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Il Farmaco 54 (1999) 695–699

IL FARMACO

Antioxidant properties of albumin: effect on oxidative metabolism of human neutrophil granulocytes

Ferdinand Kouoh *, Bernard Gressier, Michel Luyckx, Claude Brunet, Thierry Dine, Micheline Cazin, Jean Claude Cazin

Faculté des Sciences Pharmaceutiques et Biologiques, Laboratoire de Pharmacologie, Pharmacocinétique et Pharmacie Clinique, 3 rue du Professeur Laguesse, BP 83, F-59006 Lille Cedex, France

Received 7 April 1999; accepted 19 July 1999

Abstract

The present study aims at investigating the effect of bovine serum albumin (BSA) on the trial of oxidative-stress. The antioxidant effects of BSA were determined by human neutrophil granulocytes oxygen free radicals and their by-products (O_2^-, H_2O_2, HOCl) productions. BSA interacts with those reactive oxygen species (ROS) in a dose-dependent manner. The 50% inhibitory concentration (IC_{50}) of BSA estimated, after phorbol-12-myristate-13-acetate (PMA) stimulation were: 33.5 mg/ml for O_2^-, 6.5 mg/ml for H_2O_2, and 6.85 mg/ml for HOCl. When neutrophils were washed after pre-incubation with BSA, there was no significant decrease of ROS after stimulation of PMA (maximal: $15 \pm 1.2\%$). In the free cell experiments, IC_{50} for H_2O_2 and HOCl were 7.86 mg/ml and 0.67 mg/ml, respectively. The mechanism at which BSA acts may result from a simple chemical interaction with ROS rather than an intracellular mechanism by intervention in PMA oxidative metabolism. These antioxidant activities confer to BSA properties, which might be used to prevent damage inflicted by these ROS during inflammatory disorders.

Keywords: Bovine serum albumin; Antioxidant properties; Human neutrophil granulocytes

1. Introduction

The level of antioxidant enzymes in blood plasma is much lower than the intracellular ones, obviously extracellular fluids contain only small amounts of antioxidant enzymes, such as catalase, gluthation peroxidase, and superoxide dismutase. The relative lack of extracellular antioxidant enzymes may reflect the possible function of reactive oxygen species (ROS) as bioeffector molecules [1]. Unless alternative extracellular scavenging mechanisms exist, ROS released by neutrophils in pathological mechanism [2,3] would be at liberty to exert the variety of toxic actions attributed to them in vivo such as tissue damage, oxidative injury in reoxygenated and reperfused organs. However, in human plasma, albumin is the main quantitative protein with a concentration of about 40 g/l and with a half-life of about 20 days [4]. As shown by many authors, albumin possesses some antioxidant properties which need to be specified in its interaction with ROS: it has been shown to react either by a non-specific binding of free radicals or by specifically binding copper ions that otherwise catalyze the production of free radicals [5,6]. This protein, at concentrations less than physiological, is able to inhibit markedly copper-stimulated peroxidation and haemolysis of erythrocyte membranes [7]. Moreover, albumin has a protective effect against ischaemia- and hypoxia-induced hepatic injury [8,9]. Dumoulin et al. observed a relative high scavenging activity of bovine serum albumin (BSA) on isolated perfused rat heart [10].

In the present study, we have investigated, in vitro, the antioxidant properties of BSA as a direct interaction with ROS. Thus, we first established a cellular model of oxidative stress: activated neutrophils during respiratory burst. This cellular model of oxidative stress uses neutrophils stimulated by phorbol-12-myristate-13-acetate (PMA). PMA is known to activate directly the

* Corresponding author. Tel.: +33-320-964 040; fax: +33-320-969 752.

E-mail address: elferdy@caramail.com (F. Kouoh)

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PII: S0014-827X(99)00082-8

protein kinase C (PKC) and to stimulate the release of different reactive oxygen species towards NADPH-oxidase activity [11]. Moreover, neutrophils were washed before stimulation, after pre-incubation with BSA, to appreciate an eventual intracellular effect of BSA on oxidative metabolism.

Afterwards, we have evaluated the ability of BSA to scavenge ROS in an acellular medium. Then we compared the cellular and acellular activities of BSA in order to clarify its anti-oxidant activities on human neutrophil granulocytes.

