



# Natriuretic peptide receptor-C activation attenuates angiotensin II-induced enhanced oxidative stress and hyperproliferation of aortic vascular smooth muscle cells

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## Abstract

We showed previously that natriuretic peptide receptor-C (NPR-C) agonist, C-ANP<sub>4–23</sub>, attenuated the enhanced expression of G $\alpha$  proteins in vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats (SHR) through the inhibition of enhanced oxidative stress. Since the enhanced levels of endogenous angiotensin II (Ang II) contribute to the overexpression of G $\alpha$  proteins and augmented oxidative stress in VSMC from SHR, the present study was undertaken to investigate if C-ANP<sub>4–23</sub> could also attenuate angiotensin II (Ang II)-induced oxidative stress and associated signaling. Ang II treatment of aortic VSMC augmented the levels of superoxide anion (O<sub>2</sub><sup>−</sup>), NADPH oxidase activity, and the expression of NADPH oxidase subunits and C-ANP<sub>4–23</sub> treatment attenuated all these to control levels. In addition, Ang II-induced enhanced levels of thiobarbituric acid-reactive substances (TBARS) and protein carbonyl content were also attenuated toward control levels by C-ANP<sub>4–23</sub> treatment. On the other hand, Ang II inhibited the levels of nitric oxide (NO) and augmented the levels of peroxynitrite (OONO<sup>−</sup>) in VSMC which were restored to control levels by C-ANP<sub>4–23</sub> treatment. Furthermore, C-ANP<sub>4–23</sub> treatment attenuated Ang II-induced enhanced expression of G $\alpha$  proteins, phosphorylation of p38, JNK, and ERK 1,2 as well as hyperproliferation of VSMC as determined by DNA synthesis, and metabolic activity. These results indicate that C-ANP<sub>4–23</sub>, via the activation of NPR-C, attenuates Ang II-induced enhanced nitroxidative stress, overexpression of G $\alpha$  proteins, increased activation of the p38/JNK/ERK 1,2 signaling pathways, and hyperproliferation of VSMC. It may be suggested that C-ANP<sub>4–23</sub> could be used as a therapeutic agent in the treatment of vascular remodeling associated with hypertension and atherosclerosis.

**Keywords** C-ANP<sub>4–23</sub> · Angiotensin II · Oxidative stress · MAPK · VSMC · Hyperproliferation

## Introduction

Angiotensin II (Ang II), a vasoactive peptide and a key component of the renin-angiotensin system (RAS), regulates a variety of physiological functions including proliferation, hypertrophy of vascular smooth muscle cells (VSMC), and blood pressure by interacting with two distinct receptor subtypes designated as AT<sub>1</sub> and AT<sub>2</sub>, respectively [1]. The

presence of AT<sub>1</sub> receptor subtype has been shown in rat vascular tissues; however, a small proportion of AT<sub>2</sub> receptors are also present in rat aorta [2, 3]. Most of the physiological effects of Ang II are mediated through the activation of AT<sub>1</sub> receptors, which are coupled to different signaling pathways including adenylyl cyclase/cAMP inhibition through G $\alpha$  proteins [4, 5] and MAPKs [6] and PI turnover through Gq/11 $\alpha$  [7]. Ang II has also been shown to increase oxidative stress through the activation of NADPH oxidase, enhanced expression of different subunits of NADPH oxidase, and overproduction of O<sub>2</sub><sup>−</sup> in VSMC [8, 9]. Furthermore, the enhanced levels of endogenous Ang II were also shown to contribute to the enhanced oxidative stress and hyperproliferation of VSMC from spontaneously hypertensive rats (SHR) [10]. In addition, Ang II-mediated hyperproliferative effects were associated with decreased levels of NO in VSMC [11].

Padma Madiraju and Ekhtear Hossain equally contributed for this work.

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Natriuretic peptides (NPs) comprise a family of three peptide hormones: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) [12, 13] and regulate a variety of physiological functions including blood pressure through their interaction with natriuretic peptide receptors (NPRs). Three subtypes of NPRs have been identified: NPR-A [14], NPR-B [15, 16], and NPR-C [17]. NPR-A and NPR-B are membrane guanylyl cyclase receptors, whereas NPR-C does not possess guanylyl cyclase activity and is coupled to adenylyl cyclase inhibition through the inhibitory guanine nucleotide regulatory protein Gi [17, 18], or activation of phospholipase C (PLC) [19].

ANP has been shown to act as an autocrine/paracrine modulator of cardiac hypertrophy and remodeling [20–22]. Earlier studies demonstrated that C-ANP<sub>4–23</sub>, an agonist that interacts specifically with NPR-C attenuated enhanced production of superoxide anion (O<sub>2</sub><sup>-</sup>), NADPH oxidase activity, and the overexpression of different subunits of NADPH oxidase in VSMC from SHR [23]. In addition, C-ANP<sub>4–23</sub> was also shown to attenuate hyperproliferation and hypertrophy of aortic VSMC from SHR through the inhibition of reactive oxygen species (ROS) and ROS-mediated cell signaling pathways [24]. However, whether C-ANP<sub>4–23</sub> could also attenuate the enhanced oxidative stress induced by Ang II remains obscure. The present study was therefore undertaken to investigate the effect of C-ANP<sub>4–23</sub> on Ang II-evoked nitroxidative stress, overexpression of Gi $\alpha$  proteins, increased activation of the p38/JNK/ERK 1,2 signaling pathways, and subsequent hyperproliferation of VSMC.

In this study, we have provided the first experimental evidence that NPR-C activation by C-ANP<sub>4–23</sub> attenuates Ang II-induced hyperproliferation of VSMC through decreasing the enhanced nitroxidative stress and activation of the p38/JNK/ERK 1,2 signaling pathways. From these results it may be suggested that C-ANP<sub>4–23</sub>, an activator of NPR-C, may be used as a therapeutic agent for the treatment of hypertension and atherosclerosis.

## Materials and methods

### Materials

A ring-deleted analog of ANP, C-ANP<sub>4–23</sub> was purchased from Bachem (Torrance, CA). Angiotensin II (human) was purchased from Sigma-Aldrich Chemical Co. (St Louis, Missouri, USA). Western blotting primary antibodies against Gi $\alpha$ -2 (sc-13534), Gi $\alpha$ -3 (sc-373746), p38 $\alpha$  (sc-535), p-p38 (sc-7973, phosphospecific-Tyr<sup>182</sup>), JNK1 (sc-1648), p-JNK (sc-135642, phosphospecific-Thr<sup>183</sup>), ERK 1,2 (sc-135900), p-ERK 1,2 (sc-7383, phosphospecific-Tyr<sup>204</sup>), Nox-4 (sc-21860), p22<sup>phox</sup> (sc-130551), p47<sup>phox</sup> (sc-86190), dynein IC1/2 (sc-13524), secondary antibodies goat anti-mouse IgG

horseradish peroxidase (HRP) conjugate (sc-2005), donkey anti-goat IgG horseradish peroxidase (HRP) conjugate (sc-2020), and enhanced chemiluminescence (ECL) detection system kits were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The L-[4,5-<sup>3</sup>H]-Thymidine was from PerkinElmer Inc. (Waltham, Massachusetts, USA). All other chemicals used in the experiments were purchased from Sigma-Aldrich.

