Lipoxin A₄ Mediates Aortic Contraction via RHOA/RHO Kinase, Endothelial Dysfunction and Reactive Oxygen Species

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Key Words

Lipoxin A₄ · Aorta · Contractile responses · Endothelial dysfunction

Abstract

Background: Lipoxin A₄ (LXA₄) is a biologically active product generated from arachidonic acid by lipoxygenase action. The production of lipoxins is enhanced by aspirin through acetylation of cyclooxygenase-2, via a mechanism known as 'aspirin-triggered lipoxin'. LXA₄ has both anti-inflammatory and proinflammatory actions, the latter being related with reocclusion and restenosis after coronary angioplasty in patients treated with aspirin. However, little is known of the actions of LXA₄ on the vasculature. We hypothesized that LXA₄ promotes contractile responses and contributes to endothelial dysfunction. **Methods:** We used aorta from Wistar rats to assess vascular function. Reactive oxygen species (ROS) production and contractile and regulatory proteins were investigated. **Results:** LXA₄ induced concentration-dependent contractions via formyl peptide receptor-2 activation and both RhoA/Rho kinase inhibitor and ROS scavenger decreased this contraction. Also, endothelium removal, and COX-2 and NAD(P)H oxidase inhibitors attenuate the LXA₄-

induced contraction. LXA₄ potentiated phenylephrine-induced contraction and inhibited acetylcholine-induced relaxation. In the presence of LXA₄, ROS production was increased and protein expression of RhoA, phospho-myosin light chain, COX-2 and p67phox was higher. **Conclusion:** LXA₄ has a functional role in the vasculature and may contribute to further vascular damage in conditions where its production is exacerbated, such as in angioplasty-associated complications treated with aspirin.

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Introduction

Lipoxin A₄ (LXA₄) is a biologically active product generated from arachidonic acid by lipoxygenase action. It was discovered in 1984 through interaction(s) between the 5- and 15-lipoxygenase pathways in human leukocytes [1]. The generation of lipoxins is a very rapid process and aspirin does not inhibit its formation. In fact, aspirin has been shown to trigger the production of LXA₄ through acetylation of cyclooxygenase 2 (COX-2) that metabolizes arachidonic acid to 15(R)-hydroxyeicosatetraenoic acid. This metabolite is then converted, via lipox-

xygenase, to LXA₄, also known as 'aspirin-triggered lipoxin'. This process is augmented during inflammation, atherosclerosis, and thrombosis [2, 3].

Lipoxin A₄ is a potent agonist of a specific G protein-coupled receptor (GPCR) termed formyl peptide receptor-2 (FPR-2) [4, 5]. Lipoxin A₄ has both anti-inflammatory and pro-inflammatory actions. LXA₄ may play an anti-inflammatory role via inhibition of neutrophil and eosinophil recruitment and activation, and inhibition of pro-inflammatory cytokine and ROS generation. Accordingly, Nascimento-Silva et al. [6] demonstrated that LXA₄ suppresses NAD(P)H oxidase-mediated ROS generation in endothelial cells. Also, it was demonstrated that LXA₄ attenuates lipopolysaccharide-induced intracellular ROS in microglia cells by inhibiting the translocation of the cytoplasmic NADPH oxidase subunit p47(phox) to the cell membrane as well as NADPH oxidase activity [7]. On the other hand, Brezinski et al. [3] showed that LXA₄ can be associated with serious complications following percutaneous transluminal coronary angioplasty (PTCA). PTCA can achieve effective relief of coronary arterial obstruction in 90% to 95% of patients by stretching the vessel and increasing the vessel diameter. However, PTCA can induce plaque rupture, thus triggering the appearance of vasoactive compounds that induce vasoconstriction and acute reocclusion, a serious and an early complication of PTCA. Additionally, stent thrombosis can occur at later stages, causing new ischemic events. To help prevent the process of thrombosis and restenosis after PTCA, antiplatelet therapy is administered, including aspirin. Because the aspirin treatment induces formation of LXA₄, it is possible that these eicosanoids are involved in PTCA-associated untoward events. This idea is strongly supported by evidence demonstrating that PTCA triggers the intraluminal release of LXA₄ and that aspirin therapy enhances their appearance in intracoronary blood [3].