2. Materials and methods

2.1. Isolation of neutrophils

Neutrophils were purified from fresh heparinized venous blood of healthy human subjects as previously described [12]. Briefly, neutrophils were isolated by a density gradient technique [13]. Cells isolated by this technique were always $>95\%$ viable as determined by trypan blue exclusion, and 95% of the cells were neutrophils. Cells were suspended in Hanks' Hepes (HH) buffer at pH 7.4.

2.2. Assessment of reactive oxygen species

2.2.1. Measurement of O_2^-

2.2.1.1. Neutrophils production of superoxide anion. Superoxide anion (O_2^-) production by human neutrophils stimulated by PMA was measured by reduction of ferricytochrome *c* [14,15]. First, 400 µl of PMN (2.5×10^6 cells/ml) were pre-incubated with various concentration of BSA (0–72 mg/ml) in HH buffer pH 7.4. After 30 min at 37°C, some cells were washed (residual BSA was eliminated) to form the cell washing system, while some others were not washed in order to form the standard cellular system. With the cell washing system, the effect of a direct interaction BSA–ROS may be easily put in evidence. Secondly, 100 µl of PMA (160 nM), 100 µl of superoxide dismutase (2500 U/ml), in reference tube only, and 100 µl of ferricytochrome *c* (2 mg/ml) were added in a final volume in each vial of 1 ml and incubated for 15 min at 37°C. After 5 min of centrifugation at 1125 *g*, absorbance of the supernatants was measured with a Kontron Uvikon 860 spectrophotometer against a reference cuvette. The slope of the absorbance curve at 550 nm was converted to nanomoles of O_2^- using the extinction coefficient $E_{550} = 21.1$ mM/Cm . The results were expressed as the percentage of inhibited O_2^- by BSA.

2.2.2. Measurement of H_2O_2

2.2.2.1. Neutrophils production of hydrogen peroxide. The rate of hydrogen peroxide (H_2O_2) production was determined by the peroxidase-dependent oxidation of phenol red [16], as previously reported [17]. Briefly, 0.5×10^6 PMNs stimulated by 16 nM PMA were incubated for 30 min at 37°C with phenol red dye (0.2 mg/ml) containing 17 U/ml horseradish peroxidase (Sigma) in Hanks'-Hepes buffer, pH 7.4. Absorbance of the cell-free supernatants was measured at 610 nm following the addition of 50 µl of 1 N NaOH. The amount of H_2O_2 was derived from a standard curve.

2.2.2.2. Acellular model. The potentiel scavenging effect of BSA on H_2O_2 was studied in an assay system containing the appropriate concentration of BSA and 8 µM H_2O_2. After 15 min of incubation at 37°C, 1 ml of phenol red dye (0.2 mg/ml) containing 17 U/ml horseradish peroxidase (Sigma) was added. 15 min later, 50 µl of NaOH (1 N) was added to the cell-free supernatants. The amount of H_2O_2 was spectrophotometrically measured at 610 nm as previously described [18].

2.2.3. Measurement of HOCl

2.2.3.1. Neutrophils production of hypochlorous acid. The generation of hypochlorous acid (HOCl), from human neutrophils stimulated with PMA, was measured by the chlorination of taurine [19], as previously reported [20]. Briefly, a reaction mixture of 1.5×10^6 PMNs was pre-incubated with BSA for 30 min. Afterwards PMNs were stimulated by 16 nM PMA for 30 min at 37°C in the presence of 15 mM taurine (Sigma). After the addition of 10 µl of KI (2 M) to cell-free supernatants, absorbance was measured against a reference cuvette at 350 nm.

2.2.3.2. Acellular model. The effect of BSA on HOCl concentration was investigated via a taurine chlorination system as previously described [20]. Briefly, a desired concentration of BSA was mixed with 60 µM NaOCl and 15 mM taurine in a PBS buffer. After 30 min at 37°C, 10 µl of KI 2 M was added to the system and absorbance was measure against a reference cuvette at 350 nm.

2.3. Cellular viability after BSA action

In order to control the cellular viability after BSA action, the viability test by Trypan blue exclusion was realised at the end of the cellular experiment.

