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Research paper

Atrial natriuretic peptide (ANP) modulates stress-induced autophagy in endothelial cells

Maurizio Forte [a,*,1], Simona Marchitti [b,1], Flavio di Nonno [a,1], Donatella Pietrangelo [c], Rosita Stanzione [a], Maria Cotugno [a], Luca D'Ambrosio [d], Alessandra D'Amico [d], Vittoria Cammisotto [e], Gianmarco Sarto [f], Erica Rocco [f], Beatrice Simeone [f], Sonia Schiavon [a], Daniele Vecchio [d], Roberto Carnevale [a,d], Salvatore Raffa [c], Giacomo Frati [a,d], Massimo Volpe [c,g], Sebastiano Sciarretta [a,d], Speranza Rubattu [a,c,**]

[a] IRCCS Neuromed, Pozzilli, IS, Italy
[b] Department of Experimental Medicine, Sapienza University, Rome, Italy
[c] Department of Clinical and Molecular Medicine, School of Medicine and Psychology, Sapienza University, Rome, Italy
[d] Department of Medical-Surgical Sciences and Biotechnologies, Sapienza University of Rome, Latina, Italy
[e] Department of Clinical, Internal Medicine, Anesthesiological and Cardiovascular Sciences, Sapienza University of Rome
[f] ICOT Istituto Marco Pasquali, Latina, Italy
[g] IRCCS San Raffaele, Rome, Italy

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ABSTRACT

Atrial natriuretic peptide (ANP), a cardiac hormone involved in the regulation of water/sodium balance and blood pressure, is also secreted by endothelial cells, where it exerts protective effects in response to stress. Autophagy is an intracellular self-renewal process involved in the degradation of dysfunctional cytoplasmic elements. ANP was recently reported to act as an extracellular regulator of cardiac autophagy. However, its role in the regulation of endothelial autophagy has never been investigated. Here, we tested the effects of ANP in the regulation of autophagy in human umbilical vein endothelial cells (HUVECs). We found that ANP rapidly increases autophagy and autophagic flux at physiological concentrations through its predominant pathway, mediated by natriuretic peptide receptor type A (NPR-A) and protein kinase G (PKG). We further observed that ANP is rapidly secreted by HUVEC under stress conditions, where it mediates stress-induced autophagy through autocrine and paracrine mechanisms. Finally, we found that the protective effects of ANP in response to high-salt loading or tumor necrosis factor (TNF)-α are blunted by concomitant inhibition of autophagy. Overall, our results suggest that ANP acts as an endogenous autophagy activator in endothelial cells. The autophagy mechanism mediates the protective endothelial effects exerted by ANP.

1. Introduction

Atrial natriuretic peptide (ANP) is a cardiac hormone belonging to the family of natriuretic peptides (NPs), which includes BNP and CNP [1]. Once secreted by atria in response to hemodynamic stress, ANP exerts several systemic effects such as diuresis, natriuresis and vasodilation [2]. These actions contribute to decrease blood pressure levels, in opposition to the renin-angiotensin-aldosterone system (RAAS). The biological effects of ANP are prevalently mediated by type A natriuretic peptide receptor (NPR-A) and its downstream signaling, which involves the production of intracellular messenger cyclic GMP (cGMP) and the activation of protein kinase G (PKG). ANP also exerts multiple pleiotropic effects in the cardiovascular system, through autocrine and paracrine mechanisms [3]. ANP attenuates cardiac injury in preclinical models of heart failure and ischemia/reperfusion (IR) injury [4,5]. A reduction of infarct size has been reported in patients with acute myocardial infarction receiving ANP [6].

ANP plays protective functions also in endothelial cells, which are

* Corresponding author at: IRCCS Neuromed, Pozzilli, Italy.
** Correspondence to: S. Rubattu, Department of Clinical and Molecular Medicine, School of Medicine and Psychology, Sapienza University, Rome, Italy.
E-mail addresses: maurizio.forte@neuromed.it (M. Forte), speranzadonatella.rubattu@uniroma1.it (S. Rubattu).
[1] These authors equally contribute.

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able to synthetize and secrete this peptide [7]. In this regard, ANP regulates angiogenesis, vascular inflammation and permeability [3]. Other studies have demonstrated that ANP rescues blood flow after limb ischemia in mice [8]. These findings suggest that ANP is involved in the regulation of vascular homeostasis through autocrine/paracrine mechanisms. However, the molecular mechanisms underlying these effects of ANP in endothelial cells are not fully characterized. We recently demonstrated that ANP is an extracellular regulator of cardiac autophagy, both in cardiomyocytes in vitro and in mice in vivo [9]. Autophagy is an evolutionarily conserved intracellular mechanism by which cell digests and recycles senescent or dysfunctional cytoplasmic elements, including whole organelles [10]. It is generally activated in response to nutrients or oxygen deprivation. Autophagy activation contributes to preserve cardiovascular homeostasis at baseline and limits cardiac injury in response to ischemic or metabolic stress, as observed in experimental models of myocardial infarction, heart failure and metabolic cardiomyopathy [11,12]. We found that mice with heterozygous systemic deletion of ANP undergoing I/R failed to activate cardiac autophagy [9]. We also demonstrated that the cardiac protective effects of ANP were blunted in presence of autophagy inhibition [9]. However, whether ANP acts as extracellular modulator of autophagy in other cell types, such as in endothelial cells is still unknown.

In the present work, we tested for the first time whether ANP administration can modulate autophagy in endothelial cells. We also investigated the role of endogenous ANP in the modulation of endothelial autophagy in response to stress. Finally, we attempted to unravel the biological significance of autophagy activation by ANP.

2. Materials and methods

2.1. Cell cultures

Human umbilical vein endothelial cells (HUVECs) (ATCC® CRL-1730TM) were cultured in EGM2 complete medium (Lonza, Milan, Italy) at 37 °C and 5 % CO_2. HUVECs between passages 1–4 were used for all experiments.