The formation of LXA₄ within the vascular lumen and wall during inflammation, for example, after aspirin treatment, places this lipid in a strategically advantageous site for modulation of vascular function. However, there is little and contradictory published information about the vascular actions of LXA₄.

von der Weid et al. [8] demonstrated that LXA₄ induces endothelium-dependent relaxation in mesenteric arteries and aortic segments. Accordingly, this study showed that administration of LXA₄ (1 μmol/l) to rat aortic rings, which had been precontracted with phenylephrine, resulted in relaxation [8]. On the other hand, Feuerstein and Siren [9] showed that intravenous LXA₄

dose-dependently constricted mesenteric vessels but did not alter arterial blood pressure (BP) or heart rate.

Therefore, in the present study, we used LXA₄ as a pharmacological tool to investigate its vascular effects. We hypothesized that LXA₄ promotes contractile responses and contributes to endothelial dysfunction in rat aorta via mechanisms that are dependent on the RhoA/Rho kinase pathway and oxidative stress.

Methods

Animal Housing

Twelve-week-old male Wistar rats were obtained from Harlan. The rats were housed at a constant room temperature and humidity with a 12:12 h light-dark cycle and free access to standard rat chow and tap water. The investigation conformed to 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 to the guidelines of the Committee on Care and Use of Laboratory Animal Resources of the Georgia Regents University.

Vascular Reactivity Measurements

The animals were anesthetized using isoflurane (5%) and killed by exsanguination. The aorta was carefully removed and cleaned of surrounding tissue in cold physiological salt solution (PSS) 118 mmol/l NaCl, 4.7 mmol/l KCl, 25 mmol/l NaHCO₃, 2.5 mmol/l CaCl₂·2H₂O, 1.2 mmol/l KH₂PO₄, 1.2 mmol/l MgSO₄·7H₂O, 0.01 mmol/l ethylenediamine-tetraacetic acid and 11 mmol/l glucose. Segments (2 mm in length) were mounted in a pin myograph chamber (Danish Myo Tech) for isometric tension recordings. Segments were stretched to 30 mN and equilibrated in oxygenated PSS at 37°C and pH 7.4 for 30 min. The vessel contractility was then tested by exposure to a high-K⁺ (120 mmol/l) solution. Endothelium integrity was tested with phenylephrine-induced contraction (30 μmol/l), followed by endothelium-dependent relaxation with acetylcholine (30 μmol/l).

Experimental Protocols

Cumulative concentration-response curves were performed to phenylephrine (PE, 0.1 nmol/l–30 mmol/l), acetylcholine (1 nmol/l–10 μmol/l), and LXA₄ (1 nmol/l–10 μmol/l). Relaxation curves were performed after contraction with 3 μmol/l phenylephrine. Concentration-response curves to LXA₄ were performed in the presence or absence of Y27632 (Rho-associated kinase inhibitor, 1 μmol/l), indomethacin (COX inhibitor, 10 μmol/l), SC-560 (COX-1 inhibitor, 1 μmol/l), NS-398 (COX-2 inhibitor, 1 μM), SQ 29548 (thromboxane (Tx) A₂/PGH₂ receptor antagonist, 1 μmol/l), tempol (ROS scavenger, 1 mmol/l), cyclosporin H (CsH, FPR-1 antagonist, 1 μmol/l), WRW4 (FPR-2 antagonist, 10 μmol/l) and apocynin (NAD(P)H oxidase inhibitor and ROS scavenger, 10 μmol/l). The role of the endothelium in the LXA₄-induced contraction was evaluated in aortic rings subjected to mechanical endothelium removal. The absence of endothelium (E-) was confirmed by the inability of acetylcholine (10 μmol/l) to induce relaxation.

Concentration-response curves to acetylcholine and phenylephrine were performed in the presence or absence LXA₄ (10

nmol/l) and concentration-response curves to arachidonic acid (10 nmol/l–30 μ mol/l) were performed in the presence or absence of indomethacin (10 μ mol/l).

Inhibitor incubation of vascular segments was 30 min prior to concentration-response curves. Results are presented as either % KCl-induced contraction for contraction curves or % phenylephrine (%PE)-induced contraction for relaxation curves.

