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Effects of acrolein, a natural occurring aldehyde, on the anticoagulant serpin antithrombin

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ABSTRACT

We studied the effect of acrolein, an α,β-unsaturated aldehyde that causes adduct-modification of lysine, cysteine, and histidine residues, on antithrombin, a key anticoagulant serpin. Intrinsic fluorescence, functionality (anti-FXa and anti-IIa activity), heparin affinity and conformational features of plasma and purified antithrombin were evaluated. In vivo experiments were carried out in mice. Intrinsic fluorescence showed a two-step conformational change. Acrolein, even at low dose, impaired the anticoagulant function of purified antithrombin by affecting its heparin affinity. However, higher concentrations of acrolein and long incubations are required to cause mild functional effects on plasma antithrombin and mice.

1. Introduction

Thrombosis is a multifactorial disorder, with both congenital and acquired risk factors. So far, most of the research on venous thrombosis has been focused on the identification of genetic risk factors; however, few environmental risk factors have been described in the last 10 years [1–3]. Diet, healthy life conditions and consumer habits usually tip the scale in the disease or the health favours, but few studies have investigated the elements involved and their consequences on the functionality of factors of the hemostatic system.

Acrolein is a highly electrophilic α,β-unsaturated aldehyde to which humans are exposed in industrial, environmental, and therapeutic situations. Its use as an herbicide, as starting material for acrylate polymers, as well as in the production of acrylic acid makes it potentially dangerous. Moreover, acrolein is found in cigarette smoke, in the exhaust from internal combustion engines, and in the vapours of overheated cooking oil [4]. In vivo, acrolein is a metabolic product of the anticancer drug cyclophosphamide and has been found to be formed from the metabolism of the threonine by neutrophil myeloperoxidase at sites of inflammation [4]. Interestingly, it causes oxidative modification of proteins by reacting with the sulfhydryl side chain of cysteines, ε-amino groups of lysines, and the imidazole group of histidines, being lysines the preferred residues [4]. Acrolein protein adducts have been demonstrated in diabetic nephropathy [4], and Alzheimer's disease [4], both of which are associated with increased lipid peroxidation. Actually, lipid peroxidation is related to increased risk of thrombosis due to the activation of the coagulation system and the formation of thrombin by activated platelets [4]. A recent study found a significant functional effect of acrolein on antithrombin, the main endogenous anticoagulant [2]. The aim of this study was to characterize in vitro and in vivo the effect of acrolein on the functionality and conformation of antithrombin and its implication in the risk of thrombosis that has been described in situations with increased levels of this aldehyde.

2. Materials and methods

2.1. Reagents

Purified human antithrombin was obtained from commercial concentrates (Kybernin-P®, ZLB Behring, Germany). Acrolein was purchased from Sigma (Spain). Low molecular weight heparin

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(LMWH) (Bemiparine) and unfractionated heparin were obtained from Laboratorios Rovi S.A. (Spain). LMWH has a size of 3600 Da while unfractionated heparin (UFH) has an average size of 20 000 Da.

2.2. Plasma samples

Venous blood obtained from humans (who gave their informed consent to enter the study, which was approved by the local ethic committee and performed according to the declaration of Helsinki in 2000), and mice was anticoagulated with 10 mM trisodium citrate. Fresh plasma was obtained by centrifugation and stored at −70 °C.

2.3. In vitro incubation of antithrombin with acrolein

Acrolein was diluted in 10 mM sodium phosphate buffer, pH 7.4 containing 150 mM NaCl. Purified antithrombin or plasma samples were incubated in absence or presence of 0–2 mM acrolein at 37 °C for 0–24 h. In heparin protection studies, 0.1 U/μl of LMWH was used.

