Simple shifts in redox/thiol balance that perturb blood coagulation

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Received 31 October 2001; accepted 16 November 2001

First published online 4 December 2001

Edited by Pierre Jolles

Abstract The biological chemistry that underlies and regulates the blood coagulation cascade is not fully understood. To begin to understand this, we performed clotting assays under various redox conditions. By varying the amount of oxidant and/or antioxidant in these assays, we observed that both the intrinsic/tenase complex and the extrinsic pathways were susceptible to shifts in the thiol/redox balance. We established a dichotomy where blood clotting via the intrinsic pathway was sensitive to oxidation whereas the tissue factor or extrinsic pathway was more sensitive to reduction. These differential inhibitory effects present a conceptual mechanism for selective modulation of the activities of clotting factors specific for the respective pathways. These data also suggest that blood clotting may be influenced by unidentified redox or thiol equilibria.

Key words: Thiol–disulphide interchange; Coagulation; Redox equilibrium; Oxidant; Antioxidant

1. Introduction

Reduction–oxidation or redox reactions are central not only to intermediary metabolism, but also to the integration and control of a diverse array of interactions including gene regulation by transcription factors, protein–protein interactions, and DNA synthesis [1]. Examples of proteins involved in these processes include necrosis factor (NF)-κB, ribonucleotide reductase, cytochrome *c* oxidase, and protein kinase C. Most of these contain metal ion ligation sites (mainly copper or iron) that constitute redox active sites. The body is also equipped with proteins such as catalase and superoxide dismutase which buffer against oxidant stress. Reactive oxygen species (ROS) derived from phagocyte oxidative burst or UV irradiation contribute to diseases as diverse as arthritis and autoimmune diseases, for example, systemic lupus erythromatosis. Phagocytes however also use ROS to protect the cell against pathogen invasion. Thus, redox reactions seem to span the entire spectrum of cell biology.

In blood, there are a number of redox proteins including a major cellular antioxidant caeruloplasmin. Protein disulphide isomerase, a potent redox cofactor involved in disulphide-bond formation and isomerisation, has also been localised on the platelet surface and mediates platelet aggregation [2–4]. The latter can be inhibited by reduced glutathione [5]. It is well established that redox reactions involving vitamin K underlie the biology of clotting factors VII, IX, X, protein S and prothrombin [6]. Vitamin K is required for post-translational carboxylation of these proteins by converting glutamate to γ-carboxylglutamate residues. It is suggested that this process may be coupled to disulphide-bond formation [7]. It would appear that redox or thiol chemistry may have a role in controlling effector proteins of the coagulation system. Here we examine the possible role of oxidants and antioxidants in blood clotting in vitro, by inducing shifts in the thiol/redox balance in the environment of the cognate clotting factors.

2. Materials and methods

2.1. Blood coagulation assays

Prothrombin times (PT) and activated partial thromboplastin times (APTT) were determined using pooled normal plasma. For PT determination, rabbit brain thromboplastin, PT HS-Plus (Instrumentation Laboratories Warrington, UK), was reconstituted in buffer as instructed. Normal plasma (75 μl) was incubated with various concentrations of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Sigma), reduced glutathione (GSH; Calbiochem) or oxidised glutathione (GSSG; Calbiochem). Normal saline was added where necessary to bring the total reaction volume to 100 μl. The mixture was incubated for 15 min at 37°C, and 100 μl thromboplastin was added. APTT were performed using micronised silica reconstituted in distilled water, as activator. Normal plasma and DTNB, GSH or GSSG were incubated as above; 100 μl of activator and 100 μl phospholipid were then added. In a modification of the APTT assay, DTT was added to various concentrations of GSSG at a final concentration of 5 mM. Clot formation was initiated in all cases by adding 100 μl 25 mM $CaCl_2$, and monitored on a KC-10 semi-automatic coagulometer (Amelung). All assays were repeated four times.