Reactive Oxygen Species Measurement

The oxidative fluorescent dye hydroethidine was used to evaluate ROS production in situ, as previously described [10]. Briefly, aortic segments were incubated with LXA₄ (10 nmol/l; 15 min) or ethanol (0.1% in PSS) (vehicle, VEH) and frozen at –80°C. Transverse sections (10 μ m) of frozen aorta were collected on glass slides and equilibrated for 10 min in phosphate buffer in a light-protected humidified chamber at 37°C. Fresh buffer containing hydroethidine (1 mmol/l) was topically applied to each tissue section and a coverslip placed on top. The slides were incubated in a light-protected humidified chamber at 37°C for 30 min. The negative control sections received the same volume of phosphate buffer but in the absence of hydroethidine. After this period, the slides were viewed using an optical microscope equipped with a rhodamine filter and a camera, using a 20 $\times$ objective. Three different areas of each section were analyzed for the presence of ROS by examining the regions marked with red fluorescence from the oxidation products of dihydroethidium (DHE). The images were analyzed using the ImageJ software (National Institute of Health, USA).

Immunoblotting

Arteries were cleaned of perivascular adipose tissue, incubated with LXA₄ (10 nmol/l; 15 min), snapped frozen in liquid nitrogen, and then homogenized in ice-cold tissue protein extraction reagent (T-PER) (Thermo Fisher Scientific, Waltham), with protease inhibitors [sodium orthovanadate, phenylmethanesulfonylfluoride (PMSF), protease inhibitor cocktail (Sigma)] and phosphatase inhibitors [sodium fluoride and sodium pyrophosphate] (Sigma). Protein was extracted and quantified equal amounts (20–40 μ g) were separated on polyacrylamide gels using a standard SDS-PAGE western blot protocol.

The membranes were blocked with 5% nonfat dry milk and incubated overnight at 4°C with primary antibodies raised against anti-COX-1 and COX-2 (1:2,000; Upstate), anti-p67phox (1:1,000; Cell Signaling); phosphorylated myosin light chain Thr18/Ser19 (pMLC, 1:2,000; Cell Signaling) and RhoA (1:2,000; Cell Signaling).

The membranes were then washed and incubated with horseradish peroxidase-linked secondary antibodies. Immunocomplexes were detected with an enhanced chemiluminescence system on a protein simple imager. Phosphorylated protein expression was normalized to total protein expression; all other proteins were normalized to loading control, β -actin (Sigma-Aldrich). The expression is presented as arbitrary units (A.U.).

Drugs

Stock solutions of phenylephrine, acetylcholine and Y27632 were prepared in distilled water. Apocynin, cyclosporine H, LXA₄, SQ 29548 and indomethacin were dissolved in ethanol; SC-560, NS-398 and tempol were dissolved in dimethyl sulfoxide and WRW4 was dissolved in 20% acetonitrile. All drugs

were purchased from Sigma-Aldrich (Saint Louis, Mo., USA) except for NS-398 and SC560 (Calbiochem, USA), WRW4 and tempol (Tocris Bioscience, UK). The concentrations of the inhibitors and antagonists were selected based on previous studies [11–13].

Statistical Analysis

The statistical procedures used included Student's unpaired t-tests and two-way analysis of variance (ANOVA). All analyses were performed using data analysis software GraphPad Prism 5.0 (CA, USA). Statistical significance was set at $p < 0.05$. The data are presented as mean $\pm$ SEM.

Results

Lipoxin A₄ Induces Contraction via Formyl Peptide Receptor-2 (FPR-2)

To analyze the role of LXA₄ in vascular function, LXA₄ was added in an organ bath in a cumulative manner. As observed in figure 1a, this compound induced concentration-dependent contractions even at low concentrations. Also, aortic rings treated with LXA₄ (10 nmol/l) shifted the concentration curve to phenylephrine to the left indicating increased sensitivity (fig. 1b).

Lipoxin A₄-induced contraction was abolished with FPR-2 antagonist (WRW4, 10 μ mol/l) but not with FPR-1 antagonist (CsH, 1 μ mol/l) (fig. 1c, d).

Lipoxin A₄ Activates Rho/ROCK Kinase Pathway

Incubation of aortic rings with Y27632 (Rho-associated kinase inhibitor, 1 μ mol/l), significantly reduced LXA₄-induced contraction in aortas of Wistar rats (fig. 2a). In line with this result, aortic segments treated with LXA₄ (10 nmol/l; 15 min) increased RhoA protein expression and phospho-myosin light chain Thr18/Ser19 (pMLC) (fig. 2b, c).

