsperger 5 · Nikolaos Fountoulakis 5 · Luigi Gnudi 5 · Muhammed M. Yaqoob 1 · Massimo Collino 2 · Christoph Thiemermann 1 · Egle Solito 1

Received: 11 April 2017 / Accepted: 1 September 2017 / Published online: 30 October 2017 © The Author(s) 2017. This article is an open access publication

Abstract
Aims/hypothesis Microvascular complications in the heart and kidney are strongly associated with an overall rise in inflammation. Annexin A1 (ANXA1) is an endogenous anti-inflammatory molecule that limits and resolves inflammation. In this study, we have used a bedside to bench approach to investigate: (1) ANXA1 levels in individuals with type 1 diabetes; (2) the role of endogenous ANXA1 in nephropathy and cardiomyopathy in experimental type 1 diabetes; and (3) whether treatment with human recombinant ANXA1 attenuates nephropathy and cardiomyopathy in a murine model of type 1 diabetes.

Methods ANXA1 was measured in plasma from individuals with type 1 diabetes with or without nephropathy and healthy donors. Experimental type 1 diabetes was induced in mice by injection of streptozotocin (STZ; 45 mg/kg i.v. per day for 5 consecutive days) in C57BL/6 or Anxa1−/− mice. Diabetic mice were treated with human recombinant (hr)ANXA1 (1 μg, 100 μl, 50 mmol/l HEPES; 140 mmol/l NaCl; pH 7.4, i.p.) or vehicle (100 μl, 50 mmol/l HEPES; 140 mmol/l NaCl; pH 7.4, i.p.).

Results Plasma levels of ANXA1 were elevated in individuals with type 1 diabetes with/without nephropathy compared with healthy individuals (66.0 ± 4.2/64.0 ± 4 ng/ml vs 35.9 ± 2.3 ng/ml; p < 0.05). Compared with diabetic wild-type (WT) mice, diabetic Anxa1−/− mice exhibited a worse diabetic phenotype and developed more severe cardiac (ejection fraction; 76.1 ± 1.6% vs 49.9 ± 0.9%) and renal dysfunction (proteinuria; 89.3 ± 5.0 μg/mg vs 113.3 ± 5.5 μg/mg). Mechanistically, compared with non-diabetic WT mice, the degree of the phosphorylation of mitogen-activated protein kinases (MAPKs) p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) was significantly higher in non-diabetic Anxa1−/− mice in both the heart and kidney, and was further enhanced after STZ-induced type 1 diabetes. Prophylactic treatment with hrANXA1 (weeks 1–13) attenuated both cardiac (ejection fraction; 54.0 ± 1.6% vs 72.4 ± 1.0%) and renal (proteinuria; 89.3 ± 5.0 μg/mg vs 53.1 ± 3.4 μg/mg) dysfunction associated with STZ-induced diabetes, while therapeutic administration of hrANXA1 (weeks 8–13), after significant cardiac and renal dysfunction had already developed, halted the further functional decline in cardiac and renal function seen in diabetic mice administered vehicle. In addition, administration of hrANXA1 attenuated the increase in phosphorylation of p38, JNK and ERK, and restored phosphorylation of Akt in diabetic mice.

Conclusions/interpretation Overall, these results demonstrate that ANXA1 plasma levels are elevated in individuals with
type 1 diabetes independent of a significant impairment in renal function. Furthermore, in mouse models with STZ-induced type 1 diabetes, ANXA1 protects against cardiac and renal dysfunction by returning MAPK signalling to baseline and activating pro-survival pathways (Akt). We propose ANXA1 to be a potential therapeutic option for the control of comorbidities in type 1 diabetes.

**Keywords** Akt · Annexin A1 · Diabetes · Diabetic cardiomyopathy · Diabetic nephropathy · MAPK · Microvascular complications · Type 1 diabetes

**Abbreviations**

ACR Albumin-to-creatinine ratio  
ANXA1 Annexin A1  
CRP C-reactive protein  
EF Ejection fraction  
ERK Extracellular signal-regulated kinase  
FAC Fractional area change  
FPR2 Formyl peptide receptor 2  
FS Fractional shortening  
Hr Human recombinant  
JNK c-Jun N-terminal kinase  
MAPK Mitogen-activated protein kinase  
PCT Proximal convoluted tubules  
PI3K Phosphoinositide-3 kinase  
STZ Streptozotocin  
WT Wild-type

**Introduction**

Type 1 diabetes is an autoimmune disease often diagnosed in childhood that is characterised by the loss of insulin producing beta cells, which leads to hyperglycaemia [1]. Even with strict glycaemic control using rigorous insulin management, microvascular complications including diabetic nephropathy [2] and diabetic cardiomyopathy [3] develop over time. The microvascular complications associated with diabetes are key drivers of morbidity (and ultimately mortality) rates, and therefore put a significant economic burden on healthcare providers. Most notably, there are no specific therapeutic interventions that prevent, delay or reduce the microvascular complications associated with diabetes. Diabetic nephropathy is the primary cause of death in 21% of people with type 1 diabetes [4], and cardiovascular disease, which includes diabetic cardiomyopathy, accounts for 44% of all fatalities in type 1 diabetes [5]. Both pathologies are characterised by an impairment in function (kidney [proteinuria [6]], heart [impairment in systolic contractility [7]]) caused by local inflammation [8], endothelial dysfunction [9] and loss of survival pathways, the latter of which predisposes tissues to injury.

Resolution of inflammation and modulation of survival pathways are critical to normal physiology and are often altered in disease states. Annexin A1 (ANXA1) is a 37 kDa member of the multigene annexin family with anti-inflammatory and pro-resolving activities [10]. The N-terminus of each family member varies giving each its unique functionality [11]. ANXA1 binds formyl peptide receptor 2 (FPR2/ALX), downregulating the production of proinflammatory processes but activating pro-survival pathways via protein kinase C [12]. ANXA1 levels are modulated in many disease states including cancer [13], multiple sclerosis [14], cystic fibrosis [15] and obesity/the metabolic syndrome [16]. Human recombinant ANXA1 (hrANXA1) or its N-terminal peptide (Ac2-26) have therapeutic benefits in many experimental models of disease, including rheumatoid arthritis [17], atherosclerosis [18] and nonalcoholic steatohepatitis [19].