2.4. Statistical analysis

Results are expressed as mean $\pm$ standard error of the mean (SEM). The significance of differences was determined using Wilcoxon's test. The *P*-values below 0.05 were considered significant as previously described [21].

3. Results

3.1. Cellular viability after BSA reaction

BSA used in the range of concentrations from 0.3 to 72 mg/ml had no effect on cellular viability determined by trypan blue exclusion.

3.2. Effects of BSA on anion superoxide

In the cellular experimentation, BSA was able to inhibit O_2^- production by stimulated human neutrophils in a dose-dependent manner (Fig. 1). The 50% inhibitory concentrations (IC_{50}) of BSA for PMA-induced O_2^- production, calculated with a computer program [22], was 33.5 mg/ml. At a BSA concentration in the medium of 48 mg/ml, the corresponding inhibiting effect on PMA stimulation was maximal: 91.36%.

In the free cellular experiment, O_2^- was produced by the hypoxanthine–xanthine oxidase system. BSA effect in O_2^- level was shorter than the one observed in the cellular system (Fig. 1).

However, when neutrophils were washed after preincubation with BSA 48 mg/ml, there was no more significant decrease in O_2^- production: 15($\pm$1.2)% in PMA stimulated system (Fig. 2).

3.3. Effects of BSA on hydrogen peroxide

BSA inhibited hydrogen peroxide produced by human neutrophils stimulated with PMA in a dose-dependant manner with IC_{50} of 6.5 mg/ml (Fig. 3).

In acellular experiments, when H_2O_2 was incubated with BSA, a dose dependent inhibition was observed. The IC_{50} was 7.86 mg/ml.

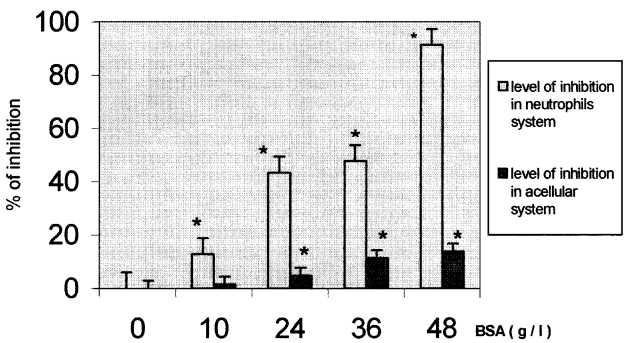

Fig. 1. Inhibiting activities of BSA on superoxide anion. The production of superoxide anion was measured as described in Section 2. The results are expressed as a percentage of anion superoxide production inhibited by BSA. Values are the means $\pm$SEM of five separated experiments. *, Significantly different from BSA-free control ($P < 0.05$).

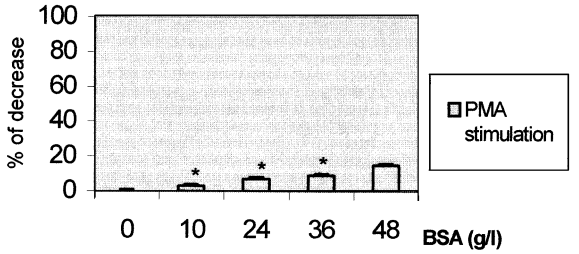

Fig. 2. Decrease of anion superoxide level in the cells washing system. Neutrophils were pre-incubated with increasing concentrations of BSA at 37°C for 30 min. Then, neutrophils were washed before the stimulation. The production of superoxide anion was measured as described in Section 2. The results are expressed as a percentage of the decrease of anion superoxide production. Values are the mean $\pm$ SEM of five separated experiments. *, Significantly different from BSA-free control ($P < 0.05$).

3.4. Effects of BSA on hypochlorous acid

When BSA was incubated with human neutrophils, the production of HOCl was inhibited by albumin in a concentration-depending manner (Fig. 4). The concentration of BSA giving 50% control activity was 6.85 mg/ml.

In the cell free system, while hypochlorous acid was incubated with BSA, a dose dependent inhibition was observed with an IC_{50} of 0.67 mg/ml.

