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Inactivation of Plasminogen Activator Inhibitor by Oxidants†

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ABSTRACT: The rapidly acting plasminogen activator inhibitor (PAI) purified from cultured bovine aortic endothelial cells (BAEs) was inactivated during iodination with chloramine T and other oxidizing iodination systems. Inactivation was observed in the absence of iodine, suggesting that the loss of activity resulted from the oxidizing conditions employed. In an attempt to further study the nature of this inactivation, the PAI was treated with chloramine T under conditions that specifically oxidize methionine and cysteine residues. Both PAI inhibitory activity and the ability of the PAI to form complexes with tissue-type PA were decreased in a dose-dependent manner by such treatment. The PAI was more sensitive to oxidative inactivation than urokinase, elastase, and α_1-protease inhibitor. Incubation of the chloramine T inactivated PAI with methionine sulfoxide peptide reductase in the presence of dithiothreitol (DTT) restored more than 90% of the PAI activity. The reductase is a DTT-dependent enzyme that specifically converts methionine sulfoxide to methionine. Little activity was restored by either the reductase or DTT alone. These results indicate that the oxidation of at least one critical methionine residue is responsible for the loss of PAI activity upon iodination. In this respect, the BAE PAI resembles α_1-protease inhibitor, a well-characterized elastase inhibitor that also is inactivated by oxidants. Both inhibitors are members of the serine protease inhibitor superfamily (Serpins), and both have a methionine residue in their reactive center.

Plasminogen activator inhibitors (PAIs)[1] have been detected in a variety of cells and tissues (Loskutoff, 1986) and thus may play a central role in regulating tissue fibrinolysis. There are at least three immunologically distinct classes of PAI, including those isolated from endothelial cells (Loskutoff & Edgington, 1977; Dosne et al., 1978; Loskutoff et al., 1983; Levin, 1983; Emeis et al., 1983; Thorsen & Philips, 1984), from placenta (Holmburg et al., 1978), and from fibroblasts (i.e., protease nexin; Baker et al., 1980; Scott et al., 1985). The endothelial cell derived PAI neutralizes both urokinase and tPA (van Mourik et al., 1984) and has been detected in human plasma and platelets (Chmielewska et al., 1983; Erickson et al., 1984a, 1985). It is released from platelets by thrombin (Erickson et al., 1984a) and other activating agents (Booth et al., 1985) and is increased in the plasma of individuals at risk of developing thrombotic problems (Chmielewska et al., 1983; Nilsson et al., 1985; Colucci et al., 1985; Hamsten et al., 1985). These results suggest that the endothelial cell PAI may also regulate vascular thrombolysis.

We recently purified this PAI from cultured BAEs (van Mourik et al., 1984) and attempted to iodinate it using standard techniques. Although we were able to incorporate ^{125}I into the PAI, we were unable to recover PAI activity. The present study was undertaken to see if this loss of PAI activity resulted from the oxidative conditions of the iodination reaction. Support for such a mechanism of oxidative inactivation is provided by studies conducted with α-1-PI (Johnson & Travis, 1979), another serine protease inhibitor (Jeppsson & Laurell, 1975). α-1-PI is an elastase inhibitor that is uniquely sensitive to oxidants (Travis & Salvesen, 1983) and is currently under intense investigation because of its potential role in the pathogenesis of lung and joint disease (Jeppsson & Laurell,

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[1] Abbreviations: PA, plasminogen activator; PAI, plasminogen activator inhibitor; tPA, tissue-type plasminogen activator; BAEs, bovine aortic endothelial cells; MEM, minimal essential medium; PBS, phosphate-buffered saline (0.14 M NaCl/0.01 M sodium phosphate, pH 7.2); SDS, sodium dodecyl sulfate; TX-100, Triton X-100; PAGE, polyacrylamide gel electrophoresis; RFA, reverse fibrin autography; DDT, dithiothreitol; α-1-PI, α_1-protease inhibitor; BSA, bovine serum albumin; CM, conditioned medium; Serpin, serine protease inhibitor; Tris, tris(hydroxymethyl)aminomethane; IgG, immunoglobulin G.

