THE EFFECTS OF DIABETES MELLITUS, CORONARY ARTERY DISEASE, EXERCISE AND STANOZOLOL ON THE FIBRINOLYTIC ENZYME SYSTEM IN MAN

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DECLARATION

The work described in this thesis was performed in the Coagulation Section of the University Department of Medicine, Royal Infirmary, Glasgow from 1981 till 1985. All the studies described were conceived, designed and analysed by the author in person, and the opinions expressed are therefore those of the author. The clinical study on stanozolol and enzyme induction was performed in association with Dr M J Brodie, Consultant in Clinical Pharmacology, of the Western Infirmary, Glasgow. Some of the studies described in this thesis have already been published or are in press in peer review journals, and copies can be found at the end of this thesis. They include:-

Small, M., McArdle, B.M., Lowe, G.D.O., Forbes, C.D. and Prentice, C.R.M. (1982). The effect of intramuscular stanozolol on fibrinolysis and blood lipids. Thrombosis Research, 28, 27-36.

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SUMMARY

This thesis reviews the literature relating vascular disease to fibrinolysis and describes a number of clinical studies which have attempted to evaluate the components of the fibrinolytic enzyme system in a variety of different medical disorders and in healthy subjects. In these clinical settings a comparison has been made between the plasma fibrinolytic activity (fibrin plate: ex vivo fibrinolysis) and BBeta 15-42 fragment (in vivo plasmin-mediated fibrinolysis) as indicators of fibrinolysis, and the relative importance of fibrinolysis and coagulation has been estimated by measuring both plasmin (BBeta 15-42) and thrombin generation (fibrinopeptide A). The effect on fibrinolysis of a number of clinical and laboratory measures have been made with fibrinolysis studied in the basal state and following pharmacological intervention and exercise.

In a double-blind, placebo-controlled study, exercise in young healthy male subjects was shown to be associated with enhanced plasma fibrinolytic activity without a concomitant activation of coagulation. The rise in plasma fibrinolytic activity was not influenced by prior administration of beta-blocker drugs and therefore adrenergic mechanisms made no contribution to the exercise enhanced plasma fibrinolytic activity. Exercise in middle-aged men was related, in part, to the workload and was associated with both thrombin and plasmin generation suggesting that the enhanced fibrinolysis may have been secondary to activation of coagulation. In men of the

same age group with coronary artery disease no alterations of haemostasis were detected. Whether such defective in vivo fibrinolysis was related to coronary artery disease per se, to the lack of changes in coagulation or to the workload intensity requires further study.

Defective fibrinolysis has been noted in coronary artery disease and in 100 men with angiographically defined coronary artery disease we have examined the inter-relationships between fibrinolysis, lipids and hormones and the relationships between these 3 factors and the severity of coronary artery disease. We found that patients with a previous myocardial infarction had raised oestradiol levels but that these were not related to the tests of fibrinolysis. While higher alpha2-antiplasmin levels were found in hypertriglyceridaemia,. lipid levels were not associated with altered fibrinolysis. We have provided evidence that BBeta 15-42 is a reliable indicator of in vivo fibrinolysis by showing significant correlations with plasminogen (negative) and FDP´s (positive). This is consistent with activation of fibrinolysis (increased BBeta levels) being associated with decreased plasminogen and raised FDP´s. Apart from fibrinogen levels, none of the laboratory measures were related to the severity of coronary artery disease which fails to provide support for defective basal fibrinolysis being associated with coronary artery disease.

The anabolic steroid, stanozolol is felt to be the most promising oral agent to induce long-term enhancement of fibrinolysis. We have performed a number of studies with this drug and have found that, given via the

intramuscular route, stanozolol did not decrease fibrinogen and alpha2-macroglobulin levels (unlike the orally administered drug) and while it led to rapid and sustained changes in plasma fibrinolytic activity and plasminogen in young healthy males, it failed to effect such changes on fibrinolysis in elderly patients with medical illness. In studies using oral stanozolol we have shown that it caused plasmin-mediated fibrinolysis in normal subjects but not in type II (non-insulin dependent) diabetic patients. We have also noted favourable effects on carbohydrate metabolism in diabetics given stanozolol, but adverse hormonal and lipid effects in other studies. The weight of evidence suggests that stanozolol is not a useful anti-thrombotic agent.

Vascular disease is the major complication affecting diabetic patients and conflicting reports abound on the function of the fibrinolytic enzyme system in diabetes mellitus. We have performed large and detailed controlled studies in diabetes and have found that poor glycaemic control was associated with reduced plasmin and enhanced thrombin generation, and that sex status, type of diabetes and diabetic therapy have an important influence on basal fibrinolysis. Failure to take these factors into account may have led to some of the confused literature on this subject. In diabetes, females had reduced plasma fibrinolytic activity compared to males while the opposite was seen in control subjects. In addition, diabetic females had raised alpha2-antiplasmin levels and augmented thrombin generation. Whether such adverse haemostatic changes could be relevant to the increased risk of

cardiovascular disease in diabetic females remains speculative. Alpha2-antiplasmin levels were both positively correlated with triglyceride levels and raised in type II diabetics and this explains why defective fibrinolysis has been found in such patients when whole blood clot lysis time assays have been employed. Basal fibrinolysis was not related to micro or macrovascular disease. Our results suggest that patients taking sulphonylurea drugs have an alteration of haemostatic balance favouring a thrombotic tendency, and that insulin therapy reversed such abnormalities. In diabetes, the trend was towards enhanced plasmin generation which may, in part, be secondary to the significantly greater thrombin generation found in all diabetic groups. The design and analysis of further studies on fibrinolysis in diabetes must allow for these differences in fibrinolysis related to sex status and type of diabetes.

Throughout these studies we have analysed the effect of beta-blockade, body weight and lipid levels on fibrinolysis and have been unable to provide any clear evidence to show that these factors have a major influence on fibrinolysis.

Plasma fibrinolytic activity, assayed on fibrin plates, has been widely used as a global measure of fibrinolysis, the assumption being that in vivo fibrinolysis is a simple function of the absolute level of circulating plasminogen activators. There are however a number of problems associated with this assay. The activity determined is much smaller than the total activator potential of the plasma, and the variation among

individuals contributes to a wide normal range which means in effect that, unless extreme and prolonged differences exist between patients and controls, a single estimation could fail to detect significant differences between such groups. In addition to these problems we have consistently been unable to show any correlation between plasma fibrinolytic activity and the BBeta 15-42 fragment which places further doubt as to the value of the fibrin plate assay giving a reliable indication of in vivo fibrinolysis. We have failed to find differences in BBeta 15-42 in situations when both the basal plasma fibrinolytic activity (subgroups of patients with diabetes and coronary artery disease) and exercise stimulated plasma fibrinolytic activity were increased (men with coronary artery disease). We have also found increased BBeta 15-42 concentrations without alterations in plasma fibrinolytic activity in healthy males given oral stanozolol. These results suggest therefore that the BBeta 15-42 fragment may be a more useful assay of fibrinolysis in the future investigation of patients with vascular disease.

CHAPTER 1

THE FIBRINOLYTIC ENZYME SYSTEM: HISTORICAL BACKGROUND AND MECHANISMS OF ACTION

1.1 INTRODUCTION

Fibrinolysis is the proteolytic digestion of solid fibrin polymer into smaller soluble fragments. It functions therefore as the physiological process capable of removing fibrin that is deposited in the vascular system or in other compartments of the body. While a variety of proteolytic mechanisms may be involved in fibrin digestion I will concentrate only on plasmin - mediated fibrinolysis and its role in the removal of fibrin from the vascular bed. Fibrinolysis may also function in several other biological phenomena such as tissue repair, neoplasia, macrophage activity, neovascularisation, ovulation and embryo implantation (Collen, 1985; Erickson et al., 1985) but this thesis is primarily concerned with its effects as part of the haemostatic system and these other sites of action will not be further discussed.

1.2 HISTORICAL BACKGROUND

Although Hippocrates, in the first century BC, noted that the blood of deer and of roe never clotted (Smith and Ross, 1912) it was John Hunter (1794) who made the first scientific observations on fibrinolysis. He noted that fluidity of post mortem blood was more likely to occur in individuals who died as a result of "fits, anger, electricity, or lightning", and later found that the blood of 2 deer that he had "run, till they dropped down and

died", failed to clot. The word fibrinolysis was first coined by Dastre (1893) who noted that fibrin lysed when left in contact with blood from dogs who had been repeatedly venesected. This proteolytic property was localised by Hedin (1904) to the globulin fraction of ox blood. In addition to this spontaneous fibrin lysis it was noted that pharmacological potentiation of fibrin lysis could be induced by chloroform (Denys and de Marbaix, 1889) probably by removing the fibrinolytic inhibitors (Delezenne and Pozerski, 1903) - the first demonstration of inhibitors of fibrinolysis. At that time, Nolf (1908) described experiments in which he induced fibrinolytic activity in vivo in dogs by means of intravenous injections of peptones (polyelectrolytes similar to dextran sulphate which is used in the modified fibrin plate technique due to its ability to activate factor XII and increase the precipitation of proactivators of fibrinolysis). He considered that the lysis of blood clots was the normal end result of the coagulation process and, like coagulation, fibrinolysis was due to proteolytic enzymes. He further suggested that the linings of blood vessels contribute components of the fibrinolytic system and that disturbances of fibrinolysis could result in deposition of fibrin on the vessel wall. This concept of the dynamic equilibrium between coagulation and fibrinolysis was restated by Astrup (1958) and still remains one of the most popular theories on haemostasis. Thus at the beginning of the century the process of fibrinolysis was well established although the exact

identity of some of the components remained unknown.

Interest in fibrinolysis was again stimulated by Tillett and Garner (1933) who found that bacteria-free filtrates of beta-haemolytic streptococci contained a substance that rapidly dissolved clotted plasma or fibrin; this agent was called streptococcal fibrinolysin. Milstone (1941) later demonstrated that the streptococcal fibrinolysin activity depended on the presence of a substance in the euglobulin fraction of plasma which was then identified to be plasminogen by Kaplan (1944). A year later this streptococcal fibrinolysin was renamed streptokinase (Christensen and MacLeod, 1945) in the mistaken belief that it was a kinase-like enzyme. It is now known that streptokinase reacts with plasminogen in a nonenzymatic fashion.

Although the spontaneous fibrinolytic activity of plasma was known from the beginning of the century and reported to be increased in liver cirrhosis (Goodpasture, 1914) the mechanism for this process was unknown. Sherry, Fletcher, and Alkjaersig (1959) reported that the spontaneous fibrinolytic activity was due to the presence of a plasminogen activator in plasma; Kwaan (1957) suggested that this activator might be derived from the venous endothelium, as had Nolf (1908), and the histological identification of the plasminogen activator was confirmed by Todd (1959).

Other important events included the demonstration of enhanced fibrinolysis by intravenous adrenaline and exercise (Biggs et al., 1947), the discovery of urokinase

(Williams, 1951) and the findings relating the fluidity of menstrual fluid with large amounts of fibrinolytic activity produced by the endometrium (Albrechtsen, 1956). This fluidity of menstrual flow is probably the most common clinical manifestation of fibrinolysis which Hunter (1794) had noted as an example of blood liquidity.

At no time in the past has there been such a rapid accumulation of knowledge in the field of fibrinolysis as during the last 10 years. Large prospective epidemiological studies and clinical studies on patients with vascular disease have started to provide evidence as to the role of fibrinolysis in thrombosis, while the molecular mechanisms of fibrinolysis have been greatly advanced. Alpha2-antiplasmin, tissue-type plasminogen activator, tissue-type plasminogen activator inhibitor, histidine-rich glycoprotein, protein C, intrinsic pathway fibrinolysis have all been discovered or their mechanisms of action defined in the last few years, and these advances will be more closely evaluated throughout this thesis.

1.3 MECHANISMS OF FIBRINOLYSIS

The current fibrinolysis cascade is illustrated in figures 1.1 and 1.2. Briefly, activation of plasminogen may occur via an intrinsic or extrinsic pathway. The intrinsic pathway includes (a) a factor XII-dependent mechanism that is initiated by negatively charged surfaces and involves factor XII, prekallikrein, and high-molecular

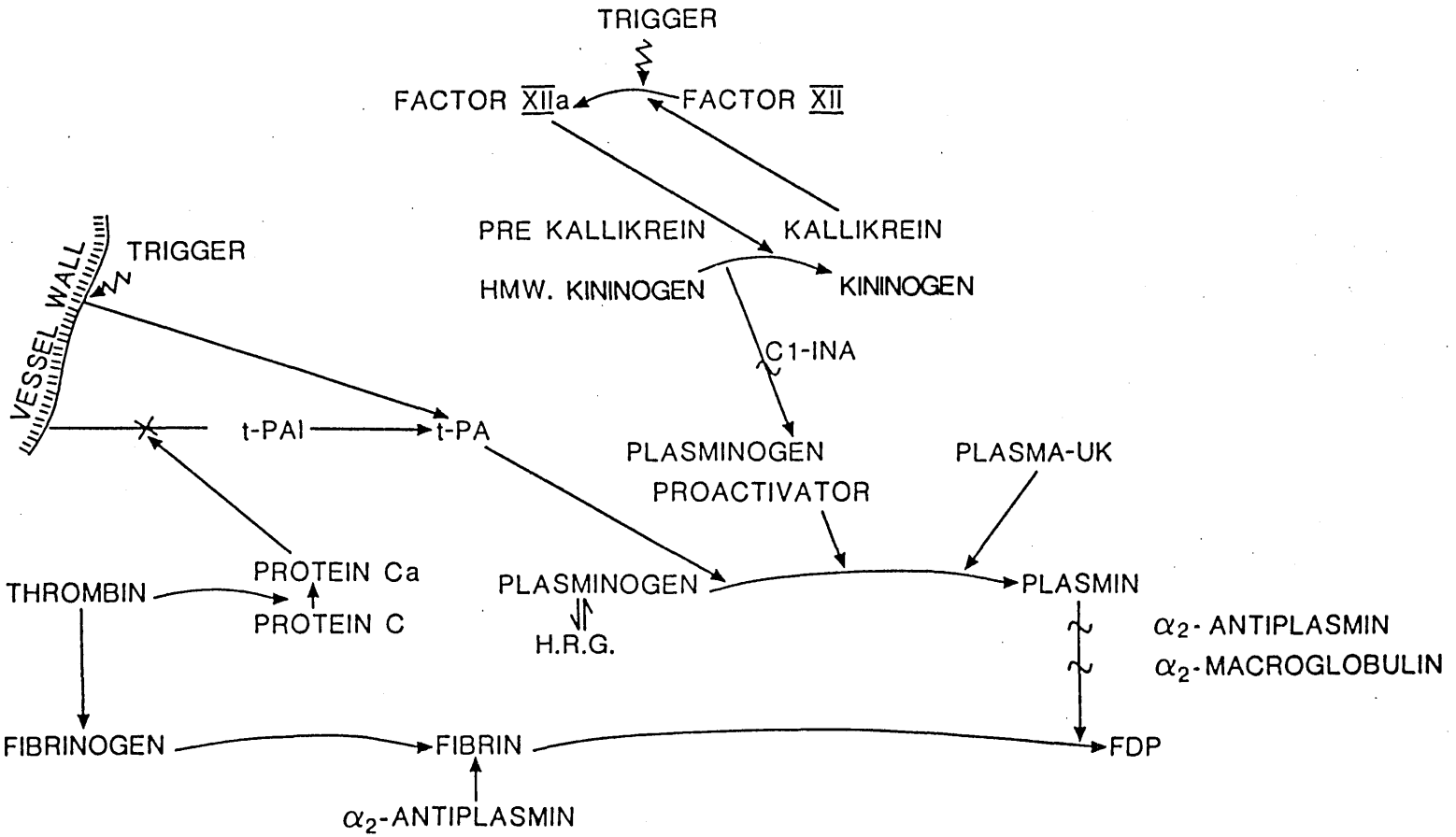

Figure 1.1

Illustrates the intrinsic and extrinsic pathways of fibrinolysis (see text)

C1-INA: C1-inactivator

t-PAI: t-PA-inhibitor

H.R.G.: Histidine-rich glycoprotein

Protein Ca: activated protein C

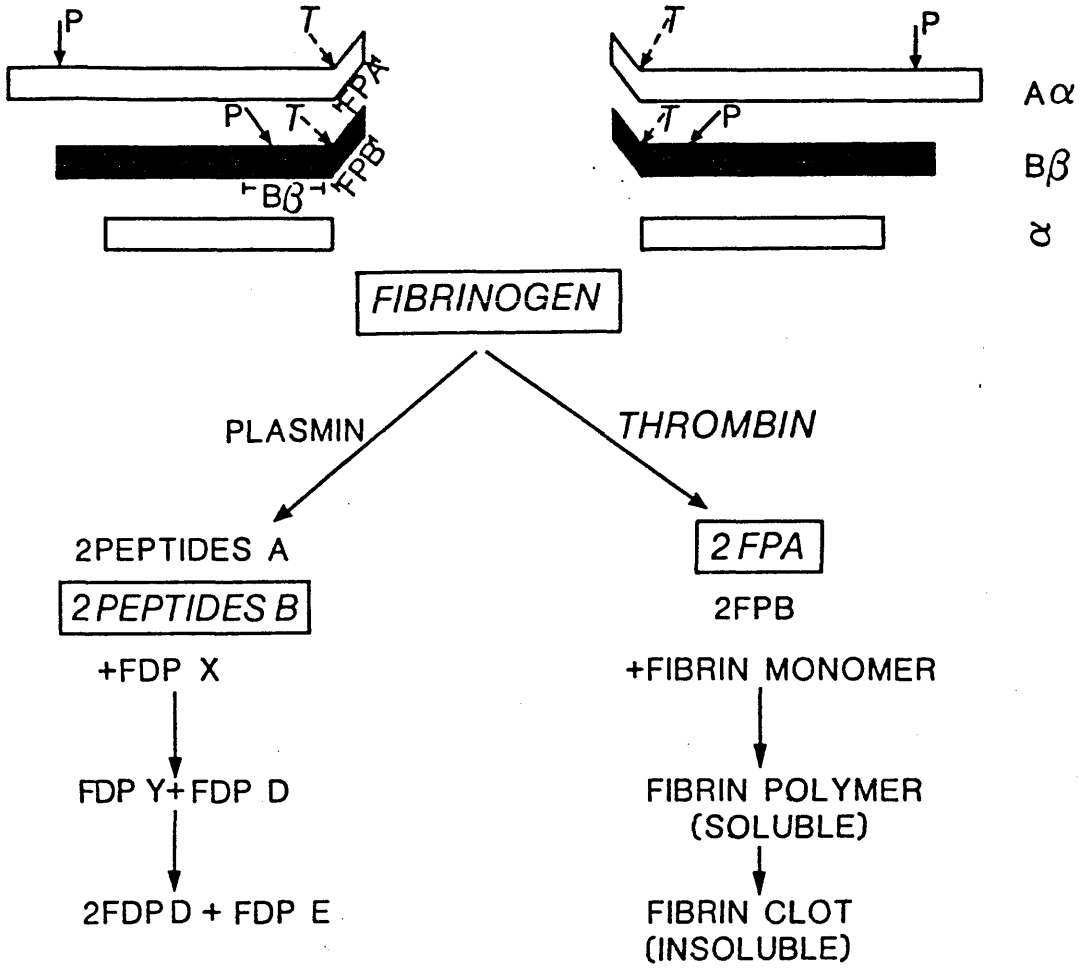

Figure 1.2

Illustrates the pathway of fibrin (ogen) degradation by thrombin or plasmin and the mechanism of production of the Bβ peptides and fibrinopeptide A (FPA).

weight kininogen and possibly other, as yet unknown proactivators and (b) a factor XII-independent mechanism that involves a urokinase-like activity. The extrinsic pathway involves a tissue-type plasminogen activator that circulates in plasma after its release from the endothelium in the vessel wall or from other tissues. This tissue-plasminogen activator acts directly on plasminogen to yield plasmin.

Inhibitors regulate the process of fibrinolysis. Alpha2- antiplasmin is the major inhibitor of plasmin, whereas alpha2-macroglobulin only comes into play when plasmin is formed in excess of the inhibitory capacity of alpha2-antiplasmin. The main inhibitor of the intrinsic pathway is Cl-inactivator, but alpha2-macroglobulin, antithrombin III and alphal-antitrypsin may also play a role in the regulation of this pathway. The most recent discovery is that of a fast-acting inhibitor to tissue-plasminogen activator which is also produced by the vascular endothelium. Protein C, activated by thrombin (Protein Ca), is known to stimulate fibrinolysis and recent evidence suggests that its mode of action could be related to a reduction of tissue-plasminogen activator inhibitor. That hereditary protein C deficiency is associated with recurrent venous thrombosis (Broekmans, 1985) suggests that protein C may have a clinically relevant effect on fibrinolysis.

Activation of fibrin (ogen) by plasmin releases small peptide fragments from the carboxy terminals of the BBeta chain which are cleaved before the X, Y, D and E fragments

are formed (figure 1.2). Measurement of BBeta 15-42 therefore allows an accurate assessment of minor changes in endogenous fibrinolysis, while changes in fibrinogen/fibrin degradation products (FDP's) only takes place when major activation of fibrinogen occurs - ie, when systematic fibrinolysis is induced.

Having given a brief synopsis of the components of the fibrinolytic enzyme system I would now like to describe these individual components in more detail. Table 1.1 lists some of the characteristiscs of these components.

1.4 PLASMINOGEN

Plasminogen, the inactive precursor of plasmin, circulates in the blood, and although in low concentrations, is present in practically all body fluids. (Hedner and Nilsson, 1981). Human plasminogen (see table 1.1) is a single chain glycoprotein consisting of 790 aminoacids which is manufactured primarily in the liver (Marsh, 1981). Native plasminogen has an amino terminal glutamic acid (glu-plasminogen) but is easily converted by plasmic digestion to amino terminal lysine, valine or methionine - called lys-plasminogen. Both glu- and lys- plasminogen exist in multiple iso-electric forms. This plasminogen is converted to plasmin by cleavage of a single peptide bond, which is brought about by urokinase or the other plasminogen activators. Activation of glu-plasminogen to plasmin by urokinase is

TABLE 1.1

COMPONENTS OF THE FIBRINOLYTIC SYSTEM

COMPONENT	~ MOLECULAR WEIGHT	~ PLASMA CONCENTRATIONS
Fibrinogen	340,000	10 µmol/l
α2-Macroglobulin	725,000	3.5 µmol/l
Plasminogen	90,000	2.4 µmol/l
α2-Antiplasmin	67,000	1.0 µmol/l
t-PA	72,000	70 pmol/l
t-PA Inhibitor	50,000	20-100 pmol/l
BBeta 15-42	3040	500 pmol/l

Legend

Approximate (~) molecular weight (daltons) and plasma concentrations of the main components of fibrinolysis.

much slower in purified systems than that of lys-plasminogen and this is reflected in the plasma half-lives (t-1/2) of the 2 forms; glu-plasminogen with a mean t-1/2 of 2.24 days, lys-plasminogen with a t-1/2 of 0.8 days (Marsh, 1981). In vivo it is unclear in which sequence the hydrolysis occurs. The plasminogen molecule contains lysine-binding sites through which it binds to fibrin. Lys-plasminogen has a higher affinity for fibrin than glu-plasminogen. These lysine-binding sites also bind 6-aminohexanoic acid which can abolish the absorption of plasminogen to fibrin (Collen, 1985) and these binding sites also mediate its interaction with alpha2-antiplasmin. More recently it has been demonstrated that histidine-rich glycoprotein has fibrinolysis-regulating properties. This protein also interacts with the lysine- binding sites of plasminogen to form a reversible complex and thereby inhibits binding of plasmin (ogen) to fibrin (Lijnen, De Cock and Collen, 1981). Changes in concentration of plasma plasminogen or histidine-rich glycoprotein may alter the concentrations of "free" (ie, available) plasminogen.

1.5 PLASMIN

Plasmin is a 2 chain molecule composed of a heavy chain or A chain, originating from the amino terminal end of plasminogen and a light chain or B chain constituting the carboxy terminal part. Plasmin is a trysin - like serine protease whose action is much more specific for fibrin (ogen) than trypsin, but plasmin can split peptide

bonds in practically all protein substrates and a wide variety of esters of arginine and lysine. It is not normally detected in human plasma as it is rapidly neutralised by inhibitors.

1.6 PLASMINOGEN ACTIVATORS

A number of substances can convert plasminogen to plasmin, and a brief classification is given in table 1.2. I will concentrate only on the physiological activators. Plasminogen activation may occur by an intrinsic or humoral pathway in which all the components involved are present in the blood, or by an extrinsic pathway in which the activators originate from the vessel wall. Alternatively, plasminogen may be activated by an exogenous pathway by administration of streptokinase or urokinase. All plasminogen activators act through hydrolysis of the arginine 560 - valine 561 bond in plasminogen (Erickson et al., 1985), although the mode of action of urokinase differs from streptokinase.

1.6.1 Intrinsic Pathway Activation

The existence of an intrinsic pathway of activation of plasminogen was first suggested over 25 years ago (Niewiarowski and Prou-Wartelle, 1959; Iatridis and Ferguson, 1962) since when most work has concentrated on the factor XII-dependent pathway. The factor XII (Hageman factor)-dependent pathway involves prekallikrien (Fletcher factor), high-molecular weight kininogen (Fitzgerald

TABLE 1.2

CLASSIFICATION OF PLASMINOGEN ACTIVATORS

Physiological

Extrinsic plasma activators

- tissue activator*
- vascular endothelial activator*

Intrinsic plasma activators

- factor XII - dependent
- factor XII - independent

Urinary activator (urokinase)

Non-physiological

Bacterial

- streptokinase
- staphylokinase

Chemical

- chloroform
- glycerol

(Taken from Marsh, 1981)

Legend

Division of plasminogen activators by physiological function.

*tissue and vascular endothelial activator are identical and are now called tissue-plasminogen activator (International Committee on Thrombosis and Haemostasis recommendation).

factor) or other components not yet identified, yet patients with these factor deficiencies do not have clinically relevant abnormalities of haemostatic function (Bennett and Ogston, 1981). The factor XII-independent pathway has received much less attention and overall the pathophysiological role of intrinsic pathway activation is largely unknown (Hedner and Nilsson, 1981; Marsh, 1981; Collen, 1985).

1.6.2 Extrinsic Pathway Activation

Plasminogen activators are present in most tissues while the plasminogen activator derived from the vessel wall, designated as extrinsic (Kluft, 1978) has received the most attention. Kluft (1978) demonstrated that the C1-inactivator-resistant activity on fibrin plates reflected extrinsic plasminogen activator and he later demonstrated that the short-term fluctuations in plasma fibrinolytic activity were due to variations in secretion from the vessel wall (Kluft, 1981). These fluctuations, such as the diurnal rhythm of fibrinolytic activity, the increase in fibrinolytic activity induced by exercise, stress, venous occlusion, or by pharmacological means (ie, DDAVP, adrenaline, nicotinic acid), had in the past been inferred from changes detected in euglobulin fibrinolytic activity. It is probable that the activator found in blood represents release of vascular plasminogen activator (Rijken et al., 1980) and a number of studies have demonstrated that vascular plasminogen activators are

similar or identical to the tissue plasminogen activator (Collen, 1985). These plasminogen activators will be referred to as tissue-type plasminogen activator (t-PA), as suggested by the International Committee on Thrombosis and Haemostasis in 1982. Refinements in the assays for t-PA have included specific quenching antibodies to t-PA on fibrin plates (Kluft, Jie and Allen, 1983) and since 1982 reports have appeared using an immunoradiometric assay. These reports have confirmed the rise in t-PA with exercise, venous occlusion and DDAVP (Rijken, Hoylaerts and Collen, 1982; Kluft et al., 1983). While basal levels of t-PA tend to be constant for a period of weeks, a large inter-individual variation was noted, and dramatic temporary fluctuations (by a factor of 50) occurs following such stimuli (Kluft et al., 1983).

Human t-PA is a single chain plasma protein consisting of 527 amino acids (see table 1.1) which requires a co-factor to express its activity, the most efficient being fibrin itself (Collen, 1985; Erickson et al., 1985). This supports the hypothesis that fibrinolysis is both triggered by and directed towards fibrin (Collen, 1985). The commercial assay for t-PA has only become available in our laboratory from 1986 and will not be evaluated in this thesis.

1.7 BBETA 15-42 FRAGMENT

To explain the mechanism whereby the BBeta 15-42 fragment is generated, and the value of the BBeta 15-42 assay, it is first necessary to briefly describe the

degradation of fibrin (ogen) (see figure 1.2). The digestive action of plasmin results in the early cleavage of two-thirds of the carboxy terminal of the AAlpha chain - peptide A, and cleavage of peptides BBeta 1-42 from the amino terminal of the BBeta chain. On the other hand thrombin cleaves fibrinopeptide A from the amino terminal of the AAlpha chain producing fibrin I and thereafter thrombin and plasmin may compete for the amino terminal end of the BBeta chain. If thrombin cleaves the BBeta 14-15 bond, fibrinopeptide B is released and fibrin II is formed, whereas if plasmin now acts on the BBeta 42-43 bond, the products are BBeta 1-42 and fragment X. Thus measurement of the BBeta peptides can indicate in vivo fibrino(geno)lysis (Nossel, 1981; Kudryk et al., 1982). If both fibrinopeptide A and BBeta peptides are simultaneously measured a differential diagnosis of thrombosis or fibrinolysis can be made. Primary fibrinolysis occurs when levels of BBeta peptides alone are elevated, while secondary fibrinolysis occurs if there is a simultaneous elevation of both fibrinopeptide A and BBeta peptides indicating both thrombin and plasmin generation.

1.8 INHIBITORS OF FIBRINOLYSIS

Since chloroform was shown to stimulate fibrinolysis by destruction of fibrinolytic inhibitors (Delezenne and Pozerski, 1903) several inhibitors of fibrinolysis have

been discovered including most recently alpha2-antiplasmin and t-PA inhibitor.

1.8.1 Intrinsic Pathway Inhibitors

Cl-inactivator is known to inhibit factor XIIa-induced fibrinolysis, and alpha2-macroglobulin and antithrombin III also inhibit this pathway (Collen, 1981). Since the role of the intrinsic fibrinolytic pathway is not established, the physiolgic role of these inhibitors remain speculative.

1.8.2 Alpha2-Antiplasmin

It is now known that there are at least six plasma protein inhibitors of plasmin, alpha2-antiplasmin, alpha2-macroglobulin, alphal-antitrypsin, inter-alpha-trypsin inhibitor, antithrombin III and complement 1-inhibitor (Marsh, 1981) although it seems likely that only the first 2 have any physiological importance. Alpha2-antiplasmin is a single chain glycoprotein which is the major plasmin inhibitor (see table 1.1). Alpha2-antiplasmin forms a stable 1:1 stoichiometric relationship with plasmin and is one of the fastest protein-protein interactions found. In addition to its major role in inhibiting plasmin, alpha2-antiplasmin inhibits plasminogen activator, inhibits the absorption of plasminogen to fibrin, and cross-links to fibrin rendering it less susceptible to

plasmin (Collen, 1980; Aoki et al., 1981). Alpha2-antiplasmin has a mean t-1/2 of 2.6 days which shortens to 0.5 days during thrombolytic therapy (Collen, 1981). There are 4 families in whom congenital alpha2-antiplasmin deficiency has been noted, and the bleeding tendancies in homozygous patients confirms the physiological importance of this inhibitor (Aoki, 1984). Alpha2-antiplasmin is also a weak acute phase reactant (Matsuda et al., 1980).

1.8.3 Alpha2-Macroglobulin

Alpha2-macroglobulin is a large glycoprotein (see table 1.1). which represents the slower reacting plasmin inhibitor, and its role seems to be inactivate plasmin formed in excess of the inhibitory capacity of alpha2-antiplasmin (Collen, 1976).

1.8.4 t-PA Inhibitor

Although it was noted that cultured endothelial cells have an anti-fibrinolytic activity (Loskutoff and Edgington, 1977) it has only been in the last 2-3 years that a fast-acting inhibitor to t-PA has been characterised (see table 1.1). The purified inhibitor is a single-chain glycoprotein which forms a 1:1 stoichometric complex with t-PA and thereby modifies its action. It is an example therefore of an antiactivator as opposed to an antiplasmin. This inhibitor is a major

secretory product of the endothelial cell accounting for between 2.5% to 12% of all the proteins released by the cell, and an immunologically related t-PA inhibitor is found in platelets (Erickson, Ginsberg and Loskutoff, 1984). Although the exact mechanism for clearing the fast-acting t-PA inhibitor is not established, its t-1/2 (in rabbits) has been reported to be about 7 minutes (Colucci, Paramo and Collen, 1985). Recent studies have shown that elevated levels of t-PA inhibitor have been found in patients with hyperlipidaemia (Brommer et al., 1984a), coronary artery disease (Paramo et al., 1985; Estelles et al., 1985), venous thrombosis (Nilsson, Ljungner and Tengborn, 1985), septicaemia (Colucci et al., 1985) and in patients who show a poor fibrinolytic response to bicycle exercise or DDAVP (Brommer et al., 1984a). The raised t-PA inhibitor levels may be relevant to the defective fibrinolysis associated with these varying conditions. In addition, improved fibrinoytic activity has been observed in association with a reduced t-PA inhibitor level induced by stanozolol (Verheijen et al., 1984) and activated protein C (Van Hinsbergh et al., 1985). This accumulating evidence points to t-PA inhibitor being an important physiological regulator of t-PA activity in vivo.

1.9 FDP´S

A brief summary of fibrinogen/fibrin degradation is illustrated in figure 1.2. FDP´s have a marked influence on coagulation by prolongation of the thrombin clotting

time and by inhibiting platelet function (Marsh, 1981). Fragment X is still susceptible to thrombin action but fragments Y, D and E form soluble complexes and are non clottable (Lowe and Prentice, 1980).