2.4. In vivo effects of acrolein on antithrombin

Non-inbred Swiss (ICR CD-1™) male mice between 8 and 10 weeks old weighing 30–35 g were used for acrolein injection. Ten milligrams of acrolein per kilogram of mouse body weight or vehicle (saline) were injected into the tail vein of the animal. Mice were anesthetized with isofluorane and blood samples were drawn in citrate through the retro-orbital plexus using heparin-coated micro-hematocrit capillaries before and 24 h after acrolein injection. Plasma samples were stored at −70 °C. All experimental procedures were performed in accordance with the approved Institutional Animal Care Guidelines from the University of Murcia.

2.5. Measurement of antithrombin activity

Antithrombin activity was determined by chromogenic methods, as previously described [5]. Anti-factor Xa (anti-FXa) assay was performed with LMWH, bovine FXa, and S-2765 chromogenic substrate (Chromogenix, IZASA, Spain), while anti-factor IIa (anti-FIIa) activity was measured with human thrombin, and S-2238 chromogenic substrate in the presence and absence of UFH (Chromogenix, IZASA, Spain). Values were expressed as a percentage of the result observed in a control pool of citrated plasma from 100 healthy subjects or a pool of plasma from 10 control mice (100%).

2.6. Electrophoretic analysis of antithrombin

Crossed immunoelectrophoresis of antithrombin in presence of heparin was carried out as previously described [6]. Separation of proteins was evaluated by SDS–PAGE, non-denaturing PAGE and immunoblotting as indicated elsewhere [7].

2.7. Fluorescence measurements

The effect of acrolein on tertiary structure of antithrombin was analyzed by fluorescence emission spectra on a Cary Eclipse spectrofluorometer. Intrinsic tryptophan emission spectra of purified antithrombin were monitored by an excitation at 295 nm and emission at 305–405 nm. The slit-widths were set at 5 nm for both excitation and emission and a constant scan rate of 400 nm/min was used. The final antithrombin concentration was 600 nM.

2.8. Statistical analysis

Statistical analysis was performed by Statistical Package for Social Science (version 15.0; SPSS, USA). Data were presented as mean ± standard error (S.E.). Differences of plasma anti-FXa activity were analysed by unpaired two tailed Student's *t*-test. Statistical significance was taken as $P < 0.05$.

3. Results

3.1. Effects of acrolein on purified antithrombin

Fig. 1 shows the time- and dose-dependent effects of the in vitro incubation of acrolein on the anticoagulant function of purified antithrombin. Anti-FXa and anti-FIIa activities were statistically reduced after incubation with acrolein at all tested concentrations and times of incubation, leading to an almost complete inhibition of the antithrombin activity at 0.5 h of incubation with 2 mM of acrolein.

Although no significant changes were observed by SDS gels (Fig. 2A), we detected significant electrophoretic changes on antithrombin by non-denaturing gels, both in the absence and presence of urea (Fig. 2B and C). Both, native and latent forms of antithrombin increased the electrophoretic mobility in a process that was time- and dose-dependent of acrolein and correlated with the loss of activity. However, we did not observe polymers neither in SDS nor in native gels (Fig. 2A and B).