The role of ANXA1 as a pro-resolving-like molecule in type 1 diabetes is unknown. Therefore, this study was designed to investigate: (1) the plasma levels of ANXA1 in individuals with diabetes with/without nephropathy; (2) the role of endogenous ANXA1 in an animal model of type 1 diabetes and in the subsequent development of microvascular complications (diabetic cardiomyopathy and diabetic nephropathy); (3) if prophylactic daily administration with hrANXA1 prevents diabetic cardiomyopathy and/or nephropathy; and (4) if therapeutic intervention with hrANXA1 (after microvascular complications have developed) can halt the progression of diabetic cardiomyopathy and/or nephropathy.

**Methods**

**Use of human participants: ethics statement** The study was approved by the research ethics committee and was undertaken in adherence to the Declaration of Helsinki. All participants gave written informed consent. Participants with type 1 diabetes were recruited from the diabetes outpatient clinic of Guy’s and St Thomas Hospitals (London, UK). Healthy donors were recruited from the William Harvey Research Institute (London, UK) and both covered by ethical approval. Non-fasting plasma was obtained from participants with type 1 diabetes with or without a diagnosis of diabetic nephropathy and healthy donors.

**Use of experimental animals: ethics statement** The animal protocols followed in this study were approved by the Animal Welfare Ethics Review Board (AWERB) of Queen Mary University of London in accordance with the derivatives of both the Home Office guidance on the Operation of Animals (Scientific Procedures Act 1986) published by Her Majesty’s
Stationery Office and the Guide for the Care and Use of Laboratory Animals of the National Research Council. This study was performed under licence issued by the Home Office (Procedure Project Licence; 70/8350) (see the electronic supplementary material [ESM] Methods for further details).

**Human study population** Details on the study population are provided in the ESM Methods and ESM Table 1. The population included healthy volunteers (n = 20), participants with type 1 diabetes (n = 20) and participants with type 1 diabetes and nephropathy (n = 22). Plasma ANXA1 and C-reactive protein (CRP) was measured using standard ELISA (described below). Correlation studies were carried out between plasma ANXA1, plasma CRP and BMI.

**Animal model of diabetes** Experimental diabetes was induced in 10-week-old wild-type (WT) male C57BL/6 or Anxa1−/− mice (Anxa1mil/sf on a C57BL/6 background) (Charles River Laboratories, Margate, UK). Diabetes was induced using streptozotocin (STZ; 45 mg/kg i.p. in 0.9 mol/l citrate buffer, 5 days, n = 8), while sham mice received vehicle only (10 ml/kg, 0.9 mol/l citrate buffer i.p., 5 days, n = 8). hrANXA1 was produced and purified as previously published [18]. Treatment groups received either prophylactic treatment (weeks 1–13 after STZ administration, n = 6), treatment during STZ administration (days 1–5, n = 6) or therapeutic treatment (weeks 8–13 after STZ administration, n = 6) with hrANXA1 (1 μg, 100 μl, 50 mmol/l HEPES; 140 mmol/l NaCl; pH 7.4, i.p.) from weeks 1–13 or vehicle. The study has been repeated in two independent experiments each time animals were randomly assigned a treatment group and all analysis of in vivo function was assessed in a blinded fashion.

**OGTT** Mice were fasted for 18 h prior to testing, given an oral bolus of glucose (1 g/kg in H2O p.o.) and blood glucose was measured at time 0 and then at 15 min intervals for 120 min by tail vein puncture.

**Assessment of cardiac function in vivo** Cardiac function was assessed in mice by echocardiography in vivo as previously described [20]. Briefly, at either 7 or 13 weeks after STZ or vehicle administration, anaesthesia was induced and two-dimensional B-mode and M-mode echocardiography images were recorded using a Vevo-770 imaging system (VisualSonics, Toronto, ON, Canada).

**Blood and biochemical analysis** Urea, creatinine and sodium were measured by a commercial veterinary testing laboratory (IDEXX, Wetherby, UK); serum insulin and urine albumin were measured using commercially available ELISA kits (Abcam, Cambridge, UK and Bethyl Laboratories, Montgomery, TX, USA).

**Results**

Participants with type 1 diabetes have elevated ANXA1 plasma levels Figure 1a shows plasma concentration of ANXA1 in healthy donors and in participants with diabetes with/without nephropathy. The median age and sex distribution of the three test groups was similar (ESM Table 1). Plasma levels of ANXA1 were higher in individuals with diabetes with or without a diagnosis of diabetic nephropathy compared with healthy individuals (Fig. 1a), with no observed sex difference in ANXA1 concentration (ESM Fig. 1). Participants with diabetic nephropathy have higher plasma levels of CRP (a marker of systemic inflammation) compared with those with no nephropathy (Fig. 1b). However, we found no significant positive correlation between CRP and ANXA1 or BMI and ANXA1 (ESM Fig. 2a, b), but there was a strong positive correlation between plasma CRP and BMI (ESM Fig. 2c).

ELISA for ANXA1 Concentrations of ANXA1 in human plasma, mouse serum or protein lysates from heart and kidney were measured by ELISA as previously reported [21–23] (see ESM Methods for further details).

**Histological analysis** Kidneys were fixed in 10% neutral-buffered formalin for 48 h before being dehydrated with 70% ethanol. Tissues were embedded in paraffin and 4 μm sections were cut. Tissue was either stained with periodic acid–Schiffs (PAS; Sigma, St Louis, MO, USA) or Sirius Red (Abcam) as previously described [24] (see ESM Methods for further details).