4. Discussion

The clinical use of albumin solution is a controversial issue, that involves albumin as a volume plasma expander, a supplement of total parenteral nutrition and a substance with pharmacological properties [23]. However, in the treatment of burned patients, albumin is currently indicated for severely burned people whose albuminemia falls to approximately 20 g/l (or proteinemia to 35 g/l). The key role of this therapeu-

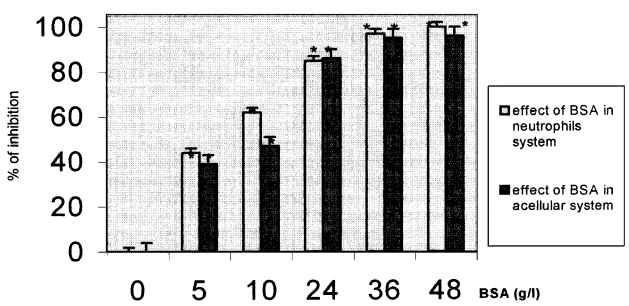

Fig. 3. Inhibiting action of BSA on hydrogen peroxide level. Neutrophils were incubated with increasing concentrations of BSA. The production of hydrogen peroxide was measured as described in Section 2. The results are expressed as percentage of hydrogen peroxide production inhibited by BSA. Values are the mean $\pm$ SEM of five separated experiments. *, Significantly different from BSA-free control ($P < 0.05$).

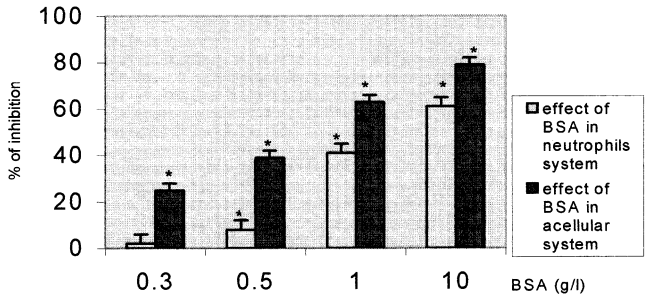

Fig. 4. Inhibiting effect of BSA on hypochlorous acid. Neutrophils were incubated with increasing concentrations of BSA. The production of hypochlorous acid was measured as described in Section 2. The results are expressed as a percentage of hypochlorous acid production inhibited by BSA. Values are the mean ± SEM of five separated experiments. *, Significantly different from BSA-free control ($P < 0.05$).

tic indication is related to hypoalbuminemia and blood volume homeostasis [24]. Moreover, inflammation processes such as activation of neutrophils take place in burned tissue [25]. This activated neutrophils lead to an oxidative-stress [26]. It is important to evaluate the ability of albumin to react in such situation as antioxidant molecule.

So, looking for albumin antioxidant properties, we had investigated on its ability to interact with O_2^-, H_2O_2, and HOCl produced by activated neutrophils in a reply to inflammatory stimulus PMA.

The inhibiting level of H_2O_2 and HOCl by BSA were nearly equivalent in cellular and acellular systems (Figs. 3 and 4). Thus, antioxidant activities of BSA might be a direct interaction of BSA with ROS. The sulfhydryl (HS–) and amine (HN–) groups of serum albumin have already been implicated in such antioxidant activities. For instance, Cyst-34 of human serum albumin had previously been implicated in the reduction of H_2O_2 [27]. HN– group may react with OCl^- to form chloramine [28].

For superoxide anion, BSA reacts with it in a dose-dependent manner as well as in cellular or acellular systems. However, the effect in acellular system was shorter than the one in cell-system experimentation (Fig. 1). The complete dismutation of O_2^- into H_2O_2, in hypoxanthine–xanthine oxidase system, may be more rapid than the rate of BSA interaction. Thus, in order to confirm the hypothetical effect of BSA on the cellular metabolism of neutrophils we had investigated BSA action in the cells washing system. Only a very small decrease of O_2^- level was observed (Fig. 2). Obviously, BSA–ROS interaction may be more evident than intracellular action of BSA on neutrophils oxidative metabolism.

By scavenging ROS produced by neutrophils in physiopathological conditions, albumin may act as a protector of tissue and biomolecules against oxidative-stress. Thus in patients with burns, albumin may also be used as an antioxidant drug in complement of its principal indications.

Acknowledgements

The authors would like to thank Mrs S. Battez Lebegue for excellent technical assistance.

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