Extra-cellular matrix induced by steroids and aging through a G-protein coupled receptor in a Drosophila model of renal fibrosis

Wenjing Zheng¹, Karen Ocorr², Marc Tatar¹

¹ Department of Ecology and Evolutionary Biology, Division of Biology and Medicine, Brown University, Providence RI 02912
² Development, Aging and Regeneration Program, SBP Medical Discovery Institute, La Jolla, CA 92037

Corresponding author:
Marc Tatar
Department of Ecology and Evolutionary Biology
Division of Biology and Medicine
Box GW, Brown University
Providence RI 02912
Marc_Tatar@Brown.edu
401-863-3455

Keywords: fibrosis, aldosterone, G-protein coupled receptor, DopEcR, aging, ecdysone

ORCID identifiers:
Marc Tatar: 0000 0003 3232 6884

Summary statement
Drosophila kidney function in impaired by steroid hormones ecdysone and by human aldosterone, and when ecdysone increases with age. Steroids induces fibrosis at the fly kidney by signaling through a recently described, membrane associated receptor, the dopamineEcR G-protein coupled receptor.
Abstract

Aldosterone is produced by the mammalian adrenal cortex to modulate blood pressure and fluid balance, however excessive, prolonged aldosterone promotes fibrosis and kidney failure. How aldosterone triggers disease may involve actions independent of its canonical mineralocorticoid receptor. Here we present a Drosophila model of renal pathology caused by excess extra-cellular matrix formation, stimulated by exogenous aldosterone and by insect ecdysone. Chronic administration of aldosterone or ecdysone induces expression and accumulation of collagen-like Pericardin at adult nephrocytes – podocyte-like cells that filter circulating hemolymph. Excess Pericardin deposition disrupts nephrocyte (glomerular) filtration and causes proteinuria in Drosophila, hallmarks of mammalian kidney failure. Steroid-induced Pericardin production arises from cardiomyocytes associated with nephrocytes, potentially reflecting an analogous role of mammalian myofibroblasts in fibrotic disease. Remarkably, the canonical ecdysteroid nuclear hormone receptor, Ecdysone Receptor EcR, is not required for aldosterone or ecdysone to stimulate Pericardin production or associated renal pathology. Instead, these hormones require a cardiomyocyte-associated G-protein coupled receptor, Dopamine-EcR (DopEcR), a membrane-associated receptor previously characterized in the fly brain as affecting behavior. DopEcR in the brain is known to affect behavior through interactions with the Drosophila epidermal growth factor receptor, dEGFR. Here we find the steroids ecdysone and aldosterone require dEGFR in cardiomyocytes to induce fibrosis of the cardiac-renal system. As well, endogenous ecdysone that becomes elevated with age is found to foster age-associated fibrosis, and to require both cardiomyocyte DopEcR and dEGFR. This Drosophila renal disease model reveals a novel signaling pathway through which steroids may modulate mammalian fibrosis through potential orthologs of DopEcR.
Introduction

Aldosterone is a primary renal regulator of sodium and potassium homeostasis, but when chronically elevated as in diabetes and primary aldosteronism (1), aldosterone promotes kidney interstitial fibrosis and glomerulosclerosis (2-4). These events are preceded by elevated inflammation through monocytes and macrophage infiltration followed by proliferation of myofibroblasts that secrete fibrinogen, collagens and elastins. Aldosterone increases reactive oxygen species (ROS) to induce profibrotic factors such as Transforming Growth Factor-β1 (TGF-β1), Plasminogen Activator Inhibitor-1, and Enothelin-1 (4). TGF-β1 contributes to fibrosis by activating myofibroblasts (5) as well as through suppressing matrix metalloproteinases, which can further promote excess extra-cellular matrix (6). Aldosterone affects these processes through its interaction with the mineralocorticoid nuclear hormone receptor (MR), as inferred from studies where blockade of MR activity prevents aldosterone-associated inflammatory and fibrotic outcomes (7-9).

Many data also suggest that aldosterone contributes to fibrosis through rapid signaling independent of MR (4). Aldosterone enhances TGF-β1 expression and fibrosis in part through stimulation of ERK1/2 (10-12), while aldosterone fosters hypertrophy in cardiomyocytes through action on ERK5 and PKC (13). As well, aldosterone effectively induces calcium influx in fibroblasts derived from MR-deficient mice (14). Angiotensin receptors crosstalk with MR to modulate NF-κB in vascular smooth muscle cells (VSMC) stimulated with aldosterone (15), suggesting that aldosterone can in part act through G-protein-coupled receptors (GPCR). With considerable debate, GPER1 has been proposed as an alternative GPCR for aldosterone (16-19). In VSMC, aldosterone was seen to activate PI3 kinase and ERK through both GPER1 and MR (20). Emerging evidence, however, shows that 17β-estradiol is the steroid agonist of GPER1 (21-23), and no pharmacological evidence demonstrates GPER1 to interact with aldosterone. The problem remains: through which receptor aside from MR might aldosterone stimulate signaling, is this a GPCR, and how does this modulate fibrosis?
Here we develop a model of steroid-induced fibrosis based on *Drosophila melanogaster*. Genetic data reveal the *Drosophila* GPRC Dopamine-EcR (DopEcR) (reviewed in (24)) is expressed in cardiomyocytes, and is necessary for exogenous aldosterone and insect ecdysone to induce excess extracellular matrix at heart-associated nephrocytes, and to disrupt fly renal function. We likewise document elevated cardiac-renal fibrosis with age and find this pathology requires endogenous synthesis of ecdysone and cardiomyocyte DopEcR. Similar requirements are found for *Drosophila* EGFR (dEGFR) in terms of exogenous hormone treatments and endogenous aging. Based on our findings we propose that mammalian homologs of DopEcR may offer a novel entrée to understand fibrotic pathology in humans.