**Western blot analysis** The degree of phosphorylation of Ser473 on Akt/p-Akt (Cell Signaling Technology, Danvers, MA, USA), Thr180/Tyr182 on p38/p-p38 (New England Biolabs, Ipswich, MA, USA), Thr185/Tyr185 on c-Jun N-terminal kinase (JNK)/p-JNK (Abcam) and Thr202/Tyr204 on extracellular signal-regulated kinase (ERK1/2)/p-ERK1/2 (New England Biolabs) was measured by semi-quantitative western blot analyses in mouse heart and kidney tissue as described previously [20, 25] (see ESM Methods for further details).

**Statistical analysis** All data in the text and figures are presented as mean and SEM of n observations, where n represents the number of animals studied. All statistical analysis was calculated using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Data without repeated measurements were assessed by a one-way ANOVA followed by Bonferroni post hoc test (multiple comparison) or unpaired Student’s t test where appropriate. OGTTs were analysed using the area under the receiver operating characteristic (ROC) curve. A p value of <0.05 was considered to be statistically significant.
Diabetic Anxa1−/− mice have a more severe diabetic phenotype as a result of STZ-induced type 1 diabetes Using a reverse translational approach, from humans to rodents, we wanted to understand the biological effect of modulating endogenous ANXA1 levels in a mouse model of type 1 diabetes in both WT and Anxa1−/− mice. When compared with non-diabetic mice, diabetic mice demonstrated a significant decrease in ANXA1 levels in both the heart and kidney (Fig. 1c, d), while secreted ANXA1 levels in the serum were unchanged (Table 1). Diabetic WT and Anxa1−/− mice both had depleted serum insulin levels, resulting in elevated non-fasted blood glucose levels (Table 1). Interestingly, diabetic Anxa1−/− mice had significantly increased non-fasted blood glucose (Table 1) and a more severe impairment of OGTT compared with diabetic WT mice, suggesting Anxa1−/− mice have a more severe diabetic phenotype (Fig. 1e, f).

Diabetic Anxa1−/− mice have more severe cardiac dysfunction as a result of STZ-induced type 1 diabetes Next, we assessed the role of endogenous ANXA1 on cardiac dysfunction associated with STZ-induced diabetes using echocardiography. When compared with non-diabetic WT mice, diabetic WT mice demonstrated a significant reduction in ejection fraction (EF) (Fig. 2a and ESM Fig. 3b), fractional shortening (FS) (Fig. 2b and ESM Fig. 3b) and fractional area change (FAC) (Fig. 2c). This indicates the development of systolic cardiac dysfunction, with even further deterioration in EF, FS and FAC observed in diabetic Anxa1−/− mice (Fig. 2a–c and ESM Fig. 3d). Diabetic WT and Anxa1−/− mice both
### Table 1  
Effects of endogenous ANXA1 and administered hrANXA1 on metabolic parameters in STZ-induced diabetes

|                          | WT+veh | WT+STZ | Anxa1−/−+veh | Anxa1−/−+STZ | Sham | STZ+veh | STZ+hrANXA1 weeks 1–13 | STZ+hrANXA1 days 1–5 | STZ+hrANXA1 weeks 8–13 |
|--------------------------|--------|--------|--------------|--------------|------|---------|------------------------|----------------------|-----------------------|
| Weight (g)               | 32.52 ± 1.00 | 27.13 ± 0.51* | 31.82 ± 0.62 | 23.50 ± 0.62† | 31.83 ± 0.83‡ | 26.67 ± 0.33 | 26.67 ± 0.33 | 27.33 ± 1.09 | 25.00 ± 0.82 |
| Heart rate (bpm)         | 52.1 ± 1 | 503 ± 3 | 504 ± 8.4 | 513 ± 6 | 493 ± 11 | 507 ± 3 | 488 ± 5 | 497 ± 6 |
| Insulin (pmol/l)         | 1375 ± 256 | 493 ± 39* | 1576 ± 493 | 420 ± 100‡ | 1736 ± 39* | 576 ± 19 | 493 ± 19 | 479 ± 39 | 534 ± 39 |
| Non-fasted blood glucose (mmol/l) | 7.86 ± 0.89 | 15.30 ± 0.21* | 7.82 ± 19.33 | 19.33 ± 1.18‡ | 7.42 ± 0.65† | 18.47 ± 1.19 | 15.76 ± 1.30 | 17.22 ± 1.69 | 16.08 ± 1.91 |
| OGTT (mmol/l × min)      | 1342 ± 78 | 2638 ± 47* | 1287 ± 54 | 3273 ± 93‡ | 1404 ± 32* | 2695 ± 210 | 2502 ± 129 | 2607 ± 100 | 2404 ± 133 |
| Serum ANXA1 (ng/ml)      | 1.35 ± 0.15 | 1.96 ± 0.13 | n/a | n/a | n/a | n/a | n/a | n/a |

Data expressed as mean ± SEM; n = 6–8 per group

Body weight, heart rate, insulin level, non-fasted blood glucose, OGTT and serum ANXA1 were assessed 13 weeks after STZ induction of type 1 diabetes

Experimental groups in study one: WT + vehicle (100 μl, 0.1 mol/l citrate buffer, i.p. for 5 days), WT + STZ (45 mg/kg, 0.1 mol/l citrate buffer, i.p. for 5 days), Anxa1−/− + vehicle (100 μl, 0.1 mol/l citrate buffer, i.p. for 5 days) and Anxa1−/− + STZ (45 mg/kg, 0.1 mol/l citrate buffer, i.p. for 5 days); in study two: sham (no STZ), WT + vehicle, STZ + hrANXA1 weeks 1–13 (1 μg hrANXA1, i.p. daily weeks 1–13), STZ + hrANXA1 days 1–5 (RED1 μg hrANXA1, i.p. daily days 1–5), and STZ + hrANXA1 weeks 8–13 (1 μg hrANXA1, i.p. daily weeks 8–13)

Data analysed by a one-way ANOVA followed by a Bonferroni post hoc test

* p < 0.05 vs WT+veh, † p < 0.05 vs Anxa1−/−+veh, ‡ p < 0.05 vs STZ+veh
demonstrated a significant increase in intra-ventricular septum thickness, suggesting the presence of severe concentric hypertrophy (Fig. 2d and ESM Fig. 3a–d).