1.10 REGULATION OF FIBRINOLYSIS

While the interaction between the various components of the fibrinolytic enzyme system have been clearly illustrated (figure 1.1) the trigger factors controlling the release of t-PA are unknown. Observations that pitressin (Schneck and von Kaulla, 1961) and later vasopression (Gader, Da Costa and Cash, 1973) or hypothalamic stimulation could enhance plasma fibrinolytic activity led to the suggestion that the hypothalamic-hypophyseal region could be a central regulator of plasminogen activator release (Cash, 1975). Recent studies however have failed both to show any abnormalities of fibrinolysis in panhypopituitary patients (Juhan-Vague et al., 1984) and to isolate a plasminogen activator releasing hormone (PARH) from the neurohypophysis (Colucci et al., 1984). The fact that intra-arterial adrenaline but not DDAVP stimulated the release of plasminogen activator from the infused local vascular bed, indicates that the catecholamine-responding and vasopression-responding receptors are both molecule specific and different in anatomical site (Cash et al., 1978). The central control or mediator system of plasminogen activator release is still therefore largely

unknown (Kluft, 1981).

CHAPTER 2

REVIEW OF THE CHARACTERISTICS AFFECTING FIBRINOLYSIS

2.1 INTRODUCTION

In this chapter I will attempt to review the previous studies on fibrinolysis which are relevant to this thesis, and to evaluate the evidence linking defective fibrinolysis to thrombosis. The effects of acute injury on fibrinolysis will not be reviewed since, in the main, the work presented in this thesis relates to fibrinolysis studied in the non-acute phase, ie, the basal state. This review covers work published up to March of 1986 and concentrates on the more recent studies. For detailed reviews, prior to 1981, of the clinical relevance of fibrinolysis to thrombosis the reader is referred to the following articles: Davidson and Walker, 1979a; Davies, Crandon and McNicol, 1981; Davidson and Walker, 1981; Hedner and Nilsson, 1981.

2.2 Venous Thrombosis

Deep vein thrombosis (DVT) and fibrinolysis has not been formally evaluated in this thesis and I will therefore only give a brief summary on the large volume of evidence on this topic. The link between recurrent DVT, thrombophlebitis and defective blood and vein wall fibrinolytic activity was first reported in 1972 (Isacson and Nilsson, 1972) and this has been confirmed in other large studies (Browse et al., 1977a; Stormorken, Lund and Holmsen, 1983). It has also been shown that the recurrence rate for patients with DVT or pulmonary embolism is less in patients with better fibrinolytic

capacity (Korninger et al., 1984). Recent studies have shown that defective fibrinolysis is due to either defective release of t-PA or an increase in t-PA inhibitor (Bergsdorf, Nilsson and Wallen, 1983; Nilsson, Ljungner and Tengborn, 1985). Post-operative DVT has also been associated with diminished fibrinolytic activity (Mansfield, 1972; Gordon-Smith, Hickman and le Quesne, 1984; Clayton, Anderson and McNicol, 1976) and pre-operatively the euglobulin clot lysis time has been used as a part of a predictive index for venous thrombosis (Clayton et al., 1976; Crandon et al., 1980). There appears to be little doubt that fibrinolysis plays a role in DVT. For a more detailed account of the accumulated evidence, the reader is referred to previous reviews on this subject (Davidson and Walker, 1979a; Vermylen and Chamone, 1979; Hedner and Nilsson, 1981).

2.3 EXERCISE AND FIBRINOLYSIS

2.3.1 Mechanisms of the Increased Fibrinolytic Activity

In his treatise on blood, John Hunter (1794) was probably the first to record the effects of exercise on haemostasis, observing the phenomenon of incoagulable blood in deer run to death. The association between exercise and fibrinolysis was first reported however in 1947 when an increase in plasma fibrinolytic activity was detected in subjects immediately after running up and down stairs (Biggs, MacFarlane and Pilling, 1947). This finding has since received widespread confirmation. The

rise in fibrinolytic activity is due to increased plasma levels of plasminogen activator (Sawyer et al., 1960) and the magnitude of this rise has been related to the duration of exercise (Bennett, Ogston, and Ogston, 1968), the intensity of exercise (Davis et al., 1976) and the time of the day that exercise is performed (Rosing et al., 1970). Although there is a significant difference in response between individuals, the fibrinolytic response to exercise is reproducible in any one individual (Cash, 1966). The increase in plasma fibrinolytic activity in these studies, as assessed by euglobulin lysis times, could be due to increases in either the intrinsic or extrinsic plasminogen activator systems. Marsh and Gaffney (1980), in a small study of 4 subjects, found no increase in the intrinsic plasminogen activators with exercise while a more detailed study (Huisveld et al., 1984) has shown that the increases in fibrinolytic activity are due to increased levels of both intrinsic and extrinsic plasminogen activators. Kluft and colleagues (1983) demonstrated that extrinsic (tissue-type) plasminogen activator activity (measured on fibrin plates with added Cl-inactivator) post exercise could be totally suppressed by the addition of specific antibodies to t-PA thus suggesting the rise in fibrinolytic activity with exercise was due to a rise in extrinsic plasminogen activators alone. Using specific assays for t-PA activity and antigen it has been noted that both increase markedly after strenuous bicycle exercise (Bergsdorf et al., 1983; Wiman, Mellbring and Ranby, 1983). The different

half-lives (t-1/2) of t-PA antigen (t-1/2 = 10 minutes) and t-PA activity (t-1/2 = 5 minutes) was taken as evidence for the existence of an inhibitor to t-PA activity which acts rapidly to inactivate about half of the exercise-related t-PA activity generated (Bergsdorf et al., 1983). Kluft and co-workers (1983) had previously noted that the rise in t-PA following exercise was short-lived (t-1/2 = 5.5 minutes) and suggested that the short half-life was due to rapid clearance by the liver. Previous studies have shown that some patients have a consistently poor response to exercise and that such patients continue to show impaired responses despite prolonged and exhaustive exercise (Cash, 1966; Cash and Woodfield, 1967). Recent work suggests that these patients have high t-PA inhibitor levels (Brommer et al., 1984a). The rise in plasma plasminogen activator is not associated with a reduction in plasminogen concentration (Bennett et al., 1968; Marsh and Gaffney, 1980; Huisveld et al., 1984) but plasminogen turnover is slightly increased by repeated strenuous exercise (Collen et al., 1977). The exact mechanism responsible for this exercise-stimulated fibrinolytic activity is still an enigma although it is thought that the increased perfusion of muscle capillaries and veins, in association with physical activity, leads to an increased release of t-PA from the endothelial cells in the vessel wall (Nilsson, Hedner and Pandolfi, 1980; Rijken, Wijngaards and Welbergen, 1980). Exercise also increases factor VIII concentrations and it is possible that a higher level of

plasminogen activator is important for maintaining a satisfactory haemostatic balance. The possible role of adrenergic mechanisms in the enhanced fibrinolytic activity after exercise will be discussed in Chapter 4.

2.3.2 Does Exercise Activate Fibrinolysis?

Despite the elevation of plasma fibrinolytic activity the evidence that actual fibrinolysis occurs, as evidenced by a rise in fibrin (ogen) degradation products (FDP's), is equivocal. A rise in FDP's has been described by some workers (Ferguson et al., 1979; Hyers et al., 1980; Mandalaki et al., 1980; Dufaux, Order and Hollman, 1984) but not others (Collen et al., 1977; Vogt, Hoffman and Straud, 1979; Marsh and Gaffney, 1982). Factors which may explain such discrepencies include the intensity and duration of exercise in combination with the different FDP assays used in these studies. Degradation of the AAlpha chain of fibrinogen has been shown to increase (Collen et al., 1977; Marsh and Gaffney, 1980) or to remain unchanged following exercise (Ferguson, Barr and Bernier, 1979). The AAlpha chain is of particular interest because it is the chain of fibrinogen most susceptible to degradation by plasmin (Ferguson, Fretto and McKee, 1975). In addition, Collen and co-workers (1977) demonstrated a small increase in the level of plasmin-antiplasmin complexes following exercise, but it seems likely that any small amount of plasmin generated by exercise would be rapidly inactivated by alpha2-antiplasmin. Overall it is felt that despite the rise in plasma fibrinolytic activity with exercise,

plasma proteolytic activity does not occur - ie, the actual fibrinolytic effect is non-existent (Marsh and Gaffney, 1982; Ogston, 1983).

2.3.3 Physical Fitness and Fibrinolysis

There appears to be no differences in the resting level of fibrinolytic activity between inactive men and men who perform regular exercise (Moxley, Brakman and Astrup, 1970; Korsan-Bengtsen, Wilhelmsen and Tibblin,1973) while fibrinolytic activity at rest is decreased by a period of physical training (Keber et al., 1979; Williams et al., 1980). Physical training does not augment the fibrinolytic response to exercise (Keber et al., 1979; Dufaux et al., 1984), but it does clearly increase the fibrinolytic response to venous occlusion which may be relevant to the beneficial effects of exercise with regard to the risk of cardiovascular disease (Williams et al., 1980). This latter study (Williams et al., 1980) involving a large group of subjects, provided the best evidence to date of a positive effect of exercise training on the fibrinolytic enzyme system.

2.4 EFFECTS OF SMOKING

While smoking is associated with higher fibrinogen concentrations (Korsan-Bengtsen, Wilhelmsen and Tibblin, 1972; Meade et al., 1979; Scarabin et al., 1982) the effects on plasma fibrinolytic activity are more controversial. Thus Korsan-Bengtsen and co-workers (1972) found no difference in plasma fibrinolytic activity

in smokers in a population study of middle-aged men, while Janzon and Nilsson (1975) found no differences in plasma or vein wall fibrinolytic activity in healthy smokers compared to non-smokers. The largest population study to date did detect a small decrease in fibrinolytic activity in smokers compared to non-smokers (Meade et al., 1979). More detailed studies on the effect of smoking on the components of fibrinolysis have shown that smoking is associated with diminished plasma fibrinolytic activity (measured on both dilute blood clot lysis times and euglobulin activity) due to diminished extrinsic plasminogen activator levels (Allen, Kluft and Brommer, 1983). This latter study also showed a significantly smaller rise in extrinsic plasminogen activator following DDAVP infusion. It appears therefore that smoking is associated with reduced fibrinolytic activity due to the extrinsic system of fibrinolysis and that smoking may alter the dynamic responses of fibrinolytic activity.

2.5 EFFECTS OF LIPIDS

There is growing evidence that hypertriglyceridaemia is associated with depressed resting plasma fibrinolytic activity (Korsan-Bengtsen et al., 1972; Simpson et al., 1983), reduced diurnal fluctuations in plasma fibrinolytic activity (Rosing et al., 1973) and reduced fibrinolytic responses to venous occlusion (Marsh and Shaper, 1977; Andersen, Arnessen and Hjermann, 1981) exercise (Epstein et al., 1970) and DDAVP (Brommer et al., 1982). Triglyceride levels have also been negatively associated

with in vivo fibrinolysis (BBeta 15-42 antigen) in an epidemiological study (Lowe et al., 1985). Plasma fibrinolytic capacity (to venous occlusion) does not improve with average reduction in hyperlipidaemia by dietary measures (Andersen et al., 1981) but successful reduction in lipid profiles, after 6 years of diet intervention, has been shown to improve fibrinolytic capacity when compared to hyperlipidaemic controls (Andersen, Arnesen and Hjermann, 1983a). Improvements in hypertriglyceridaemia, by diet and drugs, over periods of between 3-6 months have also resulted in improvements in resting fibrinolytic activity (Elkeles et al., 1980; Simpson et al., 1983). Lowe and co-workers (1982) have shown that alpha2-antiplasmin levels are raised in type II hyperlipoproteinaemia while a positive association of alpha2-antiplasmin with triglyceride concentrations has been noted in a recent epidemiological study (Lowe et al., 1985). A previous epidemiological study failed to find this association (Scarbin, Bara and Jacqueson, 1983). Furthermore, increased triglyceride levels have also been associated with increased t-PA inhibitor levels (Brommer et al., 1984a) and the evidence to date is in favour of an impairment of fibrinolytic activity by hypertriglyceridaemia due possibly to raised levels of fibrinolytic inhibitors acting at distinct sites - ie, against plasmin and t-PA.

Cholesterol concentrations, which do not show a clear relationship with plasma fibrinolytic activity (Korsan-Bengtsen et al., 1972) are positively related to

fibrinogen (Korsan-Bengtsen, 1972; Baker et al., 1982) and alpha2-antiplasmin concentrations (Scarabin et al., 1983; Lowe et al., 1985).

2.6 EFFECT OF BODY WEIGHT

Many authors have examined fibrinolytic activity in obesity and found it to be reduced (Fearnley, Chakrabarti and Avis, 1963; Bennett et al., 1966). In the large prospective population studies in middle-aged men a negative correlation of both weight and fibrinolytic activity (Korsan-Bengtsen et al., 1972) and subscapular skin fold thickness and fibrinolytic activity have been noted (Korsan-Bengtsen et al., 1972; Meade et al., 1979). The reduction in plasma fibrinolytic activity in obesity is not associated with alterations in the inhibitors of fibrinolysis suggesting a decreased production of plasminogen activator (Bennett et al., 1966) and indeed a decrease in the activity of vessel wall plasminogen activator has been noted in obese subjects (Almer, 1975a). Obese subjects have also been reported as having a poor fibrinolytic response to exercise (Ogston and McAndrew, 1964), and venous occlusion (Almer, 1975a). Obese subjects tend to have raised triglyceride concentrations and which of these 2 factors are more closely related to defective fibrinolysis is difficult to estimate. Using multiple regression statistics Korsan-Bengtsen and colleagues (1972) found obesity to be the more important variable.

2.7 FIBRINOLYSIS AND ISCHAEMIC HEART DISEASE

The effects of acute myocardial infarction on fibrinolysis have been well documented and it appears that significant changes occur in most of the components of the fibrinolytic system as part of an acute phase response rather than a cause of, or response to, thrombosis per se (Hume, 1958; Bennett, Ogston and Ogston, 1967; Knudsen et al., 1979; Haines et al., 1983; Juhan-Vague et al., 1983). The following review therefore concerns studies involving patients outwith the period of their acute myocardial infarct.

The effect of ischaemic heart disease on fibrinolysis (or vice versa) was first investigated in the early 1960's when the plasma fibrinolytic activity (clot lysis times) of men with ischaemic heart disease (known angina pectoris or a previous myocardial infarction) did not differ from that of matched control groups (Merskey et al., 1960; Ogston, 1962; Katz et al., 1963). Other control studies failed to confirm these findings reporting a greater incidence of low fibrinolytic activity among patients with ischaemic heart disease (Gajewski, 1961; Chakrabarti et al., 1966). Chakrabarti et al., (1968), in a large study of male survivors of a myocardial infarction and age-matched controls, confirmed that a greater percentage of their patients had persistently defective fibrinolytic activity which was more marked in patients under 50 years of age. Similar results were reported by Lipinska and co-workers (1979) who in addition noted reduced fibrinolytic activity in healthy subjects with a family

history of coronary disease. These previous studies assessed the resting level of plasma fibrinolytic activity in men with ischaemic heart disease, but normal physiological variations, such as the diurnal rhythm of fibrinolytic activity, and dynamic tests have also been employed. Patients with coronary artery disease were noted to have reduced diurnal variations in plasma fibrinolytic activity (Rosing et al., 1973), and reduced fibrinolytic potential was 'more commonly' found using venous occlusion (Walker et al., 1977; O'Connor et al., 1984a) or exercise (Khanna et al., 1975) when compared to controls. O'Connor and co-workers also noted higher basal protein C values in these same patients with impaired fibrinolytic capacity when compared to controls, which remains unexplained, since protein C is thought to stimulate fibrinolysis (C'Connor, Broekmans and Bertina, 1984b). Fibrinolysis has also been found to be defective in coronary artery disease when bicycle exercise was used as the dynamic stimulus to fibrinolysis (Khanna et al., 1975; Estelles et al., 1985). The alteration of diurnal rhythm and fibrinolytic potential are compatible with the hypothesis that an impairment in the responsiveness of the fibrinolytic system may be related to the development of coronary artery disease.

Two large epidemiological, prospective studies in middle-aged men have failed to show a significant difference in fibrinolytic activity in men who died of cardiovascular disease as compared to survivors (Meade et al., 1980) and in men who sustained a myocardial

infarction compared to men who remain healthy (Wilhelmsen et al., 1984). Fibrinolytic activity was however slightly lower in men who died of cardiovascular diseases compared to survivors (Meade et al., 1980).

The above studies have utilised the clot lysis time or euglobulin fibrinolytic activity as a global assessment of fibrinolysis. In a large controlled study involving patients with angiographically proven coronary artery disease, euglobulin fibrinolytic activity was not significantly different from controls, while patients had significantly elevated concentrations of plasminogen activator inhibitor activity and t-PA antigen (Paramo et al.,1985). These authors stress the possible importance of the elevated concentrations of plasminogen activator inhibitor and questioned the value of euglobulin fibrinolytic activity as a test of in vivo fibrinolysis, but ignored the findings of elevated t-PA levels which would be expected to enhance fibrinolysis. Patients with coronary artery disease performing bicycle exercise show an impaired response of t-PA compared to controls, without significant differences in t-PA inhibitor or euglobulin fibrinolytic activity (Estelles et al., 1985).

In summary there appears to be little evidence that euglobulin fibrinolytic activity, in patients with ischaemic heart disease, is different from healthy controls, while dynamic tests of fibrinolysis may show defective euglobulin fibrinolytic activity in such patients. More detailed assays have not clarified the situation by showing elevated t-PA antigen and protein C

(17-beta-hydroxy-17-alpha-methandrostano (3.2,-c) pyrazole) has proved the most promising drug available at present and has therefore been intensively studied in a variety of disorders. At the 7th International Congress on Fibrinolysis held in Venice in 1984, stanozolol remained the most widely studied oral agent to cause pharmacological potentiation of fibrinolysis.

Interest in drug stimulated fibrinolysis stemmed from the observations of poor blood fibrinolytic activity in survivors of myocardial infarction (Chapter 2.7) and it was suggested that survivors of myocardial infarction might benefit from long-term fibrinolytic therapy (Anonymous, 1968). The initial studies revealed that drugs given orally (such as biguanides or the anabolic steroid ethyloestrenol) could enhance fibrinolysis for up to 3 months but when given in combination could act for up to 3 years (Fearnley, Chakrabarti and Evans, 1969; Chakrabarti, Evans and Fearnley, 1970). In open studies on fibrinolytic enhancement using phenformin and ethyloestrenol in patients with Raynaud's disease, ischaemic heart disease, and occlusive vascular disease it was found that replacing ethyloestrenol by stanozolol, in combination with phenformin, produced similar effects on fibrinolysis (Chakrabarti et al., 1970; Menon et al., 1970). Fibrinolytic enhancement also occurred when stanozolol was combined with either of the biguanide drugs, phenformin or metformin, in patients with DVT or ischaemic heart disease (Menon, 1971). The combination of stanozolol plus phenformin has also been shown to enhance

fibrinolysis in patients with occlusive vascular disease and Behcet's syndrome (Asbeck, Meyer-Boernecke and van de Loo, 1978; Bielawiec, Mysliwiec and Perzanowski, 1978). In a double-blind randomised study involving 34 men with ischaemic heart disease it was shown that stanozolol by itself (10 mg/day for one year) was capable of stimulating fibrinolytic activity to a similar extent as the stanozolol plus phenformin combination, and that stanozolol therapy was associated with a rise in plasminogen and fall in fibrinogen (Davidson et al., 1972). The same workers later showed that anabolic steroids could lead both to a fall in alpha2-macroglobulin concentrations and to a sustained increase in fibrinolytic activity in men with ischaemic heart disease over a 5 year period (Walker et al., 1975; Walker et al., 1978). There followed a series of clinical trials which indicated that fibrinolytic enhancement by stanozolol may have therapeutic potential in a variety of situations. A double-blind, placebo-controlled study involving 25 patients with cutaneous vasculitis indicated the therapeutic benefit of phenformin plus stanozolol (Dodman et al., 1973; Cunliffe et al., 1975). Open studies by the same workers, using stanozolol as sole therapy, showed that 13 of 16 patients with idiopathic recurrent superficial thrombophlebitis had their attacks of thrombophlebitis stopped completely, and fibrinolysis enhanced, during 6 months taking stanozolol (Jarrett, Morland and Browse, 1977); that an improvement in all 14 patients with longstanding lipodermatosclerosis was noted

while patients received stanozolol (Browse et al., 1977b); and that all 20 patients with advanced Raynaud's phenomenon showed an increase in hand blood flow and a reduction in symptoms while receiving stanozolol (Jarrett, Morland and Browse, 1978). In a double-blind, cross-over trial involving 23 patients with venous lipodermatosclerosis it was found that stanozolol plus elastic stockings reduced the extravascular fibrin detected in skin biopsy specimens and doubled the rate of skin healing compared to the group taking placebo plus elastic stockings (Burnand et al., 1980). The same authors later produced evidence to show that pericapillary fibrin is the likely cause of lipodermatosclerosis and venous ulceration (Burnand et al., 1982). The therapeutic effect of stanozolol in this condition is thought to be related to improved fibrinolysis with reduced fibrin deposition in the ulcer bearing area of the leg (Burnand et al., 1982).

Interest in stanozolol was again generated by the study of Preston et al., (1981) showing that oral stanozolol could enhance plasma fibrinolytic activity, lower fibrinogen, alpha2-macroglobulin and increase plasminogen levels in normal subjects. In addition they showed that these alterations were all detected within one week of starting stanozolol. It was felt that if stanozolol could enhance fibrinolysis in normal subjects then in patients with depressed fibrinolysis a more pronounced effect was likely (Preston et al., 1981). It was at this time that our laboratory became involved with

a number of clinical studies using stanozolol, some of which are presented in this thesis. The encouraging results of fibrinolytic enhancement following a single intramuscular injection of stanozolol (Small et al., 1982b) has led others to study the effects of intramuscular stanozolol in patients undergoing major surgery. Although stanozolol prevented the fibrinolytic shutdown associated with surgery (Blamey et al., 1983) it failed to prevent DVT (McArdle et al., 1983; Blamey et al., 1984) or post-operative pulmonary dysfunction (Cuschieri et al., 1985) in a double-blind controlled trial.

The mechanism whereby stanozolol exerts its effects has also received closer study. Protein C, which may stimulate fibrinolysis in addition to its potent anti-coagulant action (Comp and Esmon, 1981) has been shown to increase follcwing stanozolol administration to healthy volunteers, surgical patients and elderly medical patients (Kluft et al., 1984a; Small et al., 1984c). However in a controlled study in patients undergoing major abdominal surgery given oral or intramuscular stanozolol protein C levels were not significantly different in patients with and without DVT (Blamey et al., 1985). Stanozolol has also been shown to stimulate in vivo fibrinolysis, as measured by increased BBeta 15-42 concentrations, in healthy male subjects (Small et al., 1983b), findings which have been confirmed in surgical patients (Douglas et al., 1985). Detailed studies by Kluft and co-workers (1984b) have shown that the increased

fibrinolytic activity following stanozolol administration is due to increased t-PA activity which in turn is due to a marked fall in the recently discovered inhibitor of t-PA activity (Verheijen et al., 1984; Sue-Ling et al., 1985). Plasminogen circulates as a reversible complex with its carrier protein histidine-rich glycoprotein (Lijnen, Hoylaerts and Collen, 1980) and stanozolol has been shown to decrease histidine-rich glycoprotein, increase plasminogen and therefore increase the concentration of "free" (ie, biologically active) plasminogen which theoretically should also contribute to the improved fibrinolysis (Kluft et al., 1984b). The effects of stanozolol on the components of fibrinolysis are illustrated in figure 2.1.

Stanozolol has also been used in hereditary coagulation disorders in an attempt to increase the coagulation factor concentration and correct the disorder. Stanozolol has been shown tc increase protein C and antithrombin III in patients with these deficiencies but to have no effects on factor VIII or IX concentrations (Mannucci et al., 1984; Winter et al., 1984; Greer et al., 1985). While stanozolol increased fibrinolysis in all 3 disorders a therapeutic effect on preventing thromboembolic problems in patients with protein C and antithrombin III deficiency remains unproven.

In summary, although stanozolol has been shown to stimulate fibrinolysis in a variety of conditions, and the mechanism for its effect has been clearly delineated, the uncontrolled clinical trials allow no clear conclusion to

be reached as to its therapeutic benefit. The controlled trials indicate that stanozolol does not prevent DVT but does improve lipodermatosclerosis and cutaneous vasculitis.

2.9 FIBRINOLYSIS AND DIABETES MELLITUS

2.9.1 Fibrinolytic Activity in Diabetes

Fibrinolysis in diabetes mellitus was first reported in 1961 when the plasma fibrinolytic activity in a small group of diabetics in England was found not to be significantly different from a matched control group (James et al., 1961). This was later confirmed from studies in Australia (Denborough and Paterson, 1962), North America (Sandberg et al., 1963; Moser and Hajjar , 1966) and Scotland (McKay and Hume, 1964). However the largest study of that time compared 100 diabetics with 100 controls and demonstrated a significantly low fibrinolytic activity in diabetic patients (Fearnley, Chakrabarti and Avis, 1963). Later controlled studies examined the dynamic response of plasma fibrinolytic activity to subcutaneous adrenaline (Tanser, 1967) and treadmill exercise (Cash and McGill, 1969) and produced further conflicting results. These studies employed a variety of modified clot lysis times, tested diabetics at different times of day (never fasting), and usually failed to differentiate patients into type I (insulin-dependent) and

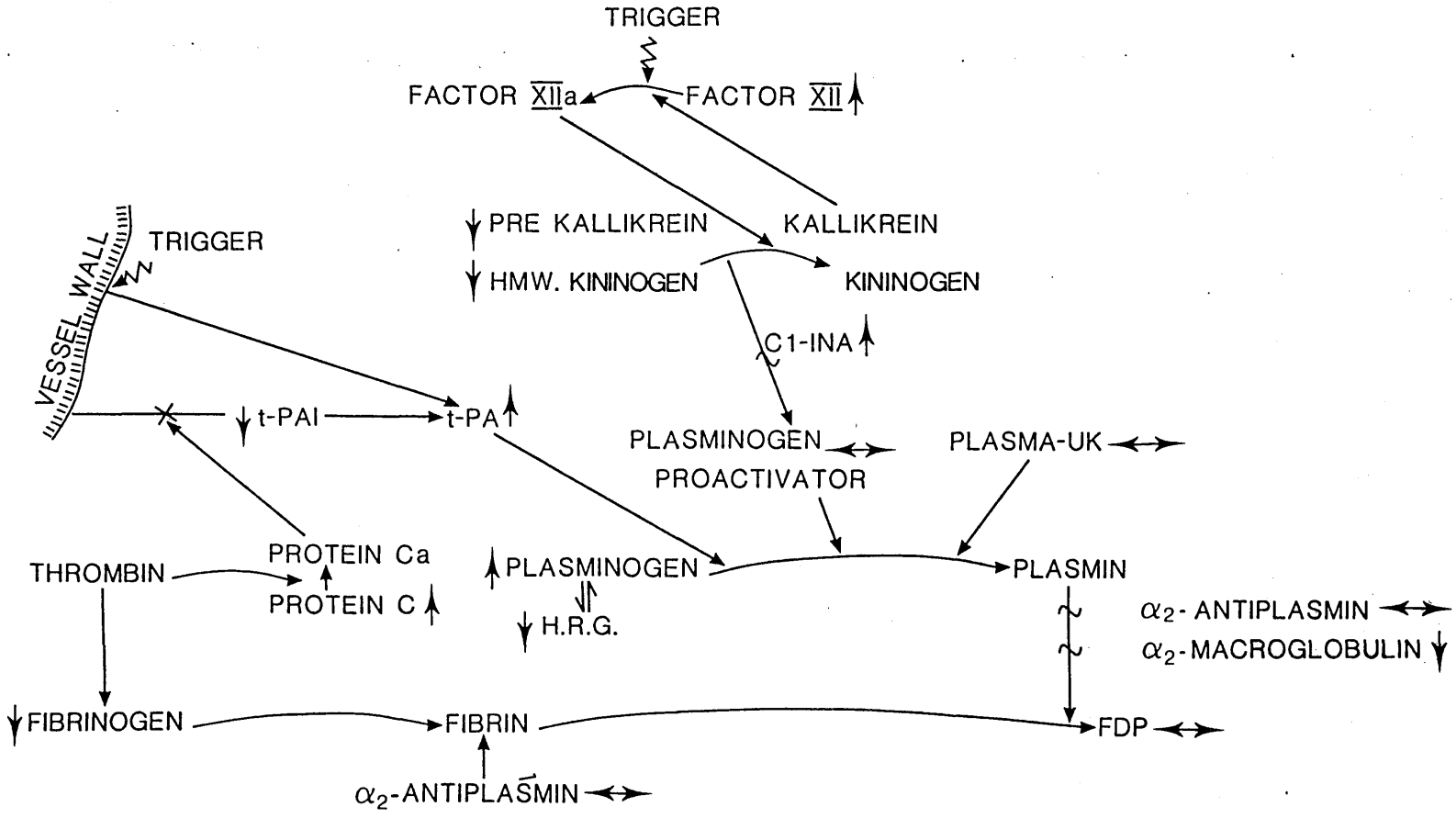

Figure 2.1

Illustrates the reported effects of stanozolol on the components of fibrinolysis

↑ : increase ↔ :no change ↓ :decrease

type II (non-insulin dependent) diabetes. It is perhaps not surprising therefore that discrepant findings were produced. There followed a number of smaller studies but the next important evidence was reported by Almer and co-workers (1975b). They compared plasma fibrinolytic activity (on fibrin plates pre and post venous occlusion) and plasminogen activator activity of the vein vessel wall in 221 diabetics with 221 control subjects and found that spontaneous plasma fibrinolytic activity and plasminogen activator content were significantly reduced in diabetic patients, and that the responses to venous occlusion were also impaired. The same workers also noted a close correlation between the plasminogen activator activity of veins and arteries in diabetes, indicating a widespread endothelial defect of the defence system against deposition of fibrin (Almer, Pandolfi and Aberg, 1975c). In addition, the defective fibrinolytic activity could be related to the degree of obesity (Almer, 1975a) and elevated triglyceride levels (Almer, 1975a; Almer et al, 1975b). The largest study to date was reported by Fuller and co-workers (1979) who examined the plasma fibrinolytic activity in 154 diabetics compared to 1079 controls and found decreased fibrinolytic activity in the diabetics. When the type of diabetes was evaluated it was found that type I diabetic females had a small, but significantly reduced fibrinolytic activity, but that marked reductions in fibrinolytic activity were noted in both male and female type II diabetic subjects, which was only partially attributable to the greater obesity in the type II

patients. In South Africa a comparison of fibrinolytic activity in Zulu diabetics (mainly type I) with Indian diabetics (mainly type II) revealed a greater depression of fibrinolysis in the Indian diabetics (Hathorn, Gillman and Campbell, 1961). The results of these studies indicated that diabetic patients, especially type II diabetics, have a depressed plasma fibrinolytic activity. More recent control studies, involving smaller number of patients, have given further conflicting results (Elder et al., 1980; Bern et al., 1981; Grignani et al., 1981; Sharma, 1981; Juhan-Vague et al., 1984; Haitas et al., 1984; Knaap et al., 1985), although depressed fibrinolytic activity has been confirmed in other studies (Gurewich et al., 1981; Christe et al., 1984). Using more specific assays, both the total and fibrin binding extrinsic plasminogen activator related antigen were found not to be significantly different in diabetic patients compared tc controls (Juhan-Vague et al., 1983; Juhan-Vague et al., 1984).

To evaluate the effect of diabetic drug therapy on fibrinolysis, cross-sectional studies have been performed and show that patients taking sulphonylurea drugs have a marked depression of plasma fibrinolytic activity (Farid et al., 1974; Almer and Nilsson, 1974) and more recently a small study has shown a defective plasminogen activator content of the vessel wall to occur commonly in patients on long-term, first generation sulphonylurea therapy (Almer, 1984). Fibrinolytic activity and the treatment of diabetes will be discussed more fully in Chapter 10.

2.9.2 Fibrinolytic Activity in Relation to the Microvascular Complications of Diabetes

Four main studies have assessed the effects of microvascular disease on fibrinolytic activity. The first and largest study of 221 diabetes and 153 controls found no difference in the spontaneous fibrinolytic activity in patients with and without diabetic retinopathy, but that patients with diabetes of greater than 10 years duration with retinopathy had significantly reduced fibrinolytic responses to venous occlusion which was even more marked in patients with nephropathy when compared to patients free of microvascular disease (Almer et al., 1975a; Almer and Pandolfi, 1976). In addition these authors also found no correlation of vessel wall plasminogen activator activity with the release of fibrinolytic activity on venous occlusion and postulated a faulty plasminogen activator release mechanism associated with microvascular disease. Haitas and colleagues (1984) compared the fibrinolytic activity pre and post venous occlusion in 13 control subjects to 10 type I diabetics without, and 11 type I diabetics with proliferative diabetic retinopathy. An impaired response to venous occlusion was noted in the proliferative group compared to both patients with no retinopathy and control subjects, while no differences between uncomplicated diabetics and control subjects were found. Christe and co-workers (1984) comparing 36 diabetics with and 44 diabetics without complications

found no differences in fibrinolytic activity either before or after venous occlusion and Lowe and colleagues (1986) found no difference in unstimulated fibrinolytic activity in 18 patients with proliferative retinopathy compared to patients with no or minimal diabetic retinopathy. These studies therefore have produced conflicting results but it appears that patients with microvascular disease have normal unstimulated fibrinolytic activity compared to diabetics without complications, but that differences can perhaps be demonstrated using a dynamic stimulus such as venous occlusion, and that the most marked changes are found in patients with severe microvascular disease (proliferative diabetic retinopathy or nephropathy). It is possible therefore that slow deposition of fibrin may occur in both the intra- and extravascular compartments in diabetic microangiopathy and that such fibrin deposition may be caused by a defective plasminogen activator release from the vascular endothelium. The demonstration of fibrin (ogen) within the glomerular capillary basement membrane in percutaneous renal biopsy samples taken at post mortem, add support to this theory (Davis, Woolf and Carstairs, 1966; Farquhar, MacDonald and Ireland, 1972). While these findings suggest an important association between defective fibrinolysis and microangiopathy, large prospective studies are needed to clarify whether this association is a causal one. Microvascular disease will occur in approximately 70-80% of diabetics after 10-15 years of type I diabetes and therefore it is likely that

any prospective study would require a long period of follow-up. There are no such studies on-going at present.

