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ORIGINAL CONTRIBUTION

EGFR trans-activation by urotensin II receptor is mediated by β-arrestin recruitment and confers cardioprotection in pressure overload-induced cardiac hypertrophy

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Abstract Urotensin II (UTII) and its seven trans-membrane receptor (UTR) are up-regulated in the heart under pathological conditions. Previous in vitro studies have shown that UTII trans-activates the epidermal growth factor receptor (EGFR), however, the role of such novel signalling pathway stimulated by UTII is currently unknown. In this study, we hypothesized that EGFR trans-activation by UTII might exert a protective effect in the overloaded heart. To test this hypothesis, we induced cardiac hypertrophy by transverse aortic constriction (TAC) in wild-type mice, and tested the effects of the UTII antagonist Urantide (UR) on cardiac function, structure, and EGFR trans-activation. After 7 days of pressure overload, UR treatment induced a rapid and significant impairment of cardiac function compared to vehicle. In UR-treated TAC mice, cardiac dysfunction was associated with reduced phosphorylation levels of the EGFR and extracellular-regulated kinase (ERK), increased apoptotic cell death and fibrosis. In vitro UTR stimulation induced membrane translocation of β-arrestin 1/2, EGFR phosphorylation/internalization, and ERK activation in HEK293 cells. Furthermore, UTII administration lowered apoptotic cell death induced by serum deprivation, as shown by reduced TUNEL/Annexin V staining and caspase 3 activation. Interestingly, UTII-mediated EGFR trans-activation could be prevented by UR treatment or knockdown of β-arrestin 1/2. Our data show, for the first time in vivo, a new UTR signalling pathway which is mediated by EGFR trans-activation, dependent by β-arrestin 1/2, promoting cell survival and cardioprotection.

Keywords Urotensin II · EGFR · Cardiac hypertrophy · β-Arrestin

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Introduction

Seven-transmembrane receptors (7TMRs) belong to the largest family of membrane receptors, regulating multiple functions in the cardiovascular system [41, 47], and accounting for a large number of prescriptions for heart failure (HF) [17, 25]. Despite optimal therapy, HF remains a relentless, progressive disease with poor prognosis, and thus novel treatments are needed to ameliorate cardiac dysfunction and prolong survival in these patients.

Urotensin II (UTII) is a conserved cyclic neuropeptide expressed in the cardiovascular, neuronal and urogenital

systems [5, 11, 29] interacting with a specific 7TMR (UTR) activating Gq and phospholipase C to generate inositol triphosphate and diacylglycerol, and thereby increasing intracellular calcium and activating protein kinase C [1, 14, 28]. UTII and UTR are up-regulated under pathological conditions, including congestive heart failure, essential hypertension, coronary artery disease and type II diabetes [36, 50]. UTII exogenous administration promotes hypertrophy of rat neonatal cardiomyocytes [27], and potentiates isoproterenol-induced cardiac growth [33, 49]. Thus, UTII might be an important determinant of cardiac dysfunction, inducing G-protein-mediated intracellular effects similar to those described for angiotensin II or endothelin-1 [4, 8, 10, 31, 32].

For a number of years, it has been known that 7TMRs activate G-protein signalling and, to limit unrestrained stimulation, G-protein-coupled receptor kinases (GRKs), leading to β-arrestins binding, which sterically interdict further G-protein signalling and scaffold receptors to the internalization machinery [43]. However, it has been recently appreciated that β-arrestins not only terminate G-protein signalling, but also function as adaptor molecules, allowing for the assembly of multiprotein signalling complexes, and promoting novel signalling pathways [24]. Among these, β-arrestins can link 7TMR stimulation to the trans-activation of epidermal growth factor receptor (EGFR) [34] through a number of undefined steps, including metalloproteinases (MMP)-mediated cleavage and extracellular shedding of heparin-binding EGF, resulting in EGFR activation [9, 16, 39, 42]. Very little is known regarding the pathophysiological effects that such novel mechanisms of signal transduction exert in the heart.

Recent studies have suggested that EGFR trans-activation plays a critical role in UTII hypertrophic signal transduction in rat cardiomyocytes [27]. The role(s) of such novel signalling pathways stimulated by UTII in the overloaded heart are currently unknown. Since β-arrestin-mediated β1-adrenergic receptor (β1AR) signalling to the EGFR has been recently shown to be beneficial in mice undergoing chronic sympathetic stimulation [28], in this study we hypothesized that UTII might exert a protective effect in the heart by trans-activating the EGFR. To test this hypothesis, we analyzed the effects of the UTR antagonist Urantide (UR) in a cellular system and in a mouse model of heart failure induced by pressure overload. Both in vivo and in vitro, UR inhibited UTR-mediated EGFR trans-activation and promoted cell death. In response to pressure overload, UR rapidly induced cardiac dysfunction, demonstrating the protective role of this signalling pathway in pathological cardiac remodelling.

Methods

Animal studies

All experiments involving animals were conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and were approved by the animal welfare regulation of University Federico II of Naples, Italy. Animals were maintained under equal conditions of temperature (21 ± 1°C), humidity (60 ± 5%) and light/dark cycle, and had free access to normal rodent chow.

Mouse model of pressure overload

Wild-type C57BL/6 male mice (8-week-old; $n = 30$) were anesthetized with an intramuscular injection of ketamine 100 mg/kg and xylazine 5 mg/kg (Sigma). Chronic pressure overload was induced by transverse aortic constriction (TAC) in 10 mice as previously described [12, 38, 45]. Additional mice underwent (the same day of the TAC procedure) the implant of mini-osmotic pumps (Alzet Model 2002; DURECT) as previously described [18] to systemically deliver UTII (TAC + UTII, 12 μmol/kg/day, $n = 13$), the UTII antagonist Urantide (TAC + UR, 2 μmol/kg/day, $n = 12$) or the EGFR inhibitor AG1478 (TAC + AG1478, 12 mg/kg/day, n = 6). Another group of animals underwent a left thoracotomy without aortic constriction (SHAM, $n = 8$). Mice from all the groups were killed 7 days after surgery to perform molecular or histological analyses. During killing, after body weight (BW) calculation, mice were anesthetized as described above, and the efficacy of the pressure overload was invasively tested by measuring the arterial pressures in the right carotid artery (proximal to the suture) and the left right carotid artery (distal to the suture). Only TAC animals with systolic pressure gradients >40 mmHg were included in the study. Next, the hearts were removed and cardiac chambers dissected to assess the left ventricular weight.

Transthoracic echocardiography

Cardiac function was not invasively monitored by transthoracic echocardiography using the Vevo 770 high resolution imaging system (VisualSonics, Toronto, Canada) before the surgery and right before termination. Briefly, the mice were anesthetized as previously described, and echocardiograms were performed with a 30-MHz RMV-707B scanning head and analyzed as previously reported [37].