2.2. ANP treatment

ANP synthetic peptide (A1663, Sigma Aldrich, Milan, Italy) was dissolved in PBS and diluted in complete medium at the final concentration of 10^{-11} M. For the evaluation of autophagy, cells were treated with ANP for different times of incubation (from 1 to 4 h). The effects of ANP on autophagy levels were also tested in NPR-A and PKG knockdown cells. Control cells were treated with vehicle (PBS 0.001 %).

2.3. Autophagic flux

Autophagic flux was evaluated by treating cells with ANP 10^{-11} M for 4 h either in the presence or in the absence of bafilomycin (B1793,100 nM, for 4 h, Sigma Aldrich). Autophagic flux was also assessed by the mRFP-GFP-LC3 probe (kindly provided by Junichi Sadoshima, Rutgers New Jersey Medical School, USA), used as reported elsewhere [9]. This probe allows to discriminate autophagosomes from autophagolysosomes. The formation of autophagosomes causes an increase in the number of GFP-positive/mRFP-positive (yellow) dots. GFP is degraded by lysosomal acids whereas mRFP is resistant. The dots become GFP-negative/mRFP-positive (red) upon fusion with lysosomes. Thus, autophagosomes were detected both in red (mRFP) and green fluorescence (GFP) whereas autophagosolysosomes were detected only in red fluorescence. The increase in both yellow and red puncta is indicative of autophagy induction. In contrast, when autophagy is inhibited, both yellow and red puncta decrease. However, if lysosomal acidification or lysosome fusion is inhibited, there is an increase in yellow puncta and a decrease in red puncta. The latter occurs when the autophagic flux is inhibited. HUVECs were plated in an 8 chamber-slide and transduced with an adenovirus overexpressing mRF-GFP-LC3 for 48 h in the presence or absence of ANP 10^{-11} M for the last 4 h. Images were acquired with an epifluorescence microscope and yellow and red dots were manually counted with Image J software.

2.4. High salt and TNFα treatment

To evaluate the level of autophagy under stress conditions HUVECs were exposed to high salt medium (NaCl, 20 mM) for 8 h. Control cells were treated with the complete medium used for the routine cultures. The effects of high NaCl environment on autophagy were also tested in cells with NPR-A or PKG knockdown.

In a separate set of experiments, the exposure to high NaCl was performed for 24 h, either in the presence or in the absence of ANP 10^{-11} M, to evaluate the effects on cell viability, LDH release, and angiogenesis. The same effects were evaluated in cells with autophagy inhibition produced by ATG7 knockdown. In additional experiments, HUVECs were treated with TNFα (20 ng/ml) for 24 h, either in the presence or in the absence of ANP 10^{-11} M. The effects of TNFα were evaluated on angiogenesis and LDH release, with or without autophagy inhibition by ATG7 knockdown.

2.5. Gene silencing

Gene silencing was performed using species-specific commercially available siRNAs [NPR-A, PKG and ATG7 (Silencer Pre-designed siRNAs, Ambion, Thermo Fisher Scientific, Waltham, Massachusetts, US)] and following the manufacturer's protocol. Briefly, a specific amount of siRNAs was incubated with a transfection agent (Lipofectamine RNAi max) (Thermo Fisher Scientific,) in OPTIMEM-reduced serum medium (Thermo Fisher Scientific). After 5 h, OPTIMEM was replaced by complete medium, and the treatments (ANP, NaCl) were started after 48 h. Control cells were incubated with only lipofectamine. The efficiency of gene silencing was assessed by RT-PCR analysis and reported in **Supplementary Fig. 1**.

2.6. NT-proANP level assay

NT-proANP levels were assayed in cell supernatants by using a colorimetric commercially available ELISA kit (Biomedica Gruppe-Pantec, Torino, Italy). HUVECs were plated on a 6-multiwell plate and, after reaching 80 % confluence, were treated for different times (4, 6, 8 h) with high salt treatment. Values were acquired by a microplate reader and the levels of ANP were calculated by following the manufacturer's protocol. We also assessed gene expression of ANP (NPPA) by RTPCR.

2.7. Cell viability and lactate dehydrogenase release

HUVECs were silenced for ATG7 and exposed to high salt medium or TNFα (20 ng/ml) for 24 h either in the presence or in the absence of 10^{-11} M ANP. Cell viability was assessed by trypan blue. Lactate dehydrogenase (LDH) release was assessed by the LDH Assay Kit (ab65393, Abcam, Cambridge, UK) following the manufacturer's instruction. Briefly, 10 μl of conditioned culture medium were added into a new plate and incubated with LDH reaction mix for 30 min at room temperature. The release of LDH was quantified with a microplate reader equipped with a 450 nm filter.

2.8. Angiogenesis

Angiogenesis was evaluated by plating HUVECs on the top of the matrigel matrix in growth factor- reduced (BD Biosciences) 35 mm culture dishes, overnight at 37 °C and 5 % CO_2. Before matrigel assay, HUVECs were silenced for ATG7 and exposed to high salt medium or TNFα (20 ng/mL) for 24 h either in the presence or in the absence of

10^{-11} ANP M.

