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GLP-1 receptor activation and Epac2 link atrial natriuretic peptide secretion to control of blood pressure

Minsuk Kim[1], Mathew J Platt[2], Tadao Shibasaki[3], Susan E Quaggin[1], Peter H Backx[4], Susumu Seino[3], Jeremy A Simpson[2] & Daniel J Drucker[1]

Glucagon-like peptide-1 receptor (GLP-1R) agonists exert antihypertensive actions through incompletely understood mechanisms. Here we demonstrate that cardiac *Glp1r* expression is localized to cardiac atria and that GLP-1R activation promotes the secretion of atrial natriuretic peptide (ANP) and a reduction of blood pressure. Consistent with an indirect ANP-dependent mechanism for the antihypertensive effects of GLP-1R activation, the GLP-1R agonist liraglutide did not directly increase the amount of cyclic GMP (cGMP) or relax preconstricted aortic rings; however, conditioned medium from liraglutide-treated hearts relaxed aortic rings in an endothelium-independent, GLP-1R–dependent manner. Liraglutide did not induce ANP secretion, vasorelaxation or lower blood pressure in *Glp1r*$^{-/-}$or *Nppa*$^{-/-}$ mice. Cardiomyocyte GLP-1R activation promoted the translocation of the Rap guanine nucleotide exchange factor Epac2 (also known as Rapgef4) to the membrane, whereas Epac2 deficiency eliminated GLP-1R–dependent stimulation of ANP secretion. Plasma ANP concentrations were increased after refeeding in wild-type but not *Glp1r*$^{-/-}$ mice, and liraglutide increased urine sodium excretion in wild-type but not *Nppa*$^{-/-}$ mice. These findings define a gut-heart GLP-1R–dependent and ANP–dependent axis that regulates blood pressure.

Type 2 diabetes mellitus (T2D) is a common metabolic disorder characterized by defects in pancreatic beta-cell function and insulin sensitivity. The increasing incidence of obesity has contributed to a rising prevalence of diabetes worldwide, focusing attention on the effectiveness of therapeutic strategies used to treat these disorders. Although several new classes of antidiabetic agents have been approved over the past decade, T2D is a progressive disease, and most patients require multiple agents to achieve effective control of their diabetes.

Attenuation of the morbidity and mortality attributable to cerebrovascular and cardiovascular disease in subjects with diabetes requires a comprehensive understanding of the cardiovascular actions of antidiabetic agents[1]. Indeed, the safety of newer medications used for the treatment of T2D is being scrutinized in large cardiovascular outcome studies. Hence, there is considerable interest in understanding the actions of glucose-lowering drugs in the cardiovascular system independent of their glucoregulatory effects. One new class of antidiabetic agents, GLP-1R agonists, exerts multiple cardiovascular actions that are potentially favorable for subjects with T2D[2–5]. GLP-1R agonists promote satiety, leading to weight loss[6]. Furthermore, GLP-1R activation decreases postprandial lipemia through direct suppression of intestinal chylomicron secretion. Notably, GLP-1R agonists reduce blood pressure in hypertensive subjects with T2D or obesity[3,5,6]; however, the mechanisms through which GLP-1 lowers blood pressure remain poorly understood[3,5].

It has been postulated that GLP-1 reduces blood pressure by acting directly on blood vessels to produce vasodilation, perhaps targeting smooth muscle or endothelial cells and activation of nitric oxide–dependent mechanisms[7–9]. GLP-1R agonists produce weight loss in most subjects, which may indirectly reduce inflammation and blood pressure[10]. GLP-1 also has actions on the kidney that could affect blood pressure: it promotes rapid natriuresis in rats, mice and humans and modulates renal hemodynamics and the phosphorylation of proteins involved in sodium handling in isolated kidney cells[11–14]. Further complicating the understanding of how GLP-1 acts on blood vessels, native GLP-1 and its primary degradation product, GLP-$1_{9–36}$, produce vasodilation and enhance blood flow in different vascular beds independently of the known GLP-1 receptor[15]. We now describe studies elucidating new mechanisms linking GLP-1R activation to the control of blood pressure.

RESULTS

Liraglutide reduces blood pressure through *Glp1r*

We infused angiotensin II (Ang II) or vehicle subcutaneously to increase blood pressure in C57BL/6 wild-type (WT) mice. Ang II increased both systolic and diastolic blood pressure (**Fig. 1a–c**), whereas the degradation-resistant GLP-1R agonist liraglutide[16] produced a rapid and significant reduction in systolic (~23 mm Hg) and diastolic (~19 mm Hg) blood pressure (**Fig. 1a–c**). As the vascular effects of GLP-1 may be transduced independently of the known

[1]Department of Medicine, Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, University of Toronto, Toronto, Ontario, Canada. [2]Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada. [3]Department of Physiology and Cell Biology, Division of Cellular and Molecular Medicine, Kobe University Graduate School of Medicine, Chuo-ku, Kobe, Japan. [4]University Health Network and Department of Physiology, University of Toronto, Toronto, Ontario, Canada. Correspondence should be addressed to D.J.D. (drucker@lunenfeld.ca).

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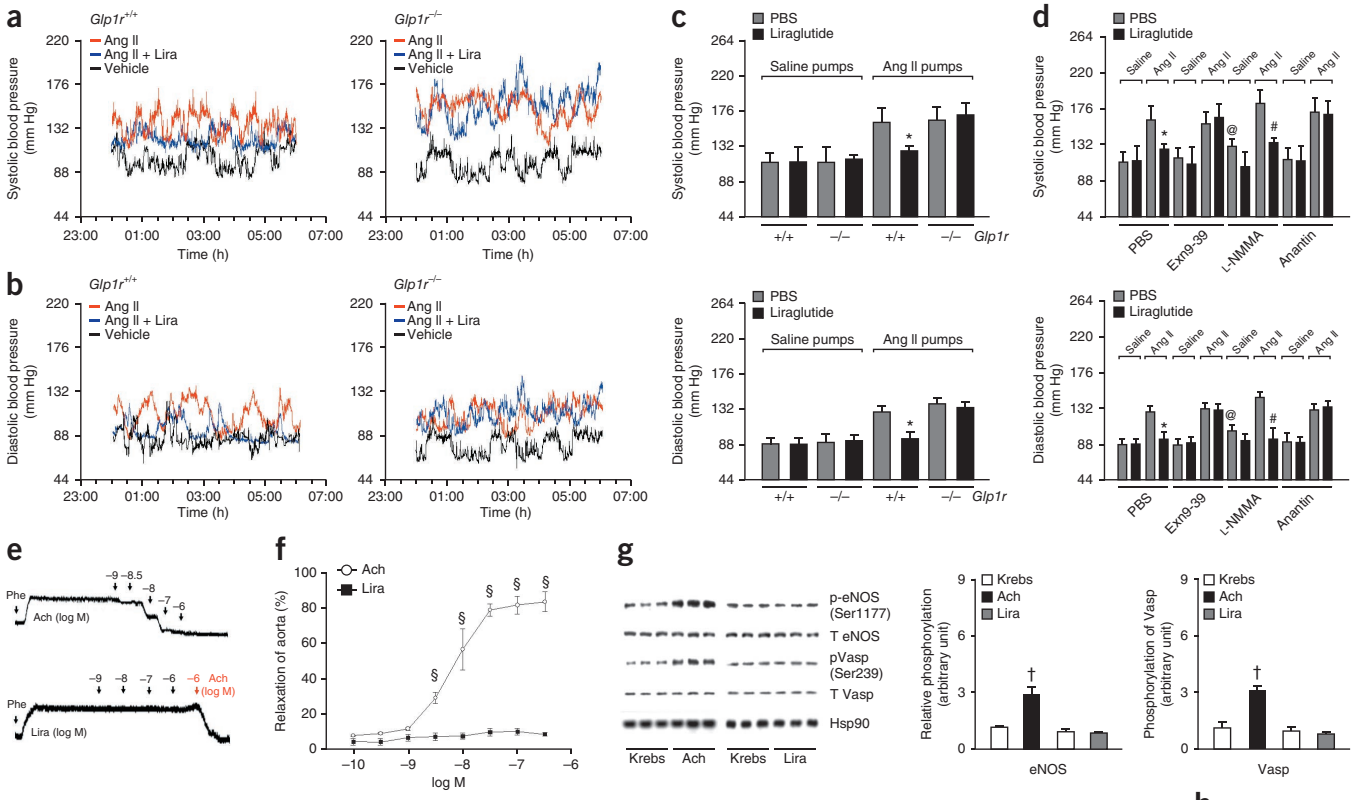

Figure 1 Liraglutide reduces blood pressure and promotes aortic relaxation. (**a**–**c**) Systolic and diastolic blood pressure in WT and *Glp1r*$^{-/-}$ mice infused with Ang II alone (red), Ang II plus acute liraglutide (Lira; blue) or saline (vehicle; black). (**d**) Systolic and diastolic blood pressure in WT mice pretreated with exendin$_{9-39}$ (Exn9–39), L-NMMA or anantin before acute liraglutide or saline (PBS) injection. (**e**,**f**) Concentration-response curves recorded in the presence of Ach (10^{-9}–10^{-6} M) or liraglutide (10^{-9}–10^{-6} M) of WT aortic rings that had been contracted with Phe (10^{-5} M). (**g**) Total (T) and phosphorylated eNOS and Vasp in aortic ring extracts after treatment with Krebs buffer, liraglutide or Ach analyzed by western blotting. Hsp90, heat shock protein 90. (**h**) cGMP content determined by enzyme-linked immunoassay (EIA) in aortic ring extracts after treatment with Krebs buffer, liraglutide or Ach. Data (**c**,**d**,**f**–**h**) are shown as the means ± s.e.m. of 3–6 mice per group, $^{*}P < 0.05$ compared to the PBS-treated Ang II–infused *Glp1r*$^{+/+}$ group, $^{\#}P < 0.05$ compared to the L-NMMA–pretreated Ang II–infused *Glp1r*$^{+/+}$ saline-treated group, $^{@}P < 0.05$ compared to the PBS-treated group, $^{\S}P < 0.05$ compared to the liraglutide-treated group, $^{\dagger}P < 0.05$ compared to the Krebs-treated groups. Statistical significance was determined by analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test.

GLP-1 receptor, GLP-1R[15], we assessed the action of liraglutide in *Glp1r*$^{-/-}$ mice[17]. Liraglutide did not reduce systolic or diastolic blood pressure after Ang II infusion in *Glp1r*$^{-/-}$ mice (**Fig. 1a**–**c**). To delineate the mechanisms through which liraglutide lowers blood pressure, we pretreated WT mice with exendin$_{9-39}$ (a GLP-1R antagonist), L-NMMA (a nitric oxide synthase inhibitor) or anantin (a natriuretic peptide receptor antagonist) for 2 d. Both exendin$_{9-39}$ and anantin blocked the antihypertensive actions of liraglutide, whereas L-NMMA had no effect (**Fig. 1d**).

The ability of anantin to block the antihypertensive actions of liraglutide implies a role for the natriuretic peptide receptor A as a target for the action of liraglutide. Consistent with the hypothesis that liraglutide relaxes vascular tone indirectly, liraglutide had no direct vasorelaxant effect on aortic rings submaximally constricted with phenylephrine (Phe), whereas the direct vasodilator acetylcholine (Ach) produced a dose-dependent relaxation of aortic rings in control experiments (**Fig. 1e**,**f**). Ach directly increased the amounts of phosphorylated endothelial nitric oxide synthase (eNOS) and vasodilator-stimulated phosphoprotein (Vasp), a downstream mediator of nitric oxide–cGMP signaling (**Fig. 1g**), and increased cGMP content in preconstricted aortic rings (**Fig. 1h**), whereas liraglutide had no direct effect on the amounts of phosphorylated eNOS (p-eNOS), phosphorylated Vasp (pVasp) or cGMP in the same experiments (**Fig. 1g**,**h**). These observations imply that liraglutide probably does not exert direct vasodilatory effects on blood vessels.

