Dual Inhibition of Cyclooxygenase and Lipoxygenase Pathways by Lipoproteins in Human Platelets

SHEIKH ARSHAD SAEED*, MUHAMMAD ANWAR WAQAR, AFAQ MOTIWALA, SYED UMER ALI, MUHAMMAD UMAIR KHAN, MUHAMMAD ATIF WAQAR AND RUSHNA PARVEZ ALI

Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan.

(Received 14th June 2007, revised 18th July 2007)

Summary: Products of arachidonic acid (AA) metabolism *i.e.*, prostaglandins (PGs), prostacyclin (PGI_2), thromboxane-A_2 (TXA_2) and lipoxygenase metabolites serve important roles in the pathogenesis of ischaemic heart disease and thrombosis. In the present study, we investigated the effects of lipoproteins on the products of AA metabolism produced by bovine seminal vesicles (BSV) and human blood platelets. Lipoproteins were separated by density gradient zonal ultracentrifugation and their effects on the synthesis of various AA metabolites were measured using radioimmunoassays. The results show that human lipoproteins VLDL, LDL and HDL inhibited AA metabolism to varying extents. VLDL, LDL and HDL inhibited the biosynthesis of prostaglandin E_2 and 6-ketoprostagladin $F_{1\alpha}$ (a stable metabolite of prostacyclin) in a concentration related manner. All three lipoproteins also exerted an inhibitory influence on the biosynthesis of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) although not in a concentration-related manner. The effects of lipoproteins on the production of AA metabolites by human platelets *i.e.* TXA_2 and 12-hydroxy-eicosatetraenoic acids (12-HETEs) were also studied using radiometric thin layer chromatography coupled with automated data integrator system. In human platelets, HDL had a concentration-dependent inhibitory effect on the production of 12-HETE and TXA_2, whereas, LDL had a strong inhibitory effect on TXA_2 production but weakly inhibited the production of 12-HETE. In contrast, VLDL did not exhibit any effect on AA metabolism by platelets. These results demonstrate an hitherto unrecognized property of human lipoproteins and point to a new facet of lipoprotein action.

Introduction

The products of arachidonic acid (AA) metabolism *i.e.*, prostaglandins (PGs), prostacyclin, thromboxane A_2 (TXA_2) and hydroxy-eicosatetraenoic acids (HETEs) are well characterized mediators of inflammatory and immunological reactions. They may act in autocrine or paracrine manner in the pathophysiology of several diseases [1]. Prostacyclin (PGI_2) is a vasodilator and inhibits platelet aggregation [2]. PGE_2 is a vasodilator and a potent inhibitor of lymphocyte and macrophage activation. It is also a mediator of fever. TXA_2 is a vasoconstrictor and stimulates platelets aggregation whereas 12-HETE is a chemotactic factor [3].

The primary function of lipoproteins is the transport of various lipids from intestine and liver to most of the tissues for oxidation and to the adipose tissue for storage Recent studies have shown that lipoproteins may also have other functions. It has been reported that high density lipoproteins (HDL) stimulate whereas low density lipoproteins (LDL) inhibit the PGI_2 synthetase activity [4-5]. On the other hand, it has been demonstrated that both LDL and HDL stimulate the formation of 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI2) in cultured human cells [6]. Since lipoprotein levels are elevated in several diseases, it is likely that they may have a modulatory effect on products of AA metabolism.

In this study we have investigated the effects of human lipoproteins on PG synthesis by isolated PG synthetase using radioimmunoassay [7]. We have also examined the effects of lipoproteins on AA metabolism by human platelets.

Results and Discussion

The effect of different concentrations of various lipoproteins on the biosynthesis of PGE_2 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ is shown in Table-1 and Fig. 1. HDL decreased PGE_2 synthesis by 25 ± 4 %, 51 ± 7 % and 83 ± 4 % at a concentration of 0.11, 1.1 and 3.2 (mg protein/ ml assay volume) respectively, whereas at the same protein concentrations HDL

*To whom all correspondence should be addressed.

