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Lipoxins stimulate prostacyclin generation by human endothelial cells

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Cultured human umbilical endothelial cells were incubated with lipoxins and their ability to generate prostacyclin (PGI_2) was assessed and compared to that induced by either leukotriene C_4 or an ionophore of divalent cations (A-23,187). When exposed to either lipoxin A_4, lipoxin B_4, or 7-*cis*,11-*trans*-lipoxin A_4, endothelial cells generated prostacyclin detected as 6-keto-$PGF_{1\alpha}$. Of the lipoxins examined, 7-*cis*,11-*trans*-lipoxin A_4 proved to be the most effective with PGI_2 production twice that induced by equimolar amounts of A-23,187 (5 μM). On a molar basis, lipoxin A_4 and lipoxin B_4 were less potent than leukotriene C_4 although they were more efficacious. When either lipoxin A_4 or lipoxin B_4 was added to cells simultaneously with leukotriene C_4, the formation of prostacyclin was greater than that induced by leukotriene C_4 alone. During the time course of exposure to lipoxins (0–20 min, 37°C), cultured endothelial cells did not further transform these compounds via ω-oxidation as determined by reverse-phase HPLC. These data indicate that lipoxins can stimulate PGI_2 generation by human endothelial cells. Moreover, they suggest a role for these lipoxygenase products of arachidonic acid in the regulation of hemostasis, inflammation and vascular reactivity.

Arachidonic acid; Lipoxygenase product

1. INTRODUCTION

Endothelial cells, in addition to serving as the interface between blood and the surrounding tissue, can influence vascular and blood cell physiology through a variety of mechanisms. For example, upon activation these cells can release and oxygenate arachidonic acid to generate prostacyclin (PGI_2), which can affect the function of smooth muscle cells, platelets, and neutrophils. A variety of other endogenous products of arachidonate metabolism including 5-lipoxygenase-derived leukotriene C_4 and leukotriene D_4 can lead to the generation and release of PGI_2 by human endothelial cells [1].

Interactions between the 5- and 15-lipoxygenase can lead to the generation of lipoxins [2–4]. Lipoxin A_4 (LXA_4) and lipoxin B_4 (LXB_4) each display unique profiles of action which are distinct from those of other oxygenated products of arachidonic acid [3–8]. Among its activities, LXA_4 possesses a vasodilatory effect. In particular, LXA_4 stimulates dilation of arterioles in vivo with vessels of the hamster cheek pouch [3,5] and induces glomerular hyperperfusion and hyperfiltration in renal microcirculation of the rat [6]. LXA_4 also induces changes in single nephron glomerular filtration rate and plasma flow rate which are due to selective relaxation of pre-glomerular resistance vessels [6]. Upon systematic administration, however, both

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Abbreviations: LXA_4, lipoxin A_4, 5*S*,6*R*,15*S*-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; LXB_4, lipoxin B_4, 5*S*,14*R*,15*S*-trihydroxy-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid; 7-*cis*-11-*trans*-LXA_4, 5*S*,6*R*,15*S*-trihydroxy-9,11,13-*trans*-7-*cis*-eicosatetraenoic acid; PGI_2, prostacyclin; RP-HPLC, reverse-phase high-pressure liquid chromatography

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LXA_4 and LXB_4 have been shown to cause endothelial-dependent relaxation in vascular tissues from a variety of species [8]. Here, we report that LXA_4, LXB_4, and the recently identified 7-*cis*-11-*trans*-LXA_4 [4] stimulate the formation of prostacyclin in cultured human endothelial cells.

2. MATERIALS AND METHODS

2.1. *Materials*

LXA_4, LXB_4, and 7-*cis*,11-*trans*-LXA_4 were prepared by total organic synthesis as described [4,9] and tested here as their free acids. Leukotriene C_4 (LTC_4) was from Biomol Research Laboratories, Inc., Plymouth Meeting, PA. All solvents were from Burdick and Jackson (HPLC grade), Muskegon, MI.

