Volume 304, number 1, 78–82 FEBS 11127 June 1992

Conversion of 5,6-dihydroxyeicosatetraenoic acids

A novel pathway for lipoxin formation by human platelets

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Received 23 March 1992

Leukotriene A_4 may be metabolized to 5(S),6(R)- and 5(S),6(S)-dihydroxy-7,9-*trans*-11,14-*cis*-eicosatetraenoic acids by enzymatic or non-enzymatic hydrolysis. Incubation of human platelet suspensions with these dihydroxy acids led to the formation of lipoxin A_4 and 6(S)-lipoxin A_4 via lipoxygenation at C-15. Furthermore, human platelets converted the two 5(R),6(S)- and 5(R),6(R)-dihydroxy-7,9-*trans*-11,14-*cis*-eicosatetraenoic acids to tetraene-containing trihydroxyeicosatetraenoic acids. In contrast, leukotrienes C_4, D_4 and E_4 were not transformed to cysteinyl-lipoxins. Time-course studies of leukotriene A_4 metabolism in human platelet suspensions indicated lipoxin formation via two pathways: (i) direct conversion of leukotriene A_4, leading to formation of the lipoxin intermediate 15-hydroxy-leukotriene A_4; and (ii) 15-lipoxygenation of the 5(S),6(R)- and 5(S),6(S)-dihydroxyeicosatetraenoic acids. The results demonstrate that lipoxygenation at C-15 of 5,6-dihydroxy-7,9,11,14-eicosatetraenoic acids may be an alternative novel pathway for platelet-dependent lipoxin formation.

Lipoxin; 5,6-Dihydroxyeicosatetraenoic acid; 12-Lipoxygenase; Human platelet

1. INTRODUCTION

Non-enzymatic hydrolysis of the unstable epoxide leukotriene (LT) A_4 leads to formation of the two C-12 epimers of 6-*trans*-LTB_4. In addition, two C-6 epimers 5(S),6(R)- and 5(S),6(S)-dihydroxy-7,9-*trans*,11,14-*cis*-eicosatetraenoic acid (5,6-diHETE) are formed [1]. Enzymatic transformation of LTA_4 to 5(S),6(R)-diHETE mediated by a liver cytosolic epoxide hydrolase has been demonstrated [2]. Furthermore, 5(S),6(R)-diHETE may also be formed from 5-HETE via a 6(R)-oxygenase activity of the 5-lipoxygenase in porcine leukocytes [3]. The biological activities of the 5,6-diHETEs are not yet fully elucidated. However, contractile effects on both lung parenchyma [4] and ileum of the guinea pig have been reported. Interestingly, 5(S),6(R)-diHETE was shown to interact with the LTD_4 receptor in guinea pig lung membranes [5]. In addition, 5,6-diHETE stimulated protein kinase C in vitro [6].

The lipoxins are trihydroxylated arachidonic acid derivatives, containing a conjugated tetraene. They are biologically active compounds, which induce e.g. bronchoconstriction, vasodilation, stimulation of protein kinase C and inhibition of NK cells [7]. In addition, lipoxin (LX) A_4 has also been suggested to antagonize leukotriene-induced effect, which implies an anti-inflammatory role of this compound [8–10]. Recently lipoxin-mediated modulation of human myeloid stem cell growth was demonstrated [11].

Lipoxin synthesis may occur via different pathways. The isolation of lipoxins from leukocyte suspensions incubated with 15(S)-hydro(pero)xyeicosatetraenoic acid (15-H(P)ETE) indicated the importance of the 5- and 15-lipoxygenases in lipoxin formation [12,13]. In accordance, we reported lipoxin synthesis from endogenous substrate in mixed incubations of human granulocytes possessing 5-lipoxygenase activity and respiratory tissue, which is rich in 15-lipoxygenase activity [14]. Furthermore, human platelets efficiently metabolized exogenous or granulocyte-derived LTA_4(5,6-epoxy-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid) to lipoxins [15–17]. This synthesis proceeded via 12-lipoxygenase-dependent hydroxylation at carbon 15 leading to formation of the unstable intermediate 15-OH-LTA_4 [17]. The present study demonstrates that human platelets possess the capacity to convert 5,6-diHETEs to lipoxins, including the biologically active LXA_4.

