Cardiac glycosides cause cytotoxicity in human macrophages and ameliorate white adipose tissue homeostasis

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Background and Purpose: Cardiac glycosides inhibit Na+/K+-ATPase and are used to treat heart failure and arrhythmias. They can induce inflammasome activation and pyroptosis in macrophages, suggesting cytotoxicity, which remains to be elucidated in human tissues.

Experimental Approach: To determine the cell-type specificity of this cytotoxicity, we used human monocyte-derived macrophages and non-adherent peripheral blood cells from healthy donors, plus omental white adipose tissue, stromal vascular fraction-derived pre-adipocytes and adipocytes from obese patients undergoing bariatric surgery. All these cells/tissues were treated with nanomolar concentrations of ouabain (50, 100, 500 nM) to investigate the level of cytotoxicity and the mechanisms leading to cell death. In white adipose tissue, we investigated ouabain-mediated cytotoxicity by measuring insulin sensitivity, adipose tissue function and extracellular matrix deposition ex vivo.

Key Results: Ouabain induced cell death through pyroptosis and apoptosis, and was more effective in monocyte-derived macrophages compared to non-adherent peripheral blood cells from healthy donors, plus omental white adipose tissue, stromal vascular fraction-derived pre-adipocytes and adipocytes from obese patients undergoing bariatric surgery. All these cells/tissues were treated with nanomolar concentrations of ouabain (50, 100, 500 nM) to investigate the level of cytotoxicity and the mechanisms leading to cell death. In white adipose tissue, we investigated ouabain-mediated cytotoxicity by measuring insulin sensitivity, adipose tissue function and extracellular matrix deposition ex vivo.

Conclusion and Implications: The use of nanomolar concentration of cardiac glycosides could be an attractive therapeutic treatment for metabolic syndrome, characterized by pathogenic infiltration and activation of macrophages.

KEYWORDS
cardiac glycosides, cell death, macrophages, obesity, ouabain, white adipose tissue
1 | INTRODUCTION

The sodium-potassium pump (Na⁺/K⁺-ATPase) is responsible for establishing Na⁺ and K⁺ concentration gradients across the plasma membrane in mammalian cells. This is the largest protein complex of the P-type family of cation pumps, which hydrolyses ATP to allow the transport of K⁺ ions inside and Na⁺ ions outside cells in a 2:3 stoichiometry (Kaplan, 2002). Cardiac glycosides are potent and highly selective inhibitors of Na⁺/K⁺-ATPase. They form a large family of naturally derived organic compounds presenting sugar and steroid moieties and have been used to treat heart conditions for more than two centuries (Prassas & Diamandis, 2008). Although toxic at a certain threshold, cardiac glycosides exert positive inotropic effects, which translate into an increase of intracellular calcium ions—essential for improving the contractile function of the heart. Calcium ions are also crucial regulators of cell death (Zhivotovsky & Orrenius, 2011), which makes cardiac glycosides cytotoxic.

Cardiac glycosides cytotoxicity causes different forms of cell death (apoptosis or necrosis) in cells other than cardiomyocytes (Xiao et al., 2002) and in particular in metastatic cells (McConkey et al., 2000). Thus, cardiac glycosides have been proposed as an alternative therapy in cancer through immunogenic cell death (Menger et al., 2012) and apoptosis of senescent cells (Guerrero et al., 2019; Triana-Martinez et al., 2019).

One particular cell type that is particularly sensitive to intracellular potassium levels is the macrophage. Low concentration of potassium activates the NLRP3 inflammasome and causes the maturation and secretion of IL-1β (Petrilli et al., 2007). The decrease in intracellular K⁺ can be induced by ATP and other damage-associated molecular patterns (Franchi et al., 2007; Munoz-Planillo et al., 2013; Petrilli et al., 2007), and K⁺ efflux is mediated through membrane channels such as P2X7 receptors (Chen & Nunez, 2010) or TWIK2 (Di et al., 2018). Activation of P2X7 receptor also induces TNF release in macrophages (Barbera-Cremades et al., 2017), suggesting that consequences of a decrease in intracellular K⁺ can be broader than NLRP3-mediated IL-1β production.

Although cardiac glycosides also decrease the intracellular K⁺, their exact role in macrophage activation and death is more controversial, presumably because of the distinct sensitivity of murine Na⁺/K⁺-ATPase to cardiac glycosides (Perne et al., 2009; Price & Lingrel, 1988) and the potential implication of Na⁺/K⁺-ATPase in signaling events independently of ion transport (Cavalcante-Silva et al., 2017). In human peripheral blood mononuclear cells, monocytes and macrophages, nanomolar concentrations of ouabain stimulate pro-inflammatory cytokine expression, including IL-1β and TNF through NF-κB (Chen et al., 2017; Foey et al., 1997; Matsumori et al., 1997; Teixeira & Rumjanek, 2014), but only recent reports linked cardiac glycoside-induced cytokine release to inflammasome-mediated cell death (i.e. pyroptosis) (Kobayashi et al., 2017; LaRock et al., 2019).

Here, we show that human monocyte-derived macrophages undergo cell death following incubation with nanomolar concentrations of cardiac glycosides and in particular with ouabain (50 nM < IC₅₀ < 100 nM). We show that non-adherent peripheral blood mononuclear cells do not show the same sensitivity to ouabain-induced cytotoxicity and the sugar moiety on the molecule is essential in driving cardiac glycoside-mediated cell death. We also report that ouabain-induced cell death is through the canonical activity of Na⁺/K⁺-ATPase. In a proof-of-principle study, we then used ouabain-mediated selective macrophage killing in a setting where these cells cause tissue damage and persistent inflammation and fibrosis. White adipose tissue explants from morbidly obese patients cultured with nanomolar concentrations of ouabain caused almost complete depletion of macrophages, decreased type VI collagen levels, while ameliorating insulin sensitivity ex vivo. These results suggest that the usage of nanomolar concentration of cardiac glycosides can be an attractive therapeutic avenue in metabolic syndrome characterized by pathogenic infiltration and activation of macrophages in the omental white adipose tissue.

2 | METHODS

2.1 | White adipose tissue explants ex vivo

Omental white adipose tissue samples were obtained from patients undergoing bariatric surgery in St Mary's Hospital, London. Informed consent was obtained from participants in accordance with ethical and Health Research Authority approval (REC 19/WM/0229). White adipose tissue samples were weighed and sectioned into 100-mg explants. They were then washed twice in pre-warmed Hank's Balanced Salt Solution (HBSS) containing 1% penicillin/streptomycin (Sigma) for 10 min each and further cultured in DMEM (Gibco) containing 10% fetal calf serum (Labtech International) and 1% penicillin/streptomycin (Sigma) with 50, 100 and 500 nM of ouabain for 48 h. For AKT, phospho-AKTser473 and cluster of...
2.2 | Primary pre-adipocyte and adipocyte culture from human white adipose tissue

Human omental white adipose tissue was digested in HBSS containing 0.5% BSA, 10-mM CaCl$_2$ and 4-mg ml$^{-1}$ collagenase type I (Sigma) in constant shaking at 37°C for 1 h. The stromal vascular fraction was isolated from the adipose fraction by centrifugation at 300g for 10 min. The stromal vascular fraction was then washed twice in HBSS and cultured with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum and 1% penicillin/streptomycin (Sigma). For stromal cell selection, the cell culture was maintained for 4 weeks and colonies were passaged once. The stromal cells (pre-adipocytes) were treated with 50 nM, 100 nM, 500 nM and 5 μM of ouabain for 24 h followed by cell viability assay. The stromal cells were also differentiated into mature adipocytes using adipocyte differentiation media (PromoCell) until 70–80% of cells became lipid-laden adipocyte-like cells (15 days). Stromal vascular fraction-derived mature adipocytes were treated with 50 nM, 100 nM, 500 nM and 5 μM of ouabain for 24 h followed by cell viability assay.