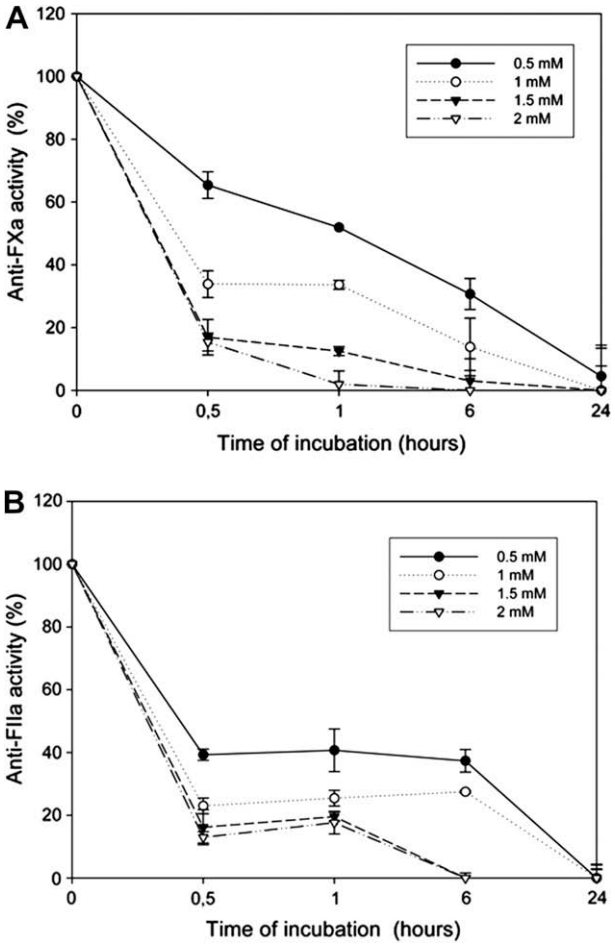

Fig. 1. Dose- and time-dependent effect of acrolein on anticoagulant activity of antithrombin. (A) Anti-FXa activity in presence of LMWH and (B) anti-FIIa activity in presence of UFH. Experiments were run in triplicate and data are presented as mean ± S.E.

