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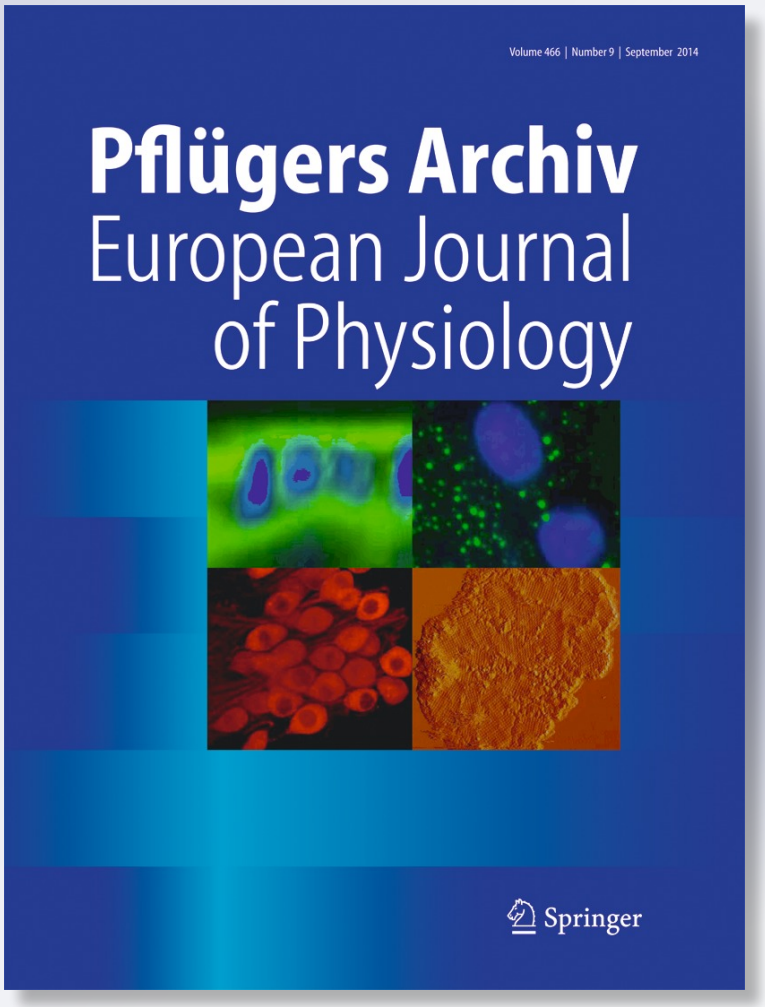

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MOLECULAR AND CELLULAR MECHANISMS OF DISEASE

Endogenous endothelin 1 mediates angiotensin II-induced hypertrophy in electrically paced cardiac myocytes through EGFR transactivation, reactive oxygen species and NHE-1

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Abstract Emerging evidence supports a key role for endothelin-1 (ET-1) and the transactivation of the epidermal growth factor receptor (EGFR) in angiotensin II (Ang II) action. We aim to determine the potential role played by endogenous ET-1, EGFR transactivation and redox-dependent sodium hydrogen exchanger-1 (NHE-1) activation in the hypertrophic response to Ang II of cardiac myocytes. Electrically paced adult cat cardiomyocytes were placed in culture and stimulated with 1 nmol l^{-1} Ang II or 5 nmol l^{-1} ET-1. Ang II increased ~45 % cell surface area (CSA) and ~37 % [^{3}H]-phenylalanine incorporation, effects that were blocked not only by losartan (Los) but also by BQ123 (AT_1 and ET_A receptor antagonists, respectively). Moreover, Ang II significantly increased ET-1 messenger RNA (mRNA) expression. ET-1 similarly increased myocyte CSA and protein synthesis, actions prevented by the reactive oxygen species scavenger MPG or the NHE-1 inhibitor cariporide (carip). ET-1 increased the phosphorylation of the redox-sensitive ERK1/2-$p90^{RSK}$ kinases, main activators of the NHE-1. This effect was prevented by MPG and the antagonist of EGFR, AG1478. Ang II, ET-1 and EGF increased myocardial superoxide production (187±9 %, 149±8 % and 163.7±6 % of control, respectively) and AG1478 inhibited these effects. Interestingly, Los inhibited only Ang II whilst BQ123 cancelled both Ang II and ET-1 actions, supporting the sequential and unidirectional activation of AT_1, ET_A and EGFR. Based on the present evidence, we propose that endogenous ET-1 mediates the hypertrophic response to Ang II by a mechanism that involves EGFR transactivation and redox-dependent activation of the ERK1/2-$p90^{RSK}$ and NHE-1 in adult cardiomyocytes.

Keywords Cardiac hypertrophy · Ang II · ET-1 · EGFR transactivation · NHE-1 · ROS

Introduction

Many cardiovascular effects initially thought to be directly produced by angiotensin II (Ang II) have been actually found to be mediated by endogenous endothelin 1 (ET-1) released in an autocrine/paracrine fashion in response to Ang II [12, 29, 34, 45, 49, 60]. The first evidence for this mechanism in cardiac hypertrophy (CH) was the seminal report of Ito et al. [29]. In this manuscript, the authors demonstrated that Ang II exerted a hypertrophic response in cultured neonatal rat myocytes that was prevented by pharmacologically blocking ET_A receptors or by using an antisense oligonucleotide to preproET-1 mRNA [29]. They also found that Ang II stimulated the release of immunoreactive ET-1 from cardiomyocytes [29]. In agreement with this, Liang and Gardner [34], also working with neonatal rat cardiomyocytes, reported that the increase in the activity of the brain natriuretic peptide gene promoter induced by Ang II was prevented by ET_A receptor blockade.

As mentioned above, these experiments were all performed in cardiomyocytes isolated from neonatal rats. Although data

Correa and Nolly contributed equally to this work

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obtained from neonatal cardiomyocytes were important, it can be challenged due to the critical differences in electrical, metabolic and pharmacological properties that exist between neonatal and adult cardiomyocytes [18, 28, 44, 54, 62]. Moreover, whilst isolated adult cardiomyocytes are quiescent cells in culture, neonatal cardiomyocytes have spontaneous beating at different rates and possess critical differences in the relative expression of calcium cycling proteins, such as the Na^+/Ca^{2+} exchanger (NCX), that play an important role in CH development after an increase in intracellular sodium [23, 36].

Primary myocardial diseases in cats are similar to those in humans, particularly, CH [26]. Morphological patterns of left ventricular hypertrophy are similar to the phenotypic expression of CH in humans. Investigators have been interested in the morphological appearance of cardiomyopathy in cats, which incorporates several of the anatomic hallmarks of CH in humans including asymmetrical left ventricular hypertrophy, cardiac muscle cell disorganization, abnormal intramural coronary arteries and myocardial fibrosis [25, 42]. Feline animal model has proven to be valuable for the study of human disease [25, 39–42, 53, 61]. For these reasons, we choose to use this experimental model.

