Biochemical Pharmacology, Vol. 37, No. 24, pp. 4743–4745, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

MODERATE ANTICOAGULATION BY SALICYLATE PREVENTS THROMBOSIS WITHOUT BLEEDING COMPLICATIONS

AN EXPERIMENTAL STUDY IN RATS

MARIA CARLA RONCAGLIONI,* INE REYERS, CHIARA CERLETTI,† MARIA BENEDETTA DONATI† and GIOVANNI DE GAETANO†

Laboratory for Haemostasis and Thrombosis Research and Laboratory of Cardiovascular Clinical Pharmacology, Istituto di Ricerche Farmacologiche "Mario Negri", via Eritrea 62, 20157 Milano, Italy

(*Received* 31 *March* 1988; *accepted* 14 *April* 1988)

Abstract—It has previously been shown that salicylate (S) acts as a vitamin K (vit K)-antagonist inducing a decrease in plasma levels of vit K-dependent clotting factors and inhibiting the vit K-dependent carboxylation reaction in the liver. In this study we evaluated whether this biochemical effect had a possible functional role. Indeed, we tested in rats the antithrombotic potency of S (175 mg/kg/i.p. twice a day for 3 days) on experimentally induced venous thrombosis. Its possible haemorrhagic effect was evaluated by measuring the bleeding time. Low-dose warfarin (W) (0.2, 0.1, 0.1 mg/kg/i.v. for 3 days) was utilized as control drug. To check for a possible potentiation between S and W, we tested the effects of their combination (S + W). Thrombotest was used to monitor the anticoagulant effect of each treatment. The incidence of thrombus formation, after venous stasis, was not significantly affected by any of the treatments used, but a significant reduction in thrombus weight was observed after either S or W treatment. Both drugs partially prolonged the Thrombotest without affecting either the bleeding time or the peri-operative mortality (mainly due to internal bleeding). When the combination S + W was used, no significant benefit was observed on the prevention of thrombus incidence or weight, although a marked Thrombotest prolongation was recorded. On the other hand this combination resulted in a pronounced bleeding tendency, as expressed in a significant prolongation of bleeding time and increased total mortality. Thus S, at doses inducing moderate anticoagulation may prevent venous thrombosis without relevant bleeding complications.

On several occasions it has been reported that aspirin and salicylate have a direct effect on the plasma levels of vitamin K (vit K)-dependent coagulation factors [1–4]. More recently it has been demonstrated that salicylate at a relatively high dosage acts as a vit K-antagonist: it lowers the plasma concentration of vit K-dependent coagulation factors and causes accumulation of microsomal substrates for vit K-dependent carboxylase in the liver (e.g. clotting factor precursor) [5]. The same effect is obtained after warfarin treatment [5, 6]. Indeed, the antithrombotic activity of coumarin derivatives is well established, as are their haemorrhagic properties, depending on the dose used [7]. So far, no data are available on the functional relevance *in vivo* of the biochemical effect of salicylate on the vit K-dependent carboxylase system.

The aim of our study was to investigate a possible functional role of salicylate as a vit K-antagonist on experimentally induced venous thrombosis (in order to assess its antithrombotic potency) and on bleeding time (in order to study its haemorrhagic properties).

* To whom all correspondence should be addressed.

† Present address: Istituto di Ricerche Farmacologiche Mario Negri, Consorzio "Mario Negri Sud", Centro di Ricerche Biomediche e Farmacologiche, 66030 S. Maria Imbaro, Italy.

Relatively high doses of salicyate are required for significant reduction of clotting factors in plasma. We thus adopted a treatment schedule in which salicylate was administered at the dose of 175 mg/kg/body wt twice a day for three days, alone or in combination with a low dose of warfarin that only slightly prolonged the Thrombotest. This enabled us to check whether the effect of the two drugs together was greater than that of the single treatments.

MATERIALS AND METHODS

Animals and drugs. Male CD COBS rats (300–350 g b.w.) were used. The rats had free access to drinking water and standard food (Altromin, Rieper, Bolzano, Italy). Sodium salicylate (Farmitalia-Carlo Erba, Milano, Italy) and racemic sodium warfarin (Coumadin, Endo Lab., Garden City, NY) were freshly dissolved in isotonic saline.

Experimental system. The animals were divided into four separate groups: Group 1—control, received only saline (C); Group 2—received only salicylate (S); Group 3—received only warfarin (W); Group 4—received both salicylate and warfarin (S + W).

The animals receiving warfarin were given the following doses i.v. at 8 a.m.: day 1—0.2 mg/kg; day 2—0.1 mg/kg; day 3—0.1 mg/kg.

The salicylate group received two doses per day of 175 mg/kg/i.p. (8 a.m. and 8 p.m.). The salicylate plus warfarin group received the combined doses.