Western blot analysis of tissue from the hearts of mice demonstrated that, compared with non-diabetic WT mice, diabetic WT mice demonstrated a significant increase in the degree of phosphorylation of Thr180/Tyr182 on p38, Thr183/Tyr185 on JNK and Thr202/Tyr204 on ERK1/2 (Fig. 2e–g). Interestingly, compared with non-diabetic WT mice, non-diabetic Anxa1−/− mice demonstrated significantly more phosphorylation of Thr180/Tyr182 on p38, Thr183/Tyr185 on JNK and Thr202/Tyr204 on ERK1/2, suggesting activation at basal state, while diabetic Anxa1−/− mice demonstrated a further increase in the degree of phosphorylation of Thr180/Tyr182 on p38, Thr183/Tyr185 on JNK and Thr202/Tyr204 on ERK1/2 (Fig. 2e–g).

Diabetic Anxa1−/− mice have more severe renal dysfunction as a result of STZ-induced type 1 diabetes To investigate the role of endogenous ANXA1 on renal dysfunction associated with STZ-induced diabetes, renal function was assessed using urinary variables. When compared with non-diabetic WT mice, diabetic WT mice demonstrated a significant increase in urinary albumin-to-creatinine ratio (ACR) (Fig. 3a) and fractional excretion of sodium (Fig. 3b), indicating the presence of proteinuria and tubular dysfunction, which was further exacerbated in diabetic Anxa1−/− mice (Fig. 3a, b).

Histological analysis of renal tissue from non-diabetic WT mice and Anxa1−/− mice demonstrated no morphological alterations, including a well-preserved brush border in the S1–S2 segment of the proximal convoluted tubules (PCT) and normal glomerular structures at basal state (Fig. 3e). However, kidneys from diabetic WT mice demonstrated PCT dilation, a marked increase in the degree of vascular degeneration and a loss of brush borders in the S1–S2 segment of the PCT, demonstrating morphological features consistent with renal dysfunction, specifically tubular dysfunction (Fig. 3e). Kidneys from diabetic WT mice also exhibited an increase in glomerular size (Fig. 3c, e), suggesting glomerular hypertrophy consistent with observed renal dysfunction and proteinuria. All histological markers were further exacerbated in the kidneys from diabetic Anxa1−/− mice (Fig. 3e), specifically the observed loss of brush border in the S1–S2 segment of the PCT. Diabetic WT mice also exhibited significant increase in Sirius Red

---

**Fig. 2** Measurements of cardiac function in WT and Anxa1−/− mice: (a) EF, (b) FS and (c) FAC at week 13, and (d) intra-ventricular septum diameter (IVSd). (e–g) Densitometric analysis of bands is expressed as relative to sham absorbance of (e) phosphorylated p38 (pThr180/Tyr182) corrected to total p38 and normalised using the WT + vehicle band, (f) phosphorylated JNK (pThr183/Tyr185) corrected to total JNK and normalised using the WT + vehicle band, and (g) phosphorylated ERK1/2 (p-Thr202/-Tyr204) corrected to total ERK1/2 and normalised using the WT + vehicle band. Experimental groups: WT + vehicle (100 μl, 0.1 mol/l citrate buffer, i.p. for 5 days), WT + STZ (45 mg/kg, 0.1 mol/l citrate buffer, i.p. for 5 days), Anxa1−/− + vehicle (100 μl, 0.1 mol/l citrate buffer, i.p. for 5 days) and Anxa1−/− + STZ (45 mg/kg, 0.1 mol/l citrate buffer, i.p. for 5 days). Black bars, vehicle; white bars, STZ. In (g) solid bars, ERK1; hatched bars, ERK2. Data analysed by a one-way ANOVA followed by a Bonferroni post hoc test and expressed as mean ± SEM. n = 6–8 per group. *p < 0.05.
staining for collagen I and III in the interstitial spaces (Fig. 3d, e), indicating the development of renal fibrosis, while kidneys from diabetic Anxa1−/− mice had significantly more Sirius Red staining specifically in the interstitial spaces and around the vasculature (Fig. 3d, e).

Western blot analysis of the tissue from the kidneys of diabetic WT mice demonstrated a significant increase in the degree of phosphorylation of Thr180/Tyr182 on p38, Thr183/Tyr185 on JNK and Thr202/Tyr204 on ERK1/2 (Fig. 4). Interestingly, compared with non-diabetic WT mice, non-diabetic Anxa1−/− mice demonstrated significantly more phosphorylation of Thr180/Tyr182 on p38 and Thr183/Tyr185 on JNK at basal state (Fig. 4a, b), while diabetic Anxa1−/− mice demonstrated a further increase in the degree of phosphorylation of Thr180/Tyr182 on p38, Thr183/Tyr185 on JNK and Thr202/Tyr204 on ERK1/2 (Fig. 4).

**Prophylactic daily treatment with hrANXA1 protects against cardiac and renal dysfunction caused by STZ-induced model of type 1 diabetes** When compared with untreated diabetic mice, diabetic mice treated with daily hrANXA1 from weeks 1−13 or during days 1−5 exhibited no change in serum insulin level, non-fasted blood glucose or OGTT (Table 1), suggesting treatment with hrANXA1 did not alter the underlying diabetic phenotype. When left ventricular function was assessed using echocardiography, diabetic mice had reduced EF, FS and FAC compared with non-diabetic mice (Fig. 5a−c). Treatment with hrANXA1 from weeks 1−13 significantly attenuated the decreases in EF, FS
and FAC (Fig. 5a–c) suggesting that daily treatment with hrANXA1 (from weeks 1–13) protects against the development of cardiac dysfunction.

