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ARTICLE

Short-term hyperglycaemia causes non-reversible changes in arterial gene expression in a fully 'switchable' in vivo mouse model of diabetes

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Abstract
Aims/hypothesis Irreversible arterial damage due to early effects of hypo- or hyperglycaemia could account for the limited success of glucose-lowering treatments in preventing cardiovascular disease (CVD) events. We hypothesised that even brief hypo- or hyperglycaemia could adversely affect arterial gene expression and that these changes, moreover, might not be fully reversible.
Methods By controlled activation of a 'switchable' c-Myc transgene in beta cells, adult p*Ins*-c-MycER^{TAM} mice were rendered transiently hypo- and then hyperglycaemic, after which they were allowed to recover for up to 3 months. Immediate and sequential changes in aortic global gene expression from normal glycaemia through hypo- and hyperglycaemia to recovery were assessed.
Results Gene expression was compared with that of normoglycaemic transgenic and tamoxifen-treated wild-type controls. Overall, expression of 95 genes was significantly affected by moderate hypoglycaemia (glucose down to 2.5 mmol/l), whereas over 769 genes were affected by hyperglycaemia. Genes and pathways activated included several involved in atherogenic processes, such as inflammation and arterial calcification. Although expression of many genes recovered to initial pre-exposure levels when hyperglycaemia was corrected (74.9%), in one in four genes this did not occur. Quantitative reverse transcriptase PCR and immunohistochemistry verified the gene expression patterns of key molecules, as shown by global gene arrays.
Conclusions/interpretation Short-term exposure to hyperglycaemia can cause deleterious and persistent changes in arterial gene expression in vivo. Brief hypoglycaemia also adversely affects gene expression, although less substantially. Together, these results suggest that early correction of hyperglycaemia and avoidance of hypoglycaemia may both be necessary to avoid excess CVD risk in diabetes.

Keywords Aorta · Cardiovascular · Diabetes · GeneChip arrays · Hyperglycaemia · Mouse

Abbreviations

CVD	Cardiovascular disease
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor

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Introduction

Hyperglycaemia has long been the prime suspect for accelerated cardiovascular disease (CVD) in type 2 and type 1 diabetes [1–4]. Large epidemiological and clinical studies suggest that blood glucose levels predict risk of CVD [3, 5–7], in the presence and absence of other CVD risk factors as reviewed by Middelbeek and Horton [8], and even in prediabetic states [9]. Moreover, CVD risk in middle age may be similar in type 2 and type 1 diabetes patients (in whom the cardio-metabolic syndrome is usually absent) [10]. However, in randomised clinical trials, glucose-lowering treatments have had little impact on CVD risk [11, 12]. Thus questions have been raised about whether hyperglycaemia is a genuinely modifiable CVD risk factor.

The effects of high glucose on gene expression and protein levels have been described in various cell-types in vitro [13–17]. However, few studies have addressed effects of hyperglycaemia in vivo [18–20]. Despite providing much important information, these studies have largely used traditional animal models, such as NOD mice and *db*/*db* mice for type 1 and type 2 diabetes mellitus respectively [21, 22], in which onset of hyperglycaemia is unpredictable and irreversible [23–25], or associated with potentially confounding CVD risk factors, such as dyslipidaemia or drug treatments.

To overcome these limitations, we used p*Ins*-c-MycER^{TAM} transgenic mice [26, 27] to investigate molecular changes in the artery caused by hyperglycaemia in vivo. In these mice, activation of a 'switchable' c-Myc chimeric protein in pancreatic beta cells induces loss of differentiation and apoptosis, resulting in predictable onset of hyperglycaemia. Importantly, as deactivation of Myc is followed by re-differentiation and regeneration of beta cells, we were able to study recovery from hyperglycaemia without the confounding effects of glucose-lowering agents. As these mice are free of dyslipidaemia and obesity, we were also able to isolate effects of hyperglycaemia. Given current interest in the atherogenic effects of hypoglycaemia [28, 29], another interesting feature of this model is that a brief period of hypoglycaemia occurs at around 24 h after Myc activation, from which mice fully recover and which can also be examined before the subsequent development of hyperglycaemia.

We therefore first sought to define the early effects of hypoglycaemia and then of hyperglycaemia on molecular changes in the artery of p*Ins*-c-MycER^{TAM} mice in vivo. We then sought to identify which of the genes affected were reversed after short- and long-term recovery time-points once glycaemia had been corrected.

Methods

Transgenic mice

Mice (p*Ins*-c-MycER^{TAM}) were generated as previously published [26, 27]. Our experiments were performed in female mice that were 3 to 6 months of age, inbred into CBA-C57Bl/6 background (B&K International, Hull, UK) and maintained under barrier conditions. Although previous studies with p*Ins*-c-MycER^{TAM} mice have shown no significant sex-related differences in duration of glycaemia and recovery, our use of female mice only minimised variation in gene expression due to age, sex or strain.

Experimental design

c-MycER^{TAM} protein is activated in pancreatic beta cells of adult transgenic mice (p*Ins*-c-MycER^{TAM}) by daily intraperitoneal administration of tamoxifen (TAM; Sigma-Aldrich, Poole, UK) (1 mg/0.2 ml in peanut oil) [27]. Inactivation of c-MycER^{TAM} protein was achieved through withdrawal of tamoxifen. All protocols conformed to UK Home Office regulations and local Ethical Committee standards. Mice were treated with tamoxifen for up to 7 days (Myc 'ON') and then monitored during recovery for more than 4 months (140 days) (Myc 'OFF'). Experimental time-points were: c-Myc 'ON'; Day 2 (hypoglycaemia), Day 4 (recovery from hypoglycaemia), Day 7 (hyperglycaemia); c-Myc 'OFF'; Day 11 (early recovery from hyperglycaemia), Day 26 (longer term recovery from hyperglycaemia). Each time-point involved replicate experiments in at least three mice (up to 6). As a further control experiment for effects of tamoxifen, wild-type mice and matching transgenic c-MycER^{TAM} mice (n=3 per group) were given tamoxifen for up to 7 days.

