Role of atrial natriuretic peptide in the suppression of lysophosphatidic acid-induced rat aortic smooth muscle (RASM) cell growth

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Abstract

Lysophosphatidic acid (LPA) is a lipid mediator with multiple biological functions. In the present study we investigated the possible role of atrial natriuretic peptide (ANP), a hormone affecting cardiovascular homeostasis and inducing antimitogenic effects in different cell types, on LPA-induced cell growth and reactive oxygen species (ROS) production in rat aortic smooth muscle (RASM) cells. Both LPA effects on cell growth and levels of ROS were totally abrogated by physiological concentrations of ANP, without modifying the overexpression of LPA-receptors. These effects were also affected by cell pretreatment with wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3K). Moreover, the LPA-induced activation of Akt, a downstream target of PI3K, was completely inhibited by physiological concentrations of ANP, which were also able to inhibit p42/p44 phosphorylation. Taken together, our data suggest that PI3K may represent an important step in the LPA signal transduction pathway responsible for ROS generation and DNA synthesis in RASM cells. At same time, the enzyme could also represent an essential target for the antiproliferative effects of ANP. (Mol Cell Biochem 272: 19–28, 2005)

Key words: atrial natriuretic peptide, lysophosphatidic acid, phosphatidylinositol 3 kinase, reactive oxygen species

Introduction

The atrial natriuretic peptide (ANP), a hormone known to affect cardiovascular homeostasis [1], has been demonstrated to elicit antiproliferative effects in several cell types, such as cardiac fibroblasts [2], hepatoblastoma cells (HepG2) [3] and vascular smooth muscle (VSM) cells [4] with mechanisms not established yet.

ANP promotes its biological effects interacting with specific receptors located on plasma membrane of target cells: NPR-A receptors, able to activate the guanylate cyclase/guanosine 3',5'-cyclic monophosphate (cGMP) system

[1], and NPR-C receptors, negatively coupled to intracellular adenosine 3'-5'-cyclic monophosphate (cAMP) modulation or positively coupled to membrane lipid turnover through the activation of specific phospholipases [1, 5]. ANP physiological concentrations (10^{-9} – 10^{-11} M) may induce antimitogenic effects in several cell types, including rat aortic smooth muscle (RASM) cells [6], through NPR-C receptors and involve different transduction pathways [7, 8].

Several molecules derived from phospholipids have been recently found to modulate cell growth [9] and among them, the lysophosphatidic acid (LPA), a bioactive lipid mainly released from activated platelets [10]. LPA displays its

biological activities through three receptors, LPA₁, LPA₂ and LPA₃ [11], and plays an important role in the modulation of cell growth in several cell types [12–15].

LPA promotes VSM cell proliferation by activating several signaling pathways, including the enhancement of phospholipase and mitogen-activated protein kinase (MAPK) activities [16], and stimulating the phosphoinositide 3-kinases (PI3Ks), a class of lipid kinases that mediate various biological activities linked to cell proliferation and survival [17]. PI3K can also stimulate ROS production [18], which, in turn, take part to transduction pathways linked to LPA [19]. Finally, LPA can play a key role in the phenotypic modulation of VSM cells from the differentiated state to the dedifferentiated one, an important step for the development of atherosclerosis [20]. This pathologic process can also be contributed by ROS production [21].

In light of the increasing interest in possible new interventions capable of ameliorating vascular responses to mitogens, we addressed the question whether: (i) ANP can interfere with LPA-induced DNA synthesis and ROS production; (ii) PI3K can be a crucial hormone target to counteract LPA-induced RASM cells growth.

Methods

Cell culture

RASM cells were obtained from the explant of thoracic aorta of 15-week old male Wistar rats by the method of Ross [22]. Cells were cultured in 25-mm plastic tissue culture flasks and grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum (FCS), 100 µg/ml streptomycin and 100 U/ml penicillin in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. RASM cells, harvested once a week with 0.25% trypsin-0.02% EDTA and refed every other day, were used as confluent monolayers after 6–8 days at passage level 4–9.

