1985

# Diet and sex effects upon icosanoid production by endothelial cells

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DIET AND SEX EFFECTS UPON ICOSANOID PRODUCTION BY ENDOTHELIAL CELLS

*Iowa State University* PH.D. 1985

# Diet and sex effects upon icosanoid production by endothelial cells

by

Ramiro Orlando Batres

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Ames, Iowa

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## ABSTRACT

An increase in the intake of linoleic acid decreases the risk of cardiovascular disease. The mechanism for this may involve the function of linoleic acid as a prostaglandin (icosanoid) precursor. The incidence of atherosclerosis is higher in men than in women. Sex differences in prostaglandin synthesis by endothelial cells and platelets may exist. In this research work, the effects of a self-selected diet and diets with 2 levels of linoleic acid upon prostaglandin (PG) synthesis by human endothelial cells were studied, and PG synthesis by these cells when isolated from either male or female babies was compared. Prostacyclin (PG $I_2$) and PG $E_2$ synthesis by endothelial cells isolated from umbilical cord veins incubated 72 hours with culture medium containing 20% plasma from women eating either, a self-selected diet, a diet with 4% (average U.S. diet) or 10% (modified diet) of energy as linoleic acid were measured by radioimmunoassay (PG $I_2$ as 6-keto PG $F_{1\alpha}$). Ten subjects (half in Iowa and half in Nebraska) ate the average U.S. diet during 4 weeks, changing to the modified diet the next 4 weeks (sequence 1), and another 10 subjects followed the inverse sequence (sequence 2). PG $I_2$ synthesis by cells stimulated with 0.125 units of thrombin was lower ($p < 0.01$) when the cells were incubated with plasma from subjects eating the diets in sequence 1. No diet effect was found when the cells were incubated with plasma from subjects eating the diets in sequence 2. PG $E_2$ followed the same pattern as PG $I_2$ but the differences were not significant. The per cent

of linoleic acid was higher in cells incubated with plasma from subjects eating the self-selected diet and the modified diet than in cells incubated with plasma drawn when the subjects were eating the average U.S. diet. In another study, PG $I_2$ and PG $E_2$ synthesis by cells from umbilical cords from male babies was higher than that by cells from female babies. These differences were not changed by incubating the cells with plasma from males or females. No differences in the total fatty acid composition of the cells were found. This study supports previous reports showing that 10 % compared to 4 % of dietary linoleic acid either decreases or has no effect upon PG $I_2$ and PG $E_2$ syntheses, and that there are sex differences in PG $I_2$ and PG $E_2$ syntheses, being both higher in males than in females.

# INTRODUCTION

Coronary heart disease is the number one cause of death in developed countries. In the Framingham study (1), it was found that in 12.5% of men 40 to 44 years of age some form of coronary heart disease developed within the first 14 years of follow up. These men initially were free of coronary heart disease. Rates for other age groups were 16.6% for men between 45 and 49 years of age, and 25% for men 55 years of age and older. In pre-menopausal women the incidence of coronary heart disease was low, but it rose in post menopausal women.

The fact that sex is a risk factor for developing coronary heart disease has been shown in prospective studies, angiographic and autopsy studies, and retrospective case control studies (2). Starting in the 15 to 24 age group, it has been shown (3) that men have about twice the number of fibrous plaques and other complicated lesions compared to women of the same age. It has been suggested that in many subjects the development of atherosclerotic lesions begins at least in the middle of the second decade and probably earlier than that (3).

In the Framingham study, it was shown that total serum cholesterol was related to the development of coronary heart disease, and in several other studies it also has been shown that LDL-cholesterol (cholesterol in low density lipoproteins) is positively associated and that HDL-cholesterol (cholesterol in high density lipoproteins) is negatively associated with the development of coronary heart disease (2).

High fat diets low in polyunsaturated fatty acids and high in cholesterol also have been associated with the development of coronary heart disease, and a diet designed for lowering serum lipid concentrations has been recommended (4). The mechanism by which decreasing serum cholesterol levels and increasing the dietary consumption of polyunsaturated fatty acids may help to prevent the development of atherosclerosis and ischemic heart disease is not known. In a similar way, even though the knowledge about atherosclerosis has increased in recent years, its pathogenesis is still a mystery.

At least three hypotheses have been proposed to explain the pathogenesis of atherosclerosis (5). The first one is the lipid infiltration hypothesis which postulates that increased LDL levels would increase the rate of LDL infiltration in the arterial wall and would increase the cholesterol uptake by endothelial cells, smooth muscle cells and macrophages, which in turn would conduce to cholesterol accumulation and the development of atheromas.

The second one is the endothelial injury hypothesis which postulates that endothelial damage causes platelets to adhere and aggregate on the endothelium. Platelets would release platelet derived growth factor (PDGF), which would stimulate smooth muscle cells to proliferate and to secrete connective tissue matrix elements. Repeated damage to the endothelium could lead to the development of atheromas.

Finally, the third one is the unified hypothesis, which puts together the first two hypotheses. This hypothesis postulates that

damaged endothelial cells become leaky and are infiltrated by LDL and platelets. Platelets release PDGF which stimulates proliferation of smooth muscle cells and, at the same time, macrophages invade the damaged area. The uptake of LDL by smooth muscle cells and macrophages and the secretion of matrix elements increases. If the levels of LDL in blood are elevated, cholesterol derived from LDL accumulates in the smooth muscle cells and macrophages which then become foam cells. Cholesterol might accumulate also in the intercellular space. The accumulated cholesterol, cells, and matrix elements constitute an atheroma (5).

A diet very low in cholesterol, by decreasing serum cholesterol levels and LDL cholesterol, helps to prevent the development of atherosclerosis (4). A diet high in linoleic acid also decreases serum levels of cholesterol (6). In addition, linoleic acid is desaturated and elongated to produce arachidonic acid, which is a precursor for synthesis of prostaglandins, thromboxanes and leukotrienes (7). It has been postulated that a balance between prostacyclin synthesis by endothelial cells and thromboxane $A_2$ synthesis by platelets is necessary in order to avoid either damage to the endothelium by platelets, or thrombosis, or bleeding problems (8). It has been suggested that if prostacyclin synthesis by endothelial cells is reduced, platelets would adhere to the cells. This process would induce liberation of thromboxane $A_2$ by platelets which in turn would induce other platelets to adhere and aggregate. Platelets would liberate lysosomal enzymes

which would digest the endothelium. These events would be conducive to development of atherosclerosis (8).

There are many different factors which may influence the balance between prostacyclin synthesis by endothelial cells and thromboxane $A_2$ synthesis by platelets. Some of them include, blood levels of glucose, cholesterol, insulin, and lipoproteins, presence of atherosclerosis, and dietary intake of polyunsaturated fats. Specific effects of these factors will be discussed later.

This research project was designed to study first, the relationship between the dietary intake of polyunsaturated fats and prostaglandin synthesis by endothelial cells, and second, whether there were differences in prostaglandin synthesis between males and females. The latter, as mentioned earlier, is a risk factor for the development of ischemic heart disease, and the first one is important because an increase in the dietary intake of polyunsaturated fats is associated with a lower risk of developing atherosclerosis.

Primary cultures of endothelial cells harvested from the vein of human umbilical cords were used for this purpose. Sex differences were studied by using cells harvested from umbilical cords from either male or female babies. Diet effects were studied by incubating the cells with tissue culture medium supplemented with 20% plasma from human subjects eating diets with different amounts of polyunsaturated fats. The subjects whose plasma samples were used were participating in a collaborative study (North Central Regional Project, NC-167, CSRS, USDA)

in which half of them were fed the diets in Iowa, and half of them in Nebraska. Details of the experimental design of that study are presented in the section on experimental design. The group of nutritionists in charge of that particular study kindly provided the plasma samples used to supplement the tissue culture medium, and the umbilical cords were collected by the nurses of the Obstetrics Department of the Mary Greeley Medical Center.

# LITERATURE REVIEW

## Biochemistry of icosanoids

Prostaglandins thromboxane and leukotrienes, collectively called icosanoids are oxygenated metabolites of polyunsaturated fatty acids. Prostaglandins are derivatives of a 20 carbon fatty acid, prostanoic acid. The 20 carbon atoms are arranged as a central five-membered ring with two side chains which have 7 and 8 carbon atoms. The carboxyl group is situated in the 7 carbon chain. Prostaglandins have several oxygens at certain positions in the molecule. These groups and their positions determine their biological activity. Two very important positions are C-9 and C-11 in the ring (Figure 1).

Leukotrienes are derivatives of hydroperoxy acids. They are characterized by three conjugated double bonds. Glutathione can be added in position 5 of leukotriene $A_4$, a derivative of 5,6 epoxide arachidonic acid, by the action of glutathione S transferase to give leukotriene $C_4$. Removal of glutamic acid by the action of glutamyl transpeptidase gives leukotriene $D_4$, the cysteinyl-glycine derivative. Removal of glycine gives leukotriene $E_4$. Now it is known that the slow reacting substance in anaphylaxis is a mixture of leukotriene $C_4$ and $D_4$ (9) (Figure 2).

## Synthesis of icosanoids

Icosanoids are synthesized from essential fatty acids after a series of complex biochemical transformations. A common precursor is

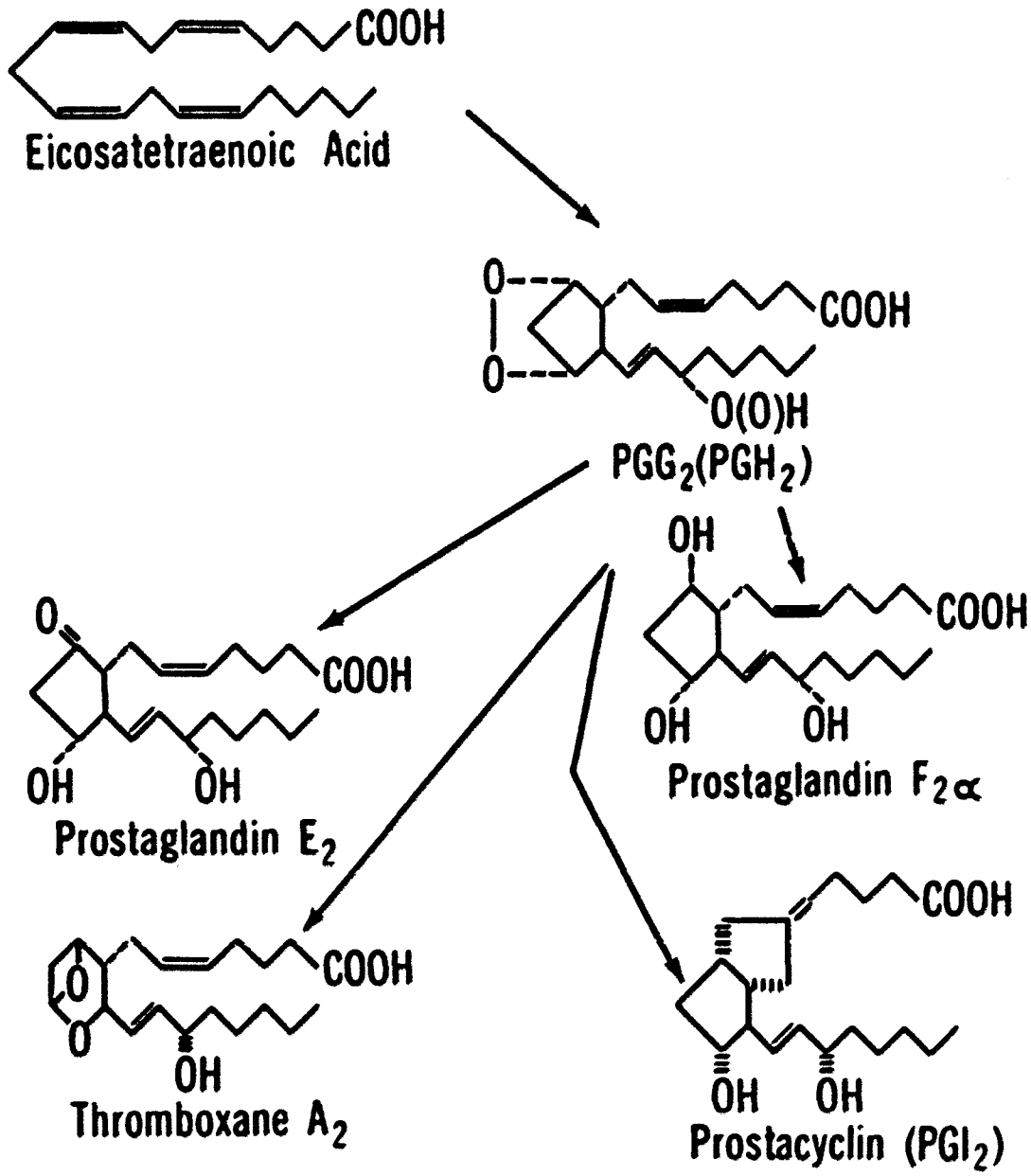


FIGURE 1. Synthesis of Prostaglandins and Thromboxanes

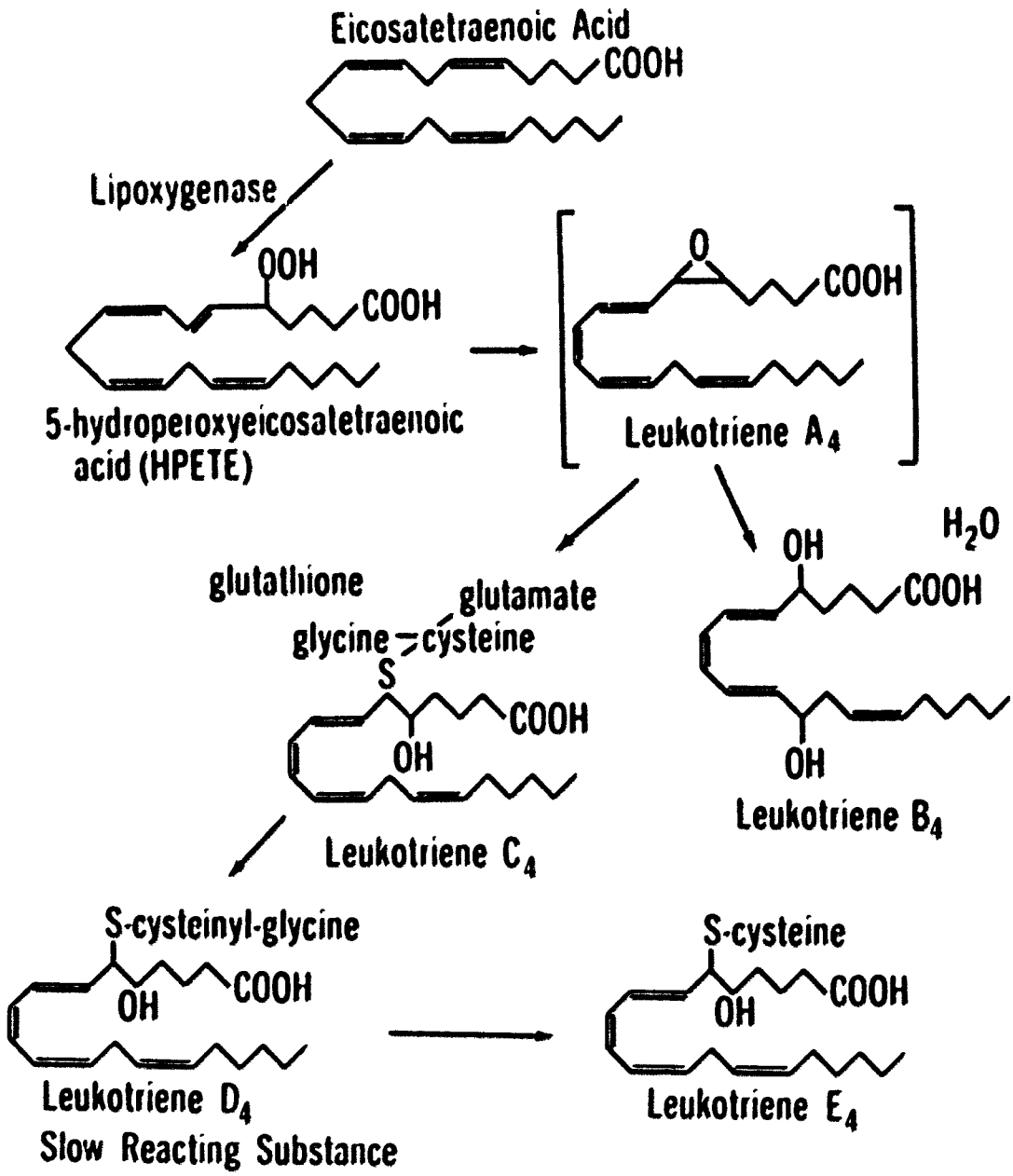


FIGURE 2. Synthesis of Leukotrienes

linoleic acid (C18:2, n-6), which by action of Δ 6 desaturase is converted to gamma-linolenic-acid (C18:3, n-6). This fatty acid is acted upon by an elongase to form di-homo-gamma-linolenic acid (C20:3 n-6). A Δ 5 desaturase forms arachidonic acid (C20:4 n-6) from di-homo-gamma-linolenic acid. Arachidonic acid can be further elongated to docosatetraenoic acid (C22:4 n-6) (Figure 3).