In a comparison of diabetic patients with and without autonomic neuropathy patients with autonomic neuropathy have been reported to have better fibrinolytic responses to venous occlusion (Almer, Sundkvist and Lilja, 1982; Almer, Sunkvist and Lilja, 1983) but others have found no correlation between fibrinolytic activity and autonomic neuropathy function tests (Haitas et al., 1984). Since autonomic neuropathy is thought to be a microvascular complication associated with both a long duration of diabetes and other microvascular complications, the improved fibrinolytic response to venous occlusion is unexpected, and the effect of autonomic neuropathy on fibrinolysis requires clarification.

2.9.3 Fibrinolysis and Glycaemic Control

Lack of accurate techniques to assess long-term glycaemic control in diabetic patients made it difficult, in the past, to evaluate the effect of glycaemic control on fibrinolysis. With the realisation that practically all proteins become glycosylated, an accurate measure of long-term glycaemic control has become available. In non-enzymatic glycosylation, glucose becomes bound to free amino-groups of plasma, membrane and cellular proteins, depending on glucose concentration, incubation time and temperature (McVerry et al., 1981). Because of this glycosylation, the tertiary or quaternary structure,

function and/or degradation of the protein molecule may be altered, as has been demonstrated for a number of proteins (Wieland, 1983). Glycosylation of haemoglobin moietes, ie, HbAl or HbAlc allows an overall assessment of glycaemic control over a period of 6-8 weeks prior to the test being taken. Clinical studies have utilised HbAl measurements from about 1978-79 and a number of studies reported on the relationship of glycaemic control in fibrinolysis. While Gunnarsson and colleagues (1980), in a cross-sectional study, found a significant inverse association of HbAl with fibrinolytic activity other workers have failed to confirm this association (Grignani et al., 1981; Dornan et al., 1983; Christe et al., 1984; Haitas et al., 1984). Longitudinal studies have shown that improvement of glycaemic control with significant lowering of HbAl concentrations have not been associated with any alterations in fibrinolytic activity (Paton et al., 1981; Knaap et al., 1985) although one study in type I diabetics treated with continuous subcutaneous insulin infusion showed a significant increase in fibrinolytic activity after 16 weeks of improved glycaemic control, while no changes in the response to venous occlusion were noted (Greaves et al., 1983). Euglycaemia over a 24 hour period using the artificial pancreas has been shown to improve fibrinolytic activity and t-PA antigen levels (Juhan-Vague et al., 1984) but this type of insulin regimen is of little clinical relevance to the management of diabetic patients.

In vitro studies have demonstrated non-enzymatic

glycosylation of fibrinogen, plasminogen and vascular plasminogen activator (McVerry et al., 1981; Geiger, Beckmann and Binder, 1983); that glycosylation reduces the susceptibility of fibrin to plasmin degradation (Brownlee, Vlassara and Cerami, 1983); and that fibrin enhanced plasmin formation by diabetic vascular plasminogen activator is significantly impaired, and can be normalised by improved glycaemic control (Geiger and Binder, 1984; Geiger and Binder, 1985). An in vivo study has demonstrated that in type I diabetics there is increased glycosylation of fibrinogen as compared to controls (Lutjens et al., 1985). It has also been shown that fibrinogen survival is reduced in diabetes (Ferguson et al., 1975b; Jones and Paterson, 1979); that such changes are more marked in patients with diabetic retinopathy (Cederholm-Williams, Dornan and Turner, 1981); and that correction of hyperglycaemia improves fibrinogen survival (Jones and Paterson, 1979). Improved glycaemic control has been shown to lower (Greaves et al., 1983) or to have no effect on fibrinogen concentrations (Paton et al., 1981; Knaap et al., 1985).

Hyperlipidaemia is common in diabetes mellitus and is closely linked to poor glycaemic control. The effect of elevated blood lipids in non-diabetics has already been discussed (Chapter 2.5) and in diabetes, hyperlipidaemia has also been associated with diminished fibrinolytic activity (Almer et al., 1975b) and, more specifically, hypertriglyceridaemia has been shown to depress fibrinolytic activity in uncomplicated type II diabetes

(Grignani et al., 1981). Whether the poor fibrinolytic activity in diabetes is more closely linked to poor glycaemic control or the resultant hyperlipidaemia has not been evaluated.

While there seems little doubt that glycosylation of proteins involved in the fibrinolytic enzyme system occurs, the lack of correlation of HbA1 with the fibrinolytic activity in clinical studies casts some doubt on the effects of glycaemic control on fibrinolysis.

2.10 COMPONENTS OF FIBRINOLYSIS IN DIABETES

2.10.1 Plasminogen

No differences in plasminogen concentration are found in either type I or type II diabetes when compared to control subjects (Cederholm-Williams, et al, 1981; Hughes et al., 1983) and plasminogen levels are not related to diabetic retinopathy (Almer et al., 1975b). Although plasminogen may be glycosylated in vitro (Geiger et al., 1983) plasminogen turnover is normal in diabetes (Cederholm- Williams et al., 1981).

2.10.2 Alpha 2-antiplasmin

Alpha 2-antiplasmin concentrations do not differ in type II diabetes when compared to controls (Grignani et al., 1981; Hughes et al., 1983), while in type I diabetes

alpha2-antiplasmin levels have been reported to be either increased (Ambrus et al., 1979) or not different from controls (Coccheri et al., 1983; Dornan et al., 1983). Dornan and colleagues (1983) found that alpha2-antiplasmin concentrations were not related to the severity of diabetic retinopathy while Christe and colleagues (1984) found raised alpha 2- antiplasmin concentrations only in patients with microvascular disease compared to controls. This requires clarification.

2.10.3 Alpha 2-macroglobulin

The alterations of alpha 2-macroglobulin in diabetes resembles those changes seen in fibrinogen levels, ie, alpha 2-macroglobulin has been noted to be higher in diabetics compared to controls (James et al., 1980; Christe et al., 1984) and still higher levels are found in advancing microvascular disease (Almer et al., 1975b; James et al., 1980; Dornan et al., 1983; Christe et al., 1984).

2.10.4 Fibrinogen

It is clear that fibrinogen concentrations are higher in type I and type II diabetes as compared to matched controls (Badawi et al., 1970; Almer et al., 1975a; Fuller et al., 1979; De Silva et al., 1979; Brooks et al., 1983; Borsey et al., 1984), and that the changes in fibrinogen are more marked in patients with increasing severity of

microvascular disease (Almer et al., 1975b; Fuller et al., 1979; Borsey et al., 1984; Christe et al., 1984; Lowe et al., 1986). In a prospective controlled trial sulphonylurea therapy was shown to increase fibrinogen concentrations (De Silva et al., 1979) but this has not received confirmation. The effect of improved glycaemia on fibrinogen concentrations is controversial (Paton et al., 1981; Simpson et al., 1982; Greaves et al., 1983).

CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

In this section, a description of the haemostatic tests used in our laboratory, and analysed in this thesis, will be presented. The assay methods, used in the various studies to be discussed in the thesis, have not changed (except for plasminogen) and, in the main, have been performed by the same expert laboratory staff over the 5 years during which this work was carried out. Special emphasis will be placed on the fibrin plate method of estimation of plasma fibrinolytic activity since laboratory techniques vary for this assay, especially in relation to the type of fibrinogen used.

3.2 Blood Sampling

Whole blood is obtained by clean venepuncture through a 19G needle into a plastic syringe, and mixed 9:1 parts with 3.8% trisodium citrate in a plastic collection tube. The sample is then placed on ice and is spun at 4^{o}C for 20 minutes at 3000G, within 30 minutes of collection. The plasma is then aliquoted into separate containers and stored at -70^{o} C until ready for assay. Plasma for fibrinogen and serum for FDP's is kept at -20^{o}C. Small but significant decreases in the concentrations of plasma fibrinolytic activity, plasminogen, alpha2-antiplasmin, alpha2-macroglobulin, FDP's and fibrinogen occurred after one month of storage at these temperatures (McArdle, 1983)

and therefore all samples were assayed as soon as possible, and usually within 3 weeks of sample collection. The anti-coagulants and storage of both BBeta 15-42 and fibrinopeptide A are different from the above components and will be discussed separately. With the restrictions on storage mentioned above comparison samples for an individual were performed, where possible, in the same assay and the intra-assay coefficient of variance for the assays used in our laboratory are listed in Table 3.1.

3.3 Plasma Fibrinolytic Activity: Fibrin Plate Method

3.3.1 Introduction

Plasminogen activators can be assayed by measuring the mean diameter of the area of lysis around a small volume of plasma euglobulin fraction placed on a plate of fibrin. The fibrin contains plasminogen as a contaminant, and so activator present in samples placed on the fibrin surface will bring about plasminogen to plasmin conversion. The advantages of the fibrin plate method include the fact that plasminogen and fibrinogen substrates are controlled and plasma can be stored at -70° C and assayed in batches whereas euglobulin clot lysis assays for example have to be performed immediately.

The fibrin plate method used in our laboratory is a modification of that described by Astrup and Mullertz (1952), Kluft et al., (1976) using Owren's buffer and Kabi fibrinogen. This area of lysis gives a measure of

TABLE 3.1

THE LABORATORY INTRA-ASSAY COEFFICIENT OF VARIANCE

	Intra-Assay C.V. (%)
Fibrinolytic Activity	5.0
BBeta 15-42 antigen	9.7
Plasminogen Activity (caseinolytic)	3.8
Plasminogen Activity (chromogenic)	5.2
α2-Antiplasmin Activity	4.2
α2-Macroglobulin Antigen	1.1
F.D.P.'s	0
Fibrinogen Activity	3.0
Fibrinopeptide A Antigen	4.4

Legend

The intra-assay coefficient of variance (C.V.) values for the tests of fibrinolysis used in our laboratory.

plasminogen activator activity although it may also detect plasmin activity.

3.3.2 METHOD

Twenty mls of fibrinogen (Human Grade L; Kabivitrium Ltd, Flow Laboratories, Irvine) is made up to 200 mls with Tris HCL buffer. Twenty mls of this mixture is placed on a level Petri dish which allows 10 fibrin plates to be made. Fibrinogen is clotted by adding 0.5 mls of thrombin solution (bovine origin, 5000 NIH units dissolved in 100 mls of Tris HCL buffer; Parke-Davies) and contents thoroughly mixed. The plates are kept on a flat surface. The 4 quadrants of the plate are marked off and 20 µl of the plasma euglobulin precipitate to be tested are accurately pipetted in triplicate. One quadrant is used for a streptokinase control (Varidase, Lederele, Hampshire, Great Britain) - 0.5 ml aliquot made from 2000 units dissolved in 50 mls of Tris HCL buffer added to 1.0 ml of Tris HCL buffer. The plates are then incubated at 37°C for 18 hours in a vibration free oven with flat shelves. The zone of lysis is then measured by taking the mean of the 2 perpendicular diameters. The area of lysis is then calculated by taking the average diameter of the 3 lysis zones, correcting it against the control values obtained using streptokinase, and using the equation-$(1/2\ d^2) \times \pi$, giving a value in mm2.

3.3.3 Euglobulin Preparation

This depends on the isoelectric precipitation of

euglobulin from diluted plasma leaving inhibitors in the supernatent (Nilsson and Olow., 1962). Add 1ml of citrated plasma to 9mls of 0.025% acetic acid and check pH is 5.9-6.0. Invert the tube and allow to stand at 4°C for 10 minutes to precipitate the euglobulins. Centrifuge at 2000G for 5 minutes at 4° C, discard supernatent and resuspend euglobulin precipitates in 1ml of Owren's buffer.

3.3.4 What Does The Fibrin Plate Measure?

The fibrin plate method is widely used as an overall assessment of fibrinolysis but detailed work has been carried out to determine the contributions that the intrinsic and extrinsic system of fibrinolysis make to the overall lysis zone. The regular euglobulin fraction (used in our laboratory) contains about 25% of the plasma level of C1-inhibitor (Kluft, 1976) and the addition of flufenamic acid nullifies the affect of C1-inactivator (Kluft, 1979) and therefore allows an assessment of the C1-inactivator fraction (Factor XII - dependent intrinsic pathway), the C1-inactivator resistant fibrinolytic activity (extrinsic and urokinase-like fibrinolysis). Kluft (1979) also found that the intrinsic system was only partially recovered during the preparation of the regular euglobulin fraction and that the addition of dextran sulphate could precipitate activators of fibrinolysis in addition to its ability to activate factor XII (a proactivator). Kluft (1979) estimated that using these 2 manoeuvres (ie, adding flufenamic acid and dextran

sulphate), the activity recovered from the modified plate was at least a two-fold increase. Further work has demonstrated that the morning level of fibrinolytic activity on the regular fibrin plate contains only 1-2% of extrinsic activator (which is completely recovered), the remainder belonging to the intrinsic system (which is incompletely recovered). The increase in fibrinolytic activity on the regular fibrin plate due to diurnal rhythm, exercise, DDAVP etc., is however due to an increase in the extrinsic pathway of fibrinolysis (Kluft, 1981; Kluft and Allen, 1983; Kluft et al., 1984).

3.4 BBeta 15-42 Fragment

The measurement of BBeta peptides allows a sensitive indicator of endogenous fibrinolysis (Nossel, 1981; Prowse et al., 1982). The assay for BBeta peptides (BBeta 1-42, BBeta 15-42) is not specific for the BBeta 15-42 since the aminoacid sequence is found in all fragments (Walenga et al., 1984). The assay however is said to quantitate the BBeta 15-42 fragment. The BBeta peptides have a short half-life, and in man BBeta 1-42 demonstrates a biphasic clearance with a t-1/2 of 10 and 100 minutes (Prowse et al., 1982).

Using a new syringe 9mls of blood was removed into 1ml of anticoagulant containing 1000 IU heparin; 1000 KIU Trasylol (Trasylol, Bayer Pharmaceuticals Ltd, Haywards Heath, Sussex, England) in sodium chloride, 0.15M. After gentle inversion three times the sample was placed on ice or centrifuged immediately at 2000g for 20 minutes at 4

°C. Plasma was assayed immediately or snap frozen and stored at -70°C until assayed. BBeta 15-42 was measured by radioimmunoassay with a commercial kit provided by IMCO Corporation Ltd (Stockholm, Sweden). Due to the cross-reaction of plasma fibrinogen with the BBeta 15-42 antisera, the protocol involves an ethanol extraction step to remove fibrinogen. The results are expressed in pmol/ml. In our laboratory the mean inter-assay coefficient of variance of BBeta 15-42 is 10.2% and BBeta 15-42 levels remain stable for at least 5 months when stored at -70°C.

3.5 Plasminogen

Plasminogen activity is measured in our laboratory by a caseinolytic assay which involves the initial acidification of the plasma sample to remove the effects of antiplasmin activity, and streptokinase activation of plasminogen. (Remmert and Cohen, 1949; Alkaersig et al., 1959). This assay has been employed in all the studies in this thesis except for the diabetic studies (excluding Chapter 13).

From 1985 however we have replaced the caseinolytic assay with a chromogenic substrate assay using substrate S-2251, (H-D-VAL-LEU-LYS-pNA; Kabi Diagnostica, Stockholm, Sweden). The principal of this assay is as follows: when an excess of streptokinase is added to plasma containing plasminogen a complex with enzymatic activity is formed which catalyses the splitting of p-nitroanaline (pNA) from

the substrate. As the streptokinase is added in excess, no free plasminogen, is left to be activated to plasmin and under these conditions the enzymatic activity is not inhibited by plasma inhibitors. The rate at which pNA is released is measured photometrically at 405 nm and the concentration of plasminogen is calculated by using standards prepared from normal pool plasma.

3.6 Alpha2-Antiplasmin

3.6.1 Introduction

Aoki and von Kaulla (1971), identified a fibrinolytic inhibitor in human serum which Morrow and Aoki (1976), then named alpha2-plasmin inhibitor having demonstrated its site of action at the level of formed plasmin. Collen and co-workers (1976) identified this plasma protein as a fast-acting inhibitor which they renamed alpha2-antiplasmin. Alpha2-antiplasmin can be accurately determined in plasma either with enzymatic assays based on the very fast inhibition of plasmin, or with immunochemical assays using specific antisera. Both of these are employed in our laboratory.

3.6.2 Alpha2-Antiplasmin Activity : Chromogenic Substrate

Plasmin catalyses the amidolytic cleavage of p-nitroanaline from the specific plasmin substrate, TOSYL-GLY-PRO- lys-pNA (Chromozym Pl, Boehringer Mannheim, Germany). The activity of a given quantity of plasmin, as well as the residual plasmin activity after incubation

with an excess of plasmin are determined. The difference between these 2 measurements corresponds to the antiplasmin activity of the plasma sample. Since the substrate also reacts slightly with thrombin, hirudin is therefore added to the test solution in order to inhibit enzymatic thrombin activity which may be present in the plasma.

3.6.3 Alpha2-Antiplasmin Antigen : Electroimmunoassay

Alpha2-antiplasmin can be quantitated in human plasma with the use of monospecific antisera, and in our laboratory an electroimmunoassay is in use (Laurell, 1966). This assay is based on the fact that during electrophoresis of an antigen in an antibody containing medium, antigen-antibody complexes will precipitate in a "rocket" shaped precipitin zone. The height of the rocket is proportional to the amount of antigen present. The monospecific antisera was obtained from Nordic Immunological Laboratories, Netherlands.

3.7 Alpha2-Macroglobulin Antigen

Norman and Hill (1958), detected a plasmin inhibitor in the alpha2-region on electrophoresis which Schwick and co-workers (1966), later identified as alpha2-macroglobulin. Alpha2-macroglobulin can be accurately quantitated by radial immunodiffusion (Mancini, 1965) using commercially available antisera (Behringwerke Laboratories).

3.8 FDP's

Degradation products of fibrinogen and fibrin can be measured by assay of their biological activation (prolongation of thrombin time, staphylococcal clumping tests) but our laboratory utilises the tanned red cell haemaglutination inhibition immunoassay described by Merskey et al., (1969). This is an automated method with commerical kits (Wellcome FDP kit) available from Wellcome Reagents, Beckenham, UK. A suspension of formalin treated sheep red cells is a very sensitive indicator of the presence of antibodies to fibrinogen. Aliquots of a specific anti-fibrinogen serum, diluted to a concentration just high enough to agglutinate the sensitised cells, are added to serial dilutions of the sample to be assayed. If sufficient fibrinogen is present in the sample it will combine with the anti- fibrinogen serum and sensitised red cells, added subsequently, will fail to agglutinate. The last completely unagglutinated well is taken as the "end point" and the amount of FDP in the sample can be assayed by comparison with a known standard.

3.9 Fibrinogen

Fibrinogen is a large glycoprotein (see Table 1.1) synthesized in the liver and to a lesser extent by platelets (Marsh, 1981). The fibrinogen molecule is a dimer, each half of the dimer consisting of 3 polypeptide chains, the AAlpha, BBeta and gamma chains - joined by disulphide bonds (see figure 1.2). Fibrinogen is an acute phase reactant, and release into blood occurs during a

variety of stimuli in addition to normal haemostatic requirements. The mean plasma t-1/2 is approximately 4 days. Fibrinogen is involved with the central event of coagulation, ie, the thrombin-catalysed conversion of fibrinogen to fibrin, and while the natural substrate for plasmin in vivo is fibrin, plasmin-mediated fibrinogenolysis may occur in conditions of systemic hyperplasminaemia. Fibrinogen is measured in our laboratory by the thrombin clotting time method (Clauss, 1957) where the thrombin clotting time is inversely proportional to the fibrinogen concentration present in the plasma sample. Automatic recording of the thrombin time is performed in duplicate using the Dade fibrometer.

3.10 Fibrinopeptide A

Fibrinopeptide A is a 16 aminoacid long peptide chain with a molecular weight of 1536 daltons. It is released by the action of thrombin on the amino terminal of the AAlpha chain of fibrinogen, leaving intermediate fibrin monomer (fibrinopeptide B is then cleaved from the BBeta chain leaving fibrin monomer). The measurement of fibrinopeptide A therefore is a sensitive index of thrombin generation in vivo (see figure 1.2). Fibrinopeptide A has a mean t-1/2 of 4 minutes and appears to be a sensitive marker of prethrombotic states (Nossel, 1981; Amiral et al., 1984; Walenga et al., 1984).

It is especially important to take blood via a clean flawless venepuncture without trauma to avoid local thrombin generation. The patient plasma is prepared as

described in the BBeta 15-42 assay, plasmin being assayed immediately or snap frozen and stored at -70° C until assayed. Fibrinopeptide A was measured by radioimmunoassay using the commercial kit supplied by IMCO Corporation Ltd (Stockholm, Sweden). As in the BBeta 15-42 assay, ethanol extraction of fibrinogen was performed. The results are expressed in pmol/ml while 1 pmol is equivalent to 1.535 ng/ml. In our laboratory the mean inter-assay coefficient of variance of fibrinopeptide A is 3.2% and levels remain stable for at least 5 months when stored at -70°C.

3.11 BODY MASS INDEX

In the various groups of patients studied in this thesis the effect of weight on the components of fibrinolysis will be assayed using the body mass index or Quetelet's index. This is defined as:- weight (kg)/height (m)2, and is a convenient and popular index of relative weight, which is almost independent of height (Truswell, 1985). Acceptable weights range from 20-25, while obesity is taken to start at values above 30 and gross obesity at values above 40.

CHAPTER 4

THE EFFECTS OF EXERCISE AND BETA-ADRENOCEPTOR BLOCKADE ON THE FIBRINOLYTIC ENZYME SYSTEM IN NORMAL VOLUNTEERS

4.1 INTRODUCTION

The effects of exercise on fibrinolysis have been reviewed in Chapter 2.3. While it is well recognised that plasminogen activator is stored in the endothelial cell lining veins and the vasa vasorum of arteries the exact mechanism by which activator is released from these storage sites remains an enigma. Since epinephrine infusion was shown to stimulate fibrinolysis (Biggs et al., 1947) the role of adrenoceptor stimulation and blockade on the pathways of plasminogen activator release has received much attention. The plasminogen activator response to catecholamines was thought to be beta2 receptor related, due to a direct effect on the endothelial cell, and could be partially blocked by propranolol (Cash, Woodfield and Allan, 1970; Cash 1975). Other studies have shown however that the increase in fibrinolytic activitiy with bicycle or treadmill exercise was not reduced by prior beta-blockade (Cohen et al., 1968; Korsan-Bengtsen and Conradson, 1974; Britton et al., 1976) and that the rise in fibrinolytic activity did not parallel the change in catecholamine levels (Hawkey et al., 1975). More recently, experiments on the fibrinolytic response to vasoactive drugs suggests that the major fibrinolytic response proceeded along non-adrenergic pathways (Rosing et al., 1978). Studies to date therefore tend to indicate that exercise induced fibrinolytic enhancement is not mediated by beta-receptor stimulation and therefore not attenuated by beta-blocking

drugs.

The resting level of plasma fibrinolytic activity has been reported to be increased (Ponari, Civardi and Poti, 1972) unchanged (Davidson et al., 1979b) or decreased (Nielson, Jessen-Jurgensen and Gormsen, 1981), by beta-blockers. At the time this study was performed the effect of exercise on alpha2-antiplasmin was unknown, although antiplasmin levels were reported not to change with exercise (Marsh and Gaffney, 1980). No previous studies on exercise and beta-blockade have included a double-blind placebo controlled design in their protocol. The aims of our study therefore were to assess whether beta-blockade had any effect on either resting or exercise stimulated plasma fibrinolytic activity or some other tests of the fibrinolytic enzyme system.

4.2 METHODS

4.2.1 Patients

Local Ethical Committee permission was obtained and 9 healthy male volunteers took part in the study. The subjects were young (age range 23-32, mean 27.6 years) and of normal body mass index (range 19.0-23.6, mean 21.5).

4.2.2 Study Design

The subjects performed maximal bicycle exercise tests on 3 occasions, with or without prior oral administration of a single dose of 2 different beta-blockers. The study

was double-blind, placebo-controlled and crossover in design and conducted in 3 stages, each subject receiving either 80 mg of the non-selective beta-blocker propranolol, 10 mg of carteolol (Berk Pharmaceuticals, non-selective, partial agonist activity) or placebo. Randomisation of treatments were made using a Latin square design. The interval between each stage was at least one month to reduce any "training effect" of repeated exercise tests. The subjects, who were on no other drug therapy, were fasted overnight and after 10 minutes at rest, blood was withdrawn for estimation of baseline haemostatic tests. The drug was administered and subjects were kept fasting for a further 2 hours to ensure maximal drug absorption. Approximately 4 hours after tablet ingestion a pre-exercise blood sample was taken. This time interval was chosen as a compromise to coincide with peak plasma levels of carteolol (Small et al., 1982a) and propranolol. Peak concentrations of propranolol are well recognised to vary markedly in normal subjects. Exercise testing was performed using an upright bicycle ergometer, peddling at a constant speed, using a graded exercise protocol with an initial workload of 300 kilopond meters/minute (300 kpm = 50 watts), increasing by 300 kpm at 3 minute intervals. Exercise was maximal. The electrocardiogram was monitored using a single lead system (modified V5 electrode) and blood pressure from the cuff sphygmomanometer. During the last 30 seconds of peak exercise a final blood sample was taken from a seperate venepuncture. The double product (heart rate x systolic

blood pressure X 10^{-3}) was calculated as an indicator of myocardial oxygen consumption and beta-blocker effect. An indirect estimate of maximal oxygen consumption (VO2 max) at peak exercise was obtained from the equation below (Blomqvist, 1973), where the workload is the kpm at the end of exercise, corrected from the duration of exercise.

$$VO2\ max = \frac{(Workload \times 2) + 300ml.\ kg.^{-1}\ min.^{-1}}{BODY\ WEIGHT\ (kg)}$$

4.2.3 Blood Tests

Plasma fibrinolytic activity and alpha2-antiplasmin were measured on 3 occasions while fibrinogen and fibrinopeptide A were measured only pre and post exercise.

4.2.4 Statistics

Statistical comparisons were performed using the unpaired Wilcoxon rank sum test, and correlations were determined by the Spearman rank order correlation coefficient.

4.3 RESULTS

4.3.1 Haemodynamic Data

The haemodynamic results are shown in table 4.1. No

TABLE 4.1

HAEMODYNAMIC RESPONSE TO EXERCISE

		Placebo	Carteolol	Propranolol
Heart Rate (beats/min)	Pre	82 ± 15	71 ± 9	67 ± 9
	Ex	172 ± 24	128** ± 12	131** ± 12
Systolic BP (mmHg)	Pre	110 ± 12	102 ± 15	106 ± 9
	Ex	177 ± 12	146* ± 24	151** ± 21
Double Product	Pre	9 ± 2	8 ± 1	7 ± 1
	Ex	31 ± 5	19** ± 4	20** ± 5
VO2 max	Ex	36 ± 9	37 ± 5	36 ± 6

Legend

Pre and exercise (Ex) values in the placebo and beta-blocker groups of the heart rate, systolic BP, double product (heart rate X systolic BP X 10^{-3}) and calculated maximal O2 consumption (VO2 max). Values are mean ± SD.

*$p < 0.05$

**$p < 0.01$, beta-blocker versus placebo

differences in the calculated VO2 max during the 3 study days were found. Reductions in exercise induced tachycardia, systolic BP and double product were noted in each beta-blocker group. No differences between the active drug groups were found. The mean exercise time was 10.9 minutes.

4.3.2 Haemostatic Tests

The haemostatic test results and the percentage change in fibrinolytic activity are listed in table 4.2, figure 4.1. In the 4-5 hour interval between 9am and pre-exercise, the fibrinolytic activity increased an average of 55% on the placebo leg and no differences were noted between the placebo and beta-blocker groups. Fibrinolytic activity increased significantly by 53% after exercise (placebo leg). The fibrinolytic activity also increased significantly in both active drug groups and no differences in the magnitude of response were noted between the 3 groups. No diurnal rhythm was noted in alpha2-antiplasmin levels and no effects of exercise on alpha2-antiplasmin, fibrinogen and fibrinopeptide A levels were noted. No significant correlations were found between the incremental change in fibrinolytic activity (on the placebo leg) and the body mass index ($r = -0.16$), peak heart rate ($r = 0.38$) or VO2 max ($r = 0.30$). The haematocrit increased by 6%, pre to post-exercise and was not affected by beta-blockade.

TABLE 4.2

EXERCISE, BETA-BLOCKADE AND FIBRINOLYSIS

	FA (mm2)	α2-AP (u/ml)	Fibrinogen (g/l)	F.P.A. (ng/ml)
Placebo				
9 am	84 ± 34	2.4 ± 0.6	-	-
Pre	131+ ± 46	2.3 ± 0.7	2.1 ± 0.3	0.66 ± 0.12
Post	201* ± 107	2.4 ± 0.7	2.3 ± 0.4	0.71 ± 0.27
Carteolol				
9 am	89 ± 35	1.9 ± 1.1	-	-
Pre	120+ ± 37	2.0 ± 1.0	2.1 ± 0.2	0.78 ± 0.48
Post	155* ± 41	1.9 ± 0.8	2.1 ± 0.4	0.66 ± 0.12
Propranolol				
9 am	73 ± 16	2.1 ± 0.7	-	-
Pre	112+ ± 47	2.1 ± 0.9	2.1 ± 0.2	0.62 ± 0.06
Post	189* ± 103	2.0 ± 1.0	2.2 ± 0.4	1.17 ± 1.55

Legend

Effect of exercise and beta-blockade on fibrinolytic activity (FA), α 2-antiplasmin (α2AP), fibrinogen and fibrinopeptide A (FPA) concentrations. Values are mean ± SD.

+ $p < 0.01$, basal versus pre-exercise

*$p < 0.01$, pre versus post-exercise.

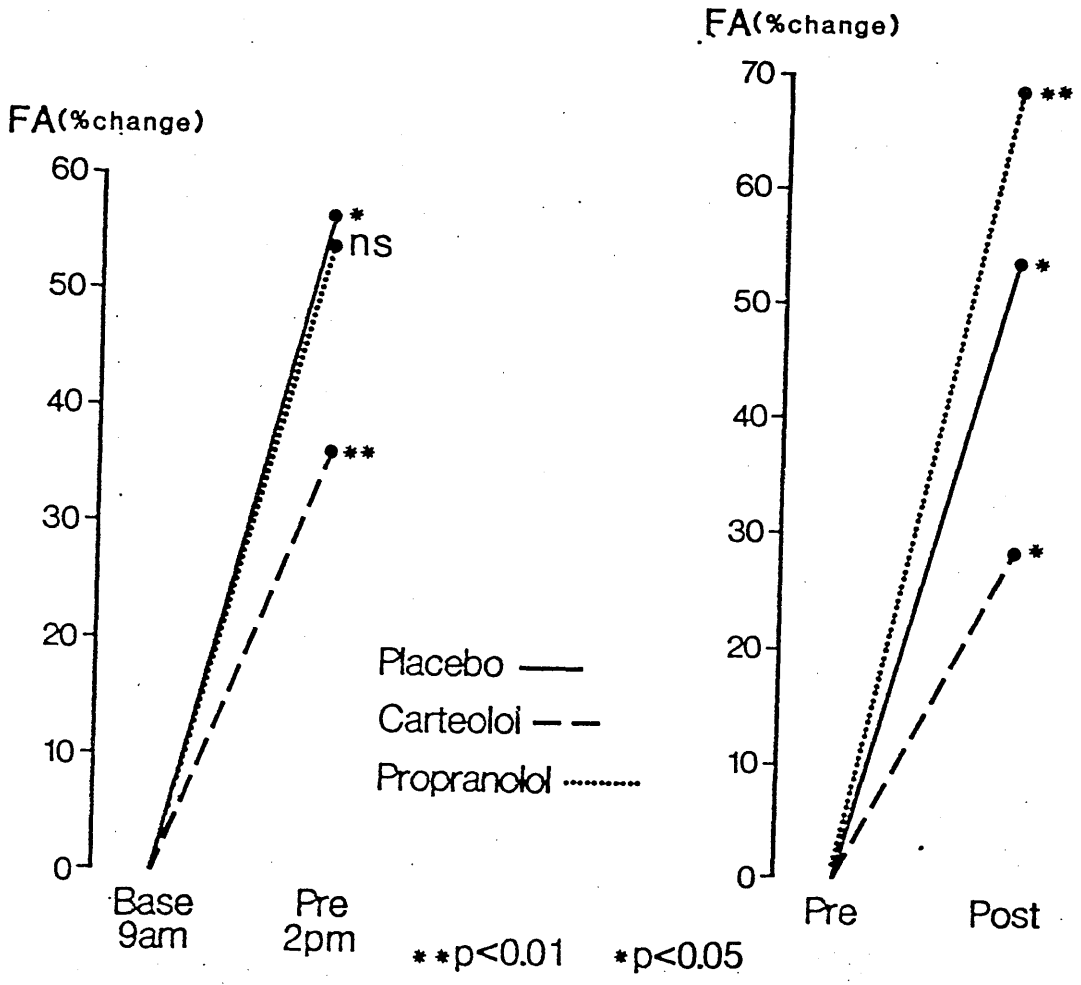

Figure 4.1

Illustrates the percentage rise in plasma fibrinolytic activity (FA) from 9am to 2pm and post exercise on the 3 study days. No differences between the 3 groups were detected. Changes are within group comparisons.