Liraglutide stimulates ANP secretion

As anantin inhibited the antihypertensive actions of liraglutide (**Fig. 1d**), we assessed whether liraglutide stimulated the secretion of atrial natriuretic peptide (ANP) or brain natriuretic peptide (BNP). Acute liraglutide administration produced a rapid 1.8-fold increase in plasma ANP concentrations in normotensive saline-infused WT mice (**Fig. 2a**). Plasma ANP concentrations were significantly higher in Ang II–infused mice than in saline-infused mice, and we found a biphasic response to liraglutide, with an initial fall in ANP concentration followed by a robust (3.7-fold) increase in plasma ANP concentration at 160 min (**Fig. 2a**). In contrast, liraglutide did not increase plasma ANP concentrations in *Glp1r*$^{-/-}$ mice (**Fig. 2a**). Although basal plasma concentrations of the structurally and functionally related BNP were increased in hypertensive mice, we found no changes in plasma BNP concentration in saline-infused or Ang II–infused WT or *Glp1r*$^{-/-}$ mice after liraglutide administration (**Supplementary Fig. 1a**,**b**). To determine whether the acute effects of liraglutide in increasing ANP concentration and lowering blood pressure were sustained with

repeated administration, we administered liraglutide twice daily for 3 weeks. Liraglutide continued to significantly ($P < 0.05$) increase ANP concentrations (**Supplementary Fig. 2a–c**) and reduce blood pressure (**Supplementary Fig. 2d–i**), predominantly during the evening lights-off period, in Ang II–infused mice after 2 and 3 weeks of repeated injection, although the magnitude of the increase in plasma ANP concentration was reduced over time. In contrast, liraglutide had no consistent effect on blood pressure during the lights-on period and

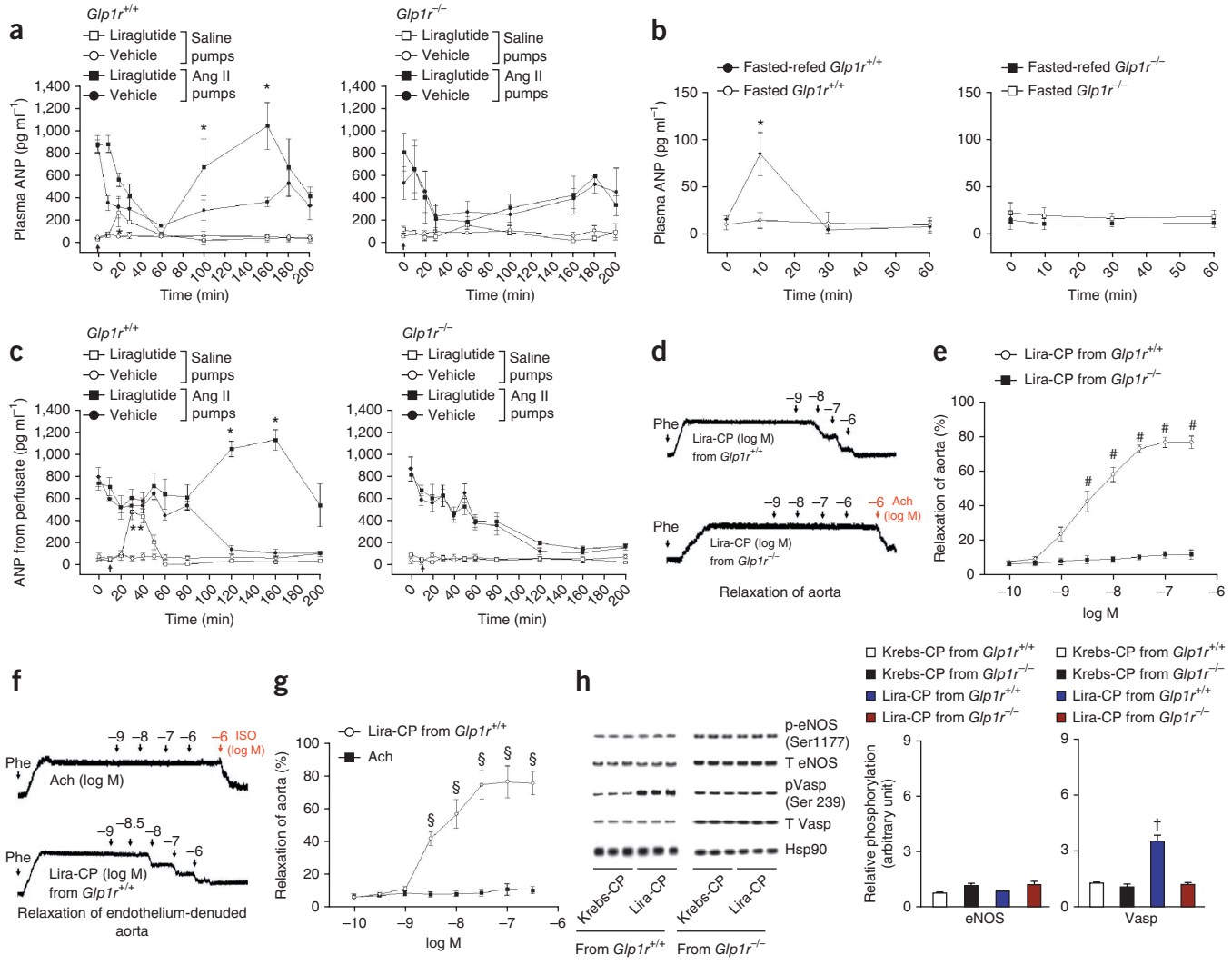

Figure 2 Liraglutide stimulates cardiac ANP secretion and elicits aortic vasodilation. (**a**) Plasma ANP concentrations in *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ mice infused with Ang II or saline for 3 weeks and treated with liraglutide (30 μg per kg body weight intraperitoneally (i.p.)) or vehicle (saline). (**b**) Plasma ANP concentrations in fasted and fasted-refed *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ mice. (**c**) ANP concentrations in coronary perfusate from normotensive or hypertensive *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ mice treated acutely with liraglutide or vehicle (saline). Arrows indicate the time of liraglutide injection. (**d**,**e**) Concentration-response curves recorded in isolated aortic rings in the presence of perfusate from *Glp1r*$^{+/+}$ or *Glp1r*$^{-/-}$ hearts perfused with liraglutide (liraglutide-conditioned perfusate (Lira-CP); 10^{-9}–10^{-6} M)). (**f**,**g**) Relaxation of endothelium-denuded aorta incubated with either Lira-CP from WT hearts or Ach. Isoproterenol (ISO; 10^{-6} M) was administered at the end of the experiment as indicated. (**h**) Total (T) and phosphorylated eNOS and Vasp, as determined by western blot analysis, in aortic rings from *Glp1r*$^{+/+}$ or *Glp1r*$^{-/-}$ mice treated with Krebs- or liraglutide-conditioned perfusate. Krebs-CP, Krebs-conditioned perfusate. (**i**) Amounts of cGMP determined by EIA in aortic extracts incubated with the indicated conditioned perfusates from *Glp1r*$^{+/+}$ or *Glp1r*$^{-/-}$ hearts. (**j**) Amounts of *Glp1r* and *Gapdh* mRNA transcripts in atrial (AT) and ventricular (VN) cardiomyocyte RNA isolated from *Glp1r*$^{+/+}$ or *Glp1r*$^{-/-}$ hearts after saline or Ang II infusion for 3 weeks. P.C., positive control (lung RNA from *Glp1r*$^{+/+}$ mice). Blots were hybridized (HyB) with an internal oligoneucleotide probe directed against mouse *Glp1r*. Data (**a**–**c**,**e**,**g**–**i**) are shown as the means ± s.e.m. of 3–6 mice per group. $^{*}P < 0.05$ compared to the vehicle-treated group, $^{\#}P < 0.05$ compared to the Lira-CP from *Glp1r*$^{-/-}$ group, $^{\S}P < 0.05$ compared to the Ach-treated group, $^{\dagger}P < 0.05$ compared to the Krebs-CP and Lira-CP from *Glp1r*$^{-/-}$ groups. Statistical significance was determined by ANOVA followed by Bonferroni's *post hoc* test.

only transiently increased plasma ANP concentrations during this period (**Supplementary Fig. 2a–i**).

The actions of liraglutide to stimulate ANP and reduce blood pressure were not restricted to the Ang II model of hypertension. We also observed these effects in a pressure overload model of hypertension secondary to transaortic constriction (TAC) (**Supplementary Fig. 3**); liraglutide administered for 2 weeks reduced systolic blood pressure, decreased heart weights and increased ANP concentrations (**Supplementary Fig. 3a–c**). Furthermore acute administration of liraglutide rapidly reduced blood pressure and increased ANP concentrations in hypertensive mice after TAC (**Supplementary Fig. 3d–f**). To assess whether ANP secretion is induced by structurally distinct GLP-1R agonists, we compared the effects of liraglutide with those of native GLP-1 and exendin-4 in Ang II–infused hypertensive mice. Both liraglutide and exendin-4 produced sustained reductions in systolic and diastolic blood pressure during the evening period (**Supplementary Fig. 4a–d**), whereas injection of native GLP-1 produced a much smaller and transient reduction in blood pressure (**Supplementary Fig. 4e,f**). Consistent with these findings, plasma ANP concentrations were robustly increased after administration of liraglutide and exendin-4 but rose only modestly after native GLP-1 administration (**Supplementary Fig. 4g,h**). We hypothesized that native GLP-1 was rapidly degraded and inactivated *in vivo*; hence, we examined ANP secretion from atrial cardiomyocytes *in vitro*. Native GLP-1, liraglutide and exendin-4 all robustly increased ANP secretion from WT cardiomyocytes, and these stimulatory effects were absent in $Glp1r^{-/-}$ cardiomyocytes (**Supplementary Fig. 4i**). In contrast, the N-terminally cleaved peptide GLP-1_{9-36}, a weak GLP-1R agonist, did not increase ANP secretion.

We next asked whether transient meal-induced increases in endogenous GLP-1 would be sufficient to increase plasma ANP concentrations in mice. Provision of food to fasted WT mice produced a rapid increase in plasma ANP concentration at 10 min (**Fig. 2b**). In contrast, food ingestion had no effect on plasma ANP concentrations in $Glp1r^{-/-}$ mice (**Fig. 2b**). Taken together, these findings demonstrate that both pharmacological and physiological activation of GLP-1R promote ANP secretion.

To determine whether GLP-1R activation directly promotes cardiac ANP secretion from the intact heart, we measured ANP concentrations in cardiac perfusate from isolated mouse hearts. Liraglutide administration to the isolated mouse heart produced a rapid rise in ANP concentrations (an approximate fivefold increase after administration of liraglutide; **Fig. 2c**) in heart perfusates from normotensive mice. Basal ANP concentrations were higher in perfusate from hearts of Ang II–treated hypertensive mice compared to saline-infused mice; however, liraglutide still significantly increased ANP concentrations in perfusate of isolated hearts from Ang II–treated mice, with approximately tenfold higher ANP concentrations after perfusion with liraglutide compared to Krebs buffer alone (**Fig. 2c**). Although Ang II infusion increased basal ANP concentrations in cardiac perfusate of hearts from $Glp1r^{-/-}$ mice, liraglutide did not increase ANP concentrations in perfusate from saline-infused or Ang II–infused $Glp1r^{-/-}$ mice (**Fig. 2c**). Furthermore, liraglutide had no effect on BNP concentrations in coronary perfusate from hearts of normotensive or hypertensive WT or $Glp1r^{-/-}$ mice (**Supplementary Fig. 1c,d**).

These data indicate that liraglutide directly enhances cardiac ANP secretion, which in turn probably reduces blood pressure through stimulating natriuresis [13,14] and vasodilatory actions on blood vessels[18]. Consistent with these mechanisms of action, perfusate from liraglutide-treated WT hearts produced a robust dose-dependent relaxation of preconstricted aortic rings, whereas perfusate from $Glp1r^{-/-}$ hearts had no effect on aortic relaxation (**Fig. 2d,e**). Endothelial denudation was associated with loss of the vasodilatory response to Ach but preservation of the vasodilatory response to perfusate from liraglutide-treated mice (**Fig. 2f,g**), consistent with the known vascular smooth muscle localization and function of the ANP receptor guanylyl cyclase A[18,19]. Perfusate from liraglutide-treated WT hearts had no effect on the amount of p-eNOS but robustly increased the amount of pVasp in aortic rings (**Fig. 2h**). In contrast, perfusate from liraglutide-treated $Glp1r^{-/-}$ hearts did not enhance Vasp phosphorylation in aortic preparations (**Fig. 2h**). These results establish a GLP-1R–ANP axis that produces vascular smooth muscle relaxation and blood pressure reduction in the hypertensive mouse. Consistent with activation of ANP-dependent signal transduction through the guanylyl cyclase-linked ANP receptor, conditioned medium from liraglutide-perfused WT but not $Glp1r^{-/-}$ hearts significantly increased aortic cGMP accumulation (**Fig. 2i**). Moreover, *Glp1r* mRNA transcripts were predominantly localized to the atria, not the ventricles, of hearts from normotensive and hypertensive mice (**Fig. 2j**), consistent with our functional data and studies of chamber-specific G protein–coupled receptor expression in the heart[20].

Liraglutide promotes natriuresis and vasorelaxation via ANP

We next determined the importance of ANP for the antihypertensive actions of liraglutide using ANP knockout ($Nppa^{-/-}$) mice. Liraglutide increased urinary sodium excretion from normotensive and hypertensive Ang II–infused WT mice but did not augment urinary sodium in $Nppa^{-/-}$ mice (**Fig. 3a**). The failure of liraglutide to increase urinary sodium excretion in $Nppa^{-/-}$ mice was not attributable to defective expression of cardiac *Glp1r*, as the amounts of atrial *Glp1r* mRNA transcripts were comparable in RNA from $Nppa^{-/-}$ and $Nppa^{+/+}$ atria (**Fig. 3b**). Baseline blood pressure was significantly higher (8–20 mm Hg) in $Nppa^{-/-}$ compared to $Nppa^{+/+}$ mice (**Fig. 3c,d**). Although liraglutide significantly reduced systolic and diastolic blood pressure in hypertensive Ang II–infused $Nppa^{+/+}$ mice, liraglutide did not reduce blood pressure in $Nppa^{-/-}$ mice (**Fig. 3c,d**).

The lack of a blood pressure response to liraglutide in $Nppa^{-/-}$ mice did not reflect a generalized defect in GLP-1R signaling, as liraglutide reduced glycemic excursion, increased plasma insulin concentrations and inhibited food intake and gastric emptying in $Nppa^{-/-}$ mice (**Supplementary Fig. 5a–h**). Perfusate from liraglutide-treated $Nppa^{+/+}$ hearts had vasorelaxant activity and increased phosphorylation of Vasp but not of eNOS in isolated preconstricted atrial rings (**Fig. 3e–i**); however, perfusate from liraglutide-treated $Nppa^{-/-}$ hearts had no effect on Vasp phosphorylation or aortic ring relaxation (**Fig. 3e–i**) and did not increase the amount of cGMP in aortic ring preparations (**Fig. 3j**). Hence, GLP-1R activation promotes ANP secretion from mouse atria, which in turn activates vascular Vasp phosphorylation, cGMP formation, aortic smooth muscle relaxation and natriuresis, contributing to a reduction in blood pressure.