2. MATERIALS AND METHODS

2.1. *Materials*

The four epimers of 5,6-dihydroxy-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (5(S),6(R)-diHETE; 5(S),6(S)-diHETE; 5(R),6(R)-diHETE; 5(R),6(S)-diHETE) were synthesized as reported [18]. Synthetic LTA_4 methyl ester was purchased from Salford Ultrafine Chemicals (Manchester, UK) and was saponified as reported [7]. Lipoxins A_4 and B_4 were purchased from Cayman Chemical Co. (Ann Arbor,

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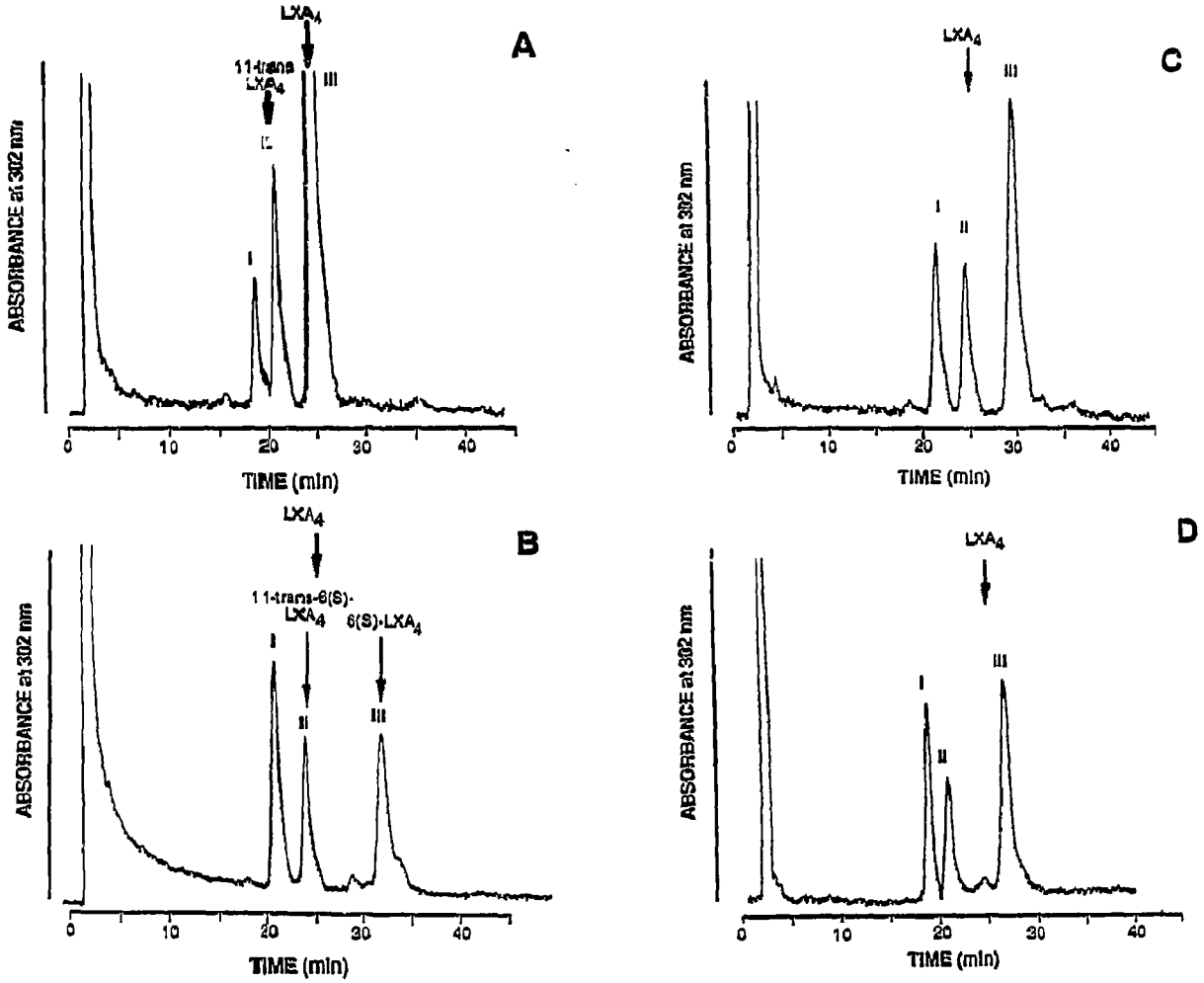

Fig. 1. Reversed-phase HPLC chromatograms of lipoxins formed by human platelet suspensions after incubation with (A) 5(S),6(R)-diHETE, (B) 5(S),6(S)-diHETE, (C) 5(R),6(R)-diHETE and (D) 5(R),6(S)-diHETE. Incubations were performed for 30 min at 37°C in the presence of ionophore A23187 (1 μM). Retention times of synthetic standards are indicated by arrows.