**Results**

**Steroid hormones induce renal dysfunction at the nephrocytes**

The tubular heart of adult *Drosophila* is lined by pericardial cells, podocyte-like nephrocytes that conduct size-selective filtration of hemolymph (25, 26) (Fig. 1A). The heart tube and the associated nephrocytes are enmeshed in an extracellular matrix composed of collagen-like proteins including Pericardin (collagen IV) (27, 28). In a first step to develop a model of *Drosophila* renal fibrosis, we measured protein in adult excreta (frass) as an analog to proteinuria seen in humans with glomerular dysfunction (29). Frass is a by-product of both digestion and discharge from renal Malpighian tubules, gut-associated structures that maintain ionic and water balance (25, 26, 30, 31). Previous work shows the appearance of frass can be modulated by diet, mating and internal metabolic state (32), and by the activity of heart-associated nephrocytes (33, 34). We asked if frass protein content could be affected by nephrocyte function. We collected frass from adult males (to exclude eggs) in microcentrifuge tubes and measured total protein content, normalized to uric acid as a way to account for excretion volume. To manipulate nephrocyte function, we depleted nephrocyte slit diaphragm genes *kirre* and *sticks-n-stones* (*sns*), which encode homologs of mammalian nefrin. Previous reports show that reduced *kirre* and *sns* impairs nephrocyte filtration measured by uptake of fluoro-dextran beads (26, 31). We replicated this result (Fig 1E, F) and likewise observed that
reduced *kirre* and *sns* also elevated protein excretion (Fig 1B). Thus, defects in nephrocyte function can induce proteinuria in *Drosophila*.

We next assessed how frass protein content was affected by nutrient and physiological conditions as occurs with human chronic kidney disease. Diets of high sugar or salt *decreased* protein excretion compared to normal diet (Fig 1C), perhaps by altering adult metabolic state. To find a treatment that might *increase* proteinuria, we fed aldosterone to adult *Drosophila*. Protein in frass was elevated in adults fed aldosterone for two weeks (Fig 1D) yet not when fed aldosterone for only 24 h (Fig S1). *Drosophila* do not synthesize aldosterone, a mammalian steroid hormone (Fig 1A) produced in the renal cortex. Rather, aldosterone likely acts in *Drosophila* as a mimic of insect steroids (Fig 1A) or by providing a precursor for the synthesis of insect steroids. 20-hydroxyecdyone (20E) is the primary active steroid in *Drosophila*. 20E is oxidized from the prohormone ecdysone by 20-hydroxylase (encoded by *shade*) at target cells. 20E activates the nuclear hormone Ecdysone Receptor (EcR) to modulate transcription. Interestingly, feeding adults 20E for two weeks did not stimulate proteinuria, but proteinuria was elevated in adults chronically fed ecdysone (Fig 1D). Likewise, chronic aldosterone and ecdysone, but not 20E, suppressed dextran filtration by nephrocytes (Fig 1G). While only aldosterone and ecdysone affected nephrocyte function and associated proteinuria, all tested steroids (aldosterone, ecdysone and 20E) reduced survival of adults on high salt diet (Fig 1H), indicating that each exogenous hormone has some capacity to impart biological activity. We found no consistent association between exogenous steroids and adult survival on normal diet (Fig 1I).

**Elevated extracellular matrix drives renal dysfunction**

Pericardial nephrocytes and the heart tube are surrounded by extracellular matrix made of collagen-like proteins including Pericardin (Fig 1A), *col4a1* and Viking (27, 28, 35). Adults fed aldosterone and ecdysone for 24 hours induced *pericardin* (*prc*) mRNA in their cardiac-nephrocyte tissue, but not when fed 20E (Fig 2A). Collagen encoding-transcripts *col4a1* and Viking mRNA were not induced by any of these steroids (Fig 2B, C). Despite
induction of prc mRNA, overnight steroid feeding itself did not elevate proteinuria (Fig S1). In contrast, aldosterone and ecdysone fed to wildtype adults for two weeks had elevated extracellular matrix Pericardin protein (PRC) around the cardiac-nephrocyte complex (Fig 2D,E). Depletion of pericardin mRNA from cardiomyocytes (tin\textasciitilde4-gal4>prc(RNAi)) (efficiency in Fig S2) but not from nephrocytes (sns-gal4>prc(RNAi)) blocked the ability of aldosterone and ecdysone to induce excess PRC deposition (Fig 2D, E). We also determined that pericardin expression in cardiomyocytes was necessary for aldosterone and ecdysone to induce proteinuria and to repress nephrocyte filtration: depletion of prc mRNA from cardiomyocytes blocked the ability of aldosterone and ecdysone to induce pathology, while depletion of prc mRNA in nephrocytes did not (Fig 2F-K). In contrast, exogenous 20E continued to produce no effects on fibrosis or nephrocyte function, independent of prc knockdown (Fig 2D, F-K). Thus, cardiomyocytes appear to be the source of Pericardin protein that accumulates in response to chronic exposure to aldosterone and ecdysone, and impairs nephrocyte function.