2.9. RT-PCR

Total RNA was extracted from cells by trizol (Thermo Fisher Scientific). 500 ng for each sample were retrotranscribed into cDNA by using the Superscript Vilo (Thermo Fisher Scientific). Then, the expression level of NPR-A, PKG, ATG7, NPPA, PDE2A and PDE3A was assessed by RTPCR using the SYBR green master mix 1× (Thermo Fisher Scientific) and the ViiA 7 Real-Time PCR System (Applied Biosystem, Foster City, CA, USA). Gene expression was calculated by the comparative delta Ct method, using GAPDH as housekeeping gene. Primers used are the following: ATG7, sense 5′-CCAAGGTCAAAGGACGAAGATAA-3′ antisense 5′-CTGGAAACTGCTACTCCATCTG-3′; NPR-A, sense 5′-CCAAATGTGGCTTTGACAAC-3′ antisense 5′-GAATCAGAATGCCGAGCAAG-3′; PKG, sense 5′-CATCAAAGAAGGAGACGTGG-3′ antisense 5′-CTGGACCCATGGTACACAAC-3′; GAPDH, sense 5′- CAAGGCTGTGGGCAAGGT -3′ antisense 5′-GGAAGGCCATGCCAGTGA-3′; NPPA, sense 5′-AGGTCAGACCAGAGCTAATC-3′ antisense 5′-GGCACGACCTCATCTTCTA-3′; PDE2A, sense 5′-GACGAGGACGAGCATGT-3′ antisense 5′-GTGGGTGAAGAGGTTCTTTG-3′; PDE3A, sense 5′-GTTCTGACTCTGAAGAGAGC-3′ antisense 5′-GTGGAAGAAACTCGTCTCAA-3′; β-ACTIN was used as housekeeping for the analysis of PDE2A and PDE3A expression: β-ACTIN, sense 5′-ATCACCATTGGCAATGAGCG-3′ antisense 5′-TTGAAGGTAGTTTCGTGGAT-3′.

2.10. Electron microscopy

HUVECs were fixed with 2 % glutaraldehyde in PBS for 2 h at 4 °C. Samples were post-fixed with 1 % osmium tetroxide in veronal acetate buffer pH 7.4 for 1 h at 25 °C, stained with uranyl acetate (5 mg/ml) for 1 h at 25 °C, dehydrated in acetone and embedded in Epon 812 (EMbed 812, Electron Microscopy Science, Hatfield, PA, USA). Ultrathin sections obtained with an Ultracut EMFCS ultramicrotome (Leica Microsystems, Wetzlar, Germany) and examined under a Morgagni 268D TEM (FEI, Hillsboro, OR, USA) equipped with a Mega View II charge-coupled device camera (SIS, Soft Imaging System GmbH, Munster, Germany). For the two-dimensional morphometric analysis of density of autophagic vacuoles (AV; total area of AV/100 μm2 of cytoplasmic area), at least 20 cell sections in ten different microscopic fields were randomly captured from ultrathin sections of each sample and digitalized at 28,000× original magnification. All AVs and total cytoplasmic area from each cell were measured with the AnalySIS software (Soft Imaging System).

2.11. Western blot analysis

Total proteins were extracted by RIPA buffer, separated by SDS PAGE and transferred onto polyvinylidene difluoride membranes (PVDF) (Amersham, Piscataway, New Jersey, USA). Membranes were blocked with 5 % non-fat milk for 2 h and incubated overnight with the following primary antibodies: LC-3 (MBL International), β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), GAPDH (Santa Cruz Biotechnology). Secondary antibodies used were anti-mouse and anti-rabbit (Millipore, Massachusetts, US). Signals were acquired with a Chemidoc (Biorad, California, US) and the intensity of the bands was quantified using Image J software.

2.12. cGMP and cAMP levels measurement

cGMP and cAMP levels in HUVECs treated or not with ANP 10^{-11} M for 4 h were evaluated by the Biotrak cGMP competitive enzyme immunoassay system (RPN226PL AK, Cytiva, Marlborough, Massachusetts) and the Direct Cyclic AMP Elisa Kit (Arbor Assay), respectively, according to the manufacturer's protocols. Values were read at 450 nm with a microplate reader.

2.13. Immunofluorescence

2×10^4 HUVECs were plated in an 8-chamber slide and fixed for 10 min with 4 % PFA, washed in PBS, blocked with 5 % normal horse serum (Vector Laboratories, Burlingame, CA, USA) and incubated overnight at 4 °C with anti-PDE2A (PD2A-112AP, FabGennix, Frisco, TX, US) or anti-PDE3A antibodies (PA5–82503, Thermo Fisher Scientific). Then, Alexa fluor 488 (Invitrogen Carlsbad, CA, USA) was used for detection in fluorescence. Cell nuclei were stained with Dapi mounting medium (DUO82040, Sigma Aldrich, Milan, Italy). Images were randomly taken with a fluorescence microscope.

2.14. Statistical analysis

All continuous variables are presented as mean ± SEM. Statistical analysis between two groups was performed by the *t*-test student. Statistical analysis between multiple groups was evaluated using one-way ANOVA followed by Bonferroni post-hoc test. Graphs and statistical analyses were performed by Graph Pad Prism (GraphPad Software, Inc. La Jolla, CA, USA) and statistical significance was set at $P < 0.05$ level. We performed at least three independent experiments for each assay.

3. Results

We evaluated the effects of physiological concentrations of ANP (10^{-11} M) on the modulation of autophagy in HUVECs (Fig. 1). We found that ANP increased LC3-II, a known marker of autophagy, after 4 h of treatment (Fig. 1A, B). In this experimental condition, ANP increased cGMP levels, without affecting levels of cAMP (**Supplementary Fig. 2A-B**). TEM analysis displayed an increased area of autophagic vacuoles following ANP exposure (Fig. 1C, D). We further analyzed autophagic flux by western blot and mRFP-GFP-LC3 experiments. HUVECs treated with ANP and bafilomycin, an inhibitor of autophagic flux, showed the highest levels of LC3-II, compared to cells treated with only ANP (Fig. 1E, F). We also analyzed the autophagic flux by performing experiments with the mRFP-GFP-LC3 tandem fluorescent probe. The latter allows to discriminate between autophagosomes (GFP-positive/mRFP-positive, yellow dots) and autophagolysosomes (GFP-negative/mRFP-positive, red dots). The increase in both yellow and red dots indicates autophagy activation. We observed that ANP increased the number of both autophagosomes and autophagolysosomes (Fig. 1G). These results suggest that exogenous administration of ANP activates autophagy and autophagic flux in endothelial cells.

To demonstrate the signaling involved in autophagy stimulation by ANP, autophagy level was evaluated in HUVECs silenced for either NPR-A or PKG. We found that ANP failed to increase LC3-II and fluorescent dots in the presence of NPR-A knockdown (Fig. 2A-C). Similar findings were obtained in endothelial cells silenced for PKG (Fig. 2D,E). The expression of phosphodiesterase PDE2A and PDE3 A, two enzymes hydrolyzing cAMP and cGMP and involved in ANP signaling [2] was not affected by ANP treatment (**Supplementary Fig. 2C-F**). These results indicate that ANP stimulates autophagy by its predominant pathway NPR-A/PKG/cGMP.