4.4 DISCUSSION

In normal subjects plasma fibrinolytic activity is lowest at 8am and peaks between 5-8pm (Rosing et al., 1970). We have noted a marked rise in fibrinolytic activity in the 4 hour interval between 9am to 1pm which emphasises the importance of the exact timing of samples when comparisons are made between fibrinolytic activity in different patient groups.

The lack of difference between VO2 max. on each drug indicates that maximum exercise was obtained on each occasion. The attenuation of exercise tachycardia, systolic BP and the double product by both beta-blockers, and the lack of difference between the drugs indicate that adequate, and equipotent, beta-blockade was achieved on each occasion. Beta-blockade therefore failed to make any significant modification to the rise of plasma fibrinolytic activity induced by exercise as has been noted before in open studies with smaller numbers of volunteer subjects. (Cohen et al., 1968; Korsan-Bengtsen and Conradson, 1974; Britton et al., 1976). Beta-blockers with partial agonist activity have been reported to enhance plasma fibrinolytic activity (Ponari et al., 1972), but carteolol, which has a weak partial agonist activity did not alter the resting level of fibrinolytic activity. Our findings are in agreement with those of Davidson and co-workers (1979b) that beta-blockers do not alter resting plasma fibrinolytic activity. Since

publication of this study, Brommer and colleagues (1984b) have shown that propranolol has no effect on either the resting fibrinolytic activity or the response to DDAVP.

We have demonstrated that exhaustive, maximal bicycle exercise of approximately 11 minutes duration causes a 54% rise in plasma fibrinolytic activity. Fibrinopeptide A concentrations did not change after exercise, and therefore thrombin generation, with significant activation of coagulation did not occur. Exercise in our healthy subjects therefore is an example of primary fibrinolysis (ie, not secondary to the activation of coagulation).

We have found that alpha2-antiplasmin levels do not significantly change with exercise even when a correction factor is applied for haemoconcentration (haematocrit rise of 6% after exercise). This finding has recently been confirmed (Huisveld et al., 1984). Theoretically any plasmin generated by exercise should be rapidly inactivated by alpha2-antiplasmin, whose concentration would then be expected to fall. The lack of change in alpha2-antiplasmin is thought to indicate that exercise fails to generate significant quantities of plasmin.

The findings from this placebo-controlled, double-blind study suggests that adrenergic mechanisms make no contributions to the rise in fibrinolytic activity with exercise and that beta-blocking drugs do not affect the resting level of fibrinolytic activity (Small et al., 1984a). While marked changes in plasma fibrinolytic activity occur with exercise, significant plasmin generation appears unlikely.

CHAPTER 5

THE EFFECTS OF EXERCISE ON THE FIBRINOLYTIC ENZYME SYSTEM IN MIDDLE-AGED MEN

5.1 INTRODUCTION

Dynamic tests of fibrinolysis using venous occlusion have shown defective responses in plasma fibrinolytic activity occur more commonly in men with coronary artery disease (Walker et al., 1977; O´Connor et al., 1984a) and that plasma fibrinolytic activity following bicycle exercise is significantly impaired in patients with coronary artery disease (Khanna et al., 1975). The change in plasma fibrinolytic activity in normal volunteers has been related to the intensity of exercise, as indicated by the maximal oxygen consumption (VO2 max), and peak heart rates (Davis et al., 1976; Marsh and Gaffney, 1982). The effect of exercise on the BBeta 15-42 fragment, and the relationship between the intensity of exercise and fibrinolysis in middle-aged men have not been reported. The main aims of our study were therefore to assess whether differences in fibrinolysis occured either at rest or following standardised exercise, in men with and without coronary artery disease, and to relate the changes in fibrinolysis to the intensity of exercise.

5.2 METHODS

5.2.1 Patients

Thirty-four males of mean age 51 years (range 40-66) gave their informed consent and took part in the study. Patients being routinely evaluated for chest pain with

exercise electrocardiography were selected provided they did not have valvular heart disease or were taking anti-coagulants as these are known to affect some tests of haemostasis. Ten patients in total had sustained a previous well documented myocardial infarction. Fifteen patients were being treated with the following groups of anti-anginal medication:- beta-adrenoceptor blockers (beta 1 selective, n = 8; non-selective, n = 5), calcium antagonists (n = 12), long-acting nitrates (n = 8). Fourteen patients were non-smokers and 20 patients were current cigarette smokers. The body mass index was used as an indicator of over or underweight.

5.2.2 Study Design

The patients performed erect bicycle exercise using the same graded exercise protocol as used in the normal volunteer study (Chapter 4), except that exercise was terminated if symptoms developed. A positive exercise test was defined as one producing ST depression of $\geqslant$ 2mm persisting 0.08 seconds after the J point in 3 consecutive cardiac cycles and/or the development of typical cardiac chest pain on exertion. All exercise tests were performed between 10-11 am and venous blood was taken from a separate venepuncture site prior to exercise, with patients seated at rest, and within one minute after peak exercise.

5.2.3 Blood Tests

Plasma fibrinolytic activity, BBeta 15-42 antigen,

fibrinogen, FDP's, alpha2-antiplasmin activity and fibrinopeptide A were assayed pre and post exercise.

5.2.4 Statistics

Statistical comparisons were performed using the unpaired Wilcoxon rank sum test, and correlations were determined by the Spearman rank order correlation co-efficient.

5.3 RESULTS

5.3.1 Assessment of patient groups

Eleven patients fulfilled the criteria for a positive exercise test. In addition to these patients, 9 patients had coronary artery disease as evidenced by coronary angiography (> 50% occlusion of a major coronary vessel), or a definite previous myocardial infarction. Coronary angiography was performed in 13 patients (10 with positive, and 3 with negative exercise tests) and all patients had significant coronary artery disease. No significant differences in the ages or body mass index of patients with or without coronary artery disease were noted.

5.3.2 BBeta 15-42 fragment

Figure 5.1 illustrates the changes in the BBeta 15-42 fragment with exercise in patients with and without

coronary artery disease. A significant increase in the BBeta 15-42 fragment occurred only in patients with no coronary artery disease. These patients exercised for a significantly longer time but no significant difference in the VO2 max was noted between the two groups (table 5.1). Neither smoking nor beta-blockade had any effects on the resting or stimulated concentrations of the BBeta 15-42 fragment.

5.3.3 Plasma fibrinolytic activity

Table 5.1 lists the effects of exercise on plasma fibrinolytic activity in the various sub- groups. Patients with or without coronary artery disease, showed similar, significant increases in plasma fibrinolytic activity after exercise and neither smoking nor beta-blockade influenced the rise in fibrinolytic activity. The 10 patients with a previous myocardial infarction had elevated resting plasma fibrinolytic concentrations compared to those patients without a prior myocardial infarction (76 $\pm$ 15 versus 58 $\pm$ 19 mm2, $p < 0.02$). The patients with a prior myocardial infarction showed no differences in their response to exercise.

5.3.4 Other results

Exercise had no effects on the concentration of alpha2-antiplasmin, fibrinogen or FDP´s (table 5.2). Significant increases in fibrinopeptide A were noted in men with no coronary artery disease (table 5.2). No correlations between plasma fibrinolytic activity and

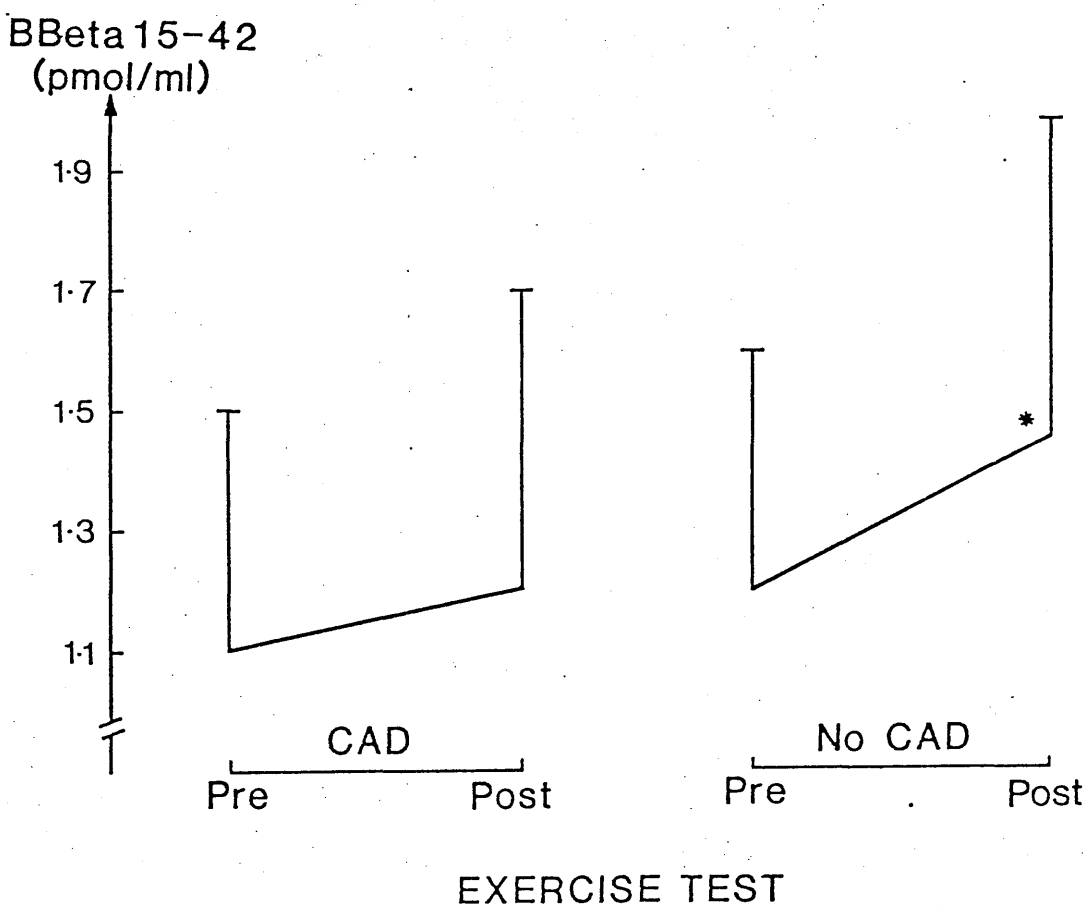

Figure 5.1

Illustrates the concentrations (mean ± 1SD) of BBeta 15-42 antigen, pre and post exercise in patients with and without coronary artery disease (CAD).

*p $<$ 0.05, pre versus post exercise

TABLE 5.1

EXERCISE AND PLASMA FIBRINOLYTIC ACTIVITY

	No	Exercise Time (mins)	VO2 max	Fibrinolytic Activity Pre	Fibrinolytic Activity Post
Coronary Disease					
Yes:	20	5.2 ± 2.1	18 ± 5	66 ± 17	81** ± 22
No:	14	6.9* ± 1.4	23 ± 5	57 ± 25	78** ± 27
Beta-Blockade:					
Yes:	13	5.8 ± 2.5	19 ± 5	70 ± 22	90** ± 26
No:	21	6.0 ± 1.7	22 ± 6	60 ± 18	74** ± 21
Smoking:					
Yes:	20	5.4 ± 2.0	18 ± 4	64 ± 22	80** ± 21
No:	14	6.6 ± 1.9	20 ± 6	61 ± 24	80** ± 29

Legend

Effect of coronary artery disease, beta-blockade and smoking on plasma fibrinolytic activity. Values are mean ± SD.

*$p < 0.05$

**$p < 0.005$

TABLE 5.2

HAEMOSTATIC TESTS AND EXERCISE

	CORONARY ARTERY DISEASE			
	YES		NO	
	PRE	POST	PRE	POST
α 2-Antiplasmin (%)	97 ± 15	104 ± 15	97 ± 21	103 ± 19
F.D.P.s (ug/ml)	3.06 ± 1.41	3.12 ± 1.47	2.75 ± 0.90	2.89 ± 1.10
Fibrinogen (g/l)	2.88 ± 0.43	3.16 ± 0.53	2.80 ± 0.55	2.95 ± 0.76
Fibrinopeptide A (pmol/ml)	2.7 ± 1.8	3.4 ± 1.7	2.5 ± 2.2	3.9 ± 2.3*

Legend

Haemostatic tests pre and post exercise in patients with and without coronary artery disease. Values are mean ± SD.

*$p < 0.005$, pre versus post exercise.

BBeta 15-42 fragment or between the body mass index and all the tests of fibrinolysis were noted.

5.3.5 Exercise intensity and fibrinolysis

The correlations between exercise time, VO2 max, peak heart rate and the incremental change in BBeta 15-42 and plasma fibrinolytic activity are shown in table 5.3. The alterations in BBeta 15-42 fragment, unlike plasma fibrinolytic activity, were related to both the exercise time and VO2 max. The fibrinolytic activity at peak exercise also failed to correlate significantly with the VO2 max ($r = 0.33$). The percentage rise of plasma fibrinolytic activity (peak - basal / basal x 100) in patients with and without coronary artery disease and in the normal volunteer study is shown in table 5.4. The rise in plasma fibrinolytic activity parallels the VO2 max, despite any significant correlation between the rise in fibrinolytic activity with VO2 max.

5.4 DISCUSSION

The effect of exercise on BBeta 15-42 in patients with coronary artery disease has not been studied before, and we have demonstrated that plasmin-mediated fibrinolysis does not occur in patients with coronary artery disease while fibrinolysis is stimulated in patients without coronary artery disease. It is possible that the defective increase in fibrinolysis in men with

TABLE 5.3

CORRELATION OF HAEMODYNAMIC DATA AND FIBRINOLYSIS

	Fibrinolytic Activity (mm^2)	BBeta 15-42 Fragment (pmol/ml)
Exercise Time (mins)	0.03	0.45*
VO2 max	0.13	0.56**
Peak heart rate (beats/min)	-0.03	0.16

Legend

Spearman correlation (r) values of ex vivo (fibrin plate) and in vivo (BBeta 15-42 fragment) fibrinolysis with haemodynamic values.

*$p < 0.01$

**$p < 0.005$

TABLE 5.4

FIBRINOLYTIC ACTIVITY AND VO2 MAX

	Fibrinolytic Activity(mm2)				VO2 max
	No.	Pre	△	%	
Controls	9	131	70	53.5	36
No CAD	14	57	20	34.9	23
CAD	20	66	15	22	18

Legend

Ex vivo fibrinolytic activity before exercise (pre), the incremental change (△), the percentage increase (%) after exercise, and the calculated maximal O2 consumption (VO2 max) in healthy subjects (controls), and patients with and without coronary artery disease (CAD). Values are mean.

coronary artery disease could predispose to thrombotic events.

The production of plasmin-antiplasmin complexes, and an increases in degradation of the AAlpha chain of fibrinogen post-exercise, drew Collen et al., (1977) to the same conclusion as the present study, that plasmin formation occurred to some extent in man following exercise. Despite the rise in BBeta 15-42 fragment with exercise, indicating plasmin generation, the lack of change in alpha2-antiplasmin levels (the major plasmin inhibitor) suggests that extensive plasmin generation does not occur. No change in FDP concentrations were detected, but this test detects only major increases in fibrin (ogen) degradation.

The measurement of fibrinopeptide A concentration allows an accurate assessment of thrombin generation (Amiral et al., 1984) and significant increases in fibrinopeptide A concentrations after exercise were noted in the men without coronary artery disease. In normal subjects, exercise has been shown to increase fibrinopeptide A concentrations (Hyers et al., 1980), although smaller studies have failed to detect this change (Vogt et al., 1979; Marsh and Gaffney, 1982). It may be that this activation of coagulation could directly lead to the stimulation of fibrinolysis which was noted in this group. The lack of thrombin generation in the patients with coronary artery disease might, in part, explain the poorer fibrinolytic responses (in vivo and ex vivo) induced by exercise.

Dynamic tests of fibrinolysis using venous occlusion and sub-maximal exercise have revealed abnormalities of fibrinolysis occur more commonly in men with coronary artery disease (Khanna et al., 1975; Walker et al., 1977; O´Connor et al., 1984a; Estelles et al., 1985), but we have been unable to show any differences in the response of plasma fibrinolytic activity between the men with and without coronary artery disease in the present study. Our study allows us to make comparisons only with the 2 previous exercise studies (Khanna et al., 1975; Estelles et al., 1985). While all 3 studies involved similar numbers of patients, the use of the euglobulin clot lysis time, treadmill exercise, a much longer exercise time and greater fibrinolytic responses may in part explain the discrepancy in the results between the present study and that of Khanna et al (1975). Using the fibrin plate assay, Estelles et al., (1985) also failed to note any difference in response to bicycle exercise between patients with a previous myocardial infarction and healthy controls, although the patients had lower t-PA activity and a reduced response of t-PA activity to exercise. These authors also suggested that this poor response may be related to increased levels of t-PA inhibitor and a previous study has shown elevated levels of t-PA inhibitor in patients with coronary disease (Paramo et al., 1985). These studies suggest a mechanism for defective fibrinolysis and provide some support for the association of defective fibrinolysis with coronary artery disease. The fibrin plate method therefore may not be sufficiently

specific to detect the differential effect of exercise in vivo, as seen using the BBeta 15-42 assay, since not all activators and inhibitors active ex vivo may be active in vivo. Indeed the lack of correlation between the fibrinolytic activity and BBeta 15-42 levels indicates that the 2 assays measure different aspects of fibrinolysis.

The relationship of exercise workload to fibrinolysis has been assessed mainly in normal healthy volunteers. The plasma fibrinolytic responses to exercise has been noted to show positive correlations with VO2 max (Davis et al., 1976) and to parallel changes in heart rate (Marsh and Gaffney, 1982). In the present study, using greater number of patients, we have been unable to confirm these associations of the plasma fibrinolytic activity, although the changes in fibrinolytic activity with exercise in our volunteers and middle-aged subjects do suggest an association with the VO2 max (table 5.4). There is a diurnal variation of the fibrinolytic response to exercise with greater responses noted around 4pm (Rosing et al., 1970). For practical reasons our studies in patients and healthy volunteers were performed at different times of day and therefore direct comparisons between these groups have not been statistically evaluated. We have shown however that the rise of in vivo fibrinolysis is clearly related to the duration and intensity of exercise. The patients with coronary artery disease exercised for a shorter period and performed slightly less work than the patients with no coronary disease, and it may be that the

differences noted in plasmin-mediated fibrinolysis are simply related to these differences in workload rather than the presence of coronary artery disease. The lack of a significant difference in VO2 max and the small difference in exercise duration between the groups argues against such a possibility. This merits further study.

We have been unable to demonstrate that beta-blockers alter the fibrinolytic responses to exercise, confirming previous studies (Cohen, et al., 1968; Korsan-Bengtsen and Conradson, 1974; Small et al., 1984a), and that the resting level of fibrinolysis is affected by beta-blockade. We have also been unable to show that smoking has any effect on either resting or exercise stimulated fibrinolysis. The effect of smoking has been discussed in detail in chapter 2.4.

In summary, we have shown that alterations of exercise induced in vivo fibrinolysis can be demonstrated in men with coronary artery disease. Whether the poor response of BBeta 15-42 is related to coronary artery disease or simply the exercise workload is unclear at present. If the response is related to coronary diseaese it may then predispose to a hypercoagulable state, which is of importance in view of the growing evidence for a close association between hypercoagulability and coronary artery disease (Meade et al., 1980; Baker et al., 1982; O´Connor et al., 1984a; Wilhelmsen et al., 1984; Sugrue et al., 1985; Yarnell et al., 1985). Epidemiological prospective studies have, to date, failed to show a significant difference in fibrinolytic potential ex vivo

in men who die of cardiovascular disease as compared to survivors (Meade et al., 1980) and in men who sustained a myocardial infarction compared to men who remain healthy (Wilhelmsen et al., 1984). The results presented in this paper suggest that tests of in vivo fibrinolysis (generation of the BBeta 15-42 fragment) may be more relevant to coronary artery disease.

CHAPTER 6

BASAL FIBRINOLYSIS AND CORONARY ARTERY DISEASE

6.1 INTRODUCTION

The established risk factors for coronary artery disease, cigarette smoking, hypertension and elevated blood lipids accounts for only 50% of the total coronary risk in population studies (Zumoff, 1981). Hormonal and haemostatic tests have been investigated to assess their contribution (if any) to coronary artery disease and it has been shown that fibrinogen and factor VII are risk associations for cardiovascular death at least as strong as that between cholesterol and cardiovascular death (Meade et al., 1980). As discussed before (Chapter 2.7) the association between defective plasma fibrinolytic activity and coronary artery disease remains unproven. The effect of the severity of coronary artery disease on plasma fibrinolytic activity and the components of fibrinolysis had not been investigated when this study was commenced. An inverse association of defective fibrinolysis with increasing severity of coronary artery disease would provide further evidence to support a role for fibrinolysis in coronary artery disease.

Recent studies have suggested that endogenous hyperoestrogenaemia may be a risk factor for ischaemic heart disease, possibly due to enhanced atherogenesis or defective fibrinolysis (for references see Small et al., 1985a). Our study allowed us to analyse these postulated mechanisms of oestradiol action.

The aims of our study were to see whether associations existed between fibrinolysis, hormones, lipids and the severity of coronary artery disease; to

Effect of Insulin Therapy on Coagulation and Platelet Function in Type II (Non-Insulin Dependent) Diabetes Mellitus

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Running Head: Insulin Therapy and Haemostasis

ABSTRACT

Twenty type II (non-insulin dependent) poorly controlled diabetics had tests of coagulation and platelet function performed while receiving high dose sulphonylurea therapy and at 1 and 3 months following their conversion to insulin. Although no overall change in glycaemic control (assessed by glycosylated haemoglobin) was noted, a reduction in thrombin generation was observed, as judged by a significant fall in fibrinopeptide A concentrations. No changes in factor VIIIC, factor VIIIR:Ag, or antithrombin III were found. Glycosylated haemoglobin concentrations showed significant correlations with antithrombin III and factor VIII:C, suggesting that improved glycaemic control might lead to an improvement of antithrombin III function and lower factor VIII:C concentrations. No changes in platelet function were detected. The introduction of an insulin regimen that improves glycaemic control might lead to a reversal of the "hypercoagulable state" found in type II diabetes.

INTRODUCTION

Abnormalities of blood coagulation and platelet function have been reported and suggest that diabetes mellitus is associated with a hypercoagulable state (1-4). It has been shown that by using a 24 hour insulin infusion, via the artificial pancreas, improvements in erythrocyte deformability and platelet function occur and that the changes in the red cell are due to a direct effect of insulin (5-7). Approximately 5% of type II diabetics each year, initially well controlled on sulphonylurea drugs, develop hyperglycaemia and require insulin to alleviate the symptoms of poorly controlled diabetes (8). In clinical practice these patients are usually given lifelong daily subcutaneous insulin injections. We wished to see if favourable changes in haemostasis could be produced by this route and method of insulin administration. The aims of our study were firstly to document the effect of insulin on some aspects of coagulation and platelet function and secondly to assess the contribution of glycaemic control to these tests in patients with secondary failure on sulphonylurea therapy.

METHODS

Twenty type II diabetic patients (15 female, 5 male) whose mean age was 62 years (range 51-72), duration of diabetes 7.5 years (1-26) and percentage of ideal body weight (Geigy scientific tables) of 103% (72-125) took part in the study. Despite high dose sulphonylurea administration (glibenclamide 15 mg.bd), the patients had constant hyperglycaemia and were therefore judged to have secondary failure of sulphonylurea therapy and to require insulin. Two patients were also taking the biguanide drug metformin. Five patients had evidence of microangiopathy (4 background and 1 proliferative retinopathy) without clinical features of nephropathy or neuropathy. Six patients were receiving antihypertensive or anti-anginal therapy but no other

medication was taken during the study period. On the morning of their first insulin injection and at 1 and 3 months thereafter a fasting venous blood sample was taken for glycosylated haemoglobin (Hb) and assessment of haemostasis.

Diabetic Assessment

All patients attended the clinic on a regular basis to be given the education appropriate to an insulin-requiring diabetic. Each patient was placed on Lentard MC (NOVO) highly purified beef/porcine lente insulin with the dosage adjusted thereafter until most urine tests before breakfast showed no glycosuria, a mid-morning clinic capillary glucose level of 10 mmol/l was attained or until hypoglycaemic symptoms had ensued. Blood for glycosylated Hb. assay was anticoagulated with EDTA and the assay performed by agar gel electrophoresis (Glytrac, Corning), the non-diabetic normal range being 5.5-8.5% in our laboratory. Residual beta cell function was assessed, in the fasting state, by measuring the C-peptide response to a 1 mg. injection of glucagon given intraveously. Serum C-peptide was determined by radioimmunoassay (RIA) using reagents supplied by NOVO Laboratories Ltd., both before and 6 minutes following glucagon injection.

Coagulation and Platelet Function Tests

Venous blood was anticoagulated with 0.13 M trisodium citrate (9:1, vol:vol) and plasma obtained for measurement of factor VIII coagulant activity (VIII:C) by a one stage method (9); factor VIII related antigen (VIIIR:Ag) and antithrombin III antigen (AT III antigen) by the Laurell method (10); AT III activity by chromogenic substrate assay ("Chromozym", Boehringer); fibrinopeptide A (FPA) and beta-thromboglobulin (B-TG) by RIA using reagents supplied by IMCO, Stockholm and the Radiochemical Centre,Amersham respectively; 6-oxo-$PGF_{1\alpha}$

was measured by RIA on unextracted plasma with antibody kindly donated by Dr.F.E. Preston (Sheffield) and thromboxane B_2 (TxB_2) by RIA with antibody provided by Upjohn (Kalamazoo).

Statistical analysis

Statistical comparisons were performed using the Wilcoxon matched-pairs signed rank test, and correlation was determined by the Spearman rank order correlation coefficient.

RESULTS

Diabetic Control

While institution of insulin therapy was associated with an improvement in well-being and reduction in glycosuria, no significant improvement in glycaemic control, as judged by glycosylated Hb levels, was noted. Prior to insulin therapy glycosylated Hb concentration was 13.2 ± S.D. 2.3% as compared with mean values of 13.1±2.1% and 12.3 ± 2.1% at 1 and 3 months respectively. No patient had a glycosylated Hb level within the normal range after 3 months of insulin administration.

Haemostatic tests

Samples for FPA were obtained at 0 and 3 months only and comparison samples were available for 13 patients. A 33% fall ($p < 0.05$) in FPA concentrations was noted at 3 months (table 1, fig. 1), but no significant change in any other haemostatic test was found (table 1). The correlations between glycosylated Hb concentrations, AT-III, C-peptide and the factor VIII complex are shown in table 2. No other significant correlations between glycaemic control and the haemostatic tests were found. With the patients on sulphonylurea therapy a positive correlation of AT III antigen and glycosylated Hb was noted with a negative trend of AT III activity with glycosylated Hb. At 3 months after introduction of insulin therapy no significiant associations were

noted, but reversals in the trends were found. Glycosylated Hb correlated positively both with VIII:C and C-peptide concentrations. Factor VIII:C also correlated significantly with C-peptide concentrations ($r=0.49$, $p<0.05$). During the study a modest correlation of AT III antigen and activity was found ($r=0.32$, $p<0.02$) with a stronger association of VIII:C and VIII R:Ag ($r = 0.58$, $p<0.001$). When glycosylated Hb, C-peptide and the haemostatic tests were compared in patients with and without microvascular disease, no significant differences were detected.

DISCUSSION

FPA is a small peptide cleaved from the N terminal end of the Aα chain of fibrinogen by thrombin, and is a sensitive indicator of thrombin generation (11) and therefore of activated coagulation. Normal plasma contains less than 2-3 pmol/ml of FPA and patients with type I diabetes have been shown to have slightly elevated levels compared to controls (12,13). The baseline FPA concentrations in our type II diabetic patients were elevated indicating increased thrombin generation. More importantly, the introduction of insulin therapy was associated with a significant reduction of FPA concentrations. The diminished thrombin generation could not be explained by improved glycaemic control since glycosylated Hb levels were unchanged. A direct effect of insulin on the coagulation system is possible since there is evidence that insulin has direct effects at other sites such as the endothelial cell (14-16) and the erythrocyte (5,6).

AT III is the major physiological inhibitor of thrombin and a number of activated coagulation enzymes and, in diabetes, levels of this coagulation inhibitor have varied (1,4,12,13,17-26). This may be related to differences in the functional assay methods, discrepancies between the functional and immunological assays which these studies have

employed, and due to a failure to distinguish between insulin-dependent (type I), insulin-treated and non-insulin-dependent (type II) diabetic patients. Some studies in type II diabetes have shown a reduction in functional activity (4,17,26) with normal immunological levels of AT III (26).

Although the concentrations of AT III activity were not significantly lower than AT III antigen in our study, the modest correlation coefficient between activity and antigen (r= 0.32), during the study is lower than expected (24) and indicates a discrepancy between the functional and immunological concentrations of AT III. This discrepancy could be explained by a structurally modified protein which retains immunoreactivity but is functionally depressed (3). There is in-vitro evidence that glycosylation of this plasma protein leads to such a defect (27), while studies on the association betwen glycaemic control and AT III have been conflicting (12,21,24,25,28-30). On the baseline sample we have shown a positive correlation of glycosylated Hb with AT III antigen and a negative trend with AT III activity; after 3 months of insulin treatment, reversal of these associations were found. These correlations noted provide further evidence that glycaemic control is a determinant of AT III concentration and function, and that insulin therapy may help improve the function of ATIII. The small, non-significant, reduction in glycosylated Hb concentrations may have lead to this alteration.

High levels of factor VIII:C may be an indicator of a hypercoagulable state (2) and have an association, in an epidemiological study, with an increased risk of subsequent cardiovascular death (31). Poor glycaemic control in type I diabetes, as assessed by HbA_1 and urinary sugar excretion, has been associated with raised factor VIII:C and VIIIR:Ag concentations (32,33) and we have confirmed a positive

association of glycaemic control and factor VIII:C in the type II diabetic patients in our study. The measurement of C-peptide allows an accurate assessemnt of endogenous insulin concentrations since C-peptide is secreted from the β-cell of the pancreas in equimolar amounts with insulin. To our knowledge, no studies have recorded the effects of insulin on the factor VIII complex. We have shown a significant correlation of the basal, fasting C-peptide with factor VIII:C concentrations which could indicate a role for insulin in the determination of circulating factor VIII:C concentrations. Alternatively, this association may reflect the underlying positive association of C-peptide with glycaemic control and this requires further study.

Improvements of coagulation and platelet function have been noted in type II diabetes with improved glycaemic control due to a high fibre diet (34), sulphonylurea therapy (4,35) and insulin therapy in patients with mild carbohydrate intolerance without glycosuria (22). In our patients with more severe carbohydrate intolerance no significant change in glycaemic control was noted following the introduction of a once daily insulin regimen and it is not surprising therefore that no alterations in the concentrations of factor VIII, AT III or the platelet assays were noted.

In summary, we have shown relationships betwen glycaemic control and factor VIII:C and AT III, which suggest that improved glycaemic control would lead to an improvement of AT III function and lower factor VIII:C concentrations. Along with the lowering of fibrinopeptide A levels demonstrated in this study, these changes in the factor VIII and AT III complex indicate that a reversal of the hypercoagulable state in type II diabetes could be achieved by an insulin regimen that improved glycaemic control.

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TABLE I

Haemostatic tests before and after insulin administration

	TIME (Months)		
	0	1	3
FPA (pmol/ml)	4.9 ± 2.5	-	3.3* ± 1.9
AT III antigen (%)	96 ± 14	103 ± 12	95 ± 15
AT III activity (%)	89 ± 17	91 ± 20	94 ± 16
VIII:C (IU/dl)	180 ± 83	197 ± 109	200 ± 98
VIIIR:Ag (IU/dl)	180 ± 69	203 ± 72	201 ± 69
β-TG (ng/ml)	41 ± 14	44 ± 26	50 ± 34
TxB_2 (pg/ml)	115 ± 39	95 ± 30	100 ± 33
$PGF_1\alpha$ (pg/ml)	17 ± 9	17 ± 9	19 ± 7

Values are mean ± ISD
* $p < 0.05$

TABLE II

Correlation of glycaemic control with AT III, factor VIII and C-peptide

	Glycosylated Hb. TIME (Months)		
	0	3	0+1+3
AT III antigen	0.47*	- 0.25	0.24
AT III activity	- 0.31	0.38	0.09
AT III antigen/activity	0.63**	- 0.33	0.10
VIII: C	0.28	0.73***	0.46***
VIIIR:Ag	- 0.04	0.41	0.15
C-peptide	0.48*	-	-

* $p < 0.05$
** $p < 0.01$
*** $p < 0.005$

reduction of the luminal diameter. To give a more accurate reflection of the degree of coronary atherosclerosis a coronary score system was devised. As no uniform method of 'scoring' coronary angiograms is available, we devised a system which divided the 3 major vessels into 9 sections to include the left main stem, proximal left anterior descending (LAD), mid LAD, distal LAD, first diagonal, proximal circumflex, distal circumflex, proximal right coronary argery (RCA) and distal RCA. Each section was scored from 0-4 points depending on the degree of vessel occlusion in each section:- no occlusion = 0; < 50% = 1; 50-74% = 2; 75-99% = 3; 100% = 4. The patients score over the total possible (9 x 4 = 36) was expressed as a ratio and called the 'coronary index'. The angiograms were reviewed blindly at the end of the study by a single cardiologist.