The GPCR dopEcR is required for steroids to drive fibrosis

It is striking that ecdysone but not 20E induces pericardin expression and associated renal pathology in Drosophila. This suggests that PRC protein in the ECM can be regulated independently of EcR, the canonical nuclear hormone ecdysone receptor of 20E. Indeed, depletion of EcR by RNAi in cardiomyocytes did not prevent the steroid-dependent induction of prc mRNA (Fig 3A), or associated ECM accumulation (Fig 3H, I) and renal pathology (Fig 3C, E).

An alternative avenue for action involves Dopamine-EcR (DopEcR, CG18314), a membrane G-protein-coupled receptor (GPCR) of ecdysone that has been described in the fly brain (36, 37) (24, 38). We detected DopEcR mRNA in adult cardiac-nephrocyte tissue, and more so in adults fed aldosterone and ecdysone (Fig 3G). Consistent with a model where DopEcR is required for aldosterone and ecdysone to stimulate renal pathology,
cardiomyocyte-specific knockdown of DopEcR (via tin4-Gal4>DopEcR(RNAi)) blocked the ability of aldosterone and ecdysone to induce prc mRNA expression (Fig 3B), elevate proteinuria and inhibit nephrocyte filtration (Fig 3D, F). Likewise, DopEcR in cardiomyocytes is required for aldosterone and ecdysone to induce excess Pericardin protein (Fig 3H, J). In contrast, while elevated deposition of PRC was prevented by cardiac-specific KD of DopEcR, PRC was not inhibited in flies with nephrocyte-specific DopEcR or EcR knockdown (Fig 3H, I, J).

Petruccelli et al. (36, 37) demonstrated that DopEcR promoted ethanol sensitivity through suppression of epidermal growth factor receptor/extracellular signal-regulated kinase signaling (EGFR/ERK) in the brain. We asked if dEGFR was required for ecdysone and aldosterone to induce fly renal fibrosis. Knockdown of dEGFR by RNAi in cardiomyocytes prevents the development of renal pathology and fibrosis in adults treated with either hormone (Fig 4A-E).

Our work suggests that ecdysone acts as an agonist of DopEcR in the heart where receptor activation modulates organ fibrosis. Nevertheless, it is possible that treatment with exogenous ecdysone antagonizes production of endogenous steroids (E or 20E), and that this loss promotes fibrosis. If true, knockdown of endogenous ecdysone should itself promote fibrosis, and addition of exogenous ecdysone should not further increase fibrosis. To test this hypothesis, we employed a mutant of the nuclear zinc finger protein encoded by molting defective, DTS-3/mld (39), which inhibits transcription of enzymes required for endogenous ecdysone synthesis. DTS-3 flies grow and emerge normally at 18°C, while adults switched to 29°C produce little ecdysone. We grew cohorts of DTS-3 females and control-wildtype females following these temperature regimes. As expected, control-wildtype females showed little prc mRNA and PRC until treated with exogenous ecdysone (Fig 4F-H). Yet, contrary to the hypothesis, DTS-3 females, with anticipated low endogenous levels of ecdysone, also had low levels of prc mRNA and PRC until stimulated by exogenous ecdysone (Fig 4F-H). Exogenous ecdysone appears to positively promote fibrosis rather than act by repressing production of endogenous steroids.
Renal fibrosis naturally occurs with age and is modulated by ecdysone and dopEcR

To this point we have induced fibrosis by treating flies with external steroids, begging the question, what is the physiological relevance in fibrosis of endogenous ecdysone acting through DopEcR and dEGFR? Endogenous ecdysone is normally elevated in aging Drosophila, where whole animal titers increase several fold between young and aged flies (40). As well, Vaughan at al. (41) found the collagen Viking accumulates in Drosophila cardiac ECM with age. We therefore measured Pericardin protein in the heart-nephrocyte ECM of untreated young and aged flies. Pericardin increased about 2-fold in 6-week old flies relative to young adults (Fig 4I, J). This age-dependent fibrosis was prevented by knockdown of endogenous ecdysone synthesis in adults, using the DTS-3 system as above (Fig 4I, J). Likewise, knockdown of both DopEcR and dEGFR in cardiomyocytes prevented fibrosis in the aged flies (Fig 4I, J). These results suggest that PRC accumulation in the nephrocyte and cardiac-associated extracellular matrix is an intrinsic property of aging flies promoted by endogenous ecdysone acting through cardiac DopEcR and EGFR receptors.