Cell proliferation studies

RASM cells were grown in 35 mm dishes and serum starved for 24 h before each experiment to rule out possible interferences with cell growth due to serum components and to promote their synchronisation. Cells were washed twice with phosphate buffer saline (PBS) and challenged with different concentrations of LPA (1–20 µM). In experiments with 10^{−6}–10^{−11} M ANP, 10^{−10} M cANP, 50 µM 8-Br-cGMP, 50 nM wortmannin, cells were pretreated for 1 h, before LPA addition. Cells were scraped off and counted after Trypan blue staining using a Neubauer modified chamber. [³H]-thymidine incorporation was used to measure the mitogenic response of RASM cells. After the specific treatments, cells were pulsed

with 1 µCi/ml [³H]-thymidine and incubated for additional 2 h. RASM cells were scraped off, harvested by centrifugation and treated with 5% TCA at 4 °C for 30 min. The TCA-insoluble fraction was resuspended in 0.1% SDS in 200 mM NaOH, and samples, after the addition of 7 ml Optifluor, were counted for radioactivity by a liquid scintillation counter (Tricarb 2180 TR, Packard Instruments, Downers Grove, IL). Cell viability was assessed by Trypan blue staining using a Neubauer modified chamber.

PCR analysis of LPA receptor expression in RASM cells

Total RNA was extracted by Proteinase K-SDS method [23] from 2 × 10⁶ RASM cells treated or not with 5 µM LPA for 6 h or 10^{−10} M ANP for 1 h. In the experiments with LPA + ANP, cells were pretreated with ANP for 1 h, then washed twice in PBS and resuspended in the same buffer before LPA addition for 6 h. Total RNA quantity and quality were assessed by measuring UV absorbance at 260 nm and by electrophoresis on agarose/formaldehyde gel. One microgram of total RNA was DNase treated (Amersham Pharmacia Biotech) and reverse-transcribed into cDNA, as previously described [24]. For PCR analysis, an equal amount (10 pg) of an internal control (L22 RNA) was added to each sample before reverse transcription and detected with specific oligonucleotides, as reported by Zalfa *et al.* [25]. An aliquot of RT products (1 µl) was PCR-amplified using 200 µM of dATP, dGTP, dTTP and 10 µM of dCTP, 0.5 U of DyNAzyme II DNA polymerase (Finnzymes), 0.2 µCi [^α-³²P]dCTP (Amersham Pharmacia Biotech; 3000 Ci/mmol) and 20 pmol of each primer designed to amplify the mRNA of rat LPA receptors: LPA₁ (NM053936), sense primer 5'-GGACACCATGATGAGCCTTC-3', antisense primer 5'-GGGCTACCAAGCAAGTTCATC-3'; LPA₂ (NM020028), sense primer 5'-ATGGGCCAGTGCTACTACAAC-3', antisense primer 5'-GCATTGACCAAGTGAGTTGGC-3'; and LPA₃ (NM023969), sense primer 5'-CCAACGT CCTCTCTCCGCAC-3', antisense primer 5'-GCAGATCAT CTTCCGCATGG-3'. All transcripts were amplified as follows: 94 °C for 4 min, unsaturated cycle numbers (25 cycles for LPA₁, 30 cycles for LPA₃, 15 cycles for GAPDH and 22 cycles for L22 cDNAs) at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The expression levels of the three receptor mRNAs in RASM cells were quantified by normalising their respective mRNA levels to L22 mRNA and the housekeeping rat GAPDH mRNA (ID: NM017008; sense primer 5'-GGACATTGTTGCCATCAACG-3', antisense primer 5'-CCTTCCACGATGCCAAAGTT-3') levels. The amplified fragments were separated on 5% polyacrylamide gel and quantified by a Phosphor-Imager (Molecular Imager FX, Bio-Rad, Hercules, CA).

Detection of ROS in RASM cells

Cells (1×10^6) were synchronised as reported earlier, resuspended in PBS buffer and loaded with the fluorescent indicator Dichlorofluorescein Diacetate (DCF-DA) at final concentrations of $10 \mu\text{M}$ for 60 min at 37°C in the dark [26]. After the incubation with the fluorescent dye, RASM cells were washed twice with PBS buffer, centrifuged for 5 min at 1500 rpm at room temperature, resuspended in PBS and challenged with LPA. In the experiments with different effectors, cells were pretreated for 1 h at 37°C in the dark. A sample of 1×10^5 cells was used to monitor ROS production. Fluorescence was measured under continuous magnetic stirring and controlled temperature (37°C) in a Perkin–Elmer luminescence spectrometer (model LS-5) equipped with a chart recorder (model R 100A), with excitation wavelength at 485 nm and emission wavelength at 530 nm using 5 and 10 nm slits respectively, for each light path. Results are expressed as fluorescence intensity, reported as fluorescence units (F.U.), of cells loaded with only DCF-DA (control) or also treated with $300 \mu\text{M}$ H_2O_2 for 1 h as positive control. When requested, RASM cells were pretreated with ANP and 50 nM wortmannin for 1 h before LPA addition.