3. Results and discussion

PT employs tissue thromboplastin to determine the integrity and activity of the extrinsic clotting system based on the levels of the vitamin K-dependent cofactors factors II, V, VII and X. The APTT on the other hand is measured using a platelet substitute (phospholipid) and an activator. It determines the activity of the contact or intrinsic pathway and the levels of factors VIII and IX, but may also indicate the activities of high molecular weight kininogen, prekallikrein and factor XI. Both the extrinsic and intrinsic pathways converge at factor X to constitute the common pathway which leads to fibrin clot formation at the site of vascular injury. The underlying biochemistry of these pathways is far from clear. We therefore

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0014-5793/02/$22.00
PII: S0014-5793(01)03209-4

initiated our study with one simple premise: that a regulatory mechanism possibly involving covalent modifications may be involved in controlling both the qualitative and quantitative response of the clotting factors to tissue injury. As a starting point, we chose to study the effects of modifications of disulphide/sulphydryl groups of the proteins on the rate of blood coagulation.

DTNB, otherwise known as Ellman's reagent, is a potent oxidant and thiol blocker that oxidises free thiols to disulphides. It is cell-impermeant and reacts with only exposed thiols. We tested it to determine what effect, if any, it had on clotting factor activity and the time course of blood coagulation. We observed that it prolonged fibrin clot formation via the intrinsic pathway (Fig. 1A) but had no inhibitory effect on the extrinsic or tissue factor pathways (Fig. 1B). Because DTNB is not ordinarily present in systemic circulation, we tested the physiological redox couple of GSH and GSSG for their ability to modulate blood coagulation. Both of these prolonged clotting time in a dose-dependent manner (Fig. 2). The extrinsic pathway (measured by PT) appeared to be sensitive both to oxidation and to reduction, since both GSSG and GSH inhibited at high concentrations (Fig. 2A,B). Whereas no component of the intrinsic pathway seemed very sensitive to reduction with GSH (Fig. 2C), it appeared that some component in this pathway was sensitive to oxidation since clotting was markedly inhibited by GSSG (Fig. 2D). This mirrored the effect of DTNB on the same pathway, even at lower concentrations (Fig. 1A).

To relieve the inhibitory effect of GSSG, we performed

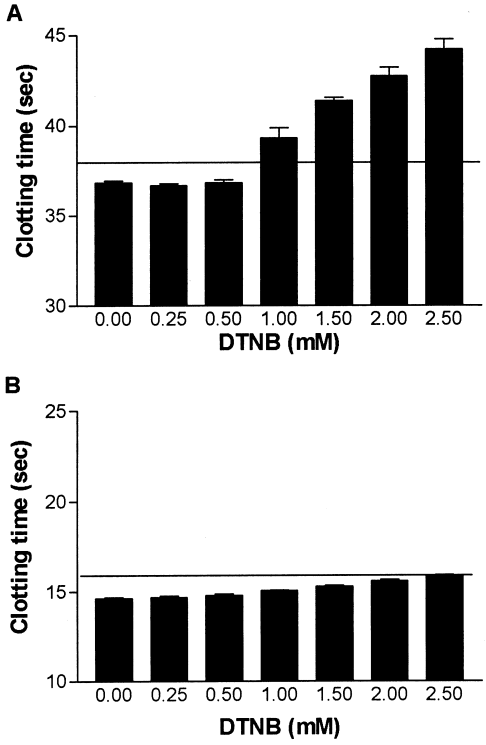

Fig. 1. Effect of DTNB on blood clotting. APTT (A) and PT (B) were determined using normal plasma as described in the text, with various concentrations of DTNB. Horizontal bar represents the upper limit for normal values. For APTT the range is 28–38 s, while the normal range for PT is 12–16 s. Each value represents the means ± S.E.M. of four independent experiments.