Discussion

Mammalian aldosterone is synthesized from cholesterol in the adrenal cortex as a 21-carbon, C21-hydroxyl steroid to control plasma Na⁺ and K⁺, water balance and blood pressure. Insect ecdysone is a 27-carbon steroid with hydroxyl groups at C21 and C27 (Fig 1A). Adult Drosophila produce ecdysone in ovaries and several somatic tissues including the Malpighian tubules (40, 42). Circulating ecdysone is converted at target cells into 20-hydroxyecdysone (20E), which induces transcriptional programs by activating the nuclear hormone Ecdysone Receptor EcR. Our data show that exogenous aldosterone and ecdysone, but not 20E, stimulate deposition of PRC in adult heart-nephrocyte extracellular matrix acting through the G-protein coupled receptor DopEcR and not the canonical nuclear hormone receptor EcR. How aldosterone mimics ecdysone in this context remains unknown. Work is needed to determine if aldosterone has affinity to DopEcR, or if aldosterone acts as precursor molecule
that can be converted to ecdysone within *Drosophila*. We likewise do not understand why exogenous 20E does not stimulate fibrosis whereas ecdysone produces a strong response. Previous work found that 20E as well as ecdysone has affinity for DopEcR in isolated Sf9 cell membranes (43), while exogenous 20E modulates DopEcR activity measured from fly brain cAMP levels, by brain nicotine-induced Ca\(^{2+}\)-responses, and by adult behavior (36-38). It also remains to determine what roles ecdysone plays in the regulation of Pericardin at the heart during normal development; perhaps, we suggest, it facilitates cardiac remodeling during molt and pupation (44).

Ecdysone circulating in adult hemolymph may act at many sites aside from EcR in fat body and ovary (45), or from DopEcR in the fly brain (36, 37). Our genetic results indicate that *DopEcR* message is required specifically in cardiomyocytes to modulate steroid-induced fibrosis. Using newly emerging tools well suited to study GPCR in *Drosophila*, we anticipate future work can directly identify which cells in this heart produce functional dopEcR proteins (46, 47). Fibrosis in human hearts arises from myofibroblasts that secrete extracellular matrix proteins including fibronectins, elastins and collagens (48-50). Based on these parallels, we propose *Drosophila* cardiomyocytes and mammalian myofibroblasts have analogous functions to produce ECM.

We find that chronic induction of pericardin by steroid hormones stimulates excess PRC protein in the ECM surrounding the myocardial-nephrocyte cells, induces proteinuria and inhibits nephrocyte filtration. Excess heart-associated ECM was previously reported in aged *Drosophila*, measured by accumulation of Pericardin and the collagen subunit Viking (41). Here we also find PRC increases in cardiac-nephrocyte ECM of old females. Remarkably, systemic knockdown of adult ecdysone synthesis, which otherwise increases with age (40), prevents elevated Pericardin in aged females, as does cardiomyocyte knockdown of DopEcR (and EGFR). From our observation that steroids elevate *pericardin* mRNA, we propose that DopEcR promotes fibrosis during aging by inducing *pericardin* mRNA, and subsequent translation and secretion of Pericardin, rather than by modulating ECM breakdown.
DopEcR is a dual agonist receptor (51). In neurons, DopEcR transduces signals from both dopamine and ecdysone to regulate mating behavior and ethanol sensitivity (36, 37). Activation by dopamine induces cAMP-mediated signal transduction. Ecdysone has greater affinity to DopEcR than does dopamine, and through unknown mechanisms will displace dopamine and induce alternative signal transduction mediated by MAP kinases (43) (38). Reports are mixed on whether ecdysone also affects cAMP via DopEcR because dopamine alone can increase cAMP in Sf9 cells expressing DopEcR (37, 43). In mammalian cells, cAMP can induce PKA to phosphorylate CREB, which then localizes to promoters. Human CREB targets include several collagen genes, and cAMP stimulation suppresses collagen-I expression in a CREB dependent manner (52-54). Accordingly, we hypothesize that dopamine-cAMP-associated transduction initiated from DopEcR may negatively regulate pericardin.