A new step in the signalling cascade triggered by Ang II/ET-1 has been described: epidermal growth factor receptor (EGFR) transactivation [3, 15, 30, 33, 59]. In 2002, Kagiyama et al. [30] demonstrated for the first time that EGFR transactivation played an essential role in Ang II-induced CH in vivo. These authors showed that EGFR downregulation by specific antisense oligonucleotide administration significantly reduced CH in adult rats with subcutaneous infusion of Ang II. In the same line, experiments performed in cardiomyocyte of transgenic mice with overexpression of a mutated AT_1 receptor were unable to transactivate the EGFR, inhibiting pathological CH development [64]. These authors demonstrated that Ang II-induced activation of EGFR by phosphorylation.

On the other hand, it is widely recognized the ability of Ang II and ET-1 to stimulate reactive oxygen species (ROS) production [12, 27, 38]. Also, it has been proposed that ROS mediates signal transduction involved in CH induced by ET-1 or phenylephrine in adult rat cardiomyocytes [57]. The main kinase cascade involved in NHE-1 phosphorylation/activation is the redox-sensitive MEK/ERK/$p90^{RSK}$.

The Na^+/H^+ exchanger isoform 1 (NHE-1) not only regulates intracellular pH and cell volume but also plays a critical role in the development of pathological CH (for review, please see Refs. [31] and [8]). Several prohypertrophic factors, such as Ang II and ET-1 as well as myocardial stretch, caused by hemodynamic overload, promotes NHE-1 phosphorylation and activation. NHE-1 hyperactivity increases intracellular Na^+ (Na^+_i) leading to Ca^{2+} overload through the NCX, activating intracellular signalling pathways involved in the hypertrophic response [11].

The increase in intracellular Ca^{2+} concentration is a well-known trigger of the hypertrophic response by activating several signalling prohypertrophic molecules such as the Ca^{2+}-calmodulin kinase II and the phosphatase calcineurin. We have previously shown that inhibition of NHE-1 was able to induce the regression of CH in spontaneously hypertensive rats effect probably due to the normalization of calcineurin/NFAT pathway activity detected [21].

Based on this background, our aim was to get insight into this signalling pathway triggered by Ang II leading to hypertrophy in adult cardiomyocytes. We were particularly interested in clarifying the potential role played by endogenous ET-1, through EGFR transactivation and ROS-dependent NHE-1 activation. We hypothesize that avoiding EGFR transactivation, ROS production or NHE-1 activation could emerge as novel strategies to successfully prevent CH in response to Ang II/ET-1. Interestingly, for this purpose we used a unique experimental model consisting of electrically paced isolated adult myocytes, intervention that helps to maintain cell morphology as well as electrical and contractile properties in culture [6].

Materials and methods

All procedures followed during this investigation conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH, Publication No. 85–23, revised 1996) and the experimental protocol was approved by the Animal Welfare Committee of La Plata School of Medicine. Cats (body weight 3–4 kg) were anaesthetized by intraperitoneal injection of sodium pentobarbital (35 mg/kg body weight) and hearts rapidly excised when plane three of phase III of anaesthesia was reached.

Isolation of adult cat ventricular myocytes

Ventricular myocytes were isolated from adult cat myocardium by collagenase digestion in combination with mechanical agitation according to the technique previously described [1]. The purity of the myocyte cultures obtained with this method was higher than 95 %. After cell isolation, Ca^{2+}-tolerant myocytes were suspended in Dulbecco's minimal essential medium (DMEM) medium (GIBCO) supplemented with (in mmol l^{-1}): creatine 5, taurine 5, L-carnitine 2. To prevent bacterial infection, the media contained 100 IU penicillin and 100 μg/ml streptomycin. β-Cytosine arabinoside (10 μmol l^{-1}) was also included in the culture medium to prevent non-muscle cell overgrowth. To facilitate adhesion to the culture dish, the myocytes were plated on laminin-coated 35 mm dishes at a density of 1.5×10^5 (laminin 10 μg/35 mm). After 1 h of incubation, cultures were rinsed to remove non-adherent myocytes.

Electrical stimulation of myocyte contraction in culture

Adult cat myocytes, which are normally quiescent in culture, were induced to develop synchronic contractions via electrical field stimulation in a device especially made to that aim. For this purpose carbon electrodes submerged in each well of a 12-well culture tray were used. Metallic wires were inserted into bored holes in the carbon to couple electrode pairs in adjacent wells and to connect the terminal electrodes to the stimulator leads. The stimulator was set to deliver pulses of 0.5 Hz. The cells were maintained under sterile conditions in an incubator in a 5 % CO_2–95 % atmospheric air at 37 °C during 18 h, the total duration of the experimental protocol.

Measurement of cell area

After 18 h of culture, cardiac myocytes were washed twice with phosphate buffered saline (PBS)/glucose and fixed in buffered 10 % formaldehyde. CSA was determined in no less than 50 randomly selected cells with rod shape and clear cross-striations. The images were captured using an analogue video camera, digitized and processed by a computer morphometry program (Image-Pro Plus for Windows 95/98 v4.5-Media Cybernetics, Silver Spring, MA, USA).

Incorporation of [^{3}H] phenylalanine

The extent of de novo protein synthesis was estimated by measuring [^{3}H] phenylalanine incorporation, normalized to the amount of DNA in isolated cat myocytes grown in laminin-coated wells and incubated in duplicate with l-[2,3,4,5,6-^{3}H] phenylalanine (10 μCi ml^{-1}, Amersham Biosciences). Cells were thoroughly washed with ice-cold PBS (pH 7.4) 18 h after culture, and 10 % trichloroacetic acid (TCA) was added. Cells were kept under these conditions at 4°C for 12 h to precipitate proteins. Each well was then scraped and the precipitate washed twice with 10 % TCA and twice with 95 % ethanol. Between washes with TCA, the suspension was briefly sonicated to improve the washing procedure. Finally, the pellet was suspended in 0.15 mol l^{-1} NaOH. Aliquots were counted by a scintillation counter and the results expressed as nmol of [^{3}H] phenylalanine incorporated per disc. DNA content/disc was fluorometrically determined in neutralized samples with the fluorescent dye bisbenzimide (Hoecht 33258). The values obtained in each experimental condition were expressed as a percentage of control, considered 100 %.