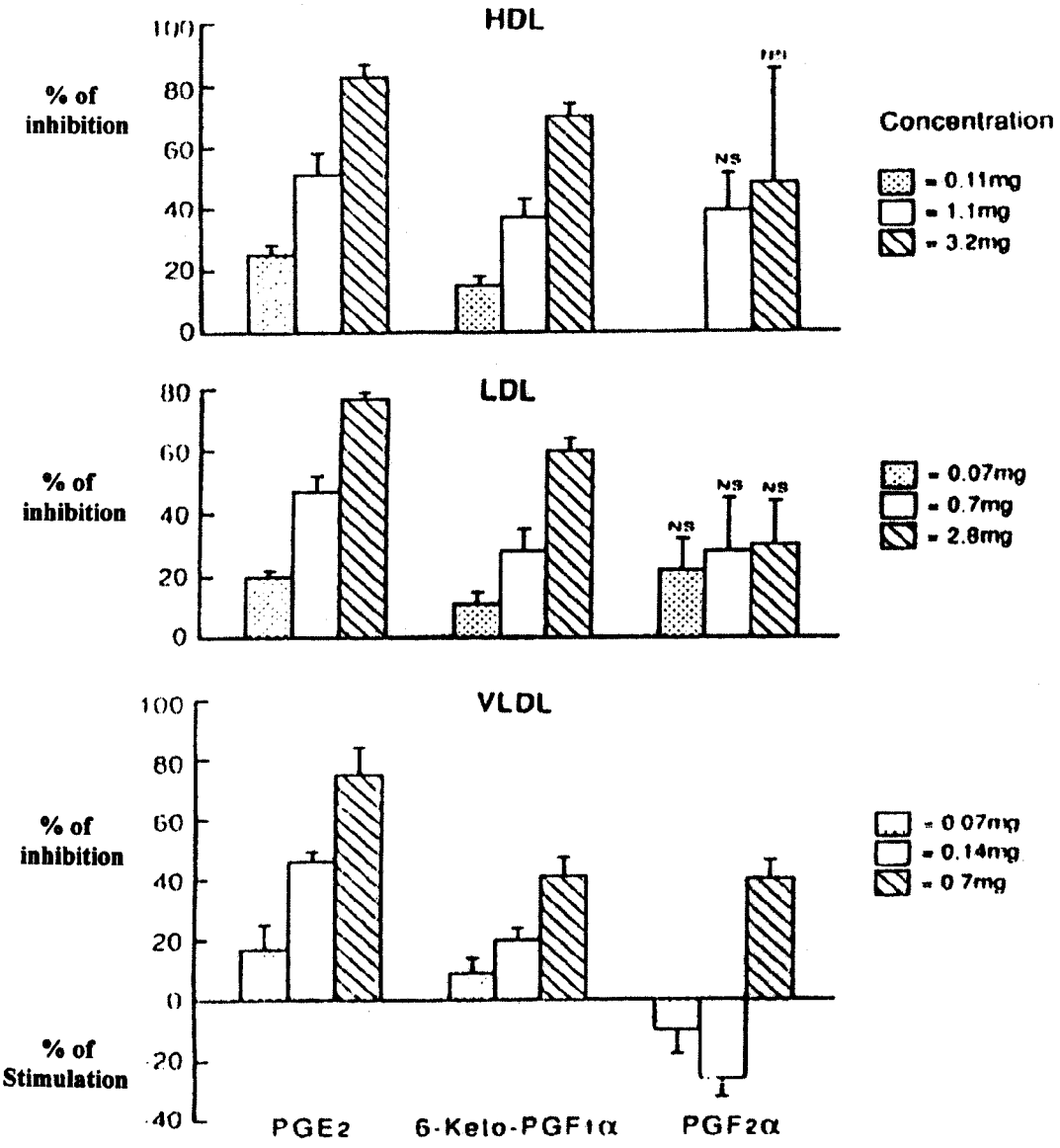

Fig. 1. Effect of various concentrations of HDL, LDL and VLDL on the biosynthesis of PGE_2, 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$. Plasma lipoprotein fractions were isolated and their effect on the biosynthesis of PGs from arachidonic acid were measured against PG synthase activity of bovine seminal vesicle (BSV) microsomes as described in materials and methods.