MI, USA). The all *trans* isomers of LXA_4, LXB_4 and 6(S)-LXA_4 were kindly provided by Dr. K.C. Nicolaou (Univ. of Penn., PA, USA), while 7-*cis*-11-*trans*-LXA_4 was a gift from Dr. C.N. Serhan (Harvard Medical School, Boston, MA, USA). Leukotrienes C_4, D_4 and E_4 were synthesized as described [19,20]. Ionophore A23187 was obtained from Calbiochem Behring (La Jolla, CA, USA), fatty acid-free human serum albumin and soybean lipoxygenase from Sigma (St. Louis, MO, USA).

2.2. *Lipoxin synthesis by soybean lipoxygenase*

Leukotrienes C_4, D_4, E_4 (2 μg) 5(S),6(R)-diHETE or 5(S),6(S)-diHETE (2 nmol) were incubated with soybean lipoxygenase (50,000 U/mg, 20 μg) as described earlier [21,22]. Incubations were performed in phosphate-buffered saline (PBS; Dulbecco's formula without Ca^{2+}/Mg^{2+}; pH 7.4). The leukotrienes and the 5,6-diHETEs were incubated for 60 min at room temperature or for 30 min at 37°C, respectively. The reaction was monitored by UV spectroscopy with scanning between 250 and 350 nm (HP 8450A, Hewlett Packard, CA, USA) and stopped by addition of 1 vol. of methanol. Thereafter $NaBH_4$ was added to reduce the hydroperoxides. After 15 min, HCl (1 M) was added to obtain pH 6, prior to addition of 1 vol. of PBS. The samples were applied on preconditioned Baker-C_{18} disposable columns (500 mg), extracted with 3 ml of water, 2 ml of 25% methanol and were finally eluted with 2 ml of methanol.

2.3. *Preparation and incubation of platelet suspensions*

Peripheral human blood from healthy donors was collected using EDTA (77 mM 7.5% v/v) as anticoagulant. The platelet-rich plasma was removed after centrifugation at 200 × *g* for 15 min and the platelets were washed twice with Tris-buffered (pH 7.4) saline containing 1.5 mM sodium EDTA. Finally the cells were resuspended in PBS to a concentration of approximately 450×10^6 platelets/ml. The platelet suspensions were equilibrated for 5 min at 37°C prior to incubation with various concentrations of the 5,6-diHETE isomers, 1 μM LXA_4 or 10 μM LTA_4 for various times or with 10 μM LTC_4, D_4 or E_4 for 30 min. All incubations were performed in the presence of ionophore A23187 (1 μM). Human serum albumin (0.3 mg/ml) was always added before incubation with LTA_4. Reactions were terminated by addition of 5 vols. of ethanol.

2.4. *Product purification and identification*

After centrifugation and evaporation of the samples, the remaining material was dissolved in 250 μl of 50:50 methanol/water (v/v). Reversed-phase HPLC was performed, using an Ultrasphere-ODS column 250 × 4.6 mm or 150 × 4.6 mm (Beckman San Ramon, CA, USA), eluted with methanol/water/acetic acid (60:40:0.01 v/v) or acetonitrile/methanol/water/acetic acid (23:15:61:0.8, v/v, apparent pH 5.6), respectively, at a flow rate of 1 ml/min. A computerized diode array spectrophotometer (HP 8451A, CA, USA) or a variable wave-