Di-homo-gamma-linolenic acid, arachidonic acid, and docosatetraenoic acid can be substrates for either cyclooxygenase or lipoxygenase. Cyclooxygenase products from di-hommo-gamma-linolenic acid are: prostaglandin $E_1$ (PG $E_1$), prostaglandin $F_1$ (PG $F_1$), and thromboxane $A_1$ (TX $A_1$). Lipoxygenase products from the same fatty acid are: leukotriene $A_3$, leukotriene $C_3$, and leukotriene $D_3$.

Cyclooxygenase products from arachidonic acid are: prostaglandin $E_2$ (PG $E_2$), prostaglandin $F_{2\alpha}$ (PG $F_{2\alpha}$), prostaglandin $D_2$, prostacyclin (PG $I_2$), and thromboxane $A_2$ (TX $A_2$). Lipoxygenase products of arachidonic acid are: leukotriene $A_4$, leukotriene $B_4$, leukotriene $C_4$, leukotriene $D_4$, and leukotriene $E_4$.

Cyclooxygenase products from docosatetraenoic acid are prostaglandins of the 3 series, PG $E_3$, PG $F_{3\alpha}$, etc. The number 3 meaning that they have 3 unsaturated carbon bonds in the chains. Lipoxygenase products of this acid are leukotrienes of the 5 series. (9, 10)

The substrate for cyclooxygenase, usually arachidonic acid, has to be in nonesterified form in order to be acted upon by the enzyme. Cells

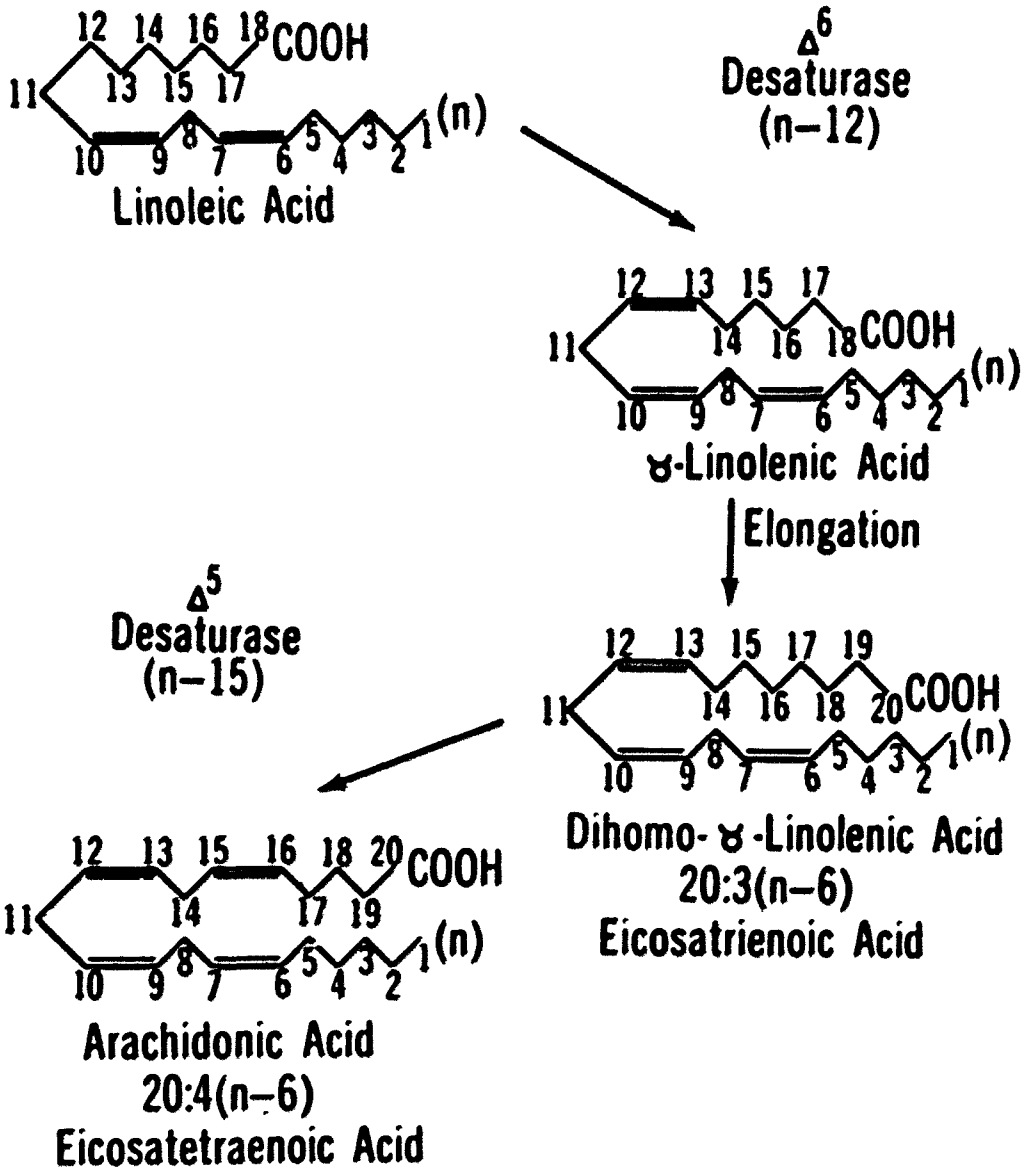


FIGURE 3. Synthesis of eicosatetraenoic acid

obtain nonesterified arachidonic acid from either the medium or by hydrolysis of phospholipids. It has been shown that normally there is a continuous release of fatty acids from membrane phospholipids. This small pool of nonesterified arachidonic acid in the cells would constitute the substrate for prostaglandin synthesis under basal conditions. When cells are stimulated, there is an increase in prostaglandin synthesis. Under this circumstance, arachidonic acid is released from cell membrane phospholipids by action of phospholipases (9,11).

Arachidonic acid is taken up actively by different phospholipids. Phosphatidylinositol has a very fast turnover rate during cell stimulation and it also has a very high concentration of arachidonic acid. Because of its fast turnover rate, it has been hypothesized that it may be the main source of arachidonic acid for prostaglandin synthesis during cell stimulation, even though it constitutes only a very small proportion of all the different phospholipid classes (11,12). It has been hypothesized that arachidonic acid from other phospholipids could be selectively transferred to lysophosphatidylinositol or to lysophosphatidic acid. Arachidonic acid would be released from inositol phospholipids through two consecutive steps; one catalyzed by phospholipase C followed by the second, catalyzed by diacylglycerol lipase (13). It also has been proposed that in platelets, arachidonic acid may be released from phosphatidic acid by a phospholipase $A_2$ (14). However, it has been shown that in many tissues a single stimulus

activates both phospholipase C and $A_2$, releasing arachidonic acid from many other phospholipids too (14).

Thrombin, a serine protease derived from prothrombin during blood coagulation, binds to cell surface platelet receptors and induces breakdown of phosphatidylinositol, synthesis of thromboxane $A_2$, and release of serotonin and lysosomal enzymes (13, 14). Thrombin also binds to endothelial cells and stimulates prostacyclin synthesis. The effect of thrombin upon prostacyclin synthesis is inhibited by mepacrine, a phospholipase inhibitor (15, 16). Prostacyclin synthesized mainly by endothelial cells, and thromboxane $A_2$, synthesized mainly by platelets, have opposite actions. Thromboxane $A_2$ produces contraction of blood vessels and platelet aggregation. Prostacyclin produces vasodilation and inhibition of platelet aggregation. They also have opposite effects on cyclic AMP (cyclic adenosine monophosphate) concentrations. An imbalance between prostacyclin and thromboxane $A_2$ synthesis has been described in several pathological conditions (16).

## Endothelial cells

Arterial vascular disease is a number one public health problem and, as mentioned before, endothelial cell injury is an important component. Thus, the study of factors that may influence the metabolism of endothelial cells may help to increase the understanding of the pathophysiology of arterial vascular disease. In this study, primary cultures of endothelial cells isolated from human umbilical cord veins were used. These cells in culture grow in characteristic monolayers,

and they inhibit platelet aggregation. It has been shown that if a suspension of endothelial cells is incubated with platelet rich plasma at 37 °C, no aggregation of platelets occurs. This characteristic is not shared by either smooth muscle cells or fibroblasts. Part of the thromboresistance of endothelial cells is explained by their ability to synthesize prostacyclin. However, it also has been shown that thromboresistance is also present in cells after they have been frozen and thawed and that it is not changed by prostaglandin inhibitors, suggesting the presence of an alternative mechanism. In addition to thromboresistance, angiotensin-converting enzyme is present in the surface of human endothelial cells, and the cells also synthesize factor VIII, and fibronectin (17).

The purpose of this research project was to study the effect of plasma from subjects eating diets providing different amounts of linoleic acid upon prostaglandin synthesis by endothelial cells growing in culture, and whether differences in prostaglandin synthesis by these cells when derived from either male or female babies could be detected. In addition to linoleic acid and sex differences, many other factors also may affect prostacyclin synthesis by endothelial cells (16). These factors are also important because a decrease in prostacyclin by the endothelium may increase the risk of developing thrombosis and ischemic heart disease by changing the balance of prostacyclin and thromboxane $A_2$ synthesis.

## Effects of diet

Effect of polyunsaturated fats upon platelet function It is known that increased ingestion of polyunsaturated fats produces changes in platelet function. Hornstra (18) found that in rats there was a positive, significant relationship between the time necessary to induce platelet thrombus and the content of sunflowerseed oil in the diet. When the greatest amounts of sunflowerseed oil were consumed, the longest time was necessary to induce a platelet thrombus. He also found that this effect was related to the linoleic acid content of the diet and that this effect was also exerted when PG $E_1$ was infused either intravenously or intraarterially (19,20). Renaud et al. (21,22) found that platelets of rats or rabbits fed a diet rich in butter or stearic acid had a higher susceptibility to thrombin induced aggregation. The opposite was found when rats or rabbits were fed a diet rich in corn oil. Hutton et al. (23) reported a reduction in cataract formation in diabetic rats when a diet high in corn oil was used. Finally, Connor (24) found that saturated fatty acids, when added to human blood, accelerated thrombus formation when tested in vitro.

In man, also, it has been shown that the type of fat ingested has an influence on platelet function. Nordoy and Rodset (25) found that the intake of a diet containing 40% of the calories as soybean oil during 21 days decreased plasma factor three activity. Hornstra et al. (26) reported that the ingestion of a diet containing 35% of the total energy as fat with 12% of the total energy as linoleic acid reduced

platelet aggregation compared to the platelet aggregation observed when a diet containing 35% of the total energy as fat with 4% of the total energy as linoleic acid was eaten. Fleischman et al. (27) reported that an increase in dietary linoleate during two weeks was associated with a decrease in platelet aggregation. A decrease in dietary linoleate during the next two weeks reversed the effect. They also reported that this effect was observed 48 hours after increasing dietary linoleate (28). The same kind of response to dietary linoleate was reported by O'Brien et al. (29).

In long term studies, King et al. (30) observed that in subjects with exudative diabetic retinopathy the consumption of a diet with a high content of linoleic acid during three years reduced the extent of the retinal exudate. Miettinen et al. (31) reported that the thrombotic tendency of platelets was lower in a group of subjects eating a diet with 12% of the energy as linoleic acid than in a group of subjects eating a diet with 4% of the energy as linoleic acid. This was a twelve year clinical study. Houtsmuller (32) and Houtsmuller et al. (33) reported a benefical effect of linoleic acid upon the progress of diabetic retinopathy. However, they did not find an effect of linoleic acid upon platelet aggregation when the platelets were stimulated with adenosine diphosphate (ADP). Renaud et al. (34) suggested that platelet function could be improved by increasing the intake of linoleic acid. They found that after one year of dietary change, platelet aggregation stimulated with thrombin was decreased by increasing the intake of

polyunsaturated fats, but not platelet aggregation stimulated with ADP. The group suggested that the response of platelets to ADP might need several years to be decreased. This statement is in agreement with the suggestion of Hornstra (20) that spontaneous platelet aggregation may be a better parameter to investigate the effect of dietary fats.

Essential fatty acid deficiency and prostaglandin synthesis In essential fatty acid deficiency, a reduction in prostaglandin synthesis has been reported. Christ and Nugteren (35) found that in adipose tissue of essential fatty acid deficient rats, PG $E_2$ synthesis was decreased. Parham et al. (36) reported that a slight increase in the dietary consumption of linoleic acid restored prostaglandin synthesis by platelets of essential fatty acid deficient rats. Friedman (37) showed that platelet aggregation was decreased in essential fatty acid deficient infants when compared to normal controls or the same infants after recovery. Similar changes were observed in the urinary excretion of prostaglandins. Vincent and Zijlstra (38) reported a decreased thromboxane $A_2$ synthesis by platelets of essential fatty acid deficient rats. deDeckere et al. (39) reported a decreased prostacyclin synthesis by hearts and aortas of essential fatty acid deficient rats. This defect was corrected by increasing the amount of calories from linoleic acid in the diet up to 3.5%, and Hoffmann and Forster (40) found that aortic prostacyclin synthesis and prostacyclin efflux from isolated hearts was higher in rats fed a diet high in linoleic acid than in rats fed a diet low in linolec acid.

Effect of polyunsaturated fats upon prostaglandin synthesis In addition to those reports which demonstrated the beneficial effect of the consumption of a diet high in polyunsaturated fatty acids upon platelet aggregation, and a reduction in prostaglandin synthesis in essential fatty acid deficiency, the effect of the intake of this kind of diet upon prostaglandin synthesis also has been studied in normal conditions. Hwang et al. (41) found that the concentration of PG $E_1$ and PG $F_{2\alpha}$ were higher in the serum of rats fed a corn oil diet during 8 weeks than those found in the serum of rats fed a beef tallow diet during the same period of time. Hwang and Kinsella (42) also reported that the concentrations of PG $E_1$ and PG $E_2$ were higher in the serum of rats fed a diet containing 11% of the energy as linoleate during 12 weeks than the concentrations found in the serum of rats fed the same amount of fat as hydrogenated coconut oil. Dupont et al. (43) found that PG $E_2$ concentrations in serum of rats increased in a linear way when the percent of calories from linoleate in the diet increased from 0 to 30%. They also found that serum PG $E_1$ decreased when dietary linoleate was increased from 0 to 2%, then PG $E_1$ increased when dietary linoleate was increased from 2 to 7% and maintained a plateau through 27%. Hulan and Kramer (44) found a significant correlation between the level of linoleic acid in the skin of rats and the capacity of this tissue to synthesize PG $E_2$. Lipinski and Mathias (45) reported that in the adipocyte norephinephrine-stimulated PG $E_2$ and PG $F_{2\alpha}$ synthesis increased with greater polyunsaturation of fat. However, Fine et al.