Liraglutide increases ANP secretion through Epac2

As GLP-1 activates cyclic AMP (cAMP)-dependent pathways in beta cells[21], we assessed GLP-1R signaling in cardiomyocytes. Liraglutide increased the amounts of cAMP in normotensive and Ang II–infused hypertensive $Glp1r^{+/+}$ atrial cardiomyocytes but not $Glp1r^{-/-}$ cardiomyocytes (**Fig. 4a**), whereas the β-adrenergic agonist isoprenaline was similarly effective in cardiomyocytes from $Glp1r^{-/-}$ and $Glp1r^{+/+}$ mice.

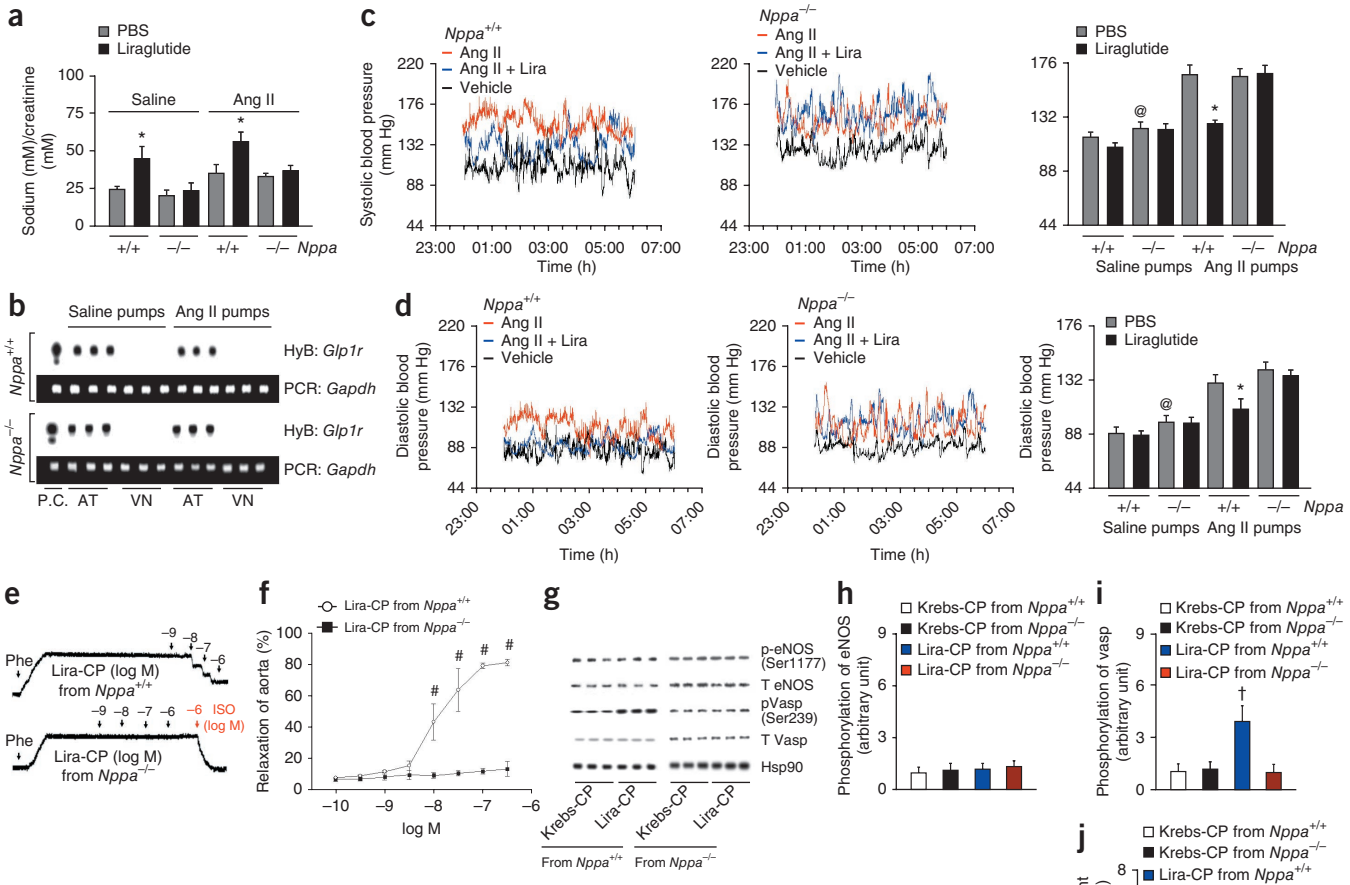

Figure 3 Liraglutide does not stimulate natriuresis or lower blood pressure in hypertensive *Nppa*$^{-/-}$ mice. (**a**) Urine sodium in *Nppa*$^{+/+}$ and *Nppa*$^{-/-}$ mice after treatment with liraglutide or saline (PBS). Data are shown as the means ± s.e.m. of 4 mice per group. $^{*}P < 0.005$ compared to the PBS-treated group. (**b**) Amounts of *Glp1r* and *Gapdh* mRNA transcripts in atrial (AT) and ventricular (VN) cardiomyocytes from saline-infused or Ang II–infused *Nppa*$^{+/+}$ and *Nppa*$^{-/-}$ mice. P.C., positive control (lung RNA from *Glp1r*$^{+/+}$ mice). (**c**,**d**) Systolic and diastolic blood pressure in *Nppa*$^{+/+}$ and *Nppa*$^{-/-}$ mice after saline or Ang II administration (red) and acute treatment with liraglutide (Lira; blue) or vehicle (black) at 9 a.m. and 4 p.m. (**e**,**f**) Aortic ring concentration-response curves in the presence of liraglutide-conditioned perfusate (Lira-CP; 10^{-9}–10^{-6} M) from *Nppa*$^{+/+}$ or *Nppa*$^{-/-}$ hearts. Isoproterenol (ISO; 10^{-6} M) was administered at the end of the experiment as indicated. (**g**–**i**) Total (T) and phosphorylated eNOS and Vasp, as determined by western blot analysis, in aortic rings from *Nppa*$^{+/+}$ or *Nppa*$^{-/-}$ mice treated with Krebs- or liraglutide-conditioned perfusate. Krebs-CP, Krebs-conditioned perfusate. (**j**) Amounts of cGMP determined by EIA in aortic extracts incubated with the indicated conditioned perfusates from *Nppa*$^{+/+}$ or *Nppa*$^{-/-}$ hearts. Data (**c**,**d**,**f**,**h**–**j**) are shown as the means ± s.e.m. of 3–5 mice per group. $^{*}P < 0.05$ compared to PBS-treated Ang II–infused *Nppa*$^{+/+}$ mice, $^{\#}P < 0.05$ compared to the Lira-CP from *Nppa*$^{-/-}$ group, $^{@}P < 0.05$ compared to PBS-treated *Nppa*$^{+/+}$ mice, $^{\dagger}P < 0.05$ compared to all other groups. Statistical significance was determined by ANOVA followed by Bonferroni's *post hoc* test.

We next perfused isolated hearts with antagonists and signal transduction inhibitors (H-89, an inhibitor of protein kinase A (PKA); SB 203580, an inhibitor of p38 MAP kinase; and 2-APB, an inositol 1,4,5-triphosphate receptor antagonist) immediately before administration of vehicle or liraglutide and collected coronary effluents for analysis of ANP. Liraglutide increased ANP secretion *ex vivo* independently of PKA, p38 MAP kinase or the inositol 1,4,5-triphosphate receptor (**Fig. 4b**,**c**). However, GLP-1R antagonism with exendin$_{9-39}$ or inhibition of phospholipase C (PLC) with U73122 reduced the induction of ANP secretion by liraglutide (**Fig. 4b**,**c**). The Epac-selective activator (8-pCPT-2-O-Me-cAMP-AM, designated ESCA-AM) alone augmented ANP secretion (**Fig. 4b**,**c**), whereas H-89 was not able to prevent ANP secretion after liraglutide perfusion (**Fig. 4b**,**c**). Notably, the stimulatory effects of ESCA-AM and liraglutide on ANP secretion were not additive. These data indicate that the effect of liraglutide in enhancing ANP secretion is PKA independent but requires both cAMP-related and PLC-dependent signals (**Fig. 4b**,**c**). As GLP-1R–induced increases in cAMP amounts activate Epac2 translocation in beta cells[22], we examined Epac2 localization in cardiomyocytes. Liraglutide reduced the amount of cytosolic Epac2 and increased that of membrane-associated Epac2 in *Glp1r*$^{+/+}$ cardiomyocytes from normotensive or hypertensive mice. Although basal amounts of cytoplasmic Epac2 protein were higher in *Glp1r*$^{-/-}$ cardiomyocytes, liraglutide did not cause Epac2 translocation in *Glp1r*$^{-/-}$ cardiomyocytes (**Fig. 4d**).

To investigate whether liraglutide directly induces ANP secretion, we isolated atrial or ventricular cardiomyocytes from normal or hypertensive hearts. Liraglutide induced a robust increase in ANP secretion from *Glp1r*$^{+/+}$ atrial but not ventricular cardiomyocytes isolated from normotensive or hypertensive mice (**Fig. 4e**,**f**), consistent with the predominantly atrial localization of *Glp1r*. In contrast, liraglutide had no effect on ANP secretion from atrial *Glp1r*$^{-/-}$ cardiomyocytes

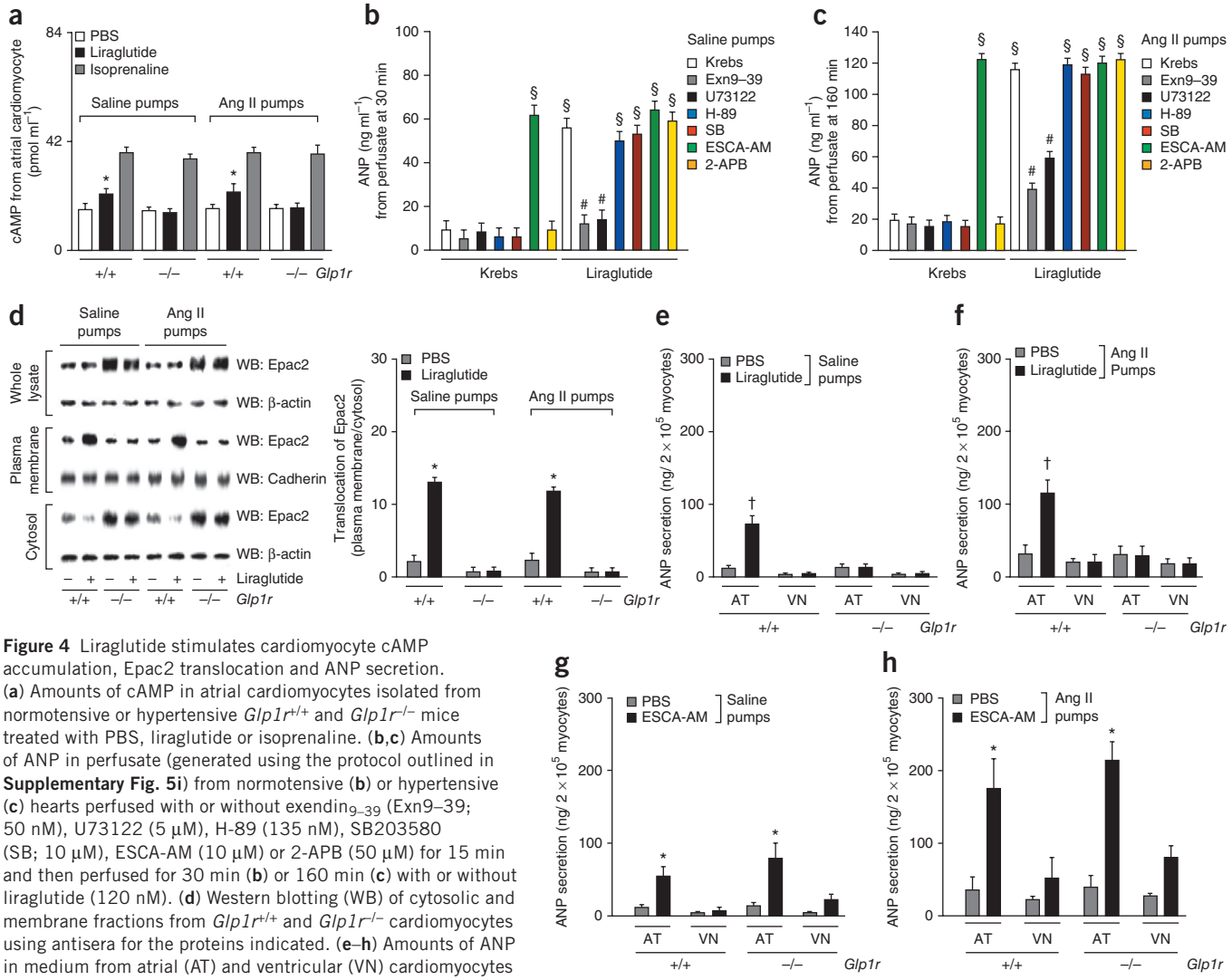

Figure 4 Liraglutide stimulates cardiomyocyte cAMP accumulation, Epac2 translocation and ANP secretion. (**a**) Amounts of cAMP in atrial cardiomyocytes isolated from normotensive or hypertensive *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ mice treated with PBS, liraglutide or isoprenaline. (**b**,**c**) Amounts of ANP in perfusate (generated using the protocol outlined in **Supplementary Fig. 5i**) from normotensive (**b**) or hypertensive (**c**) hearts perfused with or without exendin$_{9-39}$ (Exn9–39; 50 nM), U73122 (5 μM), H-89 (135 nM), SB203580 (SB; 10 μM), ESCA-AM (10 μM) or 2-APB (50 μM) for 15 min and then perfused for 30 min (**b**) or 160 min (**c**) with or without liraglutide (120 nM). (**d**) Western blotting (WB) of cytosolic and membrane fractions from *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ cardiomyocytes using antisera for the proteins indicated. (**e**–**h**) Amounts of ANP in medium from atrial (AT) and ventricular (VN) cardiomyocytes from normotensive (saline pumps) or hypertensive (Ang II pumps) *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ mice treated with PBS, liraglutide (120 nM) or ESCA-AM (10 μM) for 2 h. Data (**a**–**h**) are shown as the means ± s.e.m. for cardiomyocytes isolated from 3 mice per group. $^{*}P < 0.05$ compared to the PBS-treated *Glp1r*$^{+/+}$ group, $^{\#}P < 0.05$ compared to the liraglutide-treated Krebs-treated group, $^{\S}P < 0.05$ compared to the Krebs-treated group, $^{\dagger}P < 0.05$ compared to the PBS-treated groups. Statistical significance was determined by ANOVA followed by Bonferroni's *post hoc* test.