High-salt diet promotes vascular and endothelial dysfunction, due to hemodynamic changes or direct cellular effects [13,14]. To this aim, we investigated whether endogenous ANP can regulate stress-induced autophagy using high-salt (NaCl) treatment to mimic the effects of high-salt diet. We first observed an increased expression of ANP mRNA levels (NPPA) in HUVECs undergoing high-salt (NaCl) treatment (Fig. 3A). We also found an increased secretion of NT-proANP in endothelial cells undergoing stress (Fig. 3B). In this experimental condition, high NaCl induced autophagy whereas this effect was blunted in the presence of NPRA knockdown (Fig. 3C-E). This latter observation suggests that ANP is rapidly released by endothelial cells undergoing stress where it mediates stress-induced autophagy via autocrine/paracrine mechanisms.

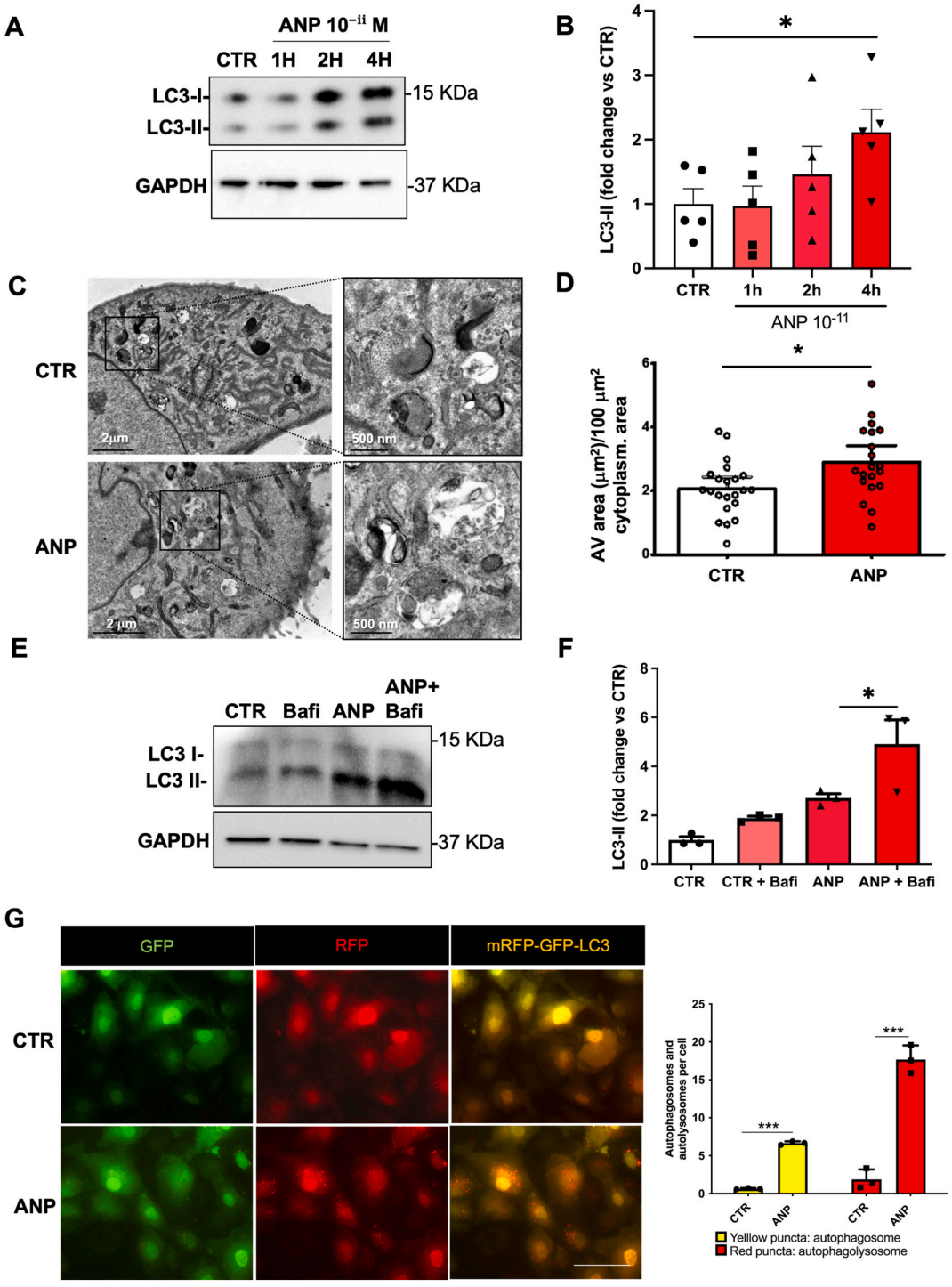

Fig. 1. ANP stimulates autophagy and autophagic flux in endothelial cells (ECs). **(A,B)** HUVECs were treated with ANP (10^{-11} M) for the indicated times in order to evaluate autophagy. Representative western blot for LC3 and corresponding densitometric analyses are reported ($N = 5$). *$p < 0.05$ obtained by using the student *t*-test. **(C, D)** Assessment of Autophagy in HUVEC cells through TEM Morphometric Analysis. **(C)** Ultrastructural analysis revealed a significant activation of the autophagic process in HUVEC cells following ANP treatment. Several degradative autophagic vacuoles were observed in the cytoplasm of these cells, distinguishing them from untreated HUVEC cells. **(D)** Quantitative morphometric analysis demonstrated a significant increase in AV structures associated with ANP treatment (Mann-Whitney test: *$p < 0.01$). **(E,F)** HUVECs were treated with bafilomycin (100 nM for 4 h) to evaluate autophagic flux either in the presence or in the absence of 10^{-11} M ANP for 4 h. Representative western blots for LC3 and corresponding quantification are shown. Values are expressed as mean ± SEM; ($N = 3$) *$p < 0.05$ obtained by using the one-way ANOVA followed by Bonferroni's Multiple Comparison Test. **(G)** HUVECs were transduced with adenovirus overexpressing mRFP-GFP-LC3 for 48 h and then treated with 10^{-11} M ANP for 4 h to evaluate LC3 puncta. Yellow dots (merged red and green) indicate autophagosomes whereas red dots indicate autolysosomes. Scale bar: 20 µm (N = 3) ***$p < 0.001$ obtained by using the student *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