2.2. *Cell preparations and incubation*

Human umbilical vein endothelial cells (HUVEC) were isolated from two to five normal term cord segments, pooled and established in primary culture using Medium 199 (M199, M.A. Bioproducts, Bethesda, MD) with 20% fetal calf serum (FCS, Gibco, Grand Island, NY) and antibiotics as previously described [10]. HUVEC cultures were serially passaged (1:3 split ratio) and maintained using M199/20% FCS supplemented with endothelial cell growth factor (50 μg/ml; Biomedical Technologies, Inc., Stoughton, MA) and porcine intestinal heparin (100 μg/ml; Sigma, St. Louis, MO) in Costar tissue culture flasks (75 cm^2, Data Packaging Corp., Cambridge, MA) coated with 0.1% gelatin (Bactogelatin 0143-02, Difco, Detroit, MI). For experimental use, HUVEC strains were replicate plated (passage levels 2–3) in microtiter wells (Costar Inc., Cambridge, MA) or 100 mm plastic dishes precoated with 0.1% gelatin. The confluence of endothelial cell layers was confirmed under microscopy before the addition of each compound.

5 min prior to the addition of the compounds, medium (M199) was decanted and Dulbecco's phosphate buffered saline (PBS) was added (pH 7.45, 37°C). After 5 min of incubation, either LXA_4 (0.5, 1, and 5 μM), LTC_4 (0.05, 0.5 and 5 μM), 7-*cis*,11-*trans*-LXA_4 (5 μM), or A-23, 187 (5 μM) were added to individual wells. The concentration and integrity of each of the eicosanoids were confirmed by UV analysis immediately before each experiment. In several experiments the cyclooxygenase inhibitor, ibuprofen (80 μg/ml), was incubated with cells (1 min, 37°C) prior to the addition of stimuli.

Following incubation (20 min, 37°C), aliquots (500 μl) of the supernatants were removed and immediately placed on ice for determination of 6-keto-$PGF_{1\alpha}$ by radioimmunoassay. Next, cold EtOH (1 ml) was added to the remaining incubations and the mixtures were taken for further analysis by HPLC. In parallel experiments, lipoxins (5 μM) were added to cells and incubated (20 min, 37°C) vide supra and cell viability was assessed by their ability to exclude trypan blue. In all reported experiments the viability was >98%. Radioimmunoassay for 6-keto-$PGF_{1\alpha}$ was performed by methods previously described [9].

2.3. *Analytical methods*

Incubations were stopped by addition of EtOH (1 ml), stored at −20°C and analyzed for lipoxygenase products as described [4]. Briefly, samples were centrifuged at 800 × g for 15 min, supernatants were removed and pellets were washed with MeOH. MeOH- and EtOH-containing fractions were combined and organic solvents were removed by rotational evaporation. Next, samples were suspended in water, acidified, and loaded into Sep-pak C18 reverse-phase cartridges (Waters Associates, Milford, MA). The cartridges were washed with water (10 ml) and hexane (10 ml), and the products were eluted from the column with methyl formate (10 ml). Samples were examined for UV absorbing materials prior to injection into a RP-HPLC system. The system consisted of an LKB dual-pump gradient HPLC equipped with a Nova-Pak C18 column (3.9 mm × 7.5 mm), injector, and solvent controller (LKB, Bromma, Sweden). The column was eluted with MeOH/H_2O/acetic acid (68:32:0.01) as solvent at a flow rate of 0.39 ml/min with an initial pressure of approximately 25 Bar. This HPLC system was equipped with a photodiode array rapid spectral detector linked to an AT&T PC6300, and post-HPLC run analyses were performed utilizing a 2140-202 Wavescan program (Bromma, Sweden) and Nelson Analytical 3000 series chromatography data system (Paramus, NJ). Products were quantitated from their UV spectra (220–340 nm) following HPLC where baselines between eluting chromophores were achieved by computer-aided manipulation. PGB_2 was included prior to extraction as an internal standard. UV spectra which were not recorded on-line during HPLC were recorded with a Hewlett-Packard model 8452 photodiode array spectrophotometer.

3. RESULTS

When added to endothelial cells in culture, each of the lipoxins stimulated PGI_2 formation as monitored by the detection of 6-keto-$PGF_{1\alpha}$ levels. All values were normalized to paired incubations in which the cells were exposed to A-23,187 (5 μM). The mean level of 6-keto-$PGF_{1\alpha}$ detected in response to A-23,187 was 4.3 ± 0.5 pmol/ml (n = 7 separate preparations of cultured endothelial cells; mean ± SEM). It can be seen that both LXA_4 and LXB_4 at a concentration of 5 μM caused a significantly greater release of PGI_2 than vehicle-treated cells ($P<0.05$). Although a tendency for LXA_4-induced PGI_2 production occurred at a concentration of 1 μM, the value of 0.44 was not statistically significant when compared to vehicle control levels. At lower concentrations of LXA_4 (0.5 μM) and LXB_4 (1.0 μM, 0.5 μM), mean values were not greater than 0.20 and were also not significantly different from those obtained with the vehicle. Lipoxin A_4-induced formation of 6-keto-$PGF_{1\alpha}$ was completely inhibited by prior treatment of the cells with the cyclo-oxygenase inhibitor ibuprofen. Results of previous studies have shown that leukotrienes can stimulate the generation of