produced 15 ± 3 %, 37 ± 6 % and 70 ± 4 % decrease in 6-keto-$PGF_{1\alpha}$ synthesis (Fig. 1). The IC_{50} values for HDL-induced inhibition of PGE_2 and 6-keto-$PGF_{1\alpha}$ were 1.05 + 0.09 and 1.95 ± 0.14 (mg protein/ ml assay volume) respectively. HDL also inhibited $PGF_{2\alpha}$ synthesis but not in a concentration manner. On the other hand, LDL produced 20 ± 2 %, 47 ± 5 % and 77 ± 2 % decrease in PGE_2 synthesis at a concentrations of 0.07, 0.7 and 2.8 (mg protein/ ml assay volume) respectively, whereas it produced 11 ± 4 %, 28 ± 7 % and 80 ± 4 % decrease in 6-keto-$PGF_{1\alpha}$ synthesis at the same protein concentration (Fig. 1). The IC_{50} values for LDL-induced inhibition of PGE_2 and 6-keto-$PGF_{1\alpha}$ were 0.92 ± 0.12 and 2.15

Table-1:Comparative inhibitory effects of human lipoproteins on the biosynthesis of prostaglandin (PG) E_2, $PGF_{2\alpha}$ and 6-keto $PFG_{1\alpha}$

Lipoprotein (L)	Concentration (mg protein/ ml assay volume)	Mean inhibition of PG biosynthesis PGE_2	6-keto $PFG_{1\alpha}$	$PGF_{2\alpha}$
.HDL	0.11	25 ± 4	15 ± 3	NA*
	1.1	51 ± 7	27 ± 6	39 ± 12
	3.2	83 ± 4	70 ± 4	48 ± 19
LDL	0.07	20 ± 2	11 ± 4	22 ± 10
	0.7	47 ± 5	28 ± 7	22 ± 10
	2.8	77 ± 2	60 ± 4	30 ± 14
VLDL	0.07	17 ± 7	9 ± 5	10 ± 8 (S)
	0.14	46 ± 3	20 ± 4	26 ± 6 (S)
	0.7	75 ± 9	41 ± 6	40 ± 6

S= Stimulation NA* = note active.

Results are mean ± S.E.M. for six determinations. Each lipoprotein had a different stock protein concentration and the concentrations for tests against PG biosynthesis were chosen to obtain a concentration-related effect upto a maximum effect.

± 0.11 (mg protein/ ml assay volume) respectively. The effect of LDL on $PGF_{2\alpha}$ synthesis, though inhibitory in nature, was not concentration dependent. VLDL also decreased PGF_2 synthesis by 17 ± 7 %, 46 ± 3 % and 75 ± 9 % in a concentration range of 0.07, 0.14 and 0.7 (mg protein/ ml assay volume) respectively (Fig. 1). The IC_{50} for VLDL induced inhibition of PGF_2 synthesis was 0.21 ± 0.01 (mg protein/ ml assay volume). VLDL inhibited 6-keto-$PGF_{1\alpha}$ by 41 ± 6 % at the maximum protein concentration obtainable through the isolation procedure. At lower protein concentrations, VLDL stimulated $PGF_{2\alpha}$ production, whereas at higher protein concentration it inhibited $PGF_{2\alpha}$ synthesis by 40 ± 6 %.