(**Fig. 4e**,**f**), whereas ESCA-AM stimulated ANP secretion from both atrial and, to a lesser extent, ventricular cardiomyocytes from normotensive and hypertensive *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ mice (**Fig. 4g**,**h**). Although liraglutide promotes ANP secretion from cardiomyocytes in a GLP-1R–dependent manner, we did not detect differences in basal levels of endogenous ANP expression in cardiomyocytes from normotensive or hypertensive *Glp1r*$^{+/+}$ compared to *Glp1r*$^{-/-}$ mice (**Supplementary Fig. 6a**).

Liraglutide requires Epac2 for blood pressure reduction

To assess the importance of Epac2 for liraglutide-stimulated ANP secretion, blood pressure and heart rate, we analyzed mice with germline disruption of the gene encoding Epac2, *Rapgef4* (ref. 23). *Glp1r* mRNA transcripts were predominantly localized to the atria and were expressed at comparable levels in RNA from *Rapgef4*$^{+/+}$ and *Rapgef4*$^{-/-}$ hearts (**Fig. 5a**). GLP-1R agonists increase heart rate in rodents and humans[5], and we detected a transient increase in heart rate and blood pressure after acute liraglutide administration in *Glp1r*$^{+/+}$ but not *Glp1r*$^{-/-}$ mice (**Supplementary Fig. 7a–f**). Although ANP also increases heart rate in hypertensive subjects[24], we observed an increase in heart rate in liraglutide-treated *Nppa*$^{-/-}$ mice (**Supplementary Fig. 6c**). Hence, ANP is not required for the chronotropic effects of GLP-1R agonists.

Liraglutide directly stimulated ANP secretion from atrial cardiomyocytes of normotensive or hypertensive *Rapgef4*$^{+/+}$ mice but not *Rapgef4*$^{-/-}$ mice (**Fig. 5b**). Although activation of cardiac vasopressin receptor signaling enhances ANP release, the stimulatory effects of liraglutide on cardiomyocyte ANP secretion were not diminished by co-treatment with an antagonist of the vasopressin V1 receptor (**Supplementary Fig. 8**). Furthermore, liraglutide increased ANP secretion from both saline-infused and Ang II–infused *Rapgef4*$^{+/+}$ intact hearts, although to different levels and with different temporal responses (**Fig. 5c**). In contrast, liraglutide did not augment ANP secretion from perfused *Rapgef4*$^{-/-}$ hearts (**Fig. 5d**). Consistent with these findings, liraglutide significantly reduced blood pressure in hypertensive *Rapgef4*$^{+/+}$ mice but had no effect on systolic or

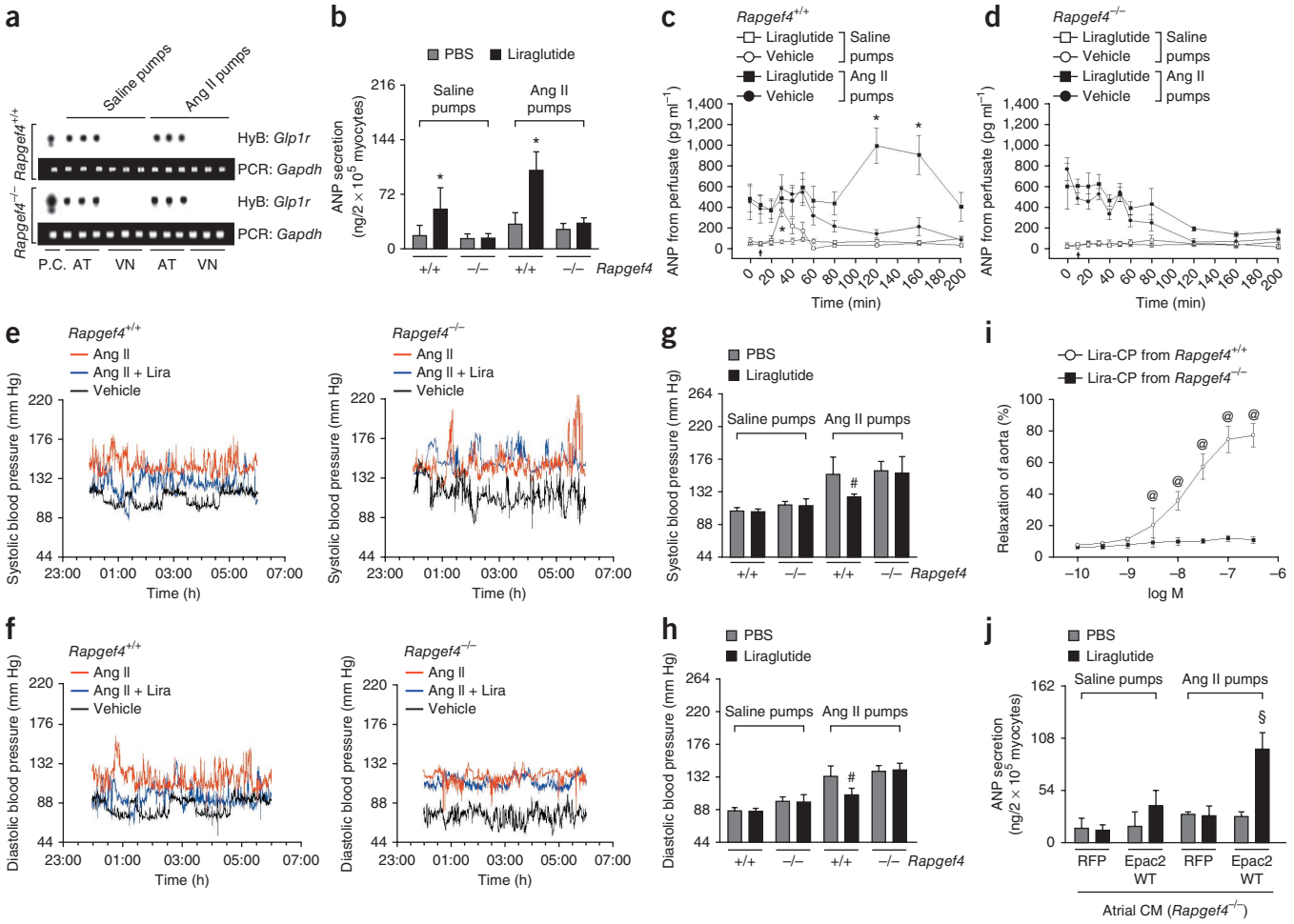

Figure 5 Liraglutide stimulates ANP secretion and lowers blood pressure in an Epac2-dependent manner. (**a**) Relative amounts of mRNA transcripts of *Glp1r* and *Gapdh* in RNA from atrial (AT) and ventricular (VN) cardiomyocytes from normotensive (saline pumps) or hypertensive (Ang II pumps) *Rapgef4*$^{+/+}$ and *Rapgef4*$^{-/-}$ mice. (**b**) ANP secretion assessed in atrial cardiomyocytes from normotensive or hypertensive *Rapgef4*$^{+/+}$ and *Rapgef4*$^{-/-}$ mice exposed to liraglutide (120 nM) or saline (PBS) for 2 h. (**c**,**d**) Amounts of ANP in perfusate of *Rapgef4*$^{+/+}$ or *Rapgef4*$^{-/-}$ hearts perfused with Krebs buffer or 120 nM liraglutide. The mice had been treated with 3 weeks of saline or Ang II infusion. Arrows indicate the start of the liraglutide or vehicle perfusion. (**e**–**h**) Systolic and diastolic blood pressure in *Rapgef4*$^{+/+}$ and *Rapgef4*$^{-/-}$ mice after 3 weeks of saline or Ang II infusion (red) followed by acute treatment with liraglutide (Lira; blue) or vehicle (black). (**i**) Aortic smooth muscle relaxation in preconstricted aortic rings using liraglutide-conditioned perfusate (Lira-CP) from *Rapgef4*$^{+/+}$ or *Rapgef4*$^{-/-}$ hearts. (**j**) ANP secretion in *Rapgef4*$^{-/-}$ atrial cardiomyocytes (CM) from normotensive or hypertensive mice transduced with recombinant adenovirus carrying Flag-tagged WT Epac2 or red fluorescent protein (RFP) as a control. After 48 h, cardiomyocytes were treated with or without liraglutide (120 nM for 2 h). Data (**b**–**d**,**g**–**h**) are shown as the means ± s.e.m. of 3–4 mice per group. $^{*}P < 0.05$ compared to the PBS-treated *Glp1r*$^{+/+}$ group, $^{\#}P < 0.05$ compared to the PBS-treated Ang II–infused *Glp1r*$^{+/+}$ group, $^{@}P < 0.05$ compared to Lira-CP from *Rapgef4*$^{-/-}$ hearts, $^{\S}P < 0.05$ compared to PBS-treated WT Epac2-infected cardiomyocytes. Statistical significance was determined by ANOVA followed by Bonferroni's *post hoc* test.

diastolic blood pressure in hypertensive *Rapgef4*$^{-/-}$ mice (**Fig. 5e**–**h**). Furthermore, perfusate from liraglutide-treated *Rapgef4*$^{+/+}$ but not *Rapgef4*$^{-/-}$ hearts produced a dose-dependent reduction in aortic ring constriction (**Fig. 5i**). We next examined whether the failure of *Rapgef4*$^{-/-}$ cardiomyocytes to respond to liraglutide (**Fig. 5d**–**h**) reflected the absence of Epac2 or developmental changes in signal transduction pathways arising from embryonic loss of Epac2. Although *Rapgef4*$^{-/-}$ atrial cardiomyocytes from normotensive or hypertensive mice transduced with a control virus encoding red fluorescent protein did not to respond to liraglutide, adenoviral Epac2 expression restored a significant ANP secretory response to liraglutide in *Rapgef4*$^{-/-}$ cardiomyocytes from hypertensive mice (**Fig. 5j**). These findings reinforce the importance of Epac2 in linking GLP-1R signaling to ANP secretion.

DISCUSSION

GLP-1R agonists lower blood pressure in preclinical studies and in human subjects with diabetes or obesity[3,5,12,13]; however, the mechanisms underlying the antihypertensive actions of GLP-1R agonists remain obscure. GLP-1 improves flow-mediated vasodilation in some but not all human studies, and preclinical studies demonstrate that these actions may be independent of the known GLP1 receptor, GLP-1R[15]. Degradation-resistant GLP-1R agonists that lower blood pressure in humans have little or no direct vasodilatory actions on blood vessels[15]. Previous studies of the natriuretic actions of GLP-1R agonists have suggested a role for GLP-1 in the kidney through activation of Na^+/H^+ exchange proteins in kidney tubules[11,25]. Although GLP-1R agonists promote phosphorylation of the renal sodium hydrogen exchanger type 3 (NHE3) at Ser552 and Ser605, independent

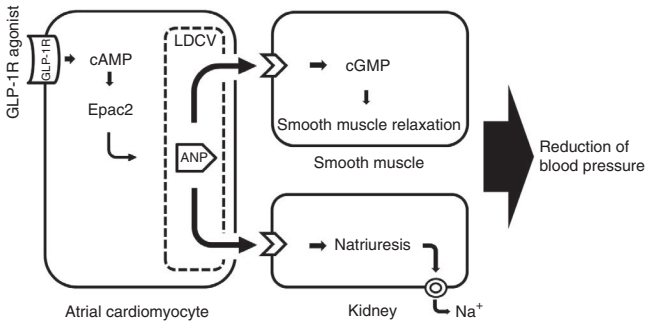

Figure 6 ANP is essential for GLP-1–stimulated urinary sodium secretion and vascular smooth muscle relaxation. Schematic mechanism for GLP-1 regulation of blood pressure. After activation of atrial cardiomyocyte GLP-1R with agonists such as liraglutide or exenatide, increased amounts of cAMP promote Epac2 membrane translocation, which then mediates ANP release from the large dense core vesicle (LDCV). ANP induces cGMP-mediated smooth muscle relaxation and natriuresis, leading to a reduction of blood pressure.

experimentation demonstrates that NHE3 phosphorylation at Ser552 and Ser605 does not directly modulate NHE3 Na^+/H^+ exchange activity[26]. The GLP-1R–ANP axis described here (**Fig. 6**) mandates re-evaluation of how GLP-1 acts on the heart, kidney and blood vessels to control blood pressure.