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1975; Travis & Salveson, 1983). The inactivation of α-1-PI by oxidants results from the conversion of a single methionine residue in the exposed reactive site of the molecule into methionine sulfoxide (Johnson & Travis, 1978, 1979; Abrams et al., 1981; Travis & Salveson, 1983; Travis et al., 1985). The data presented here suggest that a similar mechanism may be responsible for the loss of PAI activity upon iodination.

Experimental Procedures

Materials

Chemicals, proteins, and other materials were purchased from the following sources: Tris-HCl, chloramine T, *N*-chlorosuccinimide, *N*-acetyl-L-methionine, BSA, and α-1-PI (α_1-antitrypsin) from Sigma (St. Louis, MO); DTT, *N*-succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide, Trasylol (aprotinin), and fibrinogen from Calbiochem Behring (La Jolla, CA); magnesium chloride from Mallinckrodt (Paris, KY); hydrogen peroxide from Fisher Scientific (Pittsburgh, PA); *p*-aminobenzamidine–Sepharose 4B from Pierce Chemical Co. (Rockford, IL); Tween 20 and Tween 80 from Baker Chemical Co. (Phillipsburg, NJ); acrylamide and SDS from Bio-Rad (Richmond, CA); Seaplaque agarose from FMC Corp. (Rockland, ME); TX-100 from Kodak. Thrombin was a generous gift from Dr. J. Fenton (New York State Department of Health, Albany, NY), and PAI was purified from serum-free CM collected from cultured BAEs according to the method of van Mourik (van Mourik et al., 1984). Human urokinase ("Winkinase") was obtained from Sterling-Winthrop (Renselaer, NY) and was further purified by affinity chromatography on a column of *p*-aminobenzamidine–Sepharose 4B (Holmberg et al., 1976). The WHO urokinase standard (preparation 66-46) was obtained from The National Institute for Biological Standards and Control, Holly Hill, Hampstead, London, Great Britain. Human tPA was purified from CM collected from Bowes melanoma cells as described (Schleef et al., 1985a) and consisted of >90% single-chain form. The methionine sulfoxide peptide reductase employed in these studies was a partially purified preparation from *Escherichia coli* (Brot et al., 1981) and was kindly provided by Dr. Nathan Brot of the Roche Institute, Nutley, NJ. Catalase was obtained from Millipore Corp. (Freehold, NJ) and porcine pancreatic elastase from Elastin Products Co. (Pacific, MO). Goat anti-rabbit IgG was purchased from Cooper Biomedical Co. (Malvern, PA) and radiolabeled by the iodogen method (Fraker & Speck, 1978). The specific activities achieved were approximately 6×10^6 cpm/μg of protein.

Methods

PAI Assays. PAI activity was measured by three different assays. In the first, the ability of the sample to inhibit urokinase-mediated lysis of ^{125}I-labeled fibrin was evaluated as described (van Mourik et al., 1984). Samples were preincubated with urokinase (0.0025 U/mL) at 37 °C for 10–20 min, and the amount of free urokinase was then determined by monitoring the release of ^{125}I-labeled fibrin degradation products after the addition of plasminogen (4 μg/mL final concentration). PAI activity was also measured by RFA after SDS–PAGE (Loskutoff et al., 1983; Erickson et al., 1984b). The position of inhibitors in the SDS gel was revealed by the formation of opaque, lysis-resistant zones in the otherwise cleared indicator film. Finally, PAI activity was determined by measuring the ability of samples to bind to immobilized tPA by using the tPA-binding assay (Schleef et al., 1985b). Purified tPA (50 μL/well, 1 μg/mL) in PBS was bound to 96-well polyvinyl chloride microtiter plates by overnight incubation at 4 °C. The wells were washed and blocked with buffer containing BSA as described (Schleef et al., 1985b) and then incubated for 1 h at 37 °C with increasing amounts of sample. Bound PAI was detected by incubating the washed wells for 1.5 h at 37 °C with rabbit antiserum to the BAE PAI, followed by incubation for 1.5 h at 37 °C with ^{125}I-labeled goat anti-rabbit IgG [$(2.5–5) \times 10^4$ cpm/well]. The wells were removed individually and the radioactivity in each was determined.