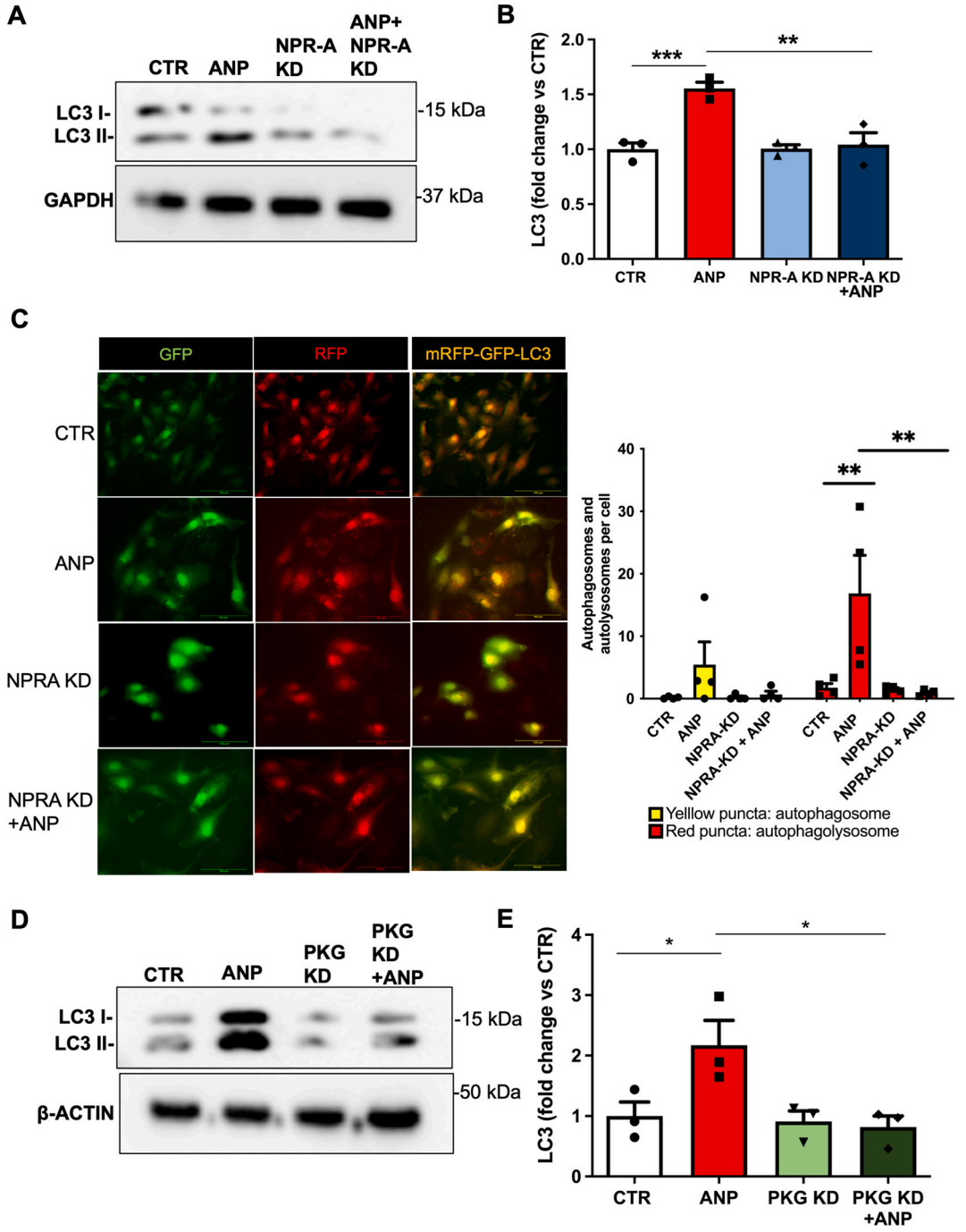

Fig. 2. ANP stimulates endothelial autophagy through type A natriuretic peptide receptor (NPR-A) and the PKG signaling. (A-C) HUVECs were silenced for NPR-A and treated with 10^{-11} M ANP for 4 h to evaluate autophagy. **(A)** Representative western blot for LC3 and corresponding densitometric analyses **(B)** are shown. (N = 3) $^{**}p < 0.01$, $^{***}p < 0.001$ obtained by using the one-way ANOVA followed by Bonferroni's Multiple Comparison Test. **(C)** Representative images of mRFP-GFP-LC3 experiments and corresponding quantification. ($N = 4$) $^{**}p < 0.01$ obtained by using the one-way ANOVA followed by Bonferroni's Multiple Comparison Test. **(D, E)** HUVECs were silenced for PKG and treated with 10^{-11} M ANP for 4 h to evaluate autophagy. Representative western blots for LC3 and corresponding densitometric analyses are shown. CTR indicates untreated and not silenced cells. Values are expressed as mean ± SEM (N = 3). $^{*}p < 0.05$, obtained by using the one-way ANOVA followed by Bonferroni's Multiple Comparison Test.