Lipoxin A₄ Induces Endothelial Dysfunction

The contribution of the endothelium in the LXA₄-induced contraction was evaluated by endothelium-denudation (E-) of aortic rings. Figure 3a shows that the endothelium removal partially decreased LXA₄-induced contraction. Also, the endothelium-dependent relaxation evoked by acetylcholine was reduced in arteries treated with LXA₄ (10 nmol/l; 15 min) compared with arteries treated with vehicle (fig. 3b).

The role of cyclooxygenase (COX) in LXA₄-induced contraction was analyzed using indomethacin (COX inhibitor, 10 μ mol/l). This nonspecific inhibitor partially decreased the LXA₄-induced contractile response in aortic segments from Wistar rats (fig. 4a). Moreover, a sim-

Fig. 1. Concentration-responses curves to lipoxin A₄ (LXA₄) and vehicle (VEH) (a), or phenylephrine in the presence or absence of LXA₄ (10 nmol/l) (b). Effects of cyclosporin H (c) CsH, FPR-1 antagonist) and WRW4 (d) FPR-2 antagonist) on LXA₄-induced

contraction. The results are expressed as the means $\pm$ SEM for the number of animals used (4–5). Two-way ANOVA: * represents statistically significant ($p < 0.05$) versus LXA₄.

ilar result was observed with COX-2 inhibitor (NS-398, 1 μ mol/l) (fig. 4b) but not with COX-1 inhibitor (SC-560, 1 μ mol/l) (fig. 4c). To investigate the role of thromboxane (Tx) A₂ and/or prostaglandin (PG) H₂ in the LXA₄-induced contraction, some rings were incubated with SQ-29548 (TXA₂/PGH₂ receptor antagonist, 1 μ mol/l). As observed in figure 4d, this compound did not change LXA₄-induced contraction. Corroborating these results, LXA₄ increased COX-2 protein expression, but did not change the COX-1 protein expression (fig. 4e, f).

Concentration-Response Curve to LXA₄ Induces an Immediate Contraction Preceding a Transient Vascular Relaxation in Pre-Contracted Rings

Concentration-response curves to LXA₄ were performed in precontracted rings with phenylephrine (1 μ mol/l). LXA₄ induced a biphasic response (fig. 5a). In low concentrations, LXA₄ induced contraction, but in higher concentrations it induced a modest relaxation (fig. 5a; table 1).

As described above, LXA₄ is a biologically active product generated from arachidonic acid (AA) by lipoxigen-

Fig. 2. Effects of Y27631 (RhoA/Rho kinase inhibitor) on LXA₄-induced contraction (a). Representative blots and densitometric analyses of protein expression of phosphorylated myosin light chain (pMLC) (b), RhoA (c) and β-actin of aorta from Wistar rats

treated with lipoxin A₄ (LXA₄) (10 nmol/l) or vehicle (VEH) for 15 min. The results are expressed as the means ± SEM for the number of animals used (4–5). Two-way ANOVA or t test: * represents statistically significant ($p < 0.05$) versus LXA₄.

Fig. 3. Effects of endothelium removal (E-) on LXA₄-induced contraction (a). Concentration response curves to acetylcholine in the presence or absence of lipoxin A₄ (LXA₄, 10 nmol/l) (b). The results are expressed as the means ± SEM for the number of animals used (4–5). Two-way ANOVA: * represents statistically significant ($p < 0.05$) versus LXA₄.

ase action. The production of LXA₄ is enhanced by aspirin through acetylation of COX-2. To investigate whether AA changes vascular function, we performed concentration-response curve to AA in precontracted rings with phenylephrine (1 μmol/l) in the presence or absence of indomethacin (10 μmol/l). In low concentrations AA induced a small contraction only in the presence of indomethacin (fig. 5b; table 1). On the other hand, it was observed that

AA induced contraction in a concentration-dependent manner in non-pre-contracted rings (fig. 5c), and indomethacin did not alter this response (fig. 5c; table 1).