Although GLP-1R expression has been localized to the rodent and human heart[27], little attention has been paid to chamber-specific localization of GLP-1R expression, and recent studies have raised a note of caution regarding the interpretation of data obtained using commercial antisera to detect the authentic GLP-1R protein[28]. The demonstration that *Glp1r* mRNA transcripts are localized predominantly to the atria is consistent with our data linking GLP-1R activation to stimulation of ANP secretion and with observations that GLP-1 promotes sodium excretion[11,13,14] (**Fig. 6**). Notably, we identify an essential role for Epac2 as a downstream target for GLP-1R signaling in cardiomyocytes. Although the exact pathways coupling GLP-1R activation to ANP secretion require further clarification, it is noteworthy that Epac proteins may couple through Rap GTPases to the activation of a new PLC isoform, PLC-ε[29], consistent with our findings that a PLC inhibitor blocks the Epac2-mediated actions of liraglutide.

Food ingestion is associated with an increase in plasma concentrations of ANP in rodents and humans[30,31], and the stimulatory effects of food on ANP concentrations are attenuated in subjects with diabetes[31]. Our data demonstrate that food ingestion increases plasma ANP concentrations in WT but not *Glp1r*$^{-/-}$ mice, revealing an essential role for endogenous GLP-1R in the transduction of a gut-heart signal promoting ANP secretion. Notably, although meal ingestion and GLP-1R agonists promote natriuresis and blood pressure reduction, ANP concentrations have not previously been examined after treatment with GLP-1R agonists. Our results demonstrate that the antihypertensive actions of GLP-1R agonists are attenuated by ANP antagonists and absent in ANP knockout mice, demonstrating an essential role for ANP as a downstream mediator of the antihypertensive actions of GLP-1.

The localization of *Glp1r* expression to atrial but not ventricular cardiomyocytes raises questions regarding the direct cardioprotective actions of GLP-1R agonists. We previously observed that pretreatment of mice with liraglutide for several days produced robust cardioprotection and improved survival after coronary artery ligation; however, acute administration of liraglutide into the coronary arteries immediately before induction of ischemia did not improve functional outcomes in isolated ischemic mouse hearts *ex vivo*[32]. Similarly, exenatide administration 15 min before revascularization and reperfusion in human subjects with myocardial infarction accompanied by ST segment elevation produced only a modest reduction in infarct size, with no functional difference in left ventricular function or clinical events detected after 30 d[33]. Our current data imply that the actions of GLP-1R agonists to improve left ventricular function and survival may be largely indirect, mediated by ANP, other atrial-derived signals, changes in blood flow or as-yet undescribed mechanisms.

The complex pathophysiology and genetics of essential human hypertension often involve changes in the functional activity of genes important for renal sodium absorption. For example, functional variation in corin activity may be associated with reduced amounts of active ANP and BNP and an increased risk of hypertension[34]. Furthermore, heterogeneity in the control of ANP secretion and action in various disease states suggests that the relative importance of a GLP-1–ANP axis may depend in part on heterogeneity in the pathophysiology contributing to hypertension in different individuals[34]. Genome-wide association studies have linked genetic variability in loci near the *NPPA*, *NPPB* and *NPR3* genes to an increased risk of hypertension[34]. Furthermore, we observed (J.A.S., unpublished data) that an experimental model of hypertension associated with reduced ANP responsivity, the SHR rat, shows a lack of blood pressure reduction pursuant to GLP-1R activation using doses of GLP-1R agonist that do not cause weight loss. It seems probable that many factors determine whether human hypertensive subjects with diabetes and renal impairment will show a robust ANP response and reduction in blood pressure after administration of GLP-1R agonists. Hence, a better understanding of the temporal and dose-response relationships and mechanisms linking GLP-1R agonists to increased ANP action in diabetic blood vessels and kidney may help optimize GLP-1 based therapies for the treatment of subjects with diabetes with ischemic heart disease or congestive heart failure.

It is increasingly recognized that ANP enhances lipolysis[35,36], promotes adipocyte thermogenesis[37], augments myocyte fat oxidation and oxidative phosphorylation[38] and stimulates glucose-stimulated insulin secretion[39]. These metabolic actions of ANP have been described predominantly in preclinical studies and overlap in part with those described for GLP-1. The identification of a GLP-1R–ANP gut-heart axis redefines our understanding of the actions of GLP-1 in the heart and cardiovascular system and should prompt further analysis of the metabolic and cardiovascular importance of ANP as a downstream target for GLP-1 action.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
M.K., M.J.P. and J.A.S. designed and carried out experiments, analyzed results and wrote the manuscript. M.K. was supported by a postdoctoral fellowship award from the Canadian Diabetes Association. S.S. provided *Rapgef4*$^{-/-}$ mice, interpreted results and wrote the manuscript. T.S. carried out experiments and reviewed the manuscript. S.E.Q. and P.H.B. helped design experiments and reviewed the manuscript. D.J.D. designed experiments, interpreted results and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Mice. *Glp1r*$^{-/-}$ (from D.J.D.), *Nppa*$^{-/-}$ (Jackson Laboratory) and *Rapgef4*$^{-/-}$ (from S.S.) mice, all in the C57BL/6 background, and corresponding WT littermate controls, were used in experimental protocols approved by the Mount Sinai Hospital Animal Care Committee. Analyses of blood pressure and ANP secretion were carried out in 3- to 4-month-old male WT littermate or homozygous *Glp1r*$^{-/-}$, *Nppa*$^{-/-}$ or *Rapgef4*$^{-/-}$ mice. Mice were genotyped by PCR using the following primers; *Glp1r* WT allele (5′-TACACAATGGGGAGCCCCTA-3′ and 5′-AAGTCATGGGATGTGTCTGGA-3′, generating a 437-bp fragment) or the neo-containing *Glp1r* knockout allele (5′-CTTGGGTGGAGAGGCTATTC-3′ and 5′-AGGTGAGATGACAGGAGATC-3′, generating a 280-bp fragment); *Nppa* WT allele (5′-CTGTCCAACACAGATCTGATG-3′ and 5′-CTGTTGCAGCCTAGTCCACT-3′, generating a 434-bp fragment) or the *Nppa* knockout allele (5′-CTGTTGCAGCCTAGTCCACT-3′ and 5′-CCTTCTATCGCCTTCTTGACG-3′, generating a 700-bp fragment); *Rapgef4* WT allele (5′-TGAACAGATTTGTGACCGGAT-3′ and 5′-CTGATCACATTAGCAAGCTC-3′, generating 345-bp fragment) or the *Rapgef4* knockout allele (5′-GCATACATTATACGAAGTTATC-3′ and 5′-CTGATCACATTAGCAAGCTC-3′, generating a 90-bp fragment). To assess the effects of liraglutide on hypertension, blood pressure in mice was measured by radiotelemetry (PA-C10 from DSI). Mice were implanted with telemetry devices, allowed to recover for 1 week and then implanted with osmotic minipumps (ALZET) for delivery of saline (0.9% NaCl) or Ang II (490 ng per kg body weight, A9525, Sigma-Aldrich) for 3 weeks[40]. After 3 weeks of Ang II or saline, liraglutide (Novo Nordisk) was injected at 9 a.m. and 4 p.m., and blood pressure data were analyzed from 12 a.m. to 6 a.m. to minimize the stress of ongoing activity in the animal care center and avoid the confounding acute transient hypertensive effects of GLP-1R agonists in rodents[41]. To assess the effects of inhibitors on blood pressure responses to liraglutide, WT mice were pretreated with exendin$_{9-39}$ (CHI Scientific; 10 μg per kg body weight three times per day; GLP-1R antagonist), L-NMMA (Cayman Chemical; 30 mg per kg body weight twice per day; nitric oxide inhibitor) or anantin (Raybiotech; 100 μg per kg body weight three times per day; antagonist of natriuretic peptide receptor), and blood pressure was determined after liraglutide injection (30 μg per kg body weight i.p.) on the third day. For analysis of plasma ANP concentrations in overnight fasted and refed mice, ANP concentrations were determined in plasma from blood samples obtained from the tail vein at 10, 30, 60 min after food ingestion using a RayBio Enzyme Immunoassay kit, EIA-ANP-1. All mice were housed under specific pathogen-free conditions in microisolator cages and maintained on a 12-h light, 12-h dark cycle with free access to food and water.

Aortic tension and relaxation. Aortic smooth muscle relaxation was measured using isometric tension[42]. The thoracic aorta was quickly removed and cut into two or three rings and placed in an organ bath containing 25 ml Krebs solution maintained at 37 °C. The rings were connected to a force transducer (MP150 from BIOPAC) to measure isometric tension. Aortic vessels were contracted submaximally using phenylephrine (Sigma Aldrich; 10^{-5} M). After reaching a stable contraction plateau, acetylcholine (Sigma Aldrich) and liraglutide (10^{-9}–10^{-6} M) were then cumulatively added to the organ bath to obtain concentration-relaxation curves. After completion of aortic tension experiments, aortic rings were quickly homogenized and centrifuged at 1,500*g* for 10 min. From the supernatants, amounts of cGMP were quantified by competition between free cGMP and acetylcholinesterase linked to cGMP using Cayman's cGMP Assay kit.

Isolated heart perfusion. Mice were anesthetized using avertin, and the heart was carefully excised. After cannulation of the aorta, hearts were perfused retrogradely with Krebs-Henseleit buffer as described[15]. Hearts from *Glp1r*$^{+/+}$ or *Glp1r*$^{-/-}$ mice were isolated and perfused in the nonrecirculating retrograde mode with Krebs buffer or liraglutide (120 nM) after a 10-min stabilization period. Coronary effluents were collected at different time points over 200 min, and the amounts of ANP were determined. The rate of coronary flow (7–8 ml/min) was controlled by a peristaltic pump. To assess the effects of various agonists and antagonists on ANP secretion from perfused hearts, isolated hearts were perfused for 15 min, followed by treatment with various antagonists and inhibitors for 15 min as outlined in **Figure 4b**,**c** and **Supplementary Figure 5i**. The reagents used were H-89 and SB 203580 (Calbiochem), 2-APB (Tocris Bioscience), U73122 (Tocris Bioscience) and 8-pCPT-2-O-Me-cAMP-AM, designated ESCA-AM (c 051, Biolog). Hearts were then perfused with Krebs buffer for 2 min, followed by perfusion with Krebs buffer alone or Krebs buffer plus liraglutide (120 nM). Perfusates were collected at 30 min from normotensive hearts and at 160 min from hypertensive hearts to determine the amount of ANP (and BNP) using Ray Bio EIA kits.

Isolation of adult cardiomyocytes. Atrial and ventricular cardiomyocytes were prepared using methods adapted from those described previously[43]. Briefly, 3- to 4-month-old mice were injected with 0.05 ml of 1000 USP/ml heparin (MedXL) for 15 min and then anesthetized using avertin. After opening the chest cavity, the heart was quickly excised and perfused using a Langendorff system. After perfusion for 2 min, the heart was digested with 2 mg/ml collagenase II (Cellutron) for 15 min. After sufficient digestion, the atrium and ventricle were isolated separately and gently agitated at 100 r.p.m. for 10 min. The tissues were gently dissociated by repeated pipetting and resuspended in stopping buffer (20% FBS in minimum essential medium (MEM); Sigma Aldrich). To induce calcium tolerance, cells were exposed to increasing calcium concentrations (100 μM, 400 μM and 900 μM) for 7 min each. The calcium-tolerant myocytes were plated onto laminin (Roche Applied Science)-coated culture dishes with 10% FBS in MEM. One hour after plating, the buffer was replaced into culture medium (serum-free MEM, 2 mM L-glutamine (Invitrogen), 10 mM 2,3-butanedione monoxime (Sigma Aldrich), insulin-transferrin sodium selenite media supplement (Sigma-Aldrich) and 100 U/ml penicillin-streptomycin (Invitrogen) and incubated for 6 h before treatment. Cells were incubated with PBS or liraglutide (0.48 μg/ml). The cell medium was collected, and the amounts of ANP were quantified using the EIA kit (Ray Bio, USA). In other experiments, cardiomyocytes were preincubated with 3-isobutyl-1-methylxanthine (Cayman; 100 μM) for 30 min. Then cells were treated with PBS, liraglutide or isoprenaline (Sigma Aldrich;10 nM) for 2 h. Samples were then collected in ice-cold ethanol and analyzed using a cAMP RIA kit (Biomedical Technologies).

TAC. TAC was performed in 8- to 9-week-old CD1 male mice (Charles River, Quebec, Canada) as previously described[44]. Two groups of mice were studied, designated series 1 and series 2. In series 1, mice were assigned randomly to receive either saline (0.9% NaCl) or liraglutide (27 μg per kg body weight twice per day at 9 a.m. and 7 p.m., with lights on/off at 8 a.m./8 p.m.) i.p. starting immediately after surgery and proceeding continuously for 2 weeks. At 2 weeks, mice were anesthetized with isoflurane and oxygen (2%:98%), and body temperature was maintained at approximately 37 °C. A 1.2F catheter (FTS-1211B-0018, Scisense Inc.) was inserted through the right carotid artery into the ascending aorta. Hemodynamic signals were digitized at a sampling rate of 1,000 Hz and recorded by computer using Spike2 software (Cambridge Electronic Design, UK). After recording baseline blood pressure, 75 μl of blood was collected for determination of baseline ANP plasma concentrations. The 2-week liraglutide-treated TAC mice were further subjected to an acute intravenous (i.v.) bolus of either saline or liraglutide (27 μg per kg body weight) given in a volume of ~45 μl; blood pressure was continuously monitored; mice were euthanized at either 15 or 30 min after intravenous injection for a terminal plasma sample to determine ANP plasma concentrations after an acute i.v. injection. For all TAC mice, heart and body weights were recorded. In series 2, blood was collected from untreated 2-week TAC mice before and 60 or 120 min after a single i.p. injection (at 4 p.m.) of saline or liraglutide (27 μg per kg body weight) for determination of ANP plasma concentrations after an acute injection in untreated TAC mice.