(46) and Steinberg et al. (47) reported that there was no difference in prostaglandin synthesis by platelets between rats fed diets containing either 20 or 40% of the calories as fat with linoleate to saturated fatty acid ratios of 0.4, 0.8, and 5.5. These diets were fed during 8 to 11 weeks. Needleman et al. (48) reported that when platelets were enriched with linoleic acid, thromboxane $B_2$ synthesis was decreased. Mathias and Dupont (49) reported that the dietary p/s ratio did not affect liver synthesis of prostaglandins, but that the lung synthesis of prostaglandins decreased as the p/s ratio in the diet increased.

In addition to these studies, Spector et al. (50,51) reported that when cultured human umbilical cord vein endothelial cells were enriched with linoleic acid, there was a decrease in prostacyclin synthesis stimulated by either thrombin or a calcium ionophore. A decrease in content of arachidonic acid in cellular phospholipids also was observed. deDeckere et al. (39) showed that feeding diets containing more than 3.5% in energy as linoleic acid did not increase prostacyclin synthesis by isolated rat aortas. Instead, a slight reduction in prostacyclin synthesis was observed. Sullivan and Mathias (52) found that $TXB_2$ in serum increased when dietary energy from linoleic acid increased from 0 to 0.4%, but from 0.4 to 29%, a decrease in TX $B_2$ was observed. Agradi et al. (53,54) and Galli et al. (55) found that feeding rabbits with diets containing either corn oil or butter (11 or 2.2 en % as linoleic acid respectively) for periods of three weeks and three months, conduced to an increase in linoleic acid and a decrease in arachidonic acid in

platelets and aortic phospholipids. Platelet aggregation stimulated by arachidonic acid but not with collagen, was decreased by the diet high in linoleic acid. This diet also decreased the synthesis of TX $B_2$ from $^{14}C$-arachidonic acid in platelets stimulated with collagen, but not the synthesis of TX $B_2$ from endogenous arachidonic acid. The synthesis of prostacyclin-like material by aortas was decreased by the diet high in linoleic acid too. The platelet and aortic cyclooxygenase appeared to be depressed in rabbits eating the corn oil diet.

These studies suggest that a high intake of linoleic acid decreases the capacity of endothelial cells to produce prostacyclin. This effect may reduce the protective action of prostacyclin. However, it has been shown that in liver, depot fat, heart, red blood cells, and some cultured cell lines, the quantity of arachidonic acid increases when the amount of linoleate is increased either in the diet or in the culture medium (56-60).

It could be that increasing the intake of linoleate would increase the tissue stores of linoleate but not necessarily the amount of arachidonate, or at least the proportion of linoleate to arachidonate would be changed in the tissues. It has been suggested that during prostaglandin synthesis, linoleate could be acting as a competitive inhibitor to arachidonate (55,61,62), decreasing prostaglandin synthesis. Fine et al. (46) were unable to change platelet prostaglandin synthesis by increasing the intake of linoleic acid, but in their experiment the fatty acid composition of the platelet

phospholipids remained constant. However, Friedman (37) and Friedman et al. (63) showed that the administration of intralipid to infants was associated with an increase in the level of linoleate and a decrease in the levels of arachidonate in lung, liver, skeletal muscle, renal medulla, renal cortex, brain, adipose tissue, red blood cells, plasma, and platelet phospholipids, and at the same time, platelet aggregation and urinary excretion of prostaglandins were decreased.

The alterations in the distribution of linoleate and arachidonate reported by Friedman (37) and Friedman et al. (63) cannot be extrapolated to normal situations because it has been reported that during the period of total parenteral nutrition, the continuous infusion of glucose, which maintains high levels of insulin in plasma, prevents the mobilization of linoleic acid from the adipose tissue (64,65).

In almost all the studies in which the effect of the intake of different quantities of polyunsaturated fatty acids upon prostacyclin synthesis have been studied, the authors have used either aortic tissues incubated in a buffer or endothelial cells in which the concentration of linoleic acid has been increased by supplementing the tissue culture medium with linoleic acid bound to albumin. These methodologies have several limitations because the effects of other factors normally present in plasma have not been taken into account. Nordoy et al. (66) found that low density lipoproteins inhibited the ability of endothelial cells to counteract platelet aggregation and that the inhibitory effect of LDL was partially reduced by HDL. Beitz and Forster (67) found a

negative correlation between LDL-cholesterol in the incubation solution and prostacyclin synthesis, but a positive correlation between HDL-cholesterol and prostacyclin synthesis. Szczeklik et al. (68) have criticized the studies in which lipoproteins have been used, suggesting that the inhibitory effect of LDL-cholesterol may be explained by the presence of lipid peroxides originated during the long process of lipoprotein isolation. Fleisher et al. (69) showed that when human HDL was incubated with endothelial cells growing in tissue culture, prostacyclin synthesis was increased, and this increase was dependent upon the concentration of HDL in the medium and inhibited by indomethacin. They also found that increasing the amount of arachidonic acid in HDL increases its stimulatory effect upon prostacyclin synthesis.

It has been suggested that increasing the concentration of linoleic acid in the medium or in endothelial cell phospholipids decreases prostacyclin synthesis because these cells do not have the capacity to synthesize arachidonic acid from linoleic acid due to a $\Delta$ 6 desaturase deficiency (51). However, some authors (70) have shown that this is not the case, and that endothelial cells do have the capacity to synthesize arachidonic acid from linoleic acid.

In this study, it was decided to use whole plasma drawn from subjects eating diets with different amounts of linoleic acid as a supplement to the tissue culture medium of endothelial cells. The effect of using these plasma samples to supplement the tissue culture

medium upon prostaglandin synthesis and the fatty acid composition of the cells was studied.

## Effects of sex

Sex differences in platelet function A difference in prostaglandin synthesis between males and females has been reported previously. It has been suggested that these differences are due to an effect of sex hormones upon prostaglandin synthesis. Uzunova et al. (71,72) found that testosterone increased the susceptibility to experimentally induced thrombogenesis in rats and rabbits. Males were more susceptible than females to the effect of testosterone, and estradiol had marginal ameliorating effects only in male rats. Flutamide, an anti-androgen, reduced the mortality rate in rats treated with testosterone.

Johnson et al. (73) reported that platelet responsiveness to adenosine diphosphate was greater in male than in female rats. They also found that castration reduced aggregability in males and increased it in females. Testosterone increased platelet aggregability in both sexes and restored the diminished responsivity observed in castrated males. The same effect was found when platelets were incubated with testosterone in vitro. Estradiol had the opposite effect and decreased aggregability.

Uzunova et al. (74) showed that male mice are more sensitive than female mice to sodium arachidonate induced pulmonary emboli formation and respiratory depression. Pre-treatment of mice with testosterone

increased the effect of arachidonate in both males and females. Pretreatment with aspirin inhibited the effect of arachidonate and pretreatment with estradiol reduced the effect of testosterone.

Chang et al. (75) found that injecting testosterone into male rats did not increase the rate of thromboxane $A_2$ synthesis by their platelets. This study suggests that the effect of testosterone upon platelet aggregability may not be related to TX $A_2$ synthesis.

Sex hormones and prostaglandins in cultured cells The effect of sex hormones upon prostaglandin synthesis have been studied in cells growing in culture. Nakao et al. (76) and Chang et al. (77,78) found that testosterone inhibits and that estradiol increases prostacyclin synthesis by rat aortic smooth muscle cells in culture. They also reported that estradiol stimulates the activity of both prostaglandin cyclooxygenase, and prostacyclin synthetase, and that induction of new protein synthesis is involved in the effect of estradiol.

Sex differences in prostacyclin synthesis Pomerantz et al. (79) reported that aortic rings isolated from male rats produced more prostacyclin than aortic rings isolated from female rats. They also found that ovariectomy increased prostacyclin synthesis, but castration did not have any effect upon prostacyclin synthesis by aortic rings in the male rats. Treatment with estradiol decreased prostacyclin synthesis in the ovariectomized rats, but treatment with progesterone increased it. Testosterone did not have any effect in gonadectomized males and females. Treatment with either estradiol or progesterone did not have any effect upon prostacyclin synthesis in castrated males.

Maggi et al. (80) reported that isolated perfused lungs from male rats synthesize more prostacyclin and thromboxane $B_2$ from $^{14}$-C arachidonic acid than lungs from female rats. They also found that aortic rings from male rats produced more prostacyclin than those from female rats. The same group of researchers (81) also suggested that there were more endoperoxide receptors in the aortas from males than in those from females. The response of the female aortas was less sensitive than those from males.

Wey et al. (82) reported similar findings to those previously published by Pomérantz et al. (79). They also found that aortic rings from male rats produced more prostacyclin than aortic rings from female rats, and that ovariectomy in rats increased prostacyclin synthesis. However, Morikawa et al. (83) did not find sex differences in arterial prostacyclin synthesis, but they reported that male rats were more susceptible to the inhibitory effect of aspirin.

Ylikorkala et al. (84) measuring the concentration of prostacyclin in plasma samples from humans did not find any difference between males and females of different age groups. But, Neri Serneri et al. (85) reported higher circulating concentrations of prostacyclin in women than in men.

In this study, it was decided to determine whether there were differences in prostaglandin synthesis between endothelial cells harvested from veins of umbilical cords from male and female babies. Also, it was decided to determine whether incubating these cells with

plasma from either male or female subjects would affect prostaglandin synthesis by the cells.

## OBJECTIVES

The purposes of this research were:

1. To study the effects of plasma from human subjects eating diets with different amounts of linoleic acid upon prostaglandin (PG $I_2$ and PG $E_2$) synthesis and fatty acid composition of human endothelial cells growing in culture.
2. To study whether there were differences in prostaglandin synthesis between human endothelial cells isolated from umbilical cord veins from male and female babies, and
3. To study the effect of plasma from males and females upon prostaglandin synthesis and fatty acid compositon of endothelial cells isolated from human umbilical cord veins from male and female babies.

## EXPERIMENTAL DESIGN

### Effects of diet

For the first study, plasma samples from a collaborative study (NC-167) were used. In that particular study, 20 women were fed two diets, one that represented the average U.S. diet (86) and one with a composition similar to the U.S. recommended pattern (modified diet) (87). The average U.S. diet contained 2000 Kcal, 40% of the calories as fat, 4% of the total calories as linoleic acid, and 700 mg of cholesterol. The modified diet contained 2000 kcal, 30% of the calories as fat, 10% of the total calories as linoleic acid, and 100 mg of cholesterol.

A cross-over experimental design was used in which the subjects first recorded their ad libitum intake during 7 days; this period was named the pre-experimental period, and the diet was named self-selected. At the end of this period, a fasting blood sample was drawn, and the subjects started the experimental period. Half of the subjects started eating the average U.S. diet during 4 weeks, and then changed to eat the modified diet during the next 4 weeks. Half of the subjects started eating the modified diet during 4 weeks, and then changed to the average U.S. diet during the next 4 weeks. Blood samples were drawn after an overnight fast at the end of the 4 weeks, before starting the new diet (Table 1).

Half of the 20 subjects participating in the study were fed their diets in the metabolic unit of Iowa, and half were fed their diets in

the metabolic unit of Nebraska. At the begining of the experimental period, all the subjects were fed the same amount of food irrespective of body weight, height, or activity. Some subjects were losing weight during the first week of this period. To stop this process, the energy intake of those individuals was adjusted by using hard candy (sugar). The seasonal time of the feeding studies were the same, with an off set of one week to facilitate sample processing.

TABLE 1. Experimental design used to study the effect of diet upon prostaglandin synthesis by endothelial cells

| | IOWA (N=10) | NEBRASKA (N=10) |
|---|---|---|
| SEQUENCE 1 | SELF-SELECTED[1] (N=10) | AVERAGE U.S. DIET[1] (N=10) |
| | MODIFIED DIET[1] (N=10) | |
| SEQUENCE 2 | SELF-SELECTED[1] (N=10) | MODIFIED DIET[1] (N=10) |
| | AVERAGE U.S. DIET[1] (N=10) | |

[1] Blood sample drawn.

In the subjects being fed their diets in Iowa, an additional blood sample was drawn two hours after starting each new diet. This sample was used to test whether changes in prostaglandin synthesis by endothelial cells could be induced by an acute change in the diet.

All the subjects participating in this study were women between 18 and 30 years of age, with no clinical disease. Intake of all medications including hormonal preparations was avoided when possible

during the entire study. In the case in which medications were taken, a record of that was kept.

In preliminary experiments, it was observed that a wide variability in prostaglandin synthesis existed between endothelial cells isolated from different umbilical cords. In order to be able to correct for variability between cords, the samples were paired by subject and cord. A pool of endothelial cells isolated from each cord was divided into 2 flasks. Because of that, 3 cords (6 flasks) were used for each subject in order to test the three diets (Table 2).

TABLE 2. Pairing of samples by subject and cord to study the effect of diet upon prostaglandin synthesis by endothelial cells

SUBJECT 1-20

| | DIET | DIET |
|---|---|---|
| CORD 1. | FLASK 1 (SELF-SELECTED) | FLASK 2 (AVERAGE U.S.) |
| CORD 2. | FLASK 1 (AVERAGE U.S.) | FLASK 2 (MODIFIED) |
| CORD 3. | FLASK 1 (SELF-SELECTED) | FLASK 2 (MODIFIED) |

A similar procedure was followed when comparisons were done between the plasma samples drawn under fasting conditions and those drawn 2 hours after breakfast (Table 3).

TABLE 3. Pairing of samples by subject and cord to study the effect of acute changes in diet upon prostaglandin synthesis by endothelial cells

SUBJECT 1-5 SEQUENCE 1

| | FASTING DIET | FED DIET |
|---|---|---|
| CORD 1. | FLASK 1 (SELF-SELECTED) | FLASK 2 (AVERAGE U.S.) |
| CORD 2. | FLASK 1 (AVERAGE U.S.) | FLASK 2 (MODIFIED) |
| CORD 3. | FLASK 1 (MODIFIED) | FLASK 2 (SELF-SELECTED) |

SUBJECT 1-5 SEQUENCE 2

| | FASTING DIET | FED DIET |
|---|---|---|
| CORD 1. | FLASK 1 (SELF-SELECTED) | FLASK 2 (MODIFIED) |
| CORD 2. | FLASK 1 (MODIFIED) | FLASK 2 (AVERAGE U.S.) |
| CORD 3. | FLASK 1 (AVERAGE U.S.) | FLASK 2 (SELF-SELECTED) |

Effect of sex

To test whether there were differences between endothelial cells isolated from umbilical cords from either male or female babies stimulated with different amounts of thrombin, the design shown in Table 4 was used.

TABLE 4. Experimental design used to study the effect of sex upon prostaglandin synthesis by endothelial cells

| | THROMBIN (UNITS) | | |
|---|---|---|---|
| | 0.000 | 0.125 | 0.500 |
| CELLS FROM MALES | N=8 | N=8 | N=8 |
| CELLS FROM FEMALES | N=8 | N=8 | N=8 |

To test whether there was an effect of plasma from either male or female subjects upon prostaglandin synthesis and fatty acid composition of endothelial cells, a 2 x 2 x 2 cross over design was used (Table 5).

The females whose plasma samples were used for this study were the same that have been described before. The males were between 18 and 32 years of age, in apparent good health, and with no history of taking medications prior to drawing the blood samples. Blood samples drawn two hours postprandial were used for this study. The male subjects were asked to keep a record of their food intake during three days (two weekdays and one weekend day). This information was used to estimate

TABLE 5. Cross over design used to study the effect of plasma from either males or females upon prostaglandin synthesis by endothelial cells

| | PLASMA | | | |
|---|---|---|---|---|
| | MALES | | FEMALES | |
| | THROMBIN (UNITS) | | THROMBIN (UNITS) | |
| | 0.000 | 0.125 | 0.000 | 0.125 |
| CELLS FROM MALES | N=6 | N=6 | N=6 | N=6 |
| CELLS FROM FEMALES | N=6 | N=6 | N=6 | N=6 |

the amount of fat, linoleic acid, and cholesterol in their diets and determine whether these were correlated with the fatty acid composition of the cells, and their prostaglandin synthesis.