Reactive Oxygen Species (ROS) Contributes to LXA₄-Induced Contraction

The small guanosine triphosphatase (GTPase) Rho plays an important role in various cellular physiologic

Fig. 4. Effects of indomethacin (Indo, COX inhibitor) (a), NS-398 (COX-2 inhibitor) (b), SC-560 (COX-1 inhibitor) (c) or SQ-29548 (d thromboxane A₂ and/or prostaglandin H₂ receptor antagonist) on LXA₄-induced contraction. Representative blots and densitometric analyses of protein expression of COX-1 (e), COX-2 (f) and β -actin of aorta from Wistar rats treated with lipoxin A₄ (LXA₄) (10 nmol/l) or vehicle (VEH) for 15 min. The results are expressed as the means $\pm$ SEM for the number of animals used (4–5). Two-way ANOVA or t test: * represents statistically significant ($p < 0.05$) versus LXA₄.

functions, including migration, motility, contraction and oxidative stress. Since LXA₄-induced contraction activates RhoA/Rho kinase and it is known that RhoA/Rho kinase induces ROS generation, it is possible that this compound can induce oxidative stress. To evaluate the contribution of ROS to the contractile responses to LXA₄, arteries were incubated with 1 mmol/l tempol, a ROS scavenger. In figure 6a, pretreatment with tempol decreased LXA₄-induced contraction. Moreover, an increase

in ethidium bromide fluorescence, reflecting the presence of ROS, was observed in aorta slices pretreated with LXA₄ (10 nmol/l; 15 min) as compared with vehicle (fig. 6b).

Lipoxin A₄ Increases ROS Generation Partially via NAD(P)H Oxidase

The contribution of NAD(P)H oxidase LXA₄-induced contraction was analyzed using apocynin (NAD(P)H oxidase inhibitor and ROS scavenger, 10 μ mol/l). Apocynin

Fig. 5. Concentration-responses curves to lipoxin A₄ (LXA₄) and vehicle (VEH) (a) or arachidonic acid (AA) (b) in pre-contracted rings with phenylephrine in the presence or absence of indomethacin (INDO, 10 μ mol/l) (b). Effects of INDO (c) on concentration-responses curves to AA in non-pre-contracted rings.

Table 1. Maximum response

	E _{max} % contraction	E _{max} % relaxation
VEH (graph A)	0.6 $\pm$ 0.4	0.1 $\pm$ 4
LXA ₄ (graph A)	4.1 $\pm$ 0.8*	13 $\pm$ 5*
AA (graph B)	-1.8 $\pm$ 0.7	-
INDO (graph B)	3.7 $\pm$ 1.6 [#]	-
AA (graph C)	80 $\pm$ 4	-
INDO (graph C)	74 $\pm$ 7.3	-

Maximum response (E_{max}) to LXA₄ or arachidonic acid (AA) in the presence or absence of indomethacin (INDO 10 μ mol/l). Results are expressed as means $\pm$ SEM (n = 4–5). t-test * vs. VEH; [#] vs. AA.

partially decreased LXA₄-induced contraction (fig. 6c). Furthermore, as observed in figure 6d, the treatment with LXA₄ (10 nmol/l; 15 min) increased the protein expression of p-67phox, a NAD(P)H oxidase isoform.

Discussion

The first important finding of the present study is powerful evidence for the role of LXA₄ as a vasoconstrictor that may contribute significantly to vascular dysfunction in conditions when its production is exacerbated, such as atherosclerosis or angioplasty, complications treated with aspirin. The second major finding is that LXA₄ induces contraction and endothelium dysfunction

mainly via RhoA/Rho kinase activation, and ROS generation.

Lipoxin A₄, bioactive product generated from arachidonic acid through the action of lipoxygenase, was first described by Serhan et al. [1] in 1984. These authors demonstrated that when LXA₄ was added to human neutrophils, it stimulated superoxide anion generation and degranulation at submicromolar concentrations without provoking a substantial aggregation response [1]. Also, they demonstrated that LXA₄ was as potent in inducing superoxide anion generation as leukotriene B₄.