Analysis of *Glp1r* mRNA transcripts. Twenty micrograms of total RNA was isolated from adult cardiomyocytes using TRI reagent (Molecular Research Center). After quantification using a Nanodrop 1000 spectrophotometer (Thermo Scientific), reverse transcription was carried out using an oligo-(dT) primer and SuperScript III Reverse Transcriptase (Invitrogen). The complementary DNA was amplified using primers for mouse *Glp1r* (5′-GTACCACGGTGTCCCTCTCA-3′ and 5′-CCTGTGTCCTTCACCTTCCCTA-3′) or *Gapdh* (5′-GACCACAGTCCATGACATCACT-3′ and 5′-TCCACCACCCTGTTGCTGTAG-3′). The amplification parameters were set at

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95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min 30 s (45 cycles total). PCR reaction products were electrophoresed in a 1% agarose gel, transferred to a Nylon membrane (Biodyne) and ultraviolet (UV) crosslinked using a UV chamber (STRATAGENE). After prehybridization for 1 h at 48 °C, membranes were hybridized with an internal ^{32}P-labeled oligonucleotide probe (5′-GCTGTATCTGAGCATAGGCT-3′) overnight at 48 °C in hybridization buffer (5% dextran sulfate, 1 mol/l NaCl and 1% SDS). Blots were washed and exposed to Kodak Biomax MS film for the indicated period of time.

Plasma membrane isolation. Cardiomyocytes were lysed using an ice-cold dounce homogenizer and centrifuged at 700*g* for 10 min. The supernatant was centrifuged at 10,000*g* for 30 min to separate the cytosol and the cellular membrane fraction. Plasma proteins were purified from the cellular membrane fraction using membrane extraction kit (Biovision). Pan-cadherin antibody (ab16505, 1:2,500, Abcam) was used as a marker of plasma membrane proteins.

Viral transduction of Epac2. To examine the role of Epac2 in ANP secretion, *Rapgef4*$^{-/-}$ atrial cardiomyocytes were prepared and infected with recombinant adenovirus vectors carrying WT Epac2 or RFP (generously provided by G. Holz, State University of New York Upstate Medical University). Mock infection as a control was performed using the adenovirus vectors carrying RFP (G. Holz). Infected cells were incubated for a further 36 h before being used for either western blot analysis or measurement of ANP secretion by EIA (Raybiotech).

Urinalysis. *Nppa*$^{+/+}$ and *Nppa*$^{-/-}$ mice were infused with saline or Ang II for 3 weeks. *Nppa*$^{+/+}$ and *Nppa*$^{-/-}$ mice were then acclimatized for 4 d in metabolic cages before 24-h urine collection. Mice had free access to rodent chow (76a diet gel). Urine was collected for 24 h after administration of liraglutide (30 μg per kg body weight i.p.) or PBS, and urine sodium was determined using an ion-selective electrode (820638-450043, Roche Diagnostics) and normalized to the values for creatinine measured using the Jaffe reaction (creatinine urinary assay kit, 500701 Cayman Chemical) in the same samples.

Western blotting. Atrial or ventricular cardiomyocytes or aortic tissues were isolated and lysed, extracts were quantified for protein content and boiled with loading dye, and 200 μg was used in gel electrophoresis. After blotting, membranes were incubated with antisera directed against p-eNOS (Ser1177; 612392, 1:2,500, BD Transduction Laboratories), total eNOS (9586, 1:2,500, Cell Signaling), pVasp (Ser239; SC-101439, 1:2,500, Santa Cruz), total Vasp (3132, 1:2,500, Cell Signaling), Hsp90 (610418, 1:2,500, BD Biosciences), Epac2 (4156, 1:2,500, Cell Signaling), β-actin (SC-130656, 1:2,500, Santa Cruz), pan-cadherin (ab16505, 1:2,500, Abcam), ANP (N-20, 1:2,500, Santa Cruz) and Flag (F3165, 1:2500, Sigma-Aldrich) and then with secondary antibodies (goat-specific donkey horseradish peroxidase (HRP)-conjugated antibody, SC-2020, 1:2,500, Santa Cruz , mouse-specific HRP-conjugated antibody, NA931V, 1:2,500, GE Health Care or rabbit-specific HRP-conjugated antibody, NA934V, 1:2,500, GE Health Care). Bands were visualized using an ECL detection kit and quantified by densitometry.

Statistics. Values are shown as the means ± s.e.m. Wherever appropriate, one-way ANOVA followed by Bonferroni's *post hoc* test was used to determine differences between group mean values. The level of statistical significance was set at $P < 0.05$.

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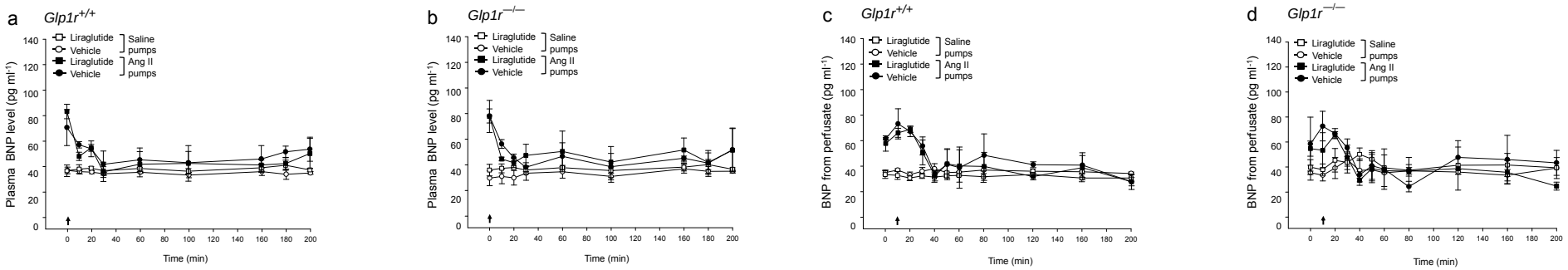
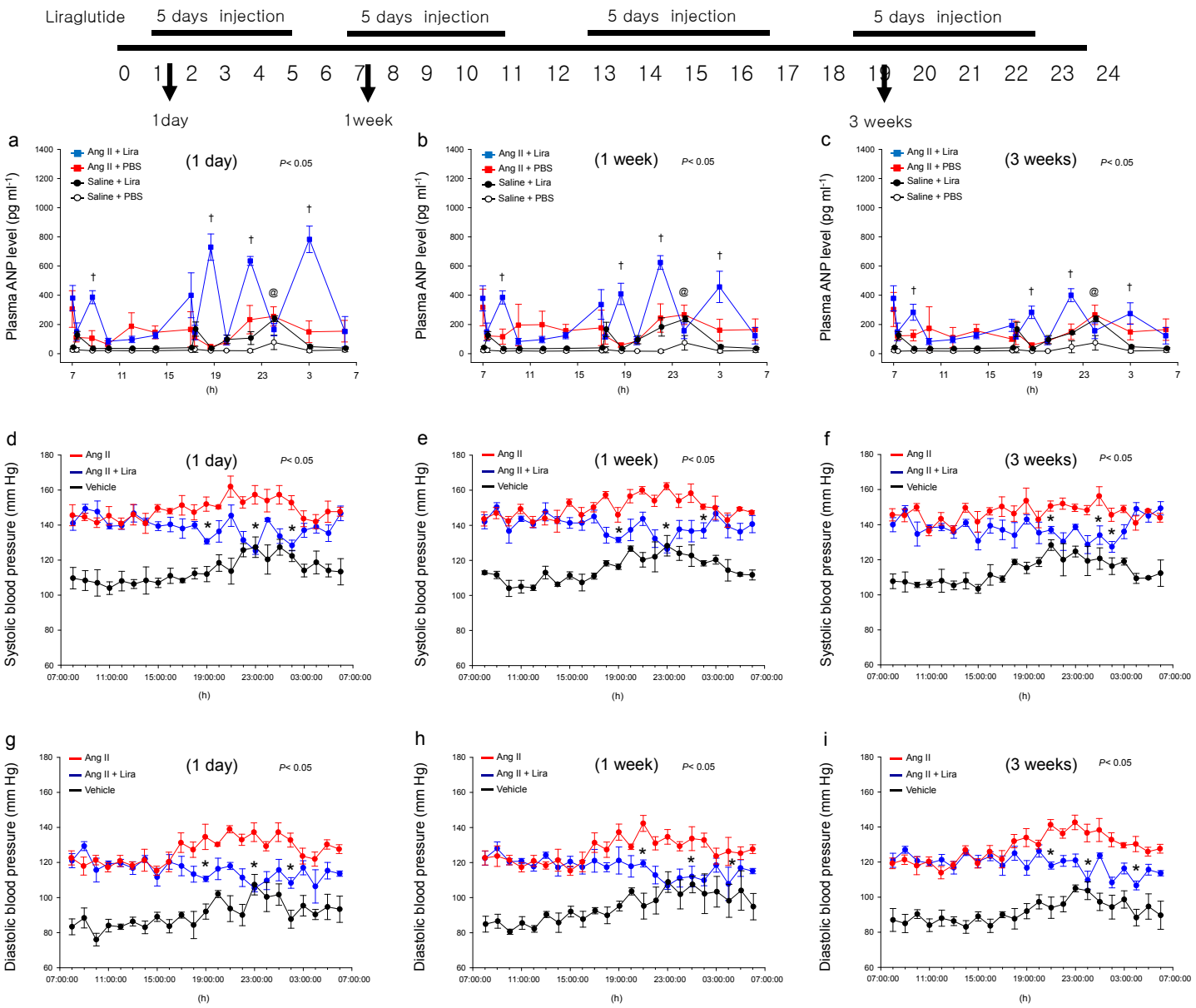
Supplementary Figures



Supplementary Fig. 1 Liraglutide has no effect on BNP secretion. *Glp1r*$^{+/+}$ or *Glp1r*$^{-/-}$ mice infused with Ang II (490 ng/kg) or saline (0.9% NaCl) for 3 weeks were treated with liraglutide (30 ug/kg; i.p.) or PBS, and blood samples were collected over a period of 200 min for analysis of plasma BNP levels using a kit from Ray Bio (a,b). Arrows indicate time of liraglutide injection. Hearts from these animals were isolated and perfused in the non-recirculating retrograde mode with Krebs buffer or liraglutide (120 nM) after a 10 min stabilization period. Coronary effluents were collected at different time points over 200 min and BNP levels were determined in perfusate collected ex vivo(c,d). Arrows designate start of liraglutide or vehicle perfusion. Data are means ± SEM of 3 to 6 mice in each group.



Supplementary Fig. 2 Repeated administration of Liraglutide for 3 weeks lowers Blood Pressure (BP) and increases ANP secretion. Wild type mice infused with Ang II (490 ng/kg) or saline (0.9% NaCl) for 3 weeks were then treated with liraglutide (30 μg/kg; i.p.) or PBS twice a day for an additional 3 weeks. Using radiotelemetry, systolic and diastolic blood pressure values (d-i) were measured for 24 hr and blood samples were collected over 24 hr for analysis of plasma ANP levels (a-c) after 1 day, 1 week or 3 weeks of saline or liraglutide administration. Data are means ± SEM of 3 to 6 mice in each group, †Significantly different from PBS-treated hypertensive group; @Significantly different from PBS-treated normotensive group, *Significantly different from PBS-treated hypertensive BP group, *P<0.05*.