Isolation of total RNA and real-time PCR

At the end of the culture period, quantitative polymerase chain reaction (PCR) to measure preproET-1, atrial and brain natriuretic peptides (ANF and BNP, respectively) expression were performed as previously reported [22]. Briefly, total RNA was isolated from cardiomyocyte suspensions using the RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA (0.8 μg) was reverse-transcribed using Omniscript RT kit (Qiagen). A dilution of the resulting cDNA was used for quantifying the relative content of mRNA by real-time PCR (iCycleriQ Real-Time PCR Detection System, Bio-Rad) using appropriate primers and SYBR Green as fluorescent probe. The following primers, designed using Primer3 software, were used:

GAPDH forward primer 5′-GGTGATGCTGGTGCTG AGTA-3′, reverse primer 5′-AGAAGGGGCAGAGA TGATGA-3′;
ANF forward primer 5′-TGTCAGCTCTTGTGGCAA AC-3′, reverse primer 5′-CTCCAAATGGTCCAGCAA AT-3′;
BNP: forward primer 5′-CAATACAGGGAGCTGCTG-3′, reverse primer 5′-CCGGAGGACATTGTCATG-3′;
preproET-1: forward primer 5-CAGACAAAGAACTC CGAGCC-3, reverse primer 5-GGTCTTGATGCTGT TGCTGA-3.

PCR reactions were performed with TaqDNA polymerase (Invitrogen). Fluorescence data were acquired at the end of extension. A melt analysis was run for all of the products to determine the specificity of the amplification. The cycle threshold values for each gene were normalized by those for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the same sample.

Western blot immunoanalysis

The antagonists MPG or AG1478 were added to the medium 15 min before ET-1. After 30 min of incubation in the presence of ET-1, cardiac myocytes were washed twice with cold PBS and lysed with RIPA buffer containing (in mmol l^{-1}) 50 Tris, 150 NaCl, 1 ethylenediaminetetraacetic acid, 1 ethylene glycol tetraacetic acid, 1 % Triton X-100, 0.5 % sodium deoxycholate, 1 sodium orthovanadate, 50 sodium fluoride, pH 7.5 with protease inhibitors (Complete Mini, Roche). After a brief centrifugation, the supernatant was kept and protein concentration determined by the Bradford method. Samples were denatured and equal amounts of protein subjected to polyacrylamide gel electrophoresis and electrotransferred o poly(vinylidene fluoride) membranes. The upper portion was used to detect p-90^{RSK}, whilst the lower portion was used for ERK1/2 detection. Membranes were incubated overnight either with the corresponding antibodies raised against P-p90^{RSK} and P-ERK1/2 (both from Cell Signalling) or p90^{RSK} or ERK2 (both from Santa Cruz Biotechnology). Total p90^{RSK} and total ERK2 were used to normalize their respective phosphorylated forms. A peroxidase-conjugated

anti-rabbit IgG (Santa Cruz Biotechnology) was used as secondary antibody and finally bands were visualized using the ECL-Plus chemiluminescence detection system (Amersham). GAPDH (Millipore) antibody was assayed as loading control. Autoradiograms were analysed by densitometric analysis (Scion Image).

Measurement of $O_2^{\cdot}$ anion

We used the lucigenin-enhanced chemiluminescence method to measure myocardial $O_2^{\cdot}$ production as previously described [7, 27]. Briefly, the chemiluminescence in arbitrary units was recorded with a luminometer (Chameleon; Hidex; Finland) for 30 s each, with a 4.5 min interval during 30 min period. The lucigenin-containing assay buffer (Krebs–HEPES buffer with 5 μM lucigenin) with the samples minus background and responses to the different drugs assayed were reported. $O_2^{\cdot}$ production was normalized to milligrams of dry weight tissue per minute.

Measurement of intracellular Na^+ (Na^+_i)

Isolated cardiomyocytes were placed into a cell chamber on the stage of an inverted microscope (TE2000-U Nikon) to measure Na^+_i by microepifluorescence. Single cardiomyocytes, under different experimental conditions, were used for each experiment. Cardiomyocytes were loaded with SBFI-AM (Molecular Probes) for 60 min, followed by three washes with dye-free solution and incubation for another 20 min to allow for dye de-esterification.

When exposed to agonists/blockers, this condition was maintained throughout the entire loading/washout and experimental protocol. All the determinations were carried out in electrically paced (0.5 Hz, starting 5 min prior to the beginning of fluorescence records) preparations at room temperature. Emitted fluorescence was monitored at 535 nm with excitation wavelengths alternating 340 nm/380 nm for SFBI. The excitation filter rotation and fluorescence recording were achieved with an IonOptix Fluorescence System coupled to the microscope. The ratio of emitted fluorescence signals were calculated offline after subtraction of the corresponding autofluorescence value at each wavelength.

Chemicals

All drugs used in the present study were analytical grade and the concentrations chose based on previous reports [12, 17, 32, 58, 59]. Ang II (1 nmol l^{-1} Sigma), ET-1 (5 nmol l^{-1} Sigma), EGF (0.1 μg ml^{-1}), *N*-(2-mercaptopropionyl)-glycine MPG, (2 mmol l^{-1}; Sigma), BQ123 (10 μmol l^{-1}; Sigma), creatine, taurine, L-carnitine and β-cytosine arabinoside were purchased from Sigma; losartan (Los, 1 μmol l^{-1}; Merck); Cariporide or HOE642 (carip, 10 μmol l^{-1}; Aventis); AG1478 (1 μmol l^{-1}; Santa Cruz Biotechnologies) and laminin from Invitrogen.

Statistics

Data was tested for normality of distribution using Kolmogorov/Smirnov test, to ensure that parametric tests were appropriate. Data is expressed as mean±SEM. Differences between groups were assessed by one-way ANOVA followed by Student–Newman–Keuls test. $P<0.05$ was considered significant.

Results

ET-1 mediates Ang II-induced hypertrophy

Electrically paced (0.5 Hz) isolated cat ventricular myocytes were cultured in the presence of 1 nmol l^{-1} Ang II alone or in combination with either the AT_1 receptor antagonist Los (1 μmol l^{-1}) or the ET_A receptor antagonist BQ123 (10 μmol l^{-1}) during 18 h. In Ang II-stimulated cardiomyocytes, CSA increased by ~45 % compared to untreated controls, effect that was cancelled not only by Los but also by specific ET_A receptor blockade with BQ123 (Fig. 1a–b). Ang II stimulated protein synthesis as revealed by the increase in [^{3}H]-phenylalanine incorporation and in agreement with the CSA results; both Los and BQ123 significantly attenuated Ang II effect (Fig. 1c).

Ang II stimulates ET-1 synthesis by the cardiomyocytes

A significant increase in ET-1 mRNA expression was detected in cardiomyocytes incubated during 18 h with 1 nmol l^{-1} Ang II (100±7.6 % vs. 171.6±29.3 %, respectively; $n=4$ each). This effect was completely prevented by Los (92.6±21.7 %, $n=4$; Fig. 2).