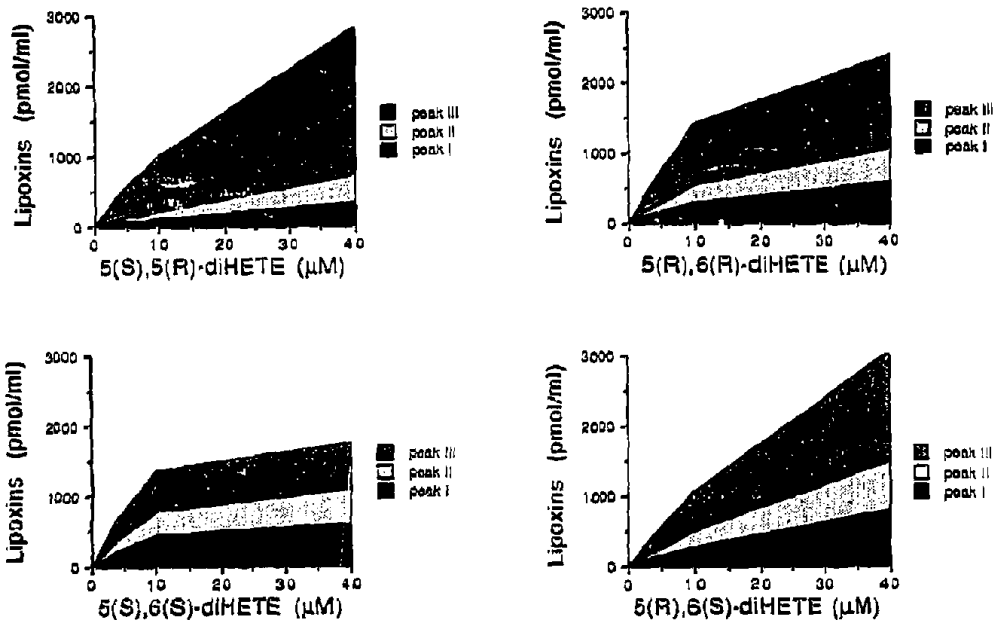

Fig. 2. Dose-response curves of platelet-dependent lipoxin formation from exogenous 5,6-diHETEs. Human platelet suspensions were incubated for 30 min at 37°C in the presence of ionophore A23187 (1 μM). The amounts of the products are successively added together and presented as an area graph (peaks I, II and III, cf. Fig. 1).

length UV detector (Kratos Spectroflow 783, Ramsey, NJ, USA) connected to an integrator (Spectra Physic 4270, San José, CA, USA) was used for UV-detection at 302 nm and quantitation of the products. The compounds were identified by co-chromatography with synthetic or biosynthetic standards and by on-line UV spectroscopy.

3. RESULTS AND DISCUSSION

Human platelet suspensions were incubated with either of the four 5,6-diHETE epimers. Each epimer was converted to three products, absorbing at 302 nm and eluting as distinct peaks on reversed-phase HPLC (Fig. 1). Ad judged by UV spectroscopy, all compounds contained conjugated tetraenes, possessing maximal absorbance at 302 nm and shoulders at 288 and 316 nm. The lipoxin formation was markedly stimulated by calcium ionophore A23187. Thus, incubation of platelets with 5(S),6(R)-diHETE (6 μM) led to the formation of 56 and 744 pmol LXA_4/ml, in the absence and presence of A23187 (1 μM), respectively. This is in accordance with the effect of A23187 on the conversion of LTA_4 to lipoxins [23]. The conversion of 5,6-diHETEs to lipoxins increased in a substrate concentration-dependent manner (0–40 μM) (Fig. 2).