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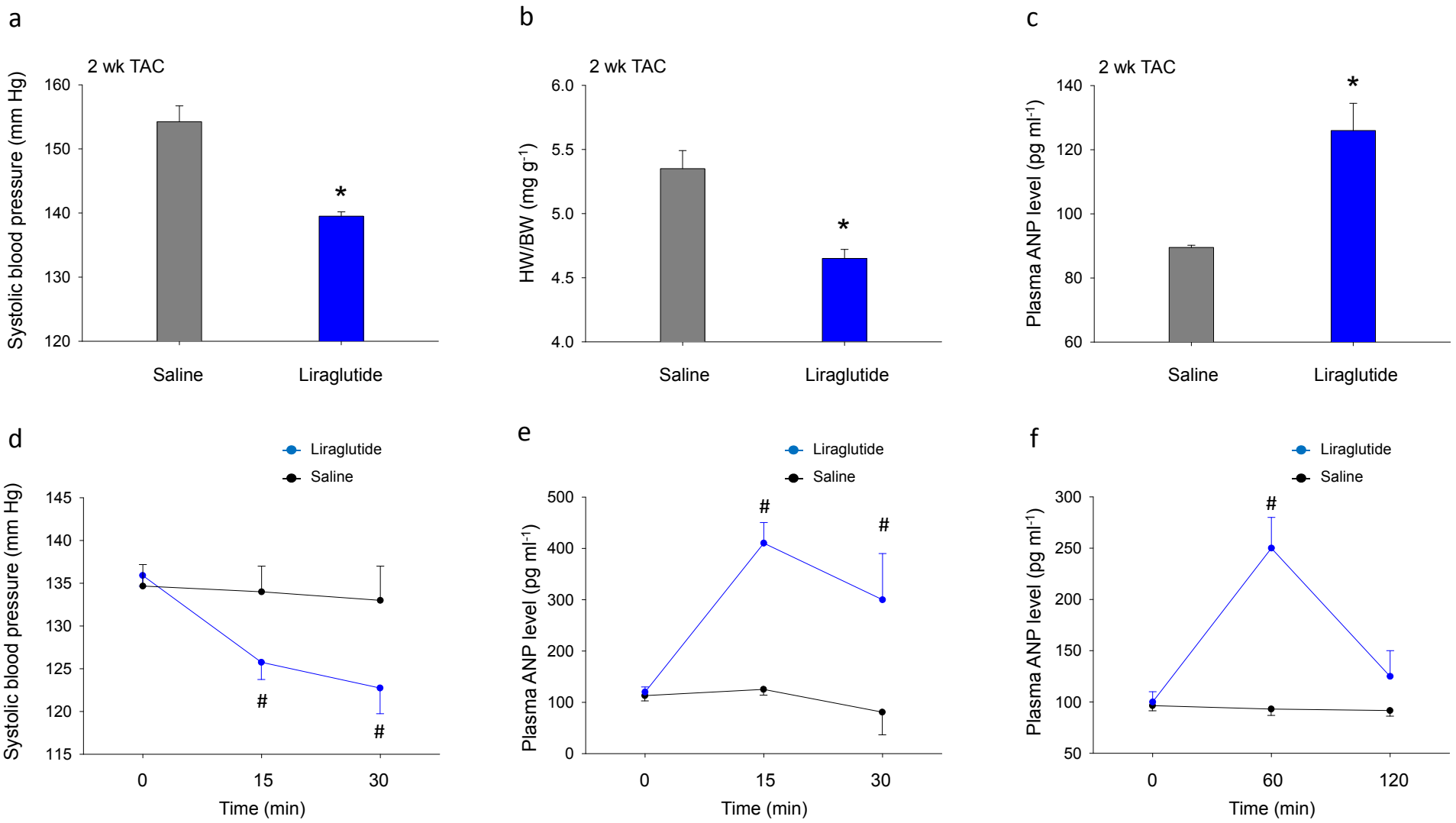

Supplementary Fig. 3 Liraglutide reduces Blood Pressure (BP) and increases ANP levels in the murine TAC model of hypertension. Male CD-1 mice were treated with liraglutide (27 ug/kg) or saline (0.9% NaCl) for two weeks (2x/day) in mice with chronic pressure-overload induced by transverse aortic constriction (TAC) in a-c. Liraglutide (Blue; 27 ug/kg; i.p.) or saline (Black; 0.9% NaCl; i.p.) was injected at 9 AM and 7 PM. Using a 1F pressure catheter, systolic blood pressure values (a) were measured in the afternoon (~ 3 - 8 PM). Heart weight, normalized to body weight, was significantly reduced in liraglutide treated animals (b). Plasma ANP levels were determined from liraglutide-and saline-treated 2 week TAC animals (c). For acute studies in d-f, 2 week TAC animals were given an acute injection of either liraglutide (Blue; 27 ug/kg i.v.) or saline (Black; 0.9% NaCl; i.v.). Blood pressure was recorded over 30 minutes (d) and blood ANP levels were determined either at time 0 (pre) and 15 min and 30 min post-injection of liraglutide or saline (e). 2 week TAC animals were given an acute injection of either liraglutide (Blue; 27 ug/kg i.p.) or saline (Black; 0.9% NaCl; i.p.) and ANP levels were determined at baseline (time 0) and 60min and 120 min post-injection. For a-c , data are means ± SEM or SD of 11 to 18 mice in each group, *Significantly different from saline treated group; for d-f, data are means ± SEM of 5 to 8 mice in each group, #Significantly different zero time point. *P<0.05*

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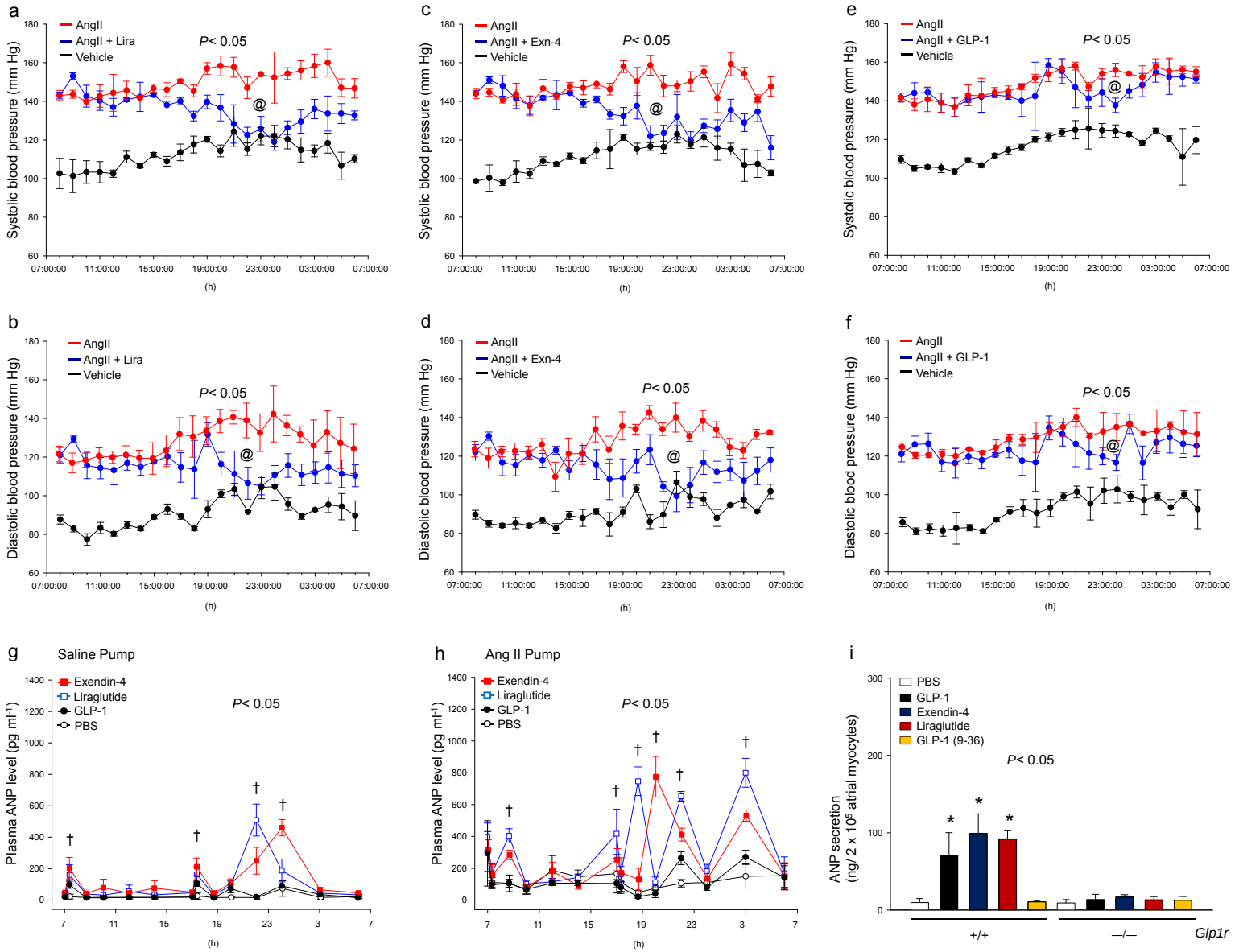
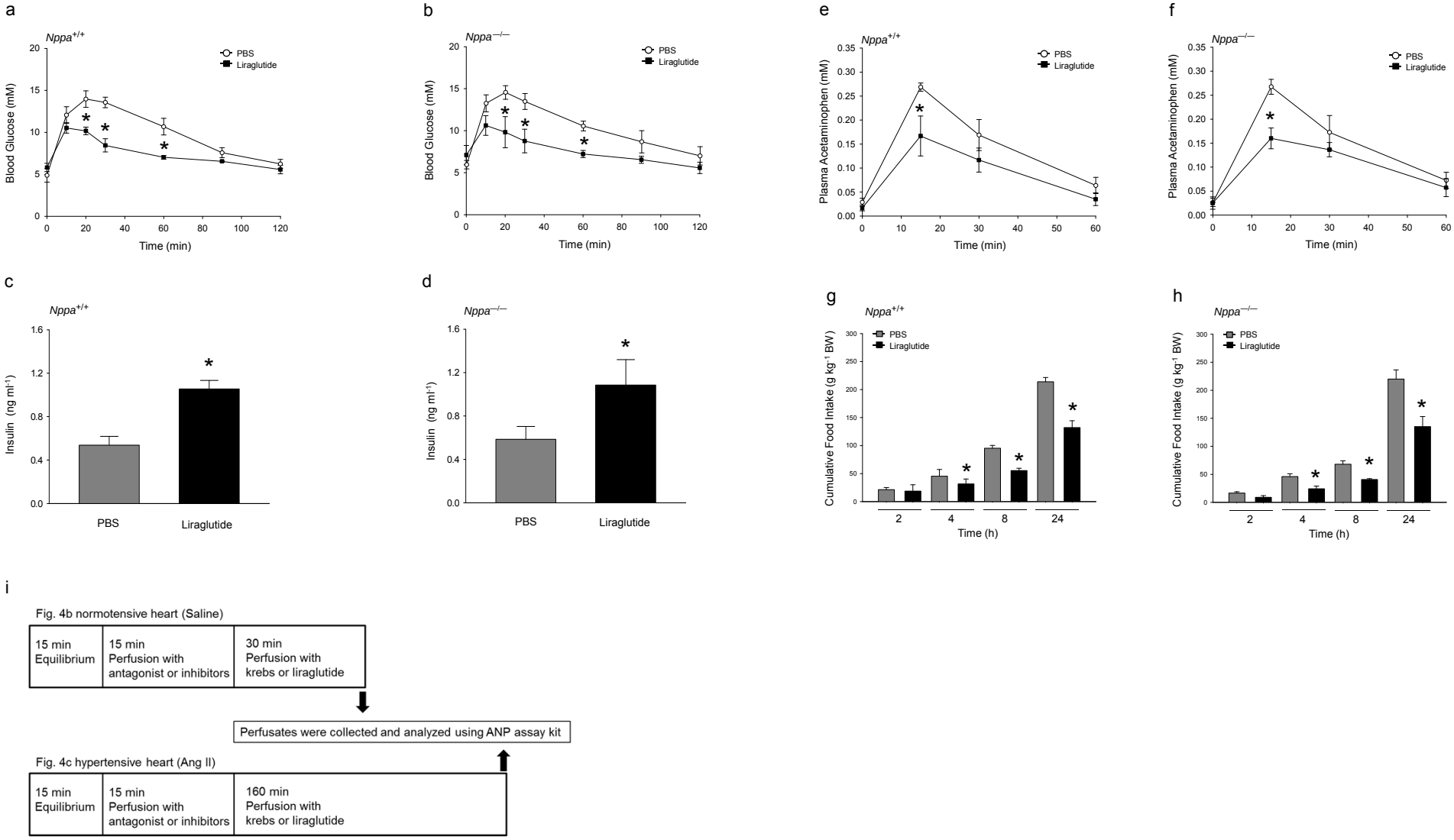
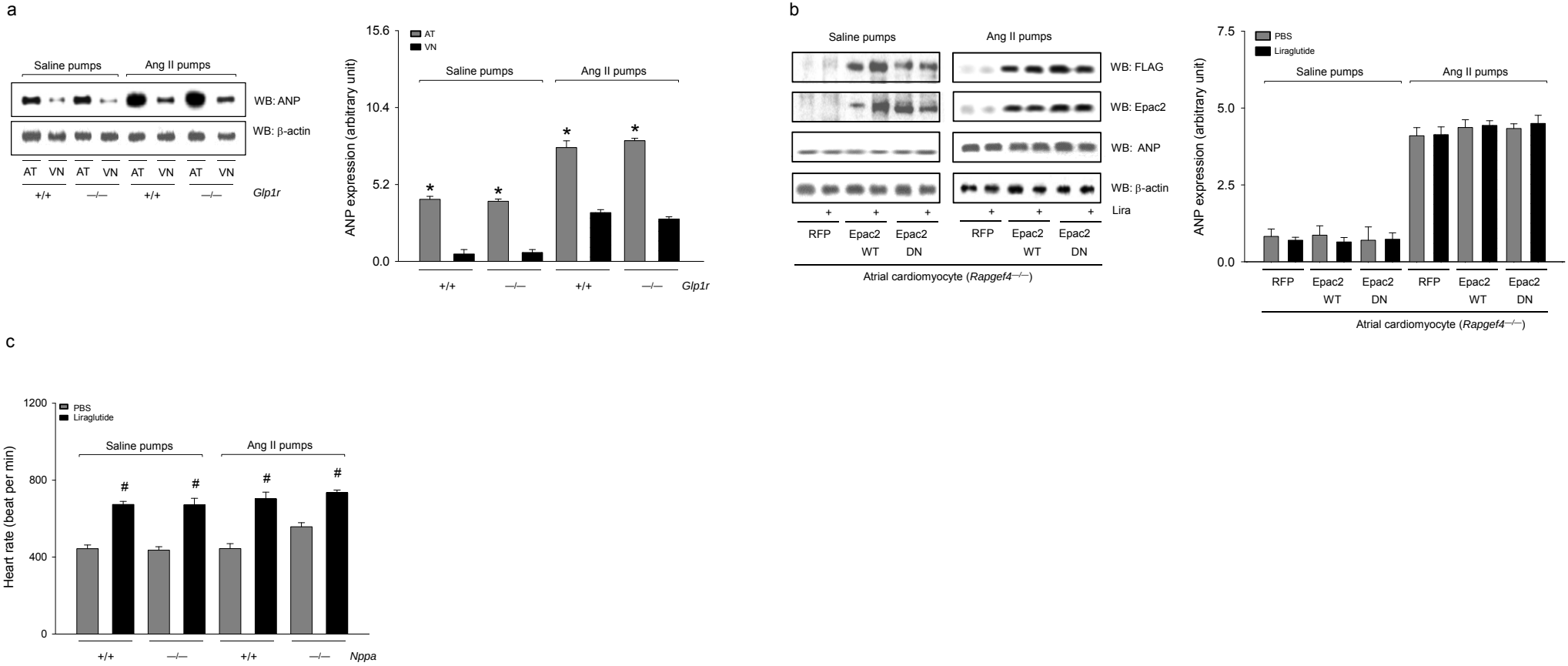


