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Cardioprotective effects of calcitonin gene-related peptide in isolated rat heart and in H9c2 cells via redox signaling

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A B S T R A C T

The calcitonin-gene-related-peptide (CGRP) release is coupled to the signaling of Angeli’s salt in determining vasodilator effects. However, it is unknown whether CGRP is involved in Angeli’s salt cardioprotective effects and which are the mechanisms of protection. We aimed to determine whether CGRP is involved in myocardial protection induced by Angeli’s salt. We also analyzed the intracellular signaling pathway activated by CGRP. Isolated rat hearts were pre-treated with Angeli’s salt or Angeli’s salt plus CGRP8-37, a specific CGRP-receptor antagonist, and subjected to ischemia (30-min) and reperfusion (120-min). Moreover, we studied CGRP-induced protection during oxidative stress (H2O2) and hypoxia/reoxygenation protocols in H9c2 cardiomyocytes. Cell vitality and mitochondrial membrane potential (ΔYm, MMP) were measured using MTT and JC-1 dyes. Angeli’s salt reduced infarct size and ameliorated post-ischemic cardiac function via a CGRP-receptor-dependent mechanism. Pre-treatment with CGRP increased H9c2 survival upon challenging with either H2O2 (redox stress) or hypoxia/ reoxygenation (H/R stress). Under these stress conditions, reduction in MMP and cell death were partly prevented by CGRP. These CGRP beneficial effects were blocked by CGRP8-37. During H/R stress, pre-treatment with either CGRP-receptor, protein kinase C (PKC) or mitochondrial KATP channel antagonists, and pre-treatment with an antioxidant (2-mercaptopropionylglycine) blocked the protection mediated by CGRP. In conclusion, CGRP is involved in the cardioprotective effects of Angeli’s salt. In H9c2 cardiomyocytes, CGRP elicits PKC-dependent and mitochondria-lKATP-redox-dependent mechanisms. Hence, CGRP is an important factor in the redox-sensible cardioprotective effects of Angeli’s salt. © 2017 Elsevier Masson SAS. All rights reserved.

Abbreviations: AS, Angeli’s salt; CHE, chelerythrine; CTRL, Control; dLVP, developed left ventricular pressure; dP/dtmax, first derivative of ventricular pressure during systole; H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase; LVEDP, left ventricular end diastolic pressure; LVP, left ventricular pressure; mKATP, mitochondrial KATP channels; mKATP, mitochondrial KATP channels; MPG, 2-mercaptopropionylglycine; MMP, mitochondrial membrane potential; 5-HD, 5-hydroxydecanoate. * Corresponding authors at: Department of Clinical and Biological Sciences, University of Turin, Regione Gonzole 10, 10043 Orbassano, Italy. E-mail addresses: claudia.penna@unito.it (C. Penna), pasquale.pagliaro@unito.it (P. Pagliaro).