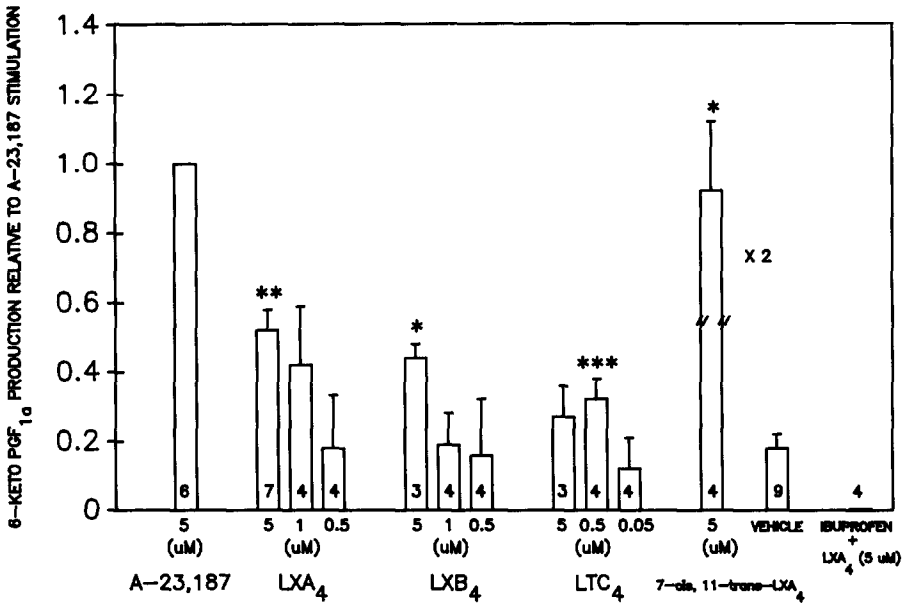

Fig.1. Summary of lipoxin-induced 6-keto-$PGF_{1\alpha}$ detected in supernates from cultured human umbilical endothelial cells. Bars and brackets represent means ± SE, respectively. Numbers within bars represent number of different endothelial cell cultures studied. * $P<0.05$ from vehicle control (ethanol); ** $P<0.01$ from vehicle control; *** $P<0.05$ from paired vehicle controls. Mean value for $PGF_{1\alpha}$ in response to A-23,187 (5 μM) was 4.3 ± 0.5 pmol/ml ($n = 7$, mean ± SE).