Cell culture

Human embryonic kidney (HEK) 293 cells and smooth muscle-derived A10 cells (SMCs) were obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 200 mg/ml L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. HEK 293 cells were transfected with 1 μg of cDNA encoding EGFR-FLAG, EGFR-GFP, β-arrestin-GFP and UTR with FuGENE6 reagent according to the manufacturer's instructions (Roche Applied Science). Transfected cells were incubated overnight in serum-free medium supplemented with 0.1% BSA, 10 mM HEPES (pH 7.4), and 1% penicillin prior to stimulation. Under serum starvation conditions, cells were pre-incubated with different UTR antagonists: UR (30 nM), BIM23127 (3 μM), UFP-803 (100 nM), [ORN^5] URP (3 μM) and SB657510 (200 nM) or with the EGFR inhibitor AG1478 (1 μM; Calbiochem) for 30 min, followed by stimulation with UTII (100 nM; Sigma-Aldrich), EGF (10 ng/ml; Upstate Biotechnology) for 15 or 5 min (immunoblotting), and 30 min (confocal microscopy).

Confocal microscopy

Following stimulation, HEK293 cells were rinsed with ice-cold PBS and fixed with 3.7% formaldehyde in PBS for 30 min. EGFR internalization and β-arrestin recruitment was visualized by green fluorescence using a single sequential line of excitation filters. The fluorescent data sets were visualized with a Zeiss 510 confocal laser scanning microscope and analyzed by LSM 510 software.

Protein extraction and immunoblot analysis

Cultured cells and left ventricular samples were lysed in a buffer containing 150 mM NaCl, 50 mM Tris pH 8.5 mM EDTA, 1% v/v NP-40, 0.5% w/v deoxycholate, 10 mM NaF, 10 mM Na_2 pyrophosphate, 2 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin; pH 7.4. Lysates were incubated on ice for 15 min and then centrifuged at 38,000×*g* for 30 min at 4°C. Protein concentrations in all lysates were measured using a dye-binding protein assay kit (Bio-Rad) and a spectrophotometer reader (Bio Rad) at a wavelength of 595 nm. Immunoblotting and immunoprecipitation were performed using commercially available antibodies (see below) as previously described [19]. Bands were visualized by enhanced chemiluminescence (ECL; Amersham Life Sciences Inc.) according to the manufacturer's instructions, and were quantified using densitometry (Chemidoc, Biorad, USA). Each experiment and densitometric quantification was separately repeated at least three times.

Antibodies

In the present study, we used commercially available primary antibodies anti-UTR (polyclonal, Sigma-Aldrich), anti-UTII (monoclonal, Phoenix Pharmaceuticals), anti-GAPDH (monoclonal, Santa Cruz), anti-Tubulin (monoclonal, Sants Cruz), anti-GRK2 (monoclonal, Santa Cruz), anti-pERK (monoclonal, Santa Cruz), anti-ERK (monoclonal, Santa Cruz), anti-FLAG (monoclonal, Sigma-Aldrich), anti-EGFR (polyclonal, Santa Cruz), anti-pEGFR Tyr845 (polyclonal, Upstate Biotechnology), anti-phosphotyrosine PY20 (polyclonal, Santa Cruz) and anti-cleaved caspase-3 (monoclonal, Cell signalling), anti-β-arrestin 1/2 (polyclonal, Santa Cruz). Secondary antibodies were purchased from Amersham Life Sciences Inc.

Histology

Left ventricular specimens were fixed in 4% formaldehyde and embedded in paraffin. After de-paraffinization and re-hydratation, 4 μm-thick sections were prepared, mounted on glass slides and stained with Masson trichrome for quantitative analysis of the infarct size as previously described [7] or with 1% Sirius red in picric acid (Carlo Erba Laboratories, Italy) to detect interstitial fibrosis. The fibrotic area (red) was measured in at least 3 hearts/group (2 sections/sample, 6–10 images/section), using a computer-assisted image analysis system (Image J, USA), and was expressed as a percentage of total area. To determine capillary density, sections were incubated with a biotinilated lectin from Bandeiraea simplicifolia (Sigma) and amplified by a Tyramide Signal Amplification (TSA) Biotin System kit (Perkin Elmer Life Sciences, MA). Staining was developed using DAB + substrate chromogen (DAKO, CA) and counterstained with Mayer's Hematoxylin. Capillary density was quantified by counting the lectin positive capillary numbers, normalized to the tissue area, in 30 randomly-chosen, high-power (200×) fields. All the sections were examined with a microscope (Leitz, DIAPLAN) and images were acquired with a digital camera (Digital JVC, TK-C1380).

TUNEL staining

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) was performed on fixed paraffin-embedded left ventricular sections (4 μm) using an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, UK), according to the manufacturer's instructions. TUNEL staining was visualized with 3,3'-diaminobenzidine (DAB, Vector), which stained brown the positive nuclei. Cardiac sections were then counterstained with methyl green (Carlo Erba). For cultured cells and AG1478-treated mice, the DNA nicks were determined

with the use of an ApopTag Fluorescein Direct In Situ Apoptosis Detection kit (Chemicon, UK) according to the manufacturer's instructions. TUNEL staining was visualized by specific green fluorescence and nuclei by 4'-6-diamidino-2-phenylindole (DAPI) (nuclear counterstain). All the sections were examined with a microscope (Leitz, DIAPLAN) and images were acquired with a digital camera (Digital JVC, TK-C1380).

Annexin V staining

Adherent A10 cells were treated for 24 h with UTII (100 nM) and UR (30 nM), harvested by trypsinization and combined with floating cells. Next, cells were stained with Annexin V Cy3 (MBL, Japan), and the percentage of apoptosis was analyzed by flow cytometry. All flow cytometric analyses were performed on a flow cytometer equipped with a 25-mW argon ion laser for excitation at 570 nm (FACSCalibur, Becton & Dickinson), and the data were analyzed with the Cell Quest software (Becton & Dickinson). All analyses were repeated 3–4 times with different preparations of cells. Gating was set to exclude debris and cellular aggregates, and 10,000 or more events were counted for each analysis.

β-Arrestin 1/2 knockdown

30–50% confluent HEK293 cells were transfected with 100 pmol siRNA for β-arrestin 1–2 (Stealth siRNA, INVITROGEN), or Stealth RNAi negative control (INVITROGEN) and 1 μg of cDNA encoding for UTR and EGFR-GFP using Lipofectamine 2000 reagent (INVITROGEN) according to the manufacturer's protocols. All assays were performed 48 h after siRNA transfection. Cells were serum-starved for 12 h, and then stimulated with UTII (100 nM) or EGF (10 ng/ml) for 15 or 5 min (immunoblotting), and 30 min (confocal microscopy).