2.3 | Isolation of macrophages and non-adherent peripheral blood mononuclear cells

Human monocyte-derived macrophages were differentiated fromuffy coats from healthy donors using gradient separation (Histopaque 1077, Sigma). Following Histopaque separation, peripheral blood mononuclear cells were resuspended in RPMI 1640 (Life Technologies) and monocytes were purified by adherence for 2 h at 37°C, 5% CO$_2$. Non-adherent peripheral blood mononuclear cells were collected and washed twice with HBSS. Non-adherent peripheral blood mononuclear cells were cultured for 5 days in RPMI 1640 containing 2-mercaptoethanol (50 nM, Sigma), HEPES (10 mM), non-essential amino acids, sodium pyruvate (0.2 mM, Thermo Fisher) and 20% fetal calf serum. Flow cytometry analysis showed that cultured non-adherent peripheral blood mononuclear cells were T-cells (CD45$^+$ and CD3$^+$ positive; ~75%) and non-haematopoietic (CD45$^-$; ~25%). They were then treated with 50 nM, 100 nM, 500 nM and 5 μM of ouabain for 24 h before performing the cell viability assay.

Adherent peripheral blood mononuclear cell monolayer was washed twice with HBSS and monocytes were differentiated into human monocyte-derived macrophages for 5 days in RPMI 1640 containing 100 ng ml$^{-1}$ macrophage colony-stimulating factor (M-CSF, PeproTech) and 10% fetal calf serum. The macrophage purity was confirmed as previously described (Papathanassi et al., 2017). For the cardiac glycoside treatment, human monocyte-derived macrophages were treated with solvent only (control, 0.01% DMSO, Sigma), 50 nM, 100 nM, 500 nM, and 5 μM of ouabain octhydrate (Sigma), digoxin, bufalin, digitoxin and ouabagenin (Sigma), in full culture media for 24 h. For ouabain and potassium chloride (KCl, Sigma) treatment, human monocyte-derived macrophages were treated with KCl (25 mM) 1 h before adding ouabain (50 nM) for 24 h. For the caspase inhibition, human monocyte-derived macrophages were treated with 50 nM, 100 nM, 500 nM and 5 μM of ouabain together with Q-VD-Oph (Sigma; 10 μM) for 24 h. For the priming experiment, human monocyte-derived macrophages were treated with LPS (Escherichia coli serotype O111:B4; Cat# L4391; 250 ng ml$^{-1}$) with or without ouabain (50, 100 and 500 nM) for 24 h.

2.4 | Cell viability assays

For human monocyte-derived macrophages, non-adherent peripheral blood mononuclear cells and pre-adipocytes, cell viability was measured using annexin V and propidium iodide (PI) staining kit (BD Biosciences) according to the manufacturer's instructions. Human monocyte-derived macrophages were detached using non-enzymatic cell dissociation buffer (Sigma) and 5×10$^4$ cells were resuspended with annexin V binding buffer. Human monocyte-derived macrophages and non-adherent peripheral blood mononuclear cells were stained with annexin V (25 μg ml$^{-1}$) and PI (125 ng ml$^{-1}$) and fluorescent cells were detected using a Fortessa X20 (BD Biosciences) flow cytometer. The results were analysed using the FlowJo (v10.6, FlowJo, RRID:SCR_008520) software. Duplets were excluded by gating FSC area versus FSC height and annexin V$^-$ and PI$^-$ cells were considered as viable cells.

For white adipose tissue-derived mature adipocytes, viability was measured by adding alamarBlue (Thermo Fisher) directly into the culture (10%) for 4 h. Light absorbance was measured using Multiskan Ascent (Thermo Fisher). The percentage of reduced alamarBlue and cell viability were quantified following the manufacturer's instructions.

2.5 | Measurement of intracellular ions

Human monocyte-derived macrophages were incubated with Asante Potassium Green-2 AM (Abcam, 2 μM), CoroNa Green, AM (Invitrogen, 5 μM) and Flu-o-4 AM (Invitrogen, 2 μM) probes for 30 min before fixation with 1% PFA for 4 h. Pictures were taken using an epifluorescent Olympus BX40 microscope equipped with a digital camera Retiga 2000R. A 5×5 square grid was overlayed to each picture and two cells per square were selected as region of interest. Intracellular K$^+$, Na$^+$ and Ca$^{2+}$ were measured from a total of 150 cells per condition from three independent experiments by quantifying the mean grey values using ImageJ software (ImageJ, RRID:SCR_003070). Cells from all donors were assigned evenly across the experimental conditions. Mean grey values were normalized as relative to a control value, which was designated as “1”.

**differentiation 36 (CD36)** Western blots, white adipose tissue explants were cultured in adipocyte maintenance media (PromoCell) containing insulin. The media were replaced with fresh media containing insulin 2 h before harvesting the white adipose tissue explants.
2.6 Western blotting

White adipose tissue explants were lysed in RIPA buffer (Thermo Fisher) supplemented with protease and phosphatase inhibitor cocktails (Thermo Fisher). Total proteins were quantified by BCA protein assay (Thermo Fisher) according to the manufacturer’s instructions. A total of 50 µg protein was diluted 1:1 with 2x Laemmli buffer (Bio-Rad), resolved by SDS-PAGE, transferred into PVDF membranes (0.2 um pore size) and blocked with 5% bovine serum albumin (Sigma). These were subjected to immunoblotting with the primary antibodies in blocking buffer against phospho-Akt-Ser473 (Cell Signaling Technology Cat# 4060, RRID:AB_2315049), panAkt (Cell Signaling Technology Cat# 4691, RRID:AB_915783), CD206 (Bio-Rad Cat# MCA2155T, RRID:AB_2144910), CD36 (Thermo Fisher Scientific Cat# PA1-16813, RRID:AB_568487), ACTB (Santa Cruz Biotechnology Cat# sc-47778, RRID:AB_626632) and HRP-labelled secondary antibodies (Thermo Fisher Scientific Cat# 31464, RRID:AB_228378 and Cat# 31450, RRID:AB_228427). The probed proteins were detected using SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher). Grey densities for phospho-AKT-Ser473 were analysed with ImageJ software and normalized by those of the total AKT. Full uncropped blots are shown in Figure S3. All immuno-related procedures involved comply with the British Journal of Pharmacology editorial on immunoblotting and immunohistochemistry (Alexander et al., 2018).