Isolation of aorta

Mice were killed by cervical dislocation. The thoracic cavity was opened rapidly and blood washed off prior to arterial arch extraction and snap-freezing in liquid nitrogen for RNA extraction. The abdominal fragment of the artery was fixed in 4% (wt/vol.) paraformaldehyde for immunohistochemistry.

Serum collection

Terminal blood collected from the right ventricle was left to coagulate for 30 min at room temperature and then centrifuged for 3 min at 10,000 *g*. Supernatant fractions were collected for biochemical analysis.

Biochemical assays Blood glucose levels were monitored daily at the same time of day during free access to food using glucose monitoring strips (Accuchek; Roche Diagnostics, Burgess Hill, UK). Lipids and NEFA were measured in serum, using a lipid profile test system (Cholestech L.D.X; Cholestech, Point of Care Services, Swindon, UK) and NEFA C system (Wako, Alpha Labs, Eastleigh, UK), respectively.

GeneChip microarray analysis

Total RNA was extracted from arteries using a kit (RNEasy Plus Mini-Kit; Qiagen, Crawley, UK) as described previously [30]. For this purpose, three mice were used per time-point, each mouse being analysed individually without pooling of RNA (as in gene expression data analysis below).

Hybridisation to Affymetrix GeneChip arrays A two-cycle protocol was performed at the beginning of our gene array analysis. Double-amplified biotin-labelled cRNA (10 μg) was hybridised to Affymetrix MOE430 2.0 GeneChips (Affymetrix, High Wycombe, UK) together with pre-labelled hybridisation controls according to the Affymetrix GeneChip Expression Analysis Technical Manual. Gene expression data, pattern and functional enrichment analyses were performed as described before [31] (see Electronic supplementary material [ESM]).

Quantitative reverse transcriptase PCR

Total RNA (100–200 ng) from arterial arch samples (used in the global gene array analysis) was also used to synthesise cDNA (Applied Biosystems, Warrington, UK) prior to quantitative PCR. Amplification was performed on the Roche Light Cycler (Roche, UK), using specific oligonucleotides, as shown on ESM Table 1. For quantification purposes, *GAPDH* was used as reference gene. Results were analysed individually per mouse (n=3 mice per time-point) using the $2^{-\Delta\Delta C_t}$ method [32]. Normalised gene expression levels were compared with levels for Day 0.

Immunohistochemistry

Methods were followed as previously described for cryo-sections [27]. Antibodies were provided by either Santa Cruz Biotech (Insight Biotech, Wembley, UK) or Dako (Ely, UK). Immunostained sections were analysed using a confocal microscope (SP2; Leica Microsystems Milton Keynes, UK) with attached image-capturing software.

Results

Blood glucose levels, p*Ins*-c-MycER^{TAM}

For the purpose of our experiment, data from at least three replicate experiments per time-point are plotted in Fig. 1. On Day 0, blood glucose levels were around 5 mmol/l, with a clear decrease down to 2.5 mmol/l (and no lower) during the first 24 h of c-Myc activation (brief hypoglycaemia window). Blood glucose levels increased from Day 2, peaking at >25 mmol/l on Day 7. Blood glucose levels fell rapidly following Myc deactivation and were largely normalised at 3 weeks, by which time glucose levels were identical to those of wild-type littermates. Glucose levels remained slightly above mean pre-treatment levels, which, as previously shown, are generally slightly lower than average for wild-type mice [33]. Importantly, transgenic and wild-type mice had an essentially normal response to glucose tolerance tests on Days 26 and 57 (3 weeks after c-Myc was 'switched off') (ESM Fig. 1).

As expected, c-Myc-induced suppression of insulin expression and destruction of beta cells was mirrored by the acute onset and maintenance of hyperglycaemia [27], with similar kinetics in female and male mice (data not shown for

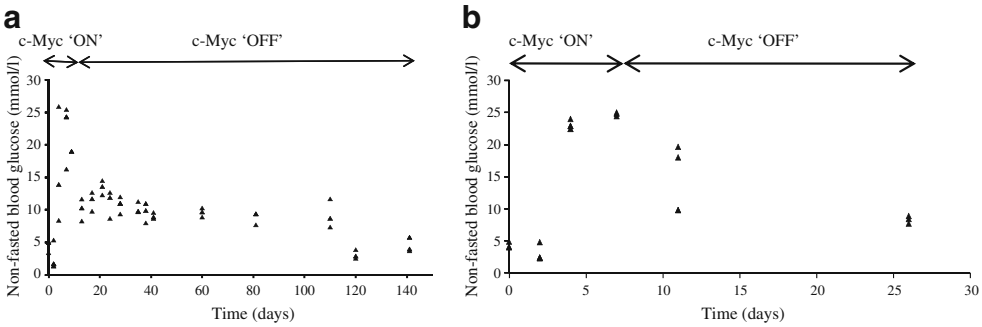

Fig. 1 Blood glucose (non-fasted). Activation of c-Myc (c-Myc 'ON') and recovery (during c-Myc 'OFF') as indicated for (**a**) up to 4 months and (**b**) shorter recovery up to 19 days. Glucose values of at least three mice are shown per time point

males). In line with this, the results obtained here were not influenced by the oestrous cycle in female mice. Wild-type mice that were treated by tamoxifen displayed blood glucose levels ranging from about 6 to 9 mmol/l (ESM Fig. 2).