Statistical analysis

Data are expressed as mean ± SE. Multi-group comparisons were made with a one-way ANOVA with a Tukey's finishing test. For all analyses, a minimum value of $p < 0.05$ was considered significant; when present, a p value < 0.001 was specified.

Results

UTR antagonism reduces EGFR trans-activation and promotes cardiac dysfunction in response to pressure overload

In order to study the role of UTII signalling in pathological cardiac remodelling, we induced cardiac hypertrophy by transverse aortic constriction (TAC) in wild-type C67BL/6 mice [13]. This surgical procedure rapidly determines cardiac hypertrophy as shown by cardiac chambers morphometry and echocardiography (Table 1). Following TAC, two additional groups of mice underwent the chronic infusion of the UTR antagonist Urantide (UR) or vehicle by micro-osmotic pumps. In additional experiments, UR circulating levels were evaluated in mice by high performance liquid chromatography–mass spectrometry (HPLC), confirming its bioavailability up to 15 days following micropump implantation (data not shown).

Table 1 Cardiac hypertrophy induced by pressure overload in mice

	SHAM ($n = 11$)	TAC ($n = 10$)	TAC + UR ($n = 7$)	TAC + UTII ($n = 13$)
Morphometry				
BW (g)	20.0 ± 3.7	18.0 ± 0.7	19.5 ± 0.5	18.7 ± 0.9
LVW (mg)	95 ± 5.4	109.1 ± 11.9*	111.7 ± 14.7*	103.8 ± 15.1*
LVW/BW (mg/g)	4.5 ± 0.6	6.0 ± 0.5*	5.8 ± 0.5*	5.7 ± 1.0*
Echocardiography				
LVEDD (mm)	2.8 ± 0.1	2.9 ± 0.2	3.2 ± 0.2	3.2 ± 0.1
LVESD (mm)	1.4 ± 0.09	1.4 ± 0.1	1.7 ± 0.2	1.5 ± 0.2
FS (%)	61.0 ± 1.3	51.0 ± 1.5*	45.0 ± 1.5**	53.0 ± 1.0*
IVSd (mm)	0.82 ± 0.08	1.10 ± 0.12*	1.18 ± 0.10*	1.14 ± 0.05*
PWd (mm)	0.78 ± 0.07	1.06 ± 0.11*	1.05 ± 0.06*	1.06 ± 0.04*
HR (bpm)	381 ± 24	412 ± 15	349 ± 20	353 ± 22

SHAM Sham-operated control animals, *TAC* Transverse aortic constriction, *TAC + UR* Transverse aortic constriction with Urantide, *TAC + UTII* Transverse aortic constriction with Urotensin II, *BW* body weight, *LVW* left ventricular weight, *LVEDD* left ventricular end-diastolic diameter, *LVESD* left ventricular end-systolic diameter, *FS* fractional shortening, *IVSd* end-diastolic interventricular septum, *PWd* end-diastolic posterior wall, *HR* heart rate

* $p < 0.001$ vs. SHAM, ** $p < 0.05$ vs. SHAM and TAC

Seven days after the surgery, TAC mice exhibited a significant increase in the expression levels of the UTR (Fig. 1a) and UTII (Fig. 1b) compared to the SHAM, while UR treatment had no effects on UTR or UTII levels. As expected, TAC mice also exhibited an increase in the left ventricle weight to body weight ratio (LVW/BW) compared to SHAM, and UR treatment did not affect cardiac hypertrophy development (LVW/BW, mg/g: SHAM = 4.51 ± 0.6; TAC = 6.04 ± 0.5; TAC + UR = 5.83 ± 0.5 not significant, Fig. 2a; Table 1). Interestingly, TAC + UR mice displayed a rapid impairment of cardiac function as shown by reduced % fractional shortening (% fractional shortening: SHAM = 61.01 ± 1.3, TAC = 51 ± 1.5, TAC + UR = 45 ± 1.5, $p < 0.05$; Fig. 2b).

Next, to assess the effects of UTR antagonism on cell survival, TUNEL staining was performed in cardiac sections from SHAM, TAC and TAC + UR mice. UR administration significantly increased apoptotic cell death