Microscopy for ROS generation

ROS generation was carried out on glass coverlips. Cells were labelled with $5 \mu\text{M}$ (final concentration) of peroxide-sensitive fluorescent dye 2',7' dichlorodihydrofluorescein diacetate (DCFH-DA) and then incubated for 6 h in the presence or absence of LPA and ANP. Intracellular fluorescence was monitored using an inverted fluorescence microscope (Leica) with a $20\times$ objective. Signal-based averaging was used to quantitate the fluorescence signal from five to six fields of cells.

PI3 kinase activity assay

Cells were grown as reported earlier and then challenged with $5 \mu\text{M}$ LPA in the presence or absence of ANP or wortmannin. The reaction was terminated by washing with 4°C PBS and lysis of cells. Lysis buffer consisted of (mM): 50 TRIS-HCl (pH 7.4), 150 NaCl, 1 Na_2VO_3 , 2 EDTA, 1 MgCl_2 , 1 CaCl_2 with 30 nM leupeptin and 1% NP-40 (v/v). Lysate was centrifuged at $12000 \times g$ for 20 min at 4°C . Lysate aliquots containing equal amounts of protein were incubated with agarose-conjugated antibody to phosphotyrosine (PY 20) for 2 h at 4°C . Immunoprecipitate was washed three times with PBS and PI3K activity was finally measured as described [27].

Immunoblots

RASM cells grown in six-well plates (10^5 cells/well) were synchronised as reported earlier, resuspended in PBS buffer and challenged with LPA. When requested, cells were pretreated with 10^{-10} M ANP and/or 50 nM wortmannin, before LPA addition for 30 min. The reaction was stopped and cells were washed twice with PBS, centrifuged at 1500 rpm for 5 min and the pellet was lysed with sample buffer containing: 10% sodium dodecyl sulfate (SDS), 10% glycerol, 100 mM dithiothreitol, 5% β -mercaptoethanol, 1% bromophenol blue, 2 mM EDTA, 1 mM sodium vanadate, 50 mM Tris-HCl (pH 6.8), plus a cocktail of protease inhibitors (1 mM phenylmethylsulfonylfluoride, 1 $\mu\text{g/ml}$ pepstatin, 10 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ aprotinin) in ice for 30 min. The lysate was centrifuged at 13000 rpm for 5 min; the supernatant was boiled for 5 min and then stored at -70°C . The protein content of cell lysates was evaluated by the method of Lowry [28]. Equal amounts of whole cell lysate (30–50 μg) were subjected to 8% acrylamide SDS-PAGE, transferred onto nitrocellulose membranes and probed with primary anti-phospho- and total p42/44 MAP kinases antibodies (both 1:1000) or anti-phospho- and total Akt antibodies overnight at 4°C (Santa Cruz, Biotechnology Inc., CA). A horseradish peroxidase-conjugated secondary antibody (1:2500) was used for 1 h at room temperature. Membranes were then analysed by ECL assay (Amersham Pharmacia Biotech, Italia, MI); each experiment was repeated at least three times.