Introduction Calcitonin gene-related peptide (CGRP) is a transmitter originated by tissue-specific splicing of the calcitonin gene mRNA and it is one of the main constituent neuropeptides of sensory nerves endings, from which it is released following a variety of stimuli [1–3]. CGRP is also present in the heart, where it induces vasodilatation and exerts positive inotropic and chronotropic effects [1,2,4,5].
Ischemic pre- and post-conditioning (i.e. short periods of ischemia applied immediately before or after ischemia) provide protection against ischemia/reperfusion (I/R) injury. Studies have involved CGRP in both pre- and post-conditioning cardioprotection [6,7]. Various studies report that the reduction I/R injury in response to ischemic conditioning or cardioprotective agents, such as adenosine, bradykinin and opioids, takes place via nitric oxide (NO)-dependent pathway [8,9]. In vivo studies have shown that CGRP release may be induced by the nitroxyl (HNO) donor Angeli's salt (AS). In normal and failing hearts, while the intravenous AS infusion increased circulating levels of CGRP, the blockade of CGRP receptor, by the specific antagonist (CGRP8-37), prevented AS inotropic/lusitropic action [10–12]. Besides HNO, AS may also release nitrite. It is well known that NO, nitrite and nitrate donors can induce preconditioning-like effects [9–16]. We have shown that HNO, produced by decomposition of AS, can induce redox-sensitive myocardial protection against reperfusion injury, which is blocked by N-acetylcysteine [17]; this thiol-donating agent can discriminate the effect of HNO from that of other nitrogen derived molecules. Indeed, AS/HNO action can be blocked by this agent, whereas the NO_ effect is potentiated [12]. Importantly, in rat coronary vasculature, AS is a potent vasodilator agent via CGRP release and potassium ATP channel activation [18–21]. Also nitroglycerin (NTG), a NO/nitrate donor, may exert protection against reperfusion injury via CGRP and protein kinase C (PKC) signaling [9,15,22]. Indeed, while NTG infusion increased CGRP release in coronary effluent, NTG-induced cardioprotection was blocked by CGRP8-37 [22]. Therefore, there is an evident cross-talk between CGRP and nitrogen derived compounds, including HNO [18–22]. Whatever the mechanism, many stimuli, including HNO, induce the release of CGRP. In particular, it has been proposed that HNO activates the sensory chemoreceptor channel and as a consequence CGRP is released, mediating local and systemic vasodilation [5]. Although it has been suggested that AS may induce the release of CGRP [11,19–21] and it has been suggested that exogenous and endogenous CGRP may have cardioprotective effects (both pre- and post-conditioning like effects) [1,4,22,23–26], no study has linked CGRP to AS-induced preconditioning. When CGRP has been infused before ischemia it resulted protective [4,27–30], even in the presence of comorbidities, such as diabetes and hypertension [4,27]. Moreover, the infusion of CGRP after ischemia induced postconditioning-like cardioprotection [25]. Since cardioprotection, induced by either ischemic pre- or post-conditioning, is mediated by the activation of PKC and mitochondrial KATP (mKATP) channels, leading to a reactive oxygen species (ROS) signaling [9,31,32], we hypothesized a similar intracellular signaling pathway for CGRP-induced myocardial protection. Therefore, here we sought to determine whether CGRP-CGRP-receptor interaction is involved in myocardial protection. Moreover, we aimed to dissect the protective intracellular signaling activated by CGRP. To this aim, we first ascertained the possibility to induce cardioprotection via CGRP-receptor activation in isolated rat hearts subjected to ischemia/reperfusion. In this experimental model AS was used as a tool to induce endogenous CGRP-CGRP-receptor interaction. Then, to test the hypothesis that CGRP protects against the myocardium injury via the activation of CGRP-receptor, PKC and mKATP/ROS signaling, we used as model the rat cardiomyocyte cell line H9c2. In these cells we studied the ability of CGRP to protect against oxidative stress and hypoxia/reoxygenation in the absence and in the presence of the specific inhibitors of aforementioned signaling molecules and CGRP-receptor antagonist.

Materials and methods

2.1. Animals

Male Wistar rats were used in compliance with the European Council directives (No. 86/609/EEC) and with Italian law (DL-116, January 27, 1992). They are in agreement with 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 follow the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines.
2.2. Study of the role of CGRP on cardioprotective effects of Angeli's salt in an ex-vivo model

The methods were similar to those previously described [17,33,34]. In brief, isolated hearts were weighed, attached to the perfusion apparatus and retrograde perfused with oxygenated Krebs–Henseleit buffer (pH 7.34), containing (in mM): 127 NaCl, 17.7 NaHCO3, 5.1 KCl, 1.5 CaCl2, 1.26 MgCl2, 11 D-glucose and gassed with 95% O2 and 5% CO2. A constant flow was adjusted with a proper pump to obtain a typical coronary perfusion pressure of 80–85 mmHg during the initial part of stabilization. Thereafter the same flow level (9 _ 1 mL/ min/g) was maintained throughout the experiment. A small hole in the left ventricular wall allowed drainage of the thebesian flow, and a polyvinyl-chloride balloon was placed into the left ventricle and connected to an electro-manometer for the recording of left ventricular pressure (LVP) during isovolumetric contraction. The balloon was filled with saline to achieve an end-diastolic LVP of 5 mmHg in baseline conditions. Coronary perfusion pressure, coronary flow and LVP were monitored to assess the preparation conditions. LVP was used to calculate the positive first derivative of pressure during systole (dP/dtmax). Developed LVP (dLVP) was obtained subtracting left ventricular end diastolic pressure (LVEDP) from peak systolic LVP. Data were analyzed in pre- and post-ischemic phase during at least 15-s of stable conditions (i.e. in the absence of arrhythmias). The hearts were electrically paced at 280 bpm and kept in a temperature-controlled chamber (37 _C).