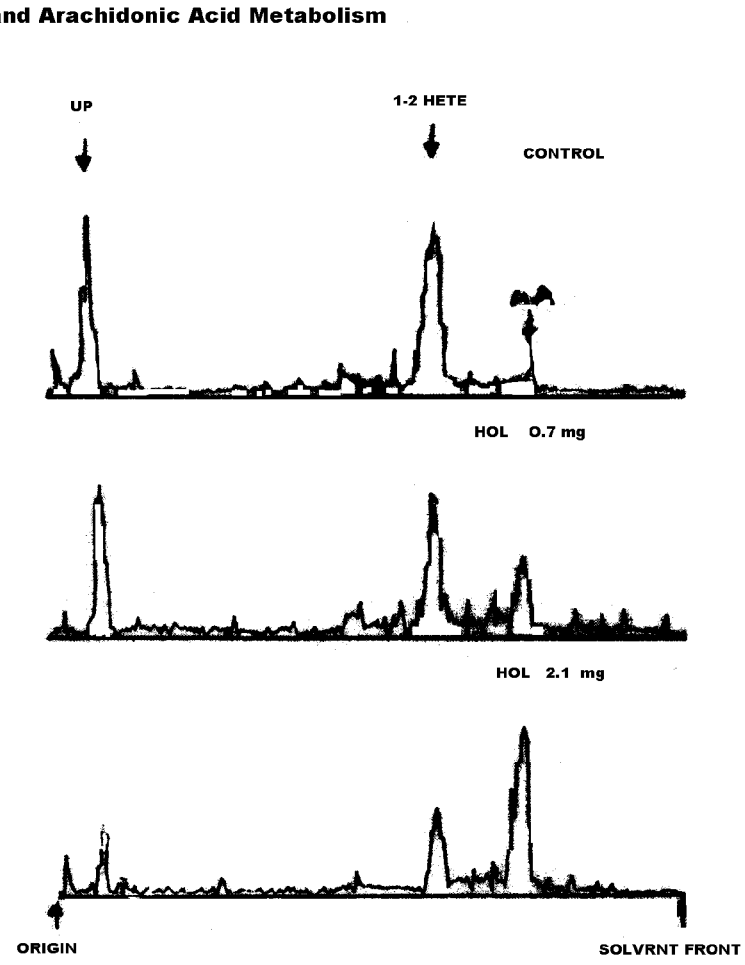

Fig. 2: Radiochromatographic scan showing the profile of lipoxygenase metabolites produced by homogenates of human platelets incubated with [1-^{14}C] AA in the absence and presence of different concentrations of HDL.

[1-^{14}C] AA is also metabolized to a more polar lipoxygenase product designated as LP1. It is possible that LP1 is tri-hydroxyeicosatrienoic acid (THETE) as described by Bryant and Bailey [8]. The effect of different concentrations of lipoproteins on [1-^{14}C] AA metabolism in human platelet is presented in the form of representative radiochromatographic scans obtained after thin layer chromatography of the products of incubation. The data presented show that HDL inhibited 12-HETE formation in a concentration-dependent manner (Fig. 2). HDL decreased 12-HETE formation by 12 ± 1 %, 58 ± 4 % and 81 ± 3 at the given concentration of 0.07, 0.7 and 2.1 (mg protein/ ml assay volume) respectively. HDL also decreased the formation of TXB_2 in human platelets in a concentration dependent manner (Fig. 3). HDL produced a 23 ± 3 %, 37 ± 6 % and 72 ± 3 % decrease in TXB_2 formation at the same protein concentrations. In similar experiments, LDL also decreased the formation of TXB_2 (Fig. 4) whereas it produced a weak inhibitory effect on the formation of 12-HETE (Fig. 5).