Enzyme Assays. Urokinase activity was determined by the ^{125}I-fibrin plate assay as described (van Mourik et al., 1984). Quantitation was achieved by comparing the amount of ^{125}I-fibrin degradation products released from wells containing the sample with fibrin degradation products released from wells containing the WHO urokinase standard. Elastase activity was determined on a Cary spectrophotometer at 410 nm with the synthetic substrate *N*-succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide according to the method of Bieth (Bieth et al., 1974). α-1-PI was quantified by incubating increasing amounts of the inhibitor for 15 min at 23 °C with elastase (1 μg/mL) prior to addition of the synthetic substrate. The α-1-PI activity was calculated by measuring the change in elastase activity.

Oxidation Reactions. All proteins to be tested for sensitivity to oxidation were brought to final concentrations of 10 or 30 μg/mL in 0.2 M Tris-HCl, pH 8.0. Under these slightly alkaline conditions, only cysteine and methionine residues are oxidized to cystine and methionine sulfoxide, respectively (Shechter et al., 1975). One-tenth volume of the oxidant at 10 times the final desired concentration was added, and the samples were incubated for 30 min at either 37 or 23 °C. The mixtures were then incubated for another 15–30 min in the presence of various agents to consume any remaining oxidant or dialyzed to remove the oxidant. For example, samples treated with chloramine T or *N*-chlorosuccinimide were incubated with *N*-acetyl-L-methionine at a final concentration that was equimolar with the highest concentration of oxidant employed in each experiment. Samples treated with hydrogen peroxide were subsequently treated with catalase (final concentration 10 μg/mL) in 0.2 M Tris-HCl, pH 8.0.

Treatment of Oxidatively Inactivated PAI with Methionine Sulfoxide Peptide Reductase. Purified BAE PAI (27 μg/mL) was incubated for 30 min at 37 °C with 500 μM chloramine T. The treated sample and an untreated control were then dialyzed overnight at 4 °C against 0.2 M Tris-HCl buffer, pH 8.0, to remove unreacted chloramine T. Aliquots (5 μL) of the dialyzed control and oxidized PAI were then added to tubes containing $MgCl_2$ and either DTT, methionine sulfoxide peptide reductase, or both. The volumes of the samples were adjusted to 30 μL by the addition of distilled H_2O, and the samples were then incubated for various times at 37 °C. The final concentrations were as follows: 0.03 M Tris-HCl, 10 mM $MgCl_2$, 15 mM DTT, and 2.03 μg/mL PAI. The methionine sulfoxide peptide reductase concentration was not determined since the enzyme employed in each case was only partially purified (Brot et al., 1981). After the incubation, the samples, together with a nonoxidized control, were assayed in the tPA-binding assay.

Miscellaneous. Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as a standard. SDS–PAGE was performed according to the method of Laemmli (1970) using 9% separating gels with 4% stacking gels. ^{125}I was measured on a γ counter (Micromedic).

Results

Inactivation of PAI by Chloramine T. The effect of chloramine T on the activity of the BAE PAI was investigated.

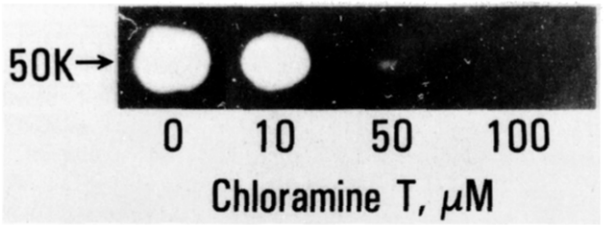

FIGURE 1: Inactivation of PAI by chloramine T. Purified BAE PAI (9 μg/mL) was incubated for 30 min at 37 °C with increasing concentrations of chloramine T. The reaction was stopped by the addition of *N*-acetyl-L-methionine as described under Methods, the samples were diluted to 1 μg/mL, and aliquots (100 μL) were removed from each tube and fractionated by SDS–PAGE. The SDS gels were then analyzed for PAI activity by RFA.