Statistical analysis

Analysis of variance was calculated, and least significant differences were estimated by using the Tukey procedure. Analysis of regression and correlation were also calculated. The statistics were calculated using the statistical analysis system (S.A.S.) (SAS Institute Cary, N.C.).

For the statistical analysis of prostaglandins it was necessary to do a square root transformation of the data, because they did not have a normal distribution. A logarithmic transformation was also tested, but it was observed that this was not adequate.

# METHODS

## Harvesting of endothelial cells

The method used to harvest the cells was a modification of the one described by Jaffe et al. (88). A sterile technique was used in all the manipulations of the umbilical cords. The umbilical cords were severed from the placenta soon after birth and placed in sterile containers (canning jars) filled with approximately 150 ml of cord buffer containing 137 mM NaCl, 4mM KCl, 0.5 mM $Na_2HPO_4$, 0.15 mM $KH_2PO_4$, 11 mM glucose, pH 7.4, and 20,000 units/liter penicillin (base), 20,000 mcg/liter streptomycin (base), and 25 mcg/liter amphotericin B (Fungizone, Grand Island Biological Co., Grand Island, N.Y.). This procedure was done by the nurses of the Obstetrics Department of The Mary Greeley Medical Center. The umbilical cords were kept at 4 °C in a refrigerator at the hospital. They were picked up within 24 to 48 hours after their collection and transported in ice to the tissue culture laboratory of the Food and Nutrition Department.

In the laboratory, the cells usually were harvested within the next two hours. Depending upon the length of the umbilical cord, either two or four 25 $cm^2$ flasks (Corning Glass Works, Corning, N.Y.) were seeded. The umbilical cord was placed on a sterile petri dish where it was inspected. All the areas with clamp marks, and a segment of approximately 2 to 3 cm at each side of the umbilical cord were excised. The umbilical cord was divided into two or four segments of approximately 15 cm each, and the segments were placed in a sterile

plastic container with approximately 150 ml of cord buffer pre-warmed at 37 °C. They were incubated at 37 °C in a water bath during 10 minutes. At the end of this period of incubation, a needle was inserted into the umbilical vein of each segment. The vein can be identified easily because it is bigger than the two arteries. The needle was held in place by clamping it with a hemostat. After this, the vein was perfused with 50 ml of cord buffer pre-warmed at 37 °C in order to wash the blood out. The cord buffer with the blood was allowed to drain. To perfuse the vein, a 50 ml sterile disposable plastic syringe containing the cord buffer was connected to the needle previously inserted inside the vein.

After washing the vein, the other end of the umbilical cord was clamped with a sterile hemostat, and 5 ml of a 0.25% sterile trypsin solution in cord buffer (Grand Island Biological Co., Grand Island, N.Y.) were infused. The trypsin solution was pre-warmed at 37 °C. The trypsin solution was infused using a 5 ml sterile disposable plastic syringe connected to the needle previously inserted into the vein. After infusing the trypsin, the plastic syringe was left connected to the needle in order to keep it closed. Then the umbilical cord was placed again in the plastic box previously mentioned, and incubated at 37 °C during 15 minutes.

At the end of this period of incubation, the umbilical cord was suspended by one of its ends, the 5 ml syringe discarded, and a three-way stopcock connected to the needle. An empty 50 ml sterile disposable plastic syringe was connected to one of the sides of the stopcock. At

the other side, it was connected to another 50 ml sterile plastic disposable syringe containing a mixture of 10 ml of modified medium 199 with 10% heat inactivated fetal bovine serum (Grand Island Biological Co., Grand Island, N.Y.) and 35 ml of cord buffer. The tissue culture medium with fetal bovine serum was included in the mixture in order to inactivate the trypsin. Modified medium 199 was prepared as follows: 9.87 g/liter medium 199 powder with Earle's salts, with L-glutamine, without sodium bicarbonate, 1 g/liter glucose, 2.2 g/liter $NaHCO_3$, 10 ml/liter BME aminoacid solution (100X), 10 ml/liter BME vitamin solution (100X), 20,000 units/liter penicillin (base), 20,000 mcg/liter streptomycin (base), 25 mcg/liter fungizone, 25 mM HEPES buffer (N'-2-Hydroxyethylpiperazine-N'-Ethanesulfonic Acid). HEPES and glucose were purchased from Sigma Chemical Co., St. Louis, MO.

The trypsin solution was aspirated into the empty syringe, and the vein was washed several times by perfusing it with the mixture of cord buffer and tissue culture medium previously mentioned. Each time the mixture was aspirated into the second syringe (initially empty). The washing of the vein is important in order to be able to flush the endothelial cells out. After finishing the washing of the vein, the content of the syringe with the cells in suspension was transferred to a sterile disposable 50 ml conical centrifuge tube (Corning Glass Works, Corning, N.Y.). The cells were sedimented at 250 x g during 10 minutes. The supernatant was discarded, and the cell pellet was resuspended in approximately 3 ml of modified medium 199 (MM-199) with 20% heat

inactivated fetal bovine serum. The yield of this procedure usually was in the range of 0.5 to 1.5 x $10^6$ cells.

The cells harvested from the two segments of the umbilical cords (the content of two centrifuge tubes, each one with the cells resuspended in 3 ml of tissue culture medium) were pooled, seeded into two 25 $cm^2$ flasks (Corning Glass Works, Corning N.Y.), and incubated at 37 °C. After 24 hours, the tissue culture medium was discarded, the cells washed with 3 ml of cord buffer pre-warmed at 37 °C, and relayered with 3 ml of tissue culture medium containing 20% heat inactivated fetal bovine serum also pre-warmed at 37 °C.

Cell incubations

In the initial set of experiments in which sex differences in prostaglandin synthesis stimulated with different doses of thrombin was studied, when the cells reached confluency, the tissue culture medium was discarded and the cells relayered with 3 ml of tissue culture medium containing 20% heat inactivated bovine serum. The cells were incubated during 72 hours with this medium and then prostaglandin synthesis was stimulated with thrombin.

In the experiments in which human plasma was incorporated into the medium, when the cells reached confluency, the tissue culture medium was discarded, the cells washed with cord buffer, and then relayered with 2.5 ml of tissue culture medium supplemented with 20% heat inactivated plasma. The cells were incubated during 72 hours with this medium and then prostaglandin synthesis was stimulated with thrombin.

Cell stimulation

To stimulate prostaglandin synthesis by the cells, the tissue culture medium first was collected, then the monolayer of cells washed twice with 1 ml of cord buffer containing 8.6 g/liter of bovine serum albumin (fatty acid poor) (Miles Laboratories Inc., Research Products, Elkhart, Indiana). After this, 1 ml of thrombin (Thrombostat. Thrombin USP bovine origin. Parke-Davis, NJ) (either 0.500 or 0.125 units/ml) in cord buffer was added to the cells and incubated with continuous agitation at 37 °C during five minutes. At the end of the period of incubation, the thrombin solution was collected. The cells were scraped with a rubber policeman, suspended in cold cord buffer, sedimented at 600 x g during 10 minutes at 4 °C, and resuspended in 1 ml of cord buffer. The cells were stored under nitrogen at -70 °C for further analysis.

Preparation of plasma and tissue culture medium

Blood samples were drawn from human subjects into sterile heparinized vacutainer tubes (Monoject, Sherwood Medical, St. Louis, Mo.). Plasma was separated by centrifuging the blood at 600 x g during 10 minutes at 4 °C. After centrifugation, the plasma was separated and stored under nitrogen at -70 °C. When blood samples were drawn from the subjects in Nebraska, the blood samples were cooled in ice and then centrifuged at room temperature because no refrigerated centrifuges were available. Immediately after centrifugation the plasma was separated and stored in a freezer at -20 °C. Approximately 24 hours later, the

samples were transported in dry ice to Iowa where they were stored under nitrogen at -70 °C for further use.

Before use, the complement was inactivated by incubating the plasma at 37 °C during 1 hour. During this process, the plasma was kept in a capped tube under nitrogen. After heat inactivation, the plasma was mixed with modified medium 199 at a final proportion of 20% plasma. The mixture was sterilized by membrane filtration. For this purpose, a 0.2 micrometer sterile disposable filter (Gelman Sciences Inc., Ann Arbor, Michigan) was used. The sterile mixture was collected in sterile disposable tubes (Corning Glass Works, Corning, N.Y.) and kept under nitrogen at -70 °C for further use.

Measurements of prostaglandins

Prostaglandins were measured using a radioimmunoassay procedure. The methodology, specificity of the antibodies and validation of the assay have been described previously (89). Prostacyclin was measured as its stable product 6-keto-PG $F_{1\alpha}$, and PG $E_2$ was directly determined. Both prostaglandins were measured in either unextracted tissue culture medium or medium after stimulation with thrombin. For this purpose, the rabbit antibody (either anti 6-keto PG $F_{1\alpha}$ or anti PG $E_2$) was incubated during 24 hours at 4 °C with anti-rabbit gamma globulin. The antibodies were added to the sample in which prostaglandins were going to be measured.

The antibodies with the sample in the presence of tritium labeled prostaglandin (either 6-keto PG $F_{1\alpha}$ or PG $E_2$ from New England Nuclear

Inc. Boston, Mass.) were incubated in a phosphate buffered saline solution (pH 7) containing 0.1% gelatin during 24 hours at 4 °C. This was enough time for the reaction to reach equilibrium. After incubation, the mixture was centrifuged and the supernatant discarded. The radioactivity present in the precipitated antibodies was measured in a liquid scintillation counter. Concentrations of prostaglandins in the samples were then estimated using a logit transformation by using a computer program designed by Duddleson et al. (90).

To prepare the antiserum, the following dilutions were made: the anti 6-keto PG $F_{1\alpha}$ antiserum was diluted to 1:400 in phosphate buffered saline containing 0.01M EDTA. The anti PG $E_2$ antiserum also was diluted to 1:400 in the same buffer above mentioned. The antibodies were further diluted to 1:6000 and 1:5000 respectively with normal rabbit serum previously diluted to 1/400 with phosphate buffered saline containing 0.01M EDTA. These dilutions were chosen because with them the binding of the tritium labeled antigens were between 25 and 45%.

For the analysis of the data, the following criteria were set: 1) the standard curve was linear, 2) the slope of the standard curve was significant, 3) two standard curves were included in each assay run; one at the beginning and the other at the end, and 4) the slopes of the standard curves and comparably transformed data from inhibition curves of the tissue culture samples and medium after stimulation with thrombin were parallel. No evidence of non-specific binding was found. To measure PG $E_2$ 100 microliters of tissue culture medium and 200

microliters of medium after stimulation with thrombin were used. To measure PG $I_2$, 100 microliters of a 1:100 dilution of tissue culture medium and 100 microliters of a 1:10 dilution of medium after stimulation with thrombin were used.

No statistical differences between either the total protein content (0.2 - 0.3 mg) or the number of cells (0.7 - 0.9 x $10^6$ cells) in the flasks assigned to the different treatments were found. Because of that, and following the example of previous papers published in the literature (51,91), the results were expressed as prostaglandin synthesis in either nanograms or picograms per flask of cells. The concentration of prostaglandins in the tissue culture medium before incubation represented less than 0.0001% of the concentration of prostaglandins found after incubation with the cells.

Determination of the fatty acid composition of the cells

This complete procedure was carried out under nitrogen. The cells were thawed and sonicated during 5 minutes. A 50 microliter aliquot was taken in order to measure the protein content using the method of Lowry et al. (92). The 0.95 ml of the cell suspension was then saponified during 60 minutes at 70 °C in 3.2 ml of 95% ethanol and 0.8 ml of 33% KOH.

After overnight refrigeration, the non-saponifiable fraction was extracted twice with 10 ml of petroleum ether and the supernatant fluid was discarded after shaking by hand during approximately 1 minute (50 complete inversions of the tubes). The samples were acidified to a pH 0

to 3, cooled at 4 °C, and the fatty acids extracted three times with 6 ml of petroleum ether and dried under a stream of nitrogen. The fatty acids were methylated by heating with 2.5 ml of Boron trifluoride methanol (Applied Science Lab. Inc., Alltech Associates, Deerfield IL) for 2 minutes in a boiling water-bath (93). The resulting esters were extracted with 4 ml of water and 6 ml of petroleum ether, dried under nitrogen, and the fatty acid methyl esters resuspended in 20 microliters of n-hexane. Quantitation was done by adding an internal standard (C 17:0, 20 micrograms) at the beginning of the saponification process.

The fatty acid methyl esters were separated by gas-liquid chromatography using a Beckman gas chromatograph GC72-5 (Beckman, Scientific Division, Fullerton, California) and a 1.83m x 3.18 mm stainless steel column packed with Alltech 10% CS-10 on 100/120 W-AW (Alltech, Deerfield, IL). Instrument settings were as follows: column temperature 180 °C, injection port temperature 220 °C, detector temperature 250 °C, nitrogen flow 20 ml/minute, sensitivity 1 x $10^{10}$ amperes.

Identification of fatty acid methyl esters was done by comparing their retention times with those of known methylated standards (Applied Science Lab., Alltech Assoc., Deerfield, IL). Relative mass amounts were determined with the integrator (Infotronics, Automatic Digital Integrator CRS-208 and Varian Aerograph model 20, Walnut Creek, California).

# RESULTS

## Effects of fasting upon prostaglandin synthesis

There were no statistically significant differences between either basal or stimulated prostacyclin synthesis by endothelial cells incubated with plasma samples drawn from fasting subjects and those drawn from the same subjects two hours after breakfast (Tables 6 and 7). Nor were there differences in prostaglandin $E_2$ synthesis (Tables 8 and 9).

Finally, as with the results described above, there were no statistically significant differences between the fasting ratio of PG $E_2$/PG $I_2$ and the two-hour post-prandial ratio of PG $E_2$/PG $I_2$ in either basal or stimulated samples (Tables 10 and 11).

## Effect of diet upon prostaglandins and cellular fatty acids

Basal prostacyclin synthesis There was a statistically significant effect ($p < 0.05$) of diet upon basal prostacyclin synthesis by endothelial cells (Table 12). It was found that endothelial cells incubated with tissue culture medium supplemented with 20% plasma from subjects eating the modified diet produced less prostacyclin than those incubated with medium supplemented with 20% plasma from the same subjects eating the self-selected diet. Basal prostacyclin synthesis by cells incubated with tissue culture medium supplemented with plasma from the subjects eating the average U.S. diet was in between and not significantly different from prostacyclin synthesis by cells incubated

TABLE 6. Effects of dietary variations in linoleic acid and fasting upon prostacyclin synthesis by endothelial cells

| DIETS | | PG $I_2$[1] | |
|---|---|---|---|
| FASTING[2] | FED[3] | FASTING | FED |
| | | (ng/flask) | |
| SELF-SELECTED | AVERAGE U.S. | 0.56 (0.07)[4] | 0.56 (0.07) |
| AVERAGE U.S. | MODIFIED | 0.53 (0.07) | 0.55 (0.07) |
| MODIFIED | SELF-SELECTED | 0.63 (0.07) | 0.54 (0.07) |
| SELF-SELECTED | MODIFIED | 0.50 (0.07) | 0.56 (0.07) |
| MODIFIED | AVERAGE U.S. | 0.47 (0.07) | 0.54 (0.07) |
| AVERAGE U.S. | SELF-SELECTED | 0.67 (0.07) | 0.68 (0.07) |

[1] PG $I_2$ was measured as 6-keto PG $F_{1\alpha}$.

[2] Plasma samples drawn after an overnight fast. The subjects had been eating the same diet for at least 4 weeks.

[3] Plasma samples drawn 2 hours after the first breakfast with this diet. The subjects had been eating the diet listed under fasting the previous 4 weeks.

[4] Mean (SEM) of the square root of the actual values. (N=5). The concentration of PG $I_2$ was measured in the tissue culture medium after 72 hours of incubation with the cells. From this value, PG $I_2$ synthesis in five minutes was estimated.