6.2.3 Blood Tests

Prior to coronary angiography, and after a 12 hour fast, a resting venous blood sample was obtained between 8-9am for a variety of tests as listed below. Plasma fibrinolytic activity, BBeta 15-42 antigen, plasminogen activity, FDP's, alpha2-antiplasmin antigen, alpha2-macroglobulin antigen, fibrinogen and fibrinopeptide A were assayed as described in chapter 3. The results of the inhibitors of fibrinolysis, alpha2-antiplasmin and alpha2-macroglobulin are expressed as the percentage of normal pool plasma. Plasma total cholesterol, HDL cholesterol and triglyceride were

assess any inter-relationships between these tests and to analyse the effects of a number of clinical factors (smoking, beta-blockade, myocardial infarction, peripheral arterial disease, body weight) on the components of fibrinolysis in a large group of men with proven coronary artery disease.

6.2 METHODS

6.2.1 Patients

We studied 100 consecutive white males, admitted for elective coronary angiography. Patients were excluded if they had sustained a myocardial infarct within 3 months of coronary investigation , or if they had valvular heart disease, as the above factors are known to affect some of the haemostatic and lipid variables measured. None of the patients had diabetes mellitus or chronic liver disease and patients had normal secondary sex characteristics. As an estimate of obesity, the body mass index was used. A history of smoking, beta-blocker administration, peripheral arterial disease and previous myocardial infarction were recorded. Peripheral arterial disease was diagnosed on a history of claudication plus absent peripheral pulses.

6.2.2 Study Design

Coronary angiography was performed by the Judkins technique (Judkins, 1967). Coronary artery disease was scored as the number of major vessels having at least 50%

measured by the Technicon SMA II and Boehringer kits (Carlson, 1973).

Oestradiol was measured in ether extracts of serum using a sensitive radioimmunoassay (RIA) based on a Steranti rabbit antiserum raised against oestradiol-6-(carboxymethyl) oxime-BSA, oestradiol-6-(0-carboxymethyl) oximino-2- (125I) iodohistamine and a double antibody separation system. This method is capable of a sensitivity of better than 20 pmol/l and yields a male reference range of 20-300 pmol/l with mean intra and inter-assay coefficients of variation of 8% and 12% respectively. Serum testosterone was measured in ether extracts of serum using a double antibody RIA based on a rabbit anti-testosterone-3- 0- carboxymethyl oxime- BSA serum and a 125I radioligand prepared from the histamine derivative of testosterone - 3- 0- carboxymethyl oxime. Oestradiol and the haemostatic samples were stored at -70° C and assayed in batches.

6.2.4 Statistics

Differences in means were analysed by Mann-Whitney U test and correlations were determined by the Spearman rank order correlation coefficient. Nominal data was analysed using Fisher's exact probability test.

6.3 RESULTS

6.3.1 Clinical Characteristics

Table 6.1 shows some of the clinical characteristics of the groups (number, age, body mass index, smoking, use of beta-blockers, prevalence of myocardial infarction, hypertension and peripheral arterial disease), in relation to the extent of coronary artery disease. No significant differences between the groups were found (Fishers test).

6.3.2 Severity of Coronary Artery Disease

Tables 6.2 and 6.3 list the concentations of the fibrinolysis, hormone and lipid tests in relation to the extent of coronary artery disease. No significant differences in the tests were seen in patients with 1, 2, or 3 vessel coronary artery disease although a trend for higher fibrinogen concentrations with increasing severity of coronary artery disease was noted. When the coronary index values were correlated with these variables, positive associations were noted between the coronary index and both age ($r = 0.35$, $p < 0.001$), and fibrinogen ($r = 0.21$, $p < 0.05$.) No correlation of age and fibrinogen was found ($r = 0.07$) and no other significant associations with the coronary index were noted.

6.3.3 Myocardial Infarction

Oestradiol concentrations were higher in men with a previous myocardial infarction in comparison to those with coronary artery disease but no previous myocardial

TABLE 6.1

PATIENT GROUP CLINICAL CHARACTERISTICS

	CORONARY VESSEL INVOLVEMENT		
	1	2	3
Number	22	25	53
Age (years)	49 ± 9	46 ± 9	51 ± 8
Body Mass Index	26 ± 2	26 ± 4	27 ± 2
Smokers	10	9	25
Beta-blocker treated	14	14	37
Myocardial Infarction	11	16	29
Peripheral Arterial Disease	3	3	7

Legend

Clinical characteristics in relation to the severity of coronary artery disease. Values are mean ± SD.

TABLE 6.2

FIBRINOLYSIS AND THE SEVERITY OF CAD

	CORONARY VESSEL INVOLVEMENT		
	1	2	3
Coronary Index	.076 ± .011	.236 ± .020	.397 ±.018
BBETA 15-42 (pmol/ml)	1.17 ± 0.55	1.33 ± 0.46	1.22 ±0.43
Fibrinolytic Activity (mm2)	79 ± 21	79 ± 22	78 ± 23
Plasminogen (cu/ml)	4.8 ± 0.8	4.7 ± 0.7	4.8 ±0.6
α 2-Antiplasmin (%)	100 ± 12	99 ± 17	103 ± 14
α 2-Macroglobulin (%)	81 ± 17	89 ± 29	83 ± 29
F.D.P.'s (ug/ml)	5.4 ± 4.5	4.4 ± 2.6	6.5 ±4.4
Fibrinogen (g/l)	3.19 ± 0.68	3.28 ± 0.69	3.40 ±0.77
Fibrinopeptide A (pmol/ml)	2.00 ± 1.07	2.85 ± 1.97	2.69 ±1.80

Legend

Components of fibrinolysis in relation to the severity of coronary artery disease (CAD), as indicated by the number of vessel involvement and the coronary index. Values are mean ± SD.

TABLE 6.3

HORMONES, LIPIDS AND THE SEVERITY OF CAD

	CORONARY VESSEL INVOLVEMENT		
	1	2	3
Oestradiol (pmol/l)	111 ± 48	127 ± 53	112 ± 56
Testosterone (nmol/l)	19.7 ± 7.2	20.0 ± 7.5	18.3 ± 5.4
Cholesterol (mmol/l)	6.3 ± 0.9	6.5 ± 1.6	6.7 ± 1.1
Triglyceride (mmol/l)	2.00 ± 1.00	2.94 ± 1.90	2.54 ± 1.50
HDL (mmol/l)	1.38 ± 0.27	1.26 ± 0.28	1.29 ± 0.26

Legend

Concentrations of hormones and lipids in relation to the severity of coronary artery disease (CAD). HDL = High Density Lipoprotein. Values are mean ± SD.

infarction (mean ± SD: 126 ± 39 versus 101 ± 62 pmol/l, $p < 0.01$), but no other differences in testosterone, lipids or the fibrinolysis tests (table 6.4) were noted.

6.3.4 Age

To assess whether abnormalities of fibrinolysis occurred in younger men, we took men under 50 years of age ($n = 47$), and found no differences in the tests of fibrinolysis in relation to the severity of coronary artery disease. Younger men with a previous myocardial infarction ($n = 28$) had higher plasma fibrinolytic activity than men without a previous myocardial infarction (83 ± 23 vs 69 ± 20 mm2, $p < 0.05$), but no other differences were noted. No correlation between age and plasma fibrinolytic activity ($r = 0.04$) or between age and the other fibrinolytic tests were noted.

6.3.5 Peripheral Arterial Disease

Peripheral arterial disease was detected in 13 patients (previous angiography confirmed the diagnosis in 3 patients). Significant elevations in plasma fibrinolytic activity, and trends to higher BBeta 15-42, fibrinogen and fibrinopeptide A concentrations were noted in this patient group compared to patients with coronary artery disease but without peripheral arterial disease (table 6.5).

6.3.6 Smoking and Beta-Blockade

No differences in the fibrinolysis tests were

TABLE 6.4

FIBRINOLYSIS AND MYOCARDIAL INFARCTION

	CORONARY ARTERY DISEASE	
	With MI	Without MI
Number	56	44
BBETA 15-42 (pmol/ml)	1.24 ± 0.45	1.23 ± 0.48
Fibrinolytic Activity (mm2)	80 ± 21	76 ± 23
Plasminogen (cu/ml)	4.8 ± 0.6	4.8 ± 0.7
∝ 2-Antiplasmin (%)	99 ± 13	104 ± 16
∝ 2-Macroglobulin (%)	86 ± 27	82 ± 27
F.D.P.'s (ug/ml)	4.9 ± 3.6	5.0 ± 4.2
Fibrinogen (g/l)	3.31 ± 0.78	3.33 ± 0.65
Fibrinopeptide A (pmol/ml)	2.53 ± 1.71	2.63 ± 1.76

Legend

Components of fibrinolysis in patients with coronary artery disease with and without a previous myocardial infarction (MI). Values are mean ± SD.

TABLE 6.5

FIBRINOLYSIS AND PERIPHERAL ARTERIAL DISEASE

	PERIPHERAL ARTERIAL DISEASE	
	YES	NO
Number	13	87
BBETA 15-42 (pmol/ml)	1.41 ± 0.54	1.21 ± 0.45
Fibrinolytic Activity (mm2)	97* ± 30	75 ± 19
Plasminogen (cu/ml)	4.3 ± 1.4	4.8 ± 0.7
α 2-Antiplasmin (%)	99 ± 15	101 ± 14
α 2-Macroglobulin (%)	82 ± 30	84 ± 21
F.D.P.'s (ug/ml)	4.7 ± 4.5	5.2 ± 2.9
Fibrinogen (g/l)	3.68 ± 0.93	3.25 ± 0.69
Fibrinopeptide A (pmol/ml)	2.90 ± 2.20	2.44 ± 1.20

Legend

Components of fibrinolysis in men with coronary artery disease with or without peripheral arterial disease. Values are mean ± SD.

*$p < 0.01$

detected in smokers compared to non-smokers (table 6.6), or in patients with and without beta-blocker administration (table 6.7). Trends for smokers to have higher fibrinogen and lower fibrinolytic activity were noted.

6.3.7 Lipids

To examine the effects of hyperlipidaemia, comparisons were made between the concentrations of the fibrinolytic tests in patients with severe hypertriglyceridaemia (plasma triglyceride > 3mmol/l) and hypercholesterolaemia (plasma cholesterol > 7 mmol/l) and patients with lower levels (tables 6.8 and 6.9). Patients with severe hypertriglyceridaemia had significantly higher concentrations of alpha2-antiplasmin but no other differences were noted. No associations between the fibrinolysis and lipid tests were found except for a correlation of alpha2-antiplasmin antigen ($r = 0.31$, $p < 0.005$), and alpha2-macroglobulin antigen ($r = -0.25$, $p < 0.05$) with the plasma triglyceride concentration.

6.3.8 Hormones

No significant associations were noted between the hormonal tests (oestradiol, testosterone) and the lipid and fibrinolysis tests

6.3.9 Correlations between the tests of fibrinolysis

Significant correlations of plasminogen ($r = -0.23$), and FDP´s ($r = 0.38$) with the BBeta 15-42 fragment were

TABLE 6.6

FIBRINOLYSIS AND SMOKING

	SMOKERS	NON-SMOKERS
Number	44	56
BBETA 15-42 (pmol/ml)	1.25 ± 0.50	1.23 ± 0.44
Fibrinolytic Activity (mm2)	75 ± 24	81 ± 20
Plasminogen (cu/ml)	4.7 ± 0.8	4.8 ± 0.6
α 2-Antiplasmin (%)	99 ± 13	103 ± 15
α 2-Macroglobulin (%)	85 ± 30	83 ± 244
F.D.P.'s (ug/ml)	5.7 ± 5.2	4.5 ± 2.5
Fibrinogen (g/l)	3.41 ± 0.69	3.22 ± 0.76
Fibrinopeptide A (pmol/ml)	2.78 ± 1.88	2.43 ± 1.62

Legend

Components of fibrinolysis in relation to smoking.
Values are mean ± SD.

TABLE 6.7

FIBRINOLYSIS AND BETA-BLOCKADE

	BETA-BLOCKADE	
	YES	NO
Number	65	35
BBETA 15-42 (pmol/ml)	1.20 ± 0.46	1.30 ± 0.47
Fibrinolytic Activity (mm2)	77 ± 23	81 ± 20
Plasminogen (cu/ml)	4.7 ± 0.8	4.8 ± 0.6
α2-Antiplasmin (%)	97 ± 18	104 ± 14
α2-Macroglobulin (%)	84 ± 31	82 ± 24
F.D.P.'s (ug/ml)	5.0 ± 4.6	4.7 ± 3.1
Fibrinogen (g/l)	3.31 ± 0.72	3.30 ± 0.78
Fibrinopeptide A (pmol/ml)	2.71 ± 1.84	2.35 ± 1.50

Legend

Components of fibrinolysis in patients with and without beta-adrenoceptor blocker administration.
Values are mean ± SD.

TABLE 6.8

FIBRINOLYSIS AND HYPERTRIGLYCERIDAEMIA

	PLASMA TRIGLYCERIDE	
	>3 mmol/l	⩽3 mmol/l
Number	24	73
Triglyceride (mmol/l)	4.5 ± 1.7	1.8 ± 0.6
BBETA 15-42 (pmol/ml)	1.27 ± 0.44	1.22 ± 0.47
Fibrinolytic Activity (mm2)	80 ± 27	76 ± 34
Plasminogen (cu/ml)	4.8 ± 0.8	4.8 ± 0.7
∝ 2-Antiplasmin (%)	107* ± 12	99 ± 15
∝ 2-Macroglobulin (%)	78 ± 24	86 ± 27
F.D.P.'s (ug/ml)	4.8 ± 4.3	5.1 ± 3.7
Fibrinogen (g/l)	3.27 ± 0.73	3.31 ± 1.00
Fibrinopeptide A (pmol/ml)	3.16 ± 2.15	2.48 ± 1.56

Legend

Components of fibrinolysis in relation to plasma triglyceride concentrations. Values are mean ± SD.

*p < 0.01

TABLE 6.9

FIBRINOLYSIS AND HYPERCHOLESTEROLAEMIA

	PLASMA CHOLESTEROL	
	> 7 mmol/l	⩽ 7 mmol/l
Number	32	66
Cholesterol (mmol/l)	7.9 ± 1.0	5.9 ± 0.6
BBETA 15-42 (pmol/ml)	1.21 ± 0.39	1.24 ± 0.50
Fibrinolytic Activity (mm2)	82 ± 25	75 ± 30
Plasminogen (cu/ml)	4.8 ± 0.8	4.7 ± 0.7
∝ 2-Antiplasmin (%)	103 ± 17	100 ± 15
∝ 2-Macroglobulin (%)	86 ± 27	84 ± 27
F.D.P.'s (ug/ml)	5.7 ± 5.3	4.6 ± 2.8
Fibrinogen (g/l)	3.32 ± 0.79	3.30 ± 0.90
Fibrinopeptide A (pmol/ml)	2.65 ± 1.96	2.56 ± 1.64

Legend

Components of fibrinolysis in relation to plasma cholesterol concentrations. Values are mean ± SD.

noted (table 6.10) but no other significant correlations between the tests of fibrinolysis were detected.

6.3.10 Body Mass Index

Correlations of triglyceride ($r = 0.36$, $p < 0.001$), cholesterol ($r = 0.28$, $p < 0.01$), testosterone ($r = -0.20$, $p < 0.05$), and plasminogen ($r = 0.20$, $p < 0.05$) with body mass index were noted. No other correlations with the fibrinolytic tests were noted, although a negative trend with BBeta 15-42 was seen ($r = -0.18$).

6.4 DISCUSSION

6.4.1 Severity of Coronary Artery Disease

We have been unable to demonstrate any difference in plasma fibrinolytic activity in relation to the severity of coronary artery disease (Small et al., 1984b), findings which have subsequently been confirmed (Paramo et al., 1985). We have, in addition, been unable to relate the severity of coronary artery disease to in vivo fibrinolysis, as indicated by the BBeta 15-42 fragment, or to the other major components of the fibrinolytic enzyme system - findings which have not previously been reported. Our study therefore fails to provide any support for the association of basal fibrinolysis with established coronary artery disease. We did not study fibrinolytic capacity which may be impaired in coronary artery disease (Khanna et al., 1975; Walker et al., 1977;

TABLE 6.10

CORRELATION OF BBETA 15-42 WITH THE TESTS OF FIBRINOLYSIS

	BBeta 15-42 antigen (pmol/ml)	
	'r'	'p'
Fibrinolytic Activity (mm2)	0.12	NS
Plasminogen (cu/ml)	-0.23	0.05
α 2-Antiplasmin (%)	0.13	NS
α 2-Macroglobulin (%)	0.04	NS
F.D.P.'s (ug/ml)	0.38	0.001
Fibrinogen (g/l)	0.14	NS
Fibrinopeptide A (pmol/ml)	0.03	NS

Legend

Spearman correlation (r) values of in vivo fibrinolysis (BBeta 15-42) with the other components of fibrinolysis

O´Connor et al., 1984a; Estelles et al., 1985) possibly due to increased levels of plasminogen activator inhibitor (Estelles et al., 1985; Paramo et al., 1985). In a more recent epidemiological study of 100 men with angina (detected by a health screening questionnaire) and 400 matched controls (Lowe et al., 1985), no relationship of angina to BBeta 15-42, alpha2-antiplasmin or alpha2-macroglobulin was observed. This is consistent with the negative findings of these 3 variables in the present, angiographic study.

For many years there has been great argument on the relative importance of the lipid and thrombogenic hypothesis for atheroma and it is probable that both play an important role in the aetiology of atheroma (Meade, 1981; Smith, 1981). Cholesterol is an established risk factor for coronary artery disease and there is also a strong inverse association between cardiovascular mortality and HDL cholesterol (Yaari et al., 1981). Angiographic studies have shown a relation of coronary artery disease with all lipid and lipoprotein concentrations (Jenkins, Harper and Nestel, 1978; Breier et al., 1985) or with only HDL cholesterol (Miller et al., 1981), but we have been unable to confirm these associations (Small et al., 1985b). Different patient populations, coronary score systems, assay and statistical analysis methods may explain these discrepant findings. We have noted however a modest, but significant correlation of fibrinogen with the severity of coronary artery disease which we previously observed in a smaller

study (Lowe et al., 1980). This is consistent with the post mortem study showing high fibrinogen levels in arterial intima and mural thrombi (Smith and Staples, 1981) and adds further support to the recent evidence showing the important association of fibrinogen with ischaemic heart disease (Meade et al., 1980; Baker et al., 1982; Wilhelmsen et al., 1984; Sugrue et al., 1985). Increasing age is probably the most powerful predictor of the risk of ischaemic heart disease (Meade, 1981) and it is not surprising therefore that we have noted a significant correlation of age with the coronary index. This association supports the use of the coronary index that we employed. No correlation of age and fibrinogen was noted ($r = 0.07$) in our study and therefore the association of both age and fibrinogen with the coronary index cannot be explained by an underlying association between age and fibrinogen in the present study. It should be noted however that fibrinogen does increase with increasing age in epidemiological studies over a wider age range (Meade et al., 1977).

6.4.2 Hormonal Associations

We have found no association between oestradiol or testosterone levels and the severity of coronary artery disease or the tests of fibrinolysis (Small et al., 1985a). The postulated mechanisms whereby raised oestradiol levels exert their harmful effect does not therefore appear to be related to either accelerated atherogenesis (Philips, 1978), or defective fibrinolysis

(Andersen, Norman and Hjermann, 1983b). We have however noted elevated oestradiol levels in men with a previous myocardial infarction compared to men without a previous myocardial infarction but with coronary artery disease. This is in keeping with the view that elevated oestradiol concentrations may be a risk factor for ischaemic heart disease (Philips et al., 1983).

6.4.3 Myocardial Infarction/Age

Apart from oestradiol levels no differences in any of the blood tests were noted for patients with or without a previous myocardial infarction. In previous controlled studies younger patients were more commonly associated with defective fibrinolysis (Chakrabarti et al., 1968; Walker et al., 1977; O´Connor et al., 1984a), and we therefore analysed patients with and without a previous myocardial infarction under 50 years of age. These younger patients had increased fasting plasma fibrinolytic activity. Patients with peripheral arterial disease also had higher plasma fibrinolytic activity and non-fasting fibrinolytic activity was higher in men with a previous myocardial infarction in our study on exercise and fibrinolysis (Chapter 5.3). These findings remain unexplained, but possibly a greater vascular endothelial damage, associated with these patients groups, could lead to a higher plasma fibrinolytic activity. Some caution must be placed on the results of the patients with peripheral arterial disease since a diagnosis of this condition made on history and examination may wrongly

classify patients in between 20-50% of cases (Marinelli, Beach and Glass, 1979; Janka, Standl and Mehnert, 1980).

6.4.4 Lipids

The relationship between hypertriglyceridaemia and depressed fibrinolytic activity has previously been discussed (Chapter 2.5) but we have been unable to confirm this association in the present study. We have however noticed significant and different associations between plasma triglyceride levels and the inhibitors of fibrinolysis, alpha2-antiplasmin antigen (positive association) and alpha2-macroglobulin antigen (negative association). Since alpha2-antiplasmin is the major fibrinolytic inhibitor it is likely that this protein would have the most important in vivo effect. An increase in alpha2-antiplasmin could promote fibrin deposition in several ways. Alpha2-antiplasmin inhibits plasminogen activator, is the major inhibitor of plasmin, inhibits the absorption of plasminogen to fibrin, and cross-links to fibrin rendering it less susceptible to plasmin (Collen, 1980; Aoki et al., 1981). When the patients were subdivided into groups with and without severe hypertriglyceridaemia the only difference between the groups was of elevated alpha2-antiplasmin levels in patients with hypertriglyceridaemia. Similar findings have been reported in patients with type II hyperlipoproteinaemia (Lowe et al., 1982), while an association of alpha2-antiplasmin with triglyceride levels has been noted in a recent epidemiological study (Lowe et

al., 1985). A previous epidemiological study failed to find such an association (Scarabin, Bara and Jacqueson, 1983). Recently, increased triglyceride levels have been associated with increased t-PA inhibitor levels (Brommer et al., 1984a). Hypertriglyceridaemia therefore is associated with a raised level of fibrinolytic inhibitors acting at distinct sites against plasmin and t-PA. The negative association between alpha2-macroglobulin and triglyceride concentrations could be a homeostatic compensatory mechanism to partially offset the raised alpha2-antiplasmin concentrations, but this requires further study. While defective fibrinolysis may be a feature of hypertriglyceridaemia there appears to be little evidence to suggest a causal association between triglyceride levels and ischaemic heart disease (Hulley et al., 1980). The role of fibrinolysis in coronary artery disease is given no support from this observation.

6.4.5 Inter-relationships of the Fibrinolysis Tests

BBeta 15-42 fragment is a new, sensitive indicator of in vivo plasmin-mediated fibrinolysis and its relation to the other components of fibrinolysis has not been evaluated. Since synthesis of various components of fibrinolysis may increase during a phase of increased fibrinolytic activity, the measurement of plasminogen and fibrinogen may, in addition, not be consistent with BBeta 15-42 levels due to these compensatory phenomena (Walenga et al., 1984). We have found that BBeta 15-42 levels show significant correlations with plasminogen (negative) and

FDP's (positive). This is consistent with the scheme: increased activation of plasminogen reduces plasminogen levels and increases plasmin digestion of fibrin (ogen), ie, BBeta 15-42 and FDP fragments. We have also found a correlation of BBeta 15-42 and FDP in stroke patients (Douglas et al., 1986). The lack of correlation between euglobulin fibrinolytic activity (ex vivo fibrinolysis) and BBeta 15-42 (in vivo fibrinolysis) indicates that these assays measure different aspects of fibrinolysis and casts doubt as to the validity of using euglobulin fibrinolytic activity as an overall assessment of fibrinolysis. We have previously described a dissociation between the BBeta 15-42 fragment and plasma fibrinolytic activity in normal volunteers given stanozolol (Small et al., 1983a; Small et al., 1983b) and in patients following major surgery (Douglas et al., 1985). The lack of correlation between euglobulin fibrinolytic activity and t-PA antigen in patients with coronary artery disease has also led others to question the value of euglobulin fibrinolytic activity as an in vivo assessment of fibrinolysis (Paramo et al., 1985).

6.4.6 Smoking

Population studies of over 700 men (Korsan-Bengtsen et al., 1972) showed no difference in fibrinolytic activity in smokers compared to non-smokers, while a slight decrease in fibrinolytic activity was noted in smokers in a population study of over 1600 men (Meade et al., 1979). While a trend towards reduced fibrinolytic

activity in smokers was seen, it is not surprising that smokers showed no significant difference in fibrinolytic activity, or the tests of fibrinolysis, in view of the smaller numbers in our study. Healthy males who are heavy smokers have defective fibrinolytic activity compared to healthy non-smoking males (Allen et al., 1983) and it is clear that to demonstrate an effect of smoking on fibrinolysis requires either large numbers or a highly selected group of subjects.

6.4.7 Body Weight

Epidemiological studies have shown a relationship between a high body mass index and coronary heart disease mortality (Jarrett, Shipley and Rose, 1982; Rhoads and Kagan, 1983). The expected correlations of body mass index and lipid levels were detected, but no correlations of obesity with fibrinolytic activity was found although a negative trend with in vivo fibrinolysis (BBeta 15-42) was seen. This association was significant in a recent epidemiological study (Lowe et al., 1985). The body mass index ranged from 20.5 to 32.4 with 90% of values between 23-28. It is possible that the narrow range of body mass index values could explain the lack of correlation with the fibrinolytic activity.

6.4.8 Beta-Blockade

While the resting level of plasma fibrinolytic activity has been reported to be altered by beta-blockade (Chapter 4.4), the present study, involving a larger group

of patients, has shown no alteration of fibrinolytic activity or the components of fibrinolysis by beta-blockade. Beta-blockade does not therefore influence basal fibrinolysis.

6.4.9 Summary and Conclusions

We have been unable to relate the tests of fibrinolysis to the severity of coronary artery disease and can therefore offer no support for an association between these two variables. Fibrinogen showed a closer association with the severity of coronary artery disease and it is possible that coagulation and not fibrinolysis is the more important haemostatic system with respect to coronary artery disease. The negative findings of this study may reflect the homogenous patient population, but the advantage of such a group is that the large patient numbers allow associations of the biochemical and haemostatic tests with the clinical characteristics of the group to be studied. Thus important observations on the severity of coronary artery disease, fibrinolysis, oestradiol and lipids have been reported (Small et al., 1985a; Small et al., 1985b) and the effect of lipid levels on the inhibitors of fibrinolysis have been recorded.

CHAPTER 7

THE EFFECTS OF INTRAMUSCULAR STANOZOLOL ON FIBRINOLYSIS IN HEALTHY MALE SUBJECTS

7.1 INTRODUCTION

The principle physiological stimulus to fibrinolysis, exercise, has already been discussed and the following chapters will concentrate on pharmacological potentiation of the fibrinolytic enzyme system by the anabolic steroid stanozolol.

The previous studies using stanozolol (17-beta-hydroxy-17-alpha-methylandrostano (3.2,-c) pyrazole) have been discussed more fully in Chapter 2.8. It was well recognised that stanozolol could enhance fibrinolysis in various forms of vascular disease when given orally (Davidson et al., 1972; Cunliffe et al., 1975; Jarrett et al., 1978; Burnand et al., 1980) but a greater interest in this drug developed following the demonstration that oral administration of stanozolol to normal subjects could lead to a rapid enhancement of fibrinolysis (Preston et al., 1981). It was felt that if stanozolol could improve fibrinolysis in normal subjects then it would have an even greater potential to improve fibrinolysis in patients with vascular disease and defective fibrinolysis. An intramuscular depot preparation of stanozolol was available whose effects on fibrinolysis had not been studied. This form of administration could offer advantages in a number of clinical settings, such as the non-compliant and/or ill patients unable to take oral medication. In particular we wished to see if this preparation could cause short-term potentiation of the fibrinolytic system which might then be tested as a prophylactic agent to prevent spontaneous or post-operative deep vein thrombosis, as abnormal fibrinolysis has been

reported in both these conditions (Chapter 2.2).

The aim of the present study was therefore to assess the onset, duration and degree of change induced in the fibrinolytic enzyme system by a single intramuscular injection of stanozolol.

7.2 METHODS

7.2.1 Patients

Local Ethical Committee permission was obtained and 12 healthy male subjects (age range 22-45, mean 30.8 years) with no history of jaundice or liver disease took part in the study.

7.2.2 Study Design

The volunteers fasted overnight and after 15 minutes at rest, blood was withdrawn, without compression by a tourniquet, for estimation of baseline fibrinolysis tests. The volunteers were then given a 1 ml (50mg) intramuscular injection of stanozolol and repeat fasting samples taken at 1, 2, 7, 14, 21, 28 and 56 days following the injection. All sampling was carried out between 9-10am and the subjects were requested to abstain from alcohol during the 24 hours prior to each test day.

7.2.3 Blood Tests

Plasma fibrinolytic activity, plasminogen activity, FDP's, alpha2-antiplasmin activity, alpha2-macroglobulin antigen and fibrinogen were measured as previously described

(Chapter 3). The results of the inhibitors of fibrinolysis are expressed as a percentage of normal pool plasma.

7.2.4 Statistics

Statistical comparisons were performed using the paired Wilcoxon rank sum test.

7.3 RESULTS

Plasma fibrinolytic activity increased significantly ($p < 0.05$) 24 hours following the injection and remained elevated for 7 days (figure 7.1). Plasminogen concentrations also increased above baseline values by day 2 and remained significantly elevated for 3 weeks following the injection (figure 7.2). No alteration in the other tests of fibrinolysis were noted (table 7.1).

7.4 DISCUSSION

The increases noted in plasma fibrinolytic activity and plasminogen in our normal volunteers are consistent with the findings of Preston and colleagues (1981) using oral stanozolol. Given via the intramuscular route this enhancement of fibrinolysis occurs earlier than any previous reports - the percentage increase from baseline being about 30% for one week and 50% over the subsequent 3 weeks. We were unable to confirm a fall in fibrinogen or

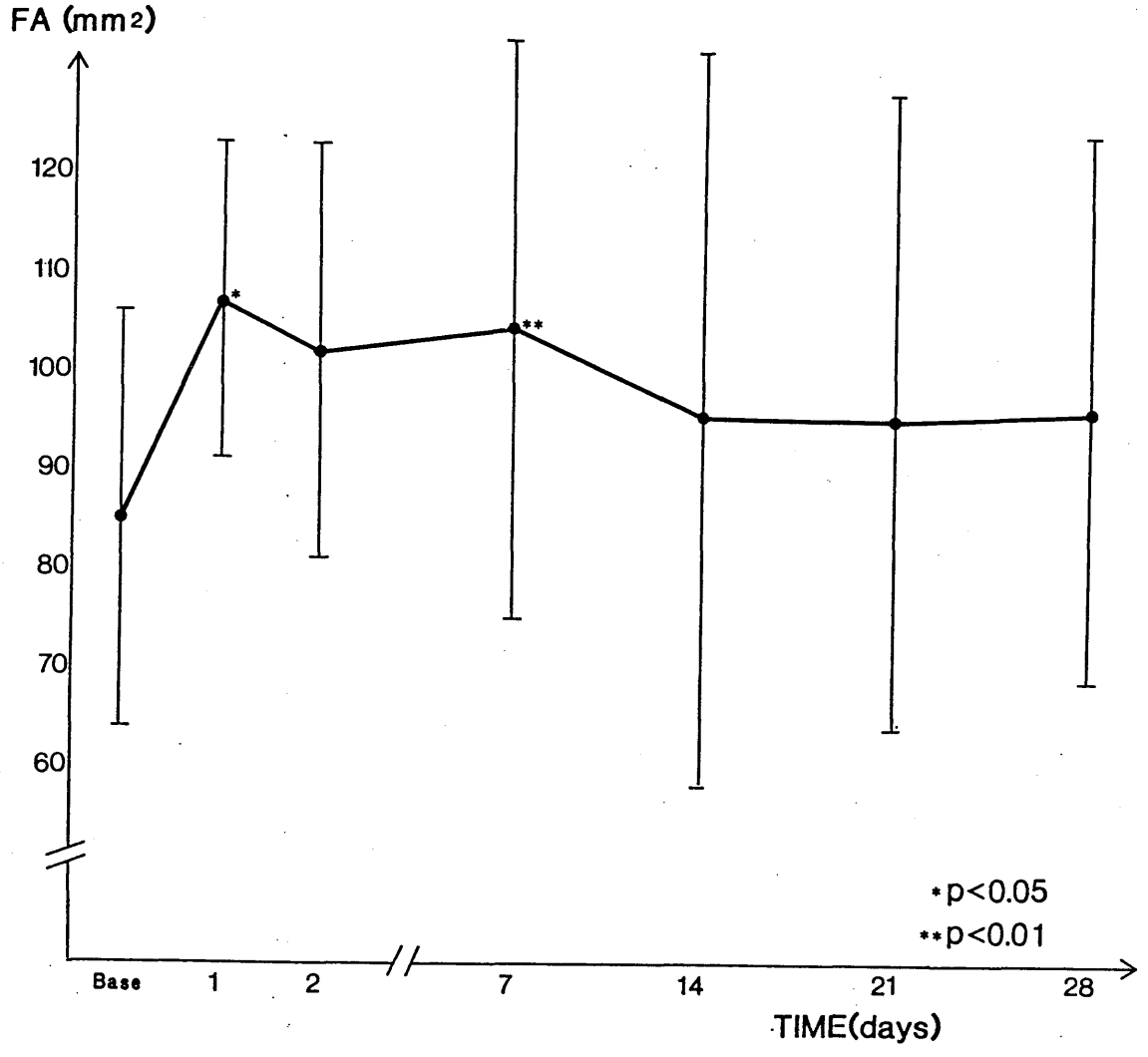

Figure 7.1

Illustrates the concentrations (mean ± SD) of fasting plasma fibrinolytic activity (FA) following the intramuscular injection (50 mg) of stanozolol in healthy male subjects. Changes are compared to basal values.