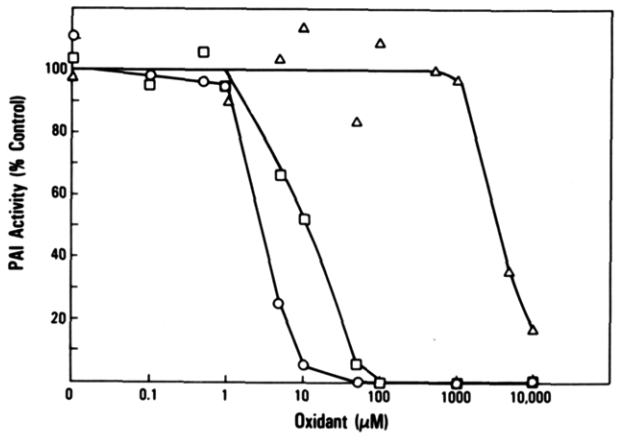

FIGURE 2: Effect of other oxidants on PAI activity as determined by the tPA-binding assay. Purified BAE PAI (9 μg/mL) was incubated for 30 min at 37 °C with increasing concentrations of *N*-chlorosuccinimide (○), chloramine T (□), or hydrogen peroxide (△). The reactions were stopped as described under Methods, and the samples were then assayed for PAI activity by using the tPA-binding assay.

Purified PAI was treated with increasing concentrations of chloramine T and then fractionated by SDS–PAGE and analyzed for PAI activity by RFA (Figure 1). PAI activity (i.e., the size of the lysis resistant zone) decreased in a dose-dependent manner as the chloramine T concentration was increased. A slight decrease in PAI activity was observed after treatment with as little as 10 μM chloramine T, and most of it had disappeared after treatment with 50 μM chloramine T. PAI activity was no longer detected in samples treated with 100 μM chloramine T.

Inactivation of PAI by Other Oxidants. The effect of other oxidants on PAI activity was examined. In these experiments, the PAI was incubated with buffer alone or with buffer containing increasing concentrations of either *N*-chlorosuccinimide, chloramine T, or hydrogen peroxide, and then residual PAI activity was quantified by using the tPA-binding assay. Figure 2 demonstrates that the PAI was inactivated by all three oxidants, with 50% inactivation occurring at 2.9 μM *N*-chlorosuccinimide, 10.2 μM chloramine T, and 3.7 mM hydrogen peroxide. Thus the inhibitor was approximately 3 times more sensitive to inactivation by *N*-chlorosuccinimide than by chloramine T. Inactivation by hydrogen peroxide required at least 1000 times more reagent than inactivation by *N*-chlorosuccinimide.

Relative Sensitivities of PAI, α-1-PI, Urokinase, and Elastase to Chloramine T. The effect of chloramine T on the biological activity of PAI, α-1-PI, urokinase, and elastase was compared. Each protein was incubated with increasing concentrations of chloramine T and then tested for remaining

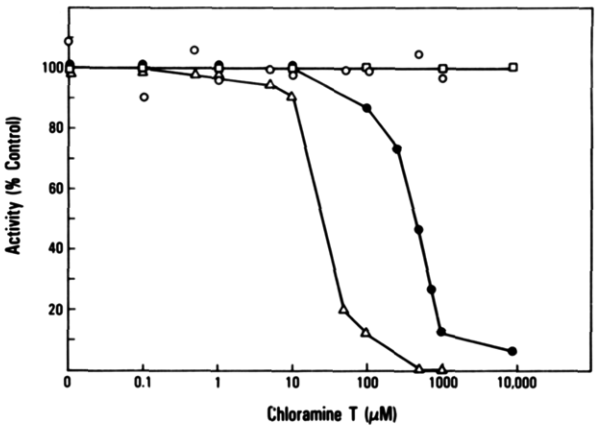

FIGURE 3: Effect of chloramine T on the biological activity of various proteins. Urokinase (○), PAI (△), α-1-PI (●), and elastase (□), each at 9 μg/mL, were incubated for 30 min at 23 °C with increasing concentrations of chloramine T. Equimolar amounts of *N*-acetyl-L-methionine were added to consume unreacted chloramine T, and the samples were assayed for remaining biological activity as described under Methods.