Materials

Dulbecco's Modified Eagle Medium (DMEM), trypsin 2.5%, glutamine, penicillin (10000 UI/ml) and streptomycin (10000 $\mu\text{g/ml}$) were from Eurobio Laboratoires (Le Ulis Cedex, France). Foetal Calf Serum (FCS) was from GIBCO (Grand Island, NY, USA). [^3H]-myristic acid (specific activity: 53 Ci/mmol) and [^3H]-thymidine (20Ci/mmol), DNase were from Amersham International (Bucks, UK). LPA (C18:1, 1-oleoyl-sn-glycerol-3-phosphate), ANP, cANP (fragment 4–23), BNP, CNP 8-Br-cGMP, wortmannin, ethylene glycol-bis(2-aminoethylether)- N,N,N',N' -tetraacetic acid (EGTA), sodium dodecyl sulfate (SDS), dithiothreitol, mercaptoethanol, glycerol, sodium vanadate, phenylmethylsulfonylfluoride, pepstatin, aprotinin, leupeptin, tris-HCl were from Sigma Chemicals (St. Louis, MO, USA). 2',7'-dichlorofluorescein diacetate (DCF-DA) and peroxide-sensitive fluorescent dye 2',7' dichlorodihydrofluorescein diacetate (DCFH-DA) were from Molecular Probes, Inc. (Eugene, OR). Anti-phosphorylated and total p42/p44 MAPK, anti-phosphorylated and total Akt and PY20 antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibody and chemiluminescence detection of

immunoreactive bands ECL reagents were obtained from Amersham Pharmacia Biotech. (Milan, Italy). Primers were purchased from Invitrogen (Milan, Italy).

Statistical methods

All data are presented as means $\pm$ S.D. Data distribution normality was preliminarily verified by the Kolmogorov–Smirnov test. Statistical analysis was performed using the Student's *t*-test and, in each group of experiments, the individual treatments were compared singularly vs. the same control. A probability of $*p < 0.05$ was accepted as significant difference.

Results

Role of ANP in LPA-induced cell growth

To assess the ability of LPA to stimulate RASM cell growth, cells were challenged with LPA in a wide concentration range (1–20 μ M) and [3 H] thymidine incorporation and cell number were evaluated at 3, 6, 9 h (Fig. 1a and b). LPA effects

Fig. 1. LPA effects on RASM cell growth. RASM cells, grown in DMEM plus 10% FCS and serum starved for 24 h to promote their synchronisation and to rule out possible interferences with cell growth due to serum components, were challenged with a wide concentration range of LPA (1–20 μ M) for 3, 6 and 9 h. [3 H] thymidine incorporation (a) and cell number (b) were assessed as reported in the Methods section. Data are reported as mean $\pm$ S.D. of four different experiments. $*p < 0.05$, as reported from Student's *t* test with respect to untreated cells (control).

were statistically significant at the lowest concentration used (1 μ M) and maximal at 5 μ M at 6 h vs. untreated cells (about twice the value obtained in the absence of LPA). For this reason, all the following experiments were carried out in the presence of 5 μ M LPA for 6 h. At higher LPA concentrations (10–20 μ M), [3 H] thymidine incorporation and cell number declined also if cell viability was $>95\%$ as monitored by Trypan blue exclusion (data not shown).

To test the effect of ANP on both LPA-induced cell growth and [3 H] thymidine incorporation, RASM cells were pretreated with ANP in a wide concentration range (10^{-6} – 10^{-11} M) (Fig. 2a and b). Our results showed a dose-dependent attenuation of LPA-induced cell growth and [3 H] thymidine incorporation at physiological concentrations (10^{-9} – 10^{-11} M) of ANP with a maximum at 10^{-10} M; conversely, pharmacological concentrations of the hormone (10^{-6} – 10^{-8} M) were ineffective. The specificity of ANP effect on LPA-induced DNA synthesis was evaluated using other natriuretic peptide such as brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). Figure 3 shows that either natriuretic peptides were ineffective to counteract LPA effects.

Fig. 2. ANP effects on LPA-induced cell growth. RASM cells, grown in DMEM plus 10% FCS and serum starved for 24 h to promote their synchronisation and to rule out possible interferences with cell growth due to serum components, were pretreated with ANP (10^{-6} – 10^{-11} M) for 1 h and then challenged with 5 μ M LPA for 6 h. [3 H] thymidine incorporation (a) and cell number (b) were assessed as reported in the Methods section. Data are reported as mean $\pm$ S.D. of four different experiments. $*p < 0.05$, as reported from Student's *t* test with respect to untreated cells (control).

Fig. 3. Effects of BNP and CNP on LPA-induced [3 H]-thymidine incorporation in RASM cells. RASM cells, grown in DMEM plus 10% FCS and serum starved for 24 h to promote their synchronisation and to rule out possible interferences with cell growth due to serum components, were pretreated with BNP (10^{-10} M) or CNP (10^{-10} M) for 1 h and then challenged with 5 μ M LPA for 6 h. [3 H] thymidine incorporation was assessed as reported in the Methods section. Data are reported as mean $\pm$ S.D. of four different experiments. * $p < 0.05$, as reported from Student's t test with respect to untreated cells (control).