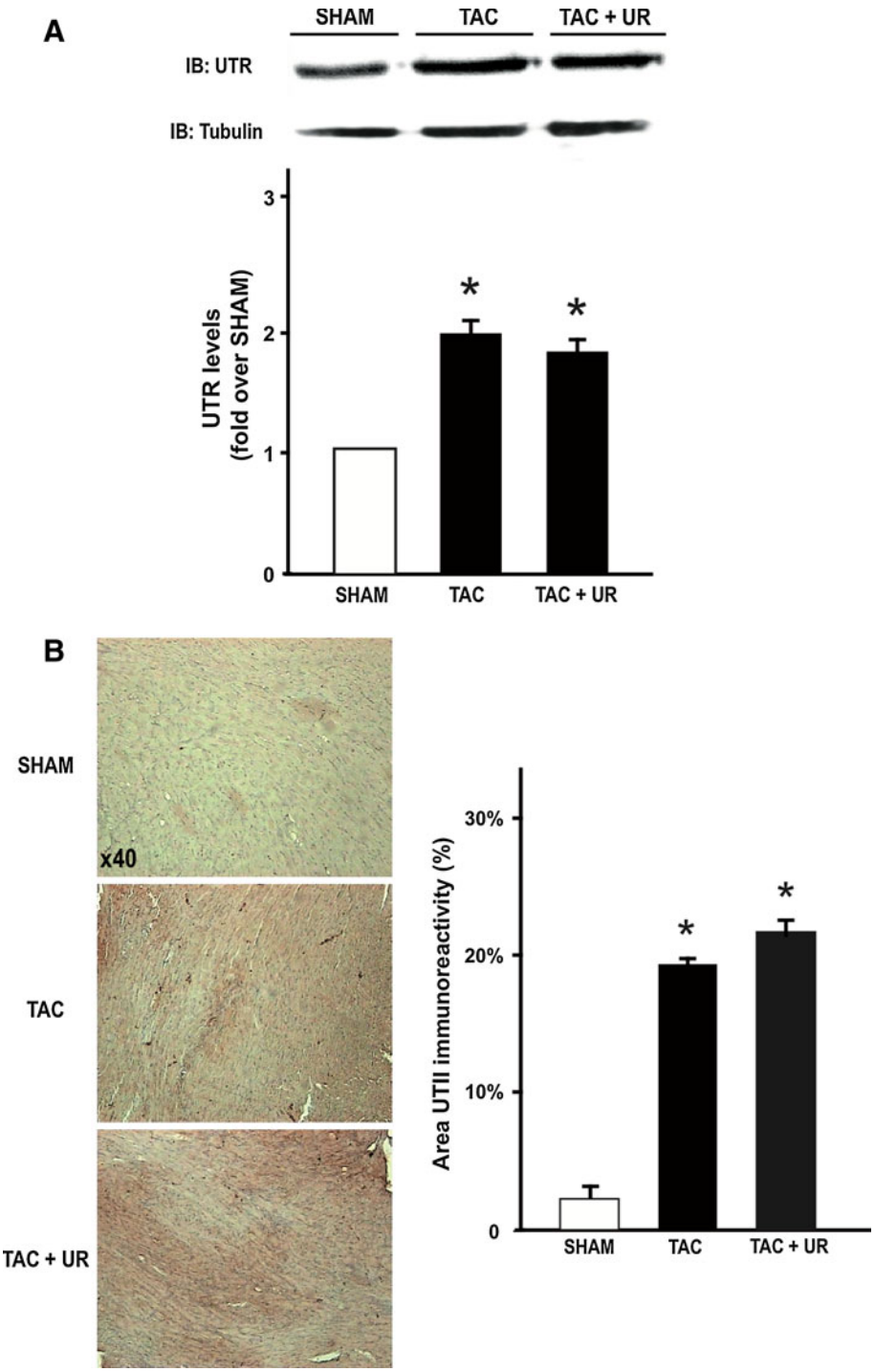


Fig. 1 Pressure overload induces endogenous UTR signalling. **a** Representative immunoblots (*upper panels*) and densitometric analysis (*lower panel*) of multiple independent experiments to evaluate UTR levels in left ventricular samples from mice undergoing the sham procedure (SHAM), transverse aortic constriction (TAC) or TAC with chronic infusion of the UTR antagonist Urantide (TAC + UR). Tubulin protein levels did not significantly change among the samples (*$p < 0.05$ vs. SHAM, $n = 10$ hearts/group). **b** *Left panels* representative UTII immunostaining of cardiac sections of mouse hearts from the different groups (×40). *Right panel* cumulative data of multiple independent experiments (*$p < 0.05$ vs. SHAM, $n = 10$ hearts/group)

in response to pressure overload, as shown by increased TUNEL-positive nuclei (% apoptotic nuclei: SHAM = 0.031 ± 0.02, TAC = 1.4 ± 0.3, TAC + UR = 2.2 ± 0.2, $p < 0.05$; Fig. 2c). Furthermore, the increased apoptotic cell death in UR-treated mice was accompanied by a greater extent of interstitial fibrosis in TAC + UR mice (% fibrosis: SHAM = 0.52 ± 0.87, TAC = 8.9 ± 0.83, TAC + UR = 14.3 ± 1.32, $p < 0.05$; Fig. 2d). Interestingly, UR treatment did not affect capillary density, that was similarly reduced in TAC and TAC + UR compared to SHAM mice (capillary density, % of SHAM: TAC = 74% ± 10.4, TAC + UR = 68% ± 13.3, not significant).

It has been previously shown that EGFR trans-activation plays a critical role in UTII signal transduction in vitro [35]. In order to determine whether UR inhibited UTR-mediated EGFR trans-activation in vivo, EGFR phosphorylation levels were analyzed in cardiac samples from SHAM, TAC and TAC + UR mice. While pressure overload induced a significant increase in EGFR phosphorylation, this activation was completely abolished by the chronic infusion of UR (Fig. 2e). Consistent with these results, ERK activation was significantly induced by TAC, and this was inhibited by UR treatment (Fig. 2f).

Fig. 2 UTR antagonism promotes cardiac dysfunction, increases apoptotic cell death and reduces EGFR trans-activation in response to pressure overload. **a** *Bar graphs* showing left ventricle weight/body weight (LVW/BW) ratios in SHAM, TAC, and TAC + UR mice (*$p < 0.05$ vs. SHAM, $n = 10$ hearts/group). **b** *Left panels* representative serial M-mode echocardiographic tracings from SHAM, TAC and TAC + UR mice. *Right panel* % fractional shortening of SHAM, TAC and TAC + UR mice (*$p < 0.05$ vs. SHAM; **$p < 0.05$ vs. SHAM and TAC, $n = 10$ hearts/group). **c** *Left panels* representative TUNEL staining of cardiac sections of mouse hearts from the different groups counterstained with methyl green. Positive nuclei appear *brown* (*arrows*; ×600). *Right panel* cumulative data of multiple independent experiments (*$p < 0.05$ vs. SHAM; ** $p < 0.05$ vs. SHAM and TAC, $n = 8$ hearts/group). **d** *Left panels* representative Sirius red staining of cardiac sections of mouse hearts from the different groups (×600). *Right panel* cumulative data of multiple independent experiments (*$p < 0.05$ vs. SHAM; ** $p < 0.05$ vs. SHAM and TAC, $n = 8$ hearts/group). **e** Representative immunoblots (*upper panels*) and densitometric analysis (*lower panel*) of multiple independent experiments to evaluate pEGFR and EGFR levels in SHAM, TAC and TAC + UR hearts. Total EGFR levels did not significantly change among the groups as shown by similar tubulin staining among the samples (*$p < 0.05$ vs. SHAM, $n = 8$ hearts/group). **f** Representative immunoblots (*upper panels*) and densitometric analysis (*lower panel*) of different repeated experiments analyzing pERK protein levels in SHAM, TAC and TAC + UR hearts. Total ERK2 or tubulin levels did not significantly change among the groups as shown by similar tubulin staining among the samples (*$p < 0.001$ vs. SHAM, $n = 8$ hearts/group)