2.2.1. Experimental protocols for isolated hearts (Fig. 1, panel A) Each heart was allowed to stabilize for 30-min. After the stabilization period, hearts were subjected to a specific protocol, which included in all groups a 30-min of global no-flow ischemia. A period of 120-min reperfusion followed the 30-min ischemia in all groups (see below). Pacing was discontinued on initiation of ischemia and restarted after the third minute of reperfusion in all groups. Hearts in the control group (I/R Group, n = 7) were perfused with buffer only for an additional 29-min after stabilization (see Fig. 1A) before to be subjected to I/R protocol. In Angeli’s salt Group (n = 7), hearts were exposed to AS (1 mM) for 19-min followed by a 10-min buffer wash-out (WO) period [17,35]. In a group, (CGRP8-37, n = 3) the effect of I/R was examined in hearts pre-exposed to only CGRP8-37 (0.1 mM), the CGRP receptor antagonist, for a total of 29-min. In another group, (AS + CGRP8-37, n = 7) the role of CGRP in AS-induced protection was examined in hearts where AS (1 mM) effects were evaluated during the co-infusion with CGRP8-37 (0.1 mM). Also in this case the antagonist (CGRP8-37, 0.1 mM) was infused for a total of 29-min, starting 5-min prior to AS administration and continued up to 5-min from the discontinuation of the 19-min AS infusion [28,29].

Assessment of myocardial injury

2.2.2.1. LDH assay. In isolated rat heart, preconditioning has been observed to lead to a 50% reduction in lactate dehydrogenase (LDH) release following 30-min ischemia. Consistently, samples of coronary effluent (2 mL) were withdrawn with a catheter inserted into the right ventricle via the pulmonary artery. Samples were extracted immediately before ischemia and at 3, 6, 10, 20 and 30 min of reperfusion and thereafter every 20 min. LDH release was measured as previously described [17,36]. Data are expressed as cumulative values for the entire reflow period.
**A. Isolated hearts**

**I/R Group**

| 120 min | 29 min | 30 min |

**AS Group**

| 120 min | 19 min wo | 30 min |

**CGRP<sub>8-37</sub> or AS+CGRP8-37 Groups**

| 120 min | 19 min wo | 30 min |

**B. H<sub>2</sub>O<sub>2</sub> protocols**

- 3 hr DMEM or CGRP or CGRP<sub>8-37</sub> → 2 hr DMEM → 3 hr WO → MTT assay or JC-1
- 3 hr DMEM → 2 hr H<sub>2</sub>O<sub>2</sub> → MTT assay or JC-1
- 3 hr CGRP or CGRP+CGRP<sub>8-37</sub> → 2 hr H<sub>2</sub>O<sub>2</sub> → MTT assay or JC-1

**C. Hypoxia/reoxygenation protocols (Normoxic subgroups are not represented)**

- Control-H/R Group
  - 24 hr DMEM → 72 hr Hypoxia (DMEM 2%) → MTT assay or JC-1
- CGRP-H/R Group
  - 24 hr CGRP → 72 hr Hypoxia (DMEM 2%) → MTT assay or JC-1
- CGRP+antagonists Groups
  - 24 hr CGRP+antagonists → 72 hr Hypoxia (DMEM 2%) → MTT assay