### Cell culture and incubation

Aortic VSMC from Male Sprague Dawley (SD) rats were cultured as described previously [25]. The cell purity was determined by immunofluorescence using  $\alpha$ -actin as described previously [25]. The cells contained high levels of smooth muscle-specific  $\alpha$ -actin [8, 26]. Cells were incubated at 37 °C in 95% air, 5% CO<sub>2</sub>, humidified atmosphere in Dulbecco's Modified Eagle's Medium, and 10% heat-inactivated fetal bovine serum (FBS). Cells were passaged upon reaching confluence with 0.5% trypsin and used between passages two and ten. Subconfluent VSMC were serum deprived 4 h in DMEM without FBS at 37 °C to reduce the interference by growth factors present in the serum. VSMC were then pre-incubated in the absence or presence of different concentrations of C-ANP<sub>4–23</sub> (10<sup>-6</sup>–10<sup>-10</sup> or 10<sup>-7</sup> M) for 1 h before stimulation with 10<sup>-6</sup> M of Ang II, a concentration previously shown to be effective in VSMC [27, 28]. After 24 h incubation, the cells were washed twice with ice-cold phosphate buffer saline (PBS) and lysed in a 200  $\mu$ l buffer containing 25 mM Tris-HCl (pH 7.5), 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/mL aprotinin, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 0.5  $\mu$ g/mL leupeptin on ice. The cell lysates were centrifuged at 12,000 $\times$ g for 15 min at 4 °C, and the supernatants were used for Western blot analysis. Protein concentrations were measured with a Bradford assay [29].

All the animal procedures used in the present study were approved by the Comité de Déontologie de l'Expérimentation sur les Animaux (CDEA) of the University of Montreal (Approval no. 99050). The investigation conforms to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH) (Guide, NRC 2011).

### Western blotting

The levels of protein expression and phosphorylation were determined by Western blotting as described previously [30]. After SDS-PAGE, the separated proteins were transferred to a nitrocellulose membrane, which were then blocked for 1 h at room temperature with 5% dry milk and

incubated overnight with specific antibodies against different proteins. Dynein was used for loading controls. The antibody–antigen complexes were detected by incubating the membranes with horseradish peroxidase-conjugated antibodies for 1 h at room temperature. The blots were then washed three times with PBS before reaction with enhanced chemiluminescence (ECL). Quantitative analysis of the proteins was performed by densitometric scanning of the autoradiographs using the enhanced laser densitometer LKB Ultrosan XL and quantified using the gel-scan XL evaluation software (version 2.1) from Pharmacia (Baie d'Urfé, Québec, Canada).

### [<sup>3</sup>H]-Thymidine incorporation assays

Aortic VSMC proliferation was quantified by DNA synthesis that was evaluated by incorporation of [<sup>3</sup>H]-Thymidine into cells as described earlier [31]. Briefly, VSMC were plated in 6-well plates (10<sup>5</sup> cells/well) and incubated at 37 °C for 24 h. After incubation, VSMC were serum-starved for 4 h (to induce cell quiescence), pre-incubated in the presence or absence of different concentrations of C-ANP<sub>4–23</sub> (10<sup>−6</sup>–10<sup>−10</sup> or 10<sup>−7</sup> M) for 60 min before stimulation with or without 10<sup>−6</sup> M Ang II for 24 h. These concentrations of different agonist/antioxidants have been used in earlier studies [10, 23, 31, 32]. The cells were then incubated with [<sup>3</sup>H] thymidine (1 μCi) for 4 h before the cells were harvested. The cells were rinsed twice with ice-cold PBS and incubated with 5% trichloroacetic acid for 1 h at 4 °C. After being washed twice with ice-cold water, the cells were incubated with 0.4 N sodium hydroxide (NaOH) solution for 30 min at room temperature, and radioactivity was determined by liquid scintillation counter.

### MTT cell proliferation assay

Aortic VSMC proliferation was also measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay using a MTT Cell Proliferation Assay Kit (Trevigen) as describe previously [33]. Briefly, VSMC were plated in 96-well culture plates (10<sup>3</sup> cells/well) and incubated at 37 °C for 24 h. After incubation, VSMC were serum-starved for 4 h (to induce cell quiescence), pre-incubated in the presence or absence of different concentrations of C-ANP<sub>4–23</sub> (10<sup>−6</sup>–10<sup>−10</sup> or 10<sup>−7</sup> M) for 60 min before stimulation with or without Ang II (10<sup>−6</sup> M) for 24 h. The cells were then incubated with 10 μL of MTT (5 mg/mL) for 4 h at 37 °C and 100 μL of cell lysis buffer was added to solubilize the crystals for 2 h at room temperature. The absorbance was determined with a spectrophotometer (TECAN infinite 200 PRO) at a wavelength of 490 nm.

### Superoxide anion (O<sub>2</sub><sup>−</sup>) measurements

Basal O<sub>2</sub><sup>−</sup> production in aortic VSMC was measured by the lucigenin-enhanced chemiluminescence method with a low concentration (5 μM/L) of lucigenin as described previously [23]. The VSMC were pre-incubated in the absence or presence of C-ANP<sub>4–23</sub> (10<sup>−7</sup> M) for 1 h and subsequently stimulated with or without Ang II (10<sup>−6</sup> M) for 24 h. After treatment with Ang II, VSMC were washed in oxygenated Krebs-HEPES buffer, scraped, and placed in scintillation vials containing lucigenin solution, and the emitted luminescence was measured with a liquid scintillation counter (Wallac 1409; Perkin Elmer Life Science, St Laurent, QC, Canada) for 5 min. The average luminescence value was estimated and the background value was subtracted, and the result was divided by the total weight of proteins in each sample.

### NADPH oxidase activity determination

The activation of NADPH oxidase activity in the samples was assessed by adding 10<sup>−4</sup> M/L NADH (Sigma) in the vials before counting. Basal O<sub>2</sub><sup>−</sup>-induced luminescence was then subtracted from the luminescence value induced by NADH [23].

### Thiobarbituric acid-reactive substances (TBARS) assay

Lipid peroxidation was determined by measuring TBARS in control and C-ANP<sub>4–23</sub>-treated aortic VSMC as described earlier [34, 35]. Aortic VSMC were pre-incubated with 0.01 mM CuCl<sub>2</sub> in 20 mM phosphate buffer (pH 7.4) at room temperature for 15 min. The reaction was started by the addition of 0.5 mM ascorbate and 1.9 mM deoxyribose and incubated for 1 h at 37 °C. Thiobarbituric acid (10 g/L) in 50 mM NaOH and concentrated acetate acid (1:1 ratio) were added to the incubation mixture, which was boiled in water for 15 min. The TBARS were quantified by spectrophotometer (TECAN infinite 200 PRO) at a wavelength of 532 nm. Phosphate buffer (20 mM; pH 7.4) was taken as blank. Protein concentration was measured with a Bio-Rad (Hercules, CA, USA) assay kit using bovine serum albumin as a standard.

### Protein carbonyl content assay

The carbonyl content of proteins in control and C-ANP<sub>4–23</sub>-treated cells was determined by the 2,4-dinitrophenylhydrazine (DNPH) method as described earlier [36]. Aortic VSMC were lysed and proteins were precipitated with 20% trichloroacetic acid. The precipitates were incubated with either 2 N HCl alone (blank) or 2 N HCl containing 10 mM

DNPH at room temperature in the dark for 1 h, being vortexed every 10 min. After the reaction, the mixture was centrifuged, and the precipitates were washed with an ethanol:ethyl acetate (1:1) mixture three times and dissolved in 6 M guanidine chloride or 8 M urea. The absorbance was determined with a spectrophotometer (TECAN infinite 200 PRO) at a wavelength of 360 nm. The concentration of protein was measured with a Bio-Rad assay kit using bovine serum albumin as a standard.