Similarly, when renal function was assessed, diabetic mice demonstrated significantly elevated ACR, serum urea and fractional excretion of sodium, while treatment with hrANXA1 from weeks 1–13 significantly attenuated these increases (Fig. 5d–f). These findings suggest that hrANXA1 protects against the development of renal dysfunction caused by STZ-induced diabetes. Treatment with hrANXA1 on days 1–5 had no effect on EF, FS, FAC, ACR, serum urea or fractional excretion of sodium (Fig. 5a–f).

**Therapeutic treatment (weeks 8–13) with hrANXA1 halts the progression of renal and cardiac dysfunction caused by STZ-induced type 1 diabetes** When compared with diabetic mice, diabetic mice given therapeutic treatment of hrANXA1 (weeks 8–13) exhibited no difference in insulin levels, non-fasted blood glucose and OGTT impairment (Table 1). When left ventricular function was assessed using echocardiography, diabetic mice demonstrated a significant reduction in EF, FS and FAC (Fig. 6a–c) from week 7 to week 13. Interestingly, mice given therapeutic treatment with hrANXA1 (weeks 8–13) demonstrated no decline in EF, FS and FAC (Fig. 6a–c) from week 7 to week 13, suggesting that late treatment with hrANXA1 can halt the progression of cardiac dysfunction.

Similarly, renal function was assessed at weeks 7 and 13, diabetic mice demonstrated a significant increase in ACR, serum urea and fractional excretion of sodium (Fig. 6d–f) from week 7 to week 13. Interestingly, mice given therapeutic treatment with hrANXA1 (weeks 8–13) demonstrated no significant increase in serum urea and fractional excretion of sodium (Fig. 6e, f), suggesting that late treatment with hrANXA1 may reduce progression of tubular dysfunction associated with STZ-induced diabetes. However, ACR did increase but not to the same degree as...
in mice treated with vehicle (Fig. 6d), suggesting a small but significant reduction in proteinuria.

Treatment with hrANXA1 restores normal signalling events in the heart and kidney after STZ-induced type 1 diabetes

Diabetic mice exhibited a significant increase in the degree of phosphorylation of Thr\(^{180}/\text{Tyr}^{182}\) on p38, Thr\(^{183}/\text{Tyr}^{185}\) on JNK and Thr\(^{202}/\text{Tyr}^{204}\) on ERK1/2 in both the heart and kidney (Fig. 7) compared with WT mice. Moreover, treatment with hrANXA1 from weeks 1–13 attenuated the increase in phosphorylation of Thr\(^{180}/\text{Tyr}^{182}\) on p38, Thr\(^{183}/\text{Tyr}^{185}\) on JNK and Thr\(^{202}/\text{Tyr}^{204}\) on ERK1/2 in both the heart and kidney (Fig. 7). Similarly, therapeutic treatment with hrANXA1 (weeks 8–13) attenuated the increase in phosphorylation of Thr\(^{183}/\text{Tyr}^{185}\) on JNK and Thr\(^{202}/\text{Tyr}^{204}\) on ERK1/2 in both the heart and kidney (Fig. 7d, f), and Thr\(^{180}/\text{Tyr}^{182}\) on p38 in the heart (Fig. 7a).

Diabetic mice exhibited significantly less phosphorylation of Ser\(^{473}\) on Akt in the heart and kidney (Fig. 7g, h) compared with non-diabetic mice. Such data suggest that Akt signalling is blunted and could be restored when mice were treated with hrANXA1 from weeks 1–13 in both the heart and kidney, while therapeutic treatment with hrANXA1 (weeks 8–13) also restored the phosphorylation of Ser\(^{473}\) of Akt in the heart but not the kidney.

Discussion

ANXA1 levels are elevated in individuals with type 1 diabetes. The main conceptually novel findings of this study are: (1) Anxa1\(^{−/−}\) mice challenged with STZ developed a more severe diabetic phenotype and more severe systolic cardiac and renal dysfunction compared with diabetic WT mice; (2) prophylactic treatment of diabetic mice with hrANXA1 did not prevent the development of a diabetic phenotype but attenuated the development of both cardiac and renal dysfunction; and (3) therapeutic administration of hrANXA1 (after both cardiac and renal dysfunction had developed) halted the progression of both cardiac and renal dysfunction seen in diabetic WT mice. Thus, endogenous ANXA1 prevents the development of end-organ injury in a murine model of type 1 diabetes and therapeutic hrANXA1 prevents (prophylactic administration) or halts (late therapeutic administration) the progression of systolic cardiac dysfunction and proteinuria caused by diabetes in these animals without altering the underlying diabetic phenotype.

Type 1 diabetes is characterised by elevated blood glucose levels, which drives low-grade chronic inflammation and thus contribute to tissue/organ damage. Healthy donors have low levels of ANXA1 in plasma [26]. Here we report that ANXA1 is elevated in individuals with type 1 diabetes with and without nephropathy (Fig. 1a). Additionally, we show that diabetic individuals have elevated systemic inflammation levels, which are further elevated by nephropathy. Elevated plasma CRP is strongly associated with adverse outcomes in myocardial infarction, stroke and coronary heart disease, which are common causes of death in people with type 1 diabetes. We hypothesise that the elevation in circulating ANXA1 is a compensatory mechanism to protect tissues from the deleterious effects of hyperglycaemia, independent of the presence of diabetic nephropathy and not as a consequence of a rise in systemic inflammation (ESM Fig. 1b). ANXA1 is found in many tissues, including the heart and kidney, but is also highly
expressed in macrophages and other immune cells [26]. Here we report that ANXA1 levels were lower in both the heart and kidney, suggesting that hyperglycaemia causes ANXA1 to be released from tissue stores. ANXA1 secretion is needed so it can signal to the FRP2 in both an autocrine and paracrine manner to activate tissue/organ protective pathways [12, 27].