In contrast to the potential action of dopamine, DopEcR stimulated by ecdysone can signal through dEGFR to ERK1/2 as seen in transfected Sf9 cells and in a neuronal analysis of ethanol induced sedation (36, 43). We now find myocardial dEGFR is also required for steroids to induce PRC and nephrocyte dysfunction, and for PRC to accumulate with age. In humans, EGFR signaling is a crucial regulator of fibrosis (55, 56). The EGFR ligands TGFα and epidermal growth factor are expressed in kidney cells where activated EGFR stimulates extracellular signal-regulated kinases1/2 (ERK1/2), Janus kinase/signal transducers and activators of transcription, and PI3-kinase/AKT. In renal interstitial fibrosis, EGFR regulates TGF-β1 via ERK1/2 to activate myofibroblasts and promote expression of ECM collagens (57). Notably, EGFR can be transactivated independent of its extracellular ligands, including by the activity of G-protein-coupled receptors (GPCR) such as the Angiotensin II receptor, and this action is mediated intracellularly by the sarcoma kinase Src. Furthermore, Src-mediated transactivation has been shown to accentuate renal fibrosis in mammals (58-60). Based on our current observations, we hypothesize that ecdysone-stimulated DopEcR might stimulate dSrc to facilitate ligand activation of dEGFR (Src42, (61)).
Studies in mammals suggest aldosterone may also signal via a membrane associated GPCR. GPER1 has been proposed to function as a non-genomic aldosterone receptor and as a potential homolog of DopEcR (21, 22, 62). GPER1-dependent induction by aldosterone is reported in renal cortical adenocarcinoma cells (17), and from mouse models with tissue specific mineralocorticoid receptor gene deletion (63). However, no data establish a mechanism of non-genomic action for aldosterone through GPER1 (23, 64), and the current steroid candidate for GPER1 is 17\(\beta\)-estradiol (65). Using the DIOPT Ortholog Prediction Tool, we identified several potential alternatives for the DopEcR homolog in the human genome including GRP52 (sequence similarity 46%) and UTS2R (sequence similarity 44%). GPR52 is an orphan G-protein coupled receptor described to modulate Huntingtin protein (HTT) through cAMP-dependent mechanisms (66). Knockdown of Gpr52 reduces HTT levels in a human tissue model, whereas neurodegeneration is suppressed by knockdown of DopEcR in Drosophila that express human Htt. The Urotensin II receptor (UTS2R) is a conserved GPCR implicated to function in renal fibrosis by trans-modulating EGFR and activating MAPK (67, 68). The kidneys of diabetic rats express elevated Urotensin II, and UTS2R is required for exogenous Urotensin to induce TGF-\(\beta\)1 and collagen in the renal ECM. If functional homology can be established between DopEcR and these mammalian candidates, Drosophila will provide a new model system to uncover mechanisms of fibrosis in humans.

Material and methods

Fly stocks. Unless noted, wildtype flies were yw (ywR). Tin\(\Delta\)4-Gal4 was a gift from the Manfred Frasch laboratory (69). sns-Gal4 was obtained from the Bloomington Stock Center (Stock #76160) and UAS-pericardin(RNAi) was obtained from the Vienna Drosophila Research Center (VDRC) (Stock #GD41321). UAS-EcR(RNAi) was from the laboratory of Neal Silverman (UMass Medical). From VDRC: DopEcR(RNAi), kk103494; dEGFR(RNAi), kk100051. Except to measure proteinuria, all assays were conducted with females because of their larger size facilitates dissection.
Steroid and diet treatment. Ecdysone (Sigma-Aldrich #E9004), 20-Hydroxyecdysone (Sigma-Aldrich #H5142) and Aldosterone (Sigma-Aldrich #A9477) were dissolved in ethanol at 5mg/ml. Flies were reared in bottles with emerging adults permitted to mate for 2-3 days. Adult were then separated by sex into 1L demography cages at ~ 120 adults per cage. Adults were fed standard laboratory cornmeal-yeast-sugar diet until age 7-10 days, at which time food media was switched to 0.5g Genesee Scientific instant fly media (Genesee Scientific #66-117) hydrated with 2ml of water containing vehicle control (150 ul ethanol) or vehicle with 150 ul of hormone solution. For chronic exposure to steroids, flies were treated for the next 14 days at 25 °C fly with media vials changed every 3 days. For overnight exposure to steroids, flies were maintained in demography cages with untreated instant fly media until age 20 days old, then exposed to diets with appropriate hormone conditions for 24 hours. In all trails, renal traits and prc mRNA were assessed in adults at 3 weeks old. The same protocols were used to expose adults to high salt or high sugar, where instant media was moistened with water containing 1.5% NaCl. To vary dietary glucose, adults were aged to 3 weeks on otherwise standard lab diet where glucose was set at 5% (control, normal) or at 34% (high sugar diet).

Proteinuria. For each biological replicate, frass of 15 males was collected for 2.5 hours in a 1.5ml centrifuge tube covered with a breathable foam plug, at 25 °C. Males were used in this assay to avoid complications of eggs also laid in the tubes by females. Deposited frass was fully dissolved with 20ul 1xPBS, providing 10ul to assess total protein and 10ul to measure uric acid, which serves as a proxy for the quantity of deposited frass. Total urine protein was determined by Pierce BCA Protein assay (Thermo Scientific #23227). Uric acid was measured by QuantiChrom Uric Acid Assay (Bioassay systems, DIUA-250).