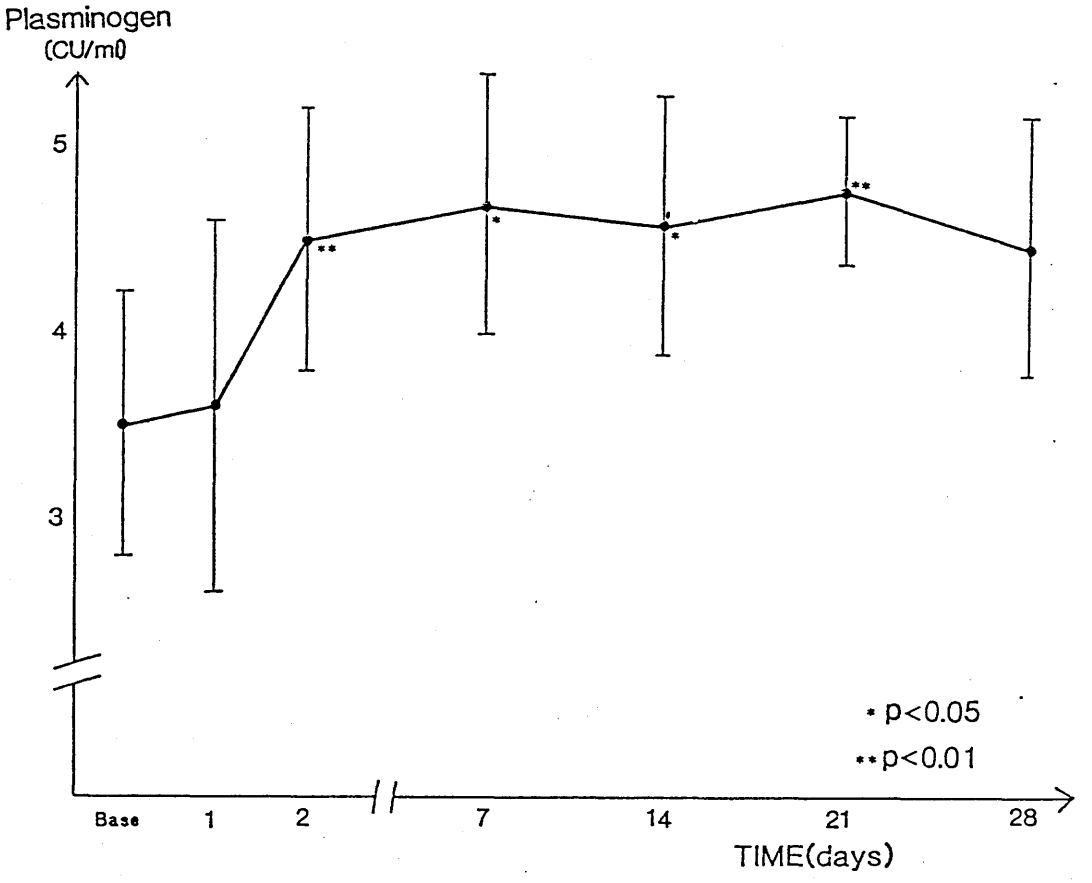

Figure 7.2

Illustrates the concentrations (mean ± SD) of plasminogen activity following the intramuscular injection (50 mg) of stanozolol to healthy male subjects. Changes are compared to basal values.

TABLE 7.1

I.M. STANOZOLOL: EFFECTS ON FIBRINOLYSIS IN NORMAL MALES

DAYS	∝2-AP (IU/ml)	∝2-MG (%)	F.D.P.'s (ug/ml)	Fibrinogen (g/l)
BASE	1.43 ± 0.42	85 ± 26	2.7 ± 0.7	2.24 ± 0.48
1	1.19 ± 0.24	84 ± 25	2.1 ± 0.7	2.48 ± 0.55
2	1.62 ± 0.59	70 ± 24	2.6 ± 0.7	2.35 ± 0.48
7	1.95* ± 0.55	90 ± 36	2.4 ± 1.0	2.06 ± 0.24
14	1.62 ± 0.80	79 ± 27	2.5 ± 0	2.32 ± 0.42
21	1.28 ± 0.59	83 ± 34	2.5 ± 0	2.20 ± 0.38
28	1.86 ± 0.76	75 ± 28	2.5 ± 0	2.28 ± 0.24

Legend

Table 7.1 lists the values (mean ± SD) of alpha2-antiplasmin (∝2-AP), alpha2-macroglobulin (∝2-MG), FDP's and fibrinogen following a single intramuscular (I.M.) injection (50 mg) of stanozolol.

*$p < 0.05$, changes relative to base.

alpha2-macroglobulin concentrations (Preston et al., 1981) which may due to the differences in dosage or to the lower baseline fibrinogen concentrations of our volunteer subjects. This enhancement of fibrinolysis via a single intramuscular injection (Small et al., 1982b) was felt to be of sufficient magnitude to warrant using such a regimen in an attempt to prevent post-operative deep vein thrombosis (Blamey et al., 1984) and to perform a further pilot study in medical patients which will be discussed in the following chapter.

CHAPTER 8

THE EFFECTS OF INTRAMUSCULAR STANOZOLOL ON FIBRINOLYSIS IN ELDERLY MEDICAL PATIENTS

8.1 INTRODUCTION

Following the demonstration of a rapid enhancement of fibrinolysis by a single intramuscular injection of stanozolol (Small et al., 1982b) a controlled trial was performed in general surgical patients which showed that while stanozolol prevented the post-operative shutdown in fibrinolysis, it failed to prevent DVT (Blamey et al., 1983; Blamey et al., 1984).

Patients in general medical wards have a high incidence of venous thrombosis, due partly to age, immobilisation, acute myocardial infarction, cardiac failure, cerebrovascular disease and chest infection (Simmons, Sheppard and Cox, 1973; Sigel et al., 1975; Belch et al., 1981; Warlow 1981). It has also been reported that medical patients with diabetes mellitus (Chapter 2.9), ischaemic heart disease (Chapter 2.7) and cerebrovascular disease (Mettinger et al., 1979) may have reduced fibrinolytic activity. In addition, studies on the relationship between defective fibrinolysis and the development of recurrent and/or idiopathic venous thrombosis suggest an aetiological association (Chapter 2.2). The effects of stanozolol on fibrinolysis in medical patients, who do not undergo the stress of major surgery, had not been studied. Prophylactic anti-thrombotic therapy in medical patients has received scant attention, despite studies showing the effectiveness of subcutaneous heparin in selected patients (McCarthy et al., 1977; Belch et al., 1981; Halkin et al., 1982). In a

pilot study of elderly medical patients at risk of venous thrombosis, and having conditions associated with defective fibrinolysis, we have examined the effects on fibrinolysis following stanozolol administration. If a single intramuscular injection were to induce favourable effects on fibrinolysis its practical advantage over twice daily, often painful, heparin injections would then merit a controlled study with respect to DVT.

8.2 METHODS

8.2.1 Patients

Local Ethical Committee permission was obtained and 12 patients (age range 60-79, mean 70.8 years) gave informed consent to receive a single intramuscular injection of stanozolol. Overall we selected 12 elderly immobilised patients to take part in the study most of whom had established vascular disease, but who were not acutely unwell. Of the 7 males and 5 females, 6 patients (5 females: 1 male) were immobilised with old, dense cerebrovascular accidents. Two of this group with cerebrovascular disease were type II diabetics, of whom one had sustained an acute myocardial infarction and the other a myocardial infarction 2 months prior to the study. Three men being treated with bedrest on day 4 following an acute myocardial infarction entered the study, and 3 immobilised men (one with severe Parkinson's disease, an ischaemic diabetic foot ulcer, and chronic

obstructive airways disease) were also included. Only 2 of the patients were current cigarette smokers and none of the patients were taking drugs known to have an effect on the fibrinolytic system. In view of the known effects of anabolic steroids, patients with liver disease, cardiac failure or prostatic tumour were excluded from the study. Patients taking anticoagulant drugs or with intercurrent infection were also excluded as these might modify the measurement of fibrinolysis and complicate data analysis.

8.2.2 Study Design

Following the baseline sample a 1 ml (50mg) intramuscular injection was given and blood samples taken 1, 2, 5 and 7 days later. All samples were taken between 8am and 9am, at rest, and following an overnight fast. The timing of samples was chosen to detect the major changes which occured in the volunteer study (Chapter 7).

8.2.3 Blood Tests

Plasma fibrinolytic activity, plasminogen activity and alpha2-antiplasmin activity were measured as previously described (Chapter 3). Protein C antigen was kindly assayed by Dr R M Bertina (Leiden, The Netherlands) by electroimmunoassay (Bertina et al., 1982). Alpha2-macroglobulin and FDP's were not assayed since no alterations in these parameters were detected in the volunteer study. Serum for liver function tests (albumin, globulin, bilirubin, alkaline phosphatse, aspartate and alanine transaminase) was analysed on the baseline and day

7 sample (Technicon SMAC).

8.2.4 Statistics

Statistical comparisons were performed using the Wilcoxon matched pairs signed-rank test.

8.3 RESULTS

The results of the fibrinolysis tests are listed in table 8.1. No change in plasma fibrinolytic activity, plasminogen, alpha2-antiplasmin or fibrinogen occurred during the study and no adverse effects on liver function tests were detected. The concentrations of plasma fibrinolytic activity were consistently low and fibrinogen consistently high during the study. Protein C levels increased significantly in the 2 days following stanozolol injection and by day 5 the highest recorded levels (29% above baseline) were noted and these remained significantly elevated at day 7.

8.4 DISCUSSION

Both the fasting plasma fibrinolytic activity and plasminogen levels showed no increment following stanozolol administration to elderly medical ptients. The results on fibrinolytic activity, which show a marked

TABLE 8.1

I.M. STANOZOLOL: EFFECTS ON FIBRINOLYSIS IN MEDICAL PATIENTS

	TIME (DAYS)				
	BASE	1	2	5	7
Fibrinolytic Activity (mm2)	64 ± 21	65 ± 23	68 ± 19	63 ± 18	55 ± 13
Plasminogen (cu/ml)	3.5 ± 0.7	3.2 ± 0.3	3.2 ± 0.3	3.3 ± 0.3	3.2 ± 0.5
α2-Antiplasmin (%)	110 ± 22	113 ± 19	113 ± 21	118 ± 11	117 ± 19
Fibrinogen (g/l)	5.6 ± 1.6	5.5 ± 1.3	5.3 ± 1.2	5.5 ± 1.1	4.8 ± 2.0
Protein C (%)	120 ± 22	127 ± 25	135* ± 23	156* ± 25	150* ± 27

Legend

Table 8.1 lists the changes in the fibrinolytic tests (mean ± SD) following a single intramuscular injection (50 mg) of stanozolol.

*$p < 0.01$, changes relative to base.

contrast to the volunteer study involving young healthy male subjects (Small et al., 1982b), are illustrated in figure 8.1. Since the sample times and assay methods were identical in both these studies, the failure of stanozolol to effect an increase in fibrinolytic activity and plasminogen may be due either to the presence of medical illness, or to the difference in age. It is well known that fibrinogen levels show a marked rise with increasing age (Hamilton et al., 1974; Meade et al., 1977) and still higher levels occur in vascular disease (Lowe, 1981). Compared to our healthy volunteers (mean age 30.8 years) our present study group (mean age 70.8 years) had significantly higher fibrinogen and lower basal levels of fibrinolytic activity. Meade and colleagues (1979) found a fall in fibrinolytic activity in subjects aged 60-65 compared to young controls while Hamilton et al., (1974) found no difference in patients 20-40 years compared to subjects 66-75 years old. Medical illness, rather than age, may be a more important factor in relation to the defective fibrinolysis demonstrated in our patients (Lowe, 1981). The difference noted between volunteers and patients illustrates the importance of documenting drug effects in patient groups. Stanozolol has been shown to increase extrinsic plasminogen activator levels (Kluft et al., 1984b) and since the resting fibrin plate lysis area reflects total plasminogen activator, of which only a small part is extrinsic plasminogen activator, it is possible that a rise in extrinsic plasminogen activator could have occurred following stanozolol administration.

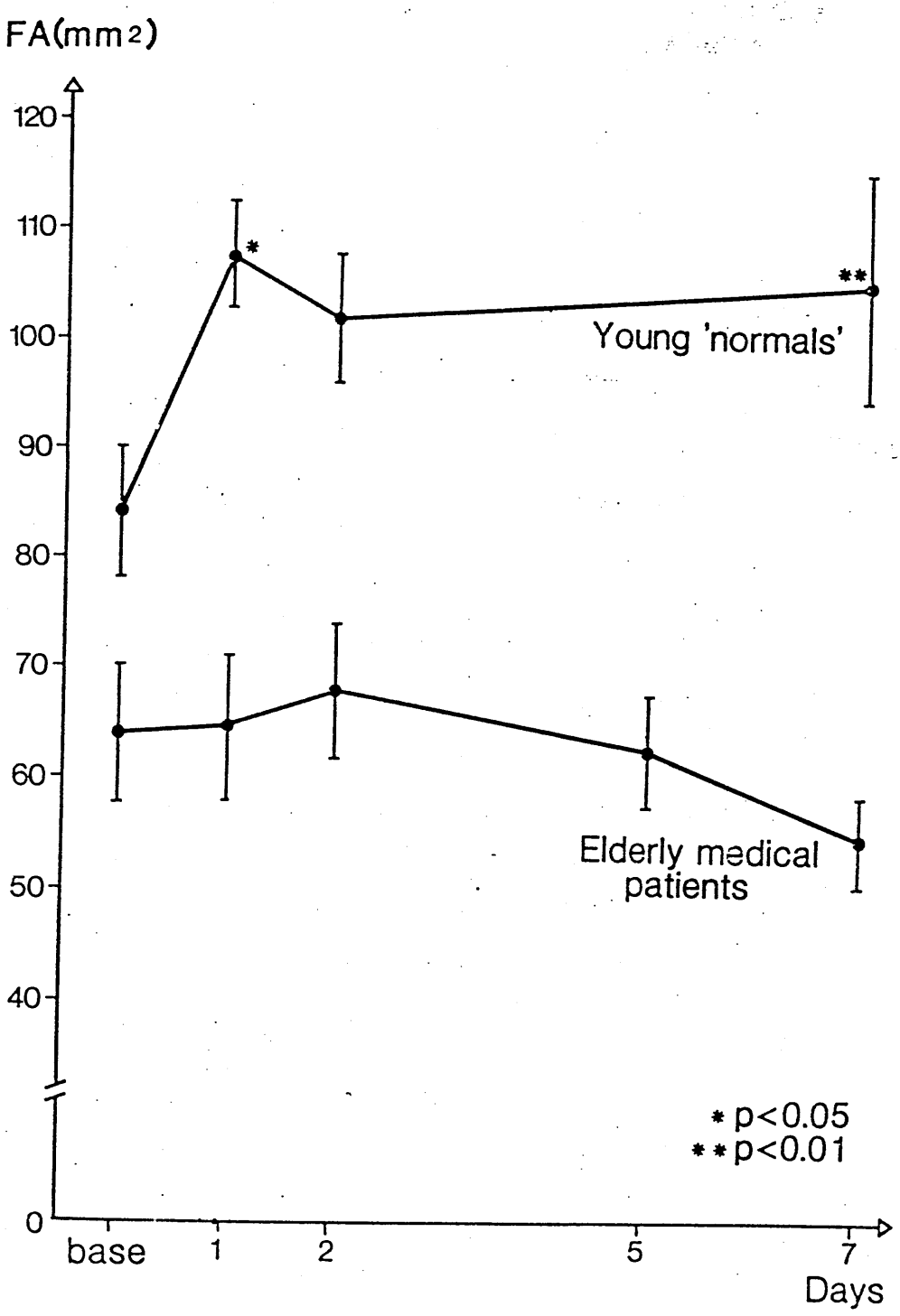

Figure 8.1

Illustrates the concentrations (mean ± SEM) of fasting plasma fibrinolytic activity (FA) in the two groups following stanozolol (50 mg I.M.) administration. Changes are compared to basal values.

This study has confirmed the pharmacological potentiation of protein C with stanozolol (Kluft et al., 1984a) in medical patients at high risk of venous thrombosis. Moreover we have illustrated the time course of onset of protein C elevation following a single intramuscular injection. Whether such elevation of protein C with its potent anticoagulant properties could prevent thrombosis is unknown. Protein C is also known to enhance fibrinolysis (Comp and Esmon, 1981) but the increased concentrations of protein C during the study were not associated with an improved plasma fibrinolytic activity.

In summary we have shown that elderly medical patients have elevated fibrinogen and depressed fibrinolytic activity and that a single intramuscular injection of stanozolol fails to improve these pro-thrombotic abnormalities (Small et al., 1984c).

CHAPTER 9

THE EFFECTS OF ORAL STANOZOLOL ON PLASMA AND SALIVARY FIBRINOLYSIS IN HEALTHY MALE SUBJECTS

9.1 INTRODUCTION

Previous studies of oral stanozolol have not investigated the immediate changes in fibrinolysis and most have concentrated on changes after one month on therapy, but Preston et al., (1981) in a study on healthy volunteers, demonstrated that significant changes in the components of fibrinolysis were detected between 7-14 days after commencing oral stanozolol. The recorded increases in fibrinolytic activity using stanozolol however may have been falsely high due to the effect of the lower fibrinogen levels (which stanozolol also induced) on the euglobulin clot lysis time which was used to measure fibrinolytic activity. Although the authors argued against such a possibility it has been shown that an increase in the fibrinogen level delays plasmin-induced clot lysis (Freidman and Clifton, 1958), and thus changes in fibrinogen levels are likely to alter the euglobulin clot lysis time results. While subjects are taking stanozolol a measurement of fibrinolytic activity which is independent of the fibrinogen concentration, such as the fibrin plate technique, may be a more appropriate assay.

At the time the study was performed there was no available assay of tissue plasminogen activator and since saliva was known to possess fibrinolytic activity (Southam, 1981; Moody, 1982) we were interested to see if stanozolol could alter tissue fibrinolytic activity. The effects of stanozolol on in vivo fibrinolysis had not been studied previously and as the BBeta 15-42 fragment became

available at this time it was used as a reflection of induced fibrinolytic activity.

The aims of our study were threefold: to examine the effects of oral stanozolol on plasma fibrinolytic activity using the more specific fibrin plate technique; to assess in vivo fibrinolysis using BBeta 15-42 measurements and to document the effects of stanozolol on the fibrinolytic activity of saliva in normal male subjects.

9.2 METHODS

9.2.1. Patients

Local Ethical Committee permission was obtained and nine healthy male subjects (age range 19-35, mean 26.8 years) gave informed consent to take part in the study.

9.2.2. Study Design

Stanozolol was administered over a 14 day period - this time interval being chosen since statistically significant changes in blood fibrinolysis had been detected during that period (Preston et al., 1981). A baseline blood sample was taken and repeat samples at 7 and 14 days while on stanozolol and 14 days after the treatment course had stopped. All sampling was carried out in the fasting state, between 9-10am, and the subjects were requested to abstain from alcohol the day before each

sample.

9.2.3 Tests

Plasma fibrinolytic activity, BBeta 15-42 antigen, plasminogen activity, alpha2-macroglobulin antigen and fibrinogen were assayed as previously described (Chapter 3). Saliva (4 mls) was collected into universal containers and, after spinning, 20 μl of the supernatant was assayed for fibrinolytic activity on fibrin plates. All the above samples were placed immediately on ice and centrifuged (at 4°c, 2000 g for 20 mins.) within 30 minutes of collection. Aliquots of plasma and saliva were then stored at -70°c until assayed in batches.

9.2.4 Statistics

Statistical comparisons were performed using the Wilcoxon matched pairs signed-rank test.

9.3 RESULTS

The results are listed in table 9.1. No change was seen in either plasma fibrinolytic activity or plasminogen levels in the 14 days on treatment. However a significant increase in salivary fibrinolytic activity to 142% of baseline was observed at 14 days returning to baseline levels off treatment. One subject consistently failed to

TABLE 9.1

ORAL STANOZOLOL: EFFECTS ON FIBRINOLYSIS IN NORMAL MALES

		DAYS ON TREATMENT		DAYS OFF TREATMENT
	BASE	7	14	14
Fibrinolytic Activity (Plasma: mm2)	73 ± 17	73 ± 11	69 ± 12	65 ± 12
Fibrinolytic Activity (Saliva: mm2)	52 ± 8	54 ± 12	74** ± 14	58 ± 13
BBeta 15-42 (pmol/ml)	0.84 ± 0.30	1.54** ± 0.45	1.18* ± 0.39	0.90 ± 0.57
Plasminogen (cu/ml)	5.2 ± 0.6	5.3 ± 0.3	4.5 ± 0.3	3.9++ ± 0.3
∝2-Macroglobulin (%)	87 ± 26	89 ± 31	75* ± 31	91+ ± 24
Fibrinogen (g/l)	2.32 ± 0.37	1.57** ± 0.30	1.71** ± 0.27	2.44 ± 0.60

Legend

Table 9.1 lists the changes in the fibrinolysis tests (mean ± SD) following a 14 day course of oral stanozolol, and also 14 days following cessation of stanozolol administration.

*p < 0.05, **p < 0.01, changes relative to base

+p < 0.05, ++p < 0.01, changes relative to day 14 on stanozolol

show any fibrinolytic activity in saliva during the study. BBeta concentrations also increased significantly on stanozolol therapy and returned to baseline values on stopping therapy. Fibrinogen levels fell to 67% and 74% of the baseline value at 7 and 14 days respectively and there was a small increase in fibrinogen to 105% off treatment. At 14 days alpha2-macroglobulin had fallen to 87% of the baseline value and there was a small increase to 105% after cessation of treatment.

9.4 DISCUSSION

We found that short-term oral stanozolol therapy had no effect on plasma fibrinolytic activity in normal males using the fibrin plate method. This difference in results from those of Preston et al., (1981), may be due to the lowering of fibrinogen decreasing the euglobulin clot lysis time. Using the fibrin plate method, fluctuations in plasminogen or fibrinogen in the euglobulin fraction do not influence the assay (Kluft et al., 1976). While more prolonged therapy with oral stanozolol may cause fibrinolytic enhancement in normal subjects this enhancement is not seen within 14 days. A number of studies with stanozolol have shown a fall in fibrinogen and, using euglobulin lysis times, a rise in plasma fibrinolytic activity (Davidson et al., 1972; Cunliffe et al., 1975; Walker et al., 1978; Burnand et al., 1980). These studies therefore may have overestimated the rise in

plasma fibrinolytic activity. Indeed following our observations Kluft et al., (1984b) have compared the changes in fibrinolytic activity as measured by fibrin plate and euglobulin lysis times, following stanozolol administration, and showed a much smaller change in fibrinolytic activity using the fibrin plate assay. Their study in addition showed a good correlation between the 2 assays before and after therapy ($r = 0.82$) but a poorer correlation while patients were taking stanozolol ($r = 0.54$). It has more recently been shown that patients given a 14 day course of oral stanozolol (10 mg/day) prior to surgery have improved euglobulin clot lysis times with no significant improvement in t-PA levels (Sue-Ling et al., 1985). Our initial observation has therefore highlighted a problem in assaying overall plasma fibrinolytic activity using euglobulin clot lysis times when changes in fibrinogen also take place.

In our study we have also failed to show that plasminogen concentrations increase during the 14 days on stanozolol therapy but have confirmed the effects, in normal subjects on plasma fibrinogen and alpha2-macroglobulin.

Previous studies on fibrinolytic enhancement by stanozolol have concentrated on the fibrinolytic activity of plasma and we thought it of importance to look at the fibrinolytic activity of saliva as a possible measure of tissue plasminogen activator activity. Saliva is known to possess fibrinolytic activity which is due to its epithelial cell component (Southam, 1981; Moody, 1982).

Our results suggested that stanozolol increases tissue activator, without a concomitant increase in plasma fibrinolytic activity. Since stanozolol has been shown to improve venous lipodermatosclerosis (Burnand et al., 1980) - a condition associated with extravascular fibrin deposition (Burnand et al., 1982) it was felt that this enhancement of tissue fibrinolysis may be relevant to its therapeutic effects.

We have also shown, for the first time, that anabolic steroid therapy with stanozolol is associated with enhanced plasmin - mediated fibrinolysis, as indicated by elevated BBeta levels while patients were on active medication, and this has recently been confirmed (Douglas et al., 1985). It is clear therefore that in normal individuals oral stanozolol is capable of inducing rapid changes in fibrinolysis in vivo. The changes in BBeta concentrations were not mirrored by the plasma fibrinolytic activity which again indicates that these 2 assays measure different aspects of fibrinolysis. This has also been noted in patients with coronary artery disease (Chapter 6).

In summary, we suggest that changes in fibrinogen concentration, while patients are taking stanozolol, may falsely exaggerate the effects on plasma fibrinolytic activity as assessed by euglobulin clot lysis times, and that stanozolol is capable of rapidly improving salivary fibrinolytic activity and plasmin-mediated fibrinolytic activity in normal subjects (Small et al., 1983a; Small et al., 1983b).

CHAPTER 10

CHARACTERISATION OF THE FIBRINOLYTIC ENZYME SYSTEM IN DIABETES MELLITUS

10.1 INTRODUCTION

Although studies on the fibrinolytic enzyme system in diabetes mellitus have produced conflicting results (Chapter 2.9) the weight of evidence seems to favour an association of defective fibrinolysis with type II diabetes (Hathorn et al., 1961; Fuller et al., 1979; Colwell et al., 1983) which is perhaps more closely linked to type II diabetics treated with sulphonylurea drugs (Farid et al., 1974; Almer and Nilsson, 1974; Almer, 1984). Previous studies on fibrinolytic activity have tested diabetics at different times of day, have often failed to differentiate between type I and type II diabetes, have involved small numbers of patients, and have utilised a variety of assays of plasma fibrinolytic activity. These factors therefore may help explain the lack of uniformity of results.

The effect of glycaemic control on fibrinolytic activity is also controversial (Chapter 2.9), while the effect of glycaemic control on the other components of fibrinolysis has received less attention. Hyperlipidaemia in non-diabetics has been associated with defective fibrinolysis while in diabetes there is a close association of poor glycaemic control with hyperlipidaemia. Whether the hyperlipidaemia, consequent upon poor glycaemic control, could be the mechanism of defective fibrinolysis in diabetes has not been formally evaluated. In vivo fibrinolysis, measured by the BBeta 15-42 antigen, has also not been evaluated in diabetes. We have therefore compared the basal components of

fibrinolysis in a large, well classified group of diabetics to a large healthy control group, and have assessed whether a variety of clinical factors (type of diabetes, diabetic treatment, body mass index, smoking, sex, microvascular disease) have any influence on these tests of fibrinolysis.

10.2 METHODS

10.2.1 Patients

Informed consent was obtained and 90 stable, non-ketotic diabetic out-patients were studied. Following Ethical Committee approval, these patients were recruited and subsequently took part in a number of studies of fibrinolysis in diabetes some of which will be presented in later chapters (eg, Myocardial Infarction Study - Chapter 11; Fibrinolytic Enhancement by Insulin and Stanozolol - Chapters 12 and 13; DDAVP stimulation - ongoing study). These studies produced balanced groups of diabetics on insulin, oral hypoglycaemics (all of whom were receiving sulphonylurea therapy), and diet alone, and some of the group characteristics are listed in table 10.1 and subsequent tables. All patients were clearly diabetic as defined by WHO criteria (WHO, 1980), and all had been diagnosed as diabetic for at least 6 months. Patients with impaired glucose tolerance were not studied. Diabetics who could not easily be clinically stratified into type I or type II groups were not studied.

TABLE 10.1

CONTROL AND PATIENT GROUP CHARACTERISTICS

	Controls	All Diabetics	Type I	Type II
Number	59	90	27	63
Age (years)	46 ± 6	56++ ± 10	47** ± 11	60++ ± 8
Diabetes Duration (years)	-	8.3 ± 8.7	13.8 ± 11.7	6.0** ± 6.2
Body Mass Index	26.1 ± 3.9	27.1 ± 4.3	25.2** ± 3.0	27.9+ ± 4.5

Legend

The number, age, duration of diabetes and body mass index of the control and diabetic groups. Values are mean ± SD.

+p < 0.05)
++p < 0.01) Controls versus Diabetics

**p < 0.01) Type I versus Type II

Microvascular disease was diagnosed by the presence of diabetic retinopathy or nephropathy. Fundal examination (pupils dilated) was carried out in all patients, in a darkened room, by a physician with formal training in the assessment of diabetic eye disease (MS), and nephropathy was defined as persistent proteinuria in a longstanding diabetic, without evidence of either urinary tract infection or non- diabetic renal disease. No patient had a history suggestive of autonomic neuropathy. A smoker was defined as either a current cigarette, pipe or cigar smoker, and non-smokers included ex-smokers who had stopped for at least 3 months prior to evaluation. The body mass index was used as an indicator of under or overweight.

As a control group 59 healthy subjects were chosen. These subjects were employees of the Imperial Tobacco Company in Glasgow, who lived in the same area as the diabetic patients. These "control" subjects were on no drug therapy and were non-hypertensive (BP < 160/95). The control group had no family history of diabetes mellitus, and random urinalysis and fasting plasma glucose levels excluded undiagnosed, asymptomatic diabetes. Neither the diabetic nor control females were receiving the oral contraceptive pill and all patients were white - ethnic differences and use of oral contraceptives are known to influence fibrinolysis (Meade et al., 1977).

10.2.2 Study Design

A single, resting, fasting blood sample was taken

from all patients with minimal venostasis, via an antecubital vein. Diabetic patients were told to withhold their insulin or sulphonylurea medication on the morning of the study. The controls were all tested at their place of employment (approximately 400 metres from our hospital).

10.2.3 Blood Tests

Plasma fibrinolytic activity, BBeta 15-42 antigen, alpha2-antiplasmin activity and fibrinogen were measured as previously described (Chapter 3). Plasminogen activity was measured by chromogenic substrate assay (S-2251, Kabi Diagnostica). Glycosylated haemoglobin was measured by the glycosylation of the haemoglobin A1 subfraction (HbA1); the non-diabetic normal range being 5.5-8.5% in our laboratory. Blood for HbA1 was anticoagulated with EDTA (1.5 mg/ml) and the assay performed by agar gel electrophoresis (Glytrac, Corning). Serum triglyceride levels were measured by the Technicon SMA II. During the course of these studies our laboratory plasminogen assay changed (from a caseinolytic to chromogenic assay) and chromogenic plasminogen levels were not available for all diabetic subjects. The data allowed comparison of plasminogen in the control groups with the diabetics as a whole, but did not allow analysis of the diabetic sub-groups.

10.2.4 Statistics

Statistical comparisons were performed by the

Mann-Whitney U test, correlation by the Spearman rank order correlation coefficient and nominal data by Fisher's exact probability test.

10.3 RESULTS

Table 10.2 lists the correlation matrix of age, body mass index, HbA1, and triglyceride levels with the tests of fibrinolysis. Alpha2-antiplasmin correlated significantly with both triglyceride levels ($r = 0.40$) and the plasma fibrinolytic activity ($r = -0.51$), while BBeta 15-42 levels were correlated with HbA1 ($r = -0.25$). The body mass index correlated with triglyceride ($r = 0.30$), but not with any of the fibrinolysis tests. Neither the duration of diabetes nor fibrinogen levels showed any significant correlations (results not listed, all r values < 0.10) while fibrinopeptide A was associated with HbA1 ($r = 0.30$, $p < 0.05$).

