J. Enzyme Inhibition, 1995, Vol. 9, pp. 253–261
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DEVELOPMENT OF A SCREENING ASSAY FOR THE *IN VITRO* EVALUATION OF THROMBOXANE A_2 SYNTHASE INHIBITORS

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(Received 23 March 1995)

Inhibitors of thromboxane A_2 (TxA_2) synthase are regarded as potentially useful agents in the treatment of cardiovascular diseases and in the prevention of tumour cell metastases. We report here a novel *in vitro* assay for the evaluation of TxA_2 synthase inhibitors. For the determination of inhibitory activity, malondialdehyde (MDA) formation by TxA_2 synthase in whole blood was utilized. After reaction with thiobarbituric acid MDA was quantified spectrofluorimetrically. The blank value was obtained by incubation with a selective TxA_2 synthase inhibitor. For the screening of compounds the simple MDA assay represents an alternative to the rather expensive and time consuming radioimmunoassay, HPLC and TLC methods. Only for compounds which have been shown to be good inhibitors in the MDA assay should a radioimmunoassay for selective inhibition of TxA_2 synthase be performed.

KEY WORDS: *In vitro* assay, thromboxane A_2 synthase, malondialdehyde formation

INTRODUCTION

Inhibitors of thromboxane A_2 (TxA_2) synthase are of special interest in the treatment of cardiovascular diseases, since TxA_2 is a potent vasoconstrictory and platelet aggregating agent. Furthermore TxA_2 seems to play a pivotal role in tumour cell metastasis.[1]

For the evaluation of TxA_2 synthase inhibitory activity of compounds besides TLC[2] and HPLC methods[3,4] a TxB_2 radioimmunoassay (RIA) method is most often used.[5] TxB_2 is the stable metabolite of TxA_2, one of the products formed by TxA_2 synthase. As shown in Figure 1 TxA_2 synthase — a cytochrome P450 enzyme[6] — catalyzes the reaction of prostaglandin H_2 (PGH_2) to yield TxA_2, malondialdehyde (MDA) and 12-OH-heptadecatrienoic acid (HHT) in a 1:1:1 ratio.[7]

The novel *in vitro* assay makes use of the MDA formation during TxA_2 synthase catalysis. Measurements of MDA have already been performed, the objective, however, was different: In the beginning of the past decade platelet MDA was thought to be only a degradation product of endoperoxide metabolism. Therefore it was common to determine MDA levels as an indicator for platelet regeneration and survival time.[8]

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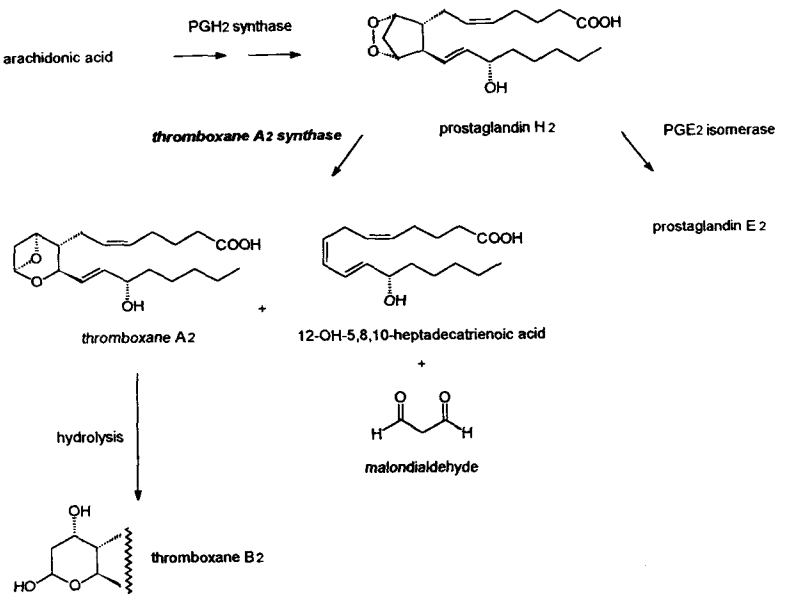

FIGURE 1 Arachidonic acid cascade: TxA_2 synthase catalyzed reaction.

The present work describes an assay which could be a reasonable alternative to TLC, HPLC and RIA methods. Since it is neither time consuming nor expensive, the assay is suitable for the screening of a large number of compounds.