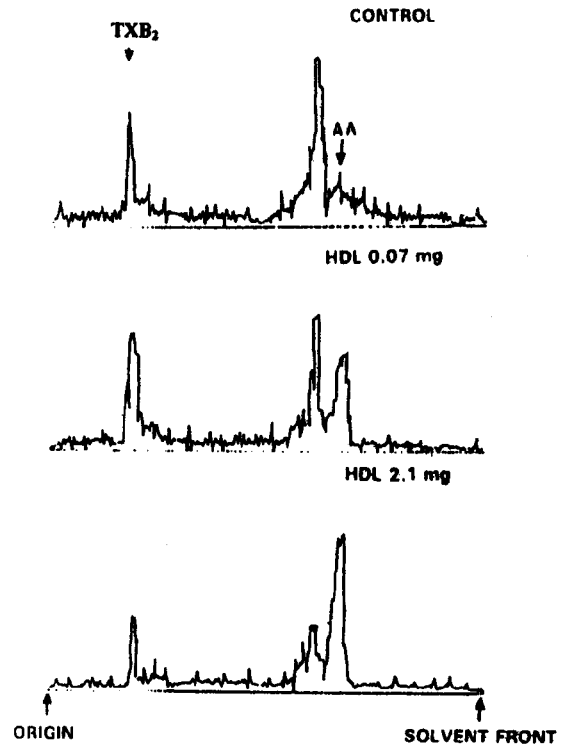

Fig. 3: Radiochromatographic scan showing the profile of COX metabolites produced by homogenates of human platelets incubated with [1-^{14}C] AA in the absence and presence of different concentrations of HDL.

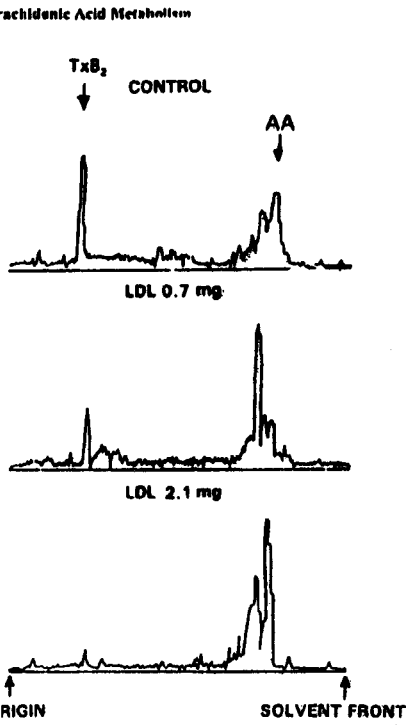

Fig. 4: Radiochromatographic scan showing the profile of cyclooxygenase metabolites produced by homogenates of human platelets incubated with [1-^{14}C] AA in the absence and presence of different concentrations of LDL.

To eliminate the possibility of artifact due to binding of different PGs by human lipoproteins we conducted the following experiment: 0.25 μCi each of $_3$H-PGE_2, ^{3}H-$PGF_{2\alpha}$ and ^{3}H-6-keto-$PGF_{1\alpha}$ (Amersham International plc, UK) were incubated with lipoproteins at (mg protein/ ml) VLDL (0.7), HDL (3.2) and or LDL (2.8) for 20 minutes. The reaction was stopped with citric acid and the PGs were extracted with ethyl acetate and quantified by liquid scintillation spectrometry. Percentage losses of titrated PGE_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ were respectively 7 ± 3, 12 ± 7 and 10 ± 3 (n = 3).

The results of present study demonstrate that human lipoproteins exert differential effects on AA metabolism by BSV and human platelets enzymes. It exhibited an inhibitory effect on PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis. The magnitude of inhibition depend upon the lipoprotein concentration indicative that it is a concentration-released effect. On the basis of protein concentration, VLDL was 4-5 times more potent in inhibiting PGE_2 synthesis when compared to HDL and LDL. In contrast, to their inhibitory