Thrombotest (Immuno, Pisa, Italy) was performed daily at 9 a.m. on 20 μl blood collected from the tail after snipping the tip. "Template" bleeding time was measured just before the last Thrombotest determination using a standardized template device [8]. Immediately thereafter, venous stasis of the inferior vena cava was induced as previously described [9], under sodium pentobarbitone anaesthesia (40 mg/kg b.w./i.p.). Briefly, the abdomen was opened and the vena cava carefully dissected. A tight ligature (with cotton thread) was placed around it just below the left renal vein. Thereafter, the abdominal incision was closed. After 2 hours the abdomen was reopened under anaesthesia, the thrombus, if present, was removed, rinsed in distilled water, blotted on filter paper and placed in a desiccator; 24 hr later the weight of the dry thrombus was recorded. The results are reported as percentage of thrombus incidence and as thrombus weight [9].

Statistical analysis. Data were statistically analyzed by Duncan's multiple range test.

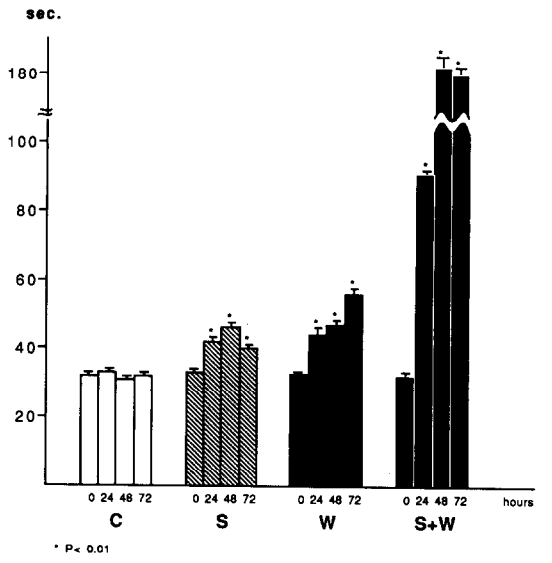

Fig. 1. Values (sec) of Thrombotest performed every 24 hr from the beginning of treatment with either warfarin or salicylate or the combination.

RESULTS

Effects of salicylate or warfarin alone

Figure 1 shows the Thrombotest values of the three drug regimens measured every 24 hr from the beginning of treatment. Either drug alone induced a moderate but significant prolongation, and the combination resulted in much greater prolongation of the Thrombotest.

In order to assess the antithrombotic potency of salicylate, alone or in combination with warfarin, we tested the efficacy of S, W and S + W treatment on an experimental model of venous thrombosis in rats. The incidence of thrombus formation after stasis was not significantly affected by any of the treatments used (Table 1).

A significant ($P < 0.05$) reduction of thrombus weight was observed after S or W treatment alone.

Overall mortality was very low during both treatments. At these doses S and W did not affect "template" bleeding time. Thus salicylate, like warfarin, appeared to protect from thrombus formation without relevant bleeding complications.

Effects of the combination salicylate plus warfarin

When the combination S + W was used instead of either drug alone, a clear though not significant benefit was observed on the reduction of thrombus incidence or weight (Table 1). Administration of S + W together resulted in a marked prolongation of Thrombotest and a pronounced bleeding tendency. Indeed, strong prolongation of bleeding time was observed associated with increased total mortality (mainly due to internal bleeding) when compared with the two drugs alone (Table 1). As 10 out of 22 rats died during the combined treatment, thrombus incidence and weight could only be measured on a small group of animals (12 and 5, respectively).

DISCUSSION

In this study salicylate at a dose which had a moderate anticoagulant effect (as reflected by a 50% prolongation of Thrombotest values) may help prevent venous thrombosis (as indicated by the reduction of thrombus weight after experimental

Table 1. Effect of salicylate (S), warfarin (W), and the combination (W + S) on death rate, stasis-induced venous thrombosis and bleeding time in rats

	Control	Salicylate	Warfarin	Warfarin + salicylate
Death rate	0/18	2/18	0/18	10/22
Thrombus incidence	10/18 (56%)	8/16 (50%)	9/18 (50%)	5/12 (42%)
Thrombus weight (mg)	4.77 ± 1.11 (N = 10)	2.02 ± 0.48* (N = 8)	1.80 ± 0.25* (N = 9)	3.13 ± 1.04 (N = 5)
Bleeding time (sec)	126 ± 11 (N = 18)	112 ± 6 (N = 18)	125 ± 10 (N = 18)	316 ± 47† (N = 22)

* $P < 0.05$ vs control.
† $P < 0.01$ vs control.

venous stasis). In this respect salicylate behaves similarly to a dose of warfarin which, given on three consecutive days, resulted in a comparable degree of anticoagulation. The antithrombotic effect of S or W was not accompanied by concomitant haemorrhagic risk, and neither treatment prolonged bleeding time or affected peri-operative mortality.