MATERIALS AND METHODS

Chemicals

Dazoxiben HCl was kindly provided by Dr. Roger P. Dickinson from Pfizer Central Research (Sandwich, Kent, U.K.). Biochemicals were obtained from Sigma (Deisenhofen, FRG), Serva (Heidelberg, FRG) and Fluka (Neu-Ulm, FRG), collagen suspension was purchased from Nycomed (München, FRG), percoll® from Pharmacia (Uppsala, Sweden).

Enzyme Sources

Three different enzyme sources were used: citrated whole blood, a platelet suspension and the 105,000×g fraction of a platelet homogenate.

Human blood was collected into sodium citrate (Monovette®, Sarstedt, FRG). Donors were healthy volunteers free of medication for at least one week. The blood has to be used within 60 min after withdrawal.

Platelets were isolated using a percoll® density gradient centrifugation. 20 ml of citrated whole blood yield 6 ml of platelet suspension. The platelet suspension has to be used within 60 min after blood withdrawal.

The 105,000×g fraction of a platelet homogenate (microsomes, nuclei and mitochondria) was prepared by the method of Needleman *et al.*[9] with slight modifications (platelet rich plasma was homogenized and immediately centrifuged at 105,000×g). Storage of the platelet homogenate at −26°C up to one week was possible (no investigations were performed as to how long the preparation retains its activity).

Incubation Procedure

The assay was performed in 1.5 ml micro test tubes (Eppendorf). Citrated whole blood (0.5 ml) was preincubated with inhibitor (in 10 μl ethanol/potassium sodium phosphate buffer, 0.01 M KH_2PO_4, 0.05 M Na_2HPO_4, pH 7.4 (1:1/V:V); control: vehicle; blank: dazoxiben HCl, 100 μM) for 10 min at 37°C. 50 μl collagen suspension (final concentration: 53.6 μg/ml) was added and incubation continued for another 10 min at 37°C. The reaction was terminated by the addition of 0.4 ml trichloroacetic acid (20% trichloroacetic acid in 0.6 M HCl). The tubes were then centrifuged for 10 min at 4,400×g.

For incubations with a platelet suspension or platelet microsomes 0.5 ml platelet suspension or 0.5 ml of a microsomal suspension (containing 1000 μg protein) was used.

Malondialdehyde Determination

0.5 ml of the supernatant was pipetted into 0.5 ml of thiobarbituric acid (TBA) solution (0.53% TBA in potassium sodium phosphate buffer, 0.01 M KH_2PO_4, 0.05 M Na_2HPO_4, pH 7.4). After heating for 30 min at 70°C and cooling for another 30 min at ambient temperature, the samples were measured spectrofluorimetrically (λ-excitation: 533 nm, λ-emission: 550 nm; Fluorescence Spectrophotometer F-2000, Hitachi).

Calibration Curve

To obtain the reaction product of malondialdehyde with thiobarbituric acid, malondialdehydebis(diethylacetal) (= 1,1,3,3-tetraethoxypropane) was used. Incubation was performed with a dilution of 1,1,3,3-tetraethoxypropane in either citrated plasma (derived from citrated whole blood by centrifugation at 2,200×g, 4°C, 15 min) or potassium sodium phosphate buffer (0.01 M KH_2PO_4, 0.05 M Na_2HPO_4, pH 7.4) instead of citrated whole blood. The procedure described above was then followed.

Selective Inhibition of TxA_2 Synthase

The selectivity of compounds for TxA_2 synthase was assessed by evaluating the production of TxB_2 and PGE_2 in citrated whole blood. The incubation procedure was slightly modified: 0.75 ml citrated whole blood was preincubated with inhibitor

