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S100A8 and S100A9 inhibit neutrophil oxidative metabolism *in-vitro*: Involvement of adenosine metabolites

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Abstract

Neutrophils are short-lived granulocytic cells of the innate immune system specialized in the production of reactive oxygen species. S100A8 and S100A9 and their heterocomplex calprotectin play a role in neutrophil recruitment and represent 40% of neutrophil cytosolic protein weight. The present study was designed to test the effect of S100A8 and S100A9 on the rate of neutrophil oxidative metabolism. It is hypothesized that the two S100 proteins inhibit neutrophil associated oxidation. Granulocytes freshly isolated from healthy volunteers were tested for their ability to oxidize dichlorofluorescin-diacetate (DCFH-DA) *in-vitro*. The data showed that S100A8 and S100A9 inhibited spontaneous and stimulated oxidation of the DCFH-DA probe by neutrophils. The inhibition of neutrophil oxidative metabolism by S100A8 and S100A9 was markedly reduced by the enzymatic activity of adenosine deaminase. Inhibitors of the P1 adenosine receptors also reduced the anti-oxidative effect of S100A8/A9 providing further support for the involvement of adenosine metabolites in S100A8/A9 anti-oxidative effect.

Keywords: *Neutrophil, ROS, S100A8, S100A9, calprotectin, adenosine, DCFH-DA.*

Abbreviations: *ROS, reactive oxygen species; PKC, Protein Kinase C; DCFH-DA, dichlorofluorescin-diacetate; CGD, Chronic Granulomatous Disease, LPS, Lipopolysaccharide.*

Introduction

The infiltration of damaged tissue by polymorphonuclear neutrophilic leukocytes (neutrophils or PMN) and their subsequent activation is critical for the host defense against microbial threats. Once recruited during acute inflammation, neutrophils produce and release copious amounts of reactive oxygen species (ROS) which target potential bacterial invaders. A failure in sufficient production of ROS leads to infections as observed in chronic granulomatous disease (CGD), a disease prompted by a deficient oxidase system in neutrophils [1]. Conversely, excess ROS production is associated with conditions such as chronic wounds [2] and cardiovascular diseases [3].

Calprotectin, a heterocomplex formed by S100A8 and S100A9, which are two calcium binding proteins, represents 40% of neutrophil cytosolic proteins by weight [4]. High serum levels of calprotectin are associated with an immune deficiency syndrome, growth retardation and arthritis [5–7]. Work done by others and confirmed by us has demonstrated that calprotectin regulates neutrophil migration [8–10]. We have shown that S100A8 and S100A9 repel neutrophils *in-vitro* and that S100A8 inhibits the recruitment of neutrophils *in-vivo* [10,11].

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Neutrophils isolated from healthy volunteers spontaneously produce and release ROS such as superoxide anion [12] *in-vitro*. Reports have indicated that exaggerated or reduced rates of spontaneous neutrophil activation are linked to viral [13] or autoimmune conditions [14], implying a physiological function for spontaneous neutrophil activation. Spontaneous and stimulated activation of neutrophil oxidative metabolism has been extensively documented *in-vitro* [15–18]. The production and release of ROS by neutrophils is dependent on the NADPH oxidase system and it can be accelerated by phorbol 12-myristate 13-acetate (PMA), a molecule which activates protein kinase C (PKC) resulting in the phosphorylation of critical sub-units of the NADPH oxidase complex [19]. Neutrophils oxidative metabolism can also be hastened by bacterial products such as lipopolysaccharides (LPS), whereas adenosine metabolites have been shown to inhibit neutrophil oxidative functions via P1 adenosine receptors [20,21]. P1 receptors are seven-transmembrane purinergic comprising A1, A2A, A2B and A3 receptors. A2A and A3 receptors are functionally expressed in neutrophils and also implicated in chemotaxis [22].

Work done by others has suggested that human and murine S100A9 inhibit the oxidative burst of macrophages contributing to the persistence of inflammatory processes in a mechanism which remains elusive [23]. Whether S100A8 and S100A9 would affect neutrophil oxidative metabolism remains unknown.

Accordingly, in this work, we tested the hypothesis that S100A8 and S100A9 negatively affected spontaneous and stimulated neutrophil oxidative metabolism. We present data supporting this hypothesis and implicating adenosine metabolites in S100A8 and S100A9 anti-oxidative effects.