Table 10.3 lists the values of HbA1, triglyceride and the tests of haemostasis in the controls, diabetics and diabetic subgroups (type I and II; insulin, sulphonylurea and diet therapy). Three diabetics were insulin-requiring (type II diabetics) and were deleted from the sub-analysis of diabetic treatment and fibrinolysis. As a group, diabetics had higher triglyceride levels compared to controls, but type I patients had lower levels than controls. HbA1 levels were higher in the sulphonylurea treated patients compared to type I ($p < 0.01$) and diet

TABLE 10.2

CORRELATION MATRIX: CLINICAL AND LABORATORY VARIABLES

	AGE	Body Mass Index	HbA_1	TG	FA	BBeta 15-42	α_2-AP
Age	-	0.03	0.10	-0.01	-0.12	-0.13	0.26
Body Mass Index		-	0.06	0.30**	-0.01	-0.06	-0.01
HbA_1			-	0.19	0.17	-0.25*	0.23
TG				-	0.17	0.06	0.40*
FA					-	0.14	0.51***
BBeta 15-42						-	0.07

Legend

Spearman rank correlations (r) values for the above clinical and laboratory variables (TG, triglyceride; FA, plasma fibrinolytic activity; α_2-AP, $alpha_2$-antiplasmin)

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

TABLE 10.3

LABORATORY TESTS IN CONTROL AND DIABETIC PATIENTS

		ALL	TYPE I	TYPE II	
	CONTROLS	DIABETICS	INSULIN	DRUGS	DIET
Number	59	90	27	38	22
Hb A_1 (%)		11.1 ± 2.7	10.1* ± 1.7	12.2 ± 2.9	10.2 ± 2.5
Triglyceride (mmol/l)	1.66 ± 1.00	2.41^{+} ± 2.50	1.05^{+} ± 0.63	3.04^{++} ± 3.20*	3.01^{++} ± 1.42*
Fibrinolytic Activity (mm^2)	82 ± 25	85 ± 19	86 ± 13	87 ± 22	81 ± 20
BBeta 15-42 (pmol/ml)	1.32 ± 0.84	1.62 ± 1.12	1.79 ± 1.34	1.53 ± 1.11	1.74 ± 0.94
α2-Antiplasmin (%)	97 ± 12	117^{+++} ± 24	104 ± 24	112^{+++} ± 22	126^{+++} ± 28
Plasminogen (%)	104 ± 19	95 ± 20			
Fibrinogen (g/l)	3.21 ± 0.75	3.34 ± 0.90	3.25 ± 0.79	3.29 ± 0.96	3.45 ± 0.95
Fibrinopeptide A (pmol/ml)	2.10 ± 1.51	3.23^{++} ± 2.12	3.27^{+} ± 2.08	3.82^{+++} ± 2.37	2.03 ± 0.88

Legend

The values of the laboratory tests are analysed with respect to type of diabetes and diabetic treatment and comparisons made to a matched control group. Values are mean ± SD

*$p < 0.05$) type I versus type II

$^{+}p < 0.05$)
$^{++}p < 0.01$) control versus diabetic
$^{+++}p < 0.001$)

control patients ($p < 0.05$). Diabetics had significantly higher alpha2-antiplasmin levels which were due to significantly higher levels in type II patients compared to controls. A trend to higher BBeta 15-42 levels was noted ($p < 0.06$) in the diet controlled diabetics compared to controls. Fibrinopeptide A levels were significantly higher in diabetes mellitus. Fibrinopeptide A levels were also significantly higher ($p < 0.03$) in the sulphonylurea treated patients compared to those treated by dietary measures alone.

No differences in the tests of haemostasis were noted in diabetics with and without microvascular complications (table 10.4).

Table 10.5 analyses the effect of sex on the haemostatic tests. No sex differences in either the control or diabetic groups were noted for body mass index, BBeta 15-42 antigen, or fibrinogen levels. Male diabetics had significantly higher plasma fibrinolytic activity than either the male controls or female diabetic patients. Alpha2-antiplasmin levels were significantly higher only in the female diabetic patients compared to female controls. We have noted that alpha2-antiplasmin levels are raised in type II diabetes but no significant difference in the proportion of male and female type II diabetic patients were noted (Fisher's test). No significant differences were found when we sub-analysed these groups to compare the concentrations of alpha2-antiplasmin (mean $\pm$ SD) in type II females (118 $\pm$ 21) to both type II males (111 $\pm$ 29) and type I females

TABLE 10.4

HAEMOSTATIC TESTS AND MICROVASCULAR DISEASE

	MICROVASCULAR DISEASE YES	NO
Number	17	73
Fibrinolytic Activity (mm2)	90 ± 20	84 ± 19
BBeta 15-42 (pmol/ml)	1.69 ± 0.99	1.62 ± 1.14
α 2-Antiplasmin (%)	113 ± 18	111 ± 25
Fibrinogen (g/l)	3.27 ± 0.78	3.33 ± 0.92
Fibrinopeptide A (pmol/ml)	2.49 ± 1.39	3.36 ± 2.19

Legend

The effect of microvascular disease on the haemostatic tests are listed. Values are mean ± SD

TABLE 10.5

EFFECT OF SEX ON HAEMOSTASIS

	CONTROLS		DIABETICS	
	MALE	FEMALE	MALE	FEMALE
Number	37	22	53	37
Type II (%)			74	65
Body Mass Index	25.7 ± 2.4	26.8 ± 5.7	26.3 ± 5.3	27.3 ± 4.4
Fibrinolytic Activity (mm2)	79 ± 25	87 ± 25	90** ± 18 +	73 ± 19
BBeta 15-42 (pmol/ml)	1.36 ± 0.97	1.25 ± 0.54	1.84 ± 1.06	1.43 ± 1.14
∝2-Antiplasmin (%)	96 ± 13	98 ± 10	106 ± 25	115++ ± 23
Fibrinogen (g/l)	3.23 ± 0.83	3.22 ± 0.59	3.31 ± 0.87	3.36 ± 0.94
Fibrinopeptide A (pmol/ml)	1.93 ± 1.08	2.38 ± 2.04	2.76 ± 1.88	3.89* ± 2.26

Legend

The sex differences in haemostatic tests and body mass index in the control and diabetic patient are listed. Values are mean ± SD

* p < 0.05)
** p < 0.01) Males versus Females

\+ p < 0.05)
++ p < 0.01) Controls versus Diabetics

(109 ± 30). Female diabetics also had significantly higher fibrinopeptide A levels than both their male counterparts and female controls.

The overall frequency of diabetic and control smokers was very similar, while slightly more diabetic males smoked (table 10.6). In the control group smokers had significantly higher alpha2-antiplasmin levels, although no differences were noted between smoking and non-smoking diabetics. Of the type II patients, 70% were non-smokers and this may explain the higher alpha2-antiplasmin levels seen in non-smoking diabetics compared to controls. Higher fibrinogen levels were found both in the diabetic ($p < 0.05$) and the non-diabetic smokers (NS) compared to their respective controls. Smoking had no effect on plasma fibrinolytic activity or BBeta 15-42 levels.

10.4 DISCUSSION

10.4.1 Effect of Type, and Treatment of Diabetes

In the epidemiclcgical study of Fuller and co-workers (1979), using the fasting dilute blood clct lysis time, diabetics, especially type II diabetics, had defective fibrinolysis. Badawi and colleagues (1970) also noted defective fibrinolytic activity, assessed by the dilute blood clot lysis time, in diabetics compared to controls, but not in the same patients using the euglobulin clot lysis time. They postulated that the defective fibrinolytic activity in diabetes mellitus depended on an

TABLE 10.6

EFFECT OF SMOKING ON HAEMOSTASIS

	'CONTROL' SMOKER		DIABETIC SMOKER	
	YES	NO	YES	NO
Number (%)	44	56	38	62
Males (%)	50	73	62	55
Fibrinolytic Activity (mm2)	88 ± 28	77 ± 22	90 ± 17	83 ± 20
BBeta 15-42 (pmol/ml)	1.22 ± 0.55	1.40 ± 0.99	1.78 ± 1.30	1.57 ± 0.96
∝2-Antiplasmin (%)	100* ± 11	93 ± 11	111 ± 22	112++ ± 24
Fibrinogen (g/l)	3.37 ± 0.79	3.10 ± 0.67	3.65 ± 0.79	3.12* ± 1.00
Fibrinopeptide A (pmol/ml)	1.79 + 1.14	2.36 + 1.74	3.25 + 2.25	3.23 + 2.03

Legend

The effect of smoking on the haemostatic tests in the control and diabetic patients are listed. Values are mean ± SD.

*$p < 0.05$, smoker versus non-smoker

++$p < 0.01$, control versus diabetic

increased anti-fibrinolytic activity detected by the whole blood clot lysis method. In the present large, cross-sectional study, we have found significantly elevated alpha2-antiplasmin levels in type II diabetes and this could explain the poor fibrinolytic activity measured on whole blood clot lysis times, since raised antiplasmin levels have been shown to influence even the euglobulin clot lysis time in a clinical study (Stegnar, Accetto and Keber, 1981).

Reports on the levels of alpha2-antiplasmin in diabetes mellitus have produced conflicting results. While some controlled studies have found no significant difference in alpha2-antiplasmin levels in type I diabetes (Coccheri et al., 1983; Dornan et al., 1983), type II diabetes (Grignani et al., 1981; Hughes et al., 1983) or in diabetes in general (Christe et al., 1984), other reports have found raised alpha2-antiplasmin levels associated with type I diabetes (Ambrus et al., 1979) and microvascular disease (Christe et al., 1984). The weight of evidence suggests that type I diabetics have normal alpha2-antiplasmin levels, while the large numbers in the present study would suppport the view that alpha2-antiplasmin levels are raised in type II diabetic patients.

We have previously noted a positive correlation of triglyceride levels and alpha2-antiplasmin in patients with coronary artery disease (Chapter 6.3), hyperlipidaemia and healthy subjects (Chapter 2.5), and this has been confirmed in the present study. We have

found that our type II diabetics have significantly higher triglyceride levels than type I diabetics and this may, in part, contribute to the increased alpha2-antiplasmin levels found in type II diabetics.

There has been some debate as to whether fibrinopeptide A levels are elevated in type I diabetes (Borsey et al., 1984; Rosove, Frank and Harwig, 1984) but we have confirmed the recent findings of Jones (1985) indicating that both type I and type II diabetes are associated with augmented thrombin activity. The slightly raised levels of BBeta 15-42 may, in part, be secondary to this activation of coagulation.

10.4.2 Effect of Glycaemic Control

In 1980 an inverse correlation of fibrinolytic activity with HbA1 levels was first noted (Gunnarsson et al., 1980) since when others have failed to confirm this finding (Grignani et al., 1980; Dornan et al., 1983; Christe et al., 1984; Haitas et al., 1984). While the present study has failed to show a significant relationship between HbA1 and plasma fibrinolytic activity, we have found that both plasmin and thrombin generation (assessed by BBeta 15-42 and fibrinopeptide A) are influenced to a small, but significant extent by glycaemic control, such that good control is associated with improved fibrinolysis and diminished thrombin generation. These findings are in keeping with the in vitro studies showing that glycosylation of haemostatic factors occurs, and that such a process may alter the

function or concentration of these factors (Chapter 2.9).

Although type I diabetics (all receiving insulin) had lower HbA1 and triglyceride concentrations than type II patients, no significant differences in the components of fibrinolysis were noted between the groups, except for raised alpha2-antiplasmin levels as previously noted.

When the type II patients were subdivided into those receiving sulphonylurea therapy and those patients treated with diet alone, the sulphonylurea patients had worse glycaemic control, slightly lower BBeta 15-42 levels and significantly higher thrombin generation. While this fails to support the other cross-sectional studies showing that fibrinolytic activity is lower and fibrinogen levels higher in patients treated with sulphonylurea drugs (Farid et al., 1974; Almer and Nilsson, 1974; De Silva et al., 1979) it does suggest that such patients have an increased pro-thrombotic tendency. It has been stated that hypertriglyceridaemia in diabetes is associated with reduced plasma fibrinolytic activity (Almer 1975a; Almer et al., 1975b) but we have been unable to confirm this and in addition, have failed to relate triglyceride levels to any of the other components of fibrinolysis. The slightly high triglyceride levels in our normal control group is in keeping with triglyceride levels found in an epidemiological study of healthy males in the West of Scotland (Lorimer et al., 1974).

10.4.3 Effect of Microvascular Disease

We have found that microvascular disease did not

effect the unstimulated tests of fibrinolysis, and this is in agreement with cther studies (Almer et al., 1975b; Fuller et al., 1979; Haitas et al., 1984; Christe et al., 1984; Lowe et al., 1986). Three studies used venous occlusion as a dynamic stimuli, and a defective response of plasma fibrinolytic activity was noted in patients with microvascular disease in 2 of these studies (Almer et al., 1975b; Haitas et al., 1984; Christe et al., 1984). No firm statement on the effect of microvascular disease on fibrinolysis can at present be given.

10.4.4 Effect of Sex

In the control group plasma fibrinolytic activity was, as expected, slightly lower in males when compared to female patients (Chakrabarti et al., 1978; Meade et al., 1979) but in diabetic patients the reverse was true. In fact, diabetic male patients had significantly higher plasma fibrinolytic activity (and slightly higher BBeta 15-42 levels) than control male subjects or diabetic females. We have fcund a negative correlation of alpha2-antiplasmin and plasma fibrinolytic activity which suggests that alpha2-antiplasmin is either associated with the levels of those plasminogen activators or other inhibitors which are precipitated in the euglobulin fraction, or else is measured in the fibrin plate assay. Which of these mechanisms is correct is unclear, since euglobulin preparation is thought to eliminate fibrinolytic inhibitors, and alpha2-antiplasmin shows no correlation with in vivo fibrinolysis, measured by BBeta

15-42 antigen. The raised alpha2-antiplasmin may explain the slightly lower levels of plasma fibrinolytic activity in our female patients. A previous study has shown significantly higher alpha2-antiplasmin levels in female subjects (Teger-Nilsson, 1979) and our female diabetics had significantly higher alpha2-antiplasmin levels than their female non-diabetic counterparts. The higher alpha2-antiplasmin levels in the female diabetics cannot be explained by a greater percentage of type II diabetics being female, since in fact the reverse is true. The sex difference in fibrinolytic activity in diabetes is in keeping with the epidemiological study of Fuller and co-wcrkers (1979) who noted type I males had normal fibrinolytic activity while a slight reduction of fibrinolytic activity was noted in type I females. Diabetic females, in addition to their poorer fibrinolysis, have more active coagulation with enhanced thrombin generation, as evidenced by elevated fibrinopeptide A levels.

In non-diabetics the general epidemiology of fibrinolytic activity and fibrinogen suggests that these may well be implicated in the pathcgenesis of ischaemic heart disease (Meade et al., 1979). In diabetics however, the protective effect of female sex on the development of cardiovascular disease is lost (Kannel and McGee. 1979; Jarrett et al., 1982; Rytter, Troelsen and Beck-Nielson, 1985; Uusitupa et al., 1985). Whether such adverse haemostatic changes of defective fibrinolysis and active coagulation in female diabetic patients could be related

to their increased risk of cardiovascular disease remains speculative.

10.4.5 Effect of Weight

Two large studies in diabetic patients have found a negative association between body weight, plasma fibrinolytic activity and plasminogen activator content of the vessel wall (Almer, 1975a; Fuller, 1981). We have been unable to confirm this association, and it may be that larger numbers are required to demonstrate such an association.

10.4.6 Effect of Smoking

As found in the coronary artery disease study (Chapter 6), we have been unable to show an effect of chronic smoking on plasma fibrinolytic activity and this has been confirmed in another large diabetic group (Almer, 1975a). Smoking did not affect the BBeta 15-42 or alpha2-antiplasmin levels in the diabetics or controls, while fibrinogen levels were greater in smokers, as has been well documented in the past (Meade et al., 1979).

10.4.7 Summary

Alpha2-antiplasmin levels are elevated in type II patients and in diabetic females and are also influenced by triglyceride levels. In addition, female diabetic patients have reduced fibrinolytic activity and enhanced coagulation which may be relevant to the increased risk of

cardiovascular disease in female subjects. Our results also suggest that sulphonylurea treatment is associated with a change in the haemostatic balance favouring a thrombotic tendency.

We have shown that glycaemic control influences both in vivo fibrinolysis and coagulation to some extent, but that overall, diabetics have no significant differences in fibrinolysis compared to a well matched control group (except for raised alpha2-antiplasmin levels). We have shown however that both sex status and type of diabetes affects fibrinolysis and by 'lumping' all the diabetics together we have cancelled out these differences. The aetiology and clinical manifestations of diabetes reveals that it is a heterogeneous condition, and it is not unreasonable therefore to divide patients into their appropriate categories. Future studies in diabetes must allow for these factors which influence fibrinolysis.

CHAPTER 11

FIBRINOLYSIS IN DIABETIC PATIENTS WITH AND WITHOUT MYOCARDIAL INFARCTION

11.1 INTRODUCTION

Atherosclerotic occlusive vascular disease is the most common complication of diabetes mellitus and accounts for approximately 75% of all deaths, mainly manifesting as cardiovascular disease (Ganda., 1980; Steiner., 1981; Aro., 1984). Coronary artery disease is common in diabetes (Shapiro, 1984; Uusitupa et al., 1985) with an increased incidence of myocardial infarction (Garcia et al., 1974) and recent studies have revealed a 2-3 fold increase in mortality following myocardial infarction (Jaffe et al., 1984; Smith et al., 1984; Gwilt et al., 1985; Rytter et al., 1985). It is generally believed that the increased risk of macrovascular disease arises from a greater frequency and/or severity of 'risk factors' (hypertension and hyperlipidaemia) in the diabetic patients, although prospective studies which have examined the predictive effect of the major risk factors have failed to establish them as the explanation for the extra risk in diabetes (Jarrett, 1984). It has been suggested that other factors, such as abnormal haemostasis could explain the excess risk (Jarrett, 1984), and to assess whether alterations in the fibrinolytic enzyme system are associated with large vessel disease in diabetes we have examined a number of tests of fibrinolysis in diabetic men with and without a previous myocardial infarction and a matched control group.

11.2 METHODS

11.2.1 Patients

Fifteen established diabetic men who had sustained a well documented myocardial infarction were studied (Group A). They were compared to 15 male diabetics, matched for age, smoking history, weight, type and duration of diabetes (Group B), and to 15 healthy non-diabetic men (controls). Group B and the controls had neither clinical nor ECG evidence of occlusive vascular disease and were not hypertensive (BP < 160/95). Group C had no family history of diabetes, no glycosuria and normal fasting glucose concentrations.

11.2.2 Study Design

A single, fasting blood sample was taken, without a tourniquet, from all subjects after informed consent was obtained. Patients with myocardial infarction were studied at least 3 months after the acute event in view of the known alterations in lipids and fibrinolysis which occur after an acute myocardial infarction.

11.2.3 Blood Tests

Plasma fibrinolytic activity, BBeta 15-42 antigen, fibrinogen and fibrinopeptide A were measured as previously described (Chapter 3). Total serum cholesterol, HDL cholesterol and triglyceride concentrations were measured by the Technicon SMA II and Boehringer kits (Carlson., 1973). Glycosylated haemoglobin (HbA1-Glytrac, Corning) and fasting plasma

glucose (Beckman II autoanalyser) were assayed as an indicator of glycaemic control. HbA1 was not measured in the control group as it is known that HbA1 is not a useful screening test for diabetes mellitus.

11.2.4 Statistics

Statistical comparisons were performed using the unpaired Wilcoxon rank sum test, correlations by the Spearman rank order correlation coefficient and nominal data by Fishers exact probability test.

11.3 RESULTS

The clinical characteristics of the groups are listed in Table 11.1. The diabetic groups A and B were well matched in all respects and no significant differences between the groups were noted (Fishers test). The control group was significantly younger than the diabetic groups ($p < 0.01$) but was well matched as regards smoking history and body mass index. No significant differences in lipid levels were noted between the 3 groups, and no differences in glycaemic control were found between groups A and B (Table 11.2) Overall the diabetic patients had significantly elevated plasma fibrinolytic activity and BBeta 15-42 levels compared to controls and no differences between groups A and B were detected (Table 11.3). Neither fibrinogen nor fibrinopeptide A levels differed significantly between groups A, B or controls (table

TABLE 11.1

CONTROL AND DIABETIC GROUP CHARACTERISTICS

		DIABETICS	
	CONTROLS	Group A	Group B
Number	15	15	15
Age (years)	51 ± 3	59* ± 8	59* ± 7
Smokers	6	4	7
Body Mass Index	26.2 ± 2.3	27.8 ± 6.8	24.9 ± 2.7
Diabetes Duration (years)	-	7.2 ± 7.9	6.3 ± 5.1
Therapy			
- insulin	-	4	3
- sulphonylurea	-	7	8
- diet	-	4	4
Complications			
- Retinopathy	-	3	3
- Hypertension	-	8	0

Legend

Clinical characteristics of the control and diabetic patients with (Group A) and without (Group B) a previous myocardial infarction. Three patients on insulin in each group were type I diabetics. Values are mean ± SD.

*$p < 0.01$, controls versus diabetics

TABLE 11.2

LIPID LEVELS AND GLYCAEMIC CONTROL

		DIABETICS	
	CONTROLS	Group A	Group B
Triglyceride (mmol/l)	1.7 ± 0.4	2.7 ± 2.6	1.9 ± 0.9
Cholesterol (mmol/l)	5.9 ± 1.0	6.2 ± 1.1	5.8 ± 0.9
HDL Cholesterol (mmol/l)	1.1 ± 0.3	1.1 ± 0.4	1.2 ± 0.3
HbA1 (%)	-	10.1 + 3.2	10.6 + 2.9
Fasting Glucose (mmol/l)	4.8 ± 0.3	9.5 ± 5.1	9.4 ± 2.6

Legend

Lipid levels and glycaemic control in the control and diabetic patients with (Group A) and without (Group B) a previous myocardial infarction. Values are mean ± SD

TABLE 11.3

HAEMOSTATIC TESTS IN CONTROL AND DIABETIC PATIENTS

	CONTROLS	D I A B E T I C S		
		Total	Group A	Group B
Fibrinolytic Activity (mm2)	71 ± 28	93++ ± 21	89 ± 21	96++ ± 21
BBeta 15-42 (pmol/ml)	1.17 ± 0.30	2.14+++ ± 1.00	2.35++ ± 0.96	1.97+ ± 1.04
Fibrinogen (g/l)	3.31 ± 0.52	3.47 ± 0.91	3.40 ± 0.89	3.53 ± 0.95
Fibrinopeptide A (pmol/ml)	2.11 ± 1.22	2.56 ± 0.36	1.96 ± 0.51	3.15 ± 2.0

Legend

Haemostatic tests in control and diabetic patients with diabetics subdivided into those with (Group A) and without (Group B) a previous myocardial infarction.
Values are mean ± SD.

\+ $p < 0.05$)
++ $p < 0.01$) Controls versus diabetics.
+++ $p < 0.001$)

11.3). No significant correlations between age and both plasma fibrinolytic activity (r = -0.28) and BBeta 15-42 (r = -0.37) were noted in the diabetic patients, and no correlation of plasma fibrinolytic activity and BBeta 15-42 was found (r = -0.05).

11.4 DISCUSSION

We have previously demonstrated that basal fibrinolysis is not influenced by microvascular disease (Chapter 10) and the aim of the present study was to assess if macrovascular disease affected fibrinolysis, which may be relevant to the increased prevalence and mortality associated with large vessel disease in diabetes. Compared to our control group, diabetics had significantly better ex vivo and in vivo fibrinolysis which could not be explained on the basis of differences in weight, smoking and lipid levels. The diabetics were slightly (but significantly) older than the control group, but since no correlations were found between age and either in vivo or ex vivo fibrinolysis, we feel this cannot explain the increased fibrinolysis in diabetes. The diabetic patients with and without a prior myocardial infarction were, in addition, closely matched for duration of diabetes, type of treatment and glycaemic control, and no significant differences in the fibrinolysis tests were noted between the groups. This implies that diabetes per se and not myocardial infarction is associated with an

enhanced fibrinolysis. This is in keeping with our previous findings that diabetic males have increased plasma fibrinolytic activity and slightly higher BBeta 15-42 levels than in male controls (Chapter 10). The interpretation of our results require caution with regard to the selection of our 2 diabetic groups. Asymptomatic coronary artery disease is common in diabetes (Shapiro, 1984) and some of our apparently healthy diabetic controls may have had coronary artery disease. Our diabetic controls however had no clinical or resting ECG evidence of coronary artery disease although coronary angiography was not performed. The patients with myocardial infarction are a highly selected group since they are survivors of an acute myocardial infarction - a condition associated with a high mortality rate. Our patients were studied at a minimum interval of 3 months following their myocardial infarction and it is known that in the first month following an acute myocardial infarction diabetics have approximately a two-fold increase in mortality of about 40% compared to non-diabetics (Rytter et al., 1985). These survivors therefore could have had better (or different) fibrinolysis compared to those who died, although the lack of difference between the survivors and healthy diabetic controls would suggest that this is unlikely.

In summary we have found that male diabetics have enhanced fibrinolysis which is not related to occlusive vascular disease. Our results suggest that fibrinolysis plays no role in the prevalance and mortality associated

with macrovascular disease. This is an agreement with the epidemiological studies showing that fibrinolysis in non-diabetics is not a risk factor for cardiovascular disease (Meade et al., 1980; Wilhelmson et al., 1984).

CHAPTER 12

ENHANCEMENT OF FIBRINOLYSIS FOLLOWING INSULIN ADMINISTRATION IN TYPE II DIABETES MELLITUS

12.1 INTRODUCTION

Cross-sectional studies on diabetic patients have suggested an association of defective fibrinolysis with the use of sulphonylurea drugs (Almer and Nilsson, 1974; Farid et al., 1974; Almer, 1984), but a prospective study on the effects on fibrinolysis of changing patients from sulphonylurea therapy to insulin has not been reported.

Approximately 5% of diabetics each year initially well controlled on sulphonylurea drugs develop hyperglycaemia and, despite maximum dose of sulphonylurea therapy, require insulin to alleviate the symptoms of poorly controlled diabetes (Anonymous, 1981). These patients, with secondary failure of sulphonylurea therapy, are usually given life-long daily subcutaneous injections of insulin. We have therefore measured a variety of tests of fibrinolysis, in a homogeneous group of connecting (C-) peptide positive, type II diabetics with secondary failure on sulphonylurea drugs, and assessed the effects of substitution of insulin.

12.2 METHODS

12.2.1. Patients

Local Ethical Committee approval was obtained and 20 (15 women, 5 men) type II diabetic patients gave informed consent and were recruited to the study. Mean age of the

patients was 62 years (range 51-72), duration of diabetes 7.5 years (1-26) and mean body mass index of 26.1 (range 17.8-31.4). All patients had been receiving sulphonylurea therapy for at least one year with adequate glycaemic control initially. Despite maximum dose sulphonylurea administration (glibenclamide 15 mg/bd), however the patients had developed constant glycosuria, with random clinic capillary glucose values between 15-25 mmol/l, and some had symptoms related to hyperglycaemia. They were therefore judged to have failed on sulphonylurea therapy and to require insulin. Five patients had clinical evidence of microangiopathy (4 background and 1 proliferative retinopathy) and 6 had evidence of macroangiopathy (4 patients with ischaemic heart disease and 2 with peripheral arterial disease). Six patients were receiving anti-hypertensive or anti- anginal therapy but no other medication was taken during the study period. All patients had normal blood urea and electrolyte concentrations and none had persistent proteinuria.

12.2.2 Study Design

On the morning of their first insulin injection and at 1 and 3 months thereafter a fasting blood sample was taken for HbA1 and assessment of fibrinolysis. The final dose of glibenclamide had been taken at 5pm on the day before starting insulin therapy.

All patients attended the clinic daily for one week,

then weekly for one month to be given the education appropriate to an insulin-requiring diabetic. Each patient was placed on Lentard MC (Novo), highly purified beef/porcine lente insulin starting at 28 units each day. Insulin dosage was adjusted thereafter until most urine tests before breakfast showed no glycosuria, a mid-morning clinic capillary glucose level of < 10 mmol/l was attained or until hypoglycaemic symptoms had ensued.

C-peptide is secreted in equimolar quantities to insulin and its measurement therefore gives an indication of endogenous insulin secretion. Residual beta-cell function was evaluated, in the fasting state by measuring C-peptide before and 6 minutes after a 1mg, intravenous injection of glucagon (Faber and Binder, 1977).

12.2.3 Blood Tests

Plasma fibrinolytic activity, BBeta 15-42 antigen, plasminogen activity, alpha 2-antiplasmin, alpha 2-macroglobulin, fibrinogen, and fibrinopeptide A were measured as previously described (Chapter 3). HbA1 was performed by agar gel electrophoresis (Glytrac, Corning), the non-diabetic normal range being 5.5-8.5% in our laboratory. Serum C-peptide was determined by radioimmunoassay (Novo Laboratories Ltd).

12.2.4 Statistics

Statistical comparisons were performed using the Wilcoxon matched- pairs signed-rank test, and correlation was determined by the Spearman rank order correlation coefficient.

12.3 RESULTS

Table 12.1 lists the haemostatic tests over the study period. Both the plasma fibrinolytic activity and BBeta 15-42 increased significantly following the introduction of insulin therapy (figures 12.1 and 12.2). No difference in the baseline, peak or increment (peak-basal) in plasma fibrinolytic activity or BBeta 15-42 were noted for patients without vascular disease when compared to those with either micro or macrovascular disease. No significant changes in the other components of the fibrinolytic system were noted except for a 12% rise in the minor inhibitor of fibrinolysis, alpha 2-macroglobulin ($p < 0.05$), after 3 months of insulin therapy. Samples for fibrinopeptide A were obtained at 0 and 3 months only and comparison samples were available for 13 patients. A 33% fall ($p < 0.05$) in fibrinopeptide A concentrations were noted at 3 months.

No major hypoglycaemic episodes occurred, but occasional symptoms of nocturnal hypoglycaemia were reported by some patients. Due to symptomatic hyperglycaemia two patients were transfered to twice-daily

TABLE 12.1

EFFECT OF INSULIN ON HAEMOSTASIS

	TIME (MONTHS)		
	0	1	3
Fibrinolytic Activity (mm2)	79 ± 20	91* ± 23	93** ± 22
BBeta 15-42 (pmol/ml)	0.96 ± 0.58	1.31 ± 0.67	1.44** ± 0.66
Plasminogen (cu/ml)	2.8 ± 0.5	3.1 ± 0.5	3.0 ± 0.5
∝ 2-Antiplasmin (%)	114 ± 29	112 ± 37	107 ± 22
∝ 2-Macroglobulin (%)	111 ± 37	112 ± 29	123* ± 36
Fibrinogen (g/l)	2.99 ± 0.77	3.16 ± 1.11	3.22 ± 0.64
Fibrinopeptide A (pmol/ml)	4.9 ± 2.5	-	3.3* ± 1.9

Legend

Table 12.1 lists the concentrations of the haemostatic tests (mean ± SD) at baseline (0 months) and after 1 and 3 months on insulin therapy.

*p < 0.05)
**p < 0.01), changes relative to base.

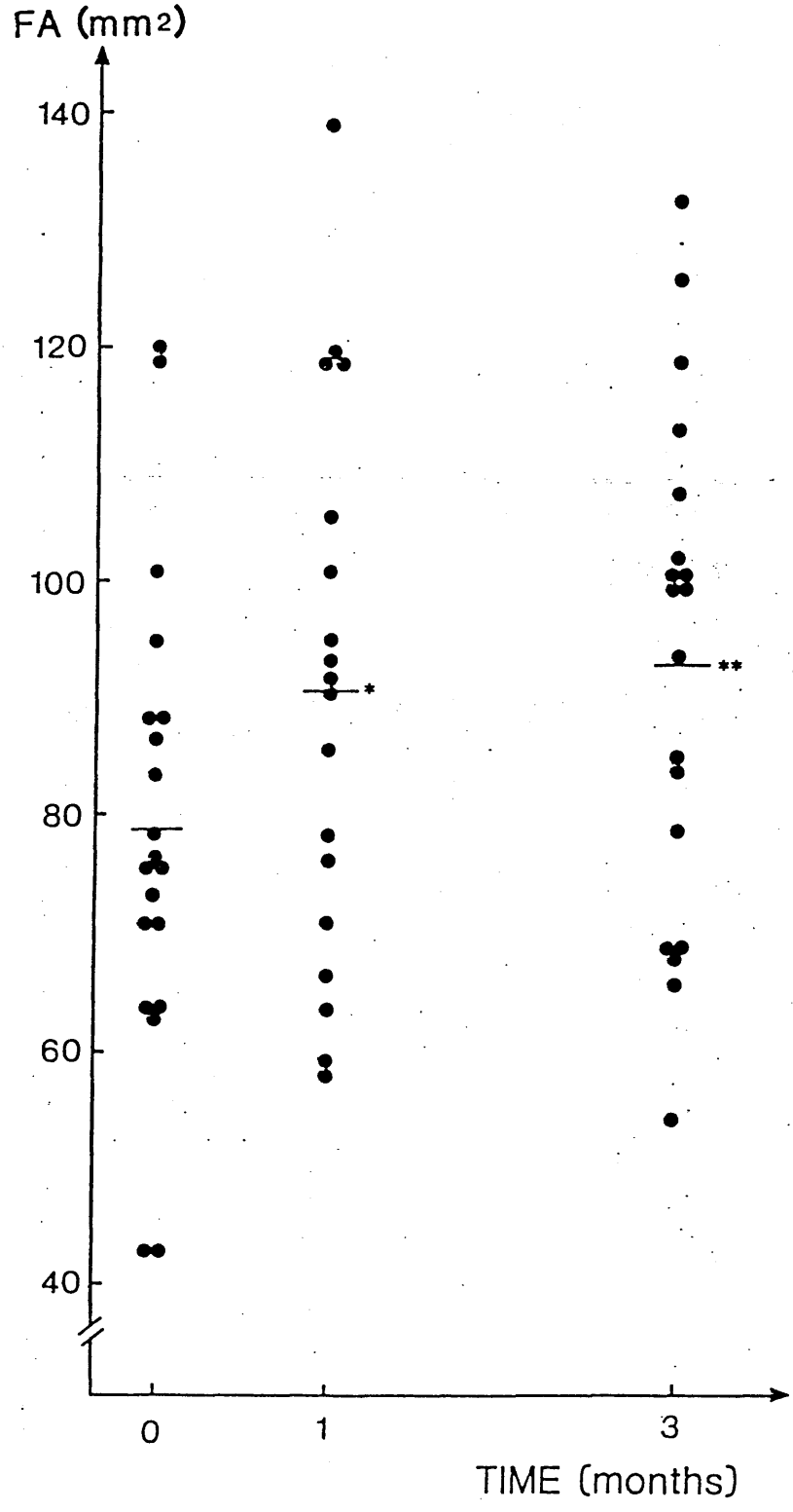

Figure 12.1

Illustrates the individual values of fasting plasma fibrinolytic activity (FA) while patients were receiving sulphonylurea drugs, and 1 and 3 months following the introduction of insulin.
The horizontal bar indicates the mean value.
*p $\prec$ 0.05, **p $\prec$ 0.01, changes are relative to time 0.