### Measurements of intracellular hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), nitric oxide (NO), and peroxynitrite ( $\text{ONOO}^-$ ) levels

The levels of intracellular  $\text{H}_2\text{O}_2$ , NO, and  $\text{ONOO}^-$  produced in VSMC were measured by using intracellular fluorescent probes, dichloro-dihydro-fluorescein diacetate (DCFH-DA), diamino fluorescein-2 diacetate (DAF-2DA), and dihydrorhodamine 123 (DHR 123), respectively, as described earlier [30, 37, 38]. Briefly, VSMC were plated in a 96-well culture plate ( $10^3$  cells/well) and incubated at  $37^\circ\text{C}$  for 24 h. After incubation, VSMC were serum-starved for 4 h (to induce cell quiescence), pre-treated in the presence or absence of C-ANP<sub>4–23</sub> ( $10^{-7}$  M) for 60 min before stimulation with or without Ang II ( $10^{-6}$  M) for 24 h. After stimulation, VSMC were washed twice with PBS and incubated at  $37^\circ\text{C}$  for 1 h with 10 mmol/L DCFH-DA for detecting  $\text{H}_2\text{O}_2$ , with both 10 mmol/L DAF-2DA and  $10^{-6}$  mol/L acetylcholine for detecting NO, and with  $5 \times 10^{-3}$  mol/L DHR 123 for detecting  $\text{ONOO}^-$ , respectively. Cells were washed twice with PBS, and fluorescence intensities were measured by a spectrophotometer (TECAN infinite 200 PRO) with excitation and emission wavelengths at 495 and 515 nm for DCFH-DA, 495 and 515 nm for DAF-2DA, and 480 and 530 nm for DHR 123, respectively. Changes in fluorescence intensities were expressed as percentages of the values obtained in the WKY group (taken as 100%).

### Measurements of extracellular NO levels

The NO concentrations in cell culture supernatant were determined by using Griess reagent as describe previously [30]. Briefly, VSMC were plated in a 96-well culture plate ( $10^3$  cells/well) and incubated at  $37^\circ\text{C}$  for 24 h. After incubation, VSMC were serum-starved for 4 h (to induce cell quiescence), pre-treated in the presence or absence of C-ANP<sub>4–23</sub> ( $10^{-7}$  M) for 60 min before stimulation with or without Ang II ( $10^{-6}$  M) for 24 h. After stimulation, 50  $\mu\text{L}$  aliquots was removed from the supernatant of the cultured cells and incubated with an equal volume of the Griess reagent at room temperature for 15 min. Sodium nitrite was used to generate a standard curve. The absorbance was

determined with a spectrophotometer (TECAN infinite 200 PRO) at a wavelength of 490 nm.

### Transfection of VSMC with siRNA

For siRNA transfection efficiency, the manufacturer's protocol was followed. Briefly, aortic VSMC were seeded in a 12-well plate or petri dishes and cultured in antibiotic free normal growth medium supplemented with 10% FBS until the cells were 60% confluent ( $\sim 48$  h). On the day of transfection, cells were washed with transfection medium (sc-36868) and incubated with 1 ml of transfection reagent (sc-29528) containing 80 pmoles of either scrambled siRNA (sc-37007), siRNA specific for  $\text{G}\alpha\text{-2}$  (sc-41753), or  $\text{G}\alpha\text{-3}$  (sc-37255) for 12 h. The medium was replaced with normal DMEM (containing 10% FBS and 1% antibiotics) for an additional 24 h (90% confluence). By using this procedure, we were able to knockdown the expression of  $\text{G}\alpha\text{-2}$  and  $\text{G}\alpha\text{-3}$  by about 90 and 80%, respectively in aortic VSMC (data not shown), which is similar to our previous report [39].

### Statistical analysis

The number of independent experiments is reported. Each experiment was conducted at least 4 times using separate cell population. All data are expressed as the mean  $\pm$  SE (standard error). Comparisons between groups were made with one-way analysis of variance (ANOVA) followed by Dunnett's tests using GraphPad Prism5 (GraphPad Software Inc., La Jolla, California, USA). Results were considered significant at a value of  $p < 0.05$ .

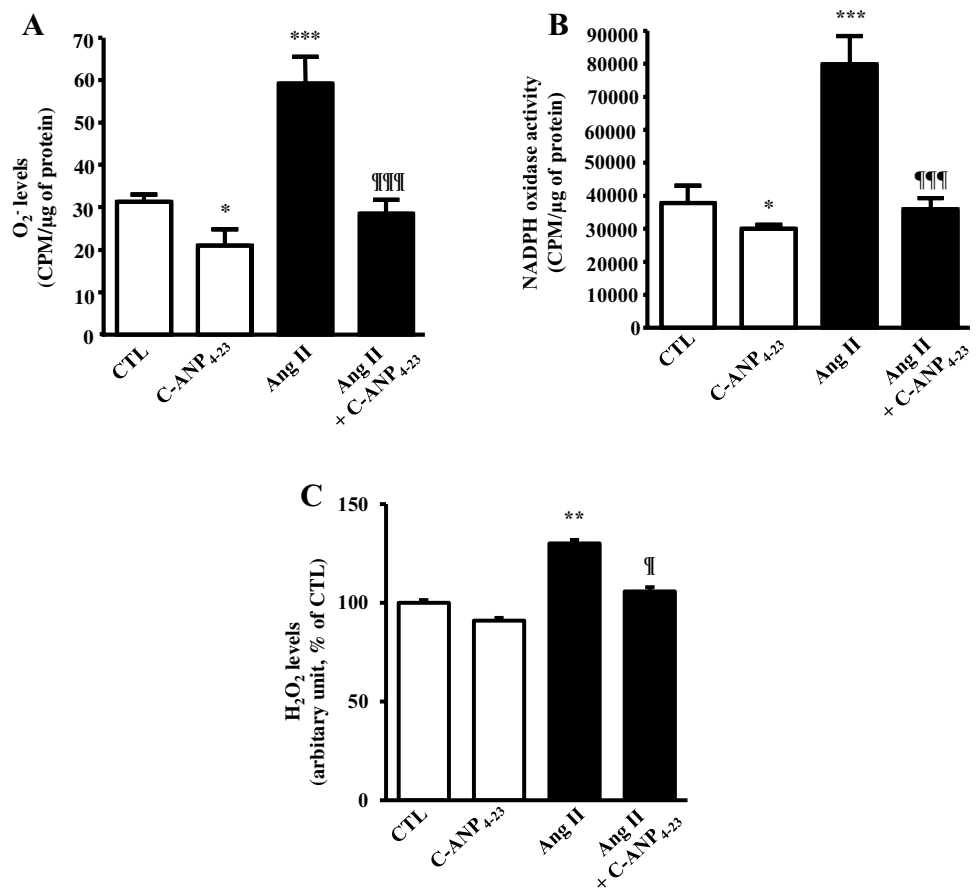
## Results

### C-ANP<sub>4–23</sub> attenuates Ang II-induced enhanced levels of $\text{O}_2^-$ , NADPH oxidase activity, and $\text{H}_2\text{O}_2$ in aortic VSMC

We earlier showed that C-ANP<sub>4–23</sub> attenuated the enhanced oxidative stress exhibited by VSMC from SHR [23]. Since the enhanced levels of endogenous Ang II were shown to contribute to the augmented oxidative stress in VSMC from SHR, it was of interest to investigate if C-ANP<sub>4–23</sub> could also attenuate Ang II-induced enhanced oxidative stress. To test this, we examined the effect of C-ANP<sub>4–23</sub> on Ang II-induced enhanced levels of  $\text{O}_2^-$ , NADPH oxidase activity, and  $\text{H}_2\text{O}_2$  in aortic VSMC. Results shown in Fig. 1 demonstrate that Ang II ( $10^{-6}$  M) treatment enhanced the basal levels of  $\text{O}_2^-$  (A), NADPH oxidase activity (B), and  $\text{H}_2\text{O}_2$  (C) in VSMC by about 100, 110, and 30%, respectively, which were attenuated towards the control levels by C-ANP<sub>4–23</sub> pre-treatment.