Fig. 4. 8-Br-cGMP and cANF effects on LPA-induced RASM cell growth. RASM cells, grown as reported in Fig. 1, were challenged with 5 μ M LPA for 6 h. 50 μ M 8-Br-cGMP or 10^{-10} M cANF or 10^{-10} M ANP were added for 1 h before LPA addition. [3 H] thymidine incorporation was assessed as reported in the Methods section. Data are reported as mean $\pm$ S.D. of four different experiments. * $p < 0.05$, as reported from Student's t test with respect to untreated cells (control).

To determine which natriuretic peptide (NP)-receptor mediates ANP inhibitory effects on LPA-induced DNA synthesis, experiments were performed with 50 μ M 8-Br-cGMP, a not hydrolyzable analogue of cGMP involved in pharmacological effects of the hormone and 10^{-10} M cANF (4–23), a ring-deleted analogue of ANP, specific activator of the C receptor. Figure 4 shows that: 8-Br-cGMP alone as well as in the presence of LPA was ineffective on [3 H] thymidine incorporation, while only the pretreatment with cANF mimicked ANP effects.

ANP effects on LPA receptor expression

Since LPA can modulate its biological effects through the involvement of three receptors, LPA₁, LPA₂ and LPA₃ [11], we carried out experiments to evaluate the role of ANP on LPA-induced receptor expression. Our results, obtained by RT-PCR analysis, showed a significant increase in mRNA levels for LPA₁ and LPA₃ receptors after LPA treatment, while no significant differences were observed in RASM cells pretreated with ANP, suggesting that ANP did not affect the LPA-induced receptor upregulation (Fig. 5). LPA₂ was expressed at an undetectable level.

Role of ANP on LPA-induced ROS production

The time-course of LPA effects on ROS production is shown in Fig. 6a (inset). LPA induced a significant increase in ROS production already at 30 min with a maximum at 6 h. To verify whether LPA effects on ROS production could be inhibited by ANP, we performed experiments in the presence or absence of 10^{-8} M (pharmacological concentration) and 10^{-10} M (physiological concentration) ANP. Figure 6a shows that 10^{-8} M as well as 10^{-10} M ANP alone were ineffective on LPA-induced ROS production, while only 10^{-10} M ANP totally abrogated LPA effects. The effects of 10^{-10} M ANP were mimicked by cANF. ROS production was also evaluated by microscopy analysis using peroxide sensitive fluorescent dye (Fig. 6b). The decrease in fluorescence in cells treated with ANP vs. cells challenged with LPA confirmed the hormone effect on ROS production.

PI3K activation is required for both ROS production and DNA synthesis stimulated by LPA: Role of ANP

To ascertain whether the ability of ANP to counteract LPA-induced ROS production and DNA synthesis in RASM cells could involve the PI3K activation, we examined LPA effects in the presence or absence of wortmannin, an inhibitor of PI3K, in cells treated or not with ANP or cANF. Our results on LPA-induced ROS production (Fig. 7a and b), as well as [3 H] thymidine incorporation (Fig. 7c and d), in the presence of wortmannin, show that: (i) LPA effects were totally abrogated; (ii) no additive effects were observed in cells treated with ANP or cANF. In addition, to confirm the role of PI3K in this mechanism, enzyme activity was analysed. LPA-induced PI3K activity was inhibited by ANP, while no additive effects were found in the presence of wortmannin plus ANP (Fig. 8). These evidences suggested a crucial role of PI3K in the mechanism by which ANP counteract LPA effects. Since Akt, an important downstream effector of PI3K, is involved in the LPA effects in several cell types

Fig. 5. ANP and LPA effects on LPA₁, LPA₂ and LPA₃ expression in RASM cells. (a) Total RNA was isolated from 2×10^6 RASM cells treated or not (CTRL) with LPA for 6 h or 10^{-10} M ANP for 1 h or pretreated with ANP for 1 h and challenged with LPA (ANP + LPA) for 6 h. RT-PCR analysis was performed as reported in the Methods Section to amplify LPA receptor mRNAs (LPA₁, LPA₂ and LPA₃) in unsaturated condition. Sizes of PCR products were as follows: LPA₁, 512 bp; LPA₂, 867 bp; and LPA₃, 277 bp. (b) The signals of LPA₁ and LPA₃ cDNAs were quantified and corrected vs. L22 and GAPDH controls. Values represent the mean $\pm$ S.E.M. of four independent experiments. * $p < 0.05$, as from data analysis with respect to untreated cells (CTRL).