2.7 Quantitative RT-PCR

Total RNA was extracted from human monocyte-derived macrophages and white adipose tissue explants using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions, and iScript cDNA Synthesis Kit (Bio-Rad) was used for cDNA synthesis. Quantitative RT-PCRs were performed on a ViiA 7 Real-Time PCR System (Life Technologies), using Brilliant II SYBR Green QPCR Master Mix (Agilent), followed by ViiA 7 RUO Software for the determination of Ct values. Target mRNA expression was normalized to HPRT reference gene. Results were analysed using a comparative Ct method and fold-change expression is shown per donor.

2.8 Immunohistochemistry and immunofluorescence

For CD68 immunostaining, 5-µm-thick paraffin white adipose tissue sections were dewaxed and rehydrated. Antigen retrieval with sodium citrate buffer (pH 6) was carried out prior to blocking. The sections were then incubated with primary antibodies against CD68 (Bio-Rad Cat# MCA1815T, RRID:AB_2074721) for 1 h. The sections were washed in PBS, followed by the incubation with HRP-labelled secondary antibody (1:10,000) for 1 h at room temperature (DAKO EnVision™ System; Agilent Technologies, UK). Diaminobenzidine (DAB) chromogen was then added and the slides were visualized using an Olympus BX40 microscope (Olympus, UK) equipped with a digital camera Retiga 2000R CCD (QImaging, Canada).

For immunofluorescence, 5-µm-thick sections were dewaxed and rehydrated. Antigen retrieval with sodium citrate buffer (pH 6) was carried out prior to blocking. Slides were then incubated overnight with Goat Anti-Type VI Collagen (Southern Biotech Cat# 1360-01, RRID:AB_2721908). After washing, slides were incubated 1 h with Donkey Anti-Goat Alexa Fluor 488 (ab150129; Abcam Cat# ab150129, RRID:AB_2687506). Slides were mounted using VECTASHIELD medium (Vector Labs). Images were taken using epifluorescent Leica DM4B microscope and the mean grey values were acquired using ImageJ software.

2.9 Adipocyte mean areas

White adipose tissue explant adipocyte mean areas were measured on Sirius red-stained sections, using ImageJ Adiposoft plug-in (Galarraga et al., 2012). The scale was set using the scale bar and the minimum and maximum areas were set to 20 and 10,000 µm², respectively. Pictures taken from each condition (4x magnification) were uploaded in an automatic directory processing mode and the adipocyte mean areas were calculated from a total of 8954 adipocyte areas across all the conditions.

2.10 ELISA

Detection of human IL-1β and TNF-α (Invitrogen) in human monocyte-derived macrophages culture supernatants was performed by sandwich ELISA, using technical duplicates, following the manufacturer’s instructions. Light absorbance was measured using Multiskan Ascent (Thermo Fisher) plate reader.

2.11 Caspase-1 and caspase-3/7 activity assays

Caspase-1 and caspase-3/7 activities were measured using Caspase-Glo® 1 and Caspase-Glo 3/7 assay (Promega), respectively, in human monocyte-derived macrophages and white adipose tissue explant culture supernatants, following the manufacturer’s instructions. Luminescence was measured using an Omega plate reader.

2.12 Data and analysis

All the experiments that were conducted to draw the main conclusions of the paper use n = 6 or n = 5 healthy human donor or patients undergoing bariatric surgery. The occasional usage of n < 5 patients reflects a confirmation of the main message, using independent assays. Data that use n < 5 patients were labelled as “preliminary” in the figure legends. Results are expressed as the mean ± SEM and were analysed using GraphPad Prism 8 software (GraphPad, RRID:SCR_002798). The
threshold of probability used to draw conclusions and statistical significance is $P \leq 0.05$. Statistical analyses were performed with Student’s t-test and paired or unpaired one-way ANOVA. In multigroup studies with parametric variables, post hoc tests were conducted only if F achieved statistical significance determined by $P \leq 0.05$ (Curtis et al., 2018). Non-parametric ANOVA was performed when results were expressed as relative to control (i.e. error bar proportional to the mean in controls). Results expressed as relative to control were analysed as such, in order to reduce unwanted source of variation between independent experiments. In these cases, we analysed paired samples across different conditions (i.e. control and ouabain-treated samples at different concentrations) by normalizing the results to the control (untreated) condition, which was designated as 1 (variables expressed as fold matched control values). Outliers were included in data analysis and presentation. The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018).

2.13 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/2 (Alexander et al., 2019).

3 | RESULTS

3.1 | Cardiac glycosides induce cell death in human monocyte-derived macrophages

We first assessed cardiac glycoside cytotoxicity using annexin V/PI staining followed by flow cytometry in human monocyte-derived macrophages. These cells were treated with either 0.01% DMSO (control) or cardiac glycosides (ouabain, digoxin, digitoxin and bufalin) at 50 nM, 100 nM, 500 nM and 5 μM for 24 h. Cardiac glycoside-treated human monocyte-derived macrophages showed reduced cell viability in a dose-dependent manner at nanomolar concentrations (Figures 1a and S1A). Among the cardiac glycosides tested, ouabain was the most cytotoxic one (50 nM < IC_{50} < 100 nM) in human monocyte-derived macrophages (Figure 1a). In order to test for the cell specificity of this cytotoxic effect, we treated the non-adherent peripheral blood mononuclear cells with the same range of concentrations of ouabain for 24 h and found that ouabain did not exert cytotoxic effects in these cells (Figure 1b). These results indicate that human monocyte-derived macrophages are particularly susceptible to the cytotoxic effects of nanomolar concentrations of cardiac glycosides when compared with non-adherent peripheral blood mononuclear cells.

It has been previously reported that ouabain, which contains a l-rhamnose unit (Figure S1B), has higher affinity to the Na$^+/K^+$-ATPase than its aglycone ouabagenin, which lacks the l-rhamnose unit (Cornelius et al., 2013). Accordingly, ouabagenin was not cytotoxic when incubated for 24 h at 50, 100 and 500 nM in human monocyte-derived macrophages (Figure S1B), suggesting that the cytotoxic effects of ouabain were caused by inhibition of Na$^+/K^+$-ATPase. To further test this hypothesis, we treated human monocyte-derived macrophages with either ouabain (50 nM) or ouabain in the presence of KCl for 24 h (Figure 1c). The usage of KCl rescued ouabain-induced cytotoxicity in human monocyte-derived macrophages, confirming that cell death is dependent on K$^+$ flux and subsequent perturbation of intracellular ion balance. This was further investigated by incubating human monocyte-derived macrophages treated with either ouabain alone or with KCl, in presence of K$^+$, Na$^+$ and Ca$^{2+}$ fluorescent probes (Figure 1d). Intracellular K$^+$ levels decreased in ouabain-treated cells compared with control human monocyte-derived macrophages and intracellular K$^+$ levels were rescued when KCl was added (Figure 1d,e). Similarly, intracellular levels of Na$^+$ and Ca$^{2+}$ increased in human monocyte-derived macrophages treated with ouabain, which were also fully rescued by adding KCl (Figure 1d,e).