**Fig. 1** Effect of C-ANP<sub>4–23</sub> on Ang II-induced enhanced levels of superoxide anion (O<sub>2</sub><sup>•-</sup>) NADPH oxidase activity and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in aortic VSMC. Serum-starved, quiescent aortic VSMC were pre-incubated in the absence or presence of C-ANP<sub>4–23</sub> (10<sup>-7</sup> M) for 60 min prior to stimulation with or without Ang II (10<sup>-6</sup> M). After 24 h later, levels of O<sub>2</sub><sup>•-</sup> (A), NADPH oxidase activity (B), and H<sub>2</sub>O<sub>2</sub> (C) were determined as described in the “Materials and Methods” section. Results are expressed as % of the control group (CTL, taken as 100%). Values are the means ± SE of at least 3 independent experiments. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 versus control (CTL) group; ¶*p* < 0.05, ¶¶*p* < 0.001 versus Ang II group



In addition, C-ANP<sub>4–23</sub> also inhibited the basal levels of O<sub>2</sub><sup>•-</sup> and NADPH oxidase activity in control cells by about 30 and 20%, respectively, without affecting the levels of H<sub>2</sub>O<sub>2</sub>.

### C-ANP<sub>4–23</sub> attenuates Ang II-induced enhanced expression of NADPH oxidase subunits in aortic VSMC

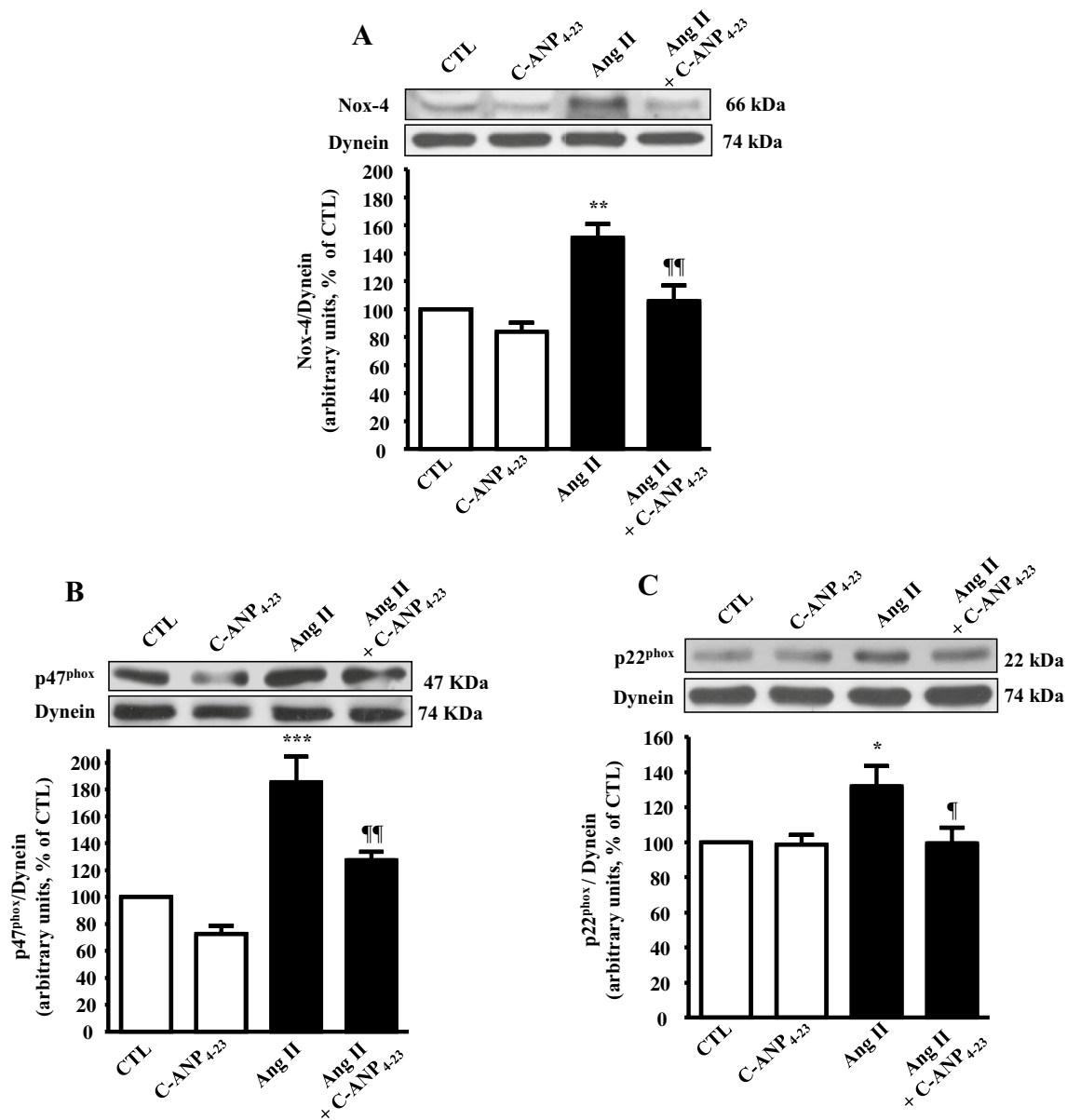
To further explore if C-ANP<sub>4–23</sub>-induced attenuation of oxidative stress was associated with the decreased expression of the NADPH oxidase subunits, we examined the effect of C-ANP<sub>4–23</sub> treatment on Ang II-induced enhanced expression of Nox-4, p47<sup>phox</sup>, and p22<sup>phox</sup> proteins, critical subunits involved in NADPH oxidase activation in aortic VSMC. Results shown in Fig. 2 indicate that, treatment of aortic VSMC with Ang II (10<sup>-6</sup> M) enhanced the expression levels of Nox-4 (A), p47<sup>phox</sup> (B), and p22<sup>phox</sup> proteins (C) by about 50, 80, and 30%, respectively, which were all attenuated to almost control levels by C-ANP<sub>4–23</sub> pre-treatment. However, C-ANP<sub>4–23</sub> treatment did not affect the levels of these proteins in control VSMC.

### C-ANP<sub>4–23</sub> attenuates Ang II-induced enhanced levels of TBARS and carbonyl content in aortic VSMC

We also examined the effect of C-ANP<sub>4–23</sub> treatment on Ang II-induced enhanced levels of MDA as well as carbonyl content (cellular markers of oxidative stress) in aortic VSMC and the results are shown in Fig. 3. Treatment of VSMC with Ang II (10<sup>-6</sup> M) enhanced the levels of TBARS (A) and carbonyl content (B) by about 40 and 70%, respectively, and these increases were completely reversed by C-ANP<sub>4–23</sub> pre-treatment. On the other hand, C-ANP<sub>4–23</sub> also decreased the levels of TBARS and carbonyl content by about 25 and 50%, respectively, in control VSMC.

### C-ANP<sub>4–23</sub> augments Ang II-induced decreased level of NO and attenuates enhanced levels of ONOO<sup>-</sup> in aortic VSMC

Ang II-induced increased oxidative stress has been shown to diminish the release and bioavailability of NO in VSMC [40]. To investigate if C-ANP<sub>4–23</sub> that has been shown to augment the levels of NO in proximal tubules [41] could also enhance the Ang II-induced decreased levels of intra- and extracellular NO in aortic VSMC, we examined the



