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Proteins released from stimulated neutrophils contain very high levels of oxidized methionine

I. Beck-Speier, L. Leuschel, G. Luippold and K.L. Maier

Projekt Inhalation der Gesellschaft für Strahlen- und Umweltforschung München, Arbeitsgruppe Biochemie, Ingolstädter Landstrasse 1, D-8042 Neuherberg, FRG

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In proteins released from quiescent human neutrophils during incubation, 21% of the methionine (Met) residues were found to be oxidized. However, the portion of oxidized Met in extracellular proteins increased to 66% after stimulating the cells with zymosan and to 75% after stimulation with phorbol myristate acetate (PMA). Generation of such high levels of oxidized Met in native proteins by activated neutrophils has, so far, not been observed. The presence of superoxide dismutase during incubation of PMA-stimulated cells produced a negligible effect on methionine oxidation, while the presence of catalase resulted in a methionine sulfoxide (Met(O)) content of only 28% in the released proteins. It is proposed that the conversion of Met to Met(O) in these proteins predominantly occurs by action of the myeloperoxidase/H_2O_2/Cl^- system in the extracellular space.

Extracellular oxidation; Methionine; Methionine sulfoxide; Myeloperoxidase; Neutrophil

1. INTRODUCTION

Met is converted to Met(O) by oxidants like hypochlorous acid, *N*-chlorosuccinimide and chloramine T [1–4]. In proteins, three main classes of Met residues are distinguished, i.e. exposed, partially exposed and buried residues. Each class exhibits a completely different reactivity towards oxidants [4,5]. Oxidation of Met in biological systems is observed during inflammation and ageing processes [5]. Fliss et al. [6] analyzed the intracellular protein of neutrophils for oxidized Met. In cells undergoing PMA-induced respiratory burst, 22% of all methionine residues in the total protein were oxidized to the sulfoxide. In contrast, about 66% of all methionine residues in proteins newly synthesized during stimulation were oxidized to the sulfoxide [6]. Newly synthesized, i.e. nascent polypeptide chains, were distinguished from completed proteins by pulse labeling with [^{35}S]Met during incubation. It was concluded that Met residues in nascent polypeptide chains of neutrophils are more susceptible to oxidation than those in completed proteins [6].

In the present study, the extracellular proteins of neutrophil incubations were analyzed for Met(O) using the cyanogen bromide cleavage method [4]. We demonstrate that about 75% of all Met residues in proteins released by stimulated neutrophils are oxidized to the sulfoxide, as compared to 21% in the protein released from resident cells. Evidence is given that the proteins are oxidized mainly in the extracellular space.

Correspondence address: K.L. Maier, Arbeitsgruppe Biochemie, Gesellschaft für Strahlen- und Umweltforschung, Ingolstädter Landstrasse 1, D-8042 Neuherberg, FRG

Abbreviations: Met, methionine; Met(O), methionine sulfoxide; PMA, phorbol myristate acetate

2. MATERIALS AND METHODS

Catalase and superoxide dismutase were purchased from Boehringer (Mannheim); PMA and zymosan from Sigma (Deisenhofen, FRG); PBS-buffer from Serva (Heidelberg);

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neutrophil isolation medium (Ficoll Hypaque) from Packard Instruments (Frankfurt); and all other chemicals were from Bender und Hobein (München).

Neutrophils were isolated from citrate-anticoagulated venous human blood by density-gradient centrifugation with neutrophil isolation medium from Packard Instruments according to Ferrante and Thong [7]. The isolated layer of neutrophils was washed with PBS-buffer (Ca^{2+}- and Mg^{2+}-free) to remove the gradient medium. Residual erythrocytes were lyzed by treating the cell pellet with distilled water for 30 s. Isotonicity was restored by adding the same volume of 1.8% (w/v) NaCl. The purified cells were resuspended in PBS-buffer, pH 7.0, containing 10 mM glucose.

Incubations of neutrophils were performed with 6×10^6 cells in a total volume of 1 ml PBS-buffer, pH 7.0, with 10 mM glucose for 60 min at 37°C and gentle shaking. Neutrophils were stimulated by PMA (300 ng/ml) or by zymosan (3 mg/ml). In some incubation mixtures catalase (10 μg) or superoxide dismutase (10 μg) was present before the stimulus was added. At the end of the incubations the cells were separated from the incubation medium by centrifugation at 400 $\times$ g for 10 min. The pellets were suspended in 0.5 ml of PBS-buffer, pH 7.4, and sonicated 4 times for 15 s to obtain cell extracts.

The proteins contained in the supernatants and cell extracts were analyzed for total Met and Met(O). Aliquots of both fractions were hydrolyzed with 6 N HCl in the presence of 5 mM dithioerythritol for 24 h at 110°C. Under these conditions oxidized Met is reduced to Met giving over 95% yield [4]. Total Met was determined by amino acid analysis of the hydrolysates performed with a Biotronik amino acid analyzer LC 6000 using a single column system [8,9]. Protein contents were calculated from amino acid analysis data. For differentiation between Met and Met(O) in proteins, the cyanogen bromide cleavage procedure was used [10]. Aliquots of the supernatants and cell extracts with 50 to 200 μg protein were lyophilized and dissolved in 840 μl of 75% (v/v) formic acid. For cleavage 60 μl of 3 M cyanogen bromide were added giving a final reagent concentration of 0.2 M. The reaction was performed under N_2-saturation in darkness at room temperature for 24 h. The Met residues are thus converted to homoserine (lactone) residues, while the Met(O) residues remain intact [10]. The cleavage products were hydrolyzed with 6 N HCl at 110°C for 48 h in the presence of 5 mM dithioerythritol. Under these conditions, Met(O) is reduced back to Met which is determined by amino acid analysis [4]. The portion of oxidized Met in the proteins analyzed was calculated as a percentage of total Met.

