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In vivo and *in vitro* inhibition of platelet aggregation by SV-IV, a major protein secreted from the rat seminal vesicle epithelium

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SV-IV* (seminal vesicle protein No. 4, according to its mobility in SDS–PAGE) is a basic, thermostable protein of low M_r (9758) synthesized and secreted under strict testosterone control from the rat seminal vesicle epithelium. The sequence of its 90 amino acids has been determined and much is known on the molecular biology of the SV-IV gene [1–8]. Computer and immunological analyses have shown that uteroglobin, a progesterone induced and binding protein widespread in many tissues and body fluids of rabbits, presents a significant degree of homology with SV-IV [9]. In addition, both uteroglobin and SV-IV were shown to possess anti-inflammatory properties [10–12] and have been found to effectively inhibit chemotaxis and phagocytosis of macrophages and neutrophils [10, 13, 14]. The mechanism of action at the basis of all these effects seems to be related, at least in part, to the inhibition of phospholipase A_2 (PLA_2, EC 3.1.1.4) [12, 15] and consequently to a block of the arachidonic acid (AA) cascade. More recently, it has been demonstrated that uteroglobin inhibits thrombin-induced platelet aggregation *in vitro* and it has been suggested that this effect was due to the inhibitory action of the protein on PLA_2 [16]. To determine whether SV-IV also possesses anti-thrombotic effects, we examined the ability of the protein to inhibit platelet aggregation induced by different agents (thrombin, ADP, collagen, platelet activating factor (PAF) and AA) both *in vivo* and *in vitro*.

Materials and Methods

Materials. The protein SV-IV was purified to homogeneity from adult rat (Fisher–Wistar) seminal vesicle secretion as described by Ostrowski *et al.* [1], and its purity was evaluated by 15% PAGE, in denaturing and non-denaturing conditions, and by amino acid composition analysis [9].

Bovine plasma thrombin (285 NIH units/mg protein), equine muscle ADP sodium salt (grade IX), human placenta collagen (type VI), porcine liver AA free acid (approx. 99%), and PAF (1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Bovine pancrease ribonuclease A was from Pharmacia (Uppsala, Sweden); indomethacin (Liometacen) was from Chiesi Farmaceutici (Milan, Italy).

In vivo *experiments of platelet aggregation in rats*. Male Wistar rats (200–250 g) were anesthetized with sodium pentobarbital (30 mg/kg, i.p.). Platelet aggregation was induced by i.v. (jugular vein) administration of 1 unit/kg of thrombin, 1 mg/kg of ADP, 2 mg/kg of collagen, 0.75 μg/kg of PAF, or 1.5 mg/kg of AA, essentially according to the method previously described by Pinon [17]. Blood samples were collected from the carotid artery 3 min after thrombin, collagen or AA injections, and 0.5 min after ADP or PAF injections, into two plastic disposable syringes (0.4 mL of blood/syringe). One syringe contained 1.6 mL of EDTA + formalin buffer (EDTA tetrasodium salt 24 mM, KH_2PO_4 1.3 mM, Na_2HPO_4 5.4 mM and formalin (40% sol) 2.5%) (sample A), whereas the second one contained 1.6 mL of EDTA buffer (EDTA 24 mM, KH_2PO_4 3.3 mM, Na_2HPO_4 13.4 mM) (sample B). After mixing, the samples were transferred to polystyrene tubes and allowed to stand for 10 min before centrifugation (350 *g* for 10 min). In sample A, aggregates were fixed by formalin, whereas in sample B they were dissociated and the platelets were made unaggregable by EDTA. Platelets were counted by light microscopy and platelet-count ratio was calculated as platelet count in the sample A/platelet count in the sample B. The anti-thrombotic effect of SV-IV was assayed by i.v. (jugular vein) injection of appropriate amount of protein dissolved in 0.2 mL of saline, 3 min before the administration of the aggregating agents. Controls were carried out by injecting saline or ribonuclease (1.5 mg/kg) under the same experimental conditions.