Hypertrophic response to ET-1: role of NHE-1

We next explored the response of adult cardiomyocytes to exogenously administered ET-1 (Fig. 3). ET-1 (5 nmol l^{-1}) induced increases in CSA and [^{3}H]-phenylalanine incorporation of similar magnitude to those elicited by Ang II. These effects were completely prevented when cardiomyocytes were co-cultured with ET-1 plus the NHE-1 specific inhibitor carip (10 μmol l^{-1}) revealing a crucial role for this exchanger in the hypertrophic response to ET-1. Since one of the main pathways involved in NHE-1 activation is the redox-sensitive kinase cascade of ERK1/2-$p90^{RSK}$ [51, 55] and ROS have been widely implicated in CH [5, 16, 24, 38, 56, 63], we decided to explore the hypertrophic response to ET-1 in the presence of the ROS scavenger MPG (2 mmol l^{-1}). As it is

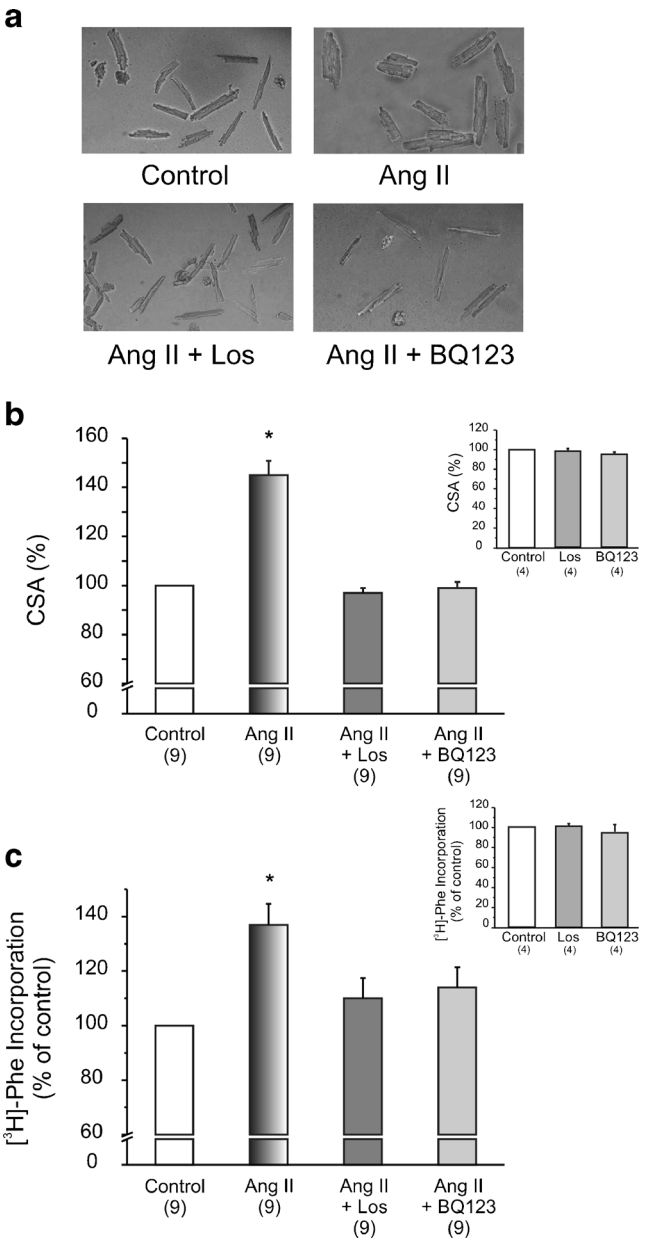

Fig. 1 Hypertrophic effect of 1 nmol l^{-1} Ang II in isolated cat cardiomyocytes cultured during 18 h under electrical pacing (0.5 Hz.). *Panel A*: Representative microphotographs of each experimental group after 18 h culture (40× magnification). *Panel B*: Ang II induced a ~45 % increase in cardiomyocytes cell surface area (*CSA*), effect that was abrogated by the AT_1 antagonist Los and even more interestingly by specifically blocking the ET_A receptor with BQ123. *Panel C*: De novo protein synthesis was measured by the incorporation of [^{3}H]-phenylalanine normalized to the amount of DNA. Ang II stimulated protein synthesis as revealed by the increase in [^{3}H]-phenylalanine incorporation and in agreement with the results obtained from CSA. Los and BQ123 significantly attenuated Ang II response, suggesting that the prohypertrophic effect of Ang II was mediated by an autocrine/paracrine effect of ET-1. The number of experiments in each group is between brackets. *Insets* show the lack of effect of all inhibitors used upon the basal measurement. * $P<0.05$ vs. all other groups, ANOVA

shown in Fig. 3, preventing the increase in ROS production completely cancelled ET-1-induced hypertrophy, evidenced

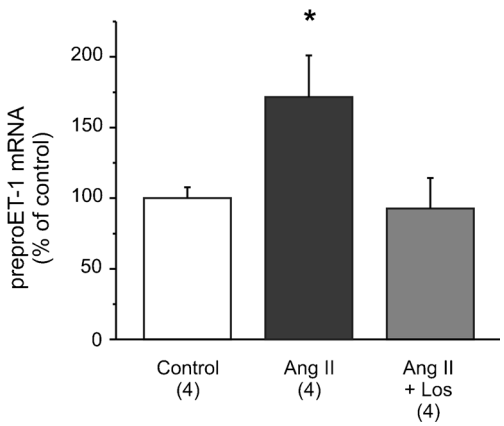

Fig. 2 Ang II (1 nmol l^{-1}) promoted preproET-1 mRNA expression. An increase in the expression of preproET-1 mRNA was detected by real-time RT-PCR in cardiomyocytes cultured in the presence of Ang II during 18 h, confirming this result the crosstalk between Ang II and ET-1. Los cancelled the increase in preproET-1 mRNA. Los alone did not influence preproET-1 mRNA expression (data not shown). The number of experimental determinations in each group is between brackets. * $P<0.05$ vs. control, ANOVA

by the absence of increase in CSA and [^{3}H]-phenylalanine incorporation. These results were confirmed in experiments in which the mRNA abundance of the cardiac hypertrophic markers ANF and BNP were assessed by real-time reverse transcription (RT)-PCR in isolated cardiomyocytes after 18 h culture. ET-1 induced significant increases in both ANF and BNP expressions that were completely cancelled by MPG and by carip (Fig. 3d).