Cholesterol, triacylglycerol and NEFA levels

HDL, total cholesterol, triacylglycerol and NEFA were measured in serum from Day 0 to Day 57 (n=3 per time-point) (Fig. 2). The first three remained unchanged when Day 0 was compared with Day 7, while NEFA concentrations were marginally affected at peak hyperglycaemia.

Global GeneChip arrays

Using Genespring GX 10.0 (Agilent Technologies, Wokingham, UK) we identified changes in gene expression by a magnitude of twofold on Day 7 (peak of glycaemia) and the presence/lack of ability to reverse by Day 26 (the longest recovery time-point available for GeneChip Array analysis). Gene expression profiles for total, reversed and non-reversed genes (Fig. 3a–c) were generated. Array data were submitted to NCBI's GEO (Accession No. GSE15401; www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15401, accessed 9 August 2010).

Genes regulated by tamoxifen in normoglycaemic control mice In control wild-type mice treated with tamoxifen, 12 genes were differentially expressed when compared with c-MycER^{TAM} up to Day 7 (ESM Fig. 3); for full list of genes, see ESM Table 2. Of the genes affected by tamoxifen, 20 were common to those altered by hyperglycaemia (18 genes) and hypoglycaemia (two genes) (ESM Table 3). No genes from this group appear in of the rest of our data analyses.

Gene changes caused by hypoglycaemia Changes in 95 genes were attributed to the brief hypoglycaemic phase (ESM Fig. 4; Pathway analysis).

Gene changes at the peak of hyperglycaemia (Day 7) At Day 7 the expression of 769 genes was altered by more than twofold (up- or downregulated) due to hyperglycaemia ($p \leq 0.05$) (Tables 1 and 2). Importantly, among the 769 altered genes at Day 7 vs Day 0, (Fig. 3a), the changes were reversed in 576 (74.9%) genes by Day 26 (Fig. 3b), whereas in the 67 (25.1%) other genes they were not reversed within the same time (Fig. 3c).

Enrichment analyses identified the following genes that changed substantially and closely mirrored changes in blood glucose levels: (1) exclusively upregulated on Day 7: cytoskeletal calmodulin, titin (*Ttn*), desmoplakin, thrombospondin 2 (*Thbs2*), protocadherin7 (*Pcdh7*), phosphodiesterase 1A (*Pde1a*), retinoic acid receptor responder 2 (*Rarres2*), adenylate kinase 3 (*Ak3*), integrin β5 (*Itgb5*), endothelin receptor A (*Ednra*), laminin γ1 (*Lamγ1* [also known as *Lamc1*]), osteoglycin (*Ogn*), caspase 12 (*Casp12*), angiopoietin 2 (*Angpt2*); (2) downregulated: myosin VB (*Myo5a*), myosin light chain kinase (*Mylk3*), troponin I (*TnnI* [also known as *Tnni1*]), myosin heavy polypeptide (*Myh* [also known as *Mutyh*]), keratin 13 (*Krt13*).

Gene ontology enrichment analysis using PatternViewer (GenoSyst, Turku, Finland) revealed that the above list includes genes involved in lipid metabolism (ESM Fig. 5,

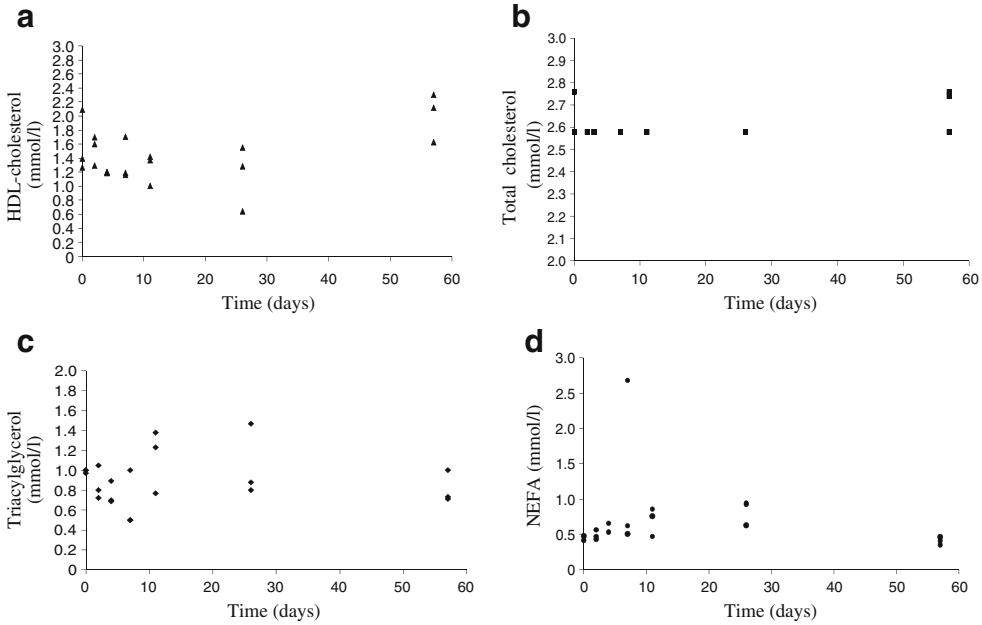

Fig. 2 Lipid profile for p*Ins*-MycER^{TAM} mice, with (**a**) HDL, (**b**) total cholesterol, (**c**) triacylglycerol and (**d**) NEFA. Results from these assays correspond to three replicates per time-point