Immunohistology. Nephrocyte-heart tissue from 3 w old females were dissected in PBS, fixed with 4% formaldehyde in PBS for 30 minutes and washed three times for 10 minutes with PBTA (1xPBS,1.5% BSA, 0.3% Tween20) at room temperature. The washed tissue was incubated with 100 ul primary antibody (mouse anti-Pericardin 1:100, Developmental Studies Hybridoma Bank) diluted in PBTA overnight at 4C, washed 3x10 minutes with 1ml PBTA at room temperature, then incubated in secondary antibody (goat anti-mouse Alexa488 1:200,
Alexa555-phalloidin 1:100, ThermoFisher Scientific) diluted in PBTA overnight, washed 3x10 minutes with 1ml PBTA at room temperature, and mounted. Confocal images were obtained with a Zeiss 800 and quantified by imageJ software. The full length of the heart tube, pericardial cells and associated ECM network was imaged from all samples at 488 nm with the same laser intensity setting to produce a Z-stack comprised of 46 optical slices.

**Nephrocyte filtration.** Adult nephrocyte-heart tissue was dissected in ADH (Artificial Drosophila Hemolymph, 108 mM Na+, 5 mM K+, 2 mM Ca²⁺, 8 mM MgCl₂, 1 mM NaH₂PO₄, 4 mM NaHCO₃, 10 mM sucrose, 5 mM trehalose, 5 mM Hepes, pH 7.1), incubated at 25°C for 15 minutes with AlexaFluor568-Dextran (10,000 MW, Life Technology) diluted in ADH at a concentration of 0.33mg/ml, washed 3x10 minutes with cold PBS at 4°C, then fixed in 4% formaldehyde for 10 minutes at room temperature, washed 3x10 minutes with PBS at room temperature, and mounted in PBS. Confocal images were obtained with a Zeiss 800 and quantified by imageJ software.

**Quantitative RT–PCR.** Total RNA was extracted from dissected renal-cardiac tissue in Trizol reagent (Invitrogen, Grand Island, NY, USA) and treated with DNase (Ambion). DNase-treated total RNA was quantified with a NanoDrop ND-1000. cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad) and measured on an ABI prism 7300 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). Three to 5 biological replicates were used for each experimental treatment (specifics in figure legends). In all figures, mRNA abundance of each gene is expressed relative to ribosomal protein L32 (rp49) mRNA from the same sample by the method of comparative CT.
Acknowledgements WZ and MT received support from NIH grant PO1 AG033561, NIDDK Diabetic Complications Consortium grant DK076169, and the office of the Dean of Biology and Medicine, Warren Alpert School of Medicine, Brown University. KO received support from NIH grant R01 HL132241. We acknowledge Erika Taylor, SBP Medical Discovery Institute, for technical assistance.

Competing interests
No competing interests declared.
References
7. G. H. Tesch, M. J. Young, Mineralocorticoid Receptor Signaling as a Therapeutic Target for Renal and Cardiac Fibrosis. Front Pharmacol 8, 313 (2017).


56. S. A.-O. Rayego-Mateos et al., Role of Epidermal Growth Factor Receptor (EGFR) and its Ligands in Kidney Inflammation and Damage.
60. Y. Qian et al., Novel Epidermal Growth Factor Receptor Inhibitor Attenuates Angiotensin II-Induced Kidney Fibrosis.
61. J. B. Cordero et al., c-Src drives intestinal regeneration and transformation. EMBO J 33, 1474-1491 (2014).
64. D. C. Rigiracciolo et al., GPER is involved in the stimulatory effects of aldosterone in breast cancer cells and breast tumor-derived endothelial cells. Oncotarget 7, 94-111 (2016).
Figures

A) [Image showing nephrocytes and other cell types]

B) [Bar graph showing protein levels for wildtype, Kirre-RNAi, and sns-RNAi]

C) [Bar graph showing protein levels for control, NaCl, and sugar]

D) [Bar graph showing protein levels for control, 20E, E, and Aldosterone]

E) [Images showing wildtype, sns-sns-RNAi, Aldosterone, and Ecdysone]

F) [Bar graph showing detrusor uptake for wildtype, sns-RNAi, kirre-RNAi]

G) [Bar graph showing detrusor uptake for control, 20E, E, and Aldosterone]

H) [Survival curve showing age vs. survival for 20E, Aldosterone, Control, and E]