Materials and methods

Expression and purification of recombinant S100 proteins

Recombinant S100A8 and S100A9 protein were produced and purified based on standard methods as previously described [10,11]. Briefly, both proteins were cloned in a pGEX-2T GST vector (Amersham, Piscataway, NJ). The proteins were expressed in Top-10 F' E-coli as GST fusion proteins. The GST tag was cleaved during the purification process. Protein concentration was assessed through a Bradford protein assay (Pierce, Rockford, IL).

Reagents

Dichlorofluorescin diacetate (DCFH-DA) and Bisindolylmaleimide I (GFX 109203X) (GFX for short) were purchased from EMD Calbiochem (San Diego, CA). Phorbol 12-myristate 13-acetate (PMA), lipopolysaccharides (LPS) from escherichia coli, N-(2-Methoxyphenyl)-N′-[2-(3-pyridinyl)-4-quinazolinyl]-urea (VUF5574) (an A3 adenosine antagonist) and Human adenosine deaminase (ADA1), from human erythrocytes were purchased from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal antibody directed against S100A8 or S100A9 were purchased from Novus Biologicals (Littleton, CO).

Isolation of peripheral neutrophils

Human peripheral neutrophils were isolated from heparinized blood donated by healthy volunteers according to a protocol approved by the University of Illinois Institutional Review Board. The cells were isolated using a histopaque gradient Sigma-Aldrich (St. Louis, MO) according to the manufacturer's instructions. Cell viability and identity was confirmed by tryptan blue staining. Live cells and neutrophils represented at least 95% of isolated leukocytes.

Assay for oxidative activation of neutrophils

The method for the measurement of oxidative activation of neutrophils was based on the ROS-dependent oxidation of DCFH-DA to DCF and was adapted from Ciapetti et al. [15]. DCFH-DA crosses the cell membrane and is hydrolysed by non-specific esterases to non-fluorescent DCFH-DA. Its oxidation by ROS results in the generation of highly fluorescent DCF [24]. DCFH-DA is therefore a widely accepted probe for the measurement of an overall index of oxidative activity. DCFH-DA was purchased from Calbiochem (Madison, WI). The assays were run in clear bottom black 96-well plates. 'Edge effects' (a higher fluorescence in edge wells) were avoided by using only centre wells. Briefly, 50 µl of Dubelco's Phosphate Buffered Saline (DPBS) containing DCFH-DA was added to each well with the final concentration of 10 µg/ml. S100 proteins or PKC inhibitors were diluted in the wells. Just before a baseline reading 100 000 neutrophils in 50 µl PBS were placed in each well; 96-well plates were incubated at 37°C and 5% CO_2 and were read at baseline (immediately after cell addition to the plates) and at indicated time points in a Spectra Max Gemini XS fluorescent plate reader. The excitation wavelength was 485 and the reading was done at 530 nm. Wells with no DCFH-DA were used to measure background fluorescence which was subtracted from each reading. Controls with no cells were also analysed and display no increased fluorescence over time. All assays were conducted in triplicate or quadruplicate wells.

Data analysis

In order to avoid differences between donors, experimental procedures and/or plate-to-plate variation,