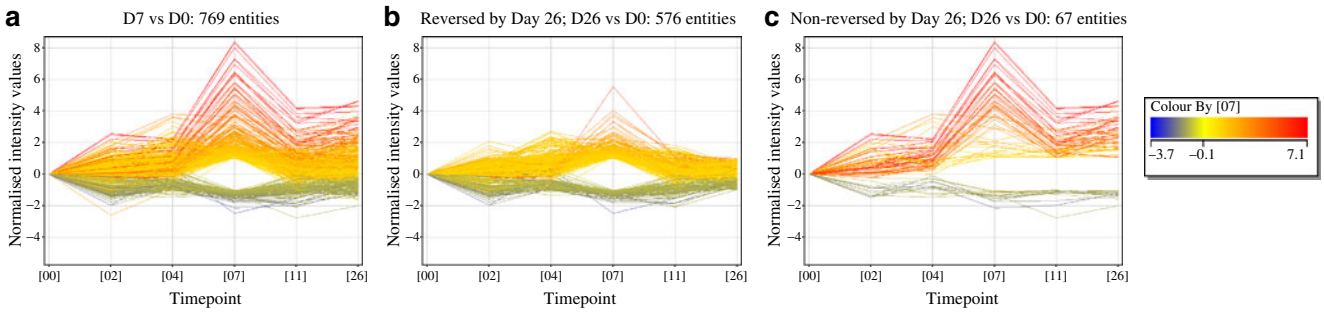

Fig. 3 Gene expression profiles from Genespring GX 10.0 (Agilent) showing expression for all altered genes. **a** Altered genes (n=769) on Day 7 vs Day 0, with 647 genes up- and 122 genes downregulated. **b** Reversed genes on Day 26 (n=576) vs Day 0. **c** Non-reversed genes on Day 26 vs Day 0 (n=67 consisting of 53 of the upregulated and 14 of the downregulated genes). Tamoxifen-affected genes were excluded. Results derived after unpaired Student's t test, $p \leq 0.05$. Intensity normalised by $\log_2$. Colour scale red to blue, highest through to lowest expression, respectively

ESM Table 4). These genes were downregulated by up to twofold from Day 2 through to Day 26.

Genes involved in arterial calcification A total of 13 genes implicated in arterial calcification and premature atherosclerosis were differentially expressed due to hyperglycaemia, but all reverted to basal levels by Day 26 (listed in Table 1 and ESM Fig. 6).

Genes that recover after restoration of normoglycaemia In addition to the calcification-related genes, examples of other genes that recovered after normalisation of blood glucose levels are shown in Tables 1 and 2 (576 genes in total). Upregulated genes on Day 7 included: (1) cell cycle-related cyclin D2 (*Ccnd2*) and insulin growth factor 1 (*Igf1*); (2) cell adhesion molecules vascular cell adhesion molecule (*Vcam1*) and matrix metalloproteinases (*Mmp3*

Table 1 Examples of genes that were altered on Day 7 and were either reversed or non-reversed by Day 26 compared with Day 0

Gene symbol by status	Gene name	Fold change	p value
Reversed			
Vcam1	Vascular cell adhesion molecule 1	2.35	1.38×10^{-3}
Mmp9	Matrix metalloproteinase 9	2.05	3.64×10^{-3}
Hsp90b1	Heat shock protein 90, beta member 1	2.63	1.16×10^{-3}
Jun	Jun oncogene	2.5	2.92×10^{-3}
Vegfc	Vascular endothelial growth factor homolog	2.41	8.06×10^{-3}
Vwf	von Willebrand factor	3.33	2.01×10^{-5}
Fabp7	Fatty acid binding protein 7, brain	−5.62	2.73×10^{-4}
Kcnq2	Potassium voltage-gated channel, subfamily Q	−2.11	3.34×10^{-4}
Calcification-related			
Bmpr2	Bone morphogenic protein receptor, type II	2.07	2.10×10^{-3}
Mmp3	Matrix metalloproteinase 3	2.78	5.10×10^{-3}
Wif1	Wnt inhibitory factor 1	2.46	1.38×10^{-2}
Ccnd2	Cyclin D2	3.89	6.97×10^{-4}
Bves	Blood vessel epicardial substance	2.2	1.74×10^{-2}
Omd	Osteomodulin	2.47	1.04×10^{-4}
Non-reversed			
Ckm	Creatine kinase, muscle	11.23	2.89×10^{-4}
Tnnt2	Troponin T2, cardiac	339.25	3.39×10^{-7}
Myoz2	Myozenin 2	24.95	1.66×10^{-6}
Fos	FBJ osteosarcoma oncogene	2.54	3.32×10^{-4}
Pln	Phospholamban	23.12	2.33×10^{-4}
Colla1	Collagen, type I, alpha 1	2.13	1.45×10^{-3}
Plg	Plasminogen	−2.64	5.09×10^{-4}
Fabp1	Fatty acid binding protein 1, liver	−2.55	3.02×10^{-5}

All genes listed were altered on Day 7; absolute fold change is shown in addition to p value for Students' t test, corresponding to significance levels for Day 7 compared with Day 0

Tamoxifen-affected genes were excluded

Table 2 Summary of changes in all entities on Day 7 compared with Day 0

Day 7	Day 11	Day 26	Definition	Entities (*n*)
Up *n*=647	Remained up	Remained up	Non-reversed	53
		Back to basal level	Reversed	34
		Reversed to lower than basal level		1
	Back to basal level	Went up		103
		Back to basal level	Early reversed	453
		Went down		2
	Reversed to lower than basal	Turned to higher than basal level		0
		Back to basal level		1
		Remained down		0
Down *n*=122	Turned to higher than basal	Remained up		0
		Back to basal level		0
		Reversed to lower than basal level		0
	Back to basal level	Went up		0
		Remained at basal level	Early reversed	53
		Went down		17
	Remained down	Turned to higher than basal level		2
		Back to basal level	Reversed	36
		Remained down	Non-reversed	14

Includes information on reversal; the pattern followed by each group of entities is also described, in addition to the numbers of entities in each group

Total entities *n*=769, reversed entities *n*=576, non-reversed entities *n*=67

$p \leq 0.05$, Student's *t* test

and *Mmp9*); and (3) atherosclerosis-related von Willebrand factor (*Vwf*), apolipoprotein D (*Apod*), *Jun*, *Myh6* and heat-shock protein *Hsp90b1*. Downregulated genes on Day 7 included: fatty acid binding protein 7 (*Fabp7*) and potassium voltage-gated channels (*Kcnh3* and *Kcnq2*). Pathway analysis showed a pivotal role in biological processes for the oncogene *Jun* in chondrogenesis, supporting an osteogenic profile, which was caused by transient hyperglycaemia. Also, *Igf1* leads to tissue growth and proliferation; other processes affected were those involving the potentially atheroprotective phospholipase *Pla2g4a*, which is responsive to hypoxia (ESM Fig. 7).