I) [Survival curve showing age vs. survival for 20E, Aldosterone, Control, and E]
Figure 1. Aldosterone and ecdysone induce renal dysfunction in adult *Drosophila*. (A) Heart-renal structure illustrated by Vinald Francis, Brown University, modeled from image of (70): cardiomyocytes within the tubular heart and connective alary muscles, red; surrounding pericardial nephrocytes, blue; cardiac extracellular matrix (ECM) comprised of collagen Pericardin, green. Structures of steroid hormones: human aldosterone, and insect ecdysone (E) and 20-hydroxyecdysone (20E). (B) Proteinuria measured as excreted protein/uric acid in 3 w old males expressing RNAi in nephrocytes to deplete slit diaphragm proteins encoded by *kirre* or *sns* (each genotype, n=6 biological replicates with 15 males each). (C) Proteinuria in 3 w old males adults fed high salt diet and high sugar diet; combined data from four independent wildtype backgrounds, each with 4 biological replicates of n=20. Values normalized to control treatment within each background. (D) Proteinuria in 3 w old males fed 20-hydroxyecdysone (20E), ecdysone (E) or aldosterone for two weeks; combined data across with three wildtype backgrounds, each with four biological replicates of n=20. (E) Dextran-bead filtration assay for nephrocyte function; confocal images (representative z-stack) of nephrocytes of 3 w old females. Efficient filtration seen in wildtype; impaired filtration occurs with depletion of slit diaphragm (*sns-RNAi*) and by treatment of wildtype with aldosterone or ecdysone. (F, G) Fluorescence intensity (A.U. arbitrary units) quantified from biological replicates of nephrocytes from dextran-bead filtration assay when slit diaphragm is depleted by RNAi, and for wildtype adults treated with 20E, ecdysone or aldosterone (each genotype, n=5). Statistics in B-D, F, G: ANOVA with Dunnett’s post hoc comparison to control, * P < 0.05, ** P < 0.001; mean± s.d. (H) Survival upon high salt diet (1.5% NaCl) for cohorts (each, n = 230-330) continuously treated with 20E, ecdysone, or aldosterone relative to control. Survival was significantly reduced by each treatment, pair-wise contrasts to control, log-rank test, P < 0.001. (I) Survival upon normal diet for adults (each cohort, n=216-280) continuously treated with 20E, ecdysone, or aldosterone. Relative to control (median life span = 42 d), survival was increased by 20E (median life span = 50 d; log-rank test, P = 0.051), but not significantly affected by aldosterone (median lifespan = 48 d, log-rank test, P = 0.742) or ecdysone (median lifespan = 46 d, log-rank test, P = 0.185).
Figure 2. Pericardin from cardiomyocytes induced by steroids produces renal dysfunction. (A) pericardin (prc) mRNA in heart-nephrocyte tissue induced in females fed ecdysone (E) and aldosterone, but not 20-hydroxyecdysone (20E), expressed relative to ribosomal protein L32 (rp49) mRNA from the same sample (each genotype, n=5 biological replicates of 10 pooled tissues). (B, C) collagen-4a1 (col4a1) and viking mRNA, expressed relative to ribosomal protein L32 (rp49) mRNA from the same sample, in heart-nephrocyte tissue.
tissue are not induced by steroid hormones (each genotype, \( n=5 \) biological replicates of 10 pooled tissues). (D) Confocal images (representative z-stacks) of heart-nephrocyte tissue of 3 w old females after two-week treatment with 20-hydroxyecdysone, ecdysone or aldosterone; wildtype and knock-down genotypes to deplete prc mRNA in nephrocytes (\( sns\text{-gal4}\text{>UAS-prc(RNAi)} \)) and cardiomyocyte (\( tin\Delta4\text{-gal4}\text{>UAS-prc(RNAi)} \)). Phalloidin (red) stains cardiomyocyte actin; secondary antibody marks (green) Pericardin protein in extra-cellular matrix around nephrocytes and heart. (E) Quantification of straining intensity (A.U., arbitrary units) for protein Pericardin (PRC) in ECM (each genotype, each treatment, \( n=6 \)). (F-H) Proteinuria in 3 w old males fed for two weeks with 20-hydroxyecdysone, ecdysone or aldosterone, assessed in wildtype background (yw/UAS-prc(RNAi)), and in genotypes that reduce pericardin (UAS-prc(RNAi)) in nephrocytes (\( sns\text{-gal4} \) or cardiomyocytes (\( tin\Delta4\text{-gal4} \)); (each genotype, each treatment: \( n=5 \) biological replicates with 15 males each). (I-K) Quantification of fluorescence intensity from biological replicates of nephrocytes in ex vivo dextran-bead filtration assay in 3 w old males fed for two weeks with 20-hydroxyecdysone, ecdysone or aldosterone, assessed in wildtype (yw/UAS-prc(RNAi)), and in genotypes that reduce pericardin (UAS-prc(RNAi)) in nephrocytes (\( sns\text{-gal4} \) or cardiomyocytes (\( tin\Delta4\text{-gal4} \)) (each genotype, each treatment: \( n=3 \)). A-C, E-K: One-way ANOVA with Dunnett’s comparison relative to control, \(* \ P < 0.05, \ P < 0.01; \) mean±s.d.
Figure 3. Cardiomyocyte DopEcR is required for steroid induction of fibrosis and renal pathology. Depletion of nuclear hormone receptor EcR by RNAi did not block ability of ecdysone and aldosterone to induce: (A) increases in heart-nephrocyte prc mRNA, relative to Rp49 (each genotype, n=5 biological replicates of 10 pooled tissues); (C) increases in proteinuria (each treatment, n=3 biological replicates with 15 males each); and (E) reduced
nephrocyte filtration (each treatment: \( n=3 \)). Depletion of GPCR DopEcR by RNAi blocked the ability of ecdysone and aldosterone to induce (B) increased heart-nephrocyte \( prc \) mRNA, relative to \( Rp49 \) (each genotype, \( n=3 \) biological replicates of 10 pooled tissues); (D) increased proteinuria (each treatment, \( n=4 \) biological replicates with 15 males each); and (F) reduced nephrocyte filtration (each treatment: \( n=3 \)). (G) \( DopEcR \) mRNA, relative to \( Rp49 \), is elevated in heart-nephrocyte of 3 w old adults treated overnight with ecdysone or aldosterone (each treatment, \( n=3 \) biological replicates of 10 pooled tissues). (H) Confocal images (representative z-stacks) of heart-nephrocyte from 3 w old females after two-week treatment with ecdysone or aldosterone, with genotypes to deplete \( EcR \) or \( DopEcR \) mRNA in nephrocytes (\( sns\)-gal4) or cardiomyocytes (\( tin\Delta4\)-gal4). Cardiomyocyte actin stained by phalloidin, red. Pericardin protein of extra-cellular matrix, green. (I, J) Quantification of PRC staining intensity (each genotype, each treatment, \( n=6 \)), with genotypes to deplete \( EcR \) mRNA (I) or \( DopEcR \) mRNA (J) in nephrocytes (\( sns\)-gal4) or cardiomyocytes (\( tin\Delta4\)-gal4). A-G, I, J: One-way ANOVA with Dunnett’s comparison relative to control, * \( p < 0.05 \), **\( p < 0.01 \); mean±s.d.
Figure 4. Ecdysone and aldosterone require dEGFR in cardiomyocytes to induce ECM Pericardin. (A) Representative z-stack confocal images of hearts from wildtype (top) and DopEcR knockdown hearts (bottom). (B) The level of proteinuria in wildtype (wt) flies was increased by ecdysone feeding but showed not increase in flies with RNAi-mediated cardiac knockdown of dEGFR (each treatment, n=5 biological replicates with 15 males each). (C)
Dextran filtration as a measure of nephrocyte function was reduced in flies fed E or Aldo; this reduction was blocked by cardiac dEGFR knockdown. (D) prc mRNA, relative to Rp49, was induced by E or Aldo feeding (each genotype, n=3 biological replicates of 10 pooled tissues). (E) Quantification of PRC staining intensity, with genotypes to deplete dEGFR mRNA from cardiomyocytes (tinΔ4-gal4) (each genotype, each treatment, n=4). F) Pericardin in ECM, confocal images from control and ecdysone treated wildtype and yw/DTS-3 females, with (G) prc mRNA relative to Rp49 (each genotype, each treatment, n=3 biological replicates of 10 pooled tissues). (H) PRC intensity quantified (each genotype, each treatment, n=4). Statistics in B-E, G, H: ANOVA with Dunnett’s post hoc comparison to within genotype control, * P < 0.05, ** P < 0.001; mean± s.d. (I, J) PRC in the ECM increases with age (between 1 to 6 w) without exogenous hormone treatments. Age-associated fibrosis is prevented in the DTS-3 mutant, and when DopEcR or dEGFR knocked down in cardiomyocytes (each knockdown genotype, each age, n=4; all wildtype controls combined, each age, n=10 ); One-way ANOVA with Dunnett’s comparison relative to 1 w, wildtype, **P < 0.01, mean±s.d.
**Fig. S1.** Overnight feeding of aldosterone to 3 week-old males did not increase proteinuria.

