TRPC5 ion channel permeation promotes weight gain in hypercholesterolaemic mice

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Transient Receptor Potential Canonical 5 (TRPC5) is a subunit of a Ca\(^{2+}\)-permeable non-selective cationic channel which negatively regulates adiponectin but not leptin in mice fed chow diet. Adiponectin is a major anti-inflammatory mediator and so we hypothesized an effect of TRPC5 on the inflammatory condition of atherosclerosis. Atherosclerosis was studied in aorta of ApoE\(^{-/-}\) mice fed western-style diet. Inhibition of TRPC5 ion permeation was achieved by conditional transgenic expression of a dominant negative ion pore mutant of TRPC5 (DNT5). Gene expression analysis in adipose tissue suggested that DNT5 increases transcript expression for adiponectin while decreasing transcript expression of the inflammatory mediator Tnf\(\alpha\) and potentially decreasing Il6, Il1\(\beta\) and Ccl2. Despite these differences there was mild or no reduction in plaque coverage in the aorta. Unexpectedly DNT5 caused highly significant reduction in body weight gain and reduced adipocyte size after 6 and 12 weeks of western-style diet. Steatosis and circulating lipids were unaffected but mild effects on regulators of lipogenesis could not be excluded, as indicated by small reductions in the expression of Srebp1c, Acaca, Scd1. The data suggest that TRPC5 ion channel permeation has little or no effect on atherosclerosis or steatosis but an unexpected major effect on weight gain.
Results

Expression of DNT5 was controlled by the doxycycline (DOX) inducible TET-ON system illustrated in Fig. 1A. All experiments were on ApoE<sup>−/−</sup> male mice on western-style diet from the age of 8 weeks (Fig. 1B). Expression of DNT5 in adipose tissue and liver was detected after induction with DOX (Fig. 1C,D; Supplementary File 1). For every parameter of the present study, observation of values for each genotype did not reveal obvious single-transgene effects (Supplementary File 2). Therefore non-transgenics and single-transgenics were pooled in the Control group as previously<sup>15</sup>. Data were analysed using two different statistical approaches: (1) a Mann-Whitney test comparing the two groups of mouse regardless of the influence of a cage; (2) a linear random effects model considering each cage as a single experiment to enable detection of small effects potentially aliased with cage (see Methods).
Reduced markers of inflammation in adipose tissue. In line with prior observations on adiponectin and leptin proteins\(^{15}\) there was elevated adiponectin transcript in the DNT5 group and no change in leptin (Fig. 2A). Consistent with elevated adiponectin, there was reduced abundance of transcript encoding the key inflammatory mediator including tumour necrosis factor \(\alpha\) (Tnf\(\alpha\)), interleukin 6 (Il6), interleukin 1\(\beta\) (Il1\(\beta\)) and C-C motif chemokine ligands 2, 3, 5 and 7 (Ccl2, 3, 5 and 7) mRNA abundance between Controls and DNT5. On the left, Mann-Whitney analysis showing mean and s.e.m (N = 10 Control, N = 14 DNT5). On the right, linear random effects model analysis showing the standardised mean difference and confidence intervals (n/N = 8/10 Control, n/N = 8/14 DNT5). Data above the zero line indicate increase in the DNT5 group. (B) Comparison of the inflammatory mediators tumour necrosis factor \(\alpha\) (Tnf\(\alpha\)), interleukin 6 (Il6), interleukin 1\(\beta\) (Il1\(\beta\)) and C-C motif chemokine ligands 2, 3, 5 and 7 (Ccl2, 3, 5 and 7) mRNA abundance between Controls and DNT5. On the left, Mann-Whitney analysis showing mean and s.e.m (N = 10 Control, N = 14 DNT5). On the right, linear random effects model analysis showing the standardised mean difference and confidence intervals. Data below the zero line indicate decrease in the DNT5 group (n/N = 8/10 Control, n/N = 8/14 DNT5).
Mild or no effect on atherosclerosis. With reciprocal effects of DNT5 on adiponectin and some inflammatory mediators it was anticipated that there may be suppression of atherosclerosis. We quantified plaque coverage in the aorta at the 12-week time point after staining of lipid deposits with Oil Red O (Fig. 3A). Analysis of the whole aorta including the aortic arch did not reveal significant effect of DNT5 but focussed analysis of the aortic conduit suggested that there may have been a small reduction in plaque coverage (Fig. 3A,B). Therefore DNT5 had no major effect on atherosclerosis in the aorta, aside perhaps for a mild region-specific effect.