TABLE 7. Effects of dietary variations in linoleic acid and fasting upon thrombin stimulated prostacyclin synthesis by endothelial cells

| DIETS | | PG $I_2$[1] | |
|---|---|---|---|
| FASTING[2] | FED[3] | FASTING | FED |
| | | (ng/flask) | |
| SELF-SELECTED | AVERAGE U.S. | 3.5 (0.48)[4] | 3.4 (0.48) |
| AVERAGE U.S. | MODIFIED | 3.9 (0.48) | 5.7 (0.48) |
| MODIFIED | SELF-SELECTED | 6.9 (0.48) | 6.9 (0.48) |
| SELF-SELECTED | MODIFIED | 3.7 (0.48) | 3.9 (0.48) |
| MODIFIED | AVERAGE U.S. | 3.5 (0.48) | 4.2 (0.48) |
| AVERAGE U.S. | SELF-SELECTED | 6.7 (0.48) | 7.4 (0.48) |

[1] PG $I_2$ was measured as 6-keto PG $F_{1\alpha}$.

[2] Plasma samples drawn after an overnight fast. The subjects had been eating the same diet for at least 4 weeks.

[3] Plasma samples drawn 2 hours after the first breakfast with this diet. The subjects had been eating the diet listed under fasting the previous 4 weeks.

[4] Mean (SEM) of the square root of the actual values. (N=5). The concentration of PG $I_2$ was stimulated with 0.5 units of thrombin during 5 minutes at 37 °C.

TABLE 8. Effects of dietary variations in linoleic acid and fasting upon prostaglandin $E_2$ synthesis by endothelial cells

| DIETS | | PG $E_2$ | |
|---|---|---|---|
| FASTING[1] | FED[2] | FASTING | FED |
| | | (pg/flask) | |
| SELF-SELECTED | AVERAGE U.S. | 1.6 (0.37)[3] | 1.6 (0.37) |
| AVERAGE U.S. | MODIFIED | 2.3 (0.37) | 2.4 (0.37) |
| MODIFIED | SELF-SELECTED | 2.0 (0.37) | 2.0 (0.37) |
| SELF-SELECTED | MODIFIED | 1.9 (0.37) | 1.8 (0.37) |
| MODIFIED | AVERAGE U.S. | 1.9 (0.37) | 1.4 (0.37) |
| AVERAGE U.S. | SELF-SELECTED | 1.4 (0.37) | 1.5 (0.37) |

[1] Plasma samples drawn after an overnight fast. The subjects had been eating the same diet for at least 4 weeks.

[2] Plasma samples drawn 2 hours after the first breakfast with this diet. The subjects had been eating the diet listed under fasting the previous 4 weeks.

[3] Mean (SEM) of the square root of the actual values. (N=5). The concentration of PG $E_2$ was measured in the tissue culture medium after 72 hours of incubation with the cells. From this value, PG $E_2$ synthesis in five minutes was estimated.

TABLE 9. Effects of dietary variations in linoleic acid and fasting upon thrombin stimulated prostaglandin $E_2$ synthesis by endothelial cells

| DIETS | | PG $E_2$ | |
|---|---|---|---|
| FASTING[1] | FED[2] | FASTING | FED |
| | | (pg/flask) | |
| SELF-SELECTED | AVERAGE U.S. | 16.6 (1.6)[3] | 16.7 (1.6) |
| AVERAGE U.S. | MODIFIED | 16.0 (1.6) | 17.9 (1.6) |
| MODIFIED | SELF-SELECTED | 20.7 (1.6) | 20.9 (1.6) |
| SELF-SELECTED | MODIFIED | 16.3 (1.6) | 16.9 (1.6) |
| MODIFIED | AVERAGE U.S. | 14.4 (1.6) | 15.6 (1.6) |
| AVERAGE U.S. | SELF-SELECTED | 22.3 (1.6) | 23.8 (1.6) |

[1] Plasma samples drawn after an overnight fast. The subjects had been eating the same diet for at least 4 weeks.

[2] Plasma samples drawn 2 hours after the first breakfast with this diet. The subjects had been eating the diet listed under fasting the previous 4 weeks.

[3] Mean (SEM) of the square root of the actual values. (N=5). The concentration of PG $E_2$ was stimulated with 0.5 units of thrombin during 5 minutes at 37 °C.

TABLE 10. Effects of dietary variations in linoleic acid and fasting upon the ratio prostaglandin $E_2/I_2$ synthesized by endothelial cells

| DIETS | | PG $E_2$/PG $I_2$[1] | |
|---|---|---|---|
| FASTING[2] | FED[3] | FASTING | FED |
| SELF-SELECTED | AVERAGE U.S. | 9.4 (3.2)[4] | 9.9 (3.2) |
| AVERAGE U.S. | MODIFIED | 13.3 (3.2) | 13.0 (3.2) |
| MODIFIED | SELF-SELECTED | 12.2 (3.2) | 13.4 (3.2) |
| SELF-SELECTED | MODIFIED | 11.6 (3.2) | 11.4 (3.2) |
| MODIFIED | AVERAGE U.S. | 14.0 (3.2) | 9.0 (3.2) |
| AVERAGE U.S. | SELF-SELECTED | 10.2 (3.2) | 8.6 (3.2) |

[1] PG $I_2$ was measured as 6-keto PG $F_{1\alpha}$.

[2] Plasma samples drawn after an overnight fast. The subjects had been eating the same diet for at least 4 weeks.

[3] Plasma samples drawn 2 hours after the first breakfast with this diet. The subjects had been eating the diet listed under fasting the previous 4 weeks.

[4] Mean (SEM) of the square root of the ratio PG $E_2$/PG $I_2$ X $10^4$. The concentration of prostaglandins was measured in the tissue culture medium after 72 hours of incubation with the cells. From this value, prostaglandin synthesis in five minutes was estimated (N=5).

TABLE 11. Effects of dietary variations in linoleic acid and fasting upon the thrombin stimulated ratio prostaglandin $E_2/I_2$ synthesized by endothelial cells

| DIETS | | PG $E_2$/PG $I_2$[1] | |
|---|---|---|---|
| FASTING[2] | FED[3] | FASTING | FED |
| SELF-SELECTED | AVERAGE U.S. | 15.1 (2.2)[4] | 15.6 (2.2) |
| AVERAGE U.S. | MODIFIED | 14.6 (2.2) | 11.0 (2.2) |
| MODIFIED | SELF-SELECTED | 9.6 (2.2) | 10.5 (2.2) |
| SELF-SELECTED | MODIFIED | 13.9 (2.2) | 15.0 (2.2) |
| MODIFIED | AVERAGE U.S. | 13.5 (2.2) | 11.9 (2.2) |
| AVERAGE U.S. | SELF-SELECTED | 11.3 (2.2) | 10.2 (2.2) |

[1] PG $I_2$ was measured as 6-keto PG $F_{1\alpha}$.

[2] Plasma samples drawn after an overnight fast. The subjects had been eating the same diet for at least 4 weeks.

[3] Plasma samples drawn 2 hours after the first breakfast with this diet. The subjects had been eating the diet listed under fasting the previous 4 weeks.

[4] Mean (SEM) of the square root of the ratio PG $E_2$/PG $I_2$ X $10^4$. The concentration of prostaglandins was measured in the tissue culture medium after 72 hours of incubation with the cells. From this value, prostaglandin synthesis in five minutes was estimated (N=5).

with medium supplemented with plasma from the subjects eating the self-selected diet and that of cells incubated with medium supplemented with plasma from the subjects eating the modified diet.

Basal prostacyclin synthesis by cells incubated with medium supplemented with plasma from the subjects eating the diets in sequence 2 (self-selected, modified, average U.S. diet) was higher ($p < 0.03$) than the amount produced by cells incubated with medium supplemented with plasma from the subjects eating the diets in sequence 1 (self-selected, average U.S. diet, modified diet) (Figure 4). There were significant differences ($p < 0.01$) between basal prostacyclin synthesis by endothelial cells incubated with tissue culture medium supplemented with plasma from the different subjects. There were no significant differences between basal prostacyclin synthesis by cells incubated with medium supplemented with plasma samples drawn in Iowa compared to Nebraska.

Thrombin stimulated prostacyclin synthesis When prostacyclin synthesis by endothelial cells was stimulated with thrombin, there was a statistically significant interaction ($p < 0.03$) between diet and sequence in which the diets were eaten (Table 13). Prostacyclin synthesis by cells incubated with medium supplemented with plasma from subjects eating the diets in sequence 1 (self-selected, average U.S. diet, modified diet) was affected by diet. It was lower in cells incubated with medium supplemented with plasma from subjects eating the modified diet than in cells incubated with medium supplemented with

TABLE 12. Prostacyclin synthesis by endothelial cells incubated with plasma from subjects eating three different diets[1]

EXPERIMENT STATION IOWA

PG $I_2$[2] ng/flask

| DIET | SELF-SELECTED | AVERAGE U.S. | MODIFIED |
|---|---|---|---|
| SEQUENCE 1 | 0.56 (0.05)[3] | 0.58 (0.05) | 0.37 (0.05) |
| DIET | SELF-SELECTED | MODIFIED | AVERAGE U.S. |
| SEQUENCE 2 | 0.59 (0.05) | 0.56 (0.05) | 0.55 (0.05) |

EXPERIMENT STATION NEBRASKA

| DIET | SELF-SELECTED | AVERAGE U.S. | MODIFIED |
|---|---|---|---|
| SEQUENCE 1 | 0.60 (0.05) | 0.48 (0.05) | 0.43 (0.05) |
| DIET | SELF-SELECTED | MODIFIED | AVERAGE U.S. |
| SEQUENCE 2 | 0.58 (0.06) | 0.57 (0.05) | 0.56 (0.05) |

[1] Diet effect ($p < 0.05$). Sequence effect ($p < 0.03$).

[2] PG $I_2$ was measured as 6-keto PG $F_{1\alpha}$.

[3] Mean (SEM) of the square root of the actual values. N=10.

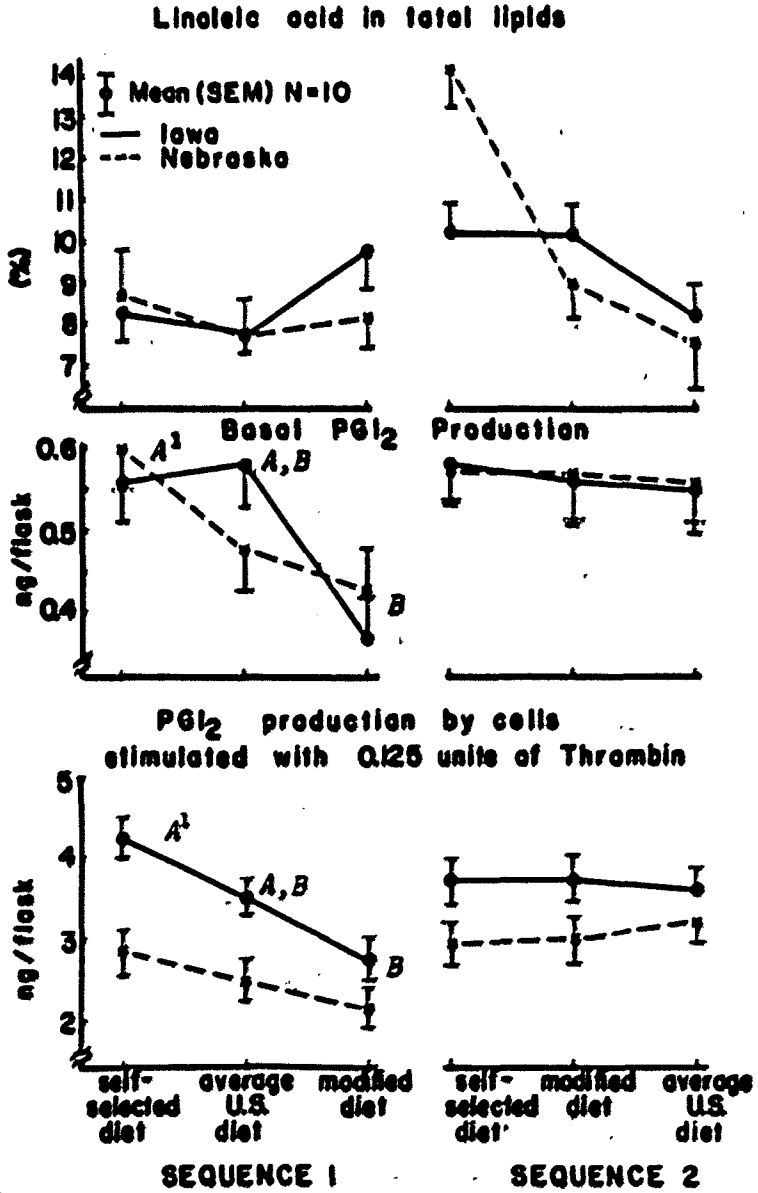


FIGURE 4. Diet effect on cell linoleate and prostacyclin synthesis
[1] Sequence effect ($p < 0.05$). Different letters ($p < 0.05$).

plasma from the same subjects eating the self-selected diet. Prostacyclin synthesis by cells incubated with medium supplemented with plasma from the subjects eating the average U.S. diet was in between and not significantly different from that of cells incubated with medium supplemented with plasma from the subjects when eating the self-selected diet or the modified diet.

There was no effect of diet upon prostacyclin synthesis by endothelial cells incubated with medium supplemented with plasma from subjects eating diets in sequence 2 (self-selected, modified, average U.S. diet) (Figure 4). There were significant differences ($p < 0.01$) between prostacyclin synthesis by endothelial cells incubated with medium supplemented with plasma from subjects eating the diets in sequence 1 and cells incubated with medium supplemented with plasma from subjects eating the diets in sequence 2. Also, there were significant differences ($p < 0.001$) between prostacyclin synthesis by cells incubated with medium supplemented with plasma samples drawn from subjects in Iowa and that of samples drawn from subjects in Nebraska.

Basal prostaglandin $E_2$ synthesis There was no significant effect of diet upon basal prostaglandin $E_2$ (PG $E_2$) synthesis by endothelial cells ($p = 0.12$). However, the average synthesis of this prostaglandin followed the same pattern as basal synthesis of prostacyclin (Table 14). Endothelial cells incubated with medium supplemented with plasma from the subjects when eating the self-selected diet had an average synthesis of PG $E_2$ higher than cells incubated with

TABLE 13. Prostacyclin synthesis by endothelial cells incubated with plasma from subjects eating three different diets, and stimulated with 0.125 units of thrombin[1]

EXPERIMENT STATION IOWA

PG $I_2$[2] ng/flask

| DIET | SELF-SELECTED | AVERAGE U.S. | MODIFIED |
|---|---|---|---|
| SEQUENCE 1 | 4.24 (0.27)[3] | 3.54 (0.27) | 2.79 (0.27) |
| DIET | SELF-SELECTED | MODIFIED | AVERAGE U.S. |
| SEQUENCE 2 | 3.76 (0.27) | 3.79 (0.27) | 3.63 (0.27) |

EXPERIMENT STATION NEBRASKA

| DIET | SELF-SELECTED | AVERAGE U.S. | MODIFIED |
|---|---|---|---|
| SEQUENCE 1 | 2.86 (0.27) | 2.52 (0.27) | 2.22 (0.27) |
| DIET | SELF-SELECTED | MODIFIED | AVERAGE U.S. |
| SEQUENCE 2 | 2.96 (0.27) | 3.04 (0.27) | 3.27 (0.27) |

[1] Place effect ($p < 0.001$). Sequence effect ($p < 0.01$). Diet x sequence interaction ($p < 0.03$).

[2] PG $I_2$ was measured as 6-keto PG $F_{1\alpha}$.

[3] Mean (SEM) of the square roots of the actual values. N=10. The cells were incubated with 0.125 units of thrombin during five minutes at 37 °C.

medium supplemented with plasma from the subjects when eating the average U.S. diet, and these in turn had a higher average synthesis of PG $E_2$ than cells incubated with medium supplemented with plasma from the subjects when eating the modified diet.