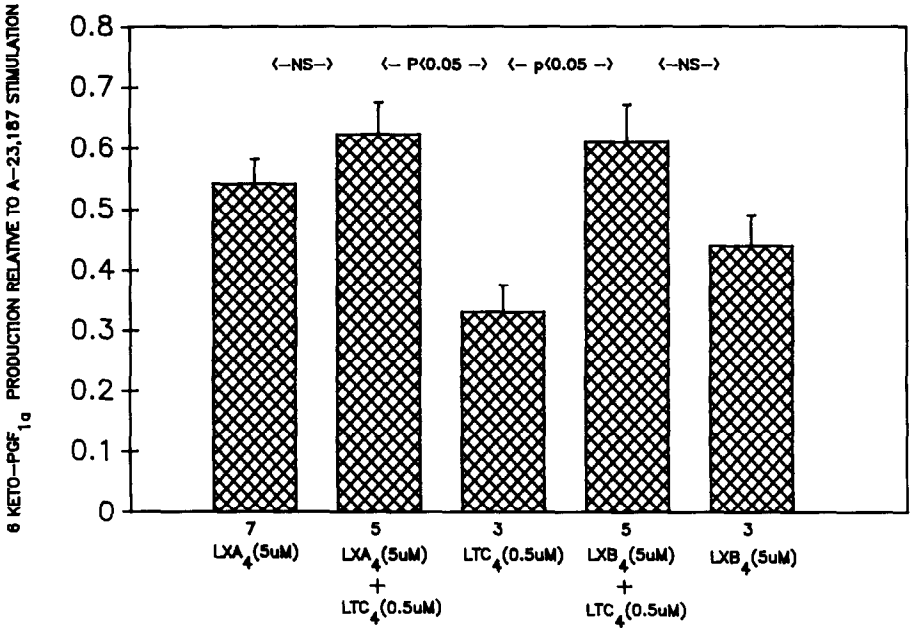

Fig.2. Effect of simultaneous addition of LTC_4 and lipoxins to human umbilical endothelial cells. Bars and brackets represent means and SE, respectively. Numbers below bars represent number of different endothelial cell cultures studied. NS, no significant difference.

PGI_2 [1]. In the present study, LTC_4 (0.5 μM) also caused an increase in the levels of PGI_2. However, increasing the LTC_4 concentration to 5 μM failed to significantly change the level of 6-keto-$PGF1_\alpha$ detected. The recently identified human neutrophil-derived product 7-*cis*,11-*trans*-LXA_4 [4] induced almost twice the amount of PGI_2 production as observed with maximal ionophore stimulation (fig.1). Thus, this compound was the most potent of the lipoxins examined.

The effect of combined addition of lipoxins and LTC_4 at maximal stimulatory concentrations is given in fig.2. In these experiments, the levels of PGI_2 generated in response to maximal stimulatory concentrations of LTD_4 were significantly less than those obtained with combined LTC_4 and lipoxin (LXB_4 or LXA_4) [*P value* < 0.05]. Although there was a tendency for the combined agonists to cause greater stimulation than lipoxins alone, these values did not prove to be statistically significant.

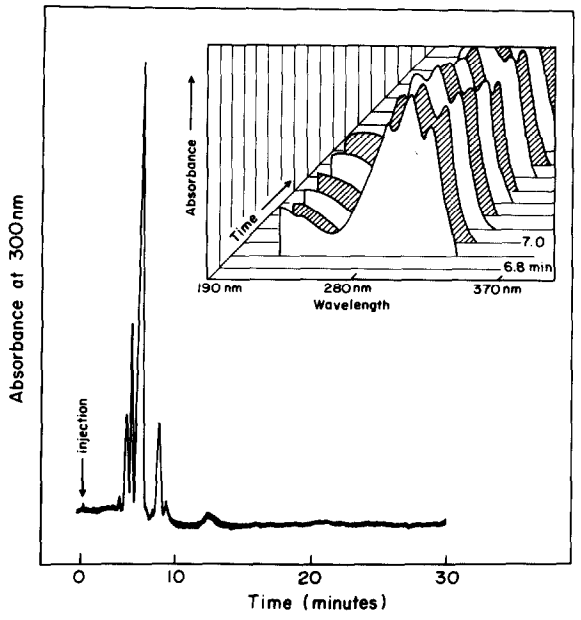

Fig.3. RP-HPLC chromatograph of lipoxin A_4 after incubation (20 min, 37°C) with endothelial cells. Following extraction, samples were injected into a RP-HPLC, Nova-Pak C18 (2.9 mm × 7.5 cm) equipped with a rapid spectral detector. Post-HPLC analyses were performed with a Wavescan 2140-202 program and a Nelson Analytical 3000 series chromatography data system. The elution times for LXA_4, all-*trans* LXA_4, and PGB_2 are 6.7, 5.6 and 9.4 min, respectively. ω-Oxidative products were not observed. In the insert, the topogram for LXA_4 is depicted. It can be seen that LXA_4 retained its tetraene chromatogram with a λ_{max} at 300 nm and shoulders at 287 and 315 nm. The chromatogram is representative of six separate incubations ($n = 3$ with separate preparations of human endothelial cells and LXA_4 and $n = 3$ with LXB_4).

Next, to determine if lipoxins were transformed by endothelial cells and to determine if their transformation products were responsible for stimulating PGI_2 formation, the products obtained following incubation were extracted and analyzed by HPLC. A representative chromatogram of LXA_4 obtained following incubation (20 min, 37°C) with endothelial cells is shown in fig.3. LXA_4 remained intact following exposure to endothelial cells. Its retention time was identical to that of the synthetic standard, and it retained its tetraene chromophore. ω-Oxidative products of LXA_4 were not observed. However, small amounts of its all-*trans*-isomer (11-*trans*-LXA_4) were detected. Previous results indicate that 15–20% of LXA_4 is converted to its all-*trans* isomer following extraction and HPLC [14]. The levels of all-*trans* LXA_4 obtained following incubation without endothelial cells, extraction and HPLC indicated that the transformation of LXA_4 to all-*trans*-LXA_4 was not a metabolic event in human endothelial cells. Similar studies with LXB_4 showed that it too was not metabolically transformed by endothelial cells during this time course.