Marked reduction in weight gain. Body weight was measured at 0-, 6- and 12-week time points (Fig. 4A). Mice in both groups appeared in similar health and gained weight. However the DNT5 group unexpectedly displayed lower body weight gain as compared to Controls at both 6- and 12-week time points (Fig. 4A,B). Histological analysis of adipocytes in fat pads revealed that adipocytes were significantly smaller in the DNT5 group (Fig. 5A,B). The data suggested that DNT5 protected against increase in body weight and adipocyte size.

No effect on steatosis and mild or no effect on lipogenesis regulators. Because of the effect on adiposity we analysed the liver to determine the relevance to steatosis (fatty liver). Histological analysis of the liver suggested no effect of DNT5 on steatosis (Fig. 6A,B). To investigate whether there might be other effects on the liver, we quantified the expression of a key determinant of lipogenesis, sterol regulatory element-binding protein 1c (Srebp1c). The expression of this gene was significantly reduced in the DNT5 group when analysed with the linear random effects model (Fig. 6C). To further explore this effect we investigated the expression of a gene downstream of Srebp1c, the gene encoding acetyl-CoA carboxylase 1 (Acaca) and the fatty acid synthase (Fasn) and the stearoyl-CoA desaturase 1 (Scd1). Expression of Acaca and Scd1 was less in the DNT5 group when using the linear random effects model and the Mann-Whitney test, respectively, consistent with down-regulated Srebp1c gene expression (Fig. 6C). Gene expression of Fasn was not significantly affected (Fig. 6C). Plasma concentrations of cholesterol, high- and low-density lipoproteins (HDL and LDL) and triglycerides were not changed (Fig. 6D). The data suggested that DNT5 had mild effects on hepatic expression of regulators of lipid metabolism but lacked effect on steatosis or plasma lipids.

Discussion
This study primarily reveals an unexpected positive effect of TRPC5 ion permeation on body weight gain and adipocyte expansion. Consistent with prior work, there was an effect on adiponectin gene expression but changes in the expression of pro-inflammatory mediators were mostly restricted to an effect on Tnf. Atherosclerosis in the aorta was largely unaffected. A mild effect on hepatic lipogenesis gene expression was apparent but there were no changes in steatosis or plasma lipids.

We analysed our data using two statistical approaches. The Mann-Whitney testing provided global analysis of the two groups of mice and was similar to the approach used in most published studies of this type. This approach allowed us to identify some effects of DNT5 but precluded the observation of effects which were small in size or
had high variability. We therefore performed additional analysis by considering each cage of mice as an individual experiment using a linear random effect model. This approach is justified by the fact that behaviour varies from one mouse cage to another, making each cage an individual entity. As expected, differences detected by the Mann-Whitney test (adiponectin and Tnfα gene expression, body weight gain and adipocyte expansion) were also detected by the linear random effect model and are likely to be strongly dependent on TRPC5 channels. Only the hepatic expression of Scd1 was significantly different with the Mann-Whitney test, suggesting this parameter is less dependent on the cage effect. The linear random effect model allowed us to pinpoint other possible effects of DNT5 (adipose tissue expression of Il6, Il1β, Ccl2, atherosclerosis in the aortic conduit, hepatic expression of Srebp1c and Acaca). The fact that these parameters show significant difference only with the linear random effect model suggests that they are less dependent on TRPC5 channels or the role of TRPC5 channels might vary from one individual to another, but could contribute to a global beneficial effect of a TRPC5 channel targeting strategy.

DNT5 was conditionally expressed and predicted to achieve effect by incorporating into native channels as they assemble from natively expressed proteins, attenuating the ion permeation in these channels. TRPC5 assembles with TRPC1 and its close relative TRPC418–20. Therefore we assume that DNT5 inhibited ion permeation through native channels which involved TRPC1 and TRPC5, the channel suggested to be functionally significant in adipocytes15. DNT5 indeed inhibited native TRPC1/TRPC5 heteromers21 and to the best of our knowledge there is no evidence for other interactions: DNT5 had no effect on two other TRP channels (TRPM2 and TRPM3) or Kv voltage-gated potassium channels which are distantly related to TRPC channels and which assemble similarly as tetramers15. Therefore DNT5 has specificity and it is likely that the natural biological rules of these channels ensure specificity.