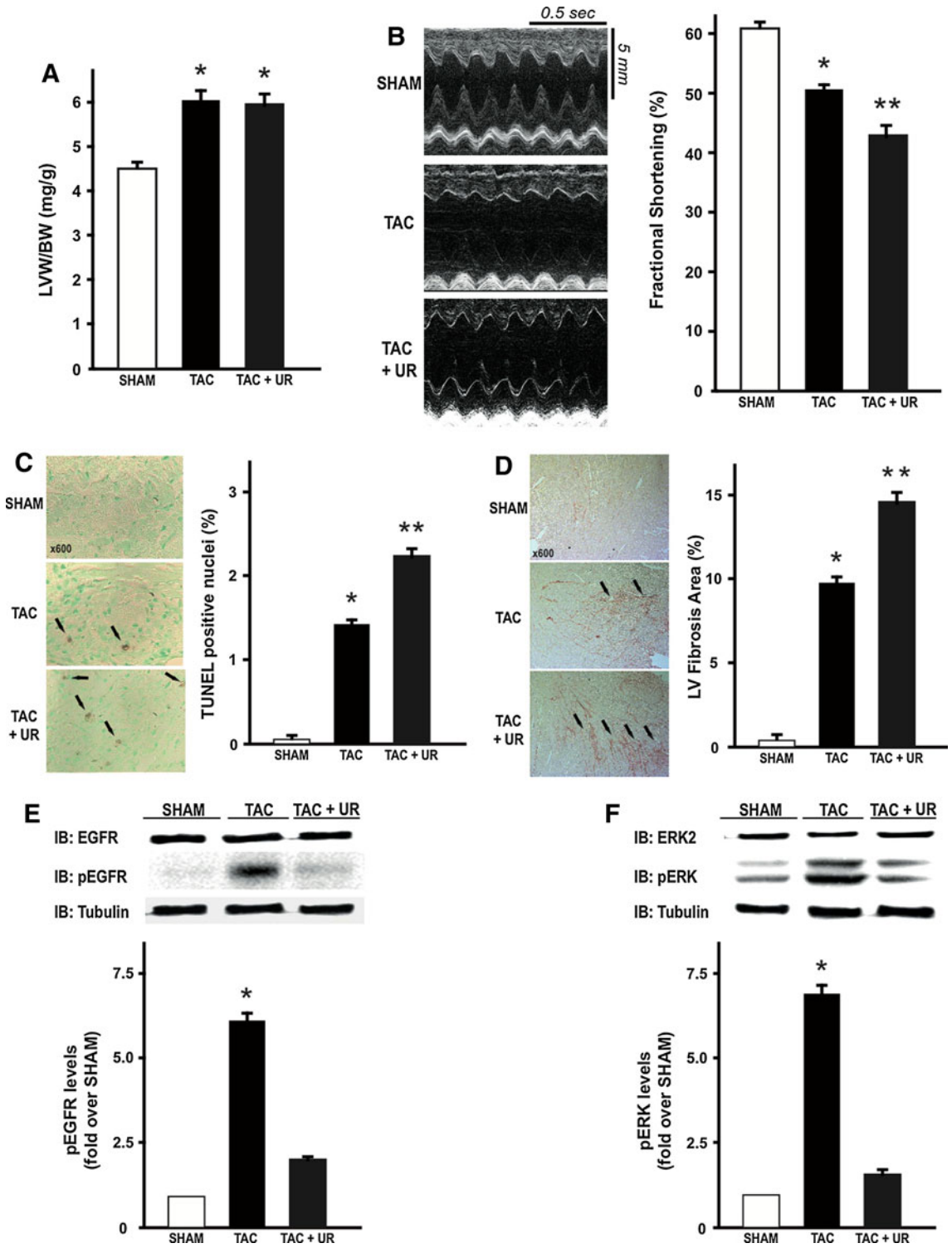
EGFR antagonism promotes apoptotic cell death and cardiac dysfunction in response to pressure overload

Since the role of 7TMR-mediated EGFR trans-activation in the heart is controversial, we next tested the effects of the selective EGFR blocker AG1478 in pressure overload-induced cardiac hypertrophy. As expected, AG1478 treatment reduced EGFR and ERK phosphorylation in TAC mice (Fig. 3a, b). Interestingly, AG1478 treatment did not affect cardiac hypertrophy development, but significantly reduced cardiac function, promoted apoptosis and increased fibrosis after 7 days of pressure overload (Fig. 3c–f), suggesting that EGFR signalling is protective under conditions of pressure overload.

β-Arrestin 1 and β-arrestin 2 are required for UTR trans-activation of EGFR

To better assess the mechanisms by which UTII stimulation could mediate EGFR signalling and induce the activation of downstream signalling pathways, HEK293 cells were transiently transfected with FLAG-EGFR and UTR, and stimulated with UTII with or without UR, or EGF. UTII stimulation resulted in a significant increase in phosphorylation of the EGFR (Fig. 4a, b) along with activation of ERK (Fig. 4a, c). Importantly, these effects could be inhibited by the knockdown of β-arrestin 1/2 (Fig. 4a–c). To determine whether UTR antagonism by molecules different from UR would affect EGFR transactivation, we tested other four different commercially available UTR antagonists on EGFR signalling and internalization (supplementary online Figure S1). Even if with different potency, all tested UTR antagonists (BIM23127, UFP-803, [ORN^5] URP, and SB657510) reduced EGFR and ERK phosphorylation, and EGFR internalization (supplementary online Figure S1).

To better determine the role of β-arrestin in EGFR trans-activation by the UTR, HEK293 cells transfected with EGFR-FLAG, UTR and β-arrestin-GFP were stimulated with UTII, EGF, UTII + UR or UR alone (Fig. 4d, panels 1–5). As expected, robust membrane translocation of β-arrestin-GFP was seen in cells stimulated with UTII (Fig. 4d, panel 2), while it was absent in cells treated with EGF, UTII + UR, or UR alone (Fig. 4d, panels 3–5). Next, HEK293 cells were transiently transfected with EGFR-GFP and UTR, and stimulated with UTII, with or without UR, or EGF. In the absence of agonist, EGFR had a uniform membrane distribution (Fig. 4d, panel 6). In contrast, UTII stimulation resulted in EGFR internalization as visualized by marked redistribution into cellular aggregates (Fig. 4d, panel 7). UTII-induced EGFR internalization was qualitatively similar to that evoked by EGF treatment (Fig. 4d, panel 8). UR treatment inhibited UTII-mediated EGFR internalization, while UR alone did not exert any effects on EGFR localization (Fig. 4d, panels 9–10). Importantly, siRNA knockdown of β-arrestin 1/2 completely blocked EGFR internalization induced by UTII, but not EGF (Fig. 4d, panels 11–15).



UTII or EGF reduce apoptotic cell death in vitro

Since β-arrestin–mediated signalling has been linked to the activation of protective signalling pathways [24], we next tested whether UTR-mediated GFR trans-activation would confer protection from apoptosis in smooth muscle-derived A10 cells (SMCs). In SMCs, UTII or EGF treatment significantly reduced apoptotic cell death induced by 24 h serum deprivation, as shown by TUNEL staining (Fig. 5a). In contrast, either UTR blockade by UR or EGFR

inhibition by AG1478 abolished this protective effect. These results were confirmed also by Annexin V and propidium iodide double staining (Fig. 5b), and by the evaluation of caspase 3 activation (Fig. 5c). Taken together, these findings suggest that EGFR trans-activation by UTR may induce activation of cytoprotective pathways that are independent of classic G-protein-dependent signalling.