Calcium is a well-known mediator of inflammasome activation in macrophages (Di et al., 2018). Ouabain has been recently reported to induce cell death by inflammasome and caspase-1 activation in THP1 macrophage-like cells (LaRock et al., 2019). We thus evaluated inflammasome activity by measuring luminescence resulting from catalytically active caspase 1 in the culture media of human monocyte-derived macrophages. Caspase-1 activity was increased in ouabain-treated human monocyte-derived macrophages (Figure 1f). We next measured the levels of mature IL-1$\beta$ in culture media by ELISA and found a dose-dependent increase of IL-1$\beta$ in ouabain-treated human monocyte-derived macrophages compared with control ones (Figure 1f). The ouabain-induced increase in IL-1$\beta$ and caspase-1 activity is conserved in LPS-primed human monocyte-derived macrophages (Figure S2A,B) and reaches IL-1$\beta$ quantities (>500 pg ml$^{-1}$) typically produced following NLRP3 inflammasome activation (Figure S2A). Ouabain also increased the activity of caspase 3/7 and the release of the inflammatory cytokine TNF-$\alpha$ in human monocyte-derived macrophages (Figure 1g). These results suggest that ouabain-induced cell death in human monocyte-derived macrophages is associated with inflammatory and apoptotic caspases. To determine whether ouabain-induced cell death in human monocyte-derived macrophages is caspase dependent, human monocyte-derived macrophages were co-treated with ouabain and pan-caspase inhibitor Q-VD-OPh (Figure 1h). Caspase inhibition blunted the cytotoxic effect of ouabain, confirming a caspase-dependent cell death in ouabain-treated human monocyte-derived macrophages.

3.2 | Ouabain depletes macrophages in the white adipose tissue ex vivo

To determine whether cardiac glycosides induce cell death in tissue macrophages, human white adipose tissue isolated from obese patients was cultured and treated with ouabain at nanomolar concentrations (Figures 1a and S1A). Among the cardiac glycosides reduced cell viability in a dose-dependent manner at nanomolar concentrations (Figures 1a and S1A). Among the cardiac glycosides tested, ouabain was the most cytotoxic one (50 nM < IC_{50} < 100 nM) in human monocyte-derived macrophages (Figure 1a). In order to test the cell specificity of this cytotoxic effect, we treated the non-adherent peripheral blood mononuclear cells with the same range of concentrations of ouabain for 24 h and found that ouabain did not exert cytotoxic effects in these cells (Figure 1b). These results indicate that human monocyte-derived macrophages are particularly susceptible to the cytotoxic effects of nanomolar concentrations of cardiac glycosides when compared with non-adherent peripheral blood mononuclear cells.
concentrations (50, 100 and 500 nM) for 48 h ex vivo. Macrophage-specific CD68 immunostaining showed a reduction of macrophages in ouabain-treated compared with control white adipose tissue explants (Figure 2a). This was confirmed by a ~70% down-regulation of CD68 mRNA expression levels, measured by quantitative RT-PCR (qRT-PCR), in ouabain-treated explants (Figure 2b). Moreover, protein levels of the macrophage mannose receptor (CD206) were assessed by western blot and found to be consistently reduced in ouabain-treated white adipose tissue explants compared with control ones (Figure 2c).

To determine whether the ouabain-induced ex vivo macrophage depletion was due to the activation of inflammatory and apoptotic caspases (as previously seen in human monocyte-derived macrophages—Figure 1), the activity of caspases 1 and 3/7 was measured in white adipose tissue explants culture media (Figure 2d). Caspases 1 and 3/7 activity was higher in ouabain-treated compared with control ones, suggesting that ouabain-induced macrophage depletion was due to the activation of these caspases.
**Figure 2** Ouabain depletes macrophages in human omental white adipose tissue (oWAT) explants. (a) Representative pictures of CD68 immunostaining in human oWAT explants treated with ouabain (50, 100 and 500 nM) for 48 h; n = 6 donors. (b) Relative CD68 mRNA expression in human oWAT explants treated with ouabain (50, 100 and 500 nM) for 48 h; n = 6 explants. (c) CD206 Western blotting in human oWAT explants treated with ouabain (50, 100 and 500 nM) for 48 h; representative from four independent experiments. (d) Caspase-1 (left) and caspase-3/7 (right) activities in human oWAT explants treated with ouabain (50, 100 and 500 nM) for 48 h; n = 6 explants. (e) Cell viability of stromal vascular fraction (SVF)-derived pre-adipocytes treated with ouabain (50, 100 and 500 nM) for 24 h (percentage of annexin V− and PI− cells); results from three independent experiments. (f) Cell viability analysis (alamarBlue reduction) of SVF-derived mature adipocytes treated with ouabain (50, 100 and 500 nM) for 24 h; results from three independent experiments. Statistical significance was calculated using paired non-parametric ANOVA followed by Dunn’s multiple comparisons (b) and paired one-way ANOVA test followed by Dunnett’s multiple comparisons (d–f). Error bars represent SEM. Scale bar = 100 μm; *P ≤ .05; **P ≤ .01; n.s. non significant.
FIGURE 3  Ouabain improves insulin sensitivity and reduces type VI collagen deposition in human omental white adipose tissue (oWAT) explants. (a) Phospho-AKTser473, total Akt and β-actin western blotting in human oWAT explants treated with insulin and ouabain (50, 100 and 500 nM) for 48 h; representative of four independent experiments. (b) Analysis of relative levels of phospho-AKTser473 protein, normalized to the loading control (total AKT); results from four independent experiments. (c) Relative ADIPOQ mRNA expression in human oWAT explants treated with ouabain (50, 100 and 500 nM) for 48 h; n = 6 explants. (d) CD36 Western blotting in human oWAT explants treated with ouabain (50, 100 and 500 nM) for 48 h; representative of n = 5 explants. (e) Representative Sirius Red pictures of human oWAT treated with ouabain (100 nM) for 48 h (top). Preliminary analysis of relative mean adipocyte areas in human oWAT explants treated with vehicle (control) and ouabain (100 nM) for 48 h (bottom); n = 4 explants. (f) Representative type VI collagen immunofluorescence pictures of human oWAT treated with ouabain (100 nM) for 48 h (top). Relative type VI collagen fluorescence (bottom left) and relative COL6A1 mRNA expression (bottom right) in human oWAT explants treated with vehicle (control) or ouabain at indicated concentrations for 48 h; n = 6 explants for immunofluorescence and n = 6 explants for qRT-PCR. Statistical significance was calculated paired non-parametric ANOVA followed by Dunn’s multiple comparisons (b, c, f). Error bars represent SEM. Scale bar = 50 μm; *P ≤ .05; n.s. non significant.
with control white adipose tissue explants (Figure 2d), suggesting that ouabain induced macrophage cell death through pyroptosis and apoptosis in white adipose tissue explants.

In order to evaluate the potential cytotoxicity of ouabain in non-immune resident cells such as adipocytes, human white adipose tissue-derived stromal cells were differentiated into pre-adipocytes and mature adipocytes and treated with ouabain (50, 100, 500 nM and 5 μM) for 24 h (Figure 2e,f). Ouabain cytotoxicity showed no differences among all the groups (Figure 2e,f), indicating that ouabain had no impact on pre-adipocyte and mature adipocyte viability.