Platelet aggregation in vitro. Blood was collected from ether-anesthetized male rabbits (2.5–3 kg) by intracardiac puncture and anticoagulated with 0.38% trisodium citrate. The platelet-rich plasma was obtained by centrifuging the blood at 350 *g* for 10 min after dilution with platelet-poor plasma to obtain 4×10^8 cells/mL, and used for ADP aggregation studies. In addition, for platelet aggregation induced by thrombin, collagen, PAF and AA, the platelet-rich plasma was preliminarily washed with 0.38% trisodium citrate and centrifuged at 550 *g* for 15 min. The platelets contained in the pellet were then resuspended in Tyrode's solution without calcium (4×10^8 cells/mL). All aggregations were performed in aggregometer cuvettes containing 0.25 mL of platelet suspension. SV-IV was added in saline and preincubated with platelets for 3 min at 37°. Thrombogenic agents were then added and the aggregation measured at 37° in a ELVI 840 aggregometer. Control aggregation curves were obtained in the absence of SV-IV

* Abbreviations: SV-IV, seminal vesicle protein No. 4; PLA_2, phospholipase A_2; AA, arachidonic acid.

with an equivalent amount of saline to make up for the decrease in assay volume. All aggregation experiments were repeated ten times and the interassay variations were <5%.

Results and Discussion

The presence of circulating platelet aggregates in the blood of rats was examined after induction of intravascular platelet aggregation by injecting different thrombogenic agents. This simple *in vivo* method was used to evaluate the anti-thrombotic effect of SV-IV. In Table 1 the values of the platelet-count ratio (platelet-count in a sample where aggregates were fixed by formalin/platelet-count in a sample where the aggregates were dissociated and the platelets were made unaggregable by EDTA) [17], obtained under different experimental conditions, are reported. The dramatic fall of the platelet-count ratio from 0.98 to 0.54, 0.51, 0.54, 0.55 and 0.55 when thrombin, ADP, collagen, PAF and AA were respectively administered, clearly demonstrates the intravascular aggregation in the control animals. Moreover, Table 1 shows that platelet aggregation triggered *in vivo* by thrombin, ADP, collagen and PAF was markedly inhibited by a preliminary treatment of the rats with SV-IV. The effect of the protein was found to be dose-dependent, the inhibition being almost total when 1.5 mg/kg of SV-IV were injected. The administration of a different protein with a similar molecular weight (ribonuclease) failed to prevent platelet aggregation, thus indicating the specific inhibitory effect of SV-IV. Finally, it should be noted that platelet aggregation induced by AA was not inhibited by SV-IV.

In contrast, the results reported in Fig. 1 show that the administration of high concentrations of SV-IV (6 mg/kg) triggered platelet aggregation. An additive aggregatory effect of the protein, when injected in conjunction with 1 unit/kg of thrombin, was also observed.

In order to determine if the SV-IV inhibition of platelet aggregation observed *in vivo* occurred only when platelets were held in their physiological surroundings, we investigated the effect of the protein also on the *in vitro* induced aggregation of isolated platelets. Figures 2 and 3 clearly show that also the aggregation of the platelets taken out of the circulatory system was inhibited by SV-IV when thrombin, ADP, collagen or PAF were added to the cells 3 min after the protein. An increase of the incubation time beyond 3 min with SV-IV did not increase the extent of inhibition. It should be noted that platelet aggregation by AA was not affected by treating the cells with SV-IV (Fig. 2, panel D), thus confirming the results previously obtained with the *in vivo* system. Furthermore, the results reported in panel B of Fig. 3 indicate that SV-IV was effective in preventing PAF-induced platelet aggregation also when the stimulation of thromboxane synthesis was blocked by indomethacin. Ribonuclease as nonspecific protein control at a concentration of 240 μg/mL was without effect in all the performed experiments (data not shown).

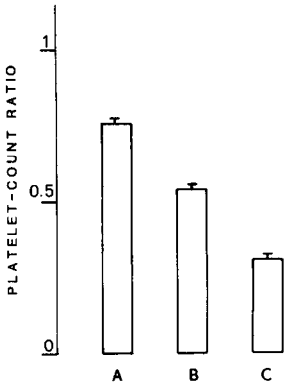

Fig. 1. Platelet aggregation induced *in vivo* by administration of high concentrations of SV-IV. Platelet aggregation was induced by i.v. administration of 6 mg/kg of SV-IV (A), 1 unit/kg of thrombin (B), or a mixture of both (C). Values are expressed as means ± SE of five experiments. Further experimental details are described in the text.