Lipoxin A₄ binds formyl peptide receptor-2 (FPR2) with high affinity, thereby stimulating arachidonate release and GTPase [14]. The FPR family, a subfamily of G protein-coupled receptors, is represented by three isoforms, FPR 1, 2 and 3. FPRs are expressed at high levels on neutrophils and monocytes, and when activated by binding of formyl peptides, they induce NADPH oxidase activation, ROS generation and cell chemotaxis [15, 16]. Besides neutrophils, FPRs are also expressed on a range of somatic cells and tissues, including the endothelium, epithelium, spleen, lung, liver, skeletal and smooth muscle [16]. Nevertheless, the biological function of FPR in other tissues is not fully understood. FPR activation in the cardiovascular system has been shown to have functional implications, such as modulation of vascular tone, even in the absence of leukocytes. Specifically, Bode et al. [17] demonstrated that N-formyl peptide derived from bacteria activates cells of unknown identity in the adventitia or media of human coronary arteries, which release vasoconstrictor cyclooxygenase-derived products. In addi-

Fig. 6. Effects of tempol (superoxide anion scavenger) (a) or apocynin (NAD(P)H oxidase inhibitor) (c) on LXA₄-induced contraction. Effect of LXA₄ (10 μmol/l) on reactive oxygen species (ROS) generation (b). Upper panel: representative fluorescence photomicrographs of microscopic sections from mesenteric resistance arteries. Lower panel: densitometric analysis of fluorescence inten-

sity. Representative blots and densitometric analyses from protein expression for p67phox (d) and β-actin of aorta from Wistar rats treated with lipoxin A₄ (LXA₄) (10 nmol/l) or vehicle (VEH) for 15 min. The results are expressed as the means ± SEM for the number of animals used (4–5). Two-way ANOVA or t test: * represents statistically significant ($p < 0.05$) versus LXA₄.

tion, Keitoku et al. [18] reported that N-formyl peptides derived from bacteria produced biphasic responses (contraction with subsequent relaxation) in human coronary arteries, mainly via generation of thromboxane A₂ and prostacyclin. In a recent publication [19], we reported that N-formyl peptides from bacteria and from mitochondria have a powerful relaxant effect in resistance ar-

teries, and that cyclosporine H (FPR-1 antagonist) inhibits this response, suggesting that FPR-1 has an important role in vascular function.

Here we observed that LXA₄ induced contraction via FPR-2 but not via FPR-1, and this contraction is both endothelium-dependent and independent. Interesting, it was observed that concentration-response curve to LXA₄

in pre-contracted rings induced a modest biphasic response. In low concentrations, LXA₄ induced a small contraction while in higher concentrations it induced a modest relaxation. Accordingly, von der Weid et al. [8] showed that one concentration of LXA₄ (1 μmol/l) resulted in relaxation of pre-contracted aortic segments rings. These authors suggested that LXA₄ induces relaxation via NO release. On the other hand, in the same study [8], it was observed that intraperitoneal administration of aspirin increased blood pressure in rats. It is possible that, aspirin, via COX inhibition, leads to LXA₄ synthesis, and induces vascular contraction leading to increased total peripheral resistance and blood pressure. Also, we performed arachidonic acid (AA) concentration-response curve in pre-contracted rings with phenylephrine with or without indomethacin. Arachidonic acid did not change the maximum phenylephrine-induced contraction. However, in the presence of indomethacin, AA induced a modest contraction at low concentrations, as observed in the LXA₄-concentration response curve in pre-contracted rings with phenylephrine. This result corroborates the data that demonstrate LXA₄-induced contraction, since indomethacin inhibited COX, but not lipoxygenase. This enzyme in the presence of AA can synthesize LXA₄. Also, as in previous studies [20], we observed that AA induced contraction in a concentration-dependent manner. Again, this response was not inhibited by indomethacin.

Regarding the endothelium-independent contraction, it was observed that LXA₄-induced contraction was abolished by RhoA/Rho kinase inhibitor. It is known that calcium sensitization of the contractile proteins is signaled by the RhoA/Rho kinase pathway to inhibit the dephosphorylation of the myosin light chain by myosin light chain phosphatase, thereby maintaining force generation [21]. Here, we observed that LXA₄ binds FPR-2 leading to RhoA/Rho kinase activation and subsequently, the phosphorylation of myosin light chain (pMLC) and contraction.