**Fig. 1.** Timeline of the three experimental protocols. A: Protocols of ischemia/reperfusion (I/R) in isolated rat hearts. Angeli’s salt (AS) was given for 19 min and CGRP8-37 was given for 29 min starting 5 min before AS and was continued 5 min after AS infusion was stopped. WO: wash out. B: Protocols of oxidative stress (H<sub>2</sub>O<sub>2</sub>) in H9c2 cells. C: Protocols of hypoxia/reoxygenation (H/R) in H9c2 cells (Normoxic subgroups are not represented). MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye is used as colorimetric assay for assessing cell metabolic activity, reflecting the number of viable cells present. JC-1: 5,50,6,60-tetraethylbenzimidazolyldicyanocyanine iodide, a cationic carbocyanine dye that accumulates in mitochondria. It is a sensitive marker for mitochondrial membrane potential. For further explanations and other abbreviations see the text.
Infarct size. At the end of the experiment, i.e. directly after reperfusion, each heart was rapidly removed from the perfusion apparatus, and the left ventricle (LV) was dissected into 2–3 mm circumferential slices. Following 15-min of incubation at 37 °C in 0.1% solution of nitro-blue tetrazolium in phosphate buffer (pH 7.2) unstained necrotic tissue was carefully separated from stained viable tissue by an independent observer who was not aware of the nature of the intervention. The weights of the necrotic and non-necrotic tissues were then determined, and the necrotic mass was expressed as a percentage of total left ventricular mass [17,35]. Study of protection against oxidative stress and study of intracellular mechanisms of CGRP protection against hypoxia/re-oxygenation in an in vitro model: H9c2 cells (Fig. 1 panels B and C) We aimed to assess the effects of alpha-CGRP (hereafter CGRP) in H9c2, a cardiac cell line that responds to different stimuli with the activation of pathways similar to adult cardiomyocytes. Commercially available H9c2 were obtained from American Type Culture Collection (ATCC; Manassas, VA). H9c2 were grown in Dulbecco’s Modified Eagle’s Medium Nutrient mixture F-12 HAM (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) streptomycin/penicillin (Wisent Inc, Quebec, Canada) at 37 °C, 5% CO2 [37]. In all sets of experiments when cells reached 80% confluence were removed from the flask, counted in the Burker chamber and plated in 96 wells plate with 5000 cells/well density. Cells were then left for 24-h in standard culture conditions, before to be subjected to H2O2 or hypoxia/reoxygenation (H/R) protocols.

H2O2 Protocols (Fig. 1 panel B) Cells were treated only with DMEM for a total of 7-h (CTRL group). Cells, after 3-h DMEM, were exposed to H2O2 (50 mM) for 2-h and then to WO with DMEM for other 3-h (H2O2 group). Groups of cells were pre-treated for 3-h with CGRP (0.1 mM, CGRP group) or CGRP8-37 (0.1 mM, CGRP8-37 group) or CGRP plus CGRP8-37, dissolved in DMEM-2% FBS. Then, in pre-treated subgroups, H2O2 (50 mM) was given for 2-h, instead of DMEM (untreated groups) [38]. At the end of H2O2 stress, the medium was changed with fresh DMEM (3-h WO). Finally, a MTT assay and an evaluation of the mitochondrial membrane potential (MMP) were performed (see infra, “Cell survival assessment” and “Measurement of mitochondrial membrane potential (JC-1)”).

Hypoxia/Reoxygenation (H/R) protocols (Fig. 1 panel C) In this set of experiments for comparative purpose groups of cells were left in normoxic conditions (not represented in figure). As Normoxia (N) we considered cell survival studied during standard H9c2 culture conditions (21% O2 and 5% CO2). As Hypoxia/Reoxygenation (H/R) we considered cell survival studied after exposition to DMEM-2% FBS with 3% O2 and 5% CO2 for 72-h and a subsequent reoxygenation (21% O2 and 5% CO2) for 3-h [39–42]. H/R protocol was obtained in an hypoxic chamber (INVIVO2 200, Belsar, Varese, Italy). During 24-h standard culture, cells were pre-treated with CGRP (0.1 µM) [42], or CGRP + antagonists (see below) dissolved in DMEM-2% FBS. After the 24-h pre-treatment, subgroups remained in normoxia (indicated by the suffix _N in the following description and not represented in Fig. 1C), whereas other cells underwent H/R (indicated by the suffix _H/R and represented in Fig. 1C). Therefore, the experimental groups were: a.) Control Groups, untreated cells: CTRL-N; CTRL-H/R. b.) CGRP Groups, cells pre-treated with CGRP only (0.1 µM): CGRP- N; CGRP-H/R. c.) CGRP + antagonists, cells pre-treated with CGRP plus antagonist, in particular: _CGRP + CGRP8-37 (0.1 µM, [42]): CGRP + CGRP8-37-N; CGRP + CGRP8-37-H/R. _CGRP + inhibitor of PKC (chelerythrine, CHE; 5 µM [4]): CGRP + CHE-N; CGRPtx+CHE-H/R. _CGRP + mKATP-channel inhibitor (5-hydroxydecanoate, 5-HD, 300 µM [40]): CGRP + 5-HD-N; CGRP + 5-HD-H/R. _CGRP + antioxidant (2-mercaptotripropionylglycine, MPG, 100 µM [15]): CGRP + MPG-N; CGRP + MPG-H/R. Moreover, all antagonists were tested alone in normoxia and H/R conditions (each group has 2 subgroups, i.e., _N and H/R, not represented in Fig. 1C).
Cell survival assessment  At the end of experiments cell survival was assessed using the cell viability test 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit. The plates were read by a spectrophotometer at 570 nm to obtain values of optical density [39]. All values were normalized to mean value of input control cells and expressed as percentage.