Table I: Effect of Methionine Sulfoxide Peptide Reductase on PAI[a]

	PAI (ng/mL)			
sample	buffer	DTT	reductase	reductase + DTT
2-h incubation				
untreated PAI	1813	1734	1088	817
chloramine T treated PAI	0 (0)	0 (0)	100 (9)	447 (55)
4-h incubation				
untreated PAI	1810	1667	840	675
chloramine T treated PAI	0 (0)	0 (0)	156 (19)	663 (94)

[a] Samples of untreated and chloramine T inactivated PAI were prepared as described under Experimental Procedures. Aliquots of the dialyzed samples were then incubated for 2 or 4 h either with buffer alone or with DTT, methionine sulfoxide peptide reductase, or methionine sulfoxide peptide reductase in the presence of DTT and tested for PAI activity by the tPA–binding assay. PAI activity was converted to nanograms per milliliter of PAI by direct comparison to a standard preparation of purified PAI of known specific activity. The numbers in parentheses indicate the percentage of PAI activity in the chloramine T treated sample as compared to its untreated but incubated control.

activity (Figure 3), as described under Experimental Procedures. The PAI was found to be approximately 10 times more sensitive than α-1-PI to inactivation by chloramine T. In order to demonstrate that the inactivation of PAI and α-1-PI by chloramine T was not simply the result of generalized, nonspecific oxidative damage, we tested the effects of chloramine T on urokinase and elastase, the respective substrates for these inhibitors. No decrease in either activity was detected at any of the chloramine T concentrations employed. It should be noted that the PAI was completely inactivated at a chloramine T concentration that was approximately 25 times lower than the concentration employed in the standard iodination reaction (12 mM).

Effect of Methionine Sulfoxide Peptide Reductase on Chloramine T Inactivated PAI. The following experiment was performed in an attempt to demonstrate that PAI inactivation depends upon the oxidative conversion of at least one methionine residue into methionine sulfoxide. Purified PAI was inactivated by treatment with chloramine T and then diluted into buffer containing either DTT, methionine sulfoxide peptide reductase, or both DTT and methionine sulfoxide peptide reductase. After incubation for 2 and 4 h at 37 °C, the PAI activity of the various samples was determined. The

chloramine T treated samples lacked detectable PAI activity (Table I) and continued to express low levels of PAI activity after incubation with buffer, DTT, or methionine sulfoxide peptide reductase. Incubation of the untreated control PAI with the reductase reduced its activity by approximately 60% over the 4-h interval, possibly because of contaminating proteases in the partially purified preparation. Methionine sulfoxide peptide reductase is specific for methionine sulfoxide and has a requirement for DTT or thioredoxin (Brot et al., 1981). When the inactivated PAI was treated with methionine sulfoxide peptide reductase in the presence of DTT, a reductase dose (not shown) and time-dependent increase in PAI activity was observed (Table I, line 4). Greater than 90% of the remaining control activity was recovered after 4-h incubation in the complete system.

Discussion

The experiments summarized in this report were designed to identify the conditions responsible for the inactivation of PAI during iodination. This loss of activity could result from the actual incorporation of the iodine moiety into critical tyrosines in the molecule (Means & Feeney, 1971) or from the conditions employed during the iodination reaction itself. The results support the latter possibility and strongly implicate oxidants in the inactivation process. These conclusions are based on the following observations: (1) Chloramine T caused a dose-dependent decrease in PAI activity in the absence of iodine (Figures 1–3, Table I). (2) The PAI was completely inactivated at a chloramine T concentration of 100–500 μM, which is considerably lower than that employed in the iodination reaction (12.5 mM). (3) Other oxidants, including *N*-chlorosuccinimide and hydrogen peroxide, caused a similar decrease in PAI activity (Figure 2). (4) The activity of the oxidatively inactivated PAI was restored by treatment with methionine sulfoxide peptide reductase (Table I), an enzyme that specifically converts methionine sulfoxide into methionine (Brot et al., 1981). (5) The activity of the inactive ^{125}I-labeled PAI was also restored upon treatment with this enzyme (data not shown).