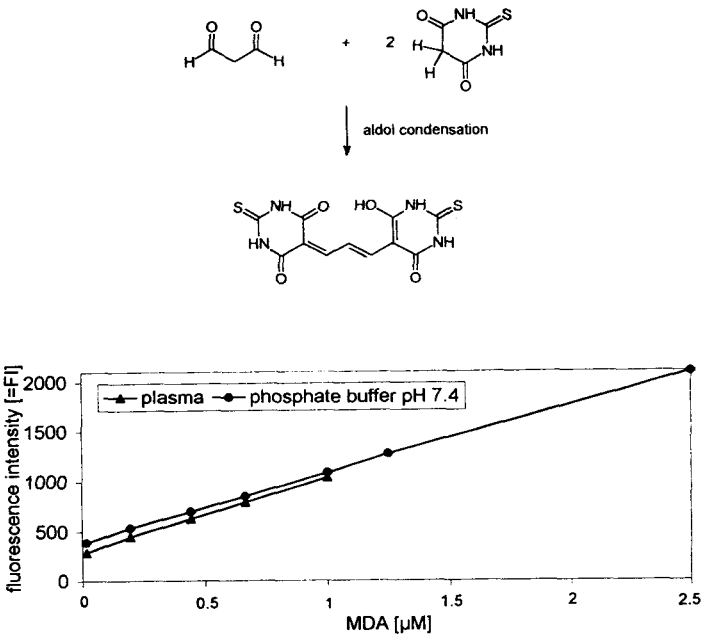

FIGURE 2 Determination of malondialdehyde with thiobarbituric acid; calibration curve of malondialdehyde in plasma and phosphate buffer, pH 7.4; SD≤0.5%; n=4.

(in 30 μl ethanol/potassium sodium phosphate buffer, 0.01 M KH_2PO_4, 0.05 M Na_2HPO_4, pH 7.4 (1:3/V:V)) for 10 min at 37°C. 30 μl collagen suspension (final concentration: 2 μg/ml) was added and incubation continued for another 10 min at 37°C. The plasma was then separated by centrifugation (2,000×g, 4°C, 15 min), collected and stored at −70°C. TxB_2 as well as PGE_2 concentration were determined by radioimmunoassay (DRG Instruments, Marburg, FRG).

RESULTS

MDA can easily be determined by reaction with thiobarbituric acid:[10] a fluorescent product (λ-ex: 533 nm; λ-em: 550 nm) is formed. The MDA calibration curve shows linearity throughout the assay range (see Figure 2). This is true for plasma as well as for phosphate buffer (pH 7.4).

As enzyme source citrated whole blood was used first, collagen served as initiator of the enzyme reaction leading to the formation of prostaglandin H_2.[11] Figure 3 shows the time course of MDA formation during the preincubation and incubation period. Since MDA is also a product of processes other than TxA_2 synthase, dazoxiben, a selective TxA_2 synthase inhibitor,[12] was used for the determination of the incubation blank.

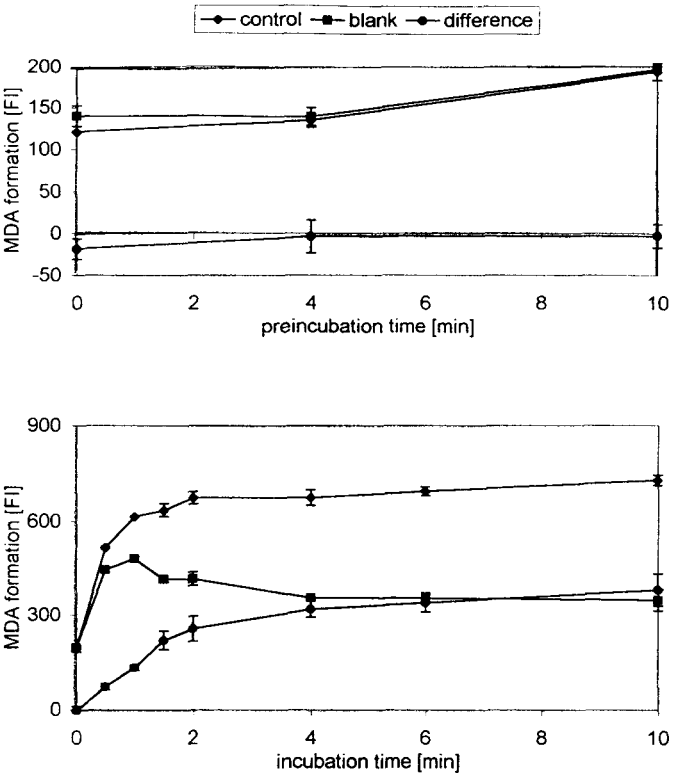

FIGURE 3 Time course of MDA formation during preincubation and incubation period; upper diagram, incubation time: 0 min; lower diagram, preincubation time: 10 min (control values: incubations with vehicle only; blank values: incubations with dazoxiben [100 μM]; difference: difference between control and blank value); FI = fluorescence intensity; n = 4.