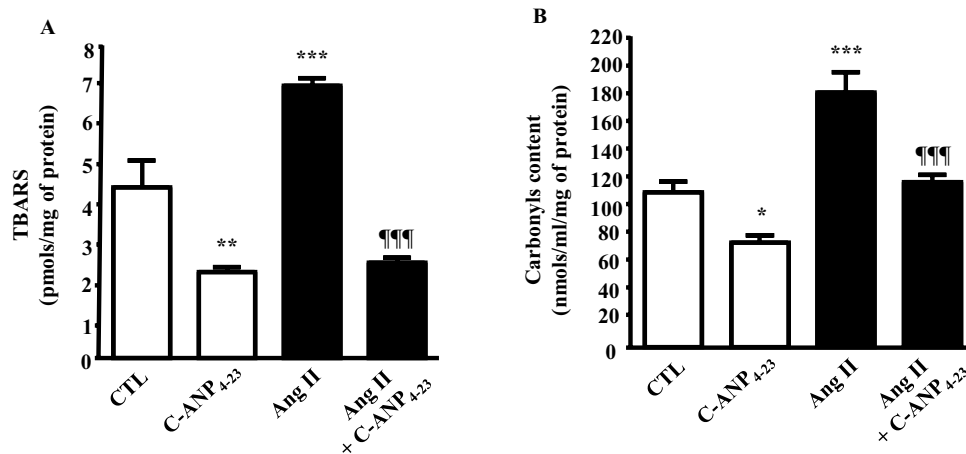
**Fig. 2** Effect of C-ANP<sub>4-23</sub> on Ang II-induced enhanced expression of NADPH oxidase subunits: Nox-4, p22<sup>phox</sup>, and p47<sup>phox</sup> in aortic VSMC. Serum-starved, quiescent aortic VSMC were pre-incubated in the absence or presence of C-ANP<sub>4-23</sub> ( $10^{-7}$  M) for 60 min prior to stimulation with or without Ang II ( $10^{-6}$  M). After 24 h later, cell lysates were prepared as described in the [Materials](#) section. 30  $\mu$ g of proteins was resolved by SDS-PAGE and was subjected to immunoblotting analysis using antibodies against Nox-4, p22<sup>phox</sup>, and p47<sup>phox</sup>. Upper panels: representative immunoblot of Nox-4 (A), p22<sup>phox</sup>

(B), and p47<sup>phox</sup> (C) with corresponding dynein. Lower panels: Protein levels of Nox-4, p22<sup>phox</sup>, and p47<sup>phox</sup> and corresponding dynein were quantified using scanning densitometry and all the values were normalized based on the corresponding dynein values. Results are expressed as % of the control group (CTL, taken as 100%). Values are the means  $\pm$  SE of at least 4 independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus control (CTL) group; ¶ $p < 0.05$ , ¶¶ $p < 0.01$  versus Ang II group

effect of C-ANP<sub>4-23</sub> treatment on intra- and extracellular levels of NO in the absence and presence of Ang II. The results shown in [Fig. 4](#) indicate that Ang II ( $10^{-6}$  M) decreased the levels of intracellular (A) and extracellular (B) NO by about 40 and 80%, respectively, which were significantly augmented by C-ANP<sub>4-23</sub> treatment. In addition, C-ANP<sub>4-23</sub> also increased the intra- and extracellular

levels of NO by about 40 and 125%, respectively, in control cells.

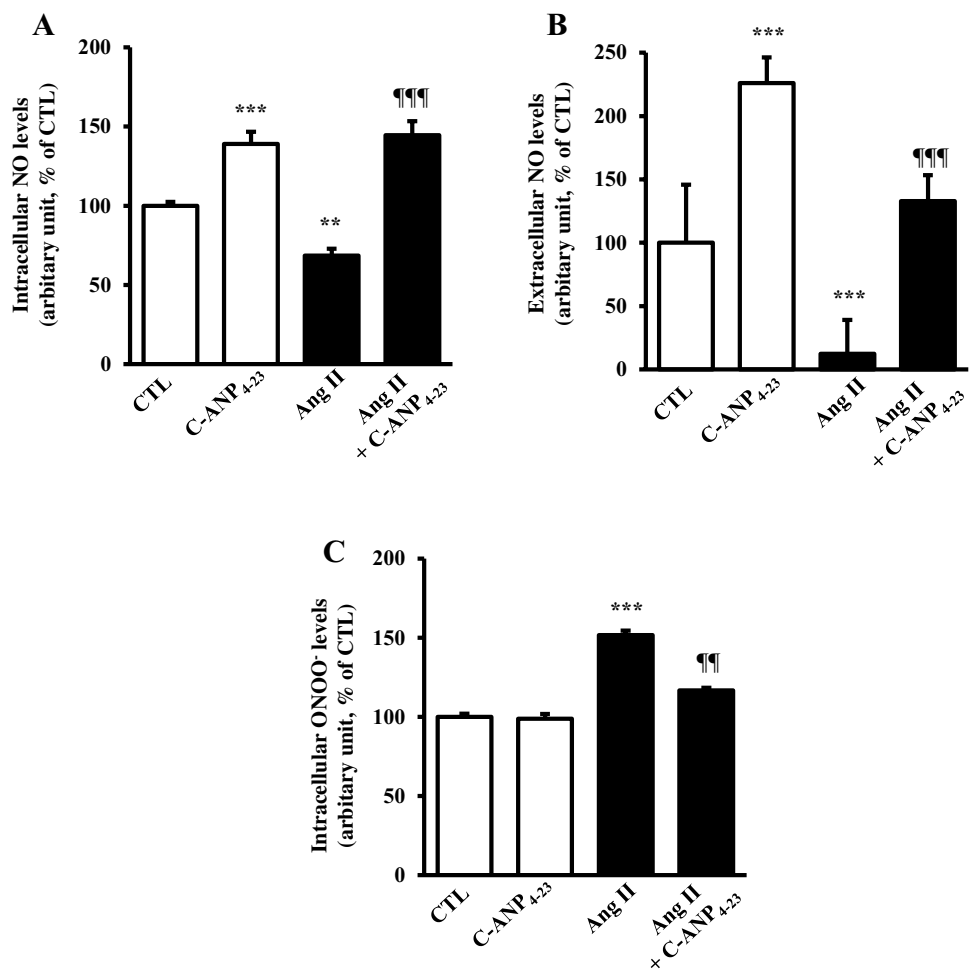
Since decreased bioavailability of NO has been shown to result in the concomitant increase in the levels of peroxynitrite (ONOO<sup>-</sup>) [42] and Ang II has been shown to enhance the levels of ONOO<sup>-</sup> in different tissues including aorta, cardiomyocytes, and endothelial cells [43–45], it



**Fig. 3** Effect of C-ANP<sub>4-23</sub> on Ang II-induced enhanced levels of thiobarbituric acid-reactive substances (TBARS) and protein carbonyl content in aortic VSMC. Serum-starved, quiescent aortic VSMC were pre-incubated in the absence or presence of C-ANP<sub>4-23</sub> ( $10^{-7}$  M) for 60 min prior to stimulation with or without Ang II ( $10^{-6}$  M). After 24 h later, TBARS (A) and protein carbonyl content (B) levels in cell

lysates were determined as described in the “Materials and Methods” section. Results are expressed as picomols/mg protein for TBARS and nmols/ml/mg protein for TBARS. Values are the means  $\pm$  SE of at least 3 independent experiments performed in triplicate. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus control (CTL) group; ¶¶¶ $p < 0.001$  versus Ang II group

**Fig. 4** Effect of C-ANP<sub>4-23</sub> on Ang II-induced decreased levels of NO and enhanced levels of ONOO<sup>-</sup> in aortic VSMC. Serum-starved, quiescent aortic VSMC were pre-incubated in the absence or presence of C-ANP<sub>4-23</sub> ( $10^{-7}$  M) for 60 min prior to stimulation with or without Ang II ( $10^{-6}$  M). After 24 h later, NO (A–B) and ONOO<sup>-</sup> (C) levels were determined as described in the “Materials and Methods” section. Results are expressed as % of the control group (CTL, taken as 100%). Values are the means  $\pm$  SE of at least 3 independent experiments. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus control (CTL) group; ¶¶ $p < 0.01$ , ¶¶¶ $p < 0.001$  versus Ang II group