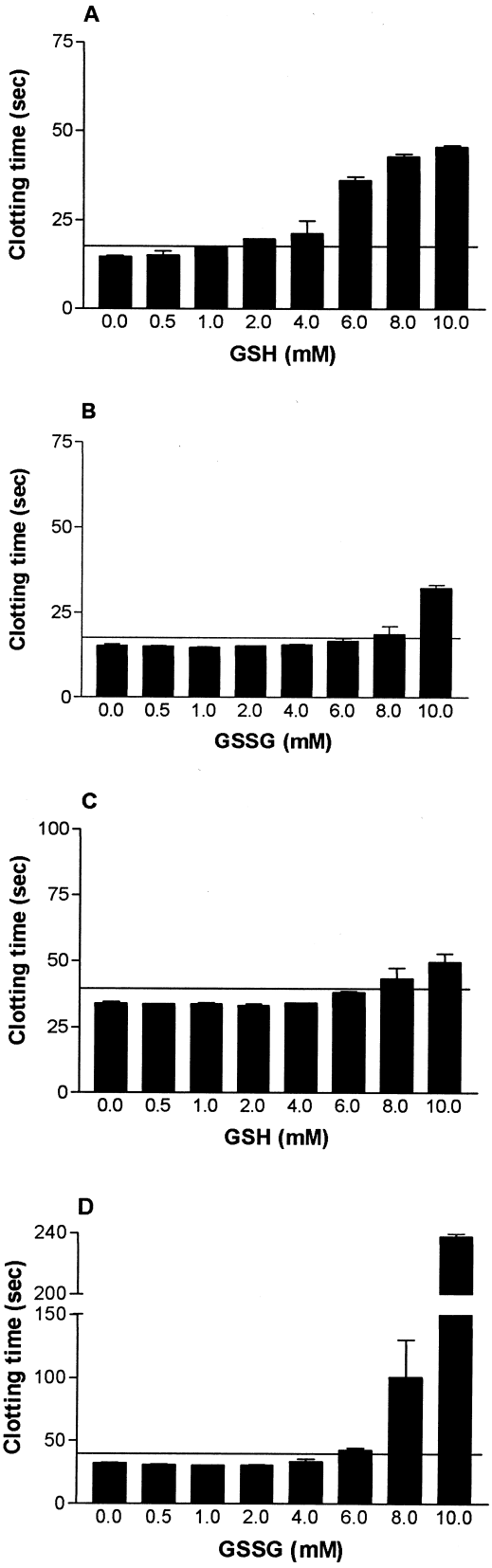

Fig. 2. GSH and GSSG have divergent effects on blood clotting. PT were determined as a function of GSH (A) or GSSG (B) concentrations. APTT were similarly performed for GSH (C) and GSSG (D) as described in the text. Horizontal bar represents upper range for normality. Each value represents the means ± S.E.M. of four independent experiments.

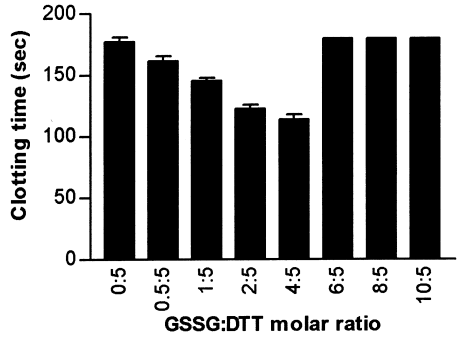

Fig. 3. DTT can partially reverse the inhibitory effects of GSSG on blood coagulation as measured by the APTT. Coagulation assays with GSSG were performed in the presence of 5 mM DTT. Clotting times were plotted as functions of the GSSG:DTT molar ratios. Values are means ± S.E.M. of four independent experiments.

APTT assays as above but also included DTT in the assay mixture. We observed that DTT could partially reverse the inhibitory effect of GSSG at a GSSG:DTT ratio of 4:5 (Fig. 3). This reversal corresponds to the reduction of factor VIII activity to between 20 and 25% of normal plasma levels. On either side of this ratio, clotting times were dramatically prolonged in a manner that reflected the effects of either of these reagents on their own. Thus, it may be assumed that a redox or thiol balance is required in order to override the effects of either inhibitor.