Non-reversed genes A group of genes (*n*=67) that were altered on Day 7 due to hyperglycaemia failed to return to basal expression levels by Day 26 (Tables 1 and 2). Examples of non-reversed genes by Day 26 were: angiogenin (*Ang1*), prostaglandin-associated genes *Hpdg* and *Alox15*, the apolipoprotein B (*Apobec1*) gene and cell turnover-associated proteins such as: (1) the upregulated FBJ osteosarcoma gene *Fos*, creatine kinase (*Ckm*), cardiac troponins *Tnn2* and *Tnn3*, and early growth response 3 (*Egr3*); and (2) the downregulated fatty acid binding proteins 1 and 4 (*Fabp1* and *Fabp4*), and plasminogen (*Plg*) (Tables 1 and 2). The expression of some genes was dramatically elevated at the peak of hyperglycaemia compared with others. Examples are the cardiac troponin 2 and 3 (*Tnn2*, *Tnn3*) genes (upregulated by more than 255- and 56-fold, respectively), *Ttn* (122-fold upregulation) and small muscle protein (*Smpx*) with a 43-fold increase on Day 7 (Tables 1 and 2, ESM Fig. 8).

Pathway analysis

The networks of genes that changed at the peak of hyperglycaemia included pro-inflammatory genes such as *S100*, the chemokine C-X-C motif and prostaglandin H synthase-2 (*Pghs-2* [also known as *Ptgs2*]) (ESM Figs 6–8), as well as genes related to oxidative stress such as paraoxonase 1 (*Pon1*) and apolipoprotein d (*Apod*). Many of these changes due to hyperglycaemia were related to genes involved in endothelial cell dysfunction such as *Vwf*, *Vcam1* and smooth muscle cell-related genes like *Tnn2* and myozenin 2 (*Myoz2*) (Table 1).

Examination of the non-reversed genes suggested ongoing tissue remodelling, with *Egr1* contributing to a wound-healing and protective mechanism, while *Egr3* is involved in cell turnover (apoptosis or proliferation). Furthermore, plasminogen (*Plg*) promotes angiogenesis, while *Fos* is potentially involved in tissue modification towards an osteogenic profile of the artery (ESM Fig. 8). The different patterns of gene expression changes are summarised in Table 2.

Table 3 Fold changes of the 30 entities forming the overlap between the genes that changed during hypoglycaemia and those that changed at peak glycaemia (Day 7)

Gene symbol	Day 0	Day 2	Day 4	Day 7	Day 11	Day 26
Apod	0	1.474	1.1003	2.066	−0.026	0.2805
Sncg	0	−1.6411	−1.165	−1.76	−2.197	−0.537
Cxcl13	0	3.213	4.079	3.976	2.837	2.333
Akap1	0	−1.364	−0.848	−1.614	−1.6259	−0.5702
Comp	0	1.283	0.755	1.956	0.8939	0.89338
Fabp4	0	−1.799	−0.337	−2.2898	−2.1526	−1.21554
Ifitm1	0	1.428	0.8188	1.053	0.927	0.10867
Igj	0	4.825	6.723	6.424	3.489	6.813
Itpr2	0	−1.0154	−0.613	−1.177	−0.8731	−0.50457
Acsm3	0	−1.288	−0.3705	−1.268	−0.7432	−0.42122
Xist	0	−1.8355	−0.0799	−1.026	−1.1511	−1.601
Igh-6	0	2.653	4.516	4.6602	3.895	4.22
Igh-6	0	4.9	5.6285	6.728	4.7308	6.277
Igk	0	3.0438	4.949	4.728	3.683	4.564
Igk	0	2.583	3.317	3.722	2.655	3.5134
Gabbr1	0	1.283	0.662	1.4366	0.8659	0.295
Car4	0	−1.193	−0.8413	−2.0376	−1.153	−0.5421
Ppara	0	−1.1158	−0.767	−0.9885	−0.462	−.0738
Xlr4a/b/c	0	−1.901	1.8709	−1.056	0.4277	−1.0978
Synj2	0	1.247	0.6407	1.154	1.2511	0.6854
Igk	0	2.119	2.997	3.451	2.4711	3.3166
Zdhh2	0	−1.0832	−0.678	−1.121	−1.083	−0.278
Rps24	0	−0.9397	0.2292	−1.359	−0.9426	−0.95638
Kank3	0	−1.328	−0.5902	−1.684	−1.272	−1.31083
Syn2	0	−1.779	−1.1061	−1.985	−1.854	−0.09891
Ret	0	−2.7131	−2.023	−3.3322	−3.155	−0.82286
Prex2	0	−1.394	−1.1882	−2.0271	−1.299	−1.07016
Pgm5	0	1.672	1.256	2.0187	0.5574	0.92584
9130214F15Rik	0	−1.4645	−0.93309	−1.973	−0.8855	0.074
Camk2n1	0	0.8971	0.444	0.976	0.30126	0.5199