Discussion

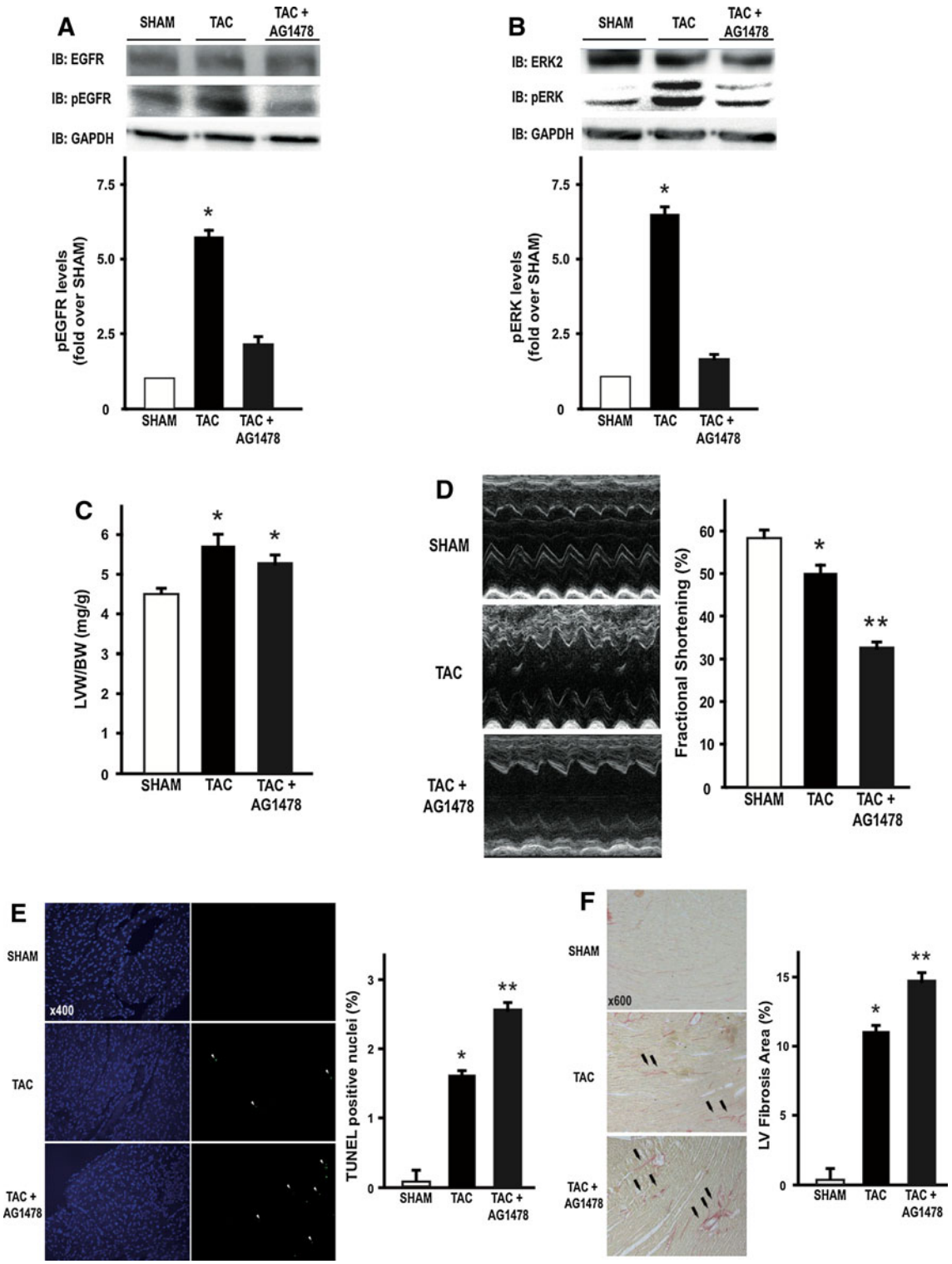
This study shows that UTR-mediated signalling can exert beneficial effects in vitro, and confer cardioprotection in a model of cardiac pathological remodelling induced by pressure overload. In vivo trans-activation of the EGFR could be responsible for such protective effects, reducing apoptotic cell death and fibrosis induced by pressure overload.

UTII has been involved in several physiological and pathological processes, such as the regulation of vascular tone, cardiac contractility and rhythm [26]. The effects of UTII/UTR signalling on cardiac remodelling and function are currently controversial. Since patients with significantly elevated plasma UTII levels displayed improved outcomes post-myocardial infarction, it has been suggested that UTII may play a cardio-protective role in cardiac remodelling [20]. Consistent with these results, UTII has been shown to dilate coronary arteries, to reduce post-ischaemia creatin kinase and atrial natriuretic peptide release [22, 40], and to exert beneficial effects on diastolic function [15]. However, UTR blockade by SB-611812 has been previously reported to attenuate cardiac dysfunction in a rat model of coronary artery ligation, and to reduce the interstitial fibrosis within the non-infarcted zone of the left ventricle [3]. These contradictory results could be explained, at least in part, by the partial agonism of some antagonists, or by differences among the different antagonists in the ability to block EGFR trans-activation as previously shown for βAR blockers [21].

UTII administration has been shown to promote hypertrophy of rat neonatal cardiomyocytes [27], and potentiate cardiac hypertrophy induced by isoproterenol in vivo [49]. Interestingly, these effects seem to be mediated, at least in vitro, by EGFR trans-activation [27]. However, in this study UTII in vivo administration under conditions of pressure overload did not exert any additional effects on cardiac mass, function, structure or apoptosis (supplementary online Figure S2). Since pressure overload markedly induced endogenous UTII levels and UTR signalling (Fig. 1), it is not surprising that further exogenous UTII administration might not exert any additional effects. Moreover, UTR antagonism by UR did not reduce cardiac hypertrophy as recently reported by in a different model with a different UTR antagonist [23]. It is possible to speculate that inhibition of UTR signalling might not be sufficient to contrast the development of cardiac hypertrophy under conditions of pressure overload.

In this study, we show for the first time the important role of UTII signalling in EGFR trans-activation and cell fate in vivo. Currently, controversy exists as to the physiological role that 7TMR-mediated EGFR trans-activation plays in the heart. While there is evidence that angiotensin II–stimulated EGFR trans-activation can contribute to pathological hypertrophy [2, 44, 48], it is also known that the EGFR family member HER2 plays a protective role in the development of dilated cardiomyopathy [6]. Here we show that UTR–mediated signalling can exert beneficial effects in vitro, and confer cardio-protection in a mouse model of cardiac pathological remodelling induced by pressure overload. Trans-activation of the EGFR could be responsible for such protective effects during heart injury, as recently demonstrated for the β1AR [34]. Future studies will be needed to better define the precise molecular mechanisms by which the EGFR signals might positively influence cardiomyocyte function. Even if we did not directly assess βAR levels or signalling after UR treatment, UR did not affect cardiac levels of the β1AR kinase GRK2 (supplementary online Figure S3), suggesting that GRK2-dependent β1AR desensitization might not be involved in these processes.