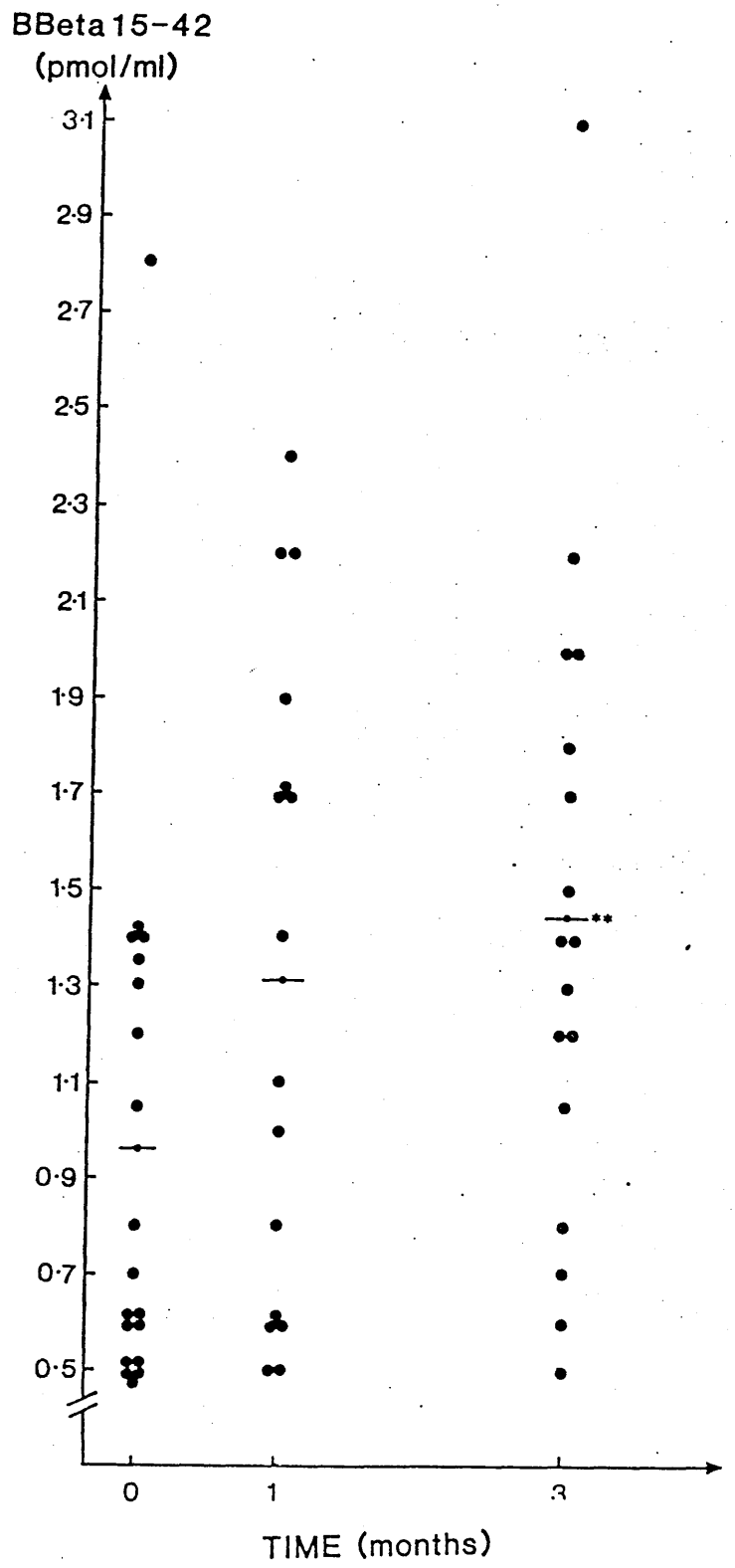

Figure 12.2

Illustrates the individual values of BBeta 15-42 antigen while patients were receiving sulphonylurea drugs, and 1 and 3 months following the introduction of insulin. The horizontal bar indicates the mean value. **p $<$ 0.01, changes relative to time 0.

insulin injections (Mixtard, Nordisk) by 3 months, while the others were still receiving Lentard MC. Mean daily insulin dosage was 42 units (range: 28-68), and mean patient weight gain at 3 months was 5.7 lbs (range: -1-21 lbs). Institution of insulin therapy was associated with an improvement in well-being, abolition of symptoms of thirst and polyuria, and reduction of diurnal glycosuria, however no significant improvement in glycaemic control, as judged by HbA1 levels, was observed. Prior to insulin therapy the mean HbA1 concentration was 13.2 ± 2.3% as compared with mean values of 13.1 ± 2.1% and 12.3 ± 2.1% at 1 and 3 months respectively. No patient had a HbA1 level within the normal range after 3 months of insulin administration. No correlations between HbA1 and plasma fibrinolytic activity or any other fibrinolytic tests were noted. Normal or elevated fasting C-peptide levels were detected in all patients confirming that these patients were not insulin-dependent. No correlations were noted between the basal, peak or incremental change in C-peptide (following glucagon) and the tests of fibrinolysis measured on the baseline sample.

12.4 DISCUSSION

We have shown that on stopping sulphonylurea drugs and substituting insulin therapy, improvement of plasma fibrinolytic activity and increased plasmin-induced fibrinolysis occurs. This fibrinolytic enhancement could not be explained by the patient's weight change following

insulin therapy, an alteration of glycaemic control, and was not influenced by the presence of pre-existing macro or microvascular disease.

It is possible that sulphonylurea therapy has a deleterious effect on fibrinolysis, with a rebound improvement in fibrinolytic activity upon cessation of such therapy. Prospective assessment of fibrinolysis in a small group of newly diagnosed type II patients, placed on sulphonylurea therapy for one year, has not shown any impairment of fibrinolysis (Bogie et al., 1976) but this does not exclude a possible effect on fibrinolysis with high dosage and prolonged treatment, as found in the present study group. If longterm sulphonylurea treatment does indeed lead to impaired fibrinolysis, then it is interesting to speculate whether the high frequency of cardiovascular deaths in sulphonylurea treated diabetic patients, observed in the University Group Diabetes Programme Study (1970), could be linked to a depression of fibrinolysis.

Alternatively, insulin treatment may stimulate fibrinolysis and there is evidence that endothelial cells bind and internalise insulin (Kaiser et al., 1982) and that insulin has a direct effect on the endothelial cell (Osterby, Gundersen and Christensen, 1978; Fortes, Garcia Leme and Scivoletto, 1984) which is the site of storage of vascular plasminogen activator. Other studies have noted that type I diabetics have an increased plasma fibrinolytic activity when compared to controls (Cash and McGill, 1969; Bern et al, 1981; Elder et al., 1981;

Sharma, 1981) although in the study by Sharma (1981) however it is not clear whether patients were receiving insulin at the time of estimation of their fibrinolysis. Further support for the role of insulin on fibrinolysis is given by the findings of Sandberg and colleagues (1963) who noted that diabetic plasma took a shorter time to lyse a standard fibrin clot, and noted an inverse correlation between total daily insulin requirements and clot lysis time - ie, the higher insulin dose the shorter the lysis time. In our study however we did not note any association between fasting insulin levels, insulin secretion (measured by glucagon stimulated C-peptide release), and either plasma fibrinolytic activity or BBeta 15-42 levels. The effect of endogenous beta-cell function on fibrinolysis in type II diabetes has not previously been evaluated. Why type I diabetics should have elevated plasminogen activator level (measured as euglobulin lysis times) is unknown, although it has been postulated that this is a physiological response to relative hypercoagulability (Cash and McGill, 1969). It should be noted however that other studies involving type I diabetics have not confirmed these results (Fuller et al., 1979; Juhan-Vague et al., 1984).

We have also found that the introduction of insulin therapy was associated with a significant reduction of fibrinopeptide A concentrations and therefore diminished thrombin generation. Improvements in coagulation and platelet function have been noted in type II diabetes with improved glycaemic control due to high fibre diet (Simpson

et al., 1982), sulphonylurea therapy (Paton et al., 1981; Hughes et al., 1983), and insulin therapy in patients with mild carbohydrate intolerance without glycosuria (Evans et al., 1982). In our patients, with more severe glucose intolerance, better glycaemic control could not explain the changes noted in fibrinopeptide A (or fibrinolysis) since overall glycaemic control was unaltered.

Our results of insulin treatment in poorly controlled, type II diabetic patients are in accordance with a similar study (Peacock and Tattersall, 1984), where a once daily insulin regimen failed to improve glycaemic control in most subjects after 6 months. It is quite clear therefore that in younger patients with secondary sulphonylurea failure a once daily lente preparation is inadequate to offer good glycaemic control. In older patients requiring insulin our aim is not to achieve normoglycaemia, but to keep patients asymptomatic and in this context a once daily lente insulin is satisfactory. The best treatment for type II diabetic patients in whom dietary measurements have failed, is currently the subject of a multicentre study in the UK (Multi-Centre Study, 1983). A fair criticism of the present study is that it is not controlled and therefore the alterations in haemostasis may have occurred with the passage of time and possibly unrelated to sulphonylurea or insulin therapy. The only relevant control group would have been similar patients with symptomatic hyperglycaemia who were failing on sulphonylurea therapy. We felt that it would be unethical to deny these patients insulin and therefore

accept that some caution must be placed on our results due to lack of a suitable control group.

In summary, we have shown that improved fibrinolysis (Small et al., 1986a) and diminished thrombin generation (Small et al., 1986c) occurs when, in our routine clinical practice, we transfer patients from high dose sulphonylurea therapy to insulin. Whether the correction of these pro-thrombotic abnormalities are due to withdrawal of sulphonylurea therapy or a direct effect of insulin requires further study. Nonetheless our observations indicate the potential advantage of insulin therapy in such diabetic patients.

CHAPTER 13

THE EFFECTS OF ORAL STANOZOLOL ON FIBRINOLYSIS AND GLYCAEMIC CONTROL IN TYPE II DIABETES MELLITUS

13.1 INTRODUCTION

The weight of current evidence suggests that type II diabetes is associated with defective fibrinolysis (Chapter 2.9). The high prevalence of vascular disease, which is more marked in type II diabetes, and the need for anti-thrombotic drugs provides a rationale for the evaluation of stanozolol in diabetes mellitus. While the combination of phenformin and the anabolic steroid, ethylestrenol, have been shown to enhance fibrinolysis in newly diagnosed type II diabetic patients (Chakrabarti and Fearnley, 1974), the effects of anabolic steroid therapy alone on fibrinolysis in diabetes have not been studied.

Steroid drugs have marked effects on carbohydrate tolerance such that corticosteroids cause severe impairment of carbohydrate tolerance and may even precipitate diabetes. Different categories of anabolic steroids have differing actions on carbohydrate metabolism although stanozolol has been reported to improve fasting blood glucose concentrations in diabetes (Tainter et al., 1964). More accurate assessment of glycaemic control is now available with measurement of glycosylated proteins which are of use in the appraisal of drugs as oral hypoglycaemic agents.

Since we have previously documented a failure of stanozolol to enhance the depressed fibrinolysis in elderly medical patients (Small et al., 1984c) and since the effects of stanozolol on fibrinolysis in diabetes have not been reported we decided to perform a

placebo-controlled pilot study on the effects of stanozolol on fibrinolysis and glycaemic control in type II diabetes mellitus.

13.2 METHODS

13.2.1 Patients

Local Ethical Committee permission was obtained and 12 type II diabetic patients gave informed consent to take part in the study. Mean age of the patients was 58 years (range 36-70) , duration of diabetes of 3.8 years (range 0.5-10) and body mass index of 29.8 (range 24.4-35.4). Four patients had ischaemic heart disease, 4 had peripheral arterial disease, 4 were hypertensive, 1 had background retinopathy and 1 patient had gout. Four patients were taking sulphonylurea drugs, 4 were taking beta-adrenoceptor blocking drugs and 1 patient was taking allopurinol.

13.2.2 Study Design

The patients attended the diabetic ward at 9.00am after an overnight fast on either 6 or 7 occasions. Patients took a matched placebo between -2 and 0 weeks after which they were started on 10 mg of stanozolol/day for either 4 weeks (n = 5) or 8 weeks (n = 7). Patients had further samples taken at 2, 4 and 8 weeks while taking

stanozolol and 4 weeks after after cessation of stanozolol administration.

13.2.3 Blood Tests

At each visit plasma fibrinolytic activity, BBeta 15-42 antigen, plasminogen activity (caseinolytic activity) alpha2-antiplasmin activity, alpha-2 macroglobulin antigen and fibrinogen were measured as previously described (Chapter 3). Glycaemic control was assessed by fasting plasma glucose and glycosylation of haemoglobin (G.Hb), plasma proteins (G.P.P.), and albumin (G.Alb). Plasma glucose was measured by a Beckman II glucose analyser and blood for G.Hb was anticoagulated with EDTA and the assay performed by agar gel electrophoresis (Glytrac, Corning). Our laboratory's non-diabetic range is 5.5-8.5%. G.P.P. and G.Alb were measured using a microcolumn technique (Leiper et al., 1985), the non-diabetic normal ranges being 2.6-3.9% and 1.3-2.0% respectively.

13.2.4 Statistics

Statistical comparisons were performed using the paired Wilcoxon rank sum test.

13.3 RESULTS

13.3.1 Fibrinolysis

Table 13.1 lists the concentrations of the tests of fibrinolysis during the study period. There were no significant changes in the tests over the 2 week interval (week -2 to week 0) while patients were taking placebo. The concentrations of the various tests while patients were receiving stanozolol were therefore compared to week 0. No significant alterations in plasma fibrinolytic activity, BBeta 15-42, alpha2-antiplasmin or alpha2-macroglobulin were noted throughout the study. Significant increases in plasminogen and decreases in fibrinogen were noted while patients were taking stanozolol, although significant changes were not detected at all time points while patients were receiving active medication (figure 13.1). Four weeks after stopping stanozolol, significant falls in plasminogen and increases in fibrinogen concentrations were detected.

13.3.2 Glycaemic Control

Fasting plasma glucose concentrations (mmol/l, mean $\pm$ SD) were as follows: 10.9 $\pm$ 2.9 at -2 weeks, 10.9 $\pm$ 2.4 at week 0, 10.0 $\pm$ 2.8 at week 2 ($p < 0.05$), 9.0 $\pm$ 2.7 at week 4 ($p < 0.05$) and 8.1 $\pm$ 3.2 at week 8. After 4 weeks off active medication glucose concentrations had risen to 11.1 $\pm$ 3.4 mmol/l ($p < 0.05$, compared to final glucose levels while taking stanozolol therapy). The alterations in G.Hb, G.P.P. and G.Alb are illustrated in figure 13.2. A significant fall in G.Hb was found after 8 weeks on

TABLE 13.1

ORAL STANOZOLOL: EFFECTS ON FIBRINOLYSIS

IN TYPE II DIABETES MELLITUS

	F.A. (mm2)	BBeta 15-42 (pmol/ml)	∝2-AP (%)	∝2-MG (%)
Weeks				
-2	76 ± 20	1.00 ± 0.53	121 ± 25	100 ± 32
0	83 ± 20	1.12 ± 0.49	126 ± 20	99 ± 28
+2	79 ± 18	1.23 ± 0.93	122 ± 24	71 ± 47
+4	82 ± 21	1.24 ± 0.84	126 ± 20	92 ± 30
+8	81 ± 30	1.09 ± 0.64	119 ± 25	93 ± 39
12	80 ± 13	1.26 ± 0.61	106 ± 30	108 ± 29

Legend

Table 13.1 lists the changes in the tests of fibrinolysis (mean ± SD), while on placebo (-2 to 0 weeks), on stanozolol (0 to 4 or 8 weeks), and 4 weeks after cessation of stanozolol (week 12). Plasma fibrinolytic activity (F.A.), alpha2-antiplasmin (∝2-AP), alpha2-macroglobulin (∝2-MG).

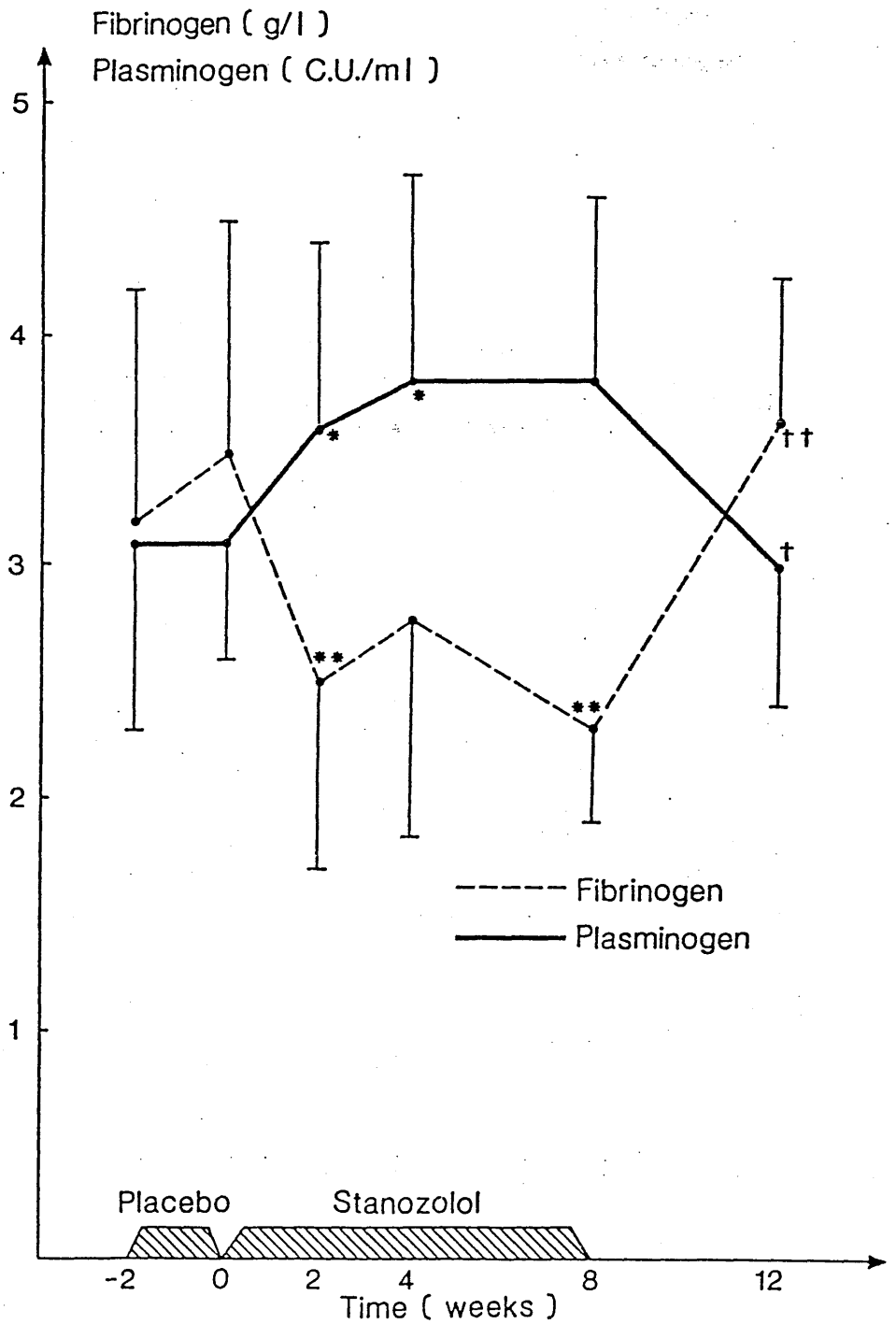

Figure 13.1

Illustrates the concentrations (mean ± 1SD) of fibrinogen and plasminogen on placebo (-2 to 0 weeks), stanozolol (0 to 4 or 8 weeks) and 4 weeks after stopping stanozolol (week 12) in type II diabetic patients.

*p < 0.05, **p < 0.01, changes relative to week 0

+p < 0.05, ++p < 0.01, changes relative to week 4 or 8

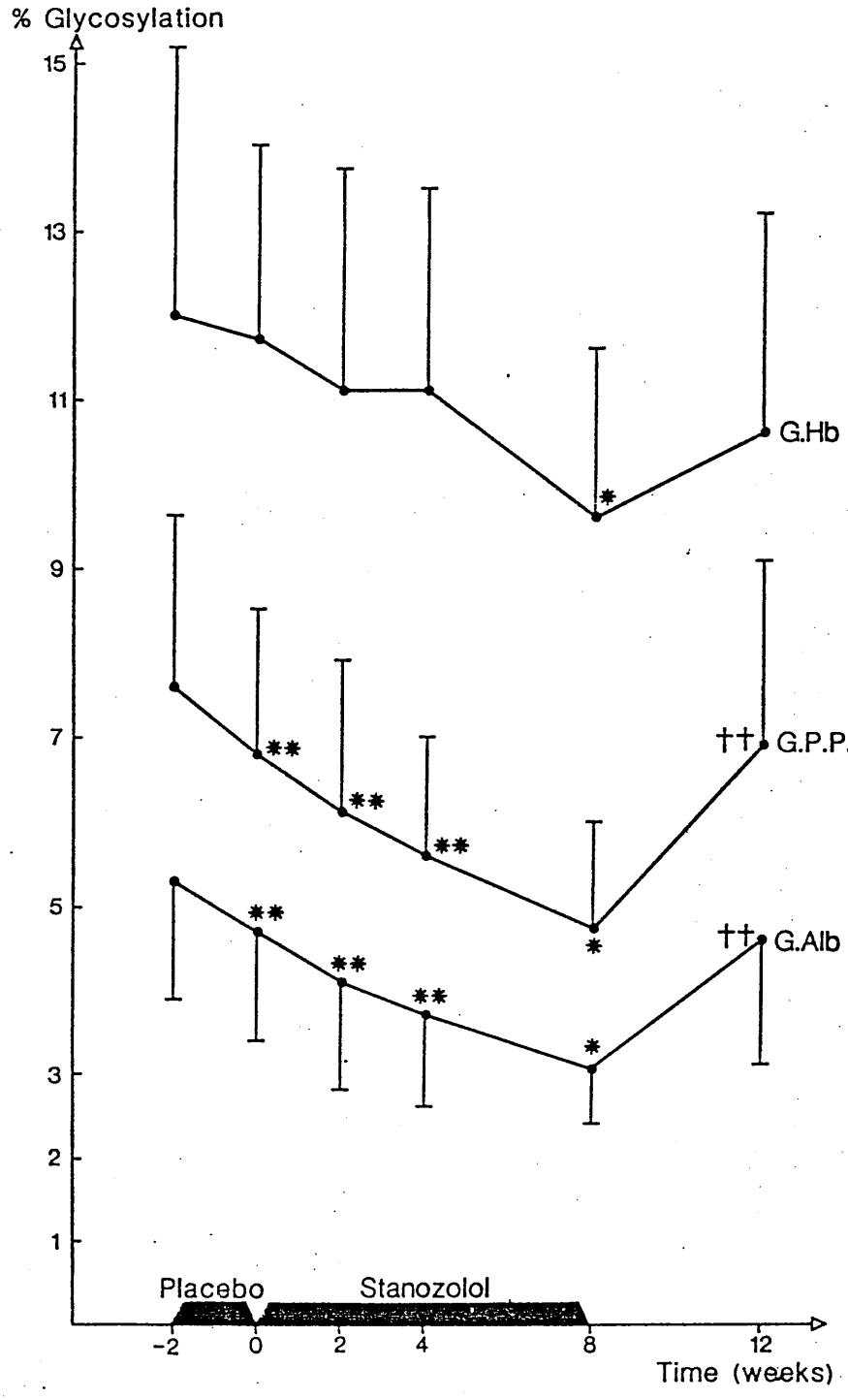

Figure 13.2

Illustrates the concentrations (mean ± SD) of glycosylated haemoglobin (G.Hb), plasma proteins (G.P.P.) and albumin (G.Alb) during the study.

*p < 0.05, **p < 0.01, changes relative to week 0

++p < 0.01, changes relative to week 4 or 8

stanozolol. Significant decreases in G.P.P. (10%), and G.Alb (9%) were noted after the 2 week placebo run-in period and therefore changes in G.P.P. and G.Alb while on stanozolol were compared to week 0. A significant and progressive fall in glycosylated proteins occurred while on active medication with significant regression towards pre-treatment levels on cessation of drug administration. Eleven of the twelve patients demonstrated improvements in the above measures of glycaemic control while the control of one subject (also receiving glibenclamide) deteriorated on stanozolol. The other three patients on sulphonylurea therapy demonstrated similar falls in the above variables as those patients with diabetes controlled on diet alone.

13.4 DISCUSSION

Oral administration of stanozolol has been shown to enhance plasma fibrinolytic activity in ischaemic heart disease, Raynaud's disease, DVT, lipodermatosclerosis cutaneous vasculitis, superficial thrombophlebitis, protein C and antithrombin III deficiency, haemophilia A and B, and normal subjects (Chapter 2.8). In our poorly controlled diabetic patients, as indicated by the high fasting plasma glucose and elevated glycosylated haemoglobin, albumin and plasma protein concentrations, stanozolol failed to exert its characteristic effects on plasma fibrinolytic activity (Kluft et al., 1984b), BBeta 15-42 concentrations (Small et al., 1983b; Douglas et al.,

1985) and alpha2-macroglobulin concentrations (Walker et al., 1975; Preston et al, 1981; Kluft et al., 1984b). Stanozolol did induce a reduction in fibrinogen and elevation of plasminogen concentrations although significant alterations in these plasma proteins were not seen at all time intervals while patients were taking stanozolol. Stanozolol does not therefore display the same pronounced effects on fibrinogen and plasminogen as seen in other patient groups. The lack of changes noted in ex vivo and in vivo fibrinolytic activity despite improved glycaemic control fails to support the view that glycamic control is a major determinant of fibrinolysis.

Recent studies have carefully evaluated the mode of action of stanozolol on fibrinolysis and it is clear that the rise in plasma fibrinolytic activity is due to a fall in t-PA inhibitor (Verheijen et al., 1984). The lack of change in fibrinolytic activity in our study suggests that changes in t-PA inhibitor may not have occurred in our diabetic patients.

We have shown that stanozolol administration to type II diabetic patients is associated with a significant improvement of glycaemic control without the occurrence of hypoglycaemic attacks. The similar falls in blood sugar levels in patients controlled by dietary measures alone compared to those on diet plus oral hypoglycaemic drugs suggests no major interaction of stanozolol with the sulphonylurea agents occurred. The improvements in fasting blood glucose and G.Hb concentrations are of a similar magnitude to those reported in obese type II

diabetics given metformin (Lord et al., 1983). The mode of action of stanozolol on carbohydrate metabolism is unknown but since the anabolic steroid methandienone has been shown to augment insulin responses (Landon, Wynn and Samols, 1963), it may be that stanozolol also possesses such a 'sulphonylurea like' hypoglycaemic action.

Due to the long life span of the red cell (120 days), G.Hb concentrations remain elevated for several weeks after the attainment of improved glycaemic control, while plasma proteins like albumin (t-1/2 of 20 days) detect glycaemic changes earlier (Jones et al., 1983). Improved control over a 2 week period, measured by glycosylated serum proteins, has been demonstrated in diabetic children without any significant change in fasting glucose concentrations (Strickland et al., 1984). In our study we noted an improvement in glycosylated protein concentrations (without alteration of fasting glucose concentrations) while the patients were on the 2 week placebo run-in period. This illustrates the well recognised phenomenon that closer medical supervision of patients by itself can lead to improved glycaemic control. It seems likely that the improved glycaemia was related not only to intensive patient surveillance but to some intrinsic effect of stanozolol, since control deteriorated sharply when patients, still under close supervision, stopped taking the drug.

The present study population was comprised of patients with hypertension, obesity, poorly controlled diabetes and, in some cases, overt occlusive vascular

disease. An anti-thrombotic drug may have therapeutic potential in such a group of patients. While stanozolol has shown favourable effects on carbohydrate metabolism the failure of stanozolol to stimulate fibrinolysis in type II diabetes suggests that anabolic steroid therapy may not modify the pro-thrombotic state associated with diabetes mellitus.

CHAPTER 14

METABOLIC EFFECTS OF STANOZOLOL

14.1 INTRODUCTION

Although stanozolol has been used since the early 1960's in numerous different clinical settings, its effects on other metabolic systems have received scant attention. While the effects of stanozolol on fibrinolysis have been considered elsewhere (Chapters 6, 7, 8, 13), these studies also allowed us to evaluate other possible sites of action of this anabolic agent. We felt it important to document its actions on hepatic function, sex-hormone status, lipid concentrations and rheology since adverse effects on these systems would be important with regard to prescribing stanozolol to different patient groups. The favourable effects on glycaemic control in type II diabetes have been discussed in chapter 13, and the following remarks summarise very briefly the metabolic effects of stanozolol we have evaluated.

14.2 Hepatic Actions

Since danazol, a closely related steroid, was shown to induce marked changes in a number of hepatic proteins (Laurell and Rannevik, 1979) it was postulated that stanozolol may alter hepatic enzyme systems (Preston et al., 1981) and we presented some evidence to support such a hypothesis (Small et al., 1982b; Small et al., 1983c). To evaluate whether stanozolol could effect such changes by inducing hepatic enzymes, we measured antipyrine kinetics and urinary 6 beta-hydroxycortisol excretion (indicators of hepatic monooxygenase activity) in normal

males given oral stanozolol. We found no change in these markers of enzyme induction and concluded that the effects of stanozolol on plasma proteins were not due to any action on the cytochrome P450 system (Thompson et al., 1984). In this study we have also shown that stanozolol was porphyrinogenic, which may be relevant to the successful treatment of aplastic anaemia with anabolic steroids (Wilson and Griffin, 1980). As far as routine liver function tests are concerned, we have noted no adverse effects in normal subjects or patients given intramuscular stanozolol, or a short course of oral stanozolol (Small et al., 1982b; Small et al., 1984c). In the study involving diabetic patients 2 of the 12 patients had disturbances in liver function tests - both were asymptomatic. A fourfold rise in alanine transaminase was found in one female after 2 weeks on stanozolol, which had improved after a further 2 weeks while the patient was still taking stanozolol and returned to normal levels on cessation of stanozolol administration. The other patient had an elevation of alanine transaminase just outwith the normal range which settled while the patient was still taking stanozolol. Our limited experience of short-term administration of stanozolol has not therefore led to any problems with liver dysfunction.

14.3 Endocrine Effects

From early work on animal studies it was reported that stanozolol had the highest anabolic to androgenic ratio of the anabolic steroids, and therefore it was less

likely to cause androgenic side-effects (Arnold, Potts and Beyler, 1963; Gribbin and Flavell Matts, 1976). No previous studies have assessed the hormonal effects of stanozolol in men, and we have found that stanozolol, given orally or intramuscularly to normal male subjects, has prolonged and pronounced effects on the pituitary-testicular and pituitary-thyroidal axis (Small et al., 1983c; Small et al., 1984d). Stanozolol reduced luteinising hormone, follicle stimulating hormone, testosterone, thyroxine (T4) and triiodothyronine (T3) concentrations. The effects on T4 and T3 were due to a reduction in thyroxine binding globulin concentrations (synthesised in the liver) while free T4 concentrations were unchanged. The reduction in total and free testosterone levels makes it likely that stanozolol has a suppressive effect on luteinising hormone secretion by the pituitary gland. All the aforementioned changes were reversible on stopping stanozolol administration but the long-term effects of anabolic steroid therapy on gonadotrophin suppression and spermatogenesis in the male are not known. Other anabolic steroids have been evaluated as a male oral contraceptive (Schurmeyer et al., 1984). Androgenic side effects are common in female patients given stanozolol (Jarrett, Morland and Browse, 1979), and in our study involving diabetic patients we have had to stop stanozolol administration because of the development of marked hirsutism and acne in one patient. Our studies therefore indicate that stanozolol is a potent androgenic agent despite previous in vitro animal studies

(Arnold et al., 1963).

14.4 Lipid Effects

Anabolic steroids have been reported to be effective in hyperlipidaemia (Ciswicka-Sznajderman, Berent and Rymaszewski, 1974; Ciswicka-Sznajderman, Berent and Rymaszewski, 1981; Wilson and Griffin, 1980) but in normal males we have noted an increase in total cholesterol, LDL cholesterol and a fall in HDL cholesterol (Small et al., 1982b) and in diabetics, an increase in LDL and fall in HDL cholesterol (Small et al., 1986b). Hyperlipidaemia is common in type II diabetes, the characteristic abnormalities being a high LDL and low HDL cholesterol (Goldberg, 1981). The changes in LDL and HDL would tend to aggrivate the abnormalities in lipoprotein levels observed in such patients, and since a high LDL and low HDL cholesterol are strong, predictive risk factors for coronary heart disease (Lewis, 1983), the lipoprotein changes could be harmful.

14.5 Rheological Effects

It was postulated that stanozolol may reduce plasma viscosity due to its actions in lowering fibrinogen and alpha2-macroglobulin concentrations (Preston et al., 1981). We have shown that stanozolol does indeed lead to a very small reduction in plasma viscosity (Small et al., 1983a) but it has been found to exert no effect on whole blood viscosity (Ayres, Jarrett and Browse, 1981). It is dubious whether such a small reduction in plasma viscosity

by stanozolol would have any therapeutic value.

14.6 Summary

In our experience, stanozolol appears to have adverse effects on lipid and sex-hormone status, and although we have not noted clinically apparent liver dysfunction it is well recognised that disturbances in liver function tests are common during stanozolol administration (Jarrett et al., 1979). It appears unlikely that the favourable effects on carbohydrate metabolism in type II diabetes outweigh these side-effects. Whether the improved fibrinolysis and anti-coagulant effects of stanozolol are clinically more relevant alterations would have to be evaluated for each individual patient. If patients are placed on long-term stanozolol administration close monitoring of the aforementioned aspects of metabolism are clearly indicated.

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