Measurement of mitochondrial membrane potential  (JC-1) The MMP was evaluated with the fluorescent dye JC-1 (5,50,6,60-tetraethylbenzimidazolylcarbocyanine iodide; Molecular Probes, Eugene, OR, USA). In brief, sub-groups of cells were incubated with JC-1-containing medium (10 µg/ml) at 37 ℃ for 20 min, washed two times with PBS and analyzed using a fluorescence microplate reader. The ratio between red and green JC-1 fluorescence was considered a quantitative index of mitochondrial membrane potential [43]. All values were normalized to mean value of control group and expressed as percentage.

Chemicals  The HNO donor Angeli’s salt (sodium trioxodinitrate, Na2N2O3) was a generous gift of Dr. Jon M. Fukuto (University of California, Los Angeles, CA). If not differently specified, chemicals and kits have been purchased from Sigma-Aldrich, St. Louis, MO, USA. Stock solutions of Angeli’s salt (100 mM in 10-mM NaOH) and of other compounds were diluted in buffer immediately prior to use, as previously described [17,44].

Statistical analysis  All values are presented as means _ SEM. All data were subjected to t-test, ANOVA and Tukey’s Multiple comparison post-tests, as appropriate. Significance was accepted at a p level of <0.05. 3.

Results  

Ex-vivo model  In isolated rat heart, Angeli’s salt (AS, 1 µM) reduced infarct size and ameliorated post-ischemic myocardial function. Baseline functional parameters and post-ischemic myocardial function are summarized in Table 1.

Angeli’s salt is a cardioprotective agent when it is infused before ischemia/reperfusion Consistently with previously reported data [17], as shown in Fig. 2A, pre-treatment with AS (1 mM) resulted in a significant reduction of infarct size (37.9 _ 3.5% of left ventricle, LV) with respect to I/R group (66.6 _ 6.4% of LV, p < 0.001). Infarct size data are corroborated by LDH release (Fig. 2B), which in the I/R hearts was about 1600 U/mg and it was significantly reduced by AS to about 900 U/mg (p < 0.01 vs I/R). CGRP receptor blockade prevents Angeli’s salt-induced reduction of myocardial injury The selective receptor blocker CGRP8-37 (0.1 mM) did not alter basal coronary resistance, cardiac performance (Table 1), and I/R injury (Fig. 2). The co-infusion of CGRP8-37 with AS prevented myocardial protection afforded by AS alone. The infarct size (63.6 _ 7.9%, Fig. 2A) was not different from the value found in I/R and CGRP8-37 groups (p = NS, AS + CGRP8-37 vs I/R and vs CGRP8-37), but significantly different from that reported in AS treated hearts (p < 0.05, AS + CGRP8-37 vs AS, Fig. 2A). Consistently, LDH release in control I/R group was superimposable to the release observed in CGRP8-37 and AS + CGRP8-37 groups (p = NS vs I/R for both groups), but significantly higher from AS alone (p < 0.05, AS + CGRP8-37 vs AS, Fig. 2B).
Infarct size and LDH release.

A: Infarct size. Infarct area expressed as percentage of the left ventricle (LV) mass resulted smaller in Angeli’s salt (AS) Group compared with ischemia/reperfusion (I/R), CGRP8-37 and AS + CGRP8-37 Groups. B: LDH release. The increase in LDH concentrations during reperfusion was lower in AS Group than in I/R, CGRP8-37 and AS + CGRP8-37 Groups. ** p < 0.01 and *** p < 0.001 vs I/R. # p < 0.05 vs AS.