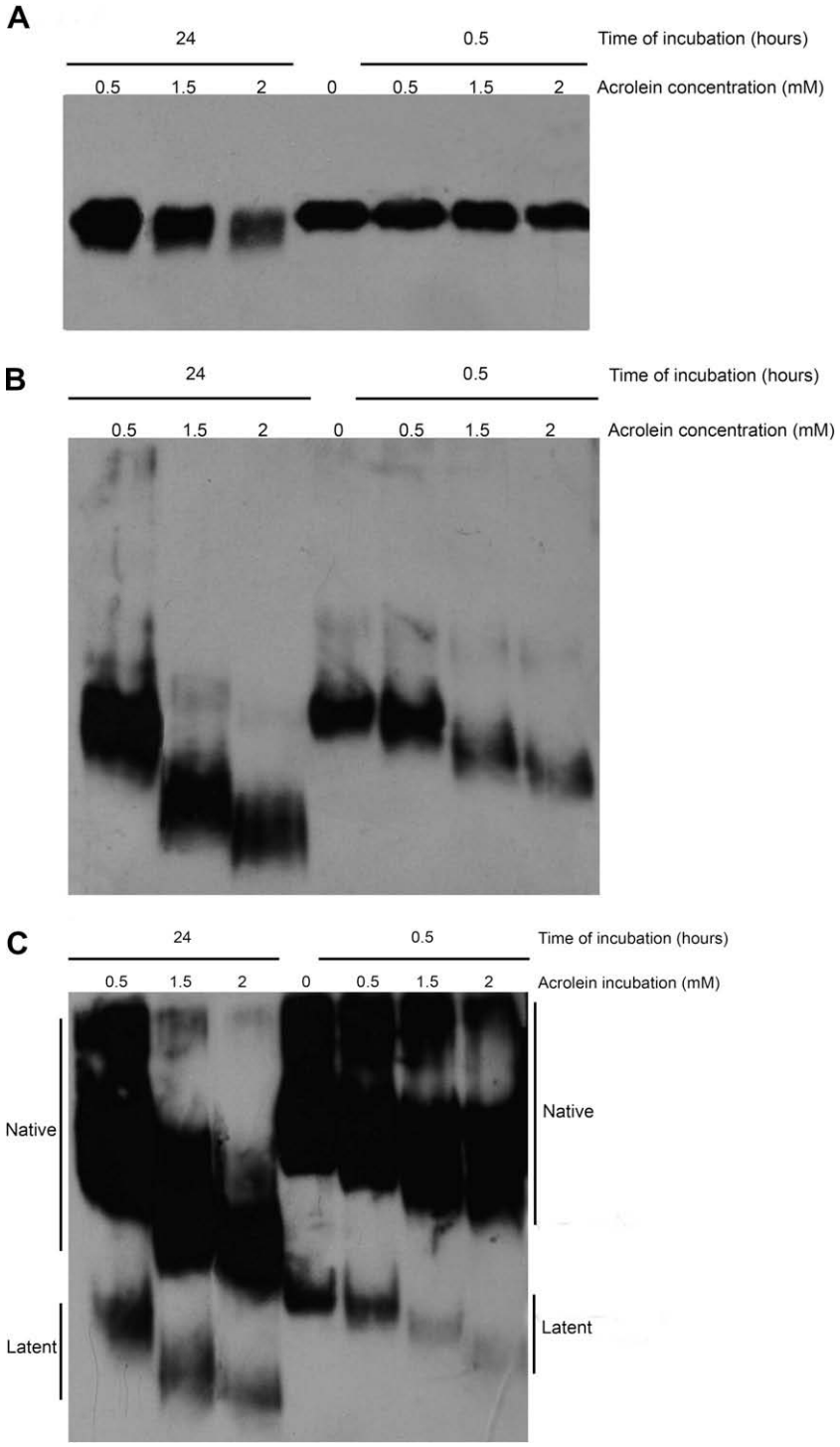

Fig. 2. Electrophoretic changes induced by acrolein on purified antithrombin. (A) SDS–PAGE, (B) non-denaturing PAGE and (C) non-denaturing PAGE + 6 M urea.

To evaluate the potential effect of acrolein on those lysine residues involved in the heparin binding, samples incubated with 0, 1 and 2 mM acrolein for 30 min were run in crossed immunoelectrophoresis gels (Fig. 3). Acrolein treatment completely transformed the protein to a form with low heparin affinity (Fig. 3). This was not the result of a transition of the native to the latent conformation, as the latent form did not increase after acrolein incubation. Thus, acrolein must affect the heparin-binding site of antithrombin. Therefore, we incubated purified antithrombin with LMWH or saline for 30 min at 22 °C before treatment with 0–2 mM acrolein. The binding of heparin to antithrombin significantly reduced the acrolein-dependent loss of anticoagulant activity (Fig. 4A). Moreover, although heparin delayed the electrophoretic shift caused by acrolein, antithrombin–heparin complex still had faster electrophoretic mobility on native gels (Fig. 4B), suggesting the affectation of other residues located out of the heparin-binding site. However, the residues affected besides those located at the heparin-binding site should not be decisive in the inhibitory function since antithrombin treated with 2 mM acrolein showed almost no impairment of its progressive anti-FIIa activity, in the absence of heparin (80.8 ± 2.5% and 76.3 ± 2.9% at 0.5 and 1.5 h of incubation with acrolein, respectively).

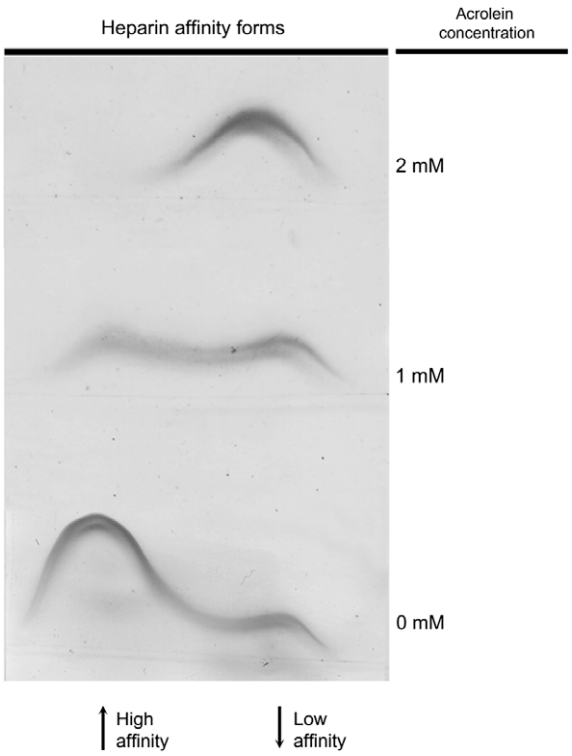

Fig. 3. Crossed immunoelectrophoresis of antithrombin in the presence of heparin. Purified antithrombin was incubated for 30 min with 0, 0.5 and 1.5 mM acrolein. The antithrombin forms of high and low heparin affinity are indicated with arrows.