3.3 | Ouabain improves insulin sensitivity and reduces type VI collagen levels in the white adipose tissue ex vivo

Macrophage infiltration is a hallmark of inflammation and insulin resistance in visceral white adipose tissue depots in mice, rats and humans (Koppaka et al., 2013; Olona et al., 2018; Wentworth et al., 2010). Insulin sensitivity was assessed by measuring the levels of AKTser473 phosphorylation, a major effector of insulin signalling, in control and ouabain-treated white adipose tissue explants (Figure 3a). Ouabain treatment increased AKTser473 phosphorylation by ∼50% compared with control white adipose tissue explants (Figure 3b), indicating that ouabain promotes insulin sensitivity in the ex vivo cultured white adipose tissue. We then confirmed this finding by measuring ADIPOQ mRNA expression and CD36 protein levels by qRT-PCR and western blot, respectively. ADIPOQ encodes for adiponectin, an adipokine that promotes insulin sensitivity (Shetty et al., 2009) and was significantly up-regulated in ouabain-treated white adipose tissue explants compared with control ones (Figure 3c). Similarly, protein levels of CD36, which have an important role in fatty acid uptake upon insulin stimulation, were also increased in ouabain-treated white adipose tissue explants (Figure 3d). We next investigated whether increased CD36 and fatty acid uptake could affect adipocyte size, as previously reported (Vroegrijk et al., 2013). Adipocyte mean areas were ∼20% bigger in ouabain-treated compared with control white adipose tissue explants (Figure 3e). These results show that ouabain improves tissue function, promoting insulin sensitivity in white adipose tissue from obese patients.

Adipose tissue macrophages have been shown to regulate collagen deposition and fibrosis in visceral white adipose tissue during obesity, inducing tissue remodelling and insulin resistance (Keophiphath et al., 2009). In line with this, type VI collagen knockout mice upon high-fat diet showed unrestrained adipocyte expansion and improved white adipose tissue insulin sensitivity (Khan et al., 2009). Type VI collagen immunostaining was performed and mean grey intensities showed a down-regulation of type VI collagen in ouabain-treated compared with control white adipose tissue explants (Figure 3f). This coincided with COL6A1 mRNA expression down-regulation (Figure 3f), confirming a decrease in type VI collagen synthesis and deposition upon ouabain treatment in the white adipose tissue.

4 | DISCUSSION

Here, we show that nanomolar concentration of cardiac glycosides compromises human macrophage viability in vitro. In the white adipose tissue explants isolated from obese patients, ouabain treatment causes macrophage depletion. We also observe that cardiac glycoside-dependent macrophage cytotoxicity results in TNF secretion in vitro, which we could not detect in the ex vivo white adipose tissue (data not shown). TNF has been shown to induce apoptosis by activating directly caspase 3 or through the Bcl-2 family protein BH3 interacting-domain death agonist (known as BID) in macrophages (Liu et al., 2004). Collectively, these results show that cardiac glycoside-mediated decrease in intracellular K⁺ and the net increase in Ca²⁺ levels trigger both pyroptotic (caspase 1-dependent IL-1β secretion) and apoptotic (cell death through caspase 3) cell death pathways in human macrophages.

Pyroptosis is caspase-1 dependent and causes the assembly of inflammasome complex, which initiates the efficient release of IL-1β (Gross et al., 2011). While pyroptosis is an inflammatory form of cell death triggered by caspase 1, apoptotic cell death is mediated by effector apoptotic caspases such as caspases 3, 6 and 7 (Man & Kanneganti, 2016). Recent evidence shows a significant crosstalk and compensation between apoptotic and pyroptotic cell death mechanisms and caspases such as caspase 6 having master regulatory role in both events (Zheng et al., 2020). Hence, it is likely that cardiac glycosides trigger broader caspase activation pathways in human macrophages, which causes IL-1β and TNF release, in line with reports showing TNF secretion following co-treatment with LPS and ATP (Barbera-Cremades et al., 2017; Di et al., 2018). Interestingly, TNF secretion has been previously shown to be caspase-1 dependent in macrophages (Miggin et al., 2007), which supports the broader effects of inflammasome activation on inducing pro-inflammatory cytokine secretion.

We report that the cell death caused by cardiac glycosides in human macrophages is dependent on K⁺ flux and requires the presence of the sugar moiety attached to the steroid part of the compound. Indeed, the addition of KCl in the media showed a complete rescue of cell viability and intracellular levels of Na⁺ and Ca²⁺. This also confirms that, similar to cardiomyocytes, macrophage survival is tightly dependent on potassium efflux and the sensitivity to cardiac glycosides is more prominent in human macrophage-like cells when compared with murine ones (LaRock et al., 2019). In macrophages, potassium efflux is mediated by membrane receptors such as P2X7 receptors and TWIK2 (Di et al., 2018), and the inhibition of Na⁺/K⁺-ATPase likely deprives the cell from potassium through the inhibition of its influx, while its export remains intact. Furthermore, our data suggest that, among other peripheral blood mononuclear cells, cardiac glycosides seem to have a particularly potent effect on macrophages. This leads to the question of why human macrophages among other cells undergo apoptosis and pyroptosis as a result of decreased intracellular potassium? Interestingly, cardiac glycosides such as digoxin and digitoxin selectively induce apoptosis of senescent cells and digitoxin has a senolytic (i.e. selective killing of senescent cells) activity at a
nanomolar range concentration that is closed to the one observed in cardiac patients treated with this drug (Guerrero et al., 2019; Lopez-Lazaro, 2007). This raises the possibility of phenotypic similarities between senescent cells and macrophages (Behmoaras & Gil, 2021), and a recent report shows that chemotherapy-induced senescent breast cancer cells are highly enriched for macrophage genes and can perform phagocytosis (Tonnesen-Murray et al., 2019). It is therefore tempting to speculate that the macrophages and senescent cells show a shared sensitivity to the cardiac glycoside-mediated cell death, although the underlying mechanisms may differ.

When used at nanomolar concentrations, cardiac glycosides showed effective depletion of macrophages from ex vivo cultured white adipose tissue explants isolated from obese patients undergoing bariatric surgery. We observed an almost complete down-regulation of CD206 (mannose receptor). Indeed, adipose tissue-infiltrating macrophages are responsible for persistent low-grade inflammation that underlies the systemic insulin resistance observed in obesity and CD206 is a specific marker for adipose tissue macrophages, which controls adipogenesis (Nawaz et al., 2017). Despite being described as an M2 macrophage marker, CD206 has been previously found to be expressed by pro-inflammatory white adipose tissue macrophages in humans (Wentworth et al., 2010). In fact, the partial depletion of CD206 macrophages enhances insulin sensitivity (Igarashi et al., 2018) and we show that this is indeed the case with the usage of ouabain (50–100 nM) in the ex vivo cultured white adipose tissue explants. This white adipose tissue macrophage depletion could be partly through caspase 1-mediated pyroptosis, though we cannot exclude apoptotic cell death. The mechanistic link between different caspases and the macrophage death remains to be identified and is a limitation of our study. Furthermore, although we could detect an increasing IL-1β upon ouabain treatment in the white adipose tissue, the overall detection range was low (<10 pg ml⁻¹, data not shown) possibly due to relatively small number of macrophages in the explants when compared with the predominant adipocyte fraction.