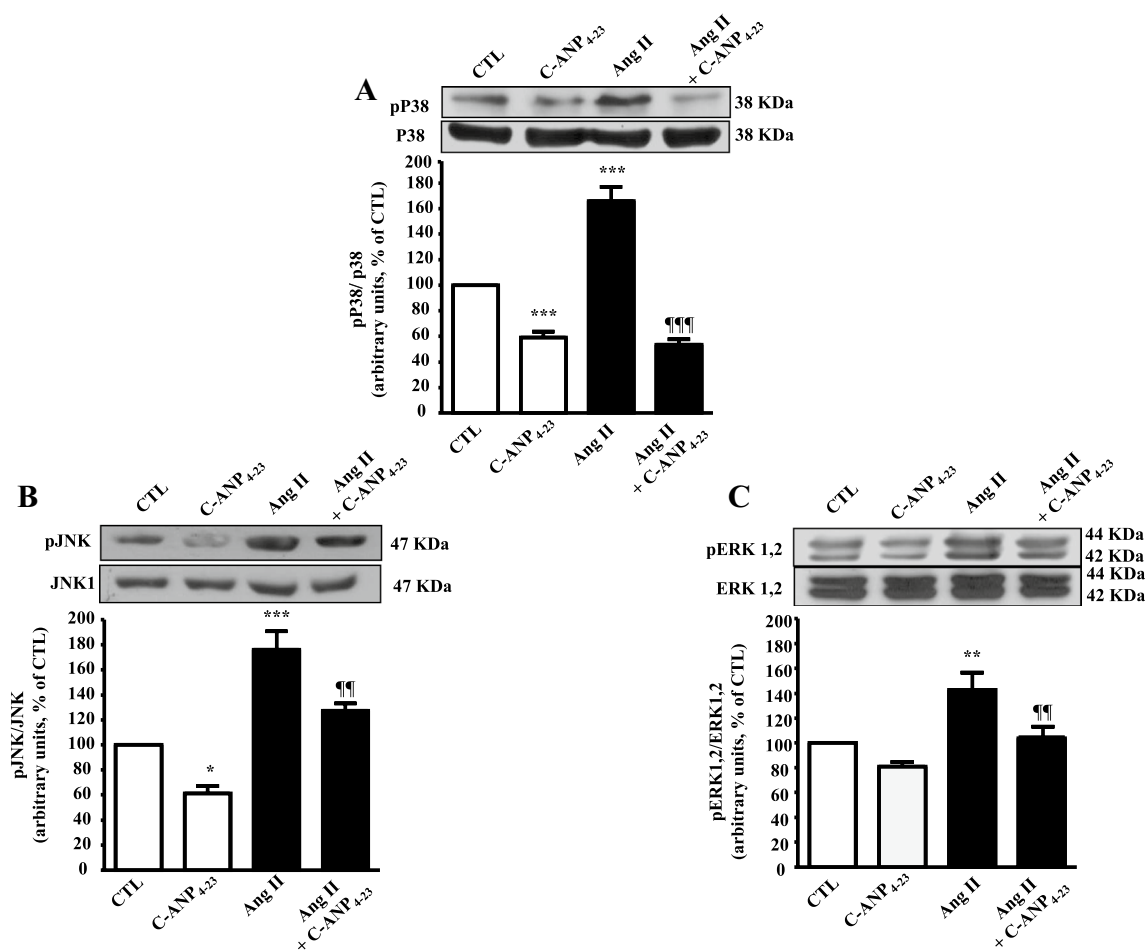


was of interest to examine if C-ANP<sub>4–23</sub> could attenuate the enhanced levels of ONOO<sup>-</sup> induced by Ang II in VSMC. Results shown in Fig. 4C indicate that Ang II increased the levels of ONOO<sup>-</sup> by about 50% in VSMC and C-ANP<sub>4–23</sub> attenuated these enhanced levels by about 80%. However, the basal ONOO<sup>-</sup> levels in control VSMC were not affected by this treatment.

### C-ANP<sub>4–23</sub> attenuates Ang II-induced enhanced phosphorylation of MAPKs: p38, JNK, ERK 1,2 in aortic VSMC

Oxidative stress due to the overproduction of ROS has been shown to activate ERKs, JNKs, or p38 MAPK

signaling pathways [46–48]. Since C-ANP<sub>4–23</sub> attenuates Ang II-induced oxidative stress, it was desirable to investigate if it could also attenuate Ang II-evoked enhanced activation of MAPK signaling pathways. To test this, the effect of C-ANP<sub>4–23</sub> treatment on Ang II-induced enhanced levels of phosphorylated p38, JNK, and ERK 1,2 were examined in aortic VSMC, and the results are shown in Fig. 5. Ang II enhanced the phosphorylation levels of p38 (A), JNK (B), and ERK 1,2 (C) by about 60, 80, and 40%, respectively, which were attenuated toward control levels by C-ANP<sub>4–23</sub> treatment. In addition, C-ANP<sub>4–23</sub> also attenuated the activation of p38 and JNK by about 40% in control VSMC without affecting the activation of ERK 1,2. However, this treatment did not affect the expression



**Fig. 5** Effect of C-ANP<sub>4–23</sub> on Ang II-induced enhanced phosphorylation of MAPK: p38, JNK, and ERK 1,2 in aortic VSMC. Serum-starved, quiescent aortic VSMC were pre-incubated in the absence or presence of C-ANP<sub>4–23</sub> ( $10^{-7}$  M) for 60 min prior to stimulation with or without Ang II ( $10^{-6}$  M). After 24 h later, cell lysates were prepared as described in the “Materials and Methods” section. 30  $\mu$ g of proteins were resolved by SDS-PAGE and were subjected to immunoblotting analysis using phosphorylated antibodies against p38, JNK, and ERK 1,2. Membranes were stripped and re-probed with p38, JNK, and ERK 1,2 antibodies. Upper panels: representa-

tive immunoblotting of phosphorylated p38 (A), JNK (B), and ERK 1,2 (C) with corresponding p38, JNK, and ERK 1,2. Lower panels: protein levels of phosphorylated p38, JNK, and ERK 1,2 were quantified using scanning densitometry and all the values were normalized based on the corresponding p38, JNK, and ERK 1,2. Results are expressed as % of the control group (CTL, taken as 100%). Values are the means  $\pm$  SE of at least 3 independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus control (CTL) group; ¶ $p < 0.01$ , ¶¶ $p < 0.001$  versus Ang II group



of total p38, JNK, and ERK 1,2 in control or treatment groups.

### C-ANP<sub>4–23</sub> attenuates Ang II-induced enhanced proliferation of aortic VSMC

Oxidative stress-induced activation of ERKs, JNKs, or p38 MAPK signaling pathways are implicated in Ang II-induced enhanced proliferation of VSMC [49–51]. Since C-ANP<sub>4–23</sub> attenuated the enhanced activation of ERKs, JNKs, or p38 MAPK signaling pathways, we investigated if C-ANP<sub>4–23</sub> could also attenuate Ang II-induced enhanced proliferation of aortic VSMC. Results shown in Fig. 6 indicate that Ang II (10<sup>-6</sup> M) treatment enhanced the DNA synthesis and metabolic activity (markers of cell proliferation) in VSMC by about 100 and 80%, respectively, compared to the control and C-ANP<sub>4–23</sub> dose-dependently attenuated the increased DNA synthesis (A) and metabolic activity (B) toward the control levels. On the other hand, C-ANP<sub>4–23</sub> (10<sup>-7</sup> M) treatment did not have any significant effect on the expression of G $\alpha$ -2 and G $\alpha$ -3 in control cells.

