ALBUMIN STABILIZES THROMBOXANE A_2

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1. Introduction

Recent work on the conversion of arachidonic acid and the prostaglandin endoperoxides in platelets into thromboxane B_2 (TXB_2) led to the discovery of an unstable, biologically very active intermediate, thromboxane A_2 (TXA_2) [1,2] (fig.1). This compound was shown to constitute the major component of the previously recognized, elusive rabbit aorta-contracting substance [3]. The conversion into thromboxanes represents the major pathway in the metabolism of prostaglandin endoperoxides in platelets [4]. TXA_2 is very potent in inducing irreversible platelet aggregation and has a half-life of around 30 s in aqueous medium at 37°C [2]. Considerably longer half-lives of the compound were found at lower temperatures [5,6]. The half-life was also increased in the presence of plasma [5,7]. Recently, a simple and sensitive radioimmunoassay was developed for the quantitative determination of TXA_2, based on its rapid conversion into a stable mono-*O*-methyl derivative of TXA_2 by excess amounts of methanol [5] (fig.1). The present paper is the first application of this method to the study of the properties of TXA_2 under various conditions. The half-life of TXA_2 was 31–35 s between pH 7.4 and pH 9 and decreased at higher or lower pH. Addition of albumin increased the half-life, probably because of binding to this protein. Certain compounds, like bilirubin, acetylsalicylic acid, phenylbutazone, sulfa and warfarin decreased the protective effect of albumin.

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2. Experimental

Platelet rich plasma (PRP), platelet poor plasma (PPP) and suspensions of washed human platelets were

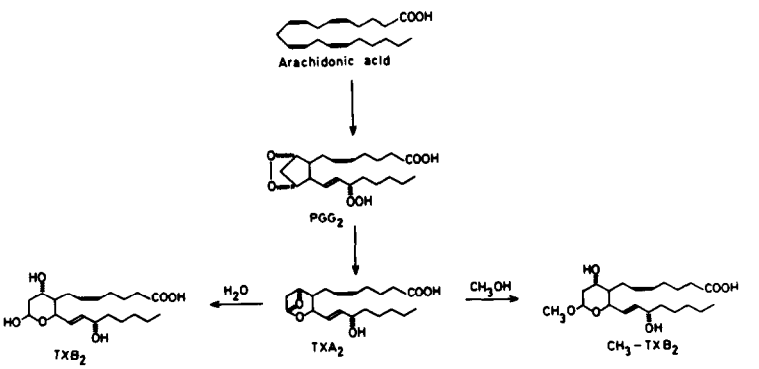

Fig.1. Formation of TXA_2 and TXB_2 from arachidonic acid, and the derivatization of TXA_2 into mono-*O*-methyl TXB_2 by addition of methanol.

prepared from blood collected from healthy donors, who had not taken any drugs for at least one week, according to the earlier described procedures [5,8,9]. The platelet suspensions contained about 500 000 platelets/μl. Studies on the generation and breakdown of thromboxanes were carried out according to the procedure in refs. [5,9]. Briefly, TXA_2 was generated by incubation of arachidonic acid with a platelet suspension, or by incubation of ammonium arachidonate with PRP, at 37°C. After 30–40 s, the platelets were rapidly removed by filtration [5]. A sample of the clear, platelet-free filtrate was added with stirring to a 37°C solution of the different compounds to be tested for effect on the half-life of TXA_2. Aliquots, 100 μl, were removed with short intervals and subjected to addition of 2.5 ml methanol. Aliquots of these methanol treated samples were taken to dryness prior to measurement of TXB_2 and mono-*O*-methyl TXB_2 by radioimmunoassay [5].

Human serum albumin (HSA), transferrin and haptoglobin were from Kabi AB, Stockholm, Sweden. Essentially fatty acid free human albumin was from Sigma Chemical Co., St Louis, MO. Prealbumin was a generous gift from Dr P. A. Pettersson, The Biomedical Center, Uppsala.