4. DISCUSSION

A number of physiologic actions of the lipoxins have been described over the last four years (for reviews, see [17]). These include inhibition of natural killer cell activity [12], endothelial-dependent relaxation of aortic strips [8], mesenteric vein constriction [7], contraction of lung parenchyma strips in a variety of species [8], vasodilation of vessels of the hamster cheek pouch [5], reduction in renal afferent arterial resistance [6], chemotaxis with human neutrophils [15] and activation of isolated protein kinase C [11]. The present results demonstrate that lipoxins can also induce PGI_2 production by human umbilical endothelial cells. These results with endothelial cells are consistent with recent findings which document that both lipoxin A_4 and lipoxin B_4 provoke an endothelial-dependent relaxation of aortic vascular strips from a variety of species [8]. Lipoxin A_4 has also been demonstrated to induce vasodilation in

the hamster cheek pouch and selective relaxation of renal afferent arteries [5,6]. These in vivo observations would suggest that the mechanism reported here, namely lipoxin-induced prostacyclin generation by endothelium, can play a role in the intact circulatory system. In addition, lipoxins may also be involved in the release of other endothelial-derived relaxing factors [20,21] such as nitric oxide [22]. On the other hand, the vasoconstriction of the splanchnic circulation observed upon systemic injection of lipoxin A_4 and lipoxin B_4 [7] may be mediated through their ability to generate other vasoactive agents such as thromboxane [16,17]. Taken together these findings suggest multiple roles for lipoxins in the regulation of vascular tone.

7-*cis*,11-*trans*-LXA_4 is a recently described lipoxin that was first isolated from human neutrophils [4]. The biological responses evoked by lipoxin A_4 and lipoxin B_4 have proven to be highly stereospecific (reviewed in [17]). For example, with airway smooth muscle changes in either orientation of alcohol groups or the geometry of double bonds in LXA_4 can delete or diminish activity, respectively [4,14–19]. When the actions of 7-*cis*,11-*trans*-LXA_4 were compared to those of LXA_4 for contractile activity in the guinea pig lung, 7-*cis*,11-*trans*-LXA_4 proved to be 10 times less active than LXA_4 [4]. In contrast, the present results indicate that 7-*cis*,11-*trans*-LXA_4 is more active than LXA_4 in stimulating the formation of PGI_2 by human endothelial cells (fig.1). Taken together, these findings provided further evidence indicating that the biological actions of lipoxins are highly stereospecific, since these two compounds (7-*cis*,11-*trans*-LXA_4 and LXA_4) differ only in the geometry of two double bonds. In this respect, it is of interest to note that LXA_4 and LXB_4 were equivalent in this system (fig.1).

Unlike leukotrienes, which can be metabolized by endothelial cells [1], the lipoxins were not further transformed via ω-oxidation following exposure to intact endothelial cells (fig.3). This finding indicates that the actions of lipoxins on endothelial cells are direct rather than requiring enzymatic transformation in order to express activity.

Recent results indicate that LXA_4 can block the binding of leukotriene D_4 to its receptor [18], and results have been presented showing that LXA_4 can inhibit leukotriene C_4-induced responses [16,17]. Taken together, these findings suggest that lipoxin A_4 may act at the receptors for cysteinyl-containing leukotrienes in certain tissues. The present findings indicate that the action of lipoxins on endothelium may involve different receptor mechanisms than in other tissues. This is supported by the finding that combined addition of maximally stimulatory concentrations of LTC_4 and LXA_4 lead to greater production of PGI_2 than that induced by LTC_4 alone (fig.2).

When exposed to leukotriene A_4, human platelets generate lipoxins and they can be generated during platelet-granulocyte interactions from endogenous sources of arachidonate [23]. Thus, the present finding that lipoxins can stimulate prostacyclin generation by human endothelial cells provides further evidence that lipoxins may serve as mediators or modulators of responses of interest in inflammation, hemostasis, and vascular reactivity.

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