### C-ANP<sub>4–23</sub> attenuates Ang II-induced enhanced expression of G $\alpha$ proteins in aortic VSMC

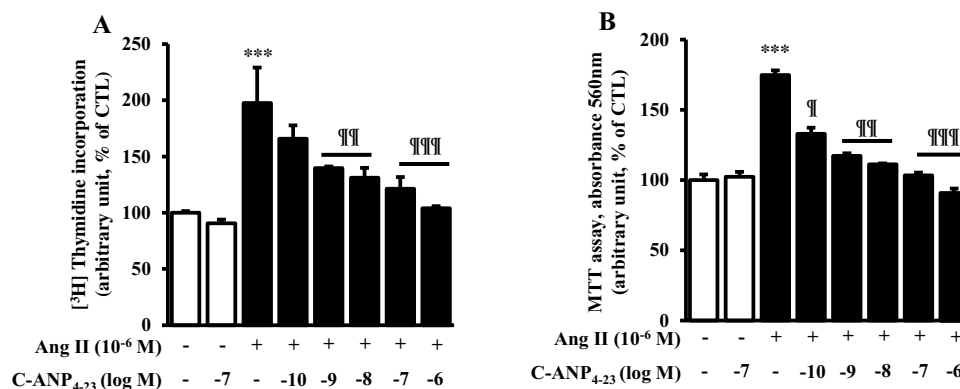
Enhanced oxidative stress has been shown to contribute to the overexpression of G $\alpha$  proteins in VSMC induced by Ang II and in VSMC from spontaneously hypertensive rats (SHR) [23, 28, 32]. In addition, the role of overexpression of G $\alpha$  proteins in hyperproliferation of VSMC from SHR has also been shown [32]. To investigate if attenuation of Ang II-induced hyperproliferation by C-ANP<sub>4–23</sub> is mediated through the inhibition of enhanced expression of G $\alpha$

proteins, we examined the effect of C-ANP<sub>4–23</sub> treatment on Ang II-induced enhanced expression G $\alpha$ -2 and G $\alpha$ -3 proteins in aortic VSMC and the results are shown in Fig. 7. As reported earlier [23], Ang II augmented the expression of G $\alpha$ -2 (A) and G $\alpha$ -3 (B) in aortic VSMC by about 60% which was inhibited by C-ANP<sub>4–23</sub> in a concentration-dependent manner and at 10<sup>-6</sup> M, the expression of both G $\alpha$ -2 and G $\alpha$ -3 was attenuated to control level. On the other hand, C-ANP<sub>4–23</sub> (10<sup>-7</sup> M) treatment did not have any significant effect on the expression of G $\alpha$ -2 (A) and G $\alpha$ -3 (B) in control cells.

In addition, we also investigated the implication of G $\alpha$  proteins in Ang II-induced enhanced proliferation of aortic VSMC by using gene knockdown experiments. Figure 7C shows that Ang II-induced enhanced metabolic activity (approximately 100%) was completely attenuated by knocking down of G $\alpha$ -2 and G $\alpha$ -3 proteins using respective siRNAs. In addition, siRNA of G $\alpha$ -2 and G $\alpha$ -3 also attenuated the basal metabolic activity by about 40 and 50%, respectively, in control cells.

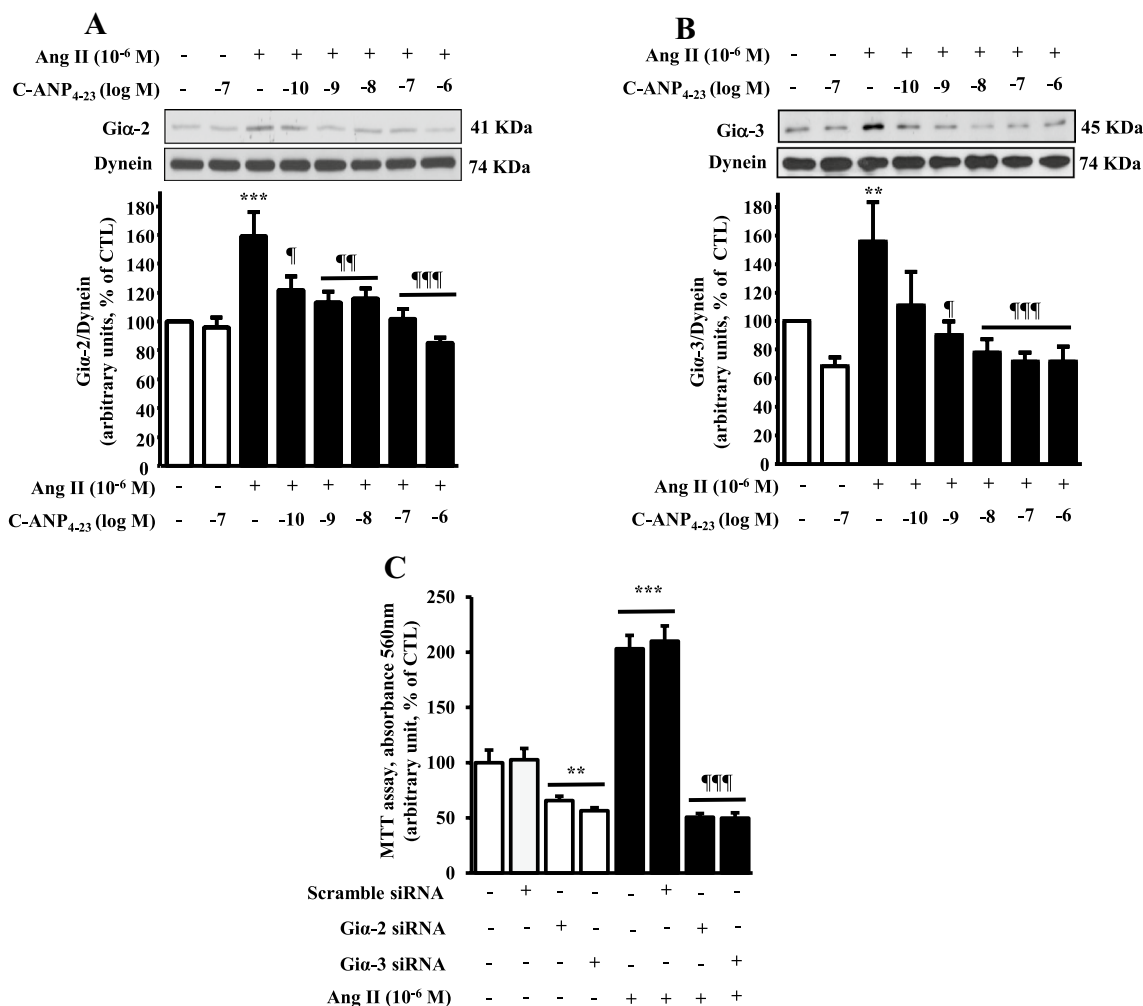
## Discussion

We earlier reported that C-ANP<sub>4–23</sub> treatment of aortic VSMC from SHR attenuated the enhanced oxidative stress by attenuating the enhanced production of superoxide anion (O<sub>2</sub><sup>-</sup>), enhanced levels of NADPH oxidase activity, and enhanced expression of NADPH oxidase subunits [23]. In addition, we also showed that the enhanced levels of endogenous Ang II contributed to the enhanced oxidative stress in VSMC from SHR [32]. However, in the present study, we report for the first time that C-ANP<sub>4–23</sub> attenuates oxidative



**Fig. 6** Effect of C-ANP<sub>4–23</sub> on Ang II-induced hyperproliferation of aortic VSMC. Serum-starved, quiescent aortic VSMC were preincubated in the absence or presence of different concentrations (10<sup>-6</sup>–10<sup>-10</sup> or 10<sup>-7</sup> M) of C-ANP<sub>4–23</sub> for 60 min prior to stimulation with or without Ang II (10<sup>-6</sup> M). After 24 h, [<sup>3</sup>H]-Thymidine (A) and MTT incorporation (B) were determined as described in the “Materi-

als and Methods” section. Results are expressed as % of the control group (CTL, taken as 100%). Values are the mean  $\pm$  SE of three separate independent experiments performed in triplicate. \*\*\**p* < 0.001 versus control (CTL) group; †*p* < 0.05, ††*p* < 0.01, †††*p* < 0.001 versus Ang II group