3.2. Effects of acrolein on spectral properties of antithrombin

Steady-state tryptophan emission spectra of antithrombin were recorded for each of the concentration of acrolein and times of incubation (data not shown). The concentration-dependent acrolein impact on antithrombin was highlighted by subtracting the differences of emission spectra of the wild-type molecule to that with the respective acrolein dose. The intrinsic fluorescence of the protein treated with acrolein showed no changes in the emission maxima wavelength (λ_{max}: 332 nm). However, we observed significant changes in the fluorescence intensity at 332 nm when antithrombin was incubated with various acrolein concentrations, suggestive of a two-steps conformational transition in antithrombin (Fig. 5). During short incubation periods (0.5 h), an unfolding transition was observed by an increase in tryptophan fluorescence (from 14% for 0.5 mM acrolein to 25% for 2 mM acrolein) (Fig. 5), probably due to a higher solvent-exposition of tryptophans. Con-

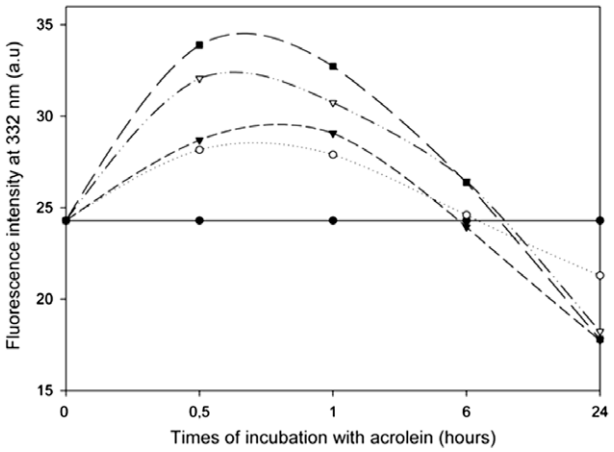

Fig. 5. Fluorescence intensity changes at 332 nm of antithrombin incubated with 0–2 mM of acrolein for 24 h. The *solid line* (—●—) represents control antithrombin and the *dashed lines* represent antithrombin treated with different acrolein concentrations: 0.5 mM (···○···), 1 mM (- - -▼- - -), 1.5 mM (-···▽···-) and 2 mM (–■–).