Basal PG $E_2$ synthesis by endothelial cells incubated with medium supplemented with plasma from subjects eating the diets in sequence 2 was higher ($p < 0.03$) than that by cells incubated with medium supplemented with plasma from subjects eating the diets in sequence 1. In addition, basal PG $E_2$ synthesis by cells incubated with medium supplemented with plasma samples drawn from subjects in Iowa was higher ($p < 0.001$) than that by cells incubated with medium supplemented with plasma samples drawn from subjects in Nebraska.

Thrombin stimulated prostaglandin $E_2$ synthesis Prostaglandin $E_2$ synthesis by endothelial cells after stimulation with thrombin followed the same pattern as prostacyclin synthesis did (Table 15). There was an interaction between sequence and diet ($p = 0.053$) similar to that observed with prostacyclin. Prostaglandin $E_2$ synthesis by cells incubated with medium supplemented with plasma from the subjects following sequence 1 when eating the modified diet was lower than that by cells incubated with medium supplemented with plasma from the same subjects when eating the self-selected diet. Prostaglandin $E_2$ synthesis by cells incubated with medium supplemented with plasma from the subjects when eating the average U.S. diet was in between the two mentioned above. However, there was no difference in PG $E_2$ synthesis

TABLE 14. Prostaglandin $E_2$ synthesis by endothelial cells incubated with plasma from subjects eating three different diets[1]

EXPERIMENT STATION IOWA

$PGE_2$ pg/flask

| DIET | SELF-SELECTED | AVERAGE U.S. | MODIFIED |
|---|---|---|---|
| SEQUENCE 1 | 3.1 (0.17)[2] | 3.1 (0.17) | 2.5 (0.17) |
| DIET | SELF-SELECTED | MODIFIED | AVERAGE U.S. |
| SEQUENCE 2 | 3.4 (0.17) | 3.2 (0.17) | 3.3 (0.17) |

EXPERIMENT STATION NEBRASKA

| DIET | SELF-SELECTED | AVERAGE U.S. | MODIFIED |
|---|---|---|---|
| SEQUENCE 1 | 2.1 (0.17) | 1.9 (0.17) | 1.7 (0.17) |
| DIET | SELF-SELECTED | MODIFIED | AVERAGE U.S. |
| SEQUENCE 2 | 1.9 (0.17) | 2.0 (0.17) | 1.9 (0.17) |

[1] Place effect ($p < 0.001$). Sequence effect ($p < 0.03$).

[2] Mean (SEM) of the square root of the actual values. N=10.

between cells incubated with medium supplemented with plasma from subjects when eating any of the three diets in sequence 2.

As with prostacyclin, PG $E_2$ synthesis by endothelial cells incubated with medium supplemented with plasma from subjects eating diets in sequence 2 was slightly higher than that by cells incubated with medium supplemented with plasma from subjects eating the diets in sequence 1, but the differences were not statistically significant ($p = 0.08$).

Prostaglandin $E_2$ synthesis by endothelial cells incubated with medium supplemented with plasma samples drawn from subjects in Nebraska was slightly higher than that by cells incubated with medium supplemented with plasma drawn from subjects in Iowa, but the difference was not statistically significant ($p = 0.09$).

Ratio PG $E_2$/PG $I_2$ in basal conditions The ratio of basal PG $E_2$/PG $I_2$ synthesis by endothelial cells incubated with medium supplemented with plasma samples from subjects when eating the modified diet was slightly higher than that of cells incubated with tissue culture medium supplemented with plasma samples from subjects eating the average U.S. diet and the self-selected diet, but the difference was not statistically significant ($p = 0.09$) (Table 16).

This ratio was higher when endothelial cells were incubated with medium supplemented with plasma samples drawn from subjects in Iowa than that found when cells were incubated with medium supplemented with plasma from subjects in Nebraska ($p < 0.001$).

TABLE 15. Prostaglandin $E_2$ synthesis by endothelial cells incubated with plasma from subjects eating three different diets, and stimulated with 0.125 units of thrombin[1]

EXPERIMENT STATION IOWA

$PGE_2$ pg/flask

| DIET | SELF-SELECTED | AVERAGE U.S. | MODIFIED |
|---|---|---|---|
| SEQUENCE 1 | 15.8 (1.08)[2] | 14.7 (1.08) | 10.4 (1.08) |
| DIET | SELF-SELECTED | MODIFIED | AVERAGE U.S. |
| SEQUENCE 2 | 16.1 (1.08) | 15.8 (1.08) | 14.3 (1.08) |

EXPERIMENT STATION NEBRASKA

| DIET | SELF-SELECTED | AVERAGE U.S. | MODIFIED |
|---|---|---|---|
| SEQUENCE 1 | 16.4 (1.08) | 15.4 (1.08) | 14.3 (1.08) |
| DIET | SELF-SELECTED | MODIFIED | AVERAGE U.S. |
| SEQUENCE 2 | 15.5 (1.08) | 15.6 (1.08) | 15.8 (1.08) |

[1] Diet x sequence interaction ($p = 0.053$).

[2] Mean (SEM) of the square root of the actual values. N=10. The cells were incubated with 0.125 units of thrombin during five minutes at 37 °C.

TABLE 16. Ratio of prostaglandin $E_2/I_2$ synthesized by endothelial cells incubated with plasma from subjects eating three different diets[1]

EXPERIMENT STATION IOWA

| DIET | SELF-SELECTED | AVERAGE U.S. | MODIFIED |
|---|---|---|---|
| SEQUENCE 1 | 18.7 (1.7)[2] | 18.0 (1.7) | 22.2 (1.7) |
| DIET | SELF-SELECTED | MODIFIED | AVERAGE U.S. |
| SEQUENCE 2 | 16.8 (1.7) | 24.1 (1.7) | 21.5 (1.8) |

EXPERIMENT STATION NEBRASKA

| DIET | SELF-SELECTED | AVERAGE U.S. | MODIFIED |
|---|---|---|---|
| SEQUENCE 1 | 11.5 (1.7) | 13.1 (1.7) | 13.6 (1.7) |
| DIET | SELF-SELECTED | MODIFIED | AVERAGE U.S. |
| SEQUENCE 2 | 13.4 (2.0) | 12.4 (1.7) | 13.5 (1.7) |

[1] Place effect ($p < 0.001$).

[2] Mean (SEM) of the square root of the ratio PG $E_2$/PG $I_2$ X $10^4$. N=10. PG $I_2$ was measured as 6-keto PG $F_{1\alpha}$.

Ratio PG $E_2$/PG $I_2$ in thrombin stimulated cells The ratio of PG $E_2$/PG $I_2$ was higher in endothelial cells stimulated with thrombin when incubated with plasma from subjects in Nebraska than that found when cells were incubated with medium supplemented with plasma from subjects in Iowa ($p < 0.001$) (Table 16). This difference was more evident when plasma from subjects eating the diets in sequence 1 were used, making the interaction, place x sequence, statistically significant ($p < 0.02$). No effect of diet was found.

Fatty acid composition of endothelial cells Endothelial cells incubated with plasma from subjects eating the self-selected diet and the modified diet had a higher percentage ($p < 0.05$) of linoleic acid than cells incubated with plasma from subjects eating the average U.S. diet irrespective of the sequence in which the diets were eaten (Figure 4). No differences were observed between the other fatty acids (Tables 18 and 19). No significant correlations were found between either basal or stimulated prostaglandin (prostacyclin or prostaglandin $E_2$) synthesis and the different fatty acids. Negative correlations were found between cellular polyunsaturated fatty acids and the saturated ones (Tables 20 and 21).

## Effect of sex

Differences between cells It was found that endothelial cells harvested from umbilical cords from male babies produced more prostacyclin than cells harvested from umbilical cords from female babies when prostacyclin synthesis was stimulated with 0.125 units of

TABLE 17. Ratio of prostaglandin $E_2/I_2$ synthesized by endothelial cells incubated with plasma from subjects eating three different diets, and stimulated with 0.125 units of thrombin[1]

EXPERIMENT STATION IOWA

| DIET | SELF-SELECTED | AVERAGE U.S. | MODIFIED |
|---|---|---|---|
| SEQUENCE 1 | 11.4 (1.29)[2] | 13.0 (1.29) | 12.1 (1.29) |
| DIET | SELF-SELECTED | MODIFIED | AVERAGE U.S. |
| SEQUENCE 2 | 14.4 (1.29) | 14.2 (1.29) | 13.4 (1.29) |

EXPERIMENT STATION NEBRASKA

| DIET | SELF-SELECTED | AVERAGE U.S. | MODIFIED |
|---|---|---|---|
| SEQUENCE 1 | 19.4 (1.29) | 21.5 (1.29) | 21.2 (1.29) |
| DIET | SELF-SELECTED | MODIFIED | AVERAGE U.S. |
| SEQUENCE 2 | 19.3 (1.29) | 19.7 (1.29) | 17.9 (1.29) |

[1] Place effect ($p < 0.001$). Place x sequence interaction ($p < 0.02$).

[2] Mean (SEM) of the square root of the ratio PG $E_2$/PG $I_2$ X $10^4$ N=10. PG $I_2$ was measured as 6-keto PG $F_{1\alpha}$. The cells were incubated with 0.125 units of thrombin during five minutes at 37 °C.

TABLE 18. Fatty acid composition of endothelial cells incubated with plasma from subjects in Iowa eating three different diets

| FATTY ACID(%) | SELF-SELECTED N=9 | AVERAGE U.S. N=8 | MODIFIED N=6 |
|---|---|---|---|
| | SEQUENCE 1 | | |
| 16:0 | 40.0 (2.4)[1] | 37.9 (2.3) | 34.8 (2.6) |
| 18:0 | 11.6 (1.5) | 13.5 (1.5) | 12.6 (1.6) |
| 18:1 | 15.0 (1.6) | 17.0 (1.6) | 16.5 (1.7) |
| 18:2 | 8.3 (0.8)A | 7.8 (0.8)B | 9.8 (0.9)A[2] |
| 18:3 | 0.8 (0.3) | 1.6 (1.0) | 1.9 (0.9) |
| 20:3 | 3.1 (1.1) | 2.4 (1.4) | 2.4 (0.8) |
| 20:4 | 20.2 (1.6) | 19.4 (1.6) | 20.5 (1.7) |
| | SEQUENCE 2 | | |
| FATTY ACID(%) | SELF-SELECTED N=10 | AVERAGE U.S. N=10 | MODIFIED N=10 |
| 16:0 | 33.7 (2.1) | 31.4 (2.0) | 32.8 (2.0) |
| 18:0 | 13.2 (1.4) | 15.3 (1.4) | 14.2 (1.3) |
| 18:1 | 16.8 (1.4) | 15.8 (1.3) | 17.9 (1.3) |
| 18:2 | 10.2 (0.7)A | 8.3 (0.7)B | 10.2 (0.7)A |
| 18:3 | 2.0 (0.8) | 0.5 (0.3) | 1.6 (1.3) |
| 20:3 | 2.1 (0.5) | 2.0 (0.7) | 2.8 (0.9) |
| 20:4 | 24.5 (1.4) | 21.5 (1.3) | 19.2 (1.3) |

[1] Values are mean (SEM).

[2] Different letters ($p < 0.05$) between diets.

TABLE 19. Fatty acid composition of endothelial cells incubated with plasma from subjects in Nebraska eating three different diets

| FATTY ACID(%) | SELF-SELECTED N=4 | AVERAGE U.S. N=7 | MODIFIED N=4 |
|---|---|---|---|
| | SEQUENCE 1 | | |
| 16:0 | 39.2 (4.7)[1] | 38.6 (1.5) | 37.3 (2.2) |
| 18:0 | 19.4 (1.7) | 19.4 (1.9) | 21.0 (1.1) |
| 18:1 | 12.0 (2.0) | 13.6 (0.8) | 14.0 (2.0) |
| 18:2 | 8.7 (1.2)A | 7.8 (0.3)B | 8.2 (0.7)A[2] |
| 18:3 | 0.7 (0.6) | 0.6 (0.2) | 0.1 (0.1) |
| 20:3 | 1.6 (0.9) | 2.3 (0.7) | 0.9 (0.6) |
| 20:4 | 17.8 (2.5) | 16.8 (1.4) | 17.9 (0.7) |
| | SEQUENCE 2 | | |
| 16:0 | 34.0 (3.4) | 30.4 (4.5) | 32.4 (2.3) |
| 18:0 | 17.6 (2.1) | 19.5 (2.8) | 18.1 (1.4) |
| 18:1 | 11.9 (2.3) | 12.5 (3.0) | 11.4 (1.5) |
| 18:2 | 14.4 (1.1)A | 7.6 (1.5)B | 9.0 (0.8)A |
| 18:3 | 1.2 (0.7) | 1.1 (0.7) | 2.4 (1.5) |
| 20:3 | 1.7 (0.4) | 1.3 (0.5) | 1.9 (0.9) |
| 20:4 | 18.1 (2.3) | 24.1 (3.0) | 22.4 (1.5) |

[1] Values are mean (SEM).

[2] Different letters ($p < 0.05$) between diets.

TABLE 20. Correlations between basal prostaglandin synthesis and cellular fatty acids

| | PG $E_2$ | PG $I_2$ | LIN | ARAC | OL | STEAR | PAL[1] |
|---|---|---|---|---|---|---|---|
| | | | | Correlation coefficient | | | |
| PG $E_2$ | 1.000 | 0.717[3] | -0.02 | 0.05 | 0.15 | -0.02 | -0.06 |
| PG $I_2$ | | 1.000 | 0.07 | -0.13 | -0.00 | 0.10 | -0.01 |
| LIN | | | 1.00 | 0.14 | -0.15 | -0.23[2] | -0.25[2] |
| ARAC | | | | 1.00 | -0.07 | -0.31[3] | -0.50[3] |
| OL | | | | | 1.00 | -0.01 | -0.32[3] |
| STEAR | | | | | | 1.00 | -0.25[2] |

[1] LIN=linoleic acid; ARAC=arachidonic acid; OL=oleic acid; STEAR=stearic acid; PAL=palmitic acid.

[2] ($p < 0.05$).

[3] ($p < 0.01$).

TABLE 21. Correlations between stimulated prostaglandin synthesis and cellular fatty acids

| | $PG\ E_2$ | $PG\ I_2$ | LIN | ARAC | OL | STEAR | PAL[1] |
|---|---|---|---|---|---|---|---|
| | | | Correlation coefficient | | | | |
| $PG\ E_2$ | 1.000 | 0.812[2] | 0.04 | -0.14 | -0.03 | 0.11 | 0.01 |
| $PG\ I_2$ | | 1.000 | 0.05 | -0.14 | 0.11 | -0.01 | 0.01 |

[1] LIN=linoleic acid; ARAC=arachidonic acid; OL=oleic acid; STEAR=stearic acid; PAL=palmitic acid.

[2] ($p < 0.01$).

thrombin ($p < 0.01$) (Table 22). Stimulating endothelial cells from male babies with 0.125 units of thrombin produced a significant increase in prostacyclin synthesis. Increasing the doses of thrombin from 0.125 to 0.500 units did not produce a further increase in prostacyclin synthesis by these cells. Stimulating endothelial cells from female babies with 0.125 units of thrombin also produced a significant increase in prostacyclin synthesis ($p < 0.01$), but up to a lower value than that observed in male cells. Increasing the amount of thrombin from 0.125 to 0.500 units produced a further increase ($p < 0.01$) in prostacyclin synthesis. This amount was not statistically different from that produced by male cells when stimulated with either 0.125 or 0.500 units of thrombin.

Prostaglandin $E_2$ synthesis also was higher in endothelial cells from male babies than in endothelial cells from female babies ($p < 0.05$) (Table 23). These differences were observed when PG $E_2$ synthesis was stimulated with either 0.125 or 0.500 units of thrombin. Unlike prostacyclin, PG $E_2$ synthesis by endothelial cells harvested from umbilical cords from male babies was further stimulated by increasing the dose of thrombin from 0.125 to 0.500 units.

No differences between the ratio of prostaglandin $E_2/I_2$ synthesized by endothelial cells from male babies and the ratio synthesized by cells from female babies were found, and no effects of stimulation with thrombin upon this ratio were observed (Table 24).