Fig. 6. ANP effect on LPA-induced ROS production. (a) RASM cells were labelled with DCF-DA, as reported in the Methods Section, and ROS production was evaluated after cell exposure to $5 \mu\text{M}$ LPA for 6 h. Cells were pretreated with ANP or cANF for 1 h before LPA addition. *Inset*: Time course of LPA-induced ROS production in RASM cells. ROS production was evaluated after cell exposure to $5 \mu\text{M}$ LPA for different experimental times. Results are expressed as fluorescence intensity, reported as fluorescence units (F.U.), of cells loaded with only DCF-DA (control) or also treated with $300 \mu\text{M}$ H₂O₂ for 1 h as positive control. Data are reported as mean $\pm$ S.D. of four different experiments. * $p < 0.05$, as reported from Student's *t* test with respect to control. (b) RASM cells were labelled with $5 \mu\text{M}$ (final concentration) of peroxide-sensitive fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and then challenged with $5 \mu\text{M}$ LPA in the presence or absence of 10^{-10} M ANP. Images were acquired as reported in Methods section. Original magnification: $20\times$.

Fig. 7. ANP effects on LPA-induced ROS production and $[^3\text{H}]$ thymidine incorporation in the presence of wortmannin in RASM cells. RASM cells were challenged with $5 \mu\text{M}$ LPA for 6 h. In the experiments with ANP or cANF or 50 nM wortmannin, cells were pretreated for 1 h before LPA addition. ROS production (a and b) and $[^3\text{H}]$ thymidine incorporation (c and d) were assessed as reported in the Methods section. Data are reported as mean $\pm$ S.D. of four different experiments. * $p < 0.05$, as reported from Student's t test with respect to untreated cells (control).

Fig. 8. ANP effects on LPA-induced specific enzyme activity of PI3K. RASM cells, grown in DMEM plus 10% FCS and serum starved for 24 h to promote their synchronisation and to rule out possible interferences with cell growth due to serum components, were pretreated with 10^{-10} M ANP or 50 nM wortmannin for 1 h and then challenged with $5 \mu\text{M}$ LPA for 6 h. PI3K specific activity was measured by kinase activity assay from cell lysates as described in the Methods Section. * $p < 0.05$, as reported from Student's t test with respect to untreated cells (control).

[29], ANP ability to counteract LPA-induced Akt phosphorylation was investigated. Immunoblots were performed after four different treatments. Our results showed that Akt phosphorylation induced by LPA was totally inhibited by cell pretreatment with wortmannin or ANP, while ANP plus wortmannin had no additive effects (Fig. 9a). The ANP effects on Akt phosphorylation were mimicked by cANF (Fig. 9b).

ANP effects on LPA-induced p42/44 MAPK phosphorylation

In order to investigate the ANP-triggered signaling via the MAPK pathways in LPA-stimulated RASM cells, LPA effects on p42/44 MAPK phosphorylation were evaluated in the presence or absence of wortmannin after pretreatment with ANP by immunoblot. Our results showed that ANP inhibited LPA-induced p42/44MAPK phosphorylation, while no differences were observed in the presence of ANP plus wortmannin (Fig. 10). The same results were obtained with cANF (data not shown).

Fig. 9. ANP effects on LPA-induced Akt phosphorylation in RASM cells. Cytosolic extracts prepared from RASM cells were separated by SDS-PAGE. Immunoblotting was performed, as described in the Methods section. Each lane represents lysates from RASM cells treated with $5 \mu\text{M}$ LPA for 30 min in the presence or absence of 10^{-10} M ANP or 50 nM wortmannin or 10^{-10} M cANF. A representative experiment is shown, which was repeated three additional times with similar results. Upper lane: phosphorylated Akt; lower lane: total Akt.