Fig. 3 EGFR antagonism promotes cardiac dysfunction and apoptotic cell death in response to pressure overload. **a** Representative immunoblots (*upper panels*) and densitometric analysis (*lower panel*) of multiple independent experiments to evaluate pEGFR and EGFR levels in SHAM, TAC and TAC + AG1478 hearts. Total EGFR levels did not significantly change among the groups, as shown by similar GAPDH staining among the samples (*$p < 0.05$ vs. SHAM, $n = 6$ hearts/group). **b** Representative immunoblots (*upper panel*) and densitometric analysis (*lower panel*) of different repeated experiments analyzing pERK protein levels in the hearts of SHAM, TAC and TAC + AG1478 mice. Total ERK2 levels did not significantly change among the groups as shown by similar GAPDH staining among the samples (*$p < 0.001$ vs. SHAM, $n = 6$ hearts/group). **c** *Bar graphs* showing left ventricle weight/body weight (LVW/BW) ratios in SHAM, TAC, and TAC + AG1478 mice (*$p < 0.05$ vs. SHAM, $n = 6$ hearts/group). **d** *Left panels* representative serial M-mode echocardiographic tracings from SHAM, TAC and TAC + AG1478 mice. *Right panel* % fractional shortening of SHAM, TAC and TAC + AG1478 mice (*$p < 0.05$ vs. SHAM; ** $p < 0.05$ vs. SHAM and TAC, $n = 6$ hearts/group). **e** *Left panels* representative DAPI (*right*) and TUNEL (*left*) staining of cardiac sections of mouse hearts from the different groups. Positive nuclei appear *green* (*arrows*; ×400). *Right panel* cumulative data of multiple independent experiments (*$p < 0.05$ vs. SHAM; **$p < 0.05$ vs. SHAM and TAC, $n = 6$ hearts/group). **f** *Left panels* representative Sirius red staining of cardiac sections of mouse hearts from the different groups (×600). *Right panel* cumulative data of multiple independent experiments (*$p < 0.05$ vs. SHAM; ** $p < 0.05$ vs. SHAM and TAC, $n = 6$ hearts/group)



Multiple mechanisms have been shown to connect 7TMRs and EGFR, including Src-mediated autocrine release of EGFR ligands such as HB-EGF [39], and the formation of 7TMR-EGFR signalling complexes [30]. Interestingly, EGFR trans-activation by UTR requires both β-arrestin 1 and 2, since elimination of either isoform in cells by siRNA prevented EGFR activation by UTII stimulation. This dual requirement might reflect distinct roles in the process played by each isoform, or perhaps the need for heterodimerization of β-arrestin 1 and 2, as previously demonstrated [43].

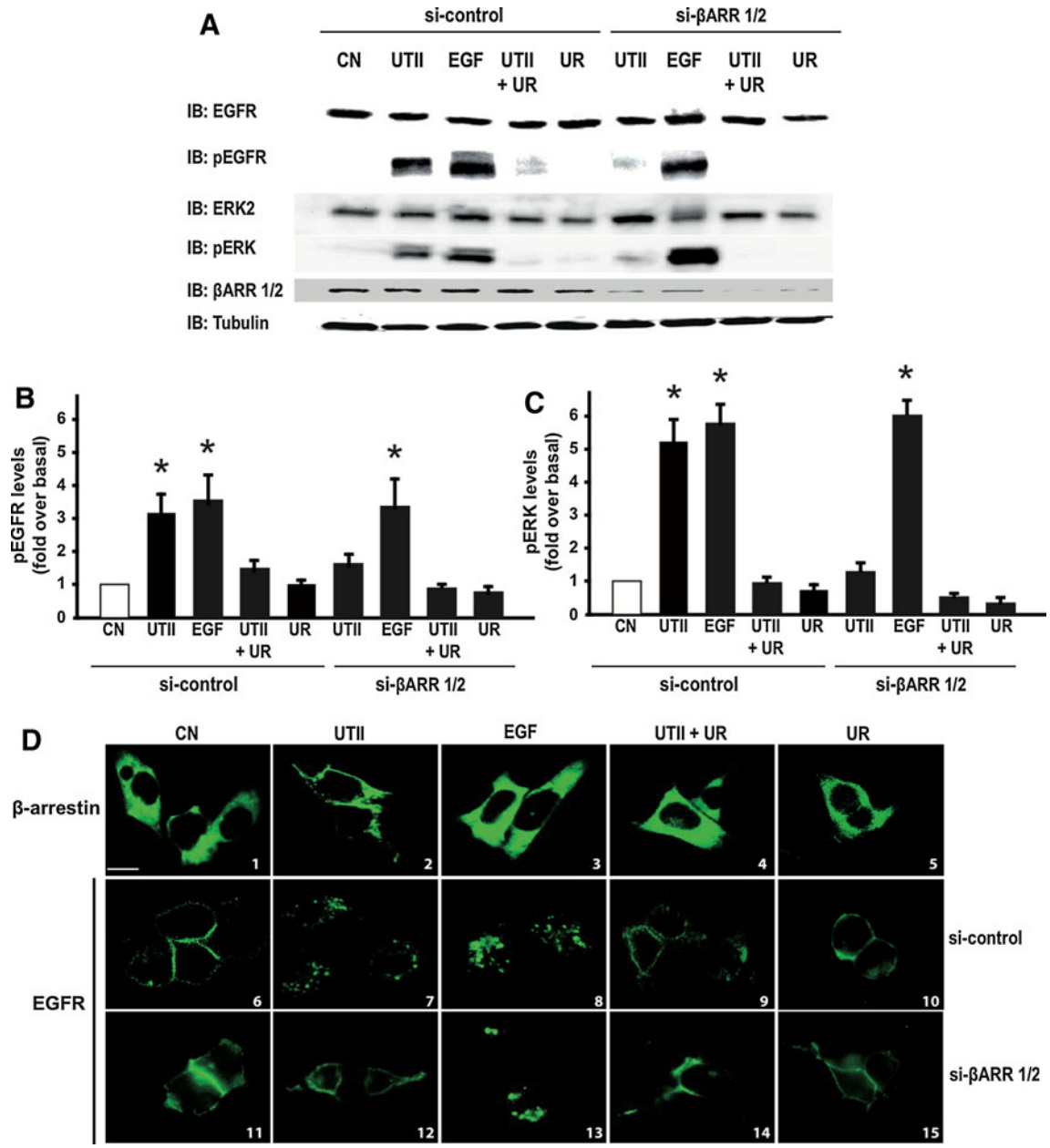


Fig. 4 UTR-mediated EGFR trans-activation is β-arrestin-dependent. **a** Representative immunoblots of multiple independent experiments to evaluate EGFR, pEGFR, ERK2, pERK, β-arrestin1/2 and tubulin levels in HEK 293 cells transfected with EGFR-FLAG and UTR, and then incubated with control siRNA or β-arrestin1/2 siRNA. Cells were unstimulated (CN) or stimulated with UTII, UTII + UR, UR alone or EGF. **b** *Bar graphs* showing cumulative data of multiple independent experiments to evaluate EGFR activation in HEK 293 cells; total EGFR levels did not significantly change among the groups ($*p < 0.05$ vs. CN). **c** *Bar graphs* showing cumulative data of multiple independent experiments to evaluate ERK activity in HEK 293 cells, total ERK2 levels did not significantly change among the groups ($*p < 0.05$ vs. CN). **d** *Panels 1–5* representative β-arrestin-GFP immunofluorescence images in HEK293 cells overexpressing EGFR-FLAG, UTR and β-arrestin-GFP. *Panels 6–15* representative EGFR immunofluorescence images in HEK293 cells overexpressing EGFR-GFP and UTR, and transfected with either control siRNA (*panels 6–10*) or β-arrestin 1/2 siRNA (*panels 11–15*). Cells were stimulated with UTII, EGFR, UR alone or UTII + UR. Each experiment was independently repeated 3–4 times. *Scale bar* 10 μm