Finally, we explored the biological significance of autophagy activation by ANP on fundamental parameters such as cell viability, LDH release, and angiogenesis (Fig. 4A-C). We demonstrated that ANP rescued cell viability, LDH release, and angiogenesis in HUVECs exposed to high-NaCl (Fig. 4A-C). The protective effects of ANP were reduced by the parallel inhibition of autophagy through ATG7 silencing, a fundamental protein required for autophagosome formation. These results suggest that ANP exerts its endothelial protective effects through autophagy activation. We also exposed HUVECs to tumor necrosis factor-alpha (TNFα) treatment, another important stress that causes endothelial dysfunction due to an increased inflammatory process [15]. We tested whether ANP may reduce TNFα-induced endothelial damage by the autophagy mechanism. The results obtained with LDH experiments (Fig. 5A) showed a trend similar to that observed with the high-salt exposure (Fig. 4B). In fact, ANP reduced the cytotoxicity induced by TNFα, although the comparisons did not reach statistical significance

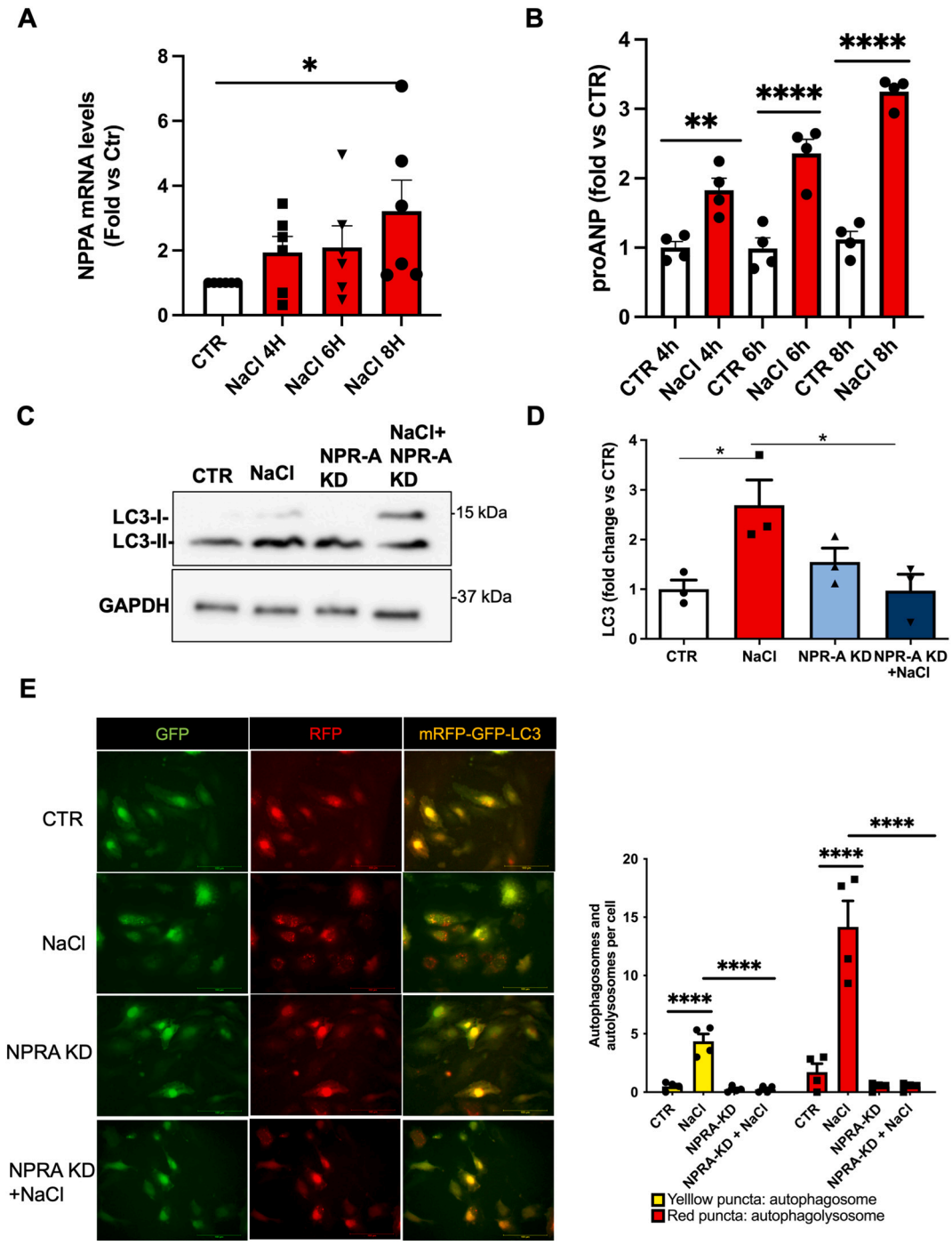

Fig. 3. ANP is released by endothelial cells in response to stress and regulates stress-induced autophagy. **(A)** NPPA gene expression in HUVECs treated with high salt (NaCl 20 mM) for the indicated time points. *p < 0.05, obtained by using the student t-test. **(B)** proANP secretion in HUVECs treated with high salt (NaCl 20 mM) for the indicated time points. Values are expressed as mean ± SEM; ($N = 4$) **$p < 0.01$, ****$p < 0.0001$ obtained by using the one-way ANOVA followed by Bonferroni's Multiple Comparison Test. **(C-E)** HUVECs were silenced for NPR-A and treated for 8 h with high salt (NaCl 20 mM) for autophagy evaluation; **(C)** Representative western blots for LC3 and corresponding densitometric analyses **(D)** are shown. Values are expressed as mean ± SEM; (N = 3). *p < 0.05, obtained by using the one-way ANOVA followed by Bonferroni's Multiple Comparison Test. **(E)** Representative images of mRFP-GFP-LC3 experiments and corresponding quantification; (N = 4) ****p < 0.0001 obtained by using the one-way ANOVA followed by Bonferroni's Multiple Comparison Test. CTR indicates untreated and not silenced cells.

($P = 0.0620$ for CTR vs TNFα and $P = 0.4644$ for TNFα vs TNFα + ANP) (Fig. 5A). We also found that ANP significantly rescued angiogenesis in HUVECs treated with TNFα (Fig. 5B). The protective effects of ANP in both sets of experiments were significantly attenuated in the presence of autophagy inhibition by ATG7 knockdown (Fig. 5A-B).