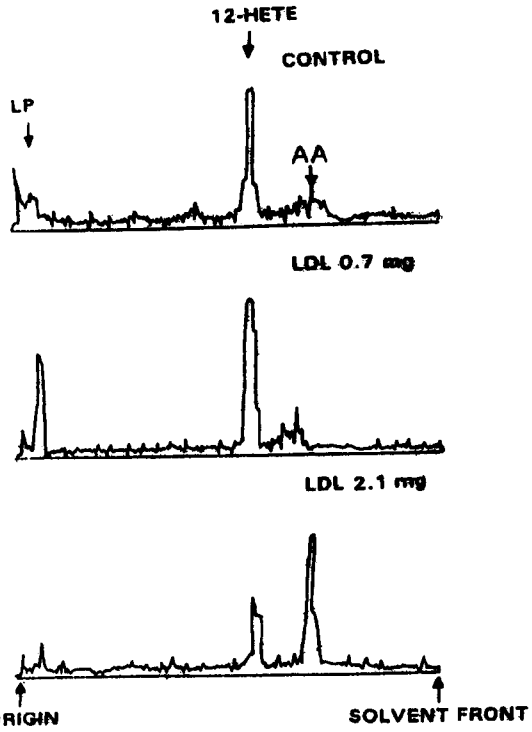

Fig. 5: Radiochromatographic scan showing the profile of lipoxygenase metabolites produced by homogenates of human platelets incubated with [1-^{14}C] AA in the absence and presence of different concentrations of LDL.

effects on PGE_2 and 6-keto-$PGF_{1\alpha}$, HDL and LDL stimulated $PGF_{2\alpha}$ synthesis, though it was not concentration-dependent. VLDL stimulated $PGF_{2\alpha}$ production at lower protein concentrations whereas it inhibited $PGF_{2\alpha}$ synthesis at higher protein concentration. The reason for this paradoxical effect of VLDL on $PGF_{2\alpha}$ synthesis is not clear. VLDL consists of mainly triglycerides as compared to LDL and HDL which contains cholesterol as their primary lipid component. It is likely that different lipid constituents of lipoproteins may exert differential effects on $PGF_{2\alpha}$ synthesis.

Our results are in marked contrast to those studies which reported that both LDL and HDL stimulate 6-keto-$PGF_{1\alpha}$ formation in mammalian smooth muscle cells. Furthermore, a stimulatory effect of HDL on prostacycline synthesis in endothelial cells has also been reported [9-10]. The reasons for these diverse stimulatory or inhibitory effects of lipoproteins in a variety of tissues are not clear, however, it is possible that PG synthetase complex from different tissues or species may have different sensitivities to various lipoproteins. Such diversity has been reported for different sensitivities of paracetamol (anti-pyretic drug) against brain COX [11].

We have also studied the effects of all the three lipoproteins on AA metabolism by platelets and found that HDL inhibits 12-HETE and TXA_2 production by human platelets in a concentration-dependent manner. LDL is a potent inhibitor of TXA_2 production in human platelets whereas it is weakly inhibitory on the formula 12-HETE. Unlike HDL and LDL, VLDL has no effect on AA metabolism in human platelets. Our results are complementary to the existing data that HDL inhibits 12-lipoxygenase activity in platelets and decreases 12-HETE formation [12]. The strong inhibitory effects of HDL on 12-HETE and TXA_2 formation may be beneficial as both of these A A metabolites play an important role in platelet aggregation and thrombus formation.

In addition, to their role in lipid transport, lipoproteins can also exert other beneficial effects. Lipoproteins can bind to endotoxin and prevent its lethal effects in rodents [13]. Lipoproteins can also decrease endotoxin-induced cytokine secretion by macrophages [14] and thereby decrease their toxic effects [15]. The ability of lipoproteins to inhibit the synthesis of A A metabolites (mediators of several inflammatory responses) may be an additional non-specific mechanism operative in host defense in several disease states and inflammatory conditions.

In conclusion, this study points to an hitherto unrecognized property of lipoproteins that may mediate some of the biological actions of these substances. Furthermore, these apparent differential inhibitory effects of lipoproteins on AA metabolism may well have physiological significance, since the biological properties of PGs E_2, $F_{2\alpha}$, prostacyclin and TXA_2 are different. Since lipoproteins levels are elevated in several diseases such as coronary heart disease and diabetes mellitus [16-17], it is likely that they may modulate a wide range of biological effects of different products of AA metabolism in the pathophysiology of these diseases.