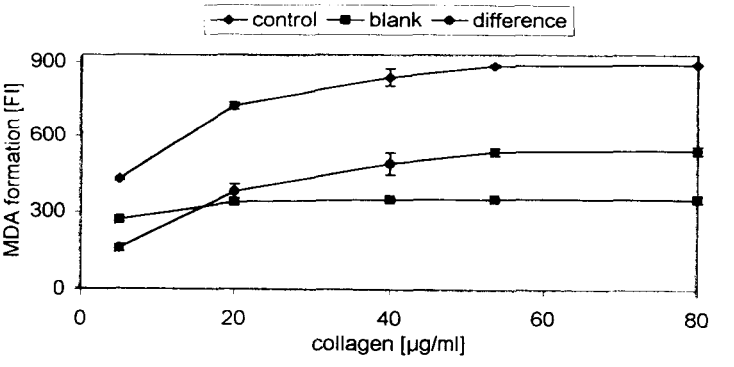

FIGURE 4 Effect of collagen concentration on MDA formation; n=4.

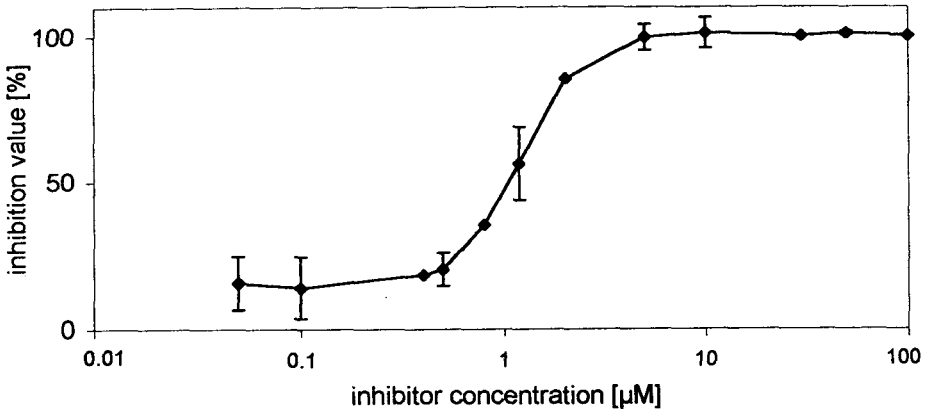

FIGURE 5 Determination of the IC_{50} value of dazoxiben (IC_{50} = 1.1 μM); the error bars represent the mean±SD of four experiments with duplicate determinations.

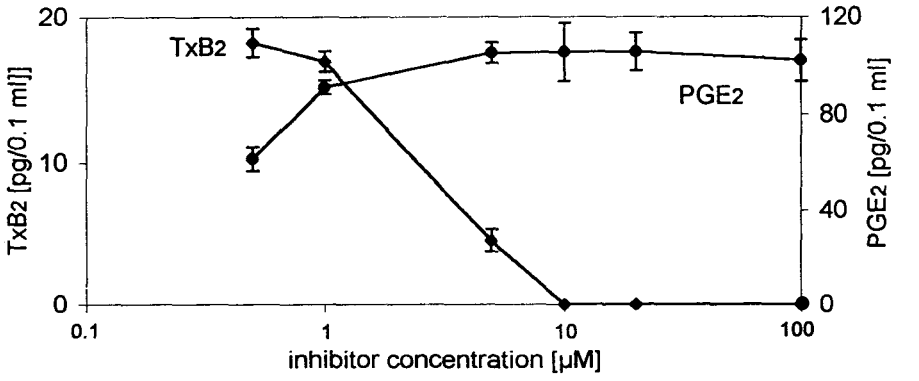

FIGURE 6 Selective TxA_2 synthase inhibition by dazoxiben (IC_{50} = 2.8 μM) leading to an increased PGE_2 level; n = 2; control values: 18.0 pg TxB_2/0.1 ml; 43 pg PGE_2/0.1 ml).

The concentration of 100 μM used is sufficient for total blockade of TxA_2 synthase (see Figure 6).

A 10 min incubation period was chosen, since MDA formation reaches a constant level by this time.