Recovery of post-ischemic left ventricle contractile function is improved by Angeli’s salt via a CGRP mechanism. In post-ischemic phase left ventricle contractile function was impaired in all groups (Table 1, row data and Fig. 3 percent variations). In control I/R group dLVP dropped from 85 ± 4 mmHg during pre-ischemia to 23 ± 4 mmHg at end of reperfusion (_67 ± 3%, p < 0.001 vs pre-ischemia values; Fig. 3A). However, this negative trend was significantly ameliorated by AS pre-treatment (dLVP dropped by 42 ± 5% only; p < 0.001 vs control group; Fig. 3A). Similarly, in control I/R group the rate of change of left ventricular pressure (peak of dP/dt max) was reduced by about 60% (p < 0.001 vs pre-ischemia, Fig. 3B) and by about 45% only in AS pretreated hearts (p < 0.05 vs controls; Fig. 3B). Although CGRP8-37 given alone in pre-ischemic phase did not avoid the reduction of dLVP and dP/dt max recovery (Table 1 and Fig. 3A and B). As can be seen in Table 1, in control I/R group LVEDP increased from about 5 mmHg during pre-ischemia to about 50 mmHg at end of reperfusion (p < 0.01 vs pre-ischemia values). However, this dramatic increase was significantly blunted by AS pre-treatment (LVEDP...
increased from about 4 mmHg to about 18 mmHg only; \( p < 0.05 \) vs control group, Table 1). The improvement on LVEDP increase induced by AS was prevented by the addition of CGRP8-37, which per se did not affect I/R-induced LVEDP elevation. Yet, the post-ischemic levels of coronary perfusion pressure were not affected by AS, CGRP and/or CGRP8-37 pre-treatment (data not shown). These results suggest that AS may trigger the endogenous release of CGRP, which may act on their own receptors to mediate cardioprotection.

Fig. 3. Left ventricle contractile function impairment. A: Developed LVP (dLVP). dLVP expressed as percentage fall resulted smaller in Angeli’s salt (AS) Group respect to ischemia/reperfusion (I/R), CGRP8-37 and AS + CGRP8-37 Groups. B: first derivative of pressure during systole (dP/dtmax). dP/dtmax expressed as percentage recovery resulted lower in AS Group respect to I/R, CGRP8-37 and AS + CGRP8-37 Groups. **p < 0.001 vs I/R. # p < 0.05 vs AS.

**In-vitro models**

Cell death by H2O2 was limited by CGRP pre-treatment In Fig. 4 cell count is reported as percent value with respect to the mean value in the control group: the oxidative stress by H2O2 reduced cell viability with respect to control group (100 \(-2\%\) to 87 \(-2\%; \ p < 0.001, \text{CTRL} \ vs \ H2O2). The pre-treatment with CGRP ameliorated the cell survival (94 \(-3\%)\) in the presence of this oxidative stress (\( p = \text{NS, CGRP+H2O2 vs CTRL group}\)). However, the co-presence of CGRP8-37 abolished the protective effect induced by CGRP (cell survival 84 \(-2\%; \ p < 0.01 \text{CGRP + H2O2 vs CGRP + CGRP8–37 + H2O2}\). No differences are observed with respect to the CTRL group when CGRP or CGRP8-37 were given alone (cell survival 101 \(-4\%\) and 97 \(-2\%, \text{respectively}\). Moreover, as can be seen in Fig. 4B, oxidative stress by H2O2 led to a reduction of JC-1 red/green ratio, which is representative of a marked reduction of MMP. Importantly, CGRP pre-treatment prevented the MMP reduction caused by oxidative stress and the co-presence of CGRP8-37 abolished the protective effect induced by CGRP pre-treatment. CGRP per se has no effect on cell proliferation in normoxic conditions, but improves cell survival after H/R In Fig. 5A, the effects of normoxia and H/R in untreated cells (Control) and after CGRP-pretreatment are reported. Data are presented as percent variation (mean \(-\text{SEM}\)) with respect to mean value of cell count in normoxia. The analysis of viability in normoxia showed that 24-h pre-treatment with CGRP did not influence cell number: we found that there were no significant differences in cell viability between untreated cells and CGRP treated cells (CTRL-N 100 \(-2\%\) vs CGRP-N = 104 \(-4\%, \ p
In the absence of pre-treatment, the H/R protocol induced a significant reduction of cell vitality with respect to CTRL-N (CTRL-N 100 ± 2% vs CTRL-H/R 87 ± 2%, p < 0.01). The pre-treatment with CGRP protected the cells and reduced significantly the mortality by H/R (Control-N 100 ± 2% vs CGRP-H/R = 98 ± 2%, p = NS). 3.2.3. Cell survival after H/R was improved by CGRP pretreatment via PKC/mKATP/ROS signaling In Fig. 5B the effects of H/R in CGRP treated cells and after CGRP + antagonists pre-treatment are reported. Either CGRP8-37, the CGRP receptor antagonist, or CHE, the inhibitor of PKC, or 5-HD, the mKATP channels blocker, abolished the protective effect induced by CGRP pre-treatment against H/R (p < 0.05 for all vs CGRP-N). Also, the treatment with the anti-oxidant MPG abolished the protective effect of CGRP in hypoxic condition (CGRP-N vs CGRP + MPG-H/R, p < 0.01). Importantly, the cell vitality in all H/R groups pre-treated with CGRP + antagonists was significantly lower than cell vitality in CGRP-H/R group. These results suggest that CGRP is an important protective factor during hypoxia able to trigger the PKC/mKATP/ROS signaling. Finally, the antagonists alone did not avoid H/R detrimental effects, with the exception of the antioxidant, MPG. In this group, in fact, there was not a reduction of cell vitality after H/R (Fig. 5C). To corroborate MTT data, we ascertained the effect of H/R on MMP. As can be seen in Fig. 5D, H/R protocol led to a reduction of JC-1 red/green ratio, i.e. H/R reduced MMP. CGRP pre-treatment prevented the MMP reduction caused by H/R.