The results of the present study provide evidence that SV-IV is able to prevent platelet aggregation induced both *in vitro* and *in vivo* by different stimuli. Although the exact role of platelets in immunological and inflammatory responses is still controversial, there can be little doubt that they are similar in many respects to other cells, as mast cells and neutrophils, which participate in these reactions. In fact, the platelets are activated by a whole host of different stimuli and release a variety of inflammatory mediators with widely differing actions [18]. It is well known that *in vivo* platelet behavior is affected by either several external factors, like ADP, PAF and prostacyclin released from other cells, or flow disturbance related to vascular defects. The ability of SV-IV to inhibit aggregation also when platelets were isolated from their physiological surroundings and studied in an *in vitro* system strongly suggests an influence of the protein directly on platelet activities. On the other hand, the observation that the effect of SV-IV was retained by holding the platelets in their physiological environment points out the protein as a potential effective drug.

Platelet activation is known to be associated with increases in the activity of PLA_2. The findings that only AA-induced platelet aggregation was unaffected by SV-IV, both *in vivo* and *in vitro*, are in line with our previously reported data [12] describing an inhibitory effect of the protein on PLA_2 activity and, as a consequence, on AA release. Therefore, exogenous AA, either administered *in vivo* or added to the isolated platelets *in vitro*, allowed to

Table 1. SV-IV inhibitory effect on platelet aggregation triggered *in vivo* by different thrombogenic agents

Treatment	Platelet-count ratio*					
	None	Thrombin	ADP	Collagen	PAF	AA
Saline (control)	0.98 ± 0.02	0.54 ± 0.01	0.51 ± 0.02	0.54 ± 0.01	0.55 ± 0.01	0.55 ± 0.01
SV-IV, 0.37 mg/kg	0.99 ± 0.01	0.71 ± 0.02†	0.71 ± 0.02†	0.70 ± 0.02†	0.64 ± 0.01†	0.57 ± 0.01
SV-IV, 0.75 mg/kg	0.97 ± 0.01	0.82 ± 0.02†	0.81 ± 0.01†	0.82 ± 0.01†	0.78 ± 0.02†	0.55 ± 0.01
SV-IV, 1.50 mg/kg	0.98 ± 0.02	0.90 ± 0.01†	0.89 ± 0.01†	0.87 ± 0.02†	0.83 ± 0.01†	0.54 ± 0.02
Ribonuclease, 1.50 mg/kg	0.97 ± 0.02	0.54 ± 0.01	0.50 ± 0.01	0.54 ± 0.01	0.55 ± 0.01	0.55 ± 0.01

* Six animals were used in each group; values are means ± SE [17].

† Significantly different from the respective control values, $P < 0.001$ (Student's *t*-test).