Fold changes were normalised for $\log_2$

Day 0 to Day 2: inclusion of the transient hypoglycaemia 'window' A set of 95 genes were differentially regulated at Day 2 following a transient window of hypoglycaemia that is first observed 24 h after c-Myc activation and extends for around 24 h. These 95 genes included peroxisome proliferator-activating factor receptor γ (*Pparγ* [also known as *Pparg*], down by twofold), *Vegfc* (up by 2.8-fold), endothelial differentiation gene 3 (*Edg3*) (down by 2.3-fold), *Apoa1* (down by 3.3-fold), *Egr* (up by twofold), immunoglobin joining chain (*Igj*, upregulated by 3.5-fold), cytochrome P450 (*Cyp3a11*, downregulated by threefold), albumin (*Alb*, downregulated by 4.9-fold) and fatty acid binding protein 1 (*Fabp1*, downregulated by 4.29-fold). The 69 of these genes that had recovered by the immediate time-point (Day 4) are regarded as exclusively regulated by the hypoglycaemia 'window'. These genes were included in the 95 genes that changed between Days 0 and 2. Among the hypoglycaemia-regulated genes are the putative homeodomain transcription factor 1 (*Phtf1*, down by twofold), collagen XII a1 (*Col12a1*, down by 2.41-fold), the genes encoding solute carrier families *Slc35a5* and *Slc9a3r2* (down by two- and 2.2-fold, respectively), and the chemokine C-X-C motif (*Cxcl2*, up by tenfold).

Day 2 to Day 4: recovery from hypoglycaemia and early effects of hyperglycaemia During this period, the mice have recovered from the brief hypoglycaemia and on Day 4 have just started to become hyperglycaemic. Of the genes that were altered on Day 2, 69 (including those that specifically changed due to hypoglycaemia) had recovered by this time point, suggesting that the effects of transient hypoglycaemia are short-lived and recovered from within 48 h. As only 26 of these genes had not recovered at Day 4, we can confidently exclude any significant residual effect of hypoglycaemia on the genes affected by hyperglycaemia.

On Day 4, a different subset of genes is now being affected, most probably due to onset of hyperglycaemia.

Thus 169 genes not previously involved were deregulated at Day 4, including cell cycle- and growth-associated genes. Other genes included the upregulated apolipoprotein E (*Apoe*), collagen 1a1 (*Cola1a1*), angiogenin (*Ang*), *Pparγ* and the downregulated S100 protein, *Cxcl2*.

Day 4 to Day 7: effects of peak hyperglycaemia As the effects of hyperglycaemia become established, dramatic upregulation of genes encoding cytoskeletal proteins occurs, e.g. α-actin (by 100-fold), *Myh6*, cardiac troponin c 1 (*Tnnc1*) and i 3 (*Tnni3*), and myozenin (*Myoz*). Other genes, e.g. apolipoprotein B (*Apobec1*), muscle creatine kinase (*Ckm*), *Wif1* and prostaglandin endoperoxide synthase (*Ptgs2*) were also increased between early and peak hyperglycaemia. In total, 510 genes were altered during this phase of the experiment. Further comparative analyses also revealed a small group of 30 genes that may have been affected by hypoglycaemia and hyperglycaemia (Table 3). Examples of these are apolipoprotein D (*Apod*, upregulated), *Pparα* (also known as *Ppara*, downregulated) and fatty acid binding protein 4 (*Fabp4*, downregulated).

Day 7 to Day 11: early events immediately after initial reduction in blood glucose levels During this period blood glucose levels are declining, but have not yet normalised. Interestingly, a large proportion of genes (400) were downregulated from Day 7 to 11 (within 4 days of switching c-Myc off and during the process of reduction in blood glucose). Included in this group are *Ccnd2*, *Mmp3*, *Vegfc*, *Myh6*, *Apobec2*, *Apod* and *Ckm*. By Day 11, although the mice were still hyperglycaemic, all 'osteogenic' genes potentially involved in arterial calcification had returned to basal levels (Table 1 'Calcification genes').

Some genes remained altered on Day 11 and Day 26, when compared with Day 0. These are the non-reversed genes such as the upregulated *Egr1* and *Fos*, and the downregulated *Plg*, *Pparγ c1a* (also known as *Ppargc1a*), leptin (*Lep*) and *Fabp1*.

Day 11 to day 26: early to established recovery This is a potentially very important group of genes (n=331), as they include those that did not recover by the end of the study period. Examples are the upregulated T-box 20, prostaglandin F receptor (*Ptger3*) and heat shock protein 90 (*Hsp90ab1*), and the downregulated beta globin and interferon regulatory factor 4 (*Irf4*) (see also next section). As mentioned in the previous section, some genes already changed on Day 11 vs Day 0 continued to be altered on Day 26. More altered genes (non-reversed by Day 26) are *Pthr1* (upregulated), *Apoa1* and *Apobec1* (downregulated).

Genes differentially expressed for the first time on Day 11 (early recovery) The expression of 57 genes, not previously altered by hyperglycaemia, changed after Day 11. This group probably includes genes involved in the recovery from hyperglycaemia. Pathway analysis showed that the following processes are involved: apoptosis and cell cycle, fatty acid oxidation and gluconeogenesis (*Pparγ c1a*), glucose import (*Fabp3*) and atherosclerosis (*Isl1*) (ESM Fig. 9).

Genes differentially expressed for the first time on Day 26 (established recovery) As the mice fully recovered from hyperglycaemia, a new group of genes changed for the first time on Day 26 (n=69), such as cell cycle-related *Rbm3* and cyclin D1 (*Ccnd1*), glutamate transporter (*Slc1a3*), retinol dehydrogenase 11 (*Rdh11*), which is involved in cell growth, and *Socs6* (involved in lipid biosynthesis) (ESM Fig. 10).