TABLE 22. Effect of sex upon prostacyclin synthesis by endothelial cells after stimulation with thrombin

| | THROMBIN (UNITS) | | |
|---|---|---|---|
| | 0.000[1] | 0.125[2] | 0.500[2] |
| | ng/flask[3] | | |
| MALE CELLS | 0.95 A (0.11) | 8.96 B (1.22) | 8.76 B[4] (0.90) |
| FEMALE CELLS | 0.57 A (0.06) | 5.43 C (0.88) | 7.75 B (0.71) |

[1] The concentration of PG $I_2$ was measured in the tissue culture medium after 72 hours of incubation with the cells. From this value, PG $I_2$ synthesis in five minutes was estimated. PG $I_2$ was measured as 6-keto PG $F_{1\alpha}$.

[2] Thrombin effect ($p < 0.01$). The cells were incubated with either 0.125 or 0.500 units of thrombin during 5 minutes at 37 °C.

[3] Mean (SEM) of the square root of the actual values (N=8).

[4] Sex effect ($p < 0.01$).

TABLE 23. Effect of sex upon prostaglandin $E_2$ synthesis by endothelial cells after stimulation with thrombin

| | THROMBIN (UNITS) | | |
|---|---|---|---|
| | 0.000[1] | 0.125[2] | 0.500[2] |
| | pg/flask[3] | | |
| MALE CELLS | 1.82 A (0.18) | 16.83 B (2.11) | 20.75 C[4] (1.48) |
| FEMALE CELLS | 1.41 A (0.24) | 11.71 D (1.84) | 14.94 E (1.19) |

[1] The concentration of PG $E_2$ was measured in the tissue culture medium after 72 hours of incubation with the cells. From this value, PG $E_2$ synthesis in five minutes was estimated.

[2] Thrombin effect ($p < 0.01$). The cells were incubated with either 0.125 or 0.500 units of thrombin during 5 minutes at 37 °C.

[3] Mean (SEM) of the square root of the actual values. (N=8).

[4] Sex effect ($p < 0.01$).

TABLE 24. Effect of sex upon the ratio PG $E_2$/PG $I_2$[1] by endothelial cells after stimulation with thrombin

| | THROMBIN (UNITS) | | |
|---|---|---|---|
| | 0.000[2] | 0.125[3] | 0.500[3] |
| | PG $E_2$/PG $I_2$ x $10^3$ | | |
| MALE CELLS | 6.26[4] (0.43) | 6.38 (0.71) | 7.23 (1.06) |
| FEMALE CELLS | 7.65 (0.84) | 8.90 (2.21) | 6.23 (0.72) |

[1] PG $I_2$ was measured as 6-keto PG $F_{1\alpha}$.

[2] The concentration of prostaglandins was measured in the tissue culture medium after 72 hours of incubation with the cells. From this value, prostaglandin synthesis in five minutes was estimated.

[3] The cells were incubated with either 0.125 or 0.500 units of thrombin during five minutes at 37 °C.

[4] Mean (SEM) of the square root of the ratio PG $E_2$/PG $I_2$ X $10^3$. (N=8).

Effects of plasma Endothelial cells harvested from umbilical cords from male babies produced more prostacyclin ($p < 0.01$) than those harvested from female babies independent of whether they were incubated with plasma from males or females (Table 25).

Endothelial cells harvested from umbilical cords from male babies also produced more PG $E_2$ ($p < 0.01$) than those harvested from female babies independent of whether they were incubated with plasma from male or female subjects (Table 26).

No differences were found between the ratio PG $E_2$/PG $I_2$ of male cells and that of female cells, and there was no effect of plasma from female or male subjects (Table 27). Even though sex differences in prostaglandin synthesis were evident, no differences were found in cellular fatty acid composition, expressed either as per cent of total fatty acids or as total concentration of fatty acids (Tables 28 and 29).

TABLE 25. Prostacyclin synthesis by either male or female cells incubated with plasma from either male or female subjects

| CELLS | PLASMA | THROMBIN (UNITS) 0.000[1] | THROMBIN (UNITS) 0.125[2] | |
|---|---|---|---|---|
| | | ng/flask[3] | | |
| MALE | MALE | 0.36 (0.07) | 5.44 (1.29) | A[4] |
| MALE | FEMALE | 0.37 (0.09) | 5.23 (0.74) | A |
| FEMALE | FEMALE | 0.24 (0.03) | 3.68 (0.51) | B |
| FEMALE | MALE | 0.21 (0.03) | 2.66 (0.30) | B |

[1] The concentration of PG $I_2$ was measured in the tissue culture medium after 72 hours of incubation with the cells. From this value, PG $I_2$ synthesis in 5 minutes was estimated. PG $I_2$ was measured as 6-keto PG $F_{1\alpha}$.

[2] Thrombin effect ($p < 0.01$). The cells were incubated with 0.125 units of thrombin during 5 minutes at 37 °C.

[3] Mean (SEM) of the square root of the actual values. (N=6).

[4] Cells effect ($p < 0.01$).

TABLE 26. Prostaglandin $E_2$ synthesis by either male or female cells incubated with plasma from either male or female subjects

| CELLS | PLASMA | THROMBIN (UNITS) 0.000[1] | 0.125[2] | |
|---|---|---|---|---|
| | | pg/flask[3] | | |
| MALE | MALE | 1.34 (0.22) | 8.27 (0.28) | A[4] |
| MALE | FEMALE | 1.26 (0.20) | 8.25 (0.29) | A |
| FEMALE | FEMALE | 0.96 (0.10) | 6.97 (0.44) | B |
| FEMALE | MALE | 0.83 (0.08) | 6.05 (0.30) | B |

[1] The concentration of PG $E_2$ was measured in the tissue culture medium after 72 hours of incubation with the cells. From this value, PG $E_2$ synthesis in 5 minutes was estimated.

[2] Thrombin effect ($p < 0.01$). The cells were incubated with 0.125 units of thrombin during 5 minutes at 37 °C.

[3] Values are Mean (SEM) of the square root of the actual values. (N=6).

[4] Cells effect ($p < 0.01$).

TABLE 27. Ratio PG $E_2$/PG $I_2$[1] synthesis by either male or female cells incubated with plasma from either males or females

| CELLS | PLASMA | THROMBIN (UNITS) 0.000[2] | THROMBIN (UNITS) 0.125[3] |
|---|---|---|---|
| | | PG $E^2$/PG $I^2$ X $10^3$ | |
| MALE | MALE | 7.93[4] (0.92) | 5.82 (0.95) |
| MALE | FEMALE | 7.29 (0.51) | 5.43 (0.65) |
| FEMALE | FEMALE | 8.29 (0.59) | 6.60 (1.00) |
| FEMALE | MALE | 7.99 (0.52) | 9.34 (1.50) |

[1] PG $I_2$ was measured as 6-keto PG $F_{1\alpha}$.

[2] The concentration of prostaglandins was measured in the tissue culture medium after 72 hours of incubation with the cells. From this value prostaglandin synthesis in 5 minutes was estimated.

[3] The cells were incubated with 0.125 units of thrombin during 5 minutes at 37 °C.

[4] Mean (SEM) of the square root of the ratio PG $E_2$/PG $I_2$ X $10^3$. (N=6).

TABLE 28. Fatty acid composition of endothelial cells harvested from veins of umbilical cords from either male or female babies and incubated with plasma from either males or females

| FATTY ACID | PLASMA MALES | PLASMA FEMALES |
|---|---|---|
| | CELLS FROM MALES | |
| | Per cent of total | |
| 16:0 | 31.7 (1.1)[1] | 37.7 (2.0) |
| 18:0 | 20.4 (1.0) | 18.2 (1.5) |
| 18:1 | 14.5 (1.3) | 13.0 (0.6) |
| 18:2 | 9.8 (0.8) | 9.3 (0.8) |
| 18:3 | 0.4 (0.2) | 0.4 (0.2) |
| 20:3 | 3.8 (1.8) | 2.6 (0.4) |
| 20:4 | 18.3 (1.8) | 18.4 (1.5) |
| | CELLS FROM FEMALES | |
| | Per cent of total | |
| 16:0 | 36.2 (1.6) | 34.3 (1.8) |
| 18:0 | 19.5 (1.1) | 19.6 (1.0) |
| 18:1 | 16.1 (1.2) | 16.2 (1.7) |
| 18:2 | 8.9 (0.6) | 9.1 (1.2) |
| 18:3 | 0.3 (0.1) | 0.6 (0.3) |
| 20:3 | 2.1 (0.4) | 1.7 (0.4) |
| 20:4 | 16.8 (2.0) | 18.4 (2.4) |

[1] Values are Mean (SEM) (N=5).

TABLE 29. Fatty acid composition of endothelial cells harvested from veins of umbilical cords from either male or female babies and incubated with plasma from either males or females

| CELLS FROM MALES | | |
|---|---|---|
| FATTY ACID | PLASMA MALES | PLASMA FEMALES |
| | ug/flask | |
| 16:0 | 6.6 (0.6)[1] | 7.1 (0.4) |
| 18:0 | 4.2 (0.2) | 3.5 (0.5) |
| 18:1 | 3.0 (0.4) | 2.5 (0.2) |
| 18:2 | 2.0 (0.2) | 1.8 (0.2) |
| 18:3 | 0.1 (0.03) | 0.1 (0.04) |
| 20:3 | 0.8 (0.5) | 0.5 (0.04) |
| 20:4 | 3.7 (0.2) | 3.5 (0.4) |
| TOTAL | 20.7 (1.3) | 19.1 (1.4) |
| **CELLS FROM FEMALES** | | |
| FATTY ACID | PLASMA MALES | PLASMA FEMALES |
| | ug/flask | |
| 16:0 | 7.0 (0.5) | 7.3 (0.9) |
| 18:0 | 4.2 (0.4) | 4.2 (0.8) |
| 18:1 | 3.1 (0.4) | 3.3 (0.5) |
| 18:2 | 2.1 (0.2) | 2.3 (0.5) |
| 18:3 | 0.1 (0.02) | 0.2 (0.08) |
| 20:3 | 0.6 (0.2) | 0.4 (0.1) |
| 20:4 | 3.9 (0.2) | 3.8 (0.3) |
| TOTAL | 20.4 (1.4) | 22.2 (3.5) |

[1] Values are Mean (SEM) (N=5).

# DISCUSSION

## Isolation of endothelial cells

The methodology used to isolate endothelial cells was a modification of the method described by Jaffe et al. (88). They used collagenase (0.2%) to harvest the cells. Some other authors also have used collagenase for this purpose (50,51,17), and there is some suggestion that collagenase may produce less damage to the cells than trypsin. Because of this information, collagenase was used first to harvest the cells, but without success. Other authors also have reported no success when trying to use collagenase (94), so trypsin was used here. In some papers, it has been reported that 45 minutes of incubation with trypsin is the optimal time to harvest endothelial cells (95). However, it was found in this study that incubation during 30 minutes produced cell damage and decreased considerably the number of viable cells when compared to a 15 minute incubation time.

Others have mentioned that the cells were isolated within 6 hours after the baby was born (95,96). But, in the present investigation, it was found that a greater number of viable cells were obtained when they were isolated 24 to 48 hours after the baby was born. Time course data are not available, but these results are in agreement with those of others (97) who reported better success when the cells were isolated 24 to 48 hours after the baby was born. After 48 hours, a considerable decrease in the number of viable cells was observed.

With the methodology used in this study, the rate of success in isolating endothelial cells was about 80%, although no exact quantitation was recorded. Only monolayers in which no fibroblast contamination was detectable were used. All the cells used grew actively, reaching confluency in an average period of 4 days, and no significant difference was found in the number of days necessary to reach confluency between the cells used in the various treatments.

Effect of fasting upon prostaglandin synthesis

In the first study, no differences in prostaglandin synthesis, either basal or thrombin stimulated, were found between endothelial cells incubated with plasma samples drawn from fasting subjects and cells incubated with plasma samples drawn two hours after breakfast. Hwang et al. (41) have reported that serum prostaglandins were higher in fed rats eating a diet with hydrogenated vegetable fat than those of fasting rats eating the same diet. The free fatty acid concentrations were higher in the serum of fasting rats than that of fed rats. However, no differences were found between fasting and fed rats when a diet with either corn oil or beef fat was eaten. A higher synthesis of prostaglandin after eating a meal is suggested by previous reports (98-101) showing an increased platelet aggregation in man after eating.

Sullivan and Mathias (52) found that serum prostaglandins were higher in fasting rats than in fed rats. The most logical explanation for this is that the higher levels of norepinephrine normally observed during fasting would be responsible for increasing the serum levels of

prostaglandins. It is known that norepinephrine increases platelet aggregation and stimulates liberation of arachidonic acid. In addition, it is known that fatty acids bound to albumin (which would represent the fraction of free fatty acids in plasma) can be utilized for prostaglandin synthesis, and it also has been reported that increased levels of free fatty acids are associated with increased platelet aggregation and thromboxane $A_2$ synthesis (102-105).

Hwang et al. (41) found an effect only when hydrogenated fat was used and no effect when other fats were used. The reason for this is not known, but they suggested that it could be due to the presence of trans-fatty acids in the hydrogenated fat. The fact that the same group of researchers (Sullivan and Mathias (52)) reported completely opposite results suggest that further investigation is needed. Prostaglandin synthesis is stimulated by tissue injury; different methodologies were used in these two studies to draw the blood samples, and an artifactual effect cannot be ruled out.

The finding of no differences in prostaglandin synthesis between endothelial cells incubated with plasma from fasting and fed subjects does not rule out the possible existence of biological differences. The time after breakfast at which the samples were drawn may be important. In the two previous studies, the fed rats were eating almost continuously until possibly a few minutes before they were killed. Further studies in which the culture medium is supplemented with plasma drawn under fasting conditions and at different times after a meal might provide an answer to this question.

Effects of diet upon prostaglandins and cellular fatty acids

There was a significant effect of diet upon prostacyclin synthesis by endothelial cells. In agreement with previous reports in the literature (50,51), there was a tendency for endothelial cells incubated with plasma drawn from subjects eating a diet with high levels of linoleate to synthesize less prostacyclin than endothelial cells incubated with plasma from subjects eating diets with lower levels of linoleate. However, the fact that an interaction between diet and sequence in which the diets were eaten was observed when prostacyclin synthesis was stimulated with thrombin suggests that the sequence in which the diets were eaten was an important factor to determine whether an effect could be observed or not, and it also suggests that a carry-over effect of the previous diet may have been present. It was observed that when the modified diet (higher linoleate) followed the average U.S. diet (lower linoleate), there was a tendency to decrease prostacyclin synthesis. But, when the average U.S. diet followed the modified diet, that tendency was not observed (Figure 4).

Several mechanisms have been suggested to explain the inhibitory effect of linoleic acid upon prostacyclin synthesis. Among those suggestions are that linoleate may decrease the amount of arachidonate in the pool used to synthesize prostaglandins, or that linoleate could be acting by inhibiting cyclooxygenase activity. Against the last hypothesis, Funk and Powell (106) reported that linoleate was not a potent inhibitor of the conversion of $^{14}$C-arachidonic acid to

prostacyclin in particulate fractions of calf aortas, suggesting then, that the effect of linoleate could be mediated by decreasing the pool of arachidonate used to synthesize prostaglandins.

In this study, it was observed that even though linoleate consumption and its concentration in the cells were decreased when the diet was changed from the modified to the average U.S. diet in sequence 2, there was no increase in prostacyclin synthesis. This could be explained if the arachidonate pool had been decreased by a previous diet high in linoleate and more than 4 weeks are required for recovery. In other studies, it has been reported that in about two weeks after increasing the amount of linoleate in the diet, the content of linoleic acid in plasma lipoproteins and free fatty acids increases and there is a decrease in plasma arachidonate (107-114). Increasing linoleate in the culture medium of endothelial cells decreases arachidonic acid in cell phospholipids and decreases prostacyclin synthesis (50,51). These observations are in agreement with the effect of the modified diet upon prostacyclin synthesis when the diets were eaten in sequence 1.