3. Results

The stability of TXA_2 was studied at different pH values. The generation of TXA_2 was carried out using washed platelets, at pH 7.4, and after filtration 0.9 ml filtrate was added to 100 μl 1 M buffer of the desired pH. The actual pH of the mixture was measured after the experiment. TXA_2 was found to be most stable between pH 7.4 and pH 9.0 ($t_{1/2}$ 31–35 s). At more alkaline pH the half-life of the compound was considerably shorter. Acidification proved to be even more deleterious: already at pH 6.9, TXA_2 was so rapidly broken down that no accurate estimation of $t_{1/2}$ was possible.

Figure 2 shows the results obtained in PRP. The half-life was found to be about 3 min, as compared to around 30 s in washed platelet suspensions. The greatly increased stability of TXA_2 found in PRP was also seen when the compound was generated by washed platelets and after filtration added to PPP. Different amounts of PPP were also tried and when

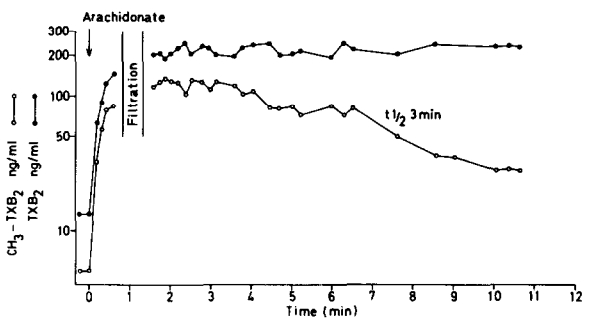

Fig.2. TXB_2 and mono-*O*-methyl TXB_2 levels after addition of ammonium arachidonate to PRP (300 μg/ml). Samples were withdrawn before and after filtration. See ref. [5] for a comparison with the results from a similar experiment carried out in the absence of albumin (washed platelets; $t_{1/2}$ 32 s).

the PPP concentration exceeded 40% of the physiological one, TXA_2 was as stable as in the PRP experiment above.

The stabilizing factor present in plasma was sought. EDTA and Ca^{2+} were without effect on the half-life of TXA_2. PPP was lyophilized and subjected to chromatography on a Sephadex G-150 column to remove the low molecular weight fraction. The remaining high molecular weight fraction was as effective in stabilizing TXA_2 as PPP itself, and the stabilizing activity of PPP seemed to be associated with the albumin fraction. No TXA_2 stabilizing activity was seen associated with the proteins eluted before albumin.

To test whether albumin was identical with the TXA_2 stabilizing factor in plasma, HSA was obtained, and also HSA essentially free from fatty acids. At pH 7.4, these protein preparations were almost as efficient as PPP in prolonging the half-life of TXA_2 ($t_{1/2}$ 2.83–2.92 min, in the presence of physiological concentration of HSA (45 mg/ml) (fig.3)). No difference was seen between the two different albumin preparations.

According to the manufacturer, the HSA preparation contained small amounts of transferrin, haptoglobin and prealbumin. These proteins were obtained and investigated in similar experiments. No stabilizing activity was observed for any of these compounds when tested in physiological concentrations. The HSA was also purified by polyacrylamide gel electropho-

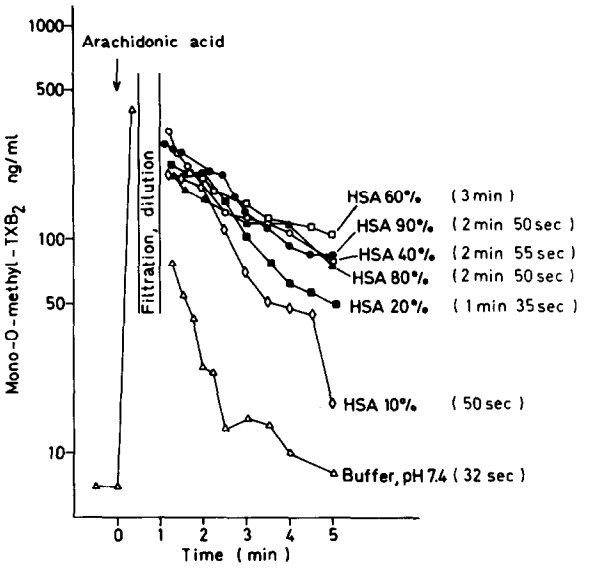

Fig.3. Half-life of TXA_2 in the presence of either buffer, pH 7.4, or human serum albumin. Percentages indicate % physiological concentrations (for HSA 45 mg/ml). $t_{1/2}$ of TXA_2 given in parentheses for each experiment.

resis [10]. After this step, no traces of the contaminating proteins could be detected. This pure albumin had a TXA_2 stabilizing capacity identical to that of the HSA before purification.