The results shown in Figure 3 indicate that the PAI is unusually sensitive to oxidative inactivation since it is approximately 1 order of magnitude more sensitive to chloramine T than is α-1-PI (Figure 3), a protease inhibitor previously characterized by its extreme sensitivity to oxidants (Jeppsson & Laurell, 1975; Johnson & Travis, 1978; Johnson & Travis, 1979; Abrams et al., 1981; Travis & Salvesen, 1983). Since both α-1-PI and the BAE PAI are protease inhibitors, and since both are inactivated by low concentrations of chloramine T and other oxidants, the mechanism of inactivation of these molecules may be similar. In the case of α-1-PI, it is the conversion of the P_1 methionine into methionine sulfoxide that is responsible for the loss of activity (Johnson & Travis, 1979; Travis & Salvesen, 1983; Travis et al., 1985). The fact that the PAI was oxidized under conditions that selectively modify methionine and cysteine residues (Shechter et al., 1975) and that the PAI has no cysteine residues (Ny et al., 1986) suggests that the loss of PAI activity is also due to the oxidation of at least one critical methionine. The studies with methionine sulfoxide peptide reductase (Table I) strongly support this hypothesis since the oxidatively inactivated PAI was reactivated by treatment with the reductase in the presence of DTT. The reductase specifically reduces methionine sulfoxide residues in proteins (Brot et al., 1981), frequently restoring biological activity. The ultimate identification of the critically modified methionine will await its isolation; however, the computer alignment of the PAI and α-1-PI cDNA sequences (Ny et al., 1986) reveals that both inhibitors have a methionine in their reactive center. The recent demonstration that the reactive site methionine of α-1-PI is responsible for its sensitivity to oxidants (Travis et al., 1985) suggests that it is the reactive site methionine in PAI that is critically oxidized.

The PAI and α-1-PI share a number of other properties. Both are high molecular weight single-chain glycoproteins that rapidly form equimolar complexes with their target enzymes (van Mourik et al., 1984; Pannell et al., 1974). In both cases, these complexes resist dissociation by denaturing agents such as SDS or urea but can be dissociated by hydroxylamine and other nucleophiles (Johnson & Travis, 1976; Levin, 1983), frequently with at least partial recovery of the activity of the bound enzyme. The formation of both tPA–PAI complexes (Schleef et al., 1985b) and of elastase–α-1-PI complexes requires the active site of the enzyme (Travis & Salvesen, 1983). These similarities raise the possibility that the BAE PAI and immunologically related PAIs (Erickson et al., 1985) may be members of the α-1-PI class of inhibitors (Hunt & Dayhoff, 1980; Chandra et al., 1983). Direct support for this hypothesis is provided by comparison of the primary sequences of the two inhibitors (Ny et al., 1986). Analysis of the cDNA sequence of the PAI reveals that it is a member of the Serpin (Carrell & Travis, 1985) superfamily of serine protease inhibitors, having approximately 30% homology with both α-1-PI and antithrombin III.

Human α-1-PI has been the subject of considerable attention recently because of its role in controlling proteolytic events in tissues. Individuals functionally deficient in this inhibitor, either because of genetic defects (Eriksson, 1965) or because of the presence of oxidants in cigarette smoke which inactivate it (Janoff & Carp, 1977), are susceptible to the development of lung emphysema at an early age. Inactive α-1-PI has also been recovered from rheumatoid synovial fluid (Beatty et al., 1982), implying a role for this inhibitor in the pathogenesis of joint disease. In each of these instances, the development of the disease state has been correlated with abnormally low α-1-PI activity (i.e., with the inability to inhibit elastase). The sensitivity of the PAI to oxidants suggests that oxidants liberated by activated inflammatory cells (Weiss & Regiani, 1984) may also inactivate PAI and thus unleash a whole cascade of tissue-destructive proteinases, including elastase, tPA, and plasmin. The recent demonstration of dramatically elevated plasmin in rheumatoid synovial fluid (Inman & Harpel, 1986) is consistent with this hypothesis.

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Registry No. I_2, 7553-56-2; L-methionine, 63-68-3; chloramine T, 127-65-1; plasminogen, 9001-91-6.

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