4. Discussion

The major finding of our work is the observation that ANP stimulates autophagy and autophagic flux in endothelial cells in vitro. We also found that ANP secretion in response to stress is required for autophagy activation, which in turn contributes to preserve cell viability and angiogenesis. NPR-A inhibition abrogates the pro-autophagic effects of

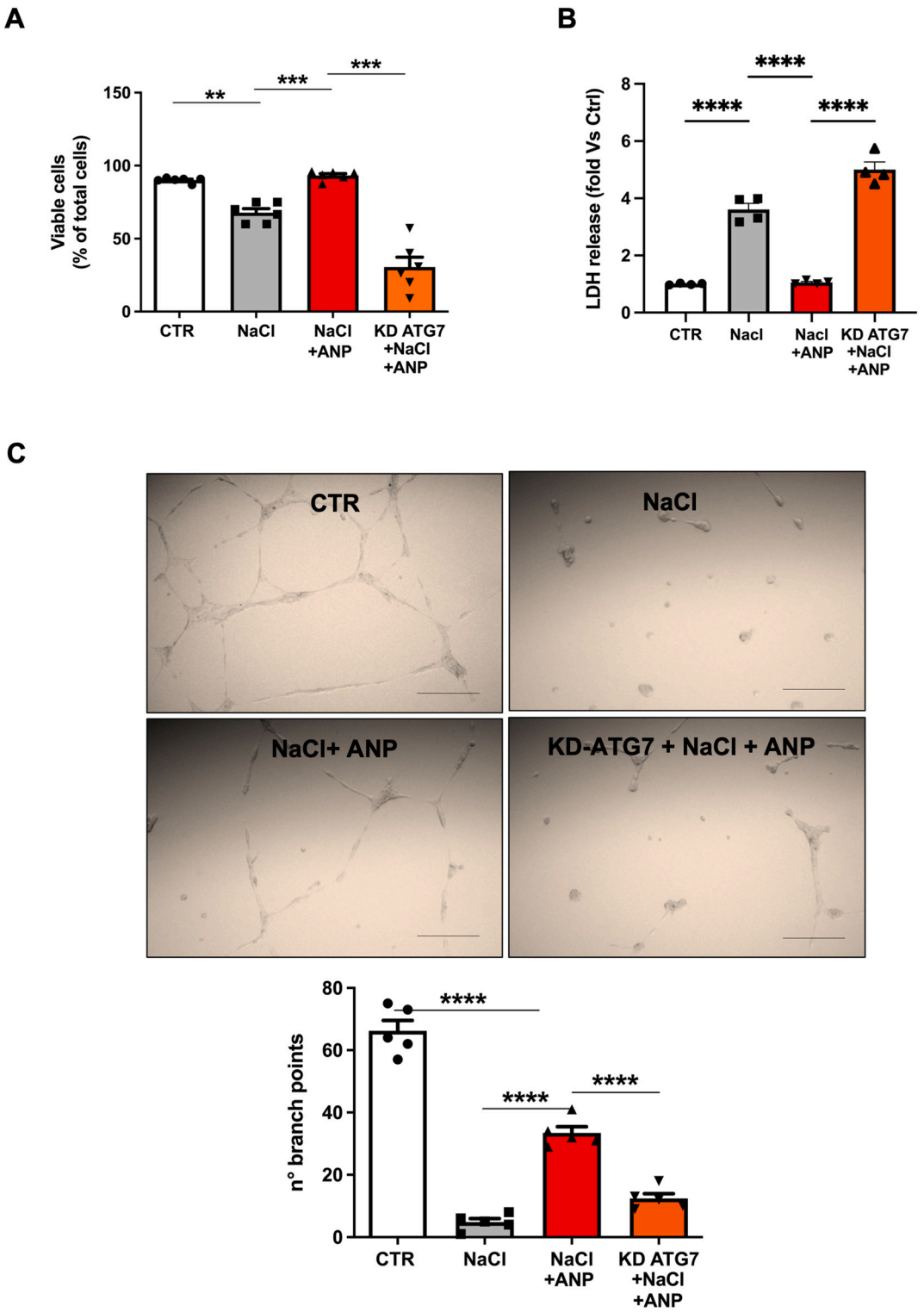

Fig. 4. ANP preserves cell viability, reduces LDH release, and improves angiogenesis through autophagy-dependent mechanism. (A-D) HUVECs were silenced for ATG7 (ATG7-KD) and treated with ANP (10^{-11} M) either in the presence or in the absence of high salt exposure (NaCl 20 mM for 24 h). Evaluation of cell viability **(A),** LDH release **(B),** and representative images of Matrigel assay and relative quantification **(C).** CTR indicates untreated and not silenced cells. Values are expressed as mean ± SEM (N = 4–6). **p < 0.01, ***p < 0.001, ****p < 0.0001 obtained by using the one-way ANOVA followed by Bonferroni's Multiple Comparison Test.

endogenous ANP whereas autophagy inhibition reduces the protective effects of ANP. To the best of our knowledge, this is the first report suggesting that ANP exerts its pleiotropic effects in the endothelium through the autophagic process.