ET-1 stimulates the ERK1/2-p90^{RSK} pathway by transactivation of the EGFR

To get further insight into the signalling pathway triggered by ET-1 leading to cardiomyocyte hypertrophy, we performed experiments in which the activation of the ERK1/2-p90^{RSK} kinase pathway was assessed by immunoblot in the presence of ET-1 alone or in combination with MPG. The increase in the phosphorylation of both kinases ERK1/2 and p90^{RSK} was induced by ROS triggered by ET-1, and it was absent when the rise in ROS was prevented by MPG (Fig. 4a). When cardiac myocytes were incubated with ET-1 in the presence of the EGFR antagonist AG1478, ERK1/2-p90^{RSK} phosphorylation was prevented, supporting a role for EGFR transactivation in the prohypertrophic signalling cascade triggered by ET-1 (Fig. 4b). In line with these findings, we explore whether a low concentration (1 μmol l^{-1}) of Ang II was able to enhance the phosphorylation of ERK1/2 and p90^{RSK}. As our results show, Ang II was able to increase the phosphorylation of both kinases. This effect was prevented by blocking the AT_1 receptor with Los and the ET_A receptor with BQ123 (Fig. 4c and d) supporting that ET-1 was a necessary

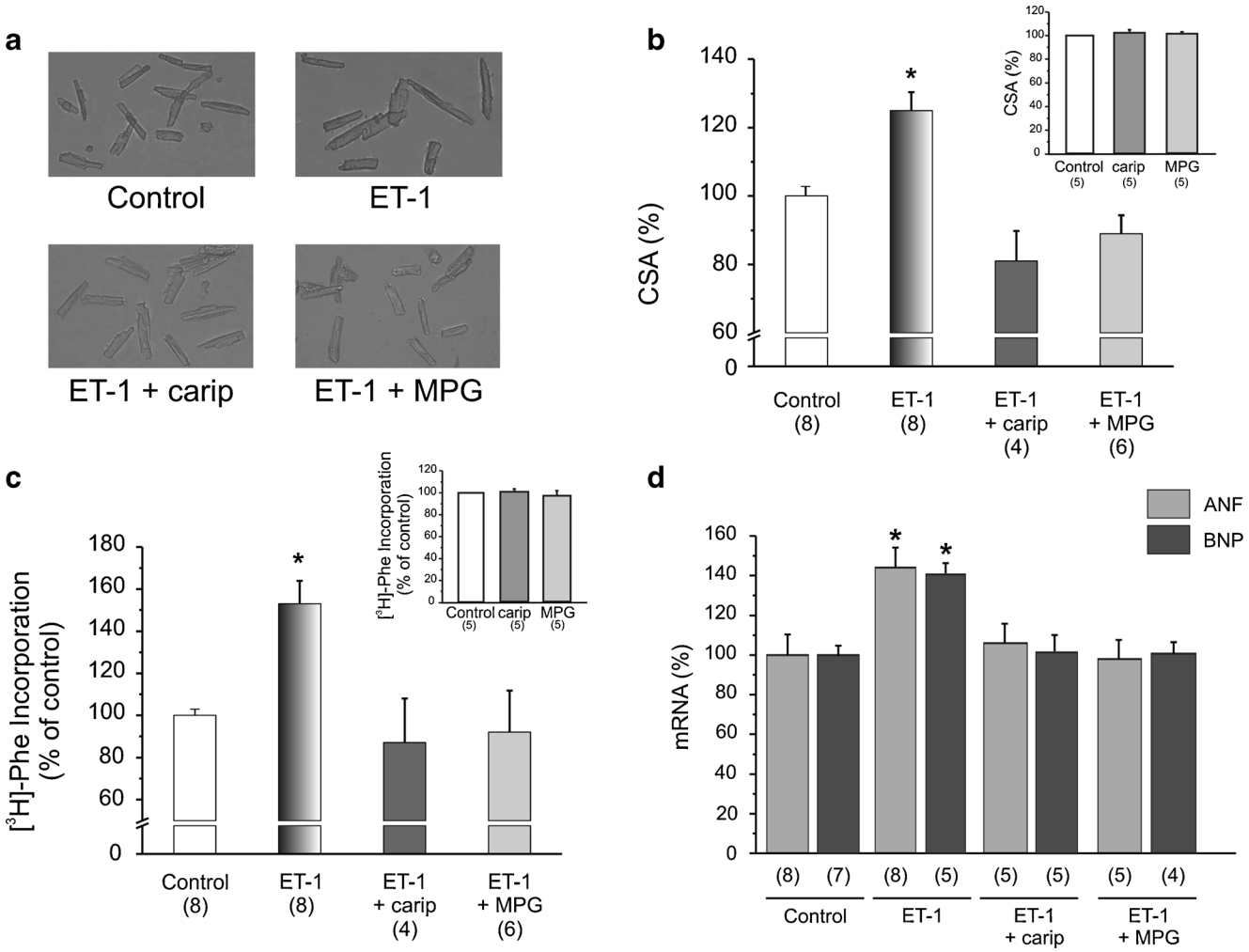

Fig. 3 Hypertrophic effect of ET-1 (5 nmol l^{-1}) in electrically paced cultured cat cardiomyocytes: participation of NHE-1 and reactive oxygen species (*ROS*). *Panel A*: Representative microphotographs of each experimental group after 18 h of culture (40× magnification). *Panel B*: ET-1 induced an increase in myocytes cell surface area (*CSA*) that was prevented by the NHE-1 inhibitor carip and also by the ROS scavenger MPG. *Panel C*: similar results to those obtained when measuring CSA were obtained when analysing de novo protein synthesis by [^{3}H]-phenylalanine incorporation normalized to the amount of DNA. *Panel D*: After 18 h culture period ET-1 significantly increased the mRNA abundance of ANF and BNP, molecular markers of pathological CH. Similarly to what happened with the CSA and the measurement of [^{3}H]-phenylalanine incorporation, this effect of ET-1 was blunted by the NHE-1 specific inhibitor carip as well as the ROS scavenger MPG. Neither carip nor MPG had an effect on ANF/BNP expressions by itself (data not shown). The number of experimental determinations in each group is between brackets. *Insets* show the lack of effect of all drugs used upon the basal measurement. * $P<0.05$ vs. all other groups, ANOVA

step in the signalling pathway triggered by (1 μmol l^{-1}) Ang II, leading to CH development.

Ang II, ET-1 and EGF stimulate myocardial superoxide production

A new set of experiments were performed to evaluate the effect of Ang II, ET-1 and EGF on myocardial superoxide production by the lucigenin chemiluminescence method. These agonists induced significant increases in superoxide production that were effectively prevented by AG1478, a selective antagonist of the EGFR. Interestingly, Los was able to prevent only the increase in superoxide production induced by Ang II, whilst BQ123 cancelled the effects of both Ang II and ET-1, but not that of EGF (Fig. 5). The inhibitors alone had no basal effects, as we have previously shown [7, 27, 59].