Fig. 4. Cell vitality and mitochondrial membrane potential in H9c2 subjected to H2O2 treatment. A: Effects of CGRP and its specific antagonist (CGRP8-37) on cellular mortality induced by oxidative stress with H2O2. Data are normalized to mean value of cell count in control conditions and expressed as percentage. B: Measurement of mitochondrial membrane potential with JC-1 in H9c2 in the presence and in the absence of H2O2 with and without CGRP and its specific antagonist (CGRP8-37). Data are normalized to mean value in control conditions and expressed as percentage. *** p < 0.001 vs Control. # p < 0.05 and ## p < 0.01 vs CGRP + H2O2.
Discussion

Here we demonstrate, for the first time, that the endogenous neuropeptide CGRP and receptor are involved in the protective effects induced by pre-treatment with AS, in cardiac ex vivo model. We also demonstrate that CGRP protects either against oxidative stress or hypoxia/reoxygenation the H9c2 cells. In this cellular model protection against H/R may occur via a PKC and mKATP/ROS-dependent signaling pathway, preserving MMP. Our results are in line with the recent observation that CGRP improves hypoxia-induced inflammation and apoptosis via NO\textsubscript{\textendash} in H9c2 [45]. In the isolated heart the infusion of AS, in a preconditioning like manner, reduces the infarct size and improves post-ischemic cardiac function [17]. Here we provide evidences that AS cardioprotective effects are abolished by co-infusion of a CGRP receptor antagonist, namely the CGRP8-37 fragment. These results suggest that AS-protection is dependent on endogenous CGRP and that its cardioprotective role is mediated by CGRP receptor activation. The H9c2 cell line was used as a bioassay to study the intracellular
mechanisms activated by CGRP-CGRP receptor interaction to be protective. This cell line responds to CGRP with the activation of pathways similar to adult cardiomyocytes [37,45]. We ascertained that CGRP-pretreatment may protect H9c2 cell line against oxidative stress induced by H2O2. We confirmed that CGRP is protective also against a hypoxia/reoxygenation protocol that may “mimic” I/R conditions. While CGRP protects against hypoxia/reoxygenation insult, it has minimal, if any, effects on H9c2 proliferation in normoxic conditions. In this in vitro setting we studied the intracellular pathway involved in CGRP induced protection using a) CHE, the large spectrum antagonist of PKC [4], b) 5-HD, the blocker of mKATP channels [40] and c) MPG, a highly effective antioxidant with mitochondrial affinity [8,9]. The application of either CHE or 5-HD abolishes the protective effect of CGRP in hypoxia/reoxygenation conditions. These results demonstrate indirectly that CGRP induces protection via activation of PKC and opening of mKATP dependent channels. It has been reported that the opening of these channels leads to a protective mitochondrial ROS-signaling [9,31,32]. Indeed, the protection induced by CGRP is likely dependent on ROS release [10]. Our results show that the co-treatment with an anti-oxidant (MPG + CGRP) also abolishes the protective effect against hypoxia/reoxygenation. Yet, ROS are double-edged swords, in fact MPG alone avoids damage by H/R. MPG seems to be specific for mitochondrial ROS [8,9] and this may explain the MPG limiting effect in CGRP-induced protection: it is likely that mitochondrial ROS are the primary species involved in both cardiac protection and injury [9,31,32]. We did not measure ROS generation due to the difficulty to measure transient production and to interpret the results obtained [46]. This is a limitation of our study to be kept in account. Nevertheless, ROS production during hypoxia/reoxygenation generally occurs in the mitochondrial electron transport chain [47–50]. In cardioprotective context, this ROS formation may depend on mKATP channel opening, and involve phosphorylation and mitochondrial translocation of PKC with subsequent generation of ROS with signaling role [32,47]. Importantly, in myocytes, the scavenging of ROS with MPG blunts the beneficial