We previously demonstrated that TRPC5 has a negative effect on adiponectin protein secretion by using DNT5, anti-TRPC5 blocking antibody and linoleic acid (a natural inhibitory of TRPC1/TRPC5 channels), in 3T3-adipocytes and adipose tissue15. Here, we provide further information by showing that the effect occurs at
Figure 6. Reduced hepatic gene expression for lipogenesis mediators in DNT5 mice. All data were from mice which had been provided with western-style diet and DOX for 12 weeks. All mice were ApoE<sup>−/−</sup>. (A) Example liver sections stained with H&E. Scale bar 100 μm. (B) Comparison of lipid content (steatosis) between Controls and DNT5. On the left, Mann-Whitney analysis showing mean and s.e.m (N = 11 Control, N = 14 DNT5). On the right, linear random effects model showing the standardised mean difference and confidence intervals. Data below the zero line indicate decrease in the DNT5 group (n/N = 8/11 Control, n/N = 8/13 DNT5). (C) All data were from quantitative real-time PCR analysis of liver RNA. Comparison of expression of sterol regulatory element-binding protein 1c gene (Srebp1c), acetyl-CoA carboxylase 1 gene (Acaca), fatty acid synthase gene (Fasn) and stearoyl-CoA desaturase 1 gene (Scd1) mRNA abundance between Controls and DNT5. On the left, Mann-Whitney analysis showing mean and s.e.m (N = 11 Control, N = 14 DNT5). On the right linear random effects model showing the standardised mean difference and confidence intervals. Data below the zero line indicate decrease in the DNT5 group (n/N = 8/11 Control, n/N = 8/13 DNT5). (D) Comparison of non-fasting
plasma concentrations of cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and triglycerides between Controls and DNT5 after 12 weeks of western-style diet and DOX administration. On the left, Mann-Whitney analysis showing mean and s.e.m (N = 11 Control, N = 14 DNT5). On the right, linear random effects model showing the standardised mean difference and confidence intervals (n/N = 8/11 Control, n/N = 8/14 DNT5).

The study encourages further investigation of the role of these ion channels in weight gain and inflammation and supports the development of new pharmacology for these channels. An important next step forward is the identification of a specific small-molecule inhibitor of the channels which could be administered effectively in vivo.
mice and other species. After initial limited progress towards such inhibitors35, highly potent and specific agents are emerging31,32,36. It will be interesting to investigate if inhibitors of this type are protective in cardiovascular and metabolic disease models and if they lack significant unwanted effects on other parameters, which Trpc5 knockout mouse studies have suggested might occur on joint inflammation37 and possibly on blood pressure38,39.

Methods

Ethical approval. All procedures were approved by the University of Leeds Ethical Review Committee and conducted under UK Home Office licence in accordance with the requirements of the Animals (Scientific Procedures) Act 1986 (UK).

Transgenic mice. Conditional expression of dominant negative ion pore mutant of TRPC5 (DNT5) in mice has been described previously15. Briefly, DNT5 cDNA cloned into the pTRE vector from Clontech was expressed dependent on doxycycline (DOX) regulation of an additional co-expressed transgene encoding reverse tetracycline DOX-regulated transactivator (rtTA) from the ROSA26 locus, designed to confer broad expression across multiple cell types. These mice were crossed with mice homozygous for disruption of ApoE (Charles River, Belgium) so that all mice were ApoE−/−. Primers used for genotyping primer are specified in Table 1. Only male mice were used for experiments. From 8-weeks of age the food for all mice was changed from chow to western-style diet for the next 12 weeks. The diet contained 21% fat from lard supplemented with 0.15% wt/wt cholesterol (#829100, SDS, Witham, Essex, UK). At the same time, all mice received 0.5 mg/mL DOX in the drinking water, sweetened with 2% sucrose. Single transgenics and non-transgenic litter-mates used as Controls also received DOX. Detailed results for each genotype are given in Supplementary File 2. As expected, DOX induced DNT5 expression only in double transgenics.

Tissue collection. Tissue was collected under terminal anaesthesia. Epididymal fat pads and liver were snap frozen on liquid nitrogen and stored at −80 °C. Mice were then perfusion-fixed with phosphate-buffered paraformaldehyde (4% wt/vol, pH 7.2). The entire aortic tree was dissected free of fat and other tissue.