Stimulation of NHE-1 activity increases intracellular sodium

Our next interest was to evaluate the effect of Ang II on NHE-1 in our experimental model, through the measurement of Na^+_i levels, as an indicator of the exchanger's activity. As shown in Fig. 6, Ang II induced a significant increase in Na^+_i detected by the rise in SBFI (340 nm/380 nm) ratio. This effect was prevented by carip, NHE-1-specific blocker, demonstrating that it was due to a stimulatory action of Ang II upon the exchanger. Note that SBFI ratio basal value was unaffected by carip.

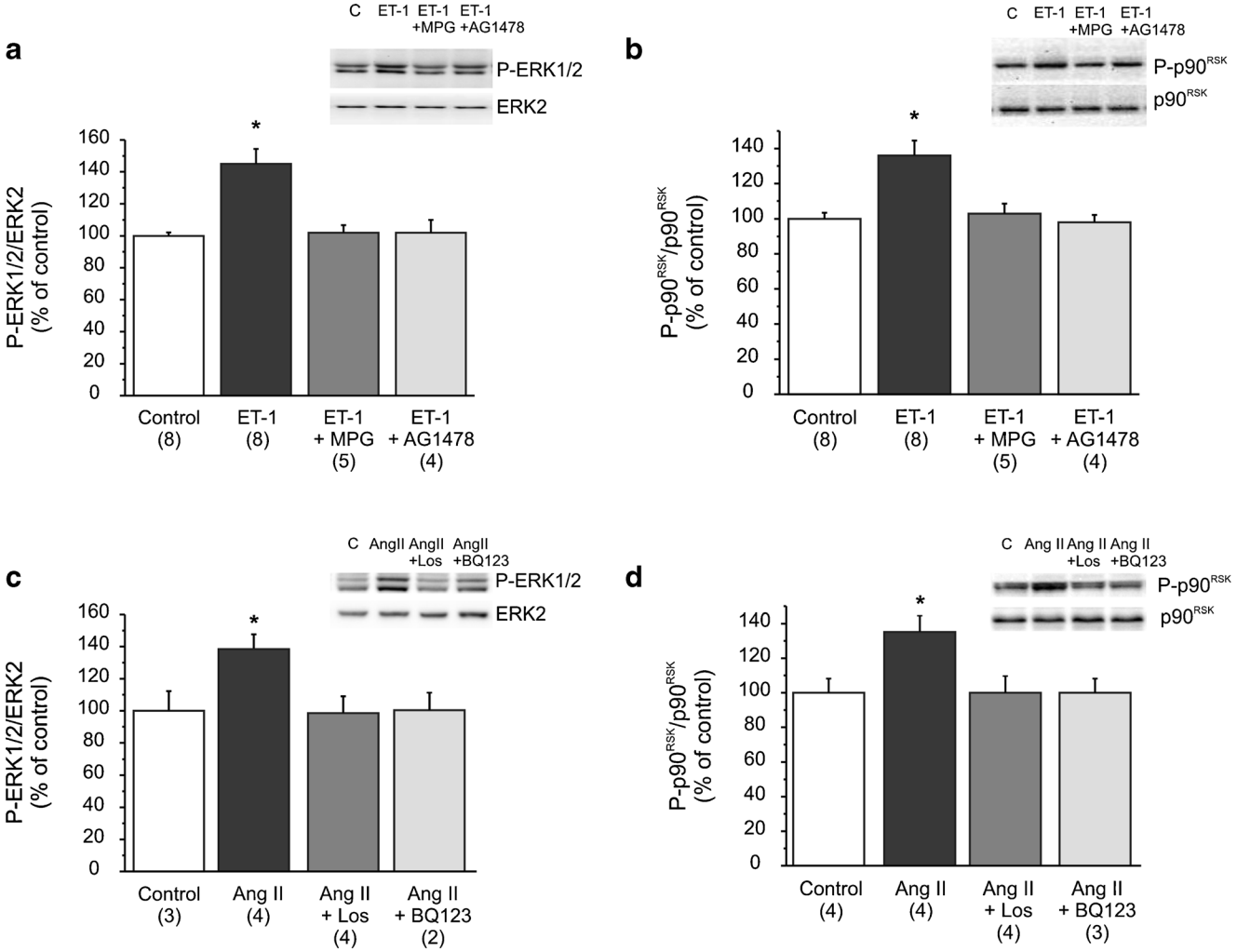

Fig. 4 ET-1-induced phosphorylation of ERK1/2 and $p90^{RSK}$ in isolated cat ventricular myocytes. *Panels A and B*: ET-1 (5 nmol l^{-1}) induced an increase in ERK1/2 and $p90^{RSK}$ phosphorylation that was prevented by the ROS scavenger MPG and by the antagonist of the EGFR, AG1478. *Panels C and D*: Ang II (1 nmol l^{-1}) augments ERK1/2 and $p90^{RSK}$ phosphorylation which was prevented by Los and BQ123. On top of each bar graph representative, Western blot images are shown. The number of experimental determinations in each group is between brackets. * $P<0.05$ vs. all other groups, ANOVA

Discussion

The following information emerge from the data presented herein: (1) A low dose of exogenous Ang II (in the same order of magnitude to that released by stretched cardiomyocytes during hypertrophy development [52]) induces hypertrophy entirely through an autocrine effect of endogenous ET-1 in adult cardiomyocytes. (2) The intracellular signalling pathway involved requires the transactivation of the EGFR. (3) The increase in myocardial ROS production and the activation of redox-sensitive kinases (ERK1/2-$p90^{RSK}$) known to stimulate the NHE-1 are key steps for hypertrophy development. (4) Stimulation of NHE-1 activity increase Na^{+}_{i}, probably favouring a subsequent increase Ca^{2+}_{i} through the reverse mode of the NCX exchanger as we have previously reported in cat papillary muscles [11]. (5) It seems to be a sequential and unidirectional activation of AT_1, ET_A and EGF receptors in the prohypertrophic signalling cascade triggered by Ang II. Our data represent, to our knowledge, the first evidence that endogenous ET-1 completely mediates the hypertrophic response to exogenous Ang II through EGFR transactivation and ROS production in isolated adult ventricular myocytes electrically paced.