Anticoagulation reduces the activity of FII, IX and X less rapidly and extensively than FVII [10]. Although we did not measure individual vit K-dependent clotting factor activities in this study, it is likely that the decrease in FVII is greater than reflected by Thrombotest measurements. On the other hand, epidemiological data have shown that moderate increases of FVII levels are significantly associated with a higher incidence of ischaemic heart disease [11]. Based on this observation it is reasonable to postulate that a moderate reduction of FVII levels may result in protection against thromboembolic events. Following this reasoning, a study is in progress (Meade *et al.*, personal communication) to test the primary prevention of coronary artery disease by low-dose warfarin. The objective is to reduce FVII levels to about 70% of the controls.

Our experimental study, despite the obvious limitations of the model, supports the concept that moderate anticoagulation results in significant protection against thrombosis. The obvious advantage of this approach is the lack of bleeding complications. As observed in our animals treated with the combination of salicylate and warfarin, full anticoagulation was associated with significant prolongation of bleeding time and almost 50% perioperative mortality. This unwanted effect may have masked any possible beneficial effect of the drug combination in thrombosis prevention.

It was not the purpose of the present study to investigate whether potentiation of the anticoagulant effect of warfarin by salicylate was due to a well-known pharmacokinetic interaction (competition for protein binding) or to the additive pharmacodynamic effect on vit K-dependent enzymatic system [7]. The observation that salicylate shares with warfarin not only its biochemical mechanism of action on vit K-dependent carboxylase [5], but also its thrombosis-prevention efficacy while inducing only a moderate anticoagulant effect is of interest.

More than 30 years ago, Craven [12] administered aspirin to about 8000 men and reported "not a single case of detectable coronary or cerebral thrombosis". Craven believed that aspirin was "a substance with less pronounced yet predictable anticoagulant action" than oral anticoagulants and also performed a successful pilot study with small amounts of dicoumarol, giving an anticoagulant effect similar to that of aspirin in the dosage he employed (325–600 mg/daily). Although Craven's fascinating paper has only historical value today, the possibility that the moderate anticoagulant effect of aspirin may contribute to its antithrombotic efficacy must be taken into serious consideration, at least in some selected clinical conditions, mainly of venous thrombosis.

Acknowledgements—This work was supported in part by the Italian National Research Council, Grant No. 8600203.04.

Judith Baggott, Ivana Garimoldi and the Gustav A. Pfeiffer Memorial Library Staff helped prepare the manuscript.

REFERENCES

1. Quick AJ and Clesceri L, Influence of acetylsalicylic acid and salicylamide on the coagulation of blood. *J Pharmacol Exp Ther* **128**: 95–98, 1960.
2. Loew D and Vinazzer M, Dose-dependent influence of acetylsalicylic acid on platelet functions and plasmatic coagulation factors. *Haemostasis* **5**: 239–249, 1976.
3. Park BK and Leck JB, On the mechanism of salicylate-induced hypothrombinaemia. *J Pharm Pharmacol* **33**: 25–28, 1981.
4. Hildebrandt EF and Suttie JW, The effects of salicylate on enzymes of vitamin K metabolism. *J Pharm Pharmac* **35**: 421–426, 1983.
5. Roncaglioni MC, Ulrich MMW, Muller AD, Soute BAM, de Boer-van den Berg MAG and Vermeer C, The vitamin K-antagonism of salicylate and warfarin. *Thromb Res* **42**: 727–736, 1986.
6. Suttie JW, Mechanism of action of vitamin K: synthesis of gamma-carboxyglutamic acid. *CRC Crit Rev Biochem* **8**: 191–223, 1980.
7. O'Reilly RA, Anticoagulant, antithrombotic, and thrombolytic-drugs. In: *The Pharmacological Basis of Therapeutics* 7th Ed (Eds. Goodman Gilman A, Goodman LS, Rall TW and Murad F), pp. 1338–1159. Macmillan, New York, 1985.
8. Dejana E, Villa S and de Gaetano G, Bleeding time in rats: a comparison of different experimental conditions, *Thromb Haemost* **48**: 108–111, 1982.
9. Reyers I, Mysliewiec M, Mussoni L and Donati MB, Stasis-induced venous thrombosis. In: *Standardization of Animal Models of Thrombosis.* (Eds. Breddin K and Zimmerman R), pp. 99–104. Schattauer, Stuttgard, 1983.
10. Loelinger EA, van der Esch B, Mattern MJ and den Brabander AS, Behaviour of factors II, VII, IX and X during long-term treatment with coumarin. *Thromb Diathes Haemorr* **9**: 74–89, 1963.
11. Meade TW, Mellows S, Brozovic M, Miller GJ, Chakrabarti RR, North WRS, Haines AP, Stirling Y, Imeson JD and Thompson SG, Haemostatic function and ischaemic heart disease: principal results of the Northwick Park Heart Study. *Lancet* **2**: 533–537, 1986.
12. Craven LL, Prevention of coronary and cerebral thrombosis. *Mississippi Valley Med J* **78**: 213–215, 1956.