**Fig. 7** Effect of C-ANP<sub>4-23</sub> on Ang II-induced enhanced expression of Giα-2 and Giα-3 proteins in aortic VSMC. Serum-starved, quiescent aortic VSMC were pre-incubated in the absence or presence of different concentrations (10<sup>-6</sup>–10<sup>-10</sup> M or 10<sup>-7</sup> M) of C-ANP<sub>4-23</sub> for 60 min prior to stimulation with or without Ang II (10<sup>-6</sup> M). After 24 h later, cell lysates were prepared as described in the “Materials and Methods” section. 30 μg of proteins was resolved by SDS-PAGE and subjected to immunoblotting analysis using antibodies against Giα-2 and Giα-3. Upper panel (A and B) representative immunoblotting of Giα-2 or Giα-3 protein with corresponding dynein. Lower

panel (A and B) protein levels of Giα-2 and Giα-3 were quantified using scanning densitometry and all the values are normalized based on the corresponding dynein values. (C) Knockdown effects of Giα-2 or Giα-3 gene on Ang II-induced hyperproliferation of aortic VSMC. Aortic VSMC hyperproliferation (cell metabolic activity) was determined by MTT assay as described in “Materials and Methods” section. Results are expressed as % of the control group (CTL, taken as 100%). Values are the mean ± SE of four separate independent experiments. \*\**p* < 0.01, \*\*\**p* < 0.001 versus control (CTL) group; †*p* < 0.05, ††*p* < 0.01, †††*p* < 0.001 versus Ang II group

stress, nitrosative stress in aortic VSMC induced by Ang II which through the inhibition of overexpression of Giα proteins and MAPKs including p38 and JNK and ERK 1,2 pathways contributes to the anti-proliferative effect of NPR-C.

Our results showing that Ang II treatment augments the production of O<sub>2</sub><sup>-</sup>, NADPH oxidase activity, and expression of Nox-4, p22<sup>phox</sup>, and p47<sup>phox</sup> in aortic VSMC, are consistent with the studies reported earlier [10, 52–54]. Furthermore, the fact that C-ANP<sub>4-23</sub>, a specific agonist of NPR-C, inhibited Ang II-induced enhanced production of O<sub>2</sub><sup>-</sup>, enhanced NADPH oxidase activity as well as the enhanced expression of Nox4, p47<sup>phox</sup>, and p47<sup>phox</sup>

subunits of NADPH oxidase in aortic VSMC suggests that C-ANP<sub>4-23</sub> acts as an antioxidant. This notion is further supported by our study showing that treatment of aortic VSMC with C-ANP<sub>4-23</sub> prevented both Ang II-induced enhanced lipid (TBARS levels) and protein (carbonyl contents) peroxidation, which are considered as important markers of oxidative cell injury [35, 38]. Several studies have shown the interaction of natriuretic peptides and NO in the regulation of BP [55–58]. In addition, NPR-C activation by C-ANP<sub>4-23</sub> was also reported to activate eNOS in various tissues including smooth muscle [59]. Furthermore, Ang II that increases blood pressure has been shown to decrease the levels of NO

and NOS activity in VSMC [11]. We also show that Ang II decreased the levels of NO in VSMC which may be attributed to Ang II-induced enhanced production of  $O_2^-$  anion because increased levels of  $O_2^-$  have been shown to decrease the production/bioavailability of NO associated with hypertension [30, 60, 61]. This notion was further supported by our earlier study demonstrating that VSMC from SHR exhibit enhanced levels of  $O_2^-$  and decreased levels of NO and eNOS [30]. Our results showing that C-ANP<sub>4–23</sub> treatment ameliorated Ang II-evoked decreased levels of both intra- and extracellular NO suggests that C-ANP<sub>4–23</sub>-induced attenuation of  $O_2^-$  may be responsible for the augmentation of NO levels. NO has been shown to react with  $O_2^-$  and produce ONOO<sup>-</sup>, which further reduces the bioavailability of NO [40]. In accordance with this report, we also show that the levels of ONOO<sup>-</sup> were increased in VSMC after Ang II treatment and C-ANP<sub>4–23</sub> treatment decreased Ang II-induced enhanced levels of ONOO<sup>-</sup> in VSMC. In this regard, *in vivo* treatment of C-ANP<sub>4–23</sub> has also been shown to decrease the enhanced levels of ONOO<sup>-</sup> in aorta and aortic VSMC from SHR [30]. Taken together, it may be suggested that C-ANP<sub>4–23</sub> decreases nitroxidative stress induced by Ang II in VSMC by decreasing the levels of  $O_2^-$  and ONOO<sup>-</sup>.

Ang II-induced oxidative stress has been shown to enhance the proliferation of A10 VSMC [62]. In addition, enhanced levels of endogenous Ang II through ROS and ROS-mediated signaling have also been reported to contribute to the hyperproliferation of VSMC from SHR [10, 32]. Our results showing that C-ANP<sub>4–23</sub> inhibits Ang II-induced enhanced proliferation of aortic VSMC are consistent with our previous study conducted in A10 cell line [31] and suggest that the anti-proliferative effect of C-ANP<sub>4–23</sub> may be attributed to its ability to inhibit the oxidative stress induced by Ang II. It should be noted that the anti-proliferative effect of C-ANP<sub>4–23</sub> is not attributed to apoptosis, because cell viability checked by trypan blue exclusion method indicated that >90–95% cells were viable and that C-ANP<sub>4–23</sub> did not inhibit DNA synthesis in control VSMC.

C-ANP<sub>4–23</sub> has been reported to suppress Ang II-induced activation of ERK 1,2 and AKT/protein kinase B (PKB) as well as subsequent hyperproliferation of A10 VSMC line [31]. In addition, Ang II-induced  $O_2^-$  and its metabolites have been reported to effectively stimulate all 3 MAPK including ERK 1,2, JNK, p38 MAPK, and VSMC proliferation [63, 64]. However, we show for the first time that C-ANP<sub>4–23</sub> inhibits Ang II-induced activation of p38 and JNK signaling pathway in aortic VSMC in addition to ERK 1,2 pathway. Taken together, it may be suggested that C-ANP<sub>4–23</sub>-induced attenuation of Ang II-evoked hyperproliferation may be mediated through the inhibition of ROS and ROS-mediated ERK1,2, JNK, and p38 MAPK signaling pathways.

The role of Gi $\alpha$  proteins in Ang II-induced hyperproliferation of VSMC is well established [62]. In addition, enhanced levels of endogenous Ang II through the overexpression of Gi $\alpha$  proteins [32] have been shown to contribute to the hyperproliferation of VSMC from SHR [10]. The fact that C-ANP<sub>4–23</sub> decreases Ang II-induced enhanced expression of Gi $\alpha$  proteins and hyperproliferation of VSMC further suggests that the attenuation of enhanced expression of Gi $\alpha$  proteins may be responsible for the decreased proliferation induced by C-ANP<sub>4–23</sub>. The mechanism by which C-ANP<sub>4–23</sub> attenuates the enhanced expression of Gi $\alpha$  proteins and hyperproliferation appears to involve C-ANP<sub>4–23</sub>-induced increased levels of NO. This notion is supported by our recent study showing that elevation of intracellular levels of NO by NO donors attenuates the overexpression of Gi $\alpha$  protein and hyperproliferation of VSMC from SHR [38].

In conclusion, we have provided the evidence that NPR-C activation by C-ANP<sub>4–23</sub> decreases Ang II-induced enhanced oxidative stress and hyperproliferation of VSMC involving ERK 1,2/JNK/p38 MAPK signaling pathways and enhanced expression of Gi $\alpha$  proteins. From these studies, it can be suggested that C-ANP<sub>4–23</sub>, an agonist of NPR-C, has the potential to be used as an antioxidant and anti-proliferative agent.

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## Compliance with ethical standards

**Conflict of interest** The authors of this manuscript do not have any conflict of interest.

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