We have previously demonstrated in this same preparation, isolated cat cardiomyocytes, the presence of preproET-1, BigET-1, and ET-1 by immunohistochemistry, confirming that cardiomyocytes have the machinery and are capable of synthesizing ET-1 [12]. Interestingly, in the present manuscript, we show that 1 nmol/L Ang II induces a significant increase in preproET-1 mRNA. This effect was prevented by Los, AT_1 specific blocker, supporting the hypothesis that Ang II stimulates ET-1 synthesis in isolated cardiomyocytes. In line with these findings, Ito et al. [29] demonstrated that Ang II upregulated preproET-1 mRNA levels and stimulated the release

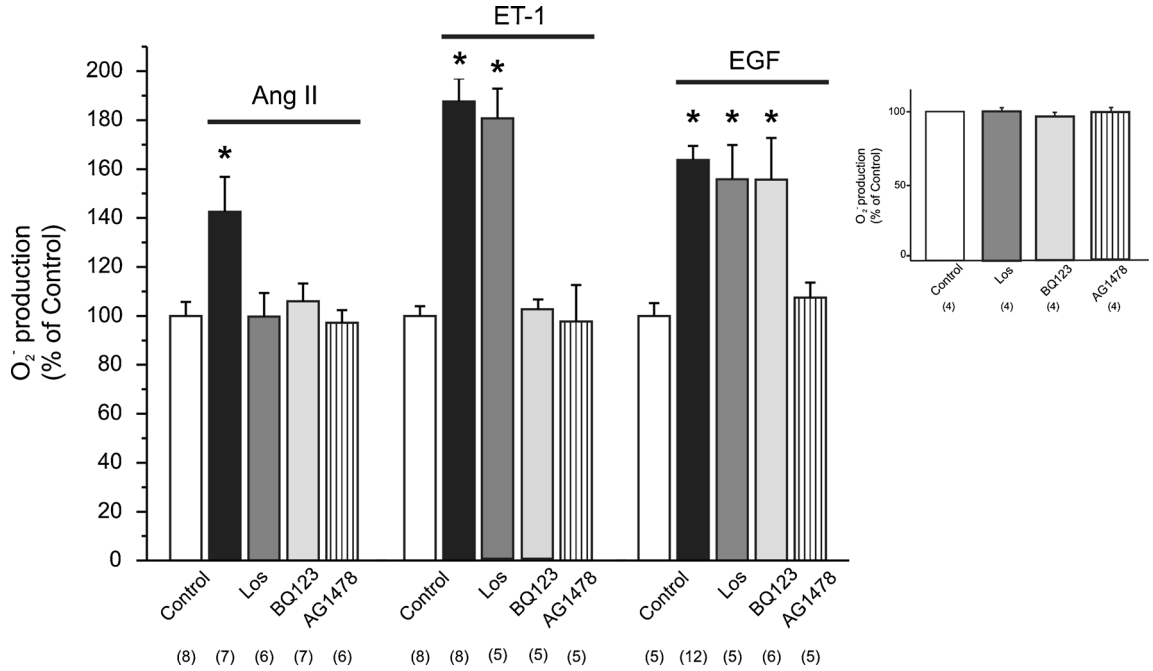

Fig. 5 Ang II/ET-1/EGFR pathway increased anion superoxide production in cat ventricular myocardium. Ang II, ET-1 and EGF significantly increased myocardial superoxide production. Ang II/ET-1 effect was prevented not only by BQ123 (antagonist of the ET_A receptor) but also by AG1478, the selective antagonist of the EGFR. Interestingly, Los only inhibited Ang II-dependent superoxide production but not that induced by ET-1. EGF-induced superoxide production was suppressed by AG1478, as expected. These findings support the unidirectional crosstalk amongst Ang II, ET-1 and EGFR; being Ang II localized before ET-1 and upstream EGFR transactivation. Between brackets there is the number of experiments performed in each group. None of the receptor antagonists used exerted an effect on superoxide production by themselves (101±8; 93.1±6.2; 99.6±12.2; for Los, BQ123 and AG1478 vs. control, respectively; $n=4$ for each). * $P<0.05$ vs. control, ANOVA

of immunoreactive ET-1, in a dose–time-dependent manner from rat neonatal cardiomyocytes. Our results show that the blockade of the ET-1 receptor (ET_A) with BQ123 prevents the increase in cardiomyocyte cross-sectional area and phenylalanine incorporation induced by Ang II, supporting the hypothesis that Ang II induces the release of ET-1 which mediates the hypertrophic response.

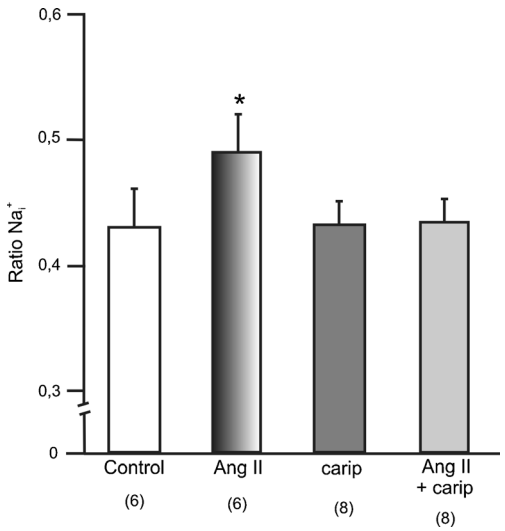

Fig. 6 NHE-1 stimulation increases intracellular sodium. Na^+_i was higher in Ang II treated than in control cells. The rise in Na^+_i was prevented by the blockade of NHE-1 with carip. This pharmacologic compound did not alter basal Na^+_i. The number of experimental determinations in each group is between brackets. * $P<0.05$ vs. all other groups, ANOVA

In the present work the hypertrophic effect of Ang II was mimicked by incubating isolated adult cardiomyocytes with exogenous ET-1 (5 nmol l^{-1}), and it was prevented either by the NHE-1-specific inhibitor carip or the ROS scavenger MPG. This signalling pathway is in agreement with that triggered by myocardial stretch increasing contractility in the so-called 'slow force response to stretch' (SFR) [9]. The SFR, characterized by an increase in contractility due to a progressive increase in calcium transient amplitude, would represent the mechanical counterpart of this autocrine/paracrine mechanism leading to cardiomyocyte hypertrophy. Interestingly, the downregulation of cardiac NHE-1 by small interference RNA completely prevents the SFR [47].

As regards whether there is persistence over time of some early intracellular signals triggered by this autocrine/paracrine mechanism such as NHE-1 activation, we have recently induced CH by transverse aortic constriction in mice. After 7 weeks of aortic constriction, CH was detected along with increased activity of redox-sensitive $p90^{RSK}$ and NHE-1 phosphorylation. AT_1 blockade with Los prevented $p90^{RSK}$ and NHE-1 activation and decreased hypertrophy development, preserving contractility despite of a higher workload [13]. The crucial role of NHE-1 activation in the cascade of events

leading to hypertrophy development is supported by evidence emerging from a variety of experimental models in which myocardial hypertrophy was prevented or reversed by NHE-1 inhibition (for review, see Refs. [8, 31]). Moreover, recently published experiments performed by the group of Wakabayashi in which cardiac overexpression of an activated form of NHE-1 was enough to activate Ca^{2+} signalling and induced CH [43].