The effect of collagen concentration on MDA formation is shown in Figure 4. 30 μg collagen (= 53.6 μg/ml) are necessary to obtain a high difference between blank and control value. Lower collagen concentrations lead to increasing standard deviations.

Figure 5 demonstrates the dose response curve for dazoxiben (IC_{50}=1.1 μM). Regarding standard deviations of the inhibition values similar results were obtained with a platelet suspension as enzyme source. The 105,000×g fraction of a platelet homogenate, however, leads to values with very high standard deviations (data not shown).

Compounds which have been shown to be good inhibitors in the MDA assay are required to be tested for selective inhibition of TxA_2 synthase, since inhibition of one of the preceding enzymes in the arachidonic acid cascade such as phospholipase A_2 (PLA_2) or prostaglandin H_2 synthase could also result in reduced MDA concentrations. Only compounds showing a concentration dependent decrease in TxB_2 formation *and* a simultaneous increase in prostaglandin E_2 (PGE_2) formation[13,14] are selective inhibitors of TxA_2 synthase, since blockade of the TxA_2 synthase leads to cumulation of PGH_2 and thus to a shift to PGE_2 formation (see Figure 1). In the case of a PGH_2 synthase or PLA_2 inhibition both, TxB_2 as well as PGE_2 concentration, would be decreased. The results of a test for selective TxA_2 synthase inhibition are illustrated by dazoxiben in Figure 6.

DISCUSSION

It has to be noted that MDA is not only formed by TxA_2 synthase; MDA is well known to be generated during fatty acid peroxidation.[15] Besides, 12-OH-5,8,10-heptadecatrienoic acid (HHT) and MDA — not TxA_2 — can be formed by reaction with hemin in the absence of TxA_2 synthase.[16] This has to be taken into account for an assay that utilizes the measurement of MDA formation by TxA_2 synthase.

In contrast to other *in vitro* assays it is not possible to obtain blank values by denaturation of the enzyme *before* the addition of substrate (here: collagen for the initiation of the arachidonic acid cascade). Incubations in which the enzyme reaction is stopped before the incubation has been started lack the MDA that is formed independently of TxA_2 synthase during the incubation (see time course of the blank in Figure 3). Thus the resulting blank would be too low.

Therefore an incubation with dazoxiben leading to a total blockade of TxA_2 synthase is used as a blank. The MDA formed by TxA_2 synthase is calculated as the difference between blank and control (see time course of the difference in Figure 3).

To our knowledge it is not yet clarified whether MDA formation by platelets is terminated within approximately 2–4 minutes or whether the reaction reaches a steady state of formation and degradation at that time.

As "substrate" of the enzyme reaction collagen is used leading to the formation of PGH_2. It is also possible to take for example PGH_2, arachidonic acid or U46619[17] — a PGH_2 analogue — as substrate. But the use of collagen imitates the situation after vessel wall injury *in vivo*, when free collagen fibrils are presented to the circulating blood.

For daily routine testing it might be more convenient to use platelet microsomes as enzyme source instead of fresh citrated blood. However, it was not possible to obtain reliable inhibition values with a platelet 105,000×g fraction using the MDA

assay, since only small differences between blank and control could be obtained as a result of low MDA formation. On the other hand the usage of whole blood offers several advantages: It is the very natural surrounding for the action of TxA_2 synthase inhibitors. Furthermore, it is important to know whether inhibitors are able to penetrate the cell membrane as well as whether their inhibitory activity is maintained in the presence of plasma proteins and blood cells. A good *in vitro-in vivo* correlation in subsequent experiments can be expected. Besides, whole blood is a very simple and convenient enzyme source.

The IC_{50} values obtained for dazoxiben — 1.1 μM in the MDA assay and 2.8 μM in the test for selective inhibition — are in the range of IC_{50} values published by other groups (0.5–9 μM).[18–20] This underlines the reliability of the MDA assay. Keeping in mind that the final experiment for inhibitors has to be the test for selective inhibition of TxA_2 synthase, the present method is a simple, time and cost saving assay for a broad screening of TxA_2 synthase inhibitors.

Acknowledgements

The helpful discussions with Dr. T. Sergejew during the course of this work are gratefully acknowledged. Thanks are also due to Pfizer Research for providing us with a sample of dazoxiben and to the Verband der Chemischen Industrie, Fonds der Chemischen Industrie for financial support.

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