Verification of gene array data

Quantitative reverse transcriptase PCR was used to verify expression patterns of a selection of genes altered by glycaemia according to the array data. Examples were genes encoding key molecules implicated in cell cycle (*Ccnd2*), diabetes/insulin resistance (*Pparγ*) and cell adhesion (*Vcam1*) (Fig. 4). All quantitative reverse transcrip-

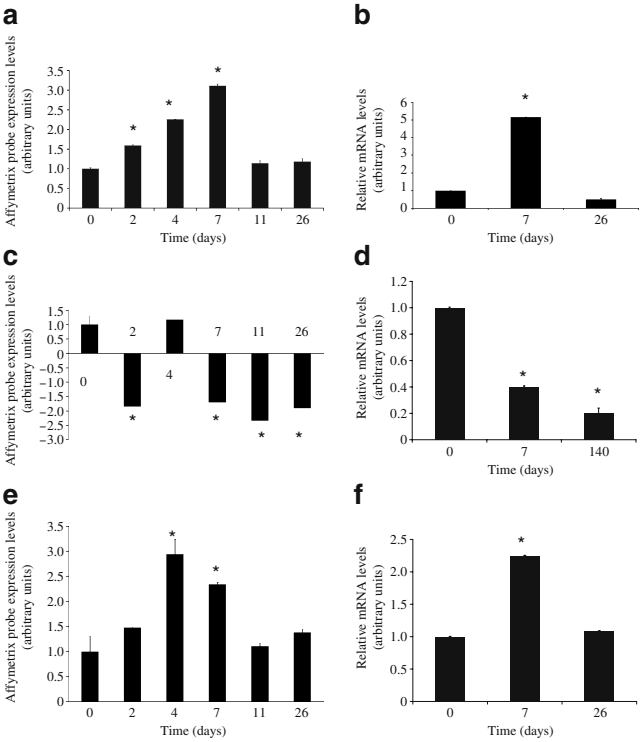

Fig. 4 Verification of GeneChip array results by quantitative real-time PCR, with gene expression profiles as Affymetrix data for (**a**) *Ccnd2*, (**c**) *Pparγ* and (**e**) *Vcam1*, and as quantitative real-time-PCR for the same genes respectively (**b, d, f**). p values for changes on Day 7 compared with Day 0 (Affymetrix data): p=2.2E-03 for *Ccnd2*, p=1.7×10^{-2} for *Pparγ* and p=1.4×10^{-3} for *Vcam1*. *p<0.05

tase PCR data confirmed the gene array results. Where necessary, later recovery time-points were analysed (Day 140).

Immunohistochemistry

We used standard fluorescence immunohistochemical techniques to study protein levels of vascular cell adhesion molecule (VCAM) 1 (cell adhesion-related), the atherosclerosis-related vascular endothelial growth factor (VEGF) and α-actin (smooth muscle cell-related). Immunofluorescent signals increased in parallel to high glucose levels (Fig. 5).

Discussion

We examined immediate and early effects of relatively brief exposure of the artery to high glucose in p*Ins*-c-MycER^{TAM} mice in vivo. Changes in arterial gene expression and to what extent these were reversible following recovery from hyperglycaemia were determined. For the first time, effects of hyperglycaemia were isolated from other potentially confounding metabolic factors and reversal studied without administration of glucose-lowering agents.

We found that 769 genes were differentially expressed by the peak of hyperglycaemia, including a very important group implicated in arterial calcification and premature atherosclerosis [34–37]. Other genes included those involved in cell cycle, cell adhesion, antioxidant processes, inflammation and angiogenesis, and those encoding molecules involved in endothelial and smooth muscle cell function. Several bone-associated proteins have been previously found in histological sections of vessels obtained from patients with diabetes or end-stage renal disease [38]. This is particularly interesting, as it was not previously thought that a calcification programme could be initiated so early after onset of a vascular insult.

We report here that although changes in expression of many of these genes reverse upon correction of hyperglycaemia, expression of one in four of these genes remains altered. In fact, non-reversing genes included those involved in cell cycle, macrophage and osteoblast differentiation, and potentially calcification, angiogenesis and wound-healing.

Our study also addressed the effects of a transient 24 h period of moderate hypoglycaemia, which occurs in the first 24 h of Myc activation, has previously been reported by us [38, 39], and from which mice recover prior to development of hyperglycaemia.