Experimental

Materials

Arachidonic acid (A A) (grade 1:99 % pure) and reduced glutathione were purchased from Sigma

Chemical Company (St. Louis, MO, USA). ^{3}H-PGE2 (SA 50-60 mCi/ mmol), ^{3}H-$PGF_{2\alpha}$ (SA 160-180 Ci/ mmol), ^{3}H-6keto- $PGF_{1\alpha}$ (SA 120-180 Ci/ mmol), [1-14C] AA (SA 58 mci/ mmol), [^{3}H] thromboxane B_2 (SA 120 Ci/ mmol) and 12-hydroxy-[^{3}H]-eicosatetroaenoic acid (12-HETE) (SA 100 Ci/ mmol) were obtained from Amersham International plc, U.K. All other chemical used were of highest purity grade.

Preparation of Lipoproteins

Blood obtained from 12 hours fasted healthy volunteers (age range 20-30 years) was collected in EDTA. The final concentration of EDTA was 1 mg/ ml. The plasma lipoproteins were separated by sequential density gradient ultracentrifugation [18]. After isolation, each lipoprotein fraction was sterilized BY filtration through a Millipore (0.45 μm) filter (Millipore Crop; Bedfor4d, MA) and stored at 4 °C. The protein content of each lipoprotein fraction was determined by using human serum albumin as standard and adjusted to initial protein concentration for each batch [19]. The homogeneity of each lipoprotein fraction was confirmed by agarose gel electrophoresis and the purified lipoproteins were used within one month of preparation.

Assay of Prostaglandin Biosynthesis

The effects of lipoproteins on the biosynthesis of various PGs from AA were measured against PG synthetase activity of bovine seminal vesicles (BSV) microsomes (Miles Laboratories Inc., USA) [7]. The standard assay mixture contained in a final volume of 1.0 ml., 50 mM phosphate buffer, pH 7.4, 1.3 mM reduced glutathione, an appropriate amount of test lipoprotein, 4 mg of BSV microsomes and 61 μM AA (as the sodium salt). After an incubation period of 20 minutes with gentle shaking at 37 °C, the reaction was terminated by addition of 0.5 ml of 0.4 M citric acid 7 ml of ethyl acetate. The reaction mixture was vortexed and centrifuged for 5 minutes at 600 x g. The ethyl acetate layer was removed, evaporated to dryness under nitrogen and the residue dissolved in 1 ml of phosphate buffer, (pH, 7.4). PGF_2, $PGF_{\alpha 2}$ and 6-keto-$PGF_{1\alpha}$ were measured by specific radioimmunoassay as described earlier [20]. All experiments were performed with appropriate controls and each lipoprotein was tested at three different protein concentrations to determine the IC_{50} values (concentrations required to inhibit PG synthesis by 50 %) to get an estimate of relative potency of different lipoproteins.

Arachidonic Acid Metabolism by Human Platelets

Human blood platelets from donors were routinely obtained in plastic bags containing 30-40 ml of concentrated platelet-rich plasma (PRP) from Dr. Panjwani Center for Molecular Medicine and Drug Research, ICCBS, University of Karachi, Karachi-75270, Pakistan. The PRP was centrifuged at 1000 g for 5 min and the supernatant was discarded. The remaining PRP was then centrifuged at 1,200 g for 20 min and the sedimented platelets were washed twice with an ice-cold phosphate buffer (50 mM, pH 7.4) containing sodium chloride (0.15 M) and EDTA (0.2 mM). After centrifugation platelets were resuspended in the same buffer without EDTA at the initial PRP cell concentration. The PRP suspension was homogenized at 4 °C using a Polytron homogenizer for 15 s and the homogenate centri-fuged 1,200 g for 20 min. 300 μl of the supernatant (containing 0.4 mg of protein) was incubated with 10μg unlabelled AA and 0.1 μCi [1-^{14}C] AA in the absence and presence of different concentrations of human lipoproteins. After 15 min with gentle shaking at 37C the reaction was stopped by adding 0.4 ml citric acid (0.4 M) and ethyl acetate (7 ml). After mixing and centrifuging at 600 g for 5 min at 4C, the organic layer was separated and evaporated to dryness under nitrogen.