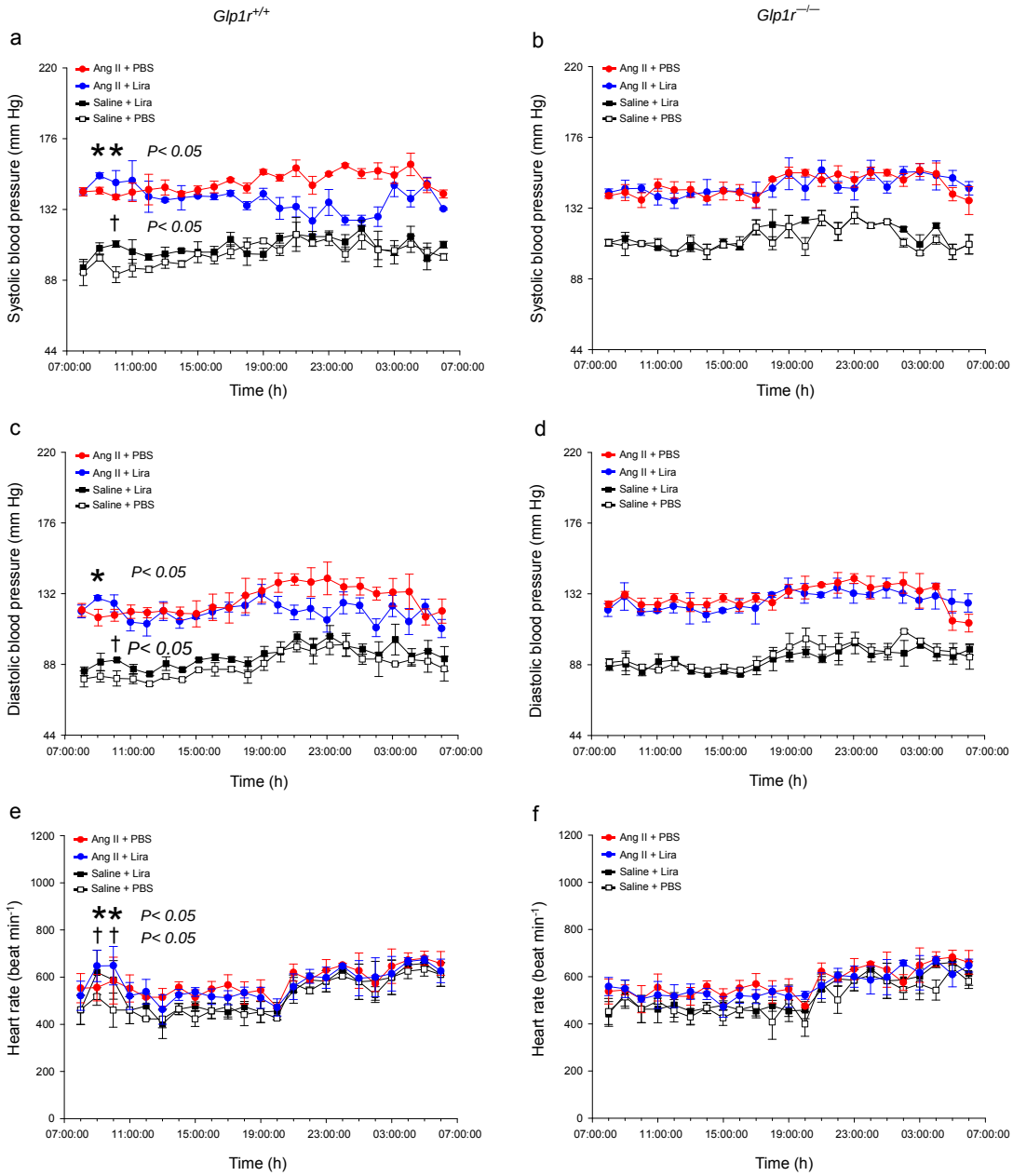
Supplementary Fig. 4 GLP-1, exendin-4 and liraglutide reduce Blood Pressure (BP) and increase plasma levels of ANP. Wild type mice infused with Ang II (490 ng/kg) or saline (0.9% NaCl) for 3 weeks were treated with liraglutide (30 μg/kg; i.p.), GLP-1 (30 μg/kg), exendin-4 (20.9 μg/kg) or PBS (vehicle) twice a day. Using radiotelemetry, systolic and diastolic blood pressure values (a-f) were measured for 24 hr. Blood samples were collected over 24 hr for analysis of plasma ANP levels (g,h). In separate experiments, cardiomyocytes were isolated from *Glp1r*$^{+/+}$ or *Glp1r*$^{-/-}$ hearts and treated with PBS, GLP-1 (120 nM), exendin-4 (120 nM), liraglutide (120 nM), or GLP-1 (9-36) (120 nM) for 2 hr (i). The cell medium was collected and assayed for ANP secretion. Data are means ± SEM of 3 to 6 mice in each group, @Significantly different from PBS-treated hypertensive group; †Significantly different from PBS-treated group, *Significantly different from PBS-treated cardiomyocyte group, *P<0.05*.



Supplementary Fig. 5 Glucoregulatory and anorectic actions of liraglutide are preserved in *Nppa*$^{-/-}$ mice. Oral glucose tolerance, gastric emptying and food intake in *Nppa*$^{+/+}$ or *Nppa*$^{-/-}$ mice in response to acute liraglutide administration. The response to an oral glucose challenge (OGTT, 1.5 g/kg of body weight) in fasted mice was assessed following injection of either PBS or liraglutide (30 µg/kg). Blood glucose levels in *Nppa*$^{+/+}$ and *Nppa*$^{-/-}$ mice were monitored throughout the experiment (a,b). Plasma insulin was determined from blood samples collected 10 min after glucose administration and measured using a kit from ALPCO (c,d). To study gastric emptying, mice fasted for 6 hrs were injected with either PBS or liraglutide (30 µg /kg), 30 min prior to an oral gavage of glucose (1.5 g/kg) and acetaminophen (0.1g/kg). The appearance of acetaminophen in plasma samples was used as a measure of gastric emptying in *Nppa*$^{+/+}$ and *Nppa*$^{-/-}$ mice (e,f). To determine food intake, overnight fasted mice were assessed following injection of either PBS or liraglutide (30 µg/kg). Cumulative food intake upon re-feeding was recorded at 2, 4, 8 and 24 hours in *Nppa*$^{+/+}$ and *Nppa*$^{-/-}$ mice mice (g,h). Data are means ± SEM of 5 mice in each group, *Significantly different from PBS treated group, *P<0.05*. The experimental protocol used to assess the effects of liraglutide on ANP secretion in isolated perfused hearts for data shown in Figure 4b,c is shown in (i).



Supplementary Fig. 6 ANP expression in *Glp1r*$^{+/+}$, *Glp1r*$^{-/-}$ or *Rapgef4*$^{-/-}$ cardiomyocytes and heart rate in *Nppa*$^{+/+}$, and *Nppa*$^{-/-}$ mice treated with liraglutide. Atrial (AT) and ventricular (VN) cardiomyocytes were isolated from *Glp1r*$^{+/+}$ and $^{-/-}$ mice infused with Ang II (490 ng/kg for 3 weeks) or saline (0.9% NaCl for 3 weeks) (a). In other experiments, atrial cardiomyocytes were isolated from normotensive or hypertensive *Rapgef4*$^{-/-}$ mice and infected with recombinant adenovirus vectors carrying FLAG tagged wild-type (WT) Epac2 or dominant-negative (DN) Epac2 (b). Infected cells were incubated for a further 36 hrs before treatment with PBS or liraglutide (120 nM) for 2 hrs. From these cells, protein was extracted and ANP expression was determined using western blot with ANP or β-actin antibodies for a&b. Heart rates in normotensive or hypertensive mice was measured using a radiotelemetry device in *Nppa*$^{-/-}$ mice after PBS or liraglutide injection for 2 hrs. (c). Data are means ± SEM of 3 to 5 mice in each group, *Significantly different from ventricular cardiomyocyte; #Significantly different from PBS treated animals group, *P<0.05*.



Supplementary Fig. 7 Acute i.v. administration of liraglutide transiently increases BP and heart rate in WT mice. *Glp1r*$^{+/+}$ (a,c,e) or *Glp1r*$^{-/-}$ (b,d,f) mice infused with Ang II (490 ng/kg) or saline (0.9% NaCl) for 3 weeks, and then acutely treated with liraglutide (30 µg/kg; i.v.) or PBS. Using radiotelemetry, systolic, diastolic blood pressure, or heart rate values were measured for 24 hr. Data are means ± SEM of 3 to 5 mice in each group, *Significantly different from PBS-treated hypertensive group; †Significantly different from PBS treated normotensive group, *P<0.05*.

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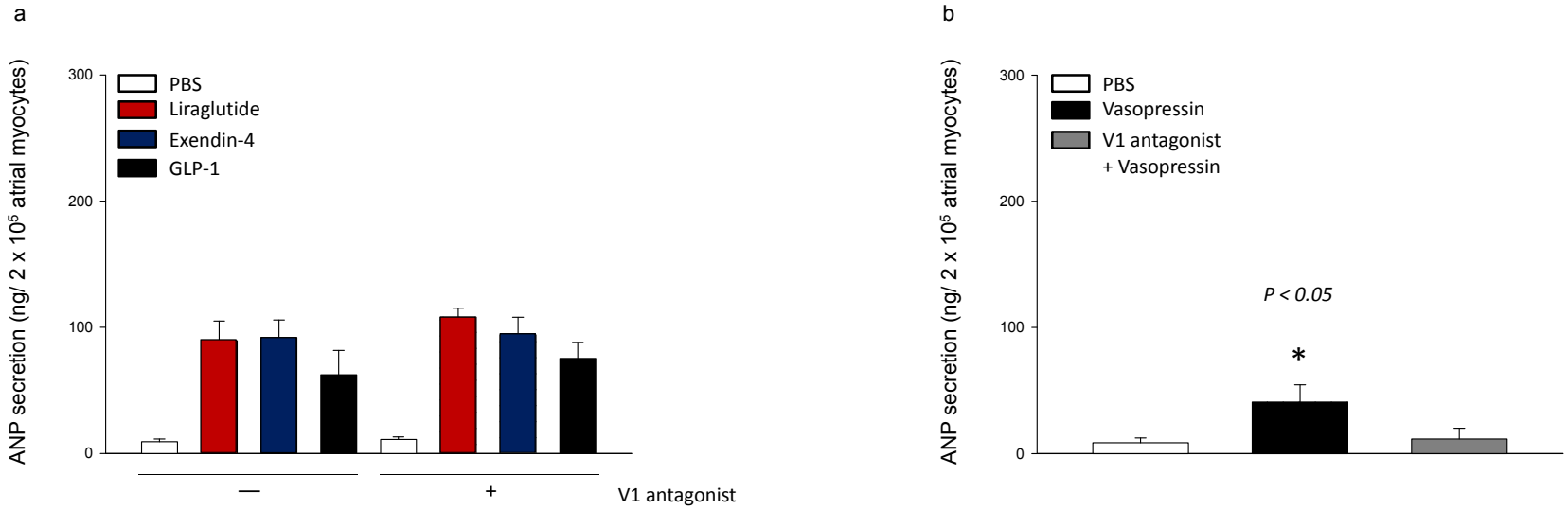

Supplementary Fig. 8 Vasopressin V1 receptor antagonists fail to attenuate GLP-1 receptor agonist-induced stimulation of ANP secretion. Atrial cardiomyocytes were isolated and preincubated for 30 min in the presence or absence of either (B-mercapto B, B-cyclopentamethylenepropionyl, O-Me-Tyr,Arg)-vasopressin (0.5 uM; V1 antagonist) before addition of arginine vasopressin (0.1uM), GLP-1 (0.12 uM), exendin-4 (0.08 uM), or liraglutide (0.12 uM) (a). For studies in (b) ANP secretion was assessed following incubation of vasopressin alone (0.1uM), or vasopressin plus O-Me-Tyr,Arg)-vasopressin (0.5 uM; V1 antagonist). The cell medium was collected and assayed for ANP secretion. Data are means ± SEM of 3 to 5 mice in each group,*Significantly different from PBS-treated cardiomyocyte group, *P<0.05*.