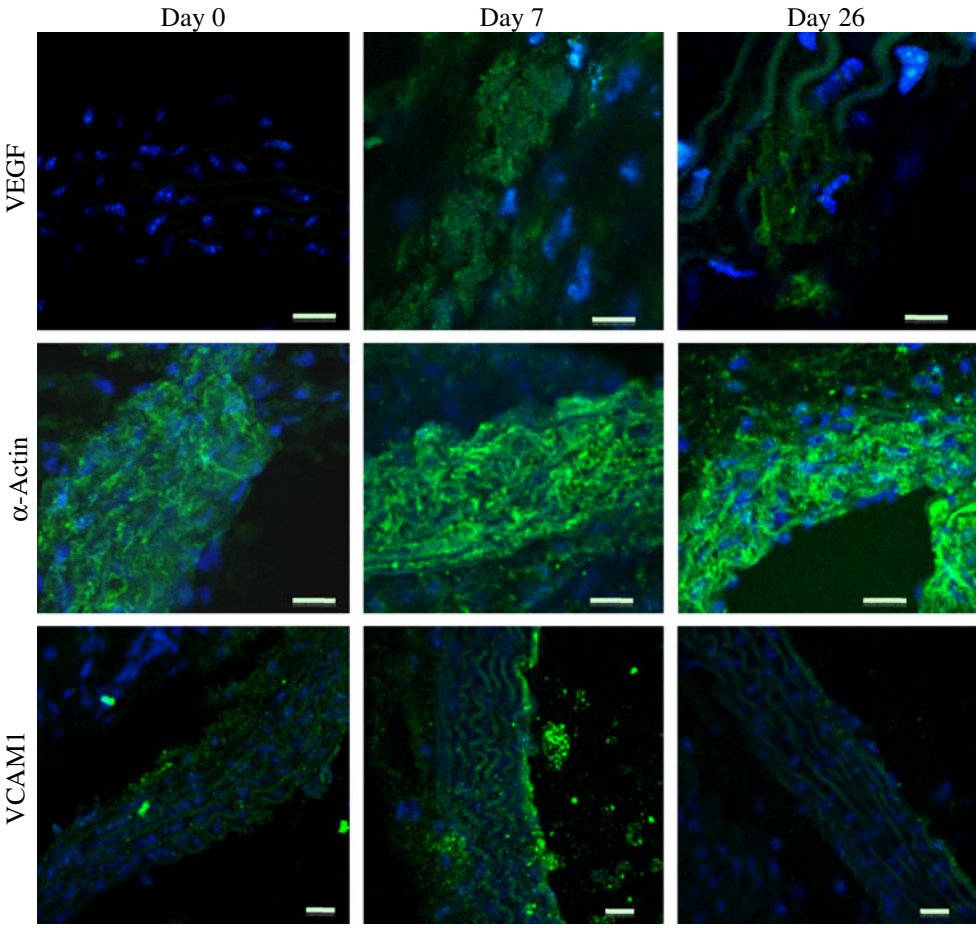

Fig. 5 Immunohistochemical analysis was performed to verify changes in protein levels of molecules shown to change at the gene level due to hyperglycaemia. These were examined on Days 0 (c-Myc 'OFF', prior to the onset of hyperglycaemia), 7 (c-Myc 'ON', hyperglycaemia) and 26 (c-Myc 'OFF', recovery). Immunofluorescence staining for VEGF, α-actin and VCAM1 is shown. Blue, DAPI; green, antibody of interest recognising mouse VEGF, α-actin and VCAM1, respectively. Scale bars 10 μm. Magnification VEGF, α-actin × 400, VCAM1 × 200

Transient hypoglycaemia significantly affects expression of 69 genes, including those involved in vascular function and differentiation, as well as in wound-healing and cholesterol metabolism, respectively [40–43]. The differential regulation of several atherosclerosis-relevant genes is very interesting in light of recent studies suggesting that symptomatic, severe hypoglycaemia is associated with an increased risk of myocardial dysfunction in the short term and in the long term may be implicated in cardiovascular dysfunction [28, 29]. It is clear that transient hypoglycaemia in our in vivo model was accompanied by changed expression of various genes involved in vascular remodelling, vascular function and lipid metabolism; however these recovered fully within 48 h of recovery from hypoglycaemia. Importantly, genes regulated by hypoglycaemia appeared to be largely separate from those affected by hyperglycaemia, apart from a small subset of genes affected by both, such as *Apod*, *Fabp4* and *Ppar*α. As the overall number of genes affected is much smaller than that identified for hyperglycaemia, it is very unlikely that long-lasting effects of recovered hypoglycaemia affected our analyses.

Intriguingly, short periods of hyperglycaemia caused permanent changes in several genes that may play supportive roles in recovery from damage induced by glucose, such as *Egr1* (involved in wound-healing) and *Slc1aj* (involved in glucose transport). However, this notion must be tempered by the persistent aberrant expression of genes that are implicated in on-going arterial damage, such as the oncogene *Fos* (which promotes an osteogenic profile in arterial cells), some which could be either beneficial or detrimental, e.g. plasminogen. It is worth noting that all of the calcification-related genes affected by hyperglycaemia had returned to normal expression in this study by Day 26.

To our knowledge, this is the first time such changes have been shown as short-term consequences of hyperglycaemia in a fully controlled in vivo animal model of diabetes. In addition, these are the first data on the uncomplicated (pure) recovery from the arterial insult of hyperglycaemia in vivo without application of any glucose-lowering agents, a crucial point given the controversy surrounding different potential actions/adverse effects of these agents themselves [12]. We can therefore use the animal model described here to begin unravelling the direct molecular effects of hyperglycaemia in vivo, as well as determining the extent to which simple reversal of the metabolic phenotype can reverse such effects.

Another point of interest is that we have shown that even after 4 months of recovery, a number of genes had still not reverted to Day 0 levels, as demonstrated by quantitative real-time PCR. This extends earlier suggestions from in vitro work with HUVECs that exposure of cells to continuous increased glucose results in a hyperglycaemic cellular memory [44]. One potential mechanism by which hyperglycaemia might cause such persistent changes has been suggested in a study showing that transient hyperglycaemia caused persistent epigenetic changes during subsequent normoglycaemia in cultured human aortic endothelial cells and after glucose infusion into non-diabetic mice [45].

In humans and most animal models, the very early stages of hyperglycaemia and diabetes have generally not been available for prospective studies. While vascular disease has previously been explored in the presence of multiple risk factors in the metabolic syndrome, we explored early changes in the artery caused solely by hyperglycaemia, the hallmark feature of diabetes, and isolated from other features of the cardiometabolic syndrome. Our findings provide a potential explanation why glucose-lowering treatments have been relatively ineffective in reducing CVD risk [11, 12], namely, that the deleterious effects of even short exposure to hyperglycaemia may be unexpectedly persistent at a molecular level.

Current guidelines for treating hyperglycaemia do not place sufficient emphasis on how quickly elevated blood glucose is corrected [46, 47]. As suggested by cell culture studies [48] and also by clinical studies, on-going arterial damage may develop undisturbed for long periods of time, leading to accelerated atherosclerosis and heart disease [49, 50]. Our results provide strong direct in vivo support for these views, while highlighting for the first time some of the immediate effects of hyperglycaemia on the artery wall in vivo without any confounding effects of glucose-lowering drugs.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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