The 5(S),6(R)-diHETE isomer was converted to one major and two minor lipoxins (Fig. 1). The major compound (peak III:A) co-eluted with synthetic LXA_4. One of the two minor peaks (peak II:A) co-eluted with 11-*trans*-LXA_4, while the second minor compound (peak I:A) eluted at a shorter retention time. This compound did not co-elute with synthetic 7-*cis*-11-*trans*-LXA_4 [24]. Incubations with 5(S),6(S)-diHETE led to the formation of three compounds in approximately similar amounts. Two of these lipoxin isomers co-chromatographed with 6(S)-LXA_4 (peak III:B) and 6(S)-11-*trans*-LXA_4 (peak II:B), respectively, while the third product displayed a shorter retention time (peak I:B). Platelets incubated with 5(R),6(R)-diHETE or 5(R),6(S)-diHETE formed one major and two minor more polar lipoxin products, from the respective substrate. The major lipoxins eluted at longer retention times than LXA_4 (peaks III:C and III:D; Fig. 1). In analogy with the conversion of 5(S),6(R)- and 5(S),6(S)-diHETE, the formation of 5(R)-LXA_4, 5(R),6(S)-LXA_4 and the 11-*trans* isomers of these compounds can be speculated. Thus, peaks II:C and III:C would most probably be 5(R)-11-*trans*-LXA_4 and 5(R)-LXA_4, whereas II:D and III:D would be 5(R),6(S)-11-*trans*-LXA_4 and 5(R),6(S)-LXA_4, respectively. The various 11-*trans*-lipoxin-isomers obtained from the four 5,6-diHETEs were probably formed by spontaneus *trans*-isomerisation of the corresponding LXA_4 isomer. Thus, isomerisation of 5,6-diHETEs to 11-*trans*-5,6-diHETEs would lead to formation of substrates, lacking the intramolecular 1,4-*cis*-pentadiene structure which is a prerequisite for substrates participating in lipoxygenase catalyzed reactions [25]. Furthermore, a significant *trans*-isomerisation to 11-*trans*-LXA_4 was observed in a buffer solution of LXA_4 kept at room temperature for 48 h (result not shown).

The structures of peaks I:A–D (Fig. 1) are unknown. Since the product formed from 5(S),6(R)-diHETE did not co-elute with 7-*cis*,11-*trans*-LXA_4, a 7-*cis*,11-*trans*

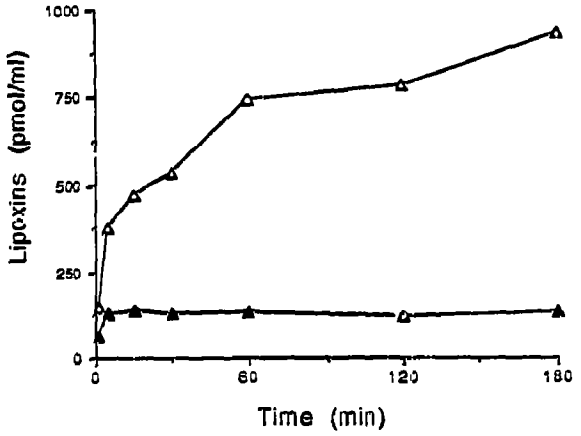

Fig. 3. Time-course of platelet-dependent conversion of LTA_4 to lipoxins. Human platelet suspensions were incubated with LTA_4 (10 μM) at 37°C in the presence of human serum albumin (0.3 mg/ml) and ionophore A23187 (1 μM). (△) LXA_4 and LXA_4-isomers; and (▲) all-*trans*-LXB_4-isomers.

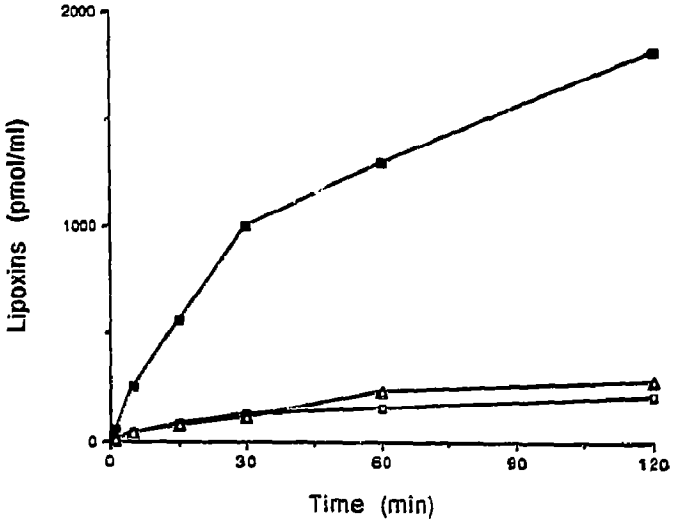

Fig. 4. Time-course of the conversion of exogenous 5(S),6(R)-diHETE (10 μM) to lipoxins in human platelets. The incubations were performed at 37°C in the presence of A23187 (1 μM). (■) LXA_4; (△) 11-*trans*-LXA_4; (□) peak I (cf. Fig. 1).