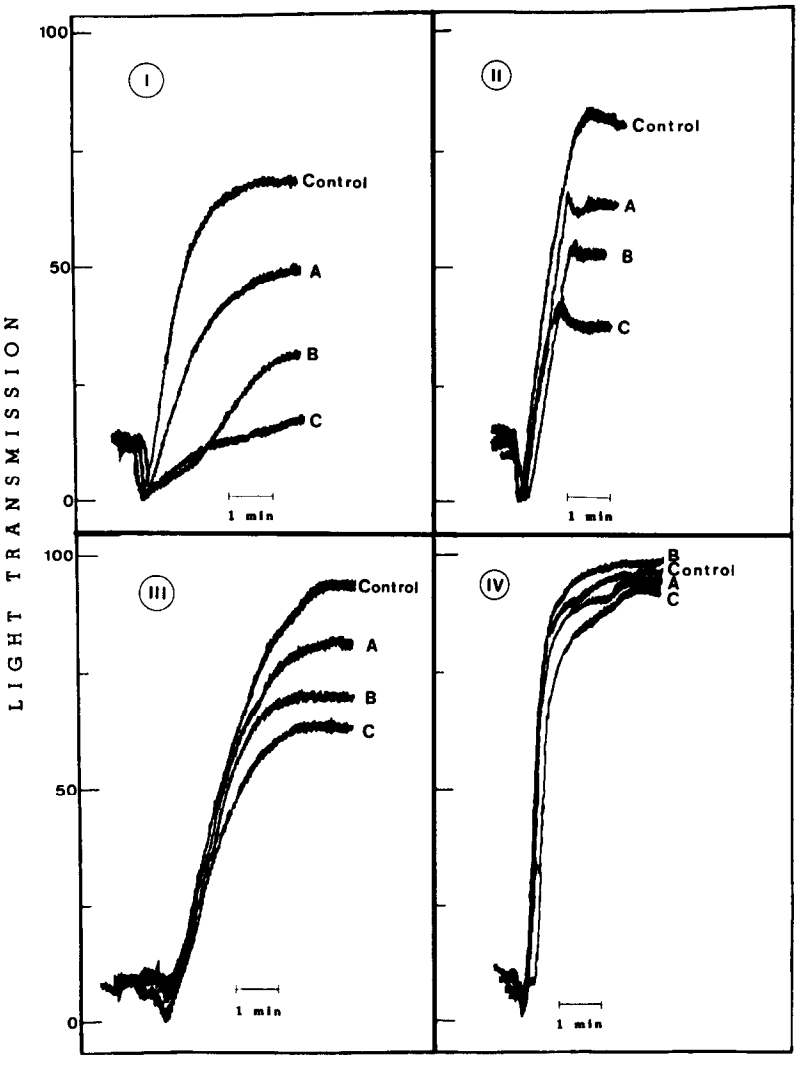

Fig. 2. Individual aggregation tracings for SV-IV-mediated inhibition of platelet aggregation induced *in vitro* by different thrombogenic agents. The platelets were incubated in the presence of either 40 (A), 80 (B), 240 (C) μg/mL SV-IV, or saline (control), before the addition of 40 mUnits/mL thrombin (panel I), 12 μg/mL ADP (panel II), 40 μg/mL collagen (panel III), or 300 μg/mL AA (panel IV). Further experimental details are described in the text.

by-pass PLA_2 inhibition and to synthesize all AA metabolites inducing platelet aggregation. Among these, PAF is known to stimulate PLA_2, being able, thus, to counteract SV-IV inhibitory effect of the enzyme. However, the inhibition by SV-IV of the PAF-induced platelet aggregation observed also in the presence of 1.4 mg/mL indomethacin, an agent blocking thromboxane biosynthesis, suggests that SV-IV specifically prevented the thromboxane-independent aggregating effect of PAF. It should be noted that the concentration of indomethacin used did not significantly affect the aggregation induced by thrombin, ADP and collagen, and that SV-IV exerts a similar inhibitory effect both in the presence and absence of indomethacin (data not shown).

Moreover, particularly intriguing was the observation of the platelet aggregating effect produced by SV-IV *in vivo* when it was administered in high amounts (6 mg/kg) to rats. A similar result was obtained also by testing *in vitro* the aggregating effect of high concentrations of the protein (data not shown). Even though we are presently unable to explain this phenomenon, it is recurrent in our studies on SV-IV the observation of either disappearance of biological activity or appearance, as in this case, of opposite effects when high concentrations of the protein were used (Ref. 12, and unpublished data). However, we can exclude that the aggregating effect of high concentrations of SV-IV was a consequence of platelet lysis since the number of platelets deriving from the treated rats, dissociated and made unaggregable by EDTA, was not different from the number of platelets detected in the rats before treatment.

Finally, since the overall response of platelets to a particular stimulus generally includes different responses other than cell aggregation, the effect of SV-IV on platelet adherence, release of granule contents and liberation of AA metabolites deserve to be investigated.

In summary, the present study documents that platelet aggregation triggered by thrombin, ADP, collagen and PAF both *in vivo* and *in vitro*, was prevented by SV-IV in

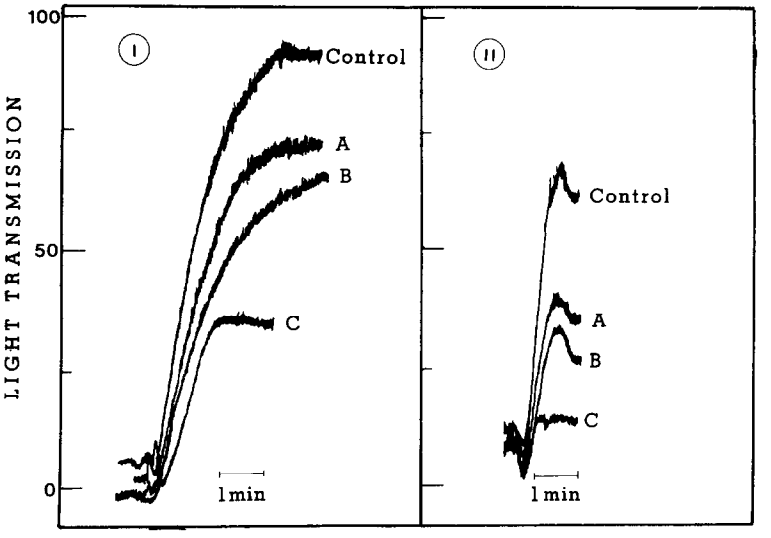

Fig. 3. SV-IV inhibition of PAF-induced platelet aggregation *in vitro*. The platelets were incubated with either 40 (A), 80 (B), 240 (C) μg/mL SV-IV or saline (control), in the absence (panel I) or presence (panel II) of 1.4 mg/mL indomethacin, before the addition of 80 pg/mL PAF. Further experimental details are described in the text.

a dose-dependent manner. Only platelet aggregation by AA was not affected by the protein, thus suggesting a possible involvement of PLA_2 inhibition in the molecular mechanism at the basis of SV-IV anti-thrombotic effect.