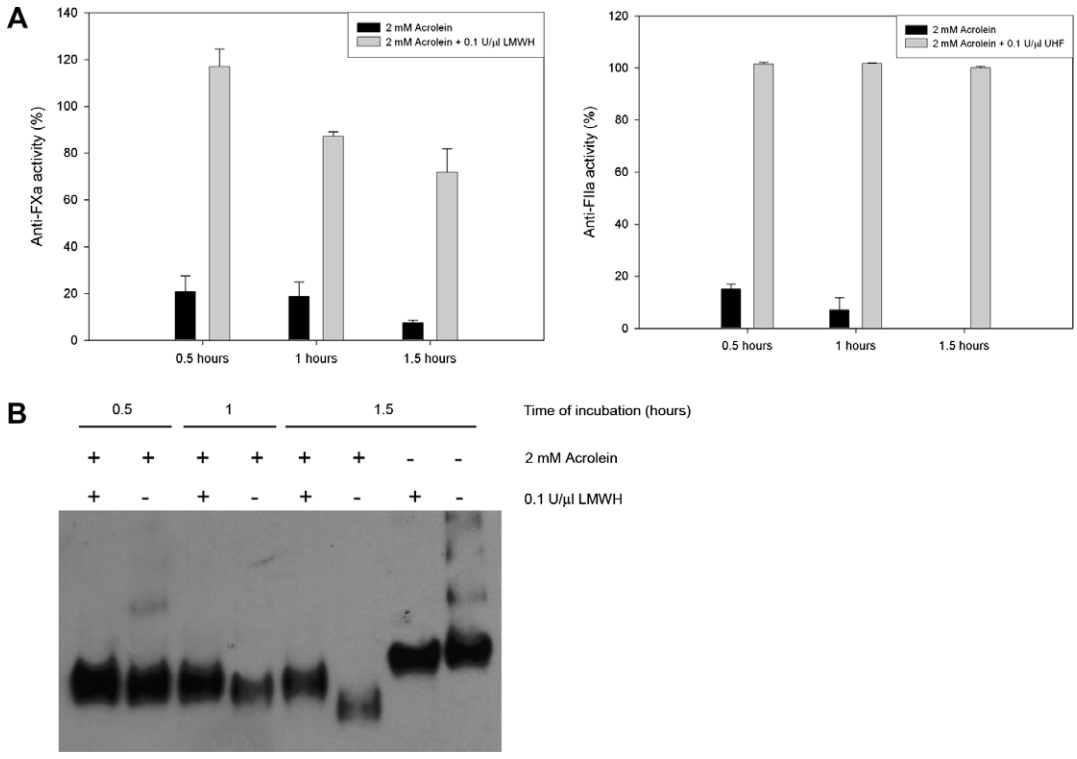

Fig. 4. Protective effect of heparin on purified antithrombin treated with acrolein. (A) Effects on the anti-FXa (left) and anti-FIIa (right) activity after acrolein treatment. The 100% activity was set with the untreated protein. The chromogenic assay was performed without adding heparin in those reactions that were incubated with heparin. Experiments were run in duplicate and data are presented as mean ± S.E. (B) Electrophoretic changes induced by acrolein in the presence and absence of heparin.