Residues were dissolved in 50 μl ethanol and 20 μl were applied to silica gel G thin layer chromatography (TLC) plates (Analtech, Delaware, USA). The AA, TXB_2 (a stable degradation product of TXA_2) and 12-HETE standards were spotted separately. The plates cm. By use of this solvent system the various lipoxygenase products (HETEs) were separated with TXB_2 and PGs remaining at the origin [21].

The solvent system used for the separation of various PGs and TXB_2 in dried organic extracts of platelet incubates, as above, was ethyl acetate: isooctane : water: acetic acid (11: 5: 10: 2, v/ v, upper phase). Radioactive zones were located and quantified by use of Berthold TLC linear analyzer and chromatography data system (Model LB 511, Berthold, Germany).

Statistical Analysis

The statistical significance was assessed by analysis of variance. Comparison of control values with those obtained with lipoproteins at different

protein concentrations (which is different for every lipoprotein) was made by the Newman-Keuls method of multiple range comparisons. A probability of $P < 0.05$ was chosen as the level of statistical significance [7].

Acknowledgment

We thank Mr. Ghulam Rasool for expert editorial assistance.

References

1. P. C. Cadler, *Am. J. Clin. Nutr.*, **83**, 1505 (2006).
2. T. Nakayama, *Curr. Med. Chem. Cardiovasc. Hematol. Agents*, **3**, 157 (2005).
3. J. D. Imig, *Clin. Sci. (Lond).*, **111**, 21 (2006).
4. G. D. Norata, A. Pirillo, F. Pellegatta, H. Inoue and A. L Catapano, *Int. J. Mol. Med.*, **14**, 353 (2004).
5. M. Vinals, J. Martinez-Gonzalez, J. J. Badimon and L. Badimon, *Arterioscler. Thromb. Vasc. Biol.*, **17**, 3481 (1997).
6. A. Barden, L. J. Beilin, K. Both, J. Ritchie, P. Leedman, B. N. Walters and C. A. Michael, *Clin. Sci. (Lond).* **97**, 697 (1999).
7. S. A. Saeed, D. M. Strickland, D. C. Young, A. Dang and M. D. Mitchell, *J. Clin. Endocrinol. Metab.*, **55**, 801 (1982).
8. R. J. Havel, H. A. Eder and J. H. Bragdon, *J. Clin. Invest.*, **34**, 1345 (1955).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **93**, 265 (1951).
10. M. D. Mitchell and A. P. F. Flint, *J. Endocrinol.* **76**, 101 (1978).
11. S. A. Saeed, R. U. Simjee, F. Mahmood and N. N. Rahman *J. Pharm. Pharmacol.*, **45**, 715 (1993).
12. R. W. Bryant and J. M. Bailey, *Prostaglandins*, **17**, 9 (1979).
13. Y. Shakhov, J. Larrue, N. Perova, B. Dorian, D. Daret, I. Shcherbakova, H. Bricaud and R. Oganov, *J. Mol. Cell Cardiol.*, **21**, 461 (1989).
14. D. N. Das, *Prostaglandins, Leukot. Essent. Fatty Acids*, **72**, 173 (2005).
15. R. J. Flower and J. R. Vane, *Nature*, **240**, 410 (1972).
16. Y. Fujimoto, M. Tsunomori, E. Muta, T. Yammamoto, H. Nishida, S. Sakuma and T. Fujita, *Res. Commun. Mol. Pathol. Pharmacol.*, **85**, 355 (1994).
17. A. M. Barcia and H. W. Harris, *Clin. Infect. Dis.*, **15**, 498 (2005).
18. A. Marson, R. M. Lawn and T. Mikita, *J. Biol. Chem.*, **279**, 28781 (2004).
19. M. C. Englund, A. L. Karlsson, O. Wiklund, G. Bondjers and B. G. Ohlsson *Atherosclerosis*, **158**, 61 (2001).
20. S. Nicholls and P. Lundman, *Semin. Vasc. Med.*, **41**, 87 (2004).
21. M. R. Taskinen, *Curr. Mol. Med.*, **5**, 297 (2005).