We therefore wanted to understand the role of endogenous ANXA1 in the development of type 1 diabetes and in tissue/organ protection in the mouse. Mice challenged with STZ developed a diabetic phenotype with elevated non-fasted blood glucose levels and impairment in OGTT secondary to a very large (>75%) reduction in serum insulin levels. In individuals with type 1 diabetes, beta cell mass is irreversibly reduced by 70–80% at the time of diagnosis [28]; similar loss of beta cells is recorded in mice administered STZ [29]. We report here that the diabetic phenotype (OGTT) caused by STZ is more severe in diabetic Anxa1−/− mice, although the serum insulin levels are reduced to a similar degree (Table 1). This finding suggests that endogenous ANXA1 limits the development of hyperglycaemia in an insulin-independent manner.

Even individuals who undergo rigorous insulin management develop microvascular complications over time [30]. Here we report that diabetic Anxa1−/− mice have more severe cardiac and renal dysfunction compared with diabetic WT mice, suggesting that endogenous ANXA1 limits the development of end-organ injury/dysfunction in mice with diabetes (Figs 2, 3). As hypertrophy is an important contributor in diabetes-associated cardiac and renal dysfunction we evaluated both intra-ventricular septum diameter [31] and glomerular size; and report that both are significantly increased in diabetic mice (Figs 2, 3). Notably, both glomerular hypertrophy and interstitial fibrosis were further significantly augmented in diabetic Anxa1−/− mice when compared with diabetic WT mice. Cardiac hypertrophy is a common response to external stressors, including hyperglycaemia and oxidative stress [32]; this compensatory process evolves into a decompensated state with profound changes in contractile dysfunction and extracellular remodelling [33]. Similarly, renal hypertrophy is also a compensatory mechanism [34]; in the case of type 1 diabetes, excessive renal hyperfiltration due to activation of the renin–angiotensin pathways [35] puts stress on glomeruli and proximal tubules [35], manifesting in functional decline.

Having found that endogenous ANXA1 limits the development of microvascular complications associated with diabetes, we wanted to investigate whether pharmacological intervention with hrANXA1 can attenuate the development of diabetic...
Fig. 7 Signalling in the heart (a, c, e, g) and kidney (b, d, f, h) was assessed 13 weeks after STZ induction of type 1 diabetes mellitus. Densitometric analysis of bands is expressed as relative to sham absorbance of (a, b) p-p38 (p-Thr180/Tyr182) corrected to total p38 and normalised using the WT + vehicle band; (c, d) p-JNK (p-Thr183/Tyr185) corrected to total JNK and normalised using the sham band; (e, f) p-ERK1/2 (p-Thr202/-Tyr204) corrected to total ERK1/2 and normalised using the sham band; and (g, h) p-Akt (p-Ser473) corrected to total Akt and normalised using sham band. Experimental groups: sham (no STZ; black bars); STZ + vehicle (white bars); STZ + hrANXA1 weeks 1–13 (1 μg hrANXA1, i.p. daily weeks 1–13; light grey bars); STZ + hrANXA1 days 1–5 (1 μg hrANXA1, i.p. for 5 days; dark grey bars) and STZ + hrANXA1 weeks 8–13 (1 μg hrANXA1, i.p. weeks 8–13, grey hatched bars). In (e, f): solid bars, ERK1; hatched bars, ERK2; for the STZ + hrANXA1 weeks 8–13 condition, ERK1, horizontal hatching, ERK2, diagonal hatching. Representative images from one of three repeated experiments are shown. Data analysed by a one-way ANOVA followed by a Bonferroni post hoc test and expressed as mean ± SEM. n = 6–8 per group. *p < 0.05
cardiomyopathy and nephropathy. Previously, both full-length ANXA1 and the Ac2-26 peptide (the N-terminal functional fragment of ANXA1) have been used in vivo and both elicit biological function [17–19]. In this study, we chose to use full-length hrANXA1, as the dose of full-length ANXA1 needed to induce biological function is up to 20 times less than that of the Ac2-26 peptide [36] and 14 times less than that needed to induce changes in gene expression in terms of molarity [37].

Treatment of mice challenged with STZ with hrANXA1 (weeks 1–13) did not alter the diabetic phenotype (reduced insulin levels, elevated blood glucose and impaired OGTT), but attenuated the cardiac dysfunction and the proteinuria caused by diabetes, suggesting that hrANXA1 reduces the cardiac and renal injury caused by hyperglycaemia and excessive oxidative stress (organ protection rather than reduction of diabetes). Most notably, we also report that a late therapeutic intervention (weeks 8–13), when a diabetic phenotype and a moderate degree of cardiac and renal dysfunction had already occurred, halted the further decline in both cardiac and renal function seen in diabetic WT mice. It should be noted that administration of ANXA1 during the administration of STZ (days 1–5) was also not sufficient to reduce the diabetic phenotype or give long-term protection to organs.

What are the mechanisms by which ANXA1 elicits protection against cardiac and renal dysfunction associated with diabetes? We show here for the first time that the development of type 1 diabetes (caused by STZ) in mice is associated with (1) a reduction in endogenous ANXA1 levels in both the heart and the kidney (increased secretion) and (2) a decline in cardiac and renal dysfunction. Thus, we speculated that endogenous ANXA1 protects both the heart and the kidney (and hence reduces organ injury/dysfunction) by acting on pro-survival and anti-inflammatory pathways.