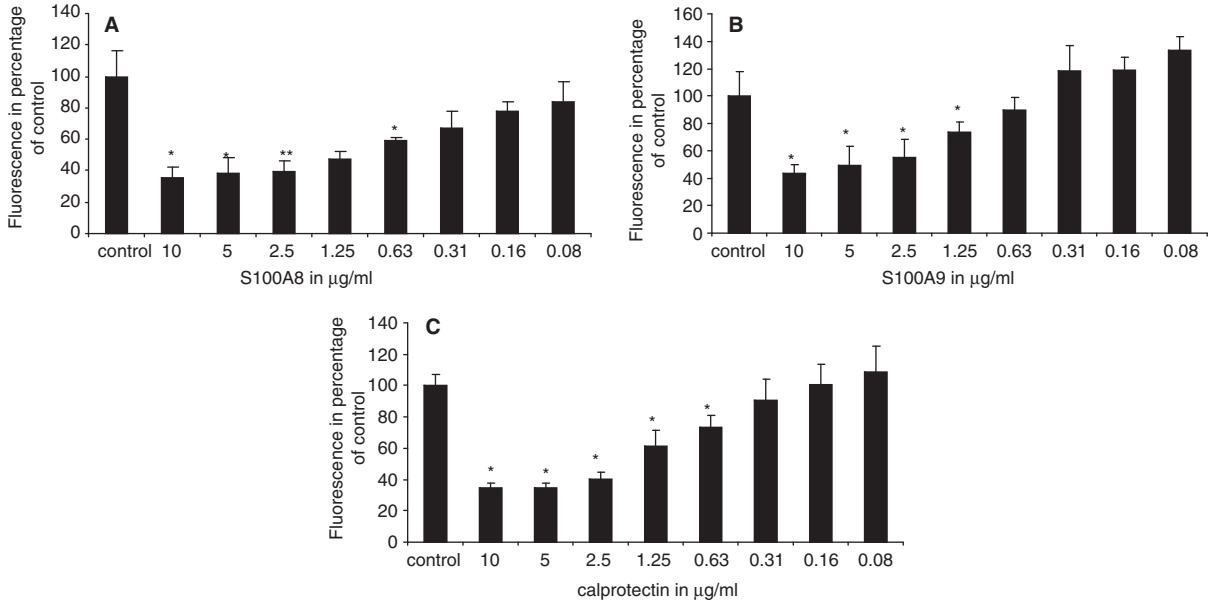

Figure 1. Fluorescence emission of neutrophils incubated with DCFH-DA probe for 1 h in the presence of various concentrations of S100A8 (A), S100A9 (B) and mixture of both S100 proteins (calprotectin) (C). Each point is the mean ± SD. This is a representative experiment of at least four experiments conducted in triplicates or quadruplicate wells. $*p < 0.05$ compared to control.

all experimental conditions and statistical analysis were run within one plate inclusive of all positive and negative controls. Single sample *t*-tests were calculated to test the S100A8, S100A9 and combined proteins in comparison to the control wells with alpha set to ≤ 0.05 to determine statistical significance. Comparisons between the S100A8, S100A9 and combined proteins were calculated with two sample *t*-tests using SPSS (SPSS Inc., Chicago, IL) with alpha set to ≤ 0.05 to determine statistical relevance.

Results

S100A8 and S100A9 decrease spontaneous oxidative activation of neutrophils

The experimental conditions in which we tested peripheral neutrophils were such that incubation resulted in the relatively slow oxidation of the DCFH-DA probe in the absence of any further stimulus. This process was followed over time in the presence and absence of different concentrations of S100A8, S100A9 and with a mixture of both (at a 1:1 weight ratio) referred to as calprotectin.

S100A8 and S100A9 together and each alone reduced the rate at which spontaneous oxidation of the DCFH-DA probe occurred in a dose-dependent manner. One hour after their incubation with DCFH-DA, the two proteins reduced the oxidation of the DCFH-DA probe by as much as 75% of control (Figures 1A–C).

In order to rule out the possibility that S100 proteins reduced the oxidation of the DCFH-DA probe in a non-specific manner, additional control experiments were conducted. First, the S100 proteins were boiled and tested for their anti-oxidative function. Whereas S100A8 or S100A9 at concentrations of 5 μg/ml inhibited the oxidative activation of neutrophils ($p < 0.05$), the same concentrations of S100A8 or S100A9 did not significantly affect neutrophil oxidative rate when the protein was first boiled (Figure 2A).

Secondly, because neutrophils produce and release S100A8 and S100A9 [25], we tested the effect of blocking the activity of endogenous S100A8/S100A9 on the oxidative metabolism of neutrophils. The data indicated that antibodies to S100A8 or S100A9 produced an increase in the rate of DCFH-DA oxidation, an effect opposite to the effect observed with the addition of extrinsic S100A8 or S100A9 (Figure 2B). Isotypic control antibodies had no measurable effect on DCFH-DA oxidation. Together those two control experiments supported the hypothesis that S100A8 and S100A9 had a specific inhibitory effect on neutrophil oxidative metabolism and that intrinsic S100A8 and S100A9 had a constitutive activity during the conduct of the DCFH-DA based oxidation assays.

The spontaneous activation of neutrophil oxidative metabolism is inhibited by GFX, a PKC inhibitor and enhanced by PMA a PKC activator

We next tested the effect of phorbol myristate acetate (PMA), a protein kinase C (PKC) activator on neutrophil oxidative activity. Not surprisingly, the data demonstrated that PMA significantly accelerated the rate of DCFH-DA oxidation. GFX (Bisindolylmaleimide I), a broad PKC inhibitor, significantly