double-bound configuration of these compounds can probably be excluded. Incubations of 5(S),6(R)- and 5(S),6(S)- with soybean lipoxygenase resulted in formation of identical products as those obtained in platelet incubations (results not shown). These findings exclude the possibility that the unknown compounds eluting in peaks 1:A–D are 15(R)-LXA_4 isomers, since the soybean lipoxygenase is specific for a 15(S)-hydroxylation [26]. Furthermore, the results indicate that the products are nor formed by a platelet-dependent isomerase. In addition, the compounds formed by 15-lipoxygenase were not reduced by $NaBH_4$, which excludes the possibility that they were 15-OOH-LXA_4 isomers. Finally, ω-metabolism of LXA_4 and LXB_4 in human leukocytes has been reported [27]. To investigate whether ω-metabolism of lipoxins could be performed by platelets, platelet suspensions were incubated with LXA_4. Except *trans*-isomerisation of LXA_4 to 11-*trans*-LXA_4 no metabolism to tetraene-containing compounds could be observed (results not shown). This is in accordance with the reported inability of human platelets to convert LTB_4 to ω-metabolites [28].

Formation of cysteinyl lipoxins in human eosinophils after incubation with 15-HETE and ionophore A23187 has been reported [22]. It was therefore of interest to investigate the capacity of human platelets to transform the cysteinyl-leukotrienes to the corresponding lipoxins. Platelet suspensions were incubated for 30 min with LTC_4, D_4 or E_4 (10 μM) in the presence of calcium ionophore A23187 (1 μM). Detectable amounts of cysteinyl lipoxins could not be observed in these experiments. Taken together the results indicate that platelet-dependent 15-hydroxylation is independent of the configuration of the hydroxyl groups at carbons 5 and 6. In contrast, the reaction is inhibited by the presence of the cysteinyl-containing substituents of leukotrienes C_4, D_4 or E_4 at carbon 6.

Addition of LTA_4 (10 μM) to human platelet suspensions induced rapid formation of LXA_4, 6(S)-LXA_4, 6(S)-11-*trans*-LXA_4, 11-*trans*-LXA_4 and all-*trans*-LXB_4 isomers during the first 5 min of incubations (Fig. 3). Thereafter the production of LXA_4 and the LXA_4-isomers continued although at a lower formation rate. In contrast, the all-*trans*-LXB_4-isomers, which can only be formed from the tetraene epoxide, ceased after 5 min. These results indicate that lipoxins are formed from LTA_4 via two different pathways in human platelets. First, a rapid conversion of LTA_4 to the unstable lipoxin intermediate, 15-OH-LTA_4, occur, followed by a subsequent hydrolysis of this tetraene epoxide to the various lipoxins [17]. Second, LTA_4 is hydrolyzed to the stable 5(S),6(R)-diHETE and 5(S),6(S)-diHETE, which are subsequently lipoxygenated at C-15, leading to formation of LXA_4 and 6(S)LXA_4, respectively. In agreement, incubation of platelets with 5(S),6(R)-diHETE (10 μM) led to formation of LXA_4 and 11-*trans*-LXA_4, which increased with time (0–120 min; Fig. 4). Interestingly, the formation rates of LXA_4-isomers were approximately the same after 30 min, whether LTA_4 or 5(S),6(R)-diHETE were used as substrate.

In summary, the present finding demonstrates that human platelets convert 5,6-diHETE to lipoxins. In contrast, the platelets were unable to transform cysteinyl-leukotrienes to the corresponding lipoxins. Furthermore, the results indicate that platelet-dependent lipoxin formation from LTA_4 proceeds both via lipoxygenation of the unstable tetraene epoxide and via initial conversion of LTA_4 to 5,6-diHETE, and subsequent lipoxygenation at C-15. The latter route of formation

may be of special importance in certain tissues, such as the liver and lung, with high epoxide hydrolase activity [29], leading to enzymatic formaten of 5(S),6(R)-di-HETE.

Acknowledgements: We thank Ms. Inger Ericsson and Ms. Barbro Näsman-Glaser for excellent technical assistance. This project was supported by the Swedish Medical Research Council (Project no. 03X-6805), the Swedish Heart and Lung Foundation, King Gustaf V 80-years Fund, Magnus Bergvalls Foundation, the National Association for the Prevention of Asthma and Allergies and Karolinska Institute's Research Funds.

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