RNA isolation and real-time RT-PCR. Total RNA was isolated using a standard TriReagent protocol and treated with DNase (TURBO DNA-free, AM1907M, Ambion). An aliquot was used for cDNA synthesis using a High Capacity RNA-to-cDNA kit (Applied Biosystems, UK) containing Oligo-dT and random primers. Real-time PCR was performed using Roche Fast Start SYBR Green I on a Lightcycler2 with Lightcycler 3.5 software or using Roche 480 SYBR Green I on a Lightcycler480II with Lightcycler 1.5.62 software. DNA amplification was for 35 cycles with an initial 10 min at 95 °C followed by 10 s at 95 °C, 6 s at 55 °C, and 14 s at 72 °C. Primers were used at 0.5 μmole/L. Sequences of PCR primer are specified in Table 1. The specificity of PCR was verified by reactions without RT (RT-) and by melt-curve analysis. PCR cycle crossing-points (Cp) were determined by fit-points methodology. Relative abundance of target RNA was calculated from (E18S/Cp)/(E target/Cp). PCR products were electrophoresed on 2% agarose gels containing ethidium bromide. All quantitative PCR reactions were performed in duplicate and the data averaged to generate one value per experiment. On Fig. 1D, data are presented as Relative abundance.

On Figs 2–6, data are presented after normalization to the Control group mean value.

| Gene          | Application        | Forward primer sequence (5′ to 3′)                     | Reverse primer sequence (5′ to 3′)                      | Product size (base pairs) |
|---------------|--------------------|--------------------------------------------------------|--------------------------------------------------------|---------------------------|
| LacZ          | Genotyping         | AATTGCTTCGCTGGCTGGTAAGC                                  | GGCTTATCATCAGCACTAAG                                    | 225                       |
| rtTA          | Genotyping         | TTTCGATCTCCTGCAACTAGTTGG                                 | GCACATTGGAAAACATCAGTC                                    | 184                       |
| ApoE-WT       | Genotyping         | GCCTAAGCCGAGGGAGAGAGCC                                   | TGTGACTTGGAGCTTGAGC                                    | 155                       |
| ApoE-KO       | Genotyping         | GCCTAAGCCGAGGGAGAGAGCC                                   | GCGCAGCCAGCTCAGT                                        | 245                       |
| 18S           | Real-time PCR      | GATCTGCTTGTGACTGTC                                      | GTCTGCTTGTGACTGTC                                       | 233                       |
| DNT5          | Real-time PCR      | CTTCATCAGCAGCTGACTG                                      | TTTCCCAGTCACAGGG                                       | 360                       |
| AdipoQ        | Real-time PCR      | GTATGGTCGCTGGTTC                                        | GTTGAGGTATGATTGCA                                       | 362                       |
| Lep           | Real-time PCR      | CTTTCATACACAGGGCAG                                      | GAGGTCCTGGCAGATATG                                      | 191                       |
| Tnfα          | Real-time PCR      | CACCCACAGCCGCTTGCTGCTC                                  | AGGGCTGCTGGCAGATG                                       | 103                       |
| Il6           | Real-time PCR      | TGATGCACTTGTGAGAAACA                                    | ACCAGAGAGAAATTCATGAGC                                   | 109                       |
| Il1b          | Real-time PCR      | GCAACAGCAGCTTAGCTGACAC                                   | ATCTTTGGGCTTGCTGCA                                       | 89                        |
| Cd32/Mcp1     | Real-time PCR      | TGAGTAGGGCTTGGACAGGTCAAAG                                 | TGTAGTGTGGCCAGATCCATGCC                                  | 126                       |
| Cd3/Mip1a     | Real-time PCR      | TGAACACAGCAGCTTGGTCAC                                   | AGGGCTGATGATCCCAGG                                       | 125                       |
| Cd5/Rantes    | Real-time PCR      | CTCAACCATATGGCTAGGGAGC                                   | ACAACACAGACTGAGAAGATG                                    | 126                       |
| Cd7/Mcp3      | Real-time PCR      | GCTGCTTTTCACATGCAACTG                                    | CCAAGGAGACAGACTGAC                                      | 135                       |
| Sreb1c        | Real-time PCR      | GGAGGGATGATGACACAC                                       | CACTGTTGCTTGTGCTGAGTG                                   | 58                        |
| Acaca         | Real-time PCR      | GCTGCTTTCTGAGAAGAC                                      | TGACTGGGAGAAGATCTG                                      | 239                       |
| Fabn          | Real-time PCR      | GCTGCGGAGAATTTTCCAAGGAG                                 | AGAGGGATGACAGACTGCA                                      | 83                        |
| Scd1          | Real-time PCR      | CCTTGGGAGATCTTCCATTC                                      | GTGTTCATTGAGAAGATCT                                      | 103                       |