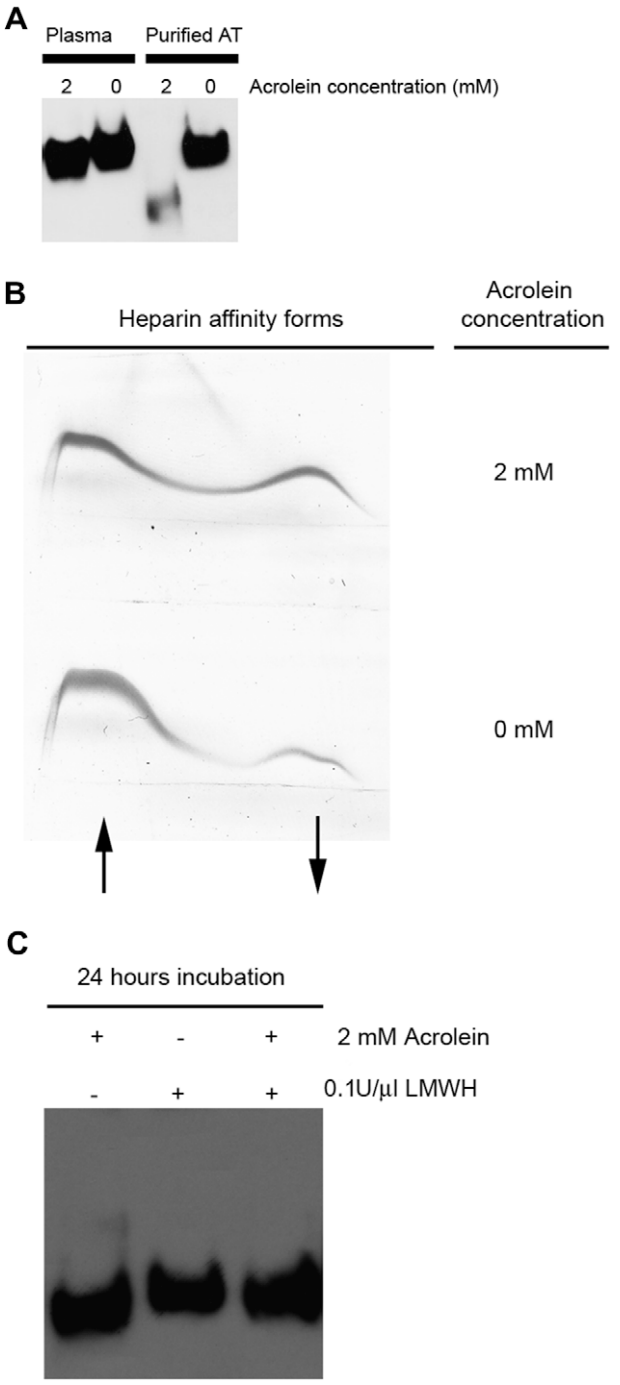

Fig. 6. Electrophoretic changes of plasma antithrombin by effect of acrolein. (A) Non-denaturing PAGE. (B) Crossed immunoelectrophoresis of samples incubated 24 h with or without acrolein. The antithrombin forms of high and low heparin affinity are indicated with arrows. (C) Protective effect of heparin on antithrombin under incubation with acrolein by non-denaturing PAGE. AT: antithrombin; LMWH: low molecular weight heparin.

currently, longer incubation periods (6 and 24 h) resulted in a marked decrease in fluorescence signal (Fig. 5), even minor than wild-type antithrombin (from −14% for 0.5 mM acrolein to −37% for 2 mM acrolein).

3.3. Effects of acrolein on plasma antithrombin

Antithrombin activity was only mildly affected at the highest concentration of acrolein and after 24 h of incubation (anti-FXa of plasma treated with acrolein 81.7 ± 6.6% vs. control plasma 103.6 ± 4.7%; $P < 0.01$). Consistent with this result, electrophoretic analysis of plasma antithrombin also found a slight electrophoretic shift, similar to that identified when using purified antithrombin (Fig. 6A). Moreover, crossed immunoelectrophoresis revealed a slight increase of the form with low heparin affinity (Fig. 6B). Finally, as for purified antithrombin, LMWH seemed to protect against the minor electrophoretic changes induced by acrolein on plasma antithrombin (Fig. 6C).

3.4. In vivo effects of acrolein on antithrombin

The decrease of antithrombin activity in plasma samples incubated with acrolein prompted us to study the in vivo effect of this compound on antithrombin in the context of a living animal. Antithrombin activity was slightly but statistically lower in mice injected with acrolein than in control mice (81.9 ± 2.9% vs. 90.6 ± 1.2%, respectively; $P = 0.039$). Thus, concentrations of acrolein in the range of 1–3 mM reduced the activity of antithrombin within 24 h from injection into a mouse.

4. Discussion

Antithrombin is a structurally modulate protein whose flexibility defines its adequate functionality to control the coagulation [8]. Any modification that concludes in a change of its flexibility or affects its functional domains provokes a decrease in its activity [9]. Genetic modifications affecting the coding sequence of this serpin may have any of these consequences leading to a state of deficiency that significantly increases the risk of thrombosis [10]. Within the environmental agents that are able to modify the func-