The comparatively marked prolongation of clotting time that we observed in the APTT indicates that dithiol–disulphide interchange may have a more significant contribution to the intrinsic than to the extrinsic pathway. The converse is true for the PT. The inhibition of clotting by GSSG via both the intrinsic and extrinsic pathways, albeit at high concentrations, may also suggest that it acts on some component of the common pathway, i.e. from factor Xa to fibrin clot formation. Thiol oxidation would appear to be the mechanism of inhibition. Previous reports have also shown that the reduction of clotting factors by the thioredoxin system (comprising thioredoxin and thioredoxin reductase) and the alkylation of thiols of these proteins completely abolished their activity [8]. Similar studies, which are supported by our observations, suggested that redox mechanisms may contribute to factor VIII function and blood clotting [9]. Disulphide interchange has also been observed to underlie the functions of fibrinogen [10] and thrombospondin [11]. Our findings also complement other studies which showed that activated polymorphonuclear leukocytes secreted potent oxidants, chloramine and hypochlorous acid, which differentially affected the functions of several clotting factors. While factors II (prothrombin), VII, IX, XII and XIII were insensitive to oxidation, factors I (fibrinogen), V, VIII and X were inactivated by oxidation [12].

One possible explanation for our observations and those preceding them is that the interaction of the clotting factors may involve the formation of transient mixed-disulphide intermediates that require critical cysteine thiols. DTNB or glutathione may block this process by competing for and sequestering those thiols and thus slowing down the process of thrombin and fibrin clot formation. On the other hand, since (all) these proteins are disulphide-bonded de novo, a change in the thiol or redox status of any one of them and the corresponding change in its activity, may be amplified down the clotting cascade. Since clotting depends on a cascade of intricately linked catalytic processes, a very small change in activity of one component could lead to measurable changes in clotting time. This may therefore indicate that the redox state of the procoagulant complex or of the constituent effector proteins, may determine its level of activity. This observation may have physiological significance that suggests that a fine redox balance may be required in vivo for blood homeostasis.

Glutathione is a major cellular constituent and acts as a buffer against oxidant stress. It modulates the redox/thiol state and function of a number of proteins including receptors and metabolic enzymes. Although we have shown that blood clotting is sensitive to the glutathione redox couple in vitro, it may not in itself be involved physiologically since its concentration in plasma is several orders of magnitude lower than that used in the above assays [13]. However, the effects that we observed would suggest that an unidentified physiological oxidant/antioxidant system may play a role in modulating blood-clotting chemistry in vivo. The thioredoxin system or a related one such as the glutataredoxin system must remain potential regulatory mechanisms for the clotting factors as indicated above, since thioredoxin can be detected in plasma [1,8,14,15]. Taking our observations together with other reports, we could speculate that thiol–disulphide interchange [16] may be a rate-limiting step in the activities of the various clotting factors and may also underpin the integration and control of the clotting cascade.

What are the clinical implications of these observations? It is known that the prolongation of clotting times by as little as 2 s can lead to a bleeding diathesis (see Figs. 1 and 2). Although our in vitro observations may not necessarily apply in vivo, some information may accrue in respect of the role of oxidants and antioxidants in blood biochemistry and how this can be modified to control a bleeding diathesis. As the differential effects of oxidants and antioxidants show, it may be possible to induce changes in clotting times in respect of either pathway. Modulation of blood-clotting times may be induced, for example, by dietary intake of antioxidants such as the carotenoids, flavonoids, tocopherols (vitamin E) and vitamin C (L-ascorbic acid). The 'blood-thinning' effects of these agents have been known for some time and may explain our observations. On the other hand, bleeding diathesis, such as haemophilia, may also be exacerbated by the intake of antioxidants. Thus, some basic information for new forms of anticoagulant therapy based on antioxidants may be derived from our observations.

Acknowledgements: This work was supported by the Katharine Dormandy Trust for Haemophilia and Allied Disorders. We thank Christine Lee for support and Dale Owen for help with coagulation assays.

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