Hyperglycaemia leads to activation (phosphorylation) of mitogen-activated protein kinases (MAPKs) p38, JNK and ERK1/2, hypertrophy and fibrosis in the heart [32–38] and kidney [39, 40]. While little evidence is available to suggest strong tissue-specific activation of proinflammatory pathways in STZ-induced diabetes, pharmacological inhibition or genetic deletion of any of these three MAPKs reduces microvascular complications (diabetic nephropathy, cardiomyopathy and retinopathy) caused by type 1 diabetes in rodents [41, 42]. Indeed we found no activation of NF-κB or the inflammasome in our model of STZ-induced diabetes (ESM Fig. 4). However, we observed a significant increase in activated (phosphorylated) p38, JNK and ERK1/2 in diabetic WT mice. Interestingly, Anxa1−/− mice, even in the absence of diabetes (no STZ challenge), had constitutive activation (phosphorylation) of p38, JNK and ERK1/2 in the heart and kidney. The degree of activation of p38, JNK and ERK1/2 was further exacerbated when Anxa1−/− mice were challenged with STZ. As diabetic Anxa1−/− mice also had excessive renal hypertrophy and fibrosis, we speculate that the excessive activation of these known proinflammatory and profibrotic signalling pathways are key drivers of the excessive pathology seen in Anxa1−/− mice. This hypothesis is supported by the following two findings: (1) treatment of diabetic WT mice with hrANXA1 attenuated the activation of p38, JNK and ERK1/2 and the cardiac and renal dysfunction caused by diabetes; and (2) even when hrANXA1 was given therapeutically (weeks 8–13) this resulted in attenuation of the activation of p38, JNK and ERK1/2 as well as the organ dysfunction caused by diabetes.

In addition, we also investigated the effect of diabetes with or without hrANXA1 treatment on the degree of activation of Akt survival pathways, which is in part regulated by IRS-1 [43]. Diabetic mice demonstrated a significant decrease in the phosphorylation of Ser473 on Akt (indicating a reduction in activity of the kinase) in the heart and kidney. One effect of this inhibition is that organs are less resistant to stressor stimuli and subsequent organ injury. In contrast, treatment with hrANXA1 attenuated the decline in Akt activation caused by diabetes. Akt is a member of the phosphoinositide-3 kinase (PI3K) signal transduction pathway. Insulin signalling through IRS-1 regulates PI3K activity and PI3K can activate Akt (phosphorylation on Ser473). Activated Akt controls inflammatory and pro-survival responses [44]. Most notably, activation of the Akt survival pathway reduces organ injury in many conditions associated with inflammation including sepsis-induced organ dysfunction [20, 45], haemorrhagic shock-induced organ dysfunction [25, 46], myocardial infarction [47] and diabetes [48]. Specifically, in cardiac and renal ischaemia/reperfusion injury treatment with the Ac2-16 peptide tissue necrosis was reduced by activation of Akt [49, 50].

In conclusion, we report for the first time that ANXA1 levels are elevated in the plasma of individuals with type 1 diabetes. We have also clearly demonstrated that endogenous ANXA1 plays a key role in protecting the heart and kidney from functional decline in an animal model of type 1 diabetes. Specifically, we have shown that key mediators of the MAPK pathway (p38, JNK and ERK1/2) are constitutively activated in Anxa1−/− mice. Administration of hrANXA1 attenuated both cardiac and renal dysfunction caused by STZ induction of diabetes. While late treatment can halt both cardiac and renal dysfunction. We propose that attenuation of MAPK pathway signalling and restoration/activation of Akt survival pathways mediates these effects. Thus, we propose that treatment with hrANXA1 may represent a novel new intervention of microvascular complications caused by type 1 diabetes.

Data availability All data generated or analysed during this study are included in this published article (and its supplementary information file).

Funding We would like to thank the following funding bodies: the British Heart Foundation (Award number: FS/13/58/30648) to GP; the Ministry of Education, Brazil (Grant number: 7326/2014-09) to RAL; University of Turin (Ricerca Locale Linea B 2015 and Linea A 2016).
to MC; the William Harvey Research Foundation to CT; Bar’ts and The London Charity Centre of Diabetic Kidney Disease (programme grant: 577/2348) to MY and CT; and FISM Fondazione Italiana Sclerosi Multipla–Cod. 2014/R/21 to ES. We acknowledge support from the National Institute for Health and Research, Biomedical Research Centre award to Guy’s and St Thomas Foundation Trust in partnership with King’s College London.

**Duality of interest**  No conflict of interest declared.

**Contribution statement**  All authors made substantial contributions to conception and design, acquisition of data and interpretation of data; GP, LG, MC, CT and ES drafted the manuscript and provided important intellectual content; all authors reviewed and approved the manuscript. GP, CT and ES are the guarantors of this work.