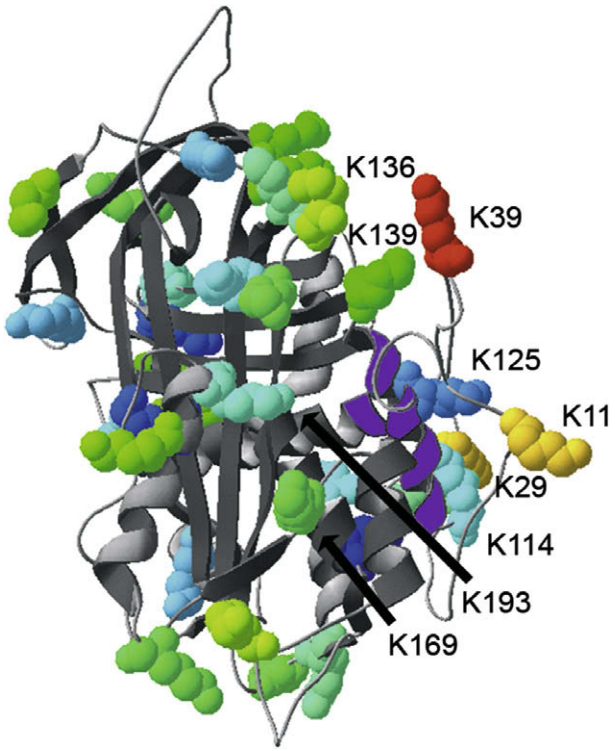

Fig. 7. Structural representation of the accessibility and position of the lysine residues in antithrombin. Dark blue colour is attributed to completely buried residues, whereas red colour is attributed to residues with at least 75% of their relative surface accessible. Helices involved in heparin binding are coloured in violet.

tion of antithrombin, acrolein is a good candidate. Increased levels of acrolein have been described in situations with thrombotic risk, such as smoking [4,11,12], treatment with cyclophosphamide or renal failure [4]. Moreover, phagocytes generate acrolein by action of myeloperoxidase on threonine residues in atheroma plaques leading to a microenvironment with high local concentrations of this aldehyde [13–15]. Additionally, a recent report described the functional effects of acrolein on antithrombin in vitro [2]. We confirmed that acrolein causes the loss of activity of antithrombin, particularly its anti-IIa activity, although no polymerization was detected, as the suggested mechanism involved in the loss of its activity [2]. Indeed, the electrophoretic data support that the loss of activity caused by acrolein on antithrombin was mainly due to the modifications caused by this agent on the serpin. Acrolein adducts reduce the positive charges in the protein since mainly lysine residues are modified. These modifications primarily affect the heparin-binding domain, where some lysine residues are exposed (Fig. 7). Modification of these residues, particularly Lys11, Lys114 and Lys125 impairs the binding of heparin, which triggers the activation of the molecule [16,17]. Indeed, most circulating antithrombin has a native state with low anticoagulant properties, because the reactive centre loop (RCL) is partially inserted into the top of the A β-sheet of the protein. The binding of glycosaminoglycans to the heparin-binding site expels the RCL leading to the active state [18,19]. According to these results, we showed that heparin protects antithrombin against the functional and electrophoretic effects caused by acrolein, but it is not able to completely abolish them, which seems to suggest that acrolein must modify other residues beside those located at the heparin-binding domain. These additional residues must not be crucial for antithrombin function, as the progressive activity is not severely disturbed, but could explain why the effect induced by acrolein on antithrombin inactivation of FIIa and FXa is slightly different. Indeed, residues other than R393 are involved in the different reactivity of antithrombin with its target proteases [20,21].

Fluorescence measurements by intrinsic tryptophan emission showed that acrolein induces two main changes in antithrombin. A first change characterized by a non-stable conformation transition associated to an increase of 14–25% of tryptophan fluorescence correlated with the loss of heparin affinity. Longer incubations must result in modification of additional residues that caused a second conformational change with a significantly decrease around of 37% fluorescence intensity, which would be associated with a reorganization of tryptophans in the core of molecule. Thus, our results suggest that the fluorescence of any of four tryptophans of antithrombin (Trp-49, -189, -225 or -307) [22] could be affected by the acrolein treatment, probably by modifying Lys, Cys or His residues located close to tryptophan residues. This analysis also revealed a dose-dependently effect (Fig. 5). This result, together with the electrophoretic analysis, suggests that low concentrations of acrolein (0.5 and 1 mM) might only form adducts with certain accessible residues, while higher concentrations would also affect buried residues.

We have also determined whether the observed in vitro effects of acrolein on antithrombin may have clinical consequences by using plasma samples and in vivo experiments performed in mice. Our results suggest that only high concentrations of acrolein (2 mM), probably barely achieved under a small amount of conditions, are required to cause minor effects (but the same identified in vitro) on antithrombin. Therefore, we think that although increased levels of acrolein may contribute to the risk of thrombosis, particularly if combined with other additive or synergistic factors, its clinical relevance in thrombosis might be minor.

Acknowledgments

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