Endothelial dysfunction is a common consequence of the exposure to several cardiovascular risk factors, such as hypertension, diabetes, dyslipidemias, smoking, obesity and metabolic syndrome. It is also an important pathogenic mechanism involved in several cardiovascular diseases, such as myocardial infarction, cardiomyopathies, or ischemic stroke [16]. Experimental evidence and observational studies also

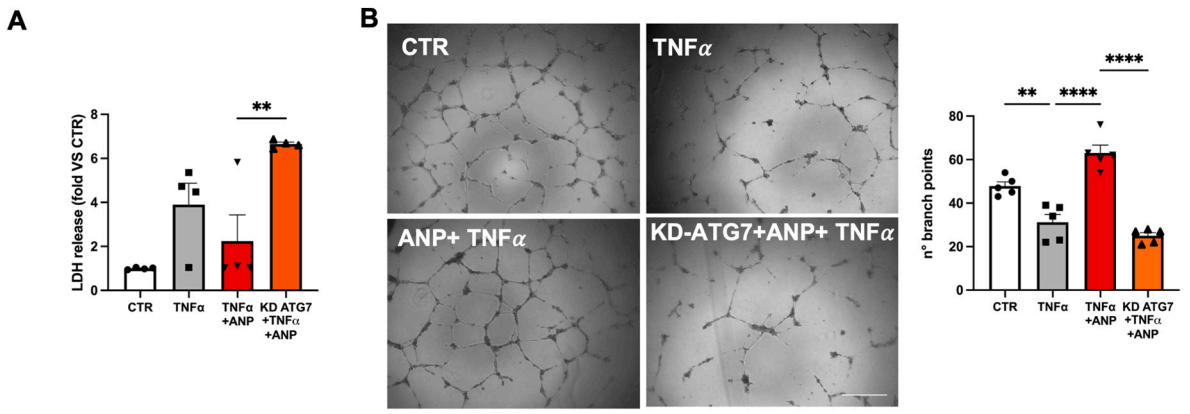

Fig. 5. ANP reduces LDH release and rescues angiogenesis in response to TNFα. (A-B) HUVECs were silenced for ATG7 (ATG7-KD) and treated with ANP (10^{-11} M) either in the presence or in the absence of TNFα (20 ng/ml) for 24 h. **(A)** Evaluation of LDH release and representative images of Matrigel assay and relative quantification **(B)**. CTR indicates untreated and not silenced cells. Values are expressed as mean ± SEM (N = 4–5). **p < 0.01, ****p < 0.0001 obtained by using the one-way ANOVA followed by Bonferroni's Multiple Comparison Test.

demonstrated that the impairment of autophagy leads to vascular dysfunction [17,18]. On the other hand, autophagy activation contributes to the reduction of endothelial dysfunction, leading to vascular protection and recovery, as observed in murine models, in hypertensive patients and in patients affected by peripheral artery disease [19–22]. However, the molecular mechanisms regulating endothelial autophagy are not well characterized, especially those regarding its extracellular modulators. Here, we demonstrated that ANP is an endogenous extracellular activator of autophagy in endothelial cells undergoing stress. This work extends our recent evidence demonstrating that ANP exerts cardiac protective effects through autophagy [9]. In a separate study, we also found that ANP improves autophagy and mitophagy (the selective form of autophagy for mitochondria) in PBMCs isolated from patients with chronic heart failure with reduced ejection fraction [23]. Remarkably, we further observed that PBMCs of HF patients treated with sacubitril/valsartan (ARNi), a drug which increases circulating ANP levels, showed increased levels of autophagy/mitophagy, associated with the improvement of left ventricular function [23]. These findings suggest that the increase of circulating levels of ANP may represent a suitable approach to activate autophagy and exert beneficial effects in condition of autophagy dysregulation, such as in HF. Patients with diabetes, obesity or metabolic syndrome show reduced levels of circulating ANP, because of reduced synthesis, down-regulation of NPR-A and increased systemic clearance [24]. In these conditions, ANP reduction may promote the development and the progression of diabetic cardiomyopathy, vascular inflammation and endothelial dysfunction. Autophagy and mitophagy are also inhibited in the cardiovascular system in the presence of diabetes and metabolic syndrome [11]. Our previous and current data suggest that restoration of ANP levels may exert protective effects by a concomitant activation of autophagy in both cardiomyocytes and endothelial cells. Mice with endothelial deletion of NPR-A undergoing pressure overload are more susceptible to cardiomyopathy development compared to wild-type mice, suggesting a major role of ANP in endothelial cells [8]. It is reasonable to hypothesize that lack of activation of endothelial autophagy in NPR-A knockout mice may exacerbate the cardiac detrimental effects of pressure overload. Of interest, we previously demonstrated that a genetic variant of ANP (C2238-αANP) is associated with increased cardiovascular risk and with endothelial dysfunction in humans [25–28]. It would be of interest to evaluate whether C2238-αANP is also associated with reduced autophagy in these individuals.

Some limitations of our study should be also acknowledged. We did not investigate the selective forms of autophagy activated by ANP. In this regard, new specific studies are required to investigate the involvement of mitophagy and whether mitophagy activation reduces mitochondrial damage. We did not test the ability of ANP to improve endothelial function through autophagy in relevant models of endothelial dysfunction in vivo. Future studies are required to test the protective effects of ANP mediated by autophagy stimulation in diabetic mice and in mice with atherosclerosis.

5. Conclusion

In conclusion, our data suggest that pharmacological strategies restoring ANP levels may provide effective therapeutic approaches to counteract the progression of cardiovascular abnormalities associated with endothelial dysfunction and may be a novel approach to broaden the range of therapies for the treatment of cardiovascular disease.

CRediT authorship contribution statement

Maurizio Forte: Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Simona Marchitti:** Methodology, Investigation, Data curation. **Flavio di Nonno:** Methodology, Investigation. **Donatella Pietrangelo:** Methodology, Investigation. **Rosita Stanzione:** Methodology, Investigation, Data curation. **Maria Cotugno:** Methodology, Investigation. **Luca D'Ambrosio:** Methodology, Investigation. **Alessandra D'Amico:** Methodology, Investigation. **Vittoria Cammisotto:** Methodology, Investigation. **Gianmarco Sarto:** Methodology. **Erica Rocco:** Data curation. **Beatrice Simeone:** Methodology. **Sonia Schiavon:** Methodology. **Daniele Vecchio:** Methodology. **Roberto Carnevale:** Supervision. **Salvatore Raffa:** Visualization, Supervision, Methodology, Investigation. **Giacomo Frati:** Writing – review & editing, Validation, Supervision. **Massimo Volpe:** Writing – review & editing, Visualization. **Sebastiano Sciarretta:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Speranza Rubattu:** Writing – review & editing, Writing – original draft, Validation, Supervision, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

None to disclose.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2024.119860.

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