Our study has important therapeutic implications for the development of novel drugs for patients with heart failure, since all currently used drugs have been developed using assays only testing G-protein-dependent signals. Thus, while G-protein-mediated intracellular effects of UTII might be similar to those described for angiotensin II or endothelin-1 and detrimental [4, 8, 10, 31, 32], our present study shows that G-protein- independent signals triggered

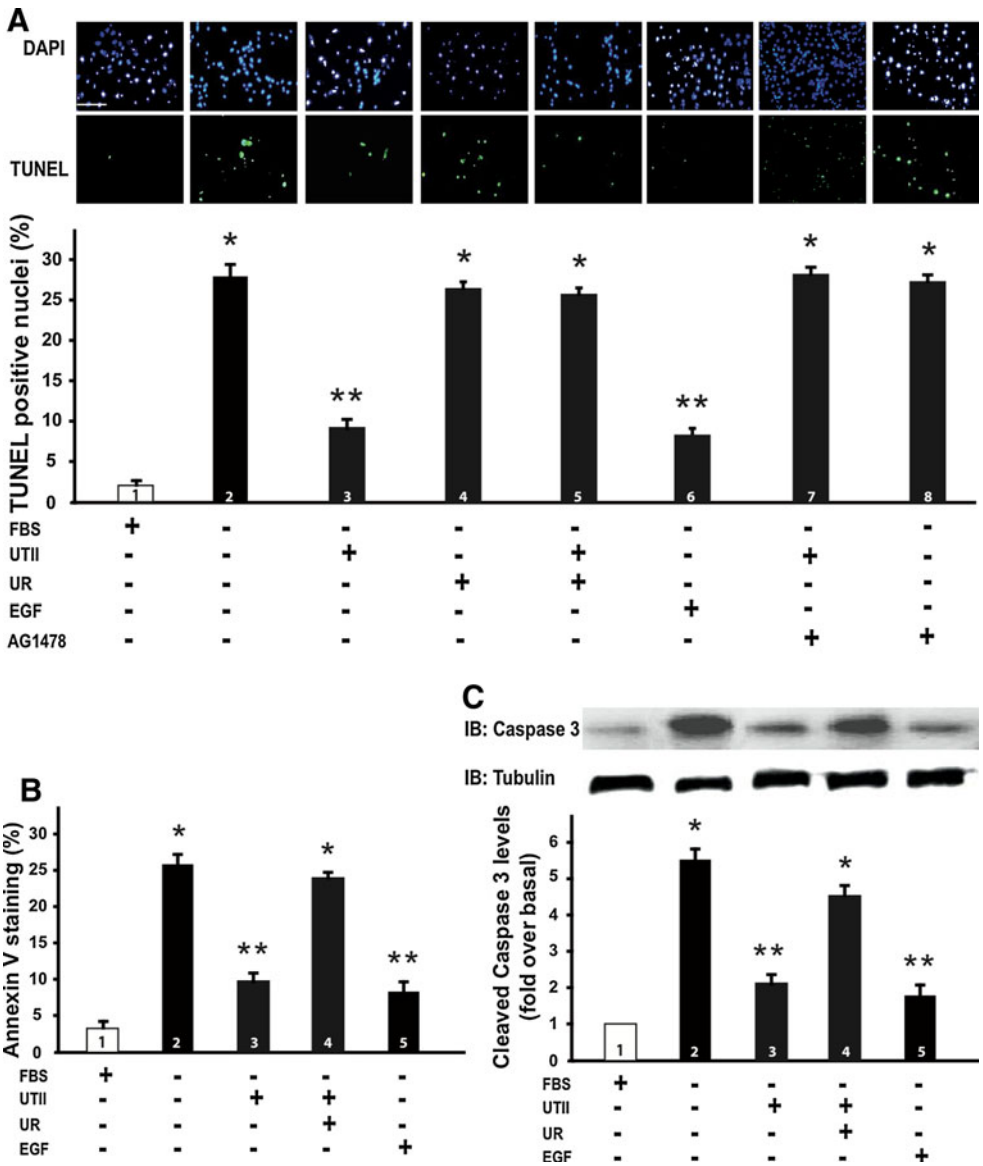


Fig. 5 UTII reduces apoptotic cell death induced by serum deprivation. **a** *Upper panels* representative DAPI (*top*) and TUNEL (bottom) stainings of smooth muscle cells (SMCs) serum-starved for 24 h, then incubated with UR (30nM) or the EGFR inhibitor AG1478 (1μM) for 30 min, and next stimulated with UTII (100 nM) or EGF (10 ng/ml). After TUNEL staining, positive nuclei appear *green* (*scale bar* 100 μm). *Bottom panel* cumulative data of multiple independent experiments (*$p < 0.05$ vs. *column 1*; **$p < 0.05$ vs. *columns 2, 4, 5, 7, 8*). **b** *Bar graphs* showing % staining of Annexin V of serum-starved SMCs incubated with UR for 30 min, and next stimulated with UTII or EGF (*$p < 0.05$ vs. *column 1*; **$p < 0.05$ vs. *columns 2, 4*). **c** representative immunoblots (*upper panels*) and densitometric analysis (*bottom panel*) of multiple independent experiments to evaluate caspase 3 cleavage/activation in serum-starved SMCs incubated with UR for 30 min, and next stimulated with UTII or EGF alone (*$p < 0.05$ vs. *column 1*; **$p < 0.05$ vs. *columns 2, 4*). Tubulin levels did not significantly change among the samples

by UTII are protective under conditions of pressure overload. Indeed, it is now becoming increasingly clear that distinct conformations of activated 7TMRs are responsible for G-protein-dependent and β-arrestin-dependent signalling pathways [44, 46]. Based on these data, it is conceivable that new generation drugs might differentially or even exclusively activate or block one or another signalling mechanism by inducing specific conformations of a wild-type receptor, resulting in improved cardiac function and prognosis in heart failure.

In conclusion this study shows, for the first time in vivo, a new UTR signalling pathway which is mediated by EGFR trans-activation and that promotes cell survival and cardioprotection. These findings provide a mechanism for the role of UTII in cardiovascular responses to injury, and might identify novel therapeutic strategies in heart failure.

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Conflict of interest None.

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