In order to explore the possible molecular interactions between other organic compounds, known to be subject to albumin binding, and TXA_2 in this respect, the following compounds were checked: testosterone, progesterone, estrone, β-estradiol, bilirubin, vitamin K_1, warfarin, barbituric acid, acetylsalicylic acid, phenylbutazone (Sigma Chemical Co.) and sulfadimidine (AB Ferrosan, Malmö, Sweden). All these compounds were preincubated overnight in a 10-fold excess on a molar basis with HSA (20 mg HSA/ml water) at pH 7.4. When these preparations were tested for effect on the TXA_2 half-life, it was found that several of these substances did not alter the stability of TXA_2. Five of the compounds, however, markedly reduced the half-life of TXA_2 in the presence of albumin, viz. acetylsalicylic acid, phenylbutazone, bilirubin, warfarin ($t_{1/2}$ of TXA_2, about 1 min in all these experiments), and sulfadimidine ($t_{1/2}$ about 1.25 min). Control experiments were carried out with all these compounds in the absence of albumin; none of them showed any influence on TXA_2 stability ($t_{1/2}$ 30–35 s in all experiments).

4. Discussion

The persistence of TXA_2 in plasma has been noted earlier [5,7]. In the experiments by Smith et al. [7], this phenomenon could to some extent be explained by a continuous production of the compound in PRP. In the present investigation, however, this possibility has been ruled out, because the platelets were removed from the incubation before the determination of the $t_{1/2}$ of TXA_2 was started, and because the same slow decline in the TXA_2 concentration was seen when PPP was added to a platelet-free filtrate containing the compound. Thus, the persistence of TXA_2 in plasma seems to be entirely caused by an increased stability of the compound under these conditions.

It was stated in ref. [7] that plasma could be replaced by serum, but not by albumin or any of the Cohn fractions I–VI. In contrast, we found in this investigation that the TXA_2-stabilizing activity of plasma could be attributed to albumin. This was shown both by Sephadex chromatography of lyophilized PPP, by comparison of the properties of PPP with those of a commercial, 98% pure HSA preparation as well as a further purified HSA where no contaminating proteins could be detected, and finally by investigating the properties of several other plasma proteins in this respect. The reason for the discrepancies between our results and those of Smith et al. is not clear. However, we found that the pH greatly influenced the stability of TXA_2. If the pH of the protein solutions was not adjusted to 7.5–8 in the experiments described in [7], this may well be the reason why these authors did not find any prolongation of the $t_{1/2}$ of TXA_2 by albumin.

The increased stability of TXA_2 in the presence of albumin is most likely caused by binding of TXA_2 to this protein. It is well known that albumin can bind fatty acids tightly and in large amounts, in contrast to almost all other proteins (review [11]). Albumin binding of prostaglandins has earlier been demonstrated by several investigators [12,13]; the phenomenon is more pronounced for less polar prostaglandins [13,14]. Thus it is likely that a compound such as TXA_2 is bound to a considerable degree by albumin.

Hormones and drugs are generally inactive when bound to albumin. However, this binding is reversible and should rather be regarded as a storage phenomenon. Thus, the biological effects of active substances are prolonged as they are protected from metabolism and excretion in the bound state (cf. [15,16]). In the case of TXA_2, also the non-enzymatic, extremely rapid degradation in aqueous medium is slowed down by albumin, and no doubt this is of a considerable importance in vivo, e.g., for the propagation of the platelet aggregation during hemostasis. For the same reason, it is likely that compounds that influence the albumin binding of TXA_2 also interfere with the biological activity of this substance. Several compounds were found to inhibit the binding of TXA_2 to albumin – whether by competition alone or by other mechanisms as well is not known at present – viz., acetylsalicylic acid, phenylbutazone, bilirubin, sulfadimidine and warfarin. Acetylsalicylic acid has earlier been found to inhibit the binding of prostaglandins to albumin [16,17]. Further studies on the influence of these compounds on the albumin binding and biological activity of TXA_2 are in progress in our laboratory.

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