The lack of effect of the modified diet when it followed the self-selected diet in sequence 2 could be explained if the amount of linoleate in the self-selected diet was similar to that in the modified diet. This could explain also the lack of effect of the average U.S. diet when it followed the self-selected diet in sequence 1. The information about the amount of linoleate in the self-selected diet presently is not available. But the percentage of linoleic acid in the

cells incubated with plasma from the subjects eating the self-selected diet was similar to that from subjects eating the modified diet, and both of them were higher than that from subjects eating the average U.S. diet. However, no differences in the arachidonic acid content of the cells were detected. This fact does not invalidate the previous possibility, because, even though no changes were detected in the total arachidonic acid content of the cells, it is possible that the amount of arachidonic acid present in the pool which is substrate for cyclooxygenase could have been decreased.

The suggestion that total arachidonic acid does not represent the pool of arachidonic acid that is used as substrate for cyclooxygenase is supported by the finding of no significant correlation between arachidonic acid and prostacyclin synthesis by endothelial cells, either in basal conditions or stimulated with thrombin. Prostacyclin synthesis was not correlated significantly with any of the other fatty acids, nor was linoleic acid correlated with arachidonic acid. These findings further support the suggestion that total fatty acids do not neccessarily represent the proportion in which they may be present in the different cellular pools.

Another possible explanation for the lack of effect observed during the change from self-selected to either modified or average U.S. diets could be that these first 4 weeks of study were a period of adaptation for the subjects. They were changed from an ad libitum intake to a fixed energy intake that was similar for all of them irrespective of

body weight, height, or activity. As a result of this, some subjects were losing weight during this first experimental period. To stop this process, the energy intake of those individuals was adjusted by using hard candy (sugar). Changes in fatty acid metabolism and possibly prostaglandin synthesis can be induced by changes in energy intake (104,115).

Prostaglandin $E_2$ synthesis followed the same pattern as prostacyclin, but differences were not statistically significant. There was no effect of diet upon the ratio PG $E_2$/PG $I_2$, and the proportion of PG $E_2$ to PG $I_2$ was very small. A lower synthesis of prostaglandin $E_2$ than prostacyclin is normally observed in endothelial cells. The ratio PG $E_2$/PG $I_2$ is larger in smooth muscle cells, and it is even greater in fibroblasts (116,117). The ratio also is increased in atherosclerotic lesions and in endothelial cells incubated with high levels of cholesterol (118,119). A change in the ratio of PG $E_2$/PG $I_2$ in endothelial cells may be the first sign of lesion. In this study, diet did not change the ratio, and independent of the diet, a significant correlation was found between prostacyclin and prostaglandin $E_2$ synthesis under basal ($R = 0.72$, $N=118$, $P<0.0001$) and stimulated conditions ($R = 0.81$, $N = 120$, $P<0.0001$).

Effects of sex

A higher prostacyclin synthesis by endothelial cells isolated from umbilical cords from male babies than that by cells isolated from female babies was found. These results are in agreement with previous studies

that have shown that male rats produce more prostacyclin than female rats (79,80,82). The latter authors have suggested that these differences are explained by the effect of sex hormones. They have found that ovariectomy in rats increased prostacyclin synthesis to levels similar to those observed in males. Using this model, an inhibitory effect of estrogens upon prostacyclin synthesis was described, but no effect of testosterone was found.

However, when cells growing in culture were incubated with sex hormones (76-78), it was found that testosterone inhibits, and that estradiol increases, prostacyclin synthesis in smooth muscle cells. In the present study, when endothelial cells were incubated with plasma from males and females, male cells still produced more prostacyclin than female cells, and incubating male cells with plasma from females did not change prostacyclin synthesis; neither was prostacyclin synthesis changed when female cells were incubated with plasma from males. These results may suggest that sex hormones do not have an effect upon prostacyclin synthesis by endothelial cells, and the results do not agree with the stimulatory effect of estradiol and the inhibitory effect of testosterone observed when they were added to smooth muscle cells growing in culture.

Several factors could be responsible for the differences in effect between these two experiments. In the studies in which sex hormones were added to the culture medium of smooth muscle cells, sex hormones already were present in the fetal calf serum used as a supplement. It

is possible that sex hormones may exert different effects depending upon the concentration at which they are present. In addition, sex hormones present in plasma are normally bound to proteins, and this binding is a way of controlling their biological effects. The sex hormone binding beta globulin also appears to influence the uptake of these hormones by the tissues (120). By adding free hormones to the culture medium of the cells, non-physiological effects could have been observed and possibly this is the explanation to the results obtained in those studies.

In the experiments done with ovariectomized rats (79,80,82), it was found that estrogens decreased prostacyclin synthesis. In those studies, estrogens were injected subcutaneously, and it could be expected that they could be delivered to the tissues in a physiological way. Estrogens also decrease thrombus formation in male rats (73). Also, it has been suggested that estrogens may have a protective effect upon the development of cardiovascular disease by their effects upon atrial myocites (121). The finding of no effect of plasma from either female or male subjects upon prostaglandin synthesis by endothelial cells isolated from umbilical cords from either male or female babies does not rule out the possibility of an effect of sex hormones. There are several points at which sex hormones may exert their effects upon prostaglandin synthesis (81). It is possible that if their effect is exerted by modifying the total amount of enzyme present in the cells (either cyclooxygenase, prostacyclin synthetase or both), it would take more than a 3 day incubation period to observe an effect.

No statistical differences between males and females were found in basal prostacyclin synthesis. This finding is in agreement with the report of Ylikorkala et al. (84) about no differences in plasma prostacyclin concentrations between men and women, but it does not support the report of Neri Serneri (85) of higher concentrations of prostacyclin in plasma from women. Interpretation of plasma concentrations of prostaglandins is very difficult. As mentioned earlier, tissue injury induces prostaglandin synthesis, and because of that, great differences in plasma concentrations of prostaglandins may only represent either different methods of blood drawing or the ability of the technician who drew the blood samples.

No differences between the fatty acid composition of female and male cells were observed, which is in agreement with previous reports (77). No correlations between either arachidonic or linoleic acid and prostaglandin synthesis were found. This finding, again, reinforces the concept that total fatty acids in the cell do not represent the composition of specific pools that may be having a direct influence upon prostaglandin synthesis.

The finding of no correlations between the amount of linoleic acid in the diet and the amount of linoleic acid in the cells or prostaglandin synthesis could be expected, since several over- or under-estimations may exist in the calculation of the composition of the diets; the 3 day period of collection of data has several limitations, and there are several different control mechanisms that cannot be

accounted for in the distribution of the fatty acids in the different cellular pools.

It would be interesting to find out whether the sex differences in prostaglandin synthesis observed in this study could be modified by incubating the cells with plasma from males and females during a more prolonged period of time, or by starting these incubations before the cells reach confluency.

A decrease in prostacyclin synthesis by the endothelium has been proposed as a factor that may increase the risk of developing thrombosis and ischemic heart disease. However, the results of this study show that endothelial cells from females and cells incubated with plasma from subjects eating a diet high in linoleic acid (factors that are associated with a lower risk of developing thrombosis and coronary heart disease) syynthesize less prostacyclin than either those from males or cells incubated with plasma from subjects eating a diet low in linoleic acid.

## SUMMARY

Atherosclerosis is a public health problem in the United States. Its pathophysiology remains a mystery, although to explain it several hypotheses have been formulated. A diet containing 10% of the total energy as linoleic acid, 30% of the total energy as fat, and no more than 100 mg of cholesterol has been recommended to decrease the risk of developing atherosclerosis. In addition to diet, several other factors have been associated with a higher risk of developing this disease, and sex is one of those, since the incidence of atherosclerosis is higher in men than in women.

Linoleic acid is elongated to arachidonic acid, which is a precursor for the synthesis of prostaglandins, thromboxanes, and leukotrienes. A balance between prostacyclin synthesis by endothelial cells and thromboxane $A_2$ synthesis by platelets is necessary in order to reduce the risk of developing thrombosis or damage to the endothelium. It has been hypothesized that continuous damage to the endothelium may lead to the development of atherosclerosis. Prostacyclin synthesis by endothelial cells and thromboxane $A_2$ synthesis by platelets can be modified by changing the amount of linoleic acid in cellular lipids.

Sex differences in prostacyclin synthesis have been reported. Male mice are more susceptible to thrombosis than female mice, and aortas from male rats produce more prostacyclin than aortas from female rats. This difference disappears when female rats are ovariectomized, but the difference is restored if the rats are treated with estradiol,

suggesting that estradiol decreases prostacyclin synthesis. However, in cells growing in culture, testosterone inhibits, and estradiol increases prostacyclin synthesis.

This research was designed to study: 1) the effect of plasma from human subjects eating diets with different amounts of linoleic acid upon prostaglandin synthesis and fatty acid composition of human endothelial cells growing in culture, 2) whether there were differences in prostaglandin synthesis between human endothelial cells isolated from umbilical cord veins from male and female babies, and 3) the effect of plasma from males and females upon prostaglandin synthesis and fatty acid composition of endothelial cells isolated from human umbilical cord veins from male and female babies.

For the first study, plasma samples from 20 healthy women, 18 to 20 years old participating in a collaborative study (NC-167), were used. They were fed two diets, one that represented the average U.S. diet and another with a composition similar to the U.S. recommended diet (Modified diet). The average U.S. diet contained 2000 Kcal, 40% of them as fat; 4% of the Kcal as linoleic acid; and 700 mg of cholesterol. The modified diet contained 2000 Kcal, 30% of the Kcalories as fat; 10% of the total Kcalories as linoleic acid; and 100 mg of cholesterol.

A cross-over experimental design was used in which, the subjects first recorded their ad libitum intake during 7 days; this period was named pre-experimental period, and the diet was named self-selected. At the end of this period, a fasting blood sample was drawn and the

subjects started the experimental period. Half of the subjects started eating the average U.S. diet during 4 weeks, and then changed to eat the modified diet during the next 4 weeks (sequence 1). Half of the subjects started eating the modified diet during 4 weeks, and then changed to the average U.S. diet during the next 4 weeks (sequence 2). Blood samples were drawn after an overnight fast at the end of the 4 weeks, before starting the new diet. Half of the 20 subjects participating in the study were fed their diets in Iowa, and half were fed their diets in Nebraska. At the begining of the experimental period, all the subjects were fed the same amount of food irrespective of body weight, height, or activity. Some subjects were losing weight during the first week of this period. To stop this process, the energy intake of those individuals was adjusted by using hard candy (sugar). In the subjects being fed their diets in Iowa, an additional blood sample was drawn two hours after starting each new diet. This sample was used to test whether changes in prostaglandin synthesis by endotnelial cells could be induced by an acute change in the diet.

To test whether there was an effect of sex upon prostaglandin synthesis, endothelial cells isolated from umbilical cords from either male or female babies were stimulated with three doses of thrombin (0.000, 0.125, and 0.500 units). To test whether there was an effect of plasma from either male or female subjects upon prostaglandin synthesis and fatty acid composition of endothelial cells, a 2 x 2 x 2 cross over design was used in which plasma from males and females was incubated with cells from males and females.

Endothelial cells were harvested from human umbilical cords using a sterile technique and grown to confluency in modified medium 199 with 20% fetal calf serum. When the cells reached confluency, plasma from the respective experimental subjects was used to supplement the modified medium 199 and the cells were incubated during 72 hours. At the end of this period, prostaglandin synthesis was stimulated with thrombin during 5 minutes at 37 °C. Prostaglandins were measured using a radioimmunoassay procedure and the fatty acids were determined by using gas-liquid chromatography.

No differences in prostaglandin synthesis (prostacyclin and prostaglandin $E_2$) between the cells incubated with plasma drawn from fasting subjects and those drawn from the same subjects 2 hours after breakfast were found. There was a significant effect of diet upon prostacyclin synthesis. Endothelial cells incubated with tissue culture medium supplemented with 20% plasma from subjects eating the modified diet produced less prostacyclin than those incubated with medium supplemented with 20% plasma from the same subjects eating the self-selected diet. Basal prostacyclin synthesis by cells incubated with tissue culture medium supplemented with plasma from the subjects eating the average U.S. diet was in between, but not statistically different from the subjects eating the self-selected diet and from that of the subjects eating the modified diet. A similar effect was found when prostacyclin synthesis was stimulated with thrombin in cells incubated with plasma from subjects eating their diets in sequence 1, but no

effect was found in cells incubated with plasma from those eating their diets in sequence 2. No effect of diet upon either prostaglandin $E_2$ synthesis or the ratio PG $E_2$/PG $I_2$ synthesis was found.

Endothelial cells incubated with plasma from subjects eating the self-selected diet and the modified diet had a higher percentage of linoleic acid than endothelial cells incubated with plasma from subjects eating the average U.S. diet, irrespective of the sequence in which the diets were eaten. No differences were observed between the other fatty acids. No significant correlations were found between either basal or stimulated prostaglandin (prostacyclin or prostaglandin $E_2$) synthesis and the different fatty acids.

It was found that endothelial cells harvested from umbilical cords from male babies produced more prostaglandins (prostacyclin and prostaglandin $E_2$) than endothelial cells harvested from umbilical cords from female babies, and no effect of plasma from either males or females upon these differences was found. Even though sex differences in prostaglandin synthesis were evident, no differences were found in cellular fatty acid composition, either expressed as per cent of total fatty acids or as total concentration of fatty acids.

The effects of linoleic acid found in this study are in agreement with previous publications that have shown no effect or an inhibitory effect of linoleic acid upon prostaglandin synthesis by endothelial cells. The effect of sex upon prostaglandin synthesis found in this study is in agreement with previous studies done in rats, but not with

studies in which sex hormones have been added to cells growing in culture. These differences possibly can be explained by the non-physiological way in which those hormones have been added to the cells.

# CONCLUSIONS

1. No differences in prostaglandin (prostacyclin and PG $E_2$) synthesis were observed between endothelial cells incubated with plasma drawn after an overnight fast and plasma drawn two hours after breakfast, even when the composition of the diet eaten at that breakfast was different from the diet being eaten before.
2. There was a tendency to decrease prostacyclin synthesis when endothelial cells were incubated with plasma from subjects eating a diet high (10% of total energy) in linoleic acid (modified diet) when it followed the intake of a diet low (4% of total energy) in linoleic acid (average U.S. diet).
3. No differences in prostacyclin synthesis were observed between endothelial cells incubated with plasma from subjects eating a diet low (4% of total energy) in linoleic acid (average U.S. diet) and endothelial cells incubated with plasma from the same subjects eating a diet high (10% of total energy) in linoleic acid (modified diet) when the diet low in linoleic acid followed the diet high in linoleic acid.
4. No statistically significant effect of diet upon PG $E_2$ or PG $E_2$/PG $I_2$ ratio was observed.
5. Endothelial cells incubated with plasma from subjects eating the self-selected diet and the modified diet had a higher percentage of linoleic acid than cells incubated with plasma

from subjects eating the average U.S. diet, irrespective of the sequence in which the diets were eaten. No differences were observed in the rest of the fatty acids.

6. No significant correlations were found between cellular fatty acids and either basal or stimulated prostaglandin (prostacyclin and prostaglandin $E_2$) synthesis.
7. The effects of linoleic acid found in this study are in agreement with previous publications that have shown an inhibitory effect of linoleic acid upon prostaglandin synthesis by endothelial cells.
8. Endothelial cells isolated from umbilical cords of male babies produced more prostaglandins (prostacyclin and PG $E_2$) than cells from umbilical cords of female babies when stimulated with 0.125 units of thrombin, and no effect of plasma from females or males upon these differences were observed.
9. No differences were found between the fatty acid composition of endothelial cells isolated from umbilical cords of male babies and those isolated from female babies.
10. No significant correlations were found between fatty acids and prostaglandin synthesis by cells from either males or females.
11. No correlations between total linoleic acid or cholesterol in the diet and either prostaglandin synthesis or fatty acid compositon of endothelial cells were found.

12. The effect of sex upon prostaglandin synthesis found in this study is in agreement with previous studies done in rats, but not with studies in which sex hormones have been added to cells growing in culture. These differences possibly can be explained by the non-physiological way in which those hormones have been added to the cells.

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