Recent studies have shown that LXA₄ has a number of immunomodulatory and anti-inflammatory actions, including in the resolution phase of inflammation [22]. LXA₄ blocks neutrophil infiltration and transmigration across mucosal epithelial cells and vascular endothelial cells through induction of NO production [14]. Contrasting these studies, here we observed that endothelium removal partially reduced LXA₄-induced contraction. These data suggest that this compound induces the release of vasoconstrictor factors from endothelium. In fact, when aortic rings were treated with indomethacin (COX

inhibitor) or NS-398 (COX-2 inhibitor), the LXA₄-induced contraction was reduced to the same magnitude as when the endothelium was removed. This suggests that LXA₄ released COX-2-derived vasoconstrictor factors, since COX-1 inhibitor did not alter LXA₄-induced contraction. Furthermore, COX-2, but not COX-1, protein expression was increased with LXA₄ treatment. Given that thromboxane A₂ and prostaglandin H₂ are COX-derived vasoconstrictor [11–13], we thought that these molecules could be involved in LXA₄-induced contraction. However, thromboxane A₂ and/or prostaglandin H₂ receptor antagonist did not change LXA₄-induced contraction. Since indomethacin and NS-398 inhibited LXA₄-induced contraction, it is possible that other (s) factor (s) derived from COX-2, but different from prostaglandins, lead to LXA₄-induced contraction. It is known that COX-2 also generates ROS in some circumstances [13, 23, 24]. Considering this fact, we can speculate that COX-2 may generate ROS in LXA₄-induced contraction.

In 1984, Serhan et al. [1] observed that LXA₄ stimulates superoxide anion generation in neutrophils. Also, FPR-2 agonist (WKYMVM) (Trp-Lys-Tyr-Met-Val-D-Met) induces ERK activation, p47phox translocation and NADPH-dependent superoxide generation [14]. Corroborating these results, it was observed that tempol, a superoxide anion scavenger, significantly decreased LXA₄-induced contraction. This demonstrates that LXA₄ induces generation of ROS in aorta. It is well established that under certain circumstances such as oxidative stress, nitric oxide degradation is increased in the presence of excessive levels of superoxide anions (O₂⁻) [25], which can lead to vasoconstriction or a decreased relaxation response.

Superoxide anion produced in vascular endothelial cells, adventitia and smooth muscle is mainly derived from NAD(P)H oxidase [25]. The phosphorylation of p47phox leads to interaction with p40phox and p67phox, which translocate to the plasma membrane to interact with the NOX-1/2 and p22phox to generate O₂⁻ [26]. Here we observed that LXA₄ induces contraction and increased ROS generation. The augmented ROS levels are also generated, in part, via NAD(P)H oxidase activation since apocynin (NAD(P)H oxidase inhibitor) decreases LXA₄-induced contraction. However, it is important to highlight that apocynin also acts like an antioxidant [27]. Thus, part of apocynin effect may be because it decreases O₂⁻ due to its antioxidant ability.

It is known that FPR-2 activation induces NADPH-dependent superoxide generation in neutrophils [14]. In the present work, we observed that LXA₄ increases

Fig. 7. Proposed effects of lipoxin A₄ (LXA₄) on vascular contraction. Formyl peptide receptor-2 (FPR-2); Cyclooxygenase-2 (COX-2); reactive oxygen reactive (ROS); nitric oxide (NO); NAD(P)H oxidase, Rho-associated protein kinase (ROCK), Ras homolog gene family, member A (RhoA), phosphorylated myosin light chain (pMLC).

p67phox protein expression in rat aorta. Collectively, these data suggest that FPR-2 activation by LXA₄ induces ROS production via, partially, NAD(P)H oxidase activation and partially via COX-2 activation.

In conclusion, the data demonstrates for the first time that LXA₄ is able to induce a moderate contraction of the aorta (fig. 7). Although in physiological conditions small changes in vascular function do not disturb the homeostasis of the whole system (because the system can compensate the initial perturbation), we believe that any minimal change in pathological conditions is very relevant since the organism may have impaired ability to compensate that stimulus. Given that we observed small changes

in vascular function from healthy animals, it is possible that these responses could be more exacerbated in disease conditions (e.g., LXA₄ can start the contraction in smaller concentration such as in patients with coronary diseases). This supports future studies in conditions when its production is exacerbated, such as vasospasm, thrombosis, atherosclerosis and aspirin-treated angioplasty-associated plaque rupture.

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