Fig. 10. ANP effects on LPA-induced p42/44 MAPK phosphorylation in RASM cells. Cytosolic extracts from RASM cells were separated by SDS-PAGE. Immunoblottings were performed as described in the Methods Section. Each lane was loaded with RASM cell lysate after treatment with $5 \mu\text{M}$ LPA for 30 min in the presence or absence of 10^{-10} M ANP or 50 nM wortmannin. A representative experiment is shown, which was repeated three additional times with similar results. Upper lane: phosphorylated p42/44 MAPK; lower lane: total p42/44 MAPK.

Discussion

ANP primary function is directed to decrease blood pressure and regulate renal sodium and water excretion, but

the hormone is also endowed with antiproliferative capability after growth factor stimulation [30]. To further investigate this aspect, we investigated the potential role of ANP in preventing LPA-induced cell growth in RASM cells. In fact, excessive VSM cell proliferation is a major feature of the hypertension and atherosclerosis pathogenesis [31].

LPA, a bioactive lipid mainly released from activated platelets [10], can participate in the development of atherosclerotic plaques stimulating VSM cell proliferation [32] and ROS production [19, 33]. Surprisingly, upstream events involved in these processes are still incompletely understood. Our results showed that physiological concentrations of ANP can counteract LPA effects, in terms of ROS generation and cell growth, very likely, involving the NPR-C receptor, as suggested by experiments carried out in the presence of cANF. This effect appears specific for ANP as demonstrated by experiments with other natriuretic peptides, such as BNP and CNP. Interestingly low concentrations of LPA significantly increased RASM cell growth which was inhibited by higher LPA concentrations. This complex response was probably due to the presence of different types of LPA receptors with different affinities for the mitogen with

opposite mitogenic and antimitogenic effects [34]. Several lines of evidence [35–37] demonstrated that ANP can counteract ROS-induced cell damage by a mechanism that involves the activation of the NPR-A/cGMP system. Our data describe for the first time the ability of physiological concentrations of ANP to contain ROS levels by the involvement of the NPR-C receptor. This appears potentially important since cells which undergo phenotypic modulation, such as RASM cells found in *in vivo* atheromatous lesions, express a relative large number of NPR-C receptors highly sensitive to physiological concentrations of hormone, while isolated and cultured cells are sensitive to pharmacological concentrations of ANP owing to the reduced NPR-C receptor expression [38]. In this context, our results could be explained by the NPR-C receptor upregulation occurring after long time exposure to LPA. Indeed, the effects herein reported occurred at hormone concentrations as low as 10^{-10} M, i.e. in the range of physiological ANP plasma concentrations, while the decreased response to higher concentrations of ANP may be explained by NPR-A receptor down regulation, as reported in other systems [39].

The biological activity of LPA is mediated by, at least, three different G-protein-coupled receptors, namely LPA₁, LPA₂ and LPA₃. Among different LPA intracellular signaling pathways, PI3K mediates various biological activities including cell survival, cytoskeletal reorganisation, cytokine gene activation, membrane trafficking and oxidative burst [40], and represents an important upstream effector coupled to VSM cell growth through MAPK activation [17]. Our study shows that physiological concentrations of ANP do not interfere with the LPA-induced receptor upregulation that is responsible for the switching from differentiated to dedifferentiated cell state, an important step for the atherogenic effect of LPA [20]. Indeed, ANP interferes with the LPA signal pathway, including PI3K/ROS, which, in turn, activate the p42/p44 MAPK. Physiological concentrations of LPA can inhibit the activity of the NPR-B receptor specific for C-type natriuretic peptide (CNP), a member of the natriuretic hormone family, suggesting a possible cross-talk between LPA and CNP in cultured fibroblast [41]. Here, we provide evidence for a new link between natriuretic hormones and LPA suggesting that physiological concentrations of ANP can keep under constant repression the atherogenic effects of LPA by inhibiting the lipid effects on PI3K. Consistently, high ANP plasma concentrations, such as observed during cardiovascular diseases, can promote the atherogenic effect of LPA. Our results open new perspectives to clarify the ANP mechanism of action candidating PI3K as a crucial target for the antimitogenic effect of the hormone. These data support the view that ANP can play a key role counteracting the potentially dangerous effects of an excess of cell growth.

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