**Open Access**  This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

**References**

1. Atkinson MA, Eisenbarth GS, Michels AW (2014) Type 1 diabetes. Lancet 383:69–82
2. Papadopoulou-Marketou N, Chrousos GP, Kanaka-Gantenbein C (2016) Diabetic nephropathy in type 1 diabetes: a review of early natural history, pathogenesis, and diagnosis. Diabetes Metab Res Rev 33:e2841
3. Bugger H, Abel ED (2014) Molecular mechanisms of diabetic cardiomyopathy. Diabetologia 57:660–671
4. The National Institute for Health and Care Excellence (2015) Type 1 diabetes in adults: diagnosis and management (Nice guideline NG17). Available from www.nice.org.uk/guidance/ng17. Accessed 16 Jan 2017
5. Martin-Timón I, Sevillano-Collantes C, Segura-Galindo A, del Cañizo-Gómez FJ (2014) Type 2 diabetes and cardiovascular disease: have all risk factors the same strength? World J Diabetes 5:444–470
6. Bhattacharjee N, Barma S, Konwar N et al (2016) Mechanistic insight of diabetic nephropathy and its pharmacotherapeutic targets: an update. Eur J Pharmacol 791:8–24
7. Niemann M, Herrmann S, Ertl G, Weidemann F (2013) Echocardiography in diabetic cardiomyopathy. Herz 38:42–47
8. Downs CA, Faulkner MS (2015) Toxie stress, inflammation and symptomatology of chronic complications in diabetes. World J Diabetes 6:554–565
9. Shi Y, Vanhoutte PM (2017) Macro- and microvascular endothelial dysfunction in diabetes. J Diabetes 9:434–449
10. Flower RJ, Blackwell GJ (1979) Anti-inflammatory steroids induce biosynthesis of a phospholipase A2 inhibitor which prevents prostaglandin generation. Nature 278:456–469
11. Rosengarth A, Gerke V, Luecke H (2001) X-ray structure of full-length annexin 1 and implications for membrane aggregation. J Mol Biol 306:489–498
12. Perretti M, D’Acquisto F (2009) Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. Nat Rev Immunol 9:62–70
13. Shen D, Nooraie F, Elshimali Y et al (2006) Decreased expression of annexin A1 is correlated with breast cancer development and progression as determined by a tissue microarray analysis. Hum Pathol 37:1583–1591
14. Cristante E, McArthur S, Mauro C et al (2013) Identification of an essential endogenous regulator of blood-brain barrier integrity, and its pathological and therapeutic implications. Proc Natl Acad Sci U S A 110:832–841
15. Bensalem N, Ventura AP, Vallée B et al (2005) Down-regulation of the anti-inflammatory protein annexin A1 in cystic fibrosis knock-out mice and patients. Mol Cell Proteomics 4:1591–1601
16. Kosieka A, Cunliffe AD, Mackenzie R et al (2013) Attenuation of plasma annexin A1 in human obesity. FASEB J 27:368–378
17. Renshaw D, Montero-Meléndez T, Dalli J et al (2010) Downstream gene activation of the receptor ALX by the agonist annexin A1. PLoS One 5:e12771
18. Kusters DHM, Chatrou ML, Willems BAG et al (2015) Pharmacological treatment with annexin A1 reduces atherosclerotic plaque burden in LDLR–/– mice on western type diet. PLoS One 10:e0130484
19. Locatelli I, Sutti S, Jindal A et al (2014) Endogenous annexin A1 is a novel protective determinant in nonalcoholic steatohepatitis in mice. Hepatology 60:531–544
20. Chen J, Kieswich JE, Chiazza F et al (2017) IsxB kinase inhibitor attenuates sepsis-induced cardiac dysfunction in CKD. J Am Soc Nephrol 28:94–105
21. Smith SF, Goulding NJ, Godolphin JL et al (1990) An assay for the assessment of lipocortin 1 levels in human lung lavage fluid. J Immunol Methods 131:119–125
22. Fang YH, Moreau EF, Getting SJ et al (2004) Modulation of inflammation and response to dexamethasone by Annexin 1 in antigen-induced arthritis. Arthritis Rheum 50:976–984
23. McArthur S, Cristante E, Paterno M et al (2010) Annexin A1: a central player in the anti-inflammatory and neuroprotective role of microglia. J Immunol 185:6317–6328
24. Johnson FL, Patel NSA, Purvis GSD et al (2017) Inhibition of IsxB kinase at 24 hours after acute kidney injury improves recovery of renal function and attenuates fibrosis. J Am Heart Assoc 6:e005092
25. Yamada N, Martin LB, Zechendorf E et al (2017) Novel synthetic, host-defense peptide protects against organ injury/dysfunction in a rat model of severe hemorrhagic shock. Ann Surg. https://doi.org/10.1097/SLA.0000000000002186
26. Gavins FNE, Hickey MJ (2012) Annexin A1 and the regulation of innate and adaptive immunity. Front Immunol 3(354):1
27. Dalli J, Consalvo AP, Ray V et al (2013) Prosolving and tissue-protective actions of annexin A1-based cleavage-resistant peptides are mediated by fomyl peptide receptor 2/ lipoxin A4 receptor. J Immunol 190:6478–6487
28. Akirav E, Kushner JA, Herold KC (2008) Beta-cell mass and type 1 diabetes: going, going, gone? Diabetes 57:2883–2888
29. Szkudelski T (2001) The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol Res 50:536–546
30. Atkinson MA (2012) The pathogenesis and natural history of type 1 diabetes. Cold Spring Harb Perspect Med 2:a007641
31. Way KJ, Ishihiki K, Suzuma K et al (2002) Expression of connective tissue growth factor is increased in injured myocardium associated with protein kinase Cβ2 activation and diabetes. Diabetes 51:2709–2718
32. Diwan A, Dom GW (2007) Decompensation of cardiac hypertrophy: cellular mechanisms and novel therapeutic targets. Physiology 22:56–64
33. Festa A, Haffner SM (2005) Inflammation and cardiovascular disease in patients with diabetes: lessons from the Diabetes Control and Complications Trial. Circulation 111:2414–2425
34. Reidy K, Kang HM, Hostetter T, Suszkat K (2014) Molecular mechanisms of diabetic kidney disease. J Clin Invest 124:2333–2340
35. Helal I, Fick-Brosnahan GM, Reed-Gitomer B, Schrier RW (2012) Glomerular hyperfiltration: definitions, mechanisms and clinical implications. Nat Rev Nephrol 8:293–300
36. Solito E, Romero IA, Marullo S et al (2000) Annexin 1 binds to U937 monocytic cells and inhibits their adhesion to microvascular endothelium: involvement of the 4 integrin. J Immunol 165: 1573–1581
37. Rodrigues-Lisoni FC, Mehemet DK, Peitl P et al (2006) In vitro and in vivo studies on CCR10 regulation by annexin A1. FEBS Lett 580:1431–1438
38. Naito Z, Takashi E, Xu G et al (2003) Different influences of hyperglycemic duration on phosphorylated extracellular signal-regulated kinase 1/2 in rat heart. Exp Mol Pathol 74:23–32
39. Komers R, Lindsley JN, Oyama TT et al (2007) Renal p38 MAP kinase activity in experimental diabetes. Lab Invest 87:548–558
40. Cheng X, Gao W, Dang Y et al (2013) Both ERK/MAPK and TGF-
41. Facio FN, Sena AA, Araújo LP et al (2011) Annexin 1 mimetic peptide protects against renal ischemia/reperfusion injury in rats. J Mol Med 89:51–63