![Graph showing proteinuria levels](image)

**Fig S2.** Validation of RNAi efficiency in cardiomyocytes, driven by tinΔ4-gal4. Mean expression levels were determined by qPCR (+/- SD, normalized to rp49) from heart-nephrocyte tissue; dissected from 20 day old females; three replicates per genotype. All differences significant at p < 0.02.

- tinΔ4>prc(RNAi)
- tinΔ4>dEGFR(RNAi)
- tinΔ4>dopEcR(RNAi)
Table 1. Primers for qPCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>F (5')</th>
<th>R (3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rp49</td>
<td>GCA CTC TCT GTT GTC GAT ACC CTT G</td>
<td>AGC GCA CCA AGC ACT TCA TC</td>
</tr>
<tr>
<td>Pericardin</td>
<td>CGG AGG ACA GGC TAC AAT AAG</td>
<td>TTC CAG GCT GAG TTT CGT ATC</td>
</tr>
<tr>
<td>Col4a1</td>
<td>GCT CTG TGC GAT TTG AGT TTG</td>
<td>CTT CTG CTC CCT TGA ATC CTT</td>
</tr>
<tr>
<td>Viking</td>
<td>GAT CTA CGA CAA CAC TGG TGA G</td>
<td>TTC GCC ACG AAG TCC AAT AG</td>
</tr>
<tr>
<td>EcR</td>
<td>TGA AGA CTC CTA TGC TGC</td>
<td>CGA CGT TGT GCT TCG TAA</td>
</tr>
<tr>
<td>dopEcR</td>
<td>CTT AGG TCC CAG CCT CAT TTC</td>
<td>AGC CAG AGC AGT TGC ATA TT</td>
</tr>
</tbody>
</table>