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Thyroid hormone analogue SKF L-94901: effects on amino acid and carbohydrate metabolism in rat skeletal muscle *in vitro*

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An analogue of T_3 [SKF L-94901 (SKF 901)] has recently been developed [1]. SKF 901 induces substantial increases in the activity of the hepatic, but not cardiac, mitochondrial enzyme cytochrome *c* 3-phosphoglycerate oxido-reductase and decreases plasma levels of both cholesterol and thyroid stimulating hormone in hypothyroid rats [1]. SKF 901 has equal affinities for receptors in heart and liver. However, SKF 901 stimulates liver, but has little effect on cardiac, function [1].

Carbohydrate, protein and amino acid metabolism is greatly influenced by thyroid hormones *in vivo*. For example, hyperthyroidism causes impaired glucose tolerance in both man and experimental animals [2–7]. Recently, it has been reported that the sensitivity of glycogen synthesis to insulin is decreased in incubated soleus muscle preparations isolated from hyperthyroid rats [8]. Furthermore, hyperthyroidism increases the responsiveness of both the rates of glycolysis and glucose transport to insulin [8]. It is not known how T_3 affects these processes. T_3 increases the rate of protein turnover either by increasing the rate of protein degradation [9, 10] or decreasing the rate of protein synthesis [11] and this largely occurs in skeletal muscle. Therefore, it is important to identify the mechanisms employed by T_3 to alter metabolic regulatory processes in skeletal muscle.

If SKF 901 has T_3-like effects in skeletal muscle then this agent may be utilised to increase the understanding of T_3 action in this tissue. No study has investigated the effects of SKF 901 on carbohydrate and amino acid metabolism in skeletal muscle. Therefore, such a study was undertaken. Rats were administered with either T_3 or SKF 901 for 5 days and the efficacy of both agents *in vivo* was similar (based on relative potency values for T_3 and SKF 901 [1]). The effects of both agents on the rates of lactate formation, glycogen synthesis and glutamine release were studied in stripped soleus muscle *in vitro*. Plasma levels of glucose and lactate and the content of glutamine and glycogen in gastrocnemius and soleus muscles, respectively, were also measured.

Materials and Methods

Rats were made hyperthyroid by daily intraperitoneal injection of T_3 (0.65 μg/g body wt) [8]. SKF 901 (3.25 μg/g body wt) was initially dissolved in small volume of alkaline H_2O before dilution to the appropriate concentration with sterile 0.9% (w/v) NaCl before intraperitoneal administration of SKF 901 at a similar efficacy as compared with T_3 (this calculation was based on the relative potency of the two agents [1]). The time between the last injection and preparation of strips of soleus muscle was 16 hr. Soleus muscle strips were prepared from 14 hr fasted male Wistar rats (Harlan-Olac, Bicester, U.K.; 160–180 g) as previously described [12–14]. The tendons of the muscles were ligated before attachment to stainless steel clips. Muscle strips were pre-incubated in Erlenmeyer flasks containing 3.5 mL Krebs–Ringer bicarbonate buffer plus (mM) *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (7), pH 7.4, glucose (5.5), pyruvate (5), 1.5% (w/v) de-fatted bovine serum albumin. In one experiment (effects of T_3 on rates of glutamine release from soleus muscle) pyruvate was omitted from the pre-incubation buffer and this resulted in lower rates of glutamine release. Other unpublished experiments (Parry-Billings *et al.*) have established that the omission of pyruvate from the pre-incubation medium dramatically reduces the variability of rates of glutamine release in control incubated isolated stripped soleus muscles but does not alter the magnitude of the effects of agents that alter rates of glutamine release. Flasks were sealed and aerated continuously with O_2:CO_2 (19:1, v/v). After pre-incubation of muscles in this medium for 30 min, at 37° in an oscillating water bath, the muscle strips were transferred to similar vials containing identical medium (except pyruvate was omitted) with added [U-^{14}C]glucose (0.5 μCi/mL) and various concentrations of insulin (see