NHE-1 hyperactivity induced by Ang II/ET-1 leads to a rise in Na^{+}_{i}. This increase in Na^{+} favours intracellular Ca^{2+} accumulation through the NCX, as we and others have previously shown [2, 10, 37, 46]. In order to evaluate this step, we determined Na^{+}_{i} levels in response to Ang II in electrically paced isolated cat cardiomyocytes. The results showed that Ang II (through NHE-1 stimulation) significantly increased Na^{+}_{i}, effect completely prevented by carip (Fig. 6).

Ang II/ET-1 stimulation or myocardial stretch by elevating Na^{+}_{i} favours the NCX reverse mode increasing Ca^{2+} influx. This could be modulated by three different pathways: a negative shift in the NCX reversal potential after a rise in Na^{+}_{i} due to NHE-1 activation, the prolongation of the action potential duration and direct stimulation of NCX by protein kinase C (Na^{+}-independent pathway). All of these mechanisms that contribute to increase intracellular Ca^{2+} are redox-sensitive and inhibited by blocking the effects of Ang II/ET-1 [9]. Due to the electrogenicity of the NCX which favours the reverse mode of operation during positive membrane potentials, namely, the plateau of the action potential, it was critical to perform the experiments of the present work in electrically paced cardiomyocytes.

Moreover, electrical pacing is of great help to maintain cardiomyocyte morphology and function in culture [6]. To this aim, a special device was designed (see 'Materials and methods' section). Cell shape and morphology are intimately linked with some aspects of cell function, such as excitation–contraction coupling [35]. Acutely isolated cells are shape as a 'brick' or 'rod' cells with rectangular 'stepped' ends and clear cross-striations. Feline cardiomyocytes are more resistant to myofibrillar atrophy in culture [14]. The contractile apparatus of feline myocytes responds more favourably to culture than rat and rabbit myocytes, although reasons for any differences between species are not clear. Rod-shape cardiomyocytes can be maintained in a relative long-term culture in serum-free media, but changes in contractile properties, gene expression and loss in protein content have been observed under these conditions [14, 19, 20, 48]. This loss of protein may be due to a reduction of mechanical activity and/or the limited mechanical load to which the cardiocytes are exposed to [6, 14]. It has been shown, in short-term culture, that mechanical activity produced by electrically stimulated contraction obviates the loss of contractile function and preserves protein synthesis rate in adult cardiomyocytes. Also, these cardiocytes presented sarcomeres as highly visible and rod-shaped. In quiescent cardiocytes, sarcomeres remained visible and the ends of the cell assumed a rounded shape. These data demonstrates that contraction is required to maintain cardiomyocyte function and protein metabolism [6].

The ability of both Ang II and ET-1 to stimulate NADPH oxidase and increase myocardial ROS production is well documented [4, 38]. We have recently reported that 1 nmol l^{-1} Ang II is able to increase ROS production in cat myocardium by inducing ET-1 release [12] and that 5 nmol l^{-1} ET-1 stimulated ROS production in a similar magnitude to 1 nmol l^{-1} Ang II [27]. In the present work, we confirmed the stimulatory effect of ET-1 on ROS production by a mechanism involving the transactivation of the EGFR, as well as the ability of EGF to exert the same effect. This rise in intracellular ROS allowed ERK1/2-$p90^{RSK}$ activation, an upstream step necessary for NHE-1 phosphorylation and stimulation.

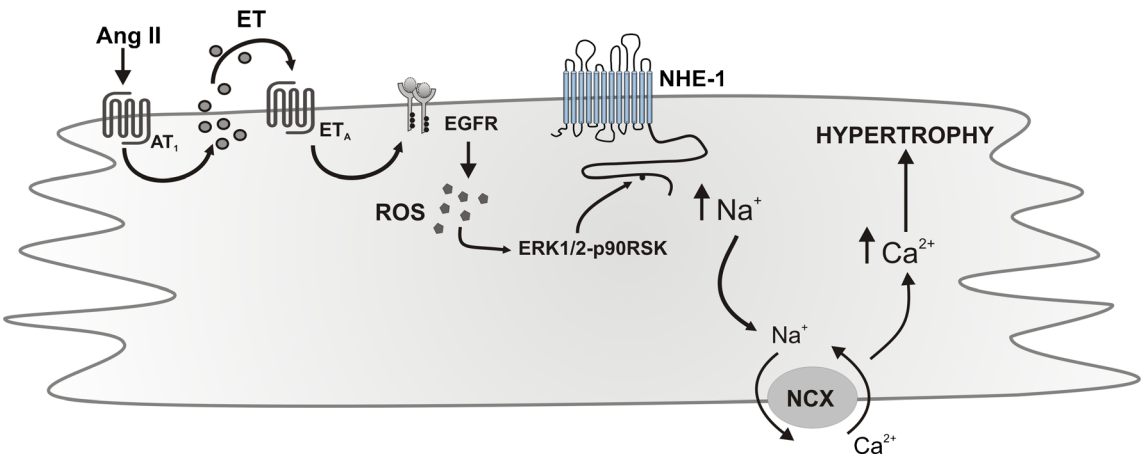

Fig. 7 Scheme of the proposed signalling pathway triggered by a low concentration of Ang II underlying the prohypertrophic effect in isolated adult cat cardiomyocytes. Endogenous ET-1 mediates the hypertrophic response to a low dose of exogenous Ang II (1 nmol l^{-1}) in isolated adult cat ventricular myocytes. The signalling cascade involves ET-1-dependent EGFR transactivation and stimulation of ROS production that activates the ERK1/2-$p90^{RSK}$ cascade. This kinase cascade is known to phosphorylate the cytosolic regulatory tail of the NHE-1 stimulating its function and favouring the increase in intracellular Na^{+} and Ca^{2+} leading to myocardial hypertrophic growth

Conclusions

Our data represent, to our knowledge, the first evidence that endogenous ET-1 through an autocrine/paracrine mechanism mediates the hypertrophic response to exogenous Ang II in isolated adult ventricular myocytes. We propose, based on previous results from other authors and our own laboratory, that the signalling cascade involves ET-1 stimulation of ROS production which activates the ERK1/2-p90^{RSK} pathway and consequently the NHE-1 by phosphorylating its cytosolic tail [27, 50, 51, 55]. In our model, NHE-1 activation plays a key role in the cascade of cellular events leading to hypertrophy development induced by Ang II/ET-1. In line with our findings, Wakabayashi's group postulated that activation of NHE-1 is sufficient to generate Ca^{2+} signals that induce CH [43]. The schematic representation of the signalling cascade that we proposed to underlie the hypertrophic effect of low concentrations of Ang II is depicted in Fig. 7.

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Ethical standards The authors declare that the experiments fully comply with the current laws of Argentina (the country in which they were performed).

Conflict of interest None

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