Table 1. Sequence of primers used for genotyping and real-time PCR.
H&E staining. Epididymal fat pad and liver tissues were fixed for 48 hr in 4% PFA at 4 °C prior to processing on a Leica ASP 200 and embedding in CellWax (Cellpath) on a Leica EG1150H embedding station. Sections of 4 μm were cut on a Leica RM2235 microtome onto Plus Frost slides (Solmeda) and allowed to dry at 37 °C overnight prior to staining. Slides were de-waxed in xylene and rehydrated in ethanol. H&E was performed by staining in Mayer’s Haematoxylin for 2 min and eosin for 2 min. Slides were imaged on an Aperio AT2 (Leica Biosystems) high definition digital pathology slide scanner with a maximal magnification of 20x. Tissue processing and imaging were performed at Section of Pathology and Tumour Biology, Leeds Institute of Cancer and Pathology. Images were analysed with ImageJ software. Adipocyte size was quantified as the cross-section surface area; for each mouse, 2 microscopic fields were analysed and 100 adipocytes per field were measured. Liver steatosis was quantified as the percentage of surface area without staining; for each mouse, 4 microscopic fields were analysed.

En face analysis of aorta. Aortae were opened longitudinally, stained with Oil Red O and mounted on a coverslip before imaging (Olympus digital camera QICAM on an Olympus SZ61 dissection microscope). Lesion area was analysed using Image-Pro Plus 7.0 (Media Cybernetics, Bethesda, MD) in aortic arch (from the heart to the end of arch curvature), thoracic and abdominal aorta (from arch terminus to iliac bifurcation).

Plasma lipid analysis. Total blood was collected, without prior fasting, on sodium-citrate from inferior vena cava, centrifuged at 13000 g, 4 °C for 10 minutes; plasma was frozen at −80 °C. Analysis was performed by the Pathology RD Laboratory at Leeds Teaching Hospitals NHS Trust with a Siemens Advia 2400 device.

Data analysis. Raw data for each parameter are presented in Supplementary File 3. All analyses were performed on a blinded basis. Two statistical approaches were used for data analysis. (1) Data were first analysed for Normality and Equality of variances (Origin Pro). As most of the parameters did not satisfy these criteria, data were analysed with a Mann-Whitney test. The minimum level of statistical significance considered in the study was U < 0.05. Significance is indicated on graphs as follows: *U < 0.05, **U < 0.01 and ***U < 0.001; U > 0.05 was considered non-significant. Data are presented as the mean ± s.e.m. (2) We fitted linear random effects models using the statistics package R 40 and the nlme library 41. Each outcome measure was modelled against the mouse group (Control or DNT5) and summarised by 95% confidence intervals for the standardised mean difference in outcome measure for DNT5 compared to Control mouse groups. Depending on model convergence, model residual diagnostics and model parameter significance, outcome measures were modelled to either include or not include: (1) a natural log transformation of the outcome measure; (2) a normally distributed random intercept for the cage in which the mice were housed; (3) an identity variance-covariance structure in the normally distributed error term that allowed a separate variance inflation factor for each cage in which the mice were housed. Model output and model structure definitions for each outcome measure are included in the Supplementary File 4. Data are presented as standardised mean difference (red dot) with 95% confidence intervals (vertical black bar), otherwise mentioned. The minimum level of statistical significance considered in the study was p < 0.05. Significance is indicated on graphs as follows: *p < 0.05, **p < 0.01 and ***p < 0.001; p > 0.05 was considered non-significant. The number of independent experiments (cages) is indicated by n and the number of mice by N.

Data and Material Availability All data generated or analysed during this study are included in this published article (and its Supplementary Information files). Materials are available on reasonable request.

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Author Contributions

B.R. prepared mice, performed RNA isolation and real-time RT-PCR and analysed histological images. N.Y.Y. collected murine tissue and performed *en face* analysis of aortae. M.S. performed adipose tissue and liver embedding and staining. P.D.B. developed statistical methods and analysed the data. A.S. prepared mice and contributed to experiments. J.F.A., M.T.K., M.A.B., S.B.W., provided intellectual input. D.J.B. initiated the project, generated research funds, led and coordinated the project. B.R. and D.J.B. generated ideas, interpreted data and wrote the paper. All authors reviewed the manuscript.

Additional Information

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