We report an improved white adipose tissue function upon treatment with ouabain. Inflammation and fibrosis are two major pathways that dysregulate white adipose tissue homeostasis in obesity (Olona et al., 2018) and current therapeutic strategies aim to target these pathways (Kusminski et al., 2016). Our findings show that ouabain-mediated macrophage cytotoxicity increases adipocyte hypertrophy and induces metabolic activity through up-regulation of CD36, which facilitates the uptake of long-chain fatty acids (Christiaens et al., 2012). Moreover, ouabain treatment reduces type VI collagen, the main collagen in the white adipose tissue, which accumulates following metabolically challenging conditions (Khan et al., 2009). Interestingly, macrophages have been shown to produce type VI collagen in the lung (Ucero et al., 2019), which suggests that ouabain-mediated macrophage depletion is the direct cause of the decreased levels of this collagen type in the white adipose tissue.

We show that stromal vascular fraction-derived cultured adipocytes and pre-adipocytes are not sensitive to ouabain-induced cell death and our data obtained in the peripheral blood suggest that macrophages are likely to be the only resident immune cells in the white adipose tissue that are cleared from the tissue through mechanisms that remain to be identified. Furthermore, the improvement of white adipose tissue homeostasis following ex vivo treatment with ouabain can be through soluble factors secreted by dying macrophages and/or direct effects of the drug on adipocytes. For instance, adipocytes treated with ouabain up-regulate Glut4, indicating a possible non-canonical role of this cardiac glycoside (Brewer et al., 2019). The identification of all soluble factors accompanying ouabain-mediated macrophage death and elucidating the pathways triggered by cardiac glycosides in adipocytes will be important in fully dissecting the mechanisms involved in the beneficial role of these compounds in inflamed tissues.

Here, we show the beneficial role of the usage of ouabain in a tissue where the increase in macrophage infiltrates is associated with tissue damage and inflammation. Notably, the white adipose tissue does not seem to act as a cardiac glycoside reservoir as pharmacokinetics of digoxin is identical between obese and non-obese patients (Abernethy et al., 1981). This suggests that local administration of cardiac glycosides could be the preferred therapeutic route for treating white adipose tissue inflammation in metabolic disease. In an infectious disease context, the use of cardiac glycosides can be detrimental because of the bactericidal role of mononuclear phagocytes (Esposito, 1985). Hence, the therapeutic advantage of nanomolar range usage of cardiac glycosides can be studied in sterile inflammation where tissue macrophage presence is generally associated with poor clinical outcome (e.g. inflammatory brain disorders and autoimmune disease). In keeping with this, the local administration of cardiac glycosides can have therapeutic effects through selective cytotoxicity towards tissue-resident macrophages, bypassing the unwanted side effects related to the reported toxicity of these compounds. Considering that some cardiac glycosides such as digoxin are frequently prescribed medicines in elderly population, these findings may also re-evaluate the usage of these in the context of tissue macrophage integrity in health and disease.

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AUTHOR CONTRIBUTIONS
J.B. supervised the studies. A.O. and J.B. conceived the experiments, analysed the data and co-wrote the manuscript. A.O., C.H., A.G., J.-H. K., M.R.J., P.K.A., D.T. and J.G. participated in performing the experiments and analysing the data. All the listed authors have seen and approved the submission of the manuscript.