effect of cardioprotective interventions [47,49–51]. In our experiments, the JC-1 fluorescence ratio supports the involvement of mitochondria and MMP maintenance in the effects mediated by CGRP, both against redox stress an H/R challenging. Mitochondria are point of convergence for cardioprotective signaling pathways and the alteration of mitochondrial integrity is associated with disruption of the MMP leading to an increased permeabilization of the mitochondrial membrane and to the dangerous ROS-induced ROS release phenomena [31,32,43]. Actually, limited ROS production and maintenance of MMP together PKC activation have been proposed as important steps of protection [48,52–55], and using inhibitors of PKC and tyrosine kinase the nexus between ROS, kinase activity and cardioprotection has been demonstrated [53,56]. All the findings of the present study support that CGRP protective pathways converge on mitochondria to maintain the MMP following oxidative stress. Nitrogen oxidative species are important components of many physiological processes and have the potential to be useful pharmacological agents in cardiovascular diseases. Angeli’s salt is a potent vasodilator agent acting by soluble guanylyl cyclase dependent CGRP release and by KATP channels activation [57]. Angeli’s salt via NO and/or CGRP release may relax the coronary vessels [18–21], an effect also observed in our model during heart pre-treatment, as revealed by a transient drop in coronary perfusion pressure, which quickly recovered during the wash-out period in the pre-ischemic phase. In pilot experiments (n = 3) we also confirmed that CGRP8–37 abolishes ischemic preconditioning’s cardioprotection (data not shown). Involvement of CGRP receptors in ischemic preconditioning’s cardioprotection has been observed several times (e.g. [4,27–30]). The results of present study suggest CGRP as redox-dependent mediator of cardioprotection. However, we should keep in mind that AS decomposition generates both HNO and nitrite [58] and that the release of nitrite may have per se a cardioprotective effect [59]. With no doubt HNO donors induces CGRP release from sensory nerve endings on from noradrenergic, non-cholinergic (NANC) neurons [5,10–12,18–21]. Paolocci et al. [44] observed an increase in circulating CGRP levels during AS treatment, which increases the contractility of the heart (positive inotropy) [44]. However, the same authors demonstrated that, under heart failure conditions, CGRP does not play a major role in the HNO induced contractility, suggesting
another molecular target [10–12,19,20]. A role for AS in the therapy of heart failure with complex redox mechanisms has been reported [60,61]. The authors, suggested that HNO targets thiol groups on selective proteins involved in calcium handling, and this mechanism is probably influenced by ROS signaling. Similar effect on redox-dependent modifications of SH group of proteins involved in calcium handling have been reported by several groups studying contractility and/or cardioprotection (for reviews see [31,62]). This redox dependency of AS/CGRP effects is in line with our present study.

Conclusions Three key observations regarding the neuropeptide CGRP were made in this study: (1) in whole heart, CGRP receptor activation is involved in the cardioprotective effects of AS, an HNO donor able to induce CGRP release [5,10,11]; (2) CGRP protects cardiac cells from redox stress and hypoxia/reoxygenation-induced injury; and (3) CGRP protective effects may depend upon the activation of the CGRP receptors and the downstream PKC and mKATP/ROS signaling, with a preservation of MMP. If confirmed in appropriate pre-clinical model, HNO and CGRP cardioprotective effects may be interesting mechanism to be further investigated for a therapeutic approaches in heart failure and cardiac ischemia/reperfusion scenarios [11]. Conflicts of interest PP is one of the inventors of “Method of treating ischemia/ reperfusion injury with nitroxy1 donors US 20040038947 A1”.

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