3. RESULTS AND DISCUSSION

Isolated neutrophils from human blood were incubated under different conditions to study the oxidation of amino acids in the proteins secreted by the cells. Both resting and stimulated neutrophils release considerable amounts of protein during incubation [11].

A sensitive parameter for oxidative modification of proteins is the Met(O) content. Differentiation between Met and oxidized Met was performed by cyanogen bromide cleavage [4,10]. It is seen from table 1 that in the proteins released from PMA-stimulated cells about 75% of all Met residues were oxidized to the sulfoxide as compared to 21% in proteins secreted from resident cells. These values were calculated from the results of 10 different incubation experiments. A similar Met(O) content in the extracellular protein was found after stimulation of neutrophils with zymosan. Generation of such high levels of oxidized Met in completed proteins by stimulated neutrophils is unique and has, so far, not been reported. Usually, Met residues are located within the hydrophobic interior of completed proteins where they seem to be more or less resistant to oxidation in contrast to Met residues in nascent polypeptide chains [6,12].

Quantitation of the Met(O) content in in-

Table 1

Met(O) content of extracellular and intracellular proteins of resting and stimulated human neutrophils

	Met(O) content (% of total methionine)			
	Extracellular proteins		Intracellular proteins	
Resting cells	21 ± 7.4	(N = 10)	10 ± 3.0	(N = 6)
Zymosan-stimulated cells	66 ± 16	(N = 4)	n.d.[a]	
PMA-stimulated cells	75 ± 11	(N = 10)	19 ± 1.5	(N = 6)

[a] Not determined

Neutrophils (6×10^6 cells/ml) were stimulated with PMA or zymosan and incubated for 60 min at 37°C. Further experimental details are described in section 2. Values are expressed as mean ± SD. N is the number of incubation experiments with neutrophils

tracellular proteins from neutrophils showed that 19% of all Met residues were oxidized in PMA-stimulated cells and 10% in resident cells (table 1). It is evident from table 1, that the extracellular proteins from PMA-stimulated cells contain an approx. 4-fold higher portion of oxidized Met than the intracellular proteins. We conclude from this observation that the proteins exported by stimulated neutrophils are oxidized at Met after release from the cells.

Two mechanisms may be responsible for the extracellular conversion of protein-bound Met to the sulfoxide: (i) oxidation by oxygen radicals, like the superoxide radical anion; and (ii) oxidation by the myeloperoxidase/H_2O_2/Cl^- system.

We stimulated neutrophils with PMA in the presence of superoxide dismutase to estimate the participation of the superoxide radical anion in the extracellular oxidation of proteins. We found that the high level of Met(O) observed in the proteins released by activated control cells was not markedly lowered in the presence of superoxide dismutase (table 2). This indicates, that the superoxide radical anion does not contribute significantly to the oxidation of Met in the extracellular proteins.

To determine the involvement of the myeloperoxidase/H_2O_2/Cl^- system in the oxidation of Met residues of extracellular proteins, catalase was added to the incubation of PMA-stimulated neutrophils. Under these conditions the portion of oxidized Met in the proteins released was found to be 28% (table 2). This value is close to the 21% level of oxidized Met found in the extracellular proteins of resting neutrophils. This result therefore implies, that the myeloperoxidase/H_2O_2/Cl^- system is mainly responsible for the extracellular oxidation of proteins released from the activated neutrophils.

Table 2

Met(O) content of the supernatant protein from neutrophils incubated in the presence of catalase or superoxide dismutase

	Met(O) content (% of total methionine)	
Resting cells	21 ± 7.4	(N = 10)
PMA-stimulated cells (− catalase)	69 ± 9.0	(N = 4)
PMA-stimulated cells (+ catalase)	28 ± 3.5	(N = 4)
PMA-stimulated cells (− SOD[a])	65	(N = 2)
PMA-stimulated cells (+ SOD[a])	61	(N = 2)

[a] Superoxide dismutase

For experimental details see section 2. Values are expressed as means ± SD. N is the number of incubation experiments with neutrophils

Our studies show that the myeloperoxidase/H_2O_2/Cl^- system released by stimulated neutrophils causes oxidation of Met sites in extracellular proteins to an unusually high degree. Myeloperoxidase catalyzes the oxidation of Cl^- by H_2O_2 to yield hypochlorous acid [13,14], which is rapidly trapped by amines to give N-chloramine derivatives (long-lived oxidants) [11,15,16]. N-Chloramines are capable of converting Met to Met(O) [16]. Grisham et al. [11] reported, that both resting and stimulated neutrophils secrete myeloperoxidase activity. Stimulated neutrophils also release considerable amounts of H_2O_2 (40 μM by 1×10^6 cells/ml), whereas quiescent cells do not [11]. During stimulation of neutrophils, extracellular N-chloramines are generated [11,15,16], which, however, are not synthesized when catalase is present in the incubation medium [16,17]. The N-chloramine derivatives may exhibit either a hydrophilic character like taurine-N-monochloramine, or a pronounced lipophilic character like NH_2Cl [11]. In contrast to hydrophilic N-chloramines, the lipophilic derivatives do not accumulate in the incubation medium because of their high reactivity [11].

We suppose that lipophilic N-chloramines are involved in the marked oxidation of completed proteins by attacking buried Met residues in the hydrophobic interior of the polypeptides. Lipophilic N-chloramines may attack hydrophobic sites in proteins demonstrated by oxidation of erythrocyte hemoglobin to methemoglobin by NH_2-Cl [18].

The results presented in this communication support the hypothesis that during inflammatory processes high levels of Met(O) may be generated in proteins from connective tissues or body fluids by an elevated number of activated neutrophils.

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