CONFLICT OF INTEREST
J.G. has acted as a consultant for Unity Biotechnology, Geras Bio, Merck KGaA and Myricx; owns equity in Unity Biotechnology and Geras Bio; and is a named inventor in Imperial College London and MRC patents related to senolytic therapies. A.G. is a named inventor in MRC patents related to senolytic therapies.
DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR
This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJR guidelines for Design & Analysis and Immunoblotting and Immunochromey and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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REFERENCES
Abernethy, D. R., Greenblatt, D. J., & Smith, T. W. (1981). Digoxin disposition in obesity. Clinical pharmacokinetic investigation. American Heart Journal, 102, 740–744. https://doi.org/10.1016/0002-8703(81)90100-9
Alexander, S. P. H., Roberts, R. E., Broughton, B. R. S., Sobe, C. G., George, C. H., Stanford, S. C., Cirino, G., Docherty, J. R., Giembycz, M. A., Hoyer, D., Insel, P. A., Izzo, A. A., Ji, Y., MacEwan, D. J., Mangum, J., Wonnacott, S., & Ahluwalia, A. (2018). Goals and practicalities of immunoblotting and immunohistochemistry: A guide for submission to the British Journal of Pharmacology. British Journal of Pharmacology, 175, 407–411. https://doi.org/10.1111/bjp.14112
Alexander, S. P. H., Kelly, E., Mathie, A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Pawson, A. J., Sharman, J. L., Southan, C., Buneman, O. P., Cidlowski, J. A., Christopoulos, A., Davenport, A. P., Fabbro, D., Speeding, M., Stiessen, J., Davies, J. A., & Collaborators, C. (2019). The Concise Guide to PHARMACOLOGY 2019/20: Introduction and other protein targets. British Journal of Pharmacology, 176(Suppl 1), 51–520.
Barbera-Cremades, M., Gomez, A. I., Baroja-Mazo, A., Martinez-Alarcón, L., Martinez, C. M., de Torre-Minguela, C., & Pelegri, P. (2017). P2X7 receptor induces tumor necrosis factor-α converting enzyme activation and release to boost TNF-α production. Frontiers in Immunology, 8, 862. https://doi.org/10.3389/fimmu.2017.00862
Behmoaras, J., & Gil, J. (2021). Similarities and interplay between senescent cells and macrophages. The Journal of Cell Biology, 220, e202010162. https://doi.org/10.1083/jcb.202010162
Brewer, P. D., Romenskaia, I., & Mastick, C. C. (2019). A high-throughput chemical–genetics screen in murine adipocytes identifies insulin-regulatory pathways. Small molecule modulators of Glut4 translocation. The Journal of Biological Chemistry, 294, 4103–4118. https://doi.org/10.1074/jbc.RA118.006986
Cavalcante-Silva, L. H. A., Lima, E. A., Carvalho, D. C. M., de Sales-Neto, J. M., Alves, A. K. A., Galvao, J., da Silva, J. S. F., & Rodrigues-Mascarenhas, S. (2017). Much more than a cardiotoxic steroid: Modulation of inflammation by ouabain. Frontiers in Physiology, 8, 895. https://doi.org/10.3389/fphys.2017.00895
Chen, G. Y., & Nunez, G. (2010). Sterile inflammation: Sensing and reacting to damage. Nature Reviews. Immunology, 10, 826–837. https://doi.org/10.1038/npj2873
Chen, Y., Huang, W., Yang, M., Xin, G., Cui, W., Xie, Z., & Silverstein, R. L. (2017). Cardiotoxic steroids stimulate macrophage inflammatory responses through a pathway involving CD36, TLR4, and Na/K-ATPase. Arteriosclerosis, Thrombosis, and Vascular Biology, 37, 1462–1469. https://doi.org/10.1161/ATVBAHA.117.309444
Christensen, V., Van Hul, M., Lijnen, H. R., & Scroyen, I. (2012). CD36 promotes adipocyte differentiation and adipogenesis. Biochimica et Biophysica Acta, 1820, 949–956. https://doi.org/10.1016/j.bbadgen.2012.04.001
Cornelius, F., Kanai, R., & Toyoshima, C. (2013). A structural view on the functional importance of the sugar moiety and steroid hydroxyls of cardiotoxic steroids in binding to Na,K-ATPase*. The Journal of Biological Chemistry, 288, 6602–6616. https://doi.org/10.1074/jbc.M112.442137
Curtis, M. J., Alexander, S., Cirino, G., Docherty, J. R., George, C. H., Giembycz, M. A., Hoyer, D., Insel, P. A., Izzo, A. A., Ji, Y., MacEwan, D. J., Sobe, C. G., Stanford, S. C., Teixeira, M. M., Wonnacott, S., & Ahluwalia, A. (2018). Experimental design and analysis and their reporting II: Updated and simplified guidance for authors and peer reviewers. British Journal of Pharmacology, 175, 987–993. https://doi.org/10.1111/bph.14153
Di, A., Xiong, S., Ye, Z., Maireddi, R. K. S., Kometani, S., Zhong, M., Mittal, M., Hong, Z., Kanneganti, T. D., Rehman, J., & Malik, A. B. (2018). The TWIK2 potassium efflux channel in macrophages mediates NLRP3 inflammasome-induced inflammation. Immunity, 49(56–65), e54.
Esposito, A. L. (1985). Digoxin disrupts the inflammatory response in experimental pneumococcal pneumonia. The Journal of Infectious Diseases, 152, 14–23. https://doi.org/10.1093/infdis/152.1.14
Foye, A. D., Crawford, A., & Hall, N. D. (1997). Modulation of cytokine production by human mononuclear cells following impairment of Na,K-ATPase activity. Biochimica et Biophysica Acta, 1355, 43–49. https://doi.org/10.1016/S0167-8889(96)00116-4
Franchi, L., Kanneganti, T. D., Dubayk, G. R., & Nunez, G. (2007). Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria. The Journal of Biological Chemistry, 282, 18810–18818. https://doi.org/10.1074/jbc.M610762200
Galarraga, M., Campion, J., Munoz-Barrutia, A., Boque, N., Moreno, H., Martinez, J. A., Milagro, F., & Ortiz-de-Solorzano, C. (2012). Adiposoft: Automated software for the analysis of white adipose tissue cellularity in histological sections. Journal of Lipid Research, 53, 2791–2796. https://doi.org/10.1194/jlr.D023788
Gross, O., Thomas, C. J., Guarda, G., & Tschopp, J. (2011). The inflammasome: An integrated view. Immunological Reviews, 243, 136–151. https://doi.org/10.1111/j.1600-065X.2011.01046.x
Guerrero, A., Herranz, N., Sun, B., Wagner, V., Gallage, S., Guiho, R., Wolter, K., Pombo, J., Irvine, E. E., Innes, A. J., Birch, J., Neeley, J., Manshaei, S., Heide, D., Dharmalingam, G., Harbig, J., Olona, A., Behmoaras, J., Dauch, D., & Gil, J. (2019). Cardiac glycosides are broad-spectrum senolytics. Nature Metabolism, 1, 1074–1088. https://doi.org/10.1038/s42255-019-0122-z
Igarashi, Y., Nawaz, A., Kado, T., Bilal, M., Kuwano, T., Yamamoto, S., Sasahara, M., Juxiang, X., Inuijma, A., Koizumi, K., Imura, J., Shibahara, N., Usui, I., Fujisaka, S., & Toke, K. (2018). Partial depletion of CD206-positive M2-like macrophages induces proliferation of beige progenitors and enhances browning after cold stimulation. Scientific Reports, 8, 14567. https://doi.org/10.1038/s41598-018-32803-0
Kaplan, J. H. (2002). Biochemistry of Na,K-ATPase. Annual Review of Biochemistry, 71, 511–535. https://doi.org/10.1146/annurev.biochem.71.102201.141218
Keophiphath, M., Achard, V., Henegar, C., Rouault, C., Clement, K., & Lacasa, D. (2009). Macrophage-secreted factors promote a profibrotic phenotype in human preadipocytes. Molecular Endocrinology, 23, 11–24. https://doi.org/10.1210/me.2008-0183
Khan, T., Muise, E. S., Iyengar, P., Wang, Z. V., Chandalia, M., Abate, N., Zhang, B. B., Bonaldo, P., Chua, S., & Scherer, P. E. (2009). Metabolic
disregulation and adipose tissue fibrosis: Role of collagen VI. 
Molecular and Cellular Biology, 29, 1575–1591. https://doi.org/10.1128/MCB.01300-08

Kobayashi, M., Usui-Kawanishi, F., Karasawa, T., Kimura, H., Watanabe, S., Mise, N., Kayama, F., Kasahara, T., Hasebe, N., & Takahashi, M. (2017). The cardiac glycoside ouabain activates NLRP3 inflammasomes and promotes cardiac inflammation and dysfunction. PLoS One, 12, e0176676. https://doi.org/10.1371/journal.pone.0176676

Koppaka, S., Kehlenbrink, S., Carey, M., Li, W., Sanchez, E., Lee, D. E., Lee, H., Chen, J., Carrasco, E., Kishore, P., Zhang, K., & Hawkins, M. (2013). Reduced adipose tissue macrophage content is associated with improved insulin sensitivity in thiazolidinedione-treated diabetic humans. Diabetes, 62, 1843–1854. https://doi.org/10.2337/db12-0868

Kusminski, C. M., Bickel, P. E., & Scherer, P. E. (2016). Targeting adipose tissue in the treatment of obesity-associated diabetes. Nature Reviews. Drug Discovery, 15, 639–660. https://doi.org/10.1038/ndr.2016.75

LaRock, D. L., Sands, J. S., Ettouati, E., Richard, M., Bushway, P. J., Adler, E. D., Nizet, V., & LaRock, C. N. (2019). Inflammasome inhibition blocks cardiac glyside cell toxicity. The Journal of Biological Chemistry, 294, 12846–12854. https://doi.org/10.1074/jbc.RA119.008330

Liu, H., Ma, Y., Pagliari, L. J., Perelman, H., Yu, C., Lin, A., & Pope, R. M. (2004). TNF-α-induced apoptosis of macrophages following inhibition of NF-κB: A central role for disruption of mitochondria. Journal of Immunology, 172, 1907–1915. https://doi.org/10.4049/jimmunol.172.3.1907

Lopez-Lazaro, M. (2007). Digitoxin as an anticancer agent with selectivity for cancer cells: Possible mechanisms involved. Expert Opinion on Therapeutic Targets, 11, 1043–1053. https://doi.org/10.1517/14728222.11.3.1043

Man, S. M., & Kanneganti, T. D. (2016). Converging roles of caspases in inflammasome activation, cell death and innate immunity. Nature Reviews. Immunology, 16, 7–21. https://doi.org/10.1038/nri.2015.7

Matsumori, A., Ono, K., Nishio, R., Iigata, H., Shioi, T., Matsui, S., Furukawa, Y., Iwasaki, A., Nose, Y., & Sasayama, S. (1997). Modulation of cytokine production and protection against lethal endotoxemia by the cardiac glycoside ouabain. Circulation, 96, 1501–1506. https://doi.org/10.1161/01.CIR.96.5.1501

McConkey, D. J., Lin, Y., Nutt, L. K., Ozel, H. Z., & Newman, R. A. (2000). Cardiac glycosides stimulate Ca ++ increase and apoptosis in androgen-independent, metastatic human prostate adenocarcinoma cells. Cancer Research, 60, 3807–3812.

Menger, L., Vacchelli, E., Adjemian, S., Martins, I., Ma, Y., Shen, S., Yamazaki, T., Sukkurwala, A. Q., Michaud, M., Mignot, G., Schlemmer, F., Sulpice, E., Locher, C., Gidrol, X., Ghiringhelli, F., Modjtahedi, N., Galluzzi, L., Andre, F., Zitvogel, L., Kroemer, G. (2012). Cardiac glycosides exert anticancer effects by inducing immunogenic cell death. Science Translational Medicine, 4, 143ra199.

Miggin, S. M., Passon-McDermott, E., Dunne, A., Jefferies, C., Pinteaux, E., Banahan, K., Murphy, C., Moyañ, P., Yamamoto, M., Akira, S., Rothwell, N., Golenbock, D., Fitzgerald, K. A., & O'Neill, L. A. (2007). NF-κB activation by the Toll-I-1 receptor domain protein MyD88 adapter-like is regulated by caspase-1. Proceedings of the National Academy of Sciences of the United States of America, 104, 3372–3377. https://doi.org/10.1073/pnas.0608100104

Munoz-Plasillo, R., Kuffa, P., Martinez-Colon, G., Smith, B. L., Rajendiran, T. M., & Nunez, G. (2013). K+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. Immunity, 38, 1142–1153. https://doi.org/10.1016/j.immuni.2013.05.016

Nawaz, A., Aminuddin, A., Kado, T., Takikawa, A., Yamamoto, S., Tsuneyama, K., Igarashi, Y., Ikutani, M., Nishida, Y., Nagai, Y., Takatsu, K., Imura, J., Sasahara, M., Okazaki, Y., Ueki, K., Okamura, T., Tokuyama, K., Ando, A., Matsumoto, M., ... Toke, K. (2017). CD206+ M2-like macrophages regulate systemic glucose metabolism by inhibiting proliferation of adipocyte progenitors. Nature Communications, 8, 286. https://doi.org/10.1038/s41467-017-00231-1

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Ozawa, A., Terra, X., Koj, H., Grau-Bove, C., Pinent, M., Ardevol, A., Diaz, A. G., Moreno-Moral, A., Edin, M., Bishop-Bailey, D., Zeldin, D. C., Aitman, T. J., Petretto, E., Blay, M., & Behmoaras, J. (2018). Epoxidegenase inactivation exacerbates diet and aging-associated metabolic dysfunction resulting from impaired aegisogenesis. Molecular Metabolism, 11, 18–32. https://doi.org/10.1016/j.molmet.2018.03.003

Papathannou, A. E., Ko, J. H., Imprialou, M., Bagnati, M., Srivastava, P. K., Vu, H. A., Cucchi, D., McAdoo, S. P., Ananieva, E. A., Mauro, C., & Behmoaras, J. (2017). BCAT1 controls metabolic reprogramming in activated human macrophages and is associated with inflammatory diseases. Nature Communications, 8, 16040. https://doi.org/10.1038/ncomms16040

Perne, A., Mueller, M. K., Steinruetz, M., Krauss-Mueller, N., Mayerhofer, J., Schwarzinger, I., Sloane, M., Uras, I. Z., Hoermann, G., Nijman, S. M., & Mayerhofer, M. (2009). Cardiac glycosides induce cell death in human cells by inhibiting general protein synthesis. PLoS One, 4, e8292. https://doi.org/10.1371/journal.pone.008292

Petriill, V., Papin, S., Dostert, C., Mayor, A., Martinon, F., & Tschopp, J. (2007). Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. Cell Death and Differentiation, 14, 1583–1589. https://doi.org/10.1038/sj.cdd.4402195

Prassas, I., & Diamandis, E. P. (2008). Novel therapeutic applications of cardiac glycosides. Nature Reviews. Drug Discovery, 7, 926–935. https://doi.org/10.1038/ndr2682

Price, E. M., & Lingrel, J. B. (1988). Structure-function relationships in the sodium-potassium ATPase. alpha. subunit: Site-directed mutagenesis of glutamine-111 to arginine and asparagine-122 to aspartic acid generates a ouabain-resistant enzyme. Biochemistry, 27, 8400–8408. https://doi.org/10.1021/bi00422a016

Shetty, S., Kusminski, C. M., & Scherer, P. E. (2009). Adiponectin in health and disease: Evaluation of adiponectin-targeted drug development strategies. Trends in Pharmacological Sciences, 30, 234–239. https://doi.org/10.1016/j.tips.2009.02.004

Teixeira, M. P., & Rujumaneck, V. M. (2014). Ouabain affects the expression of activation markers, cytokine production, and endocytosis of human monocytes. Mediators of Inflammation, 2014, 20146388.

Voshol, P. J., Rensen, P. C., van Dijk, K. W., & van Harmelen, V. (2013). Adiponectin improves insulin sensitivity in thiazolidinedione-treated diabetic tissue in the treatment of obesity-associated diabetes. Nature Reviews. Drug Discovery, 12, 857–870. https://doi.org/10.1038/nrd4098

Vroegrijk, I. O., van Klinken, J. B., van Diepen, J. A., van den Berg, S. A., Febbraio, M., Steinbusch, L. K., Glatz, J. F., Havekes, L. M., Voshol, P. J., Rensen, P. C., van Dijk, K. W., & van Harmelen, V. (2013).
CD36 is important for adipocyte recruitment and affects lipolysis. Obesity (Silver Spring), 21, 2037–2045. https://doi.org/10.1002/oby.20354

Wentworth, J. M., Naselli, G., Brown, W. A., Doyle, L., Phipson, B., Smyth, G. K., Wabitsch, M., O'Brien, P. E., & Harrison, L. C. (2010). Pro-inflammatory CD11c⁺CD206⁺ adipose tissue macrophages are associated with insulin resistance in human obesity. Diabetes, 59, 1648–1656. https://doi.org/10.2337/db09-0287

Xiao, A. Y., Wei, L., Xia, S., Rothman, S., & Yu, S. P. (2002). Ionic mechanism of ouabain-induced concurrent apoptosis and necrosis in individual cultured cortical neurons. The Journal of Neuroscience, 22, 1350–1362. https://doi.org/10.1523/JNEUROSCI.22-04-01350.2002

Zheng, M., Karki, R., Vogel, P., & Kanneganti, T. D. (2020). Caspase-6 is a key regulator of innate immunity, inflammasome activation, and host defense. Cell, 181, 674–687.

Zhivotovsky, B., & Orrenius, S. (2011). Calcium and cell death mechanisms: A perspective from the cell death community. Cell Calcium, 50, 211–221. https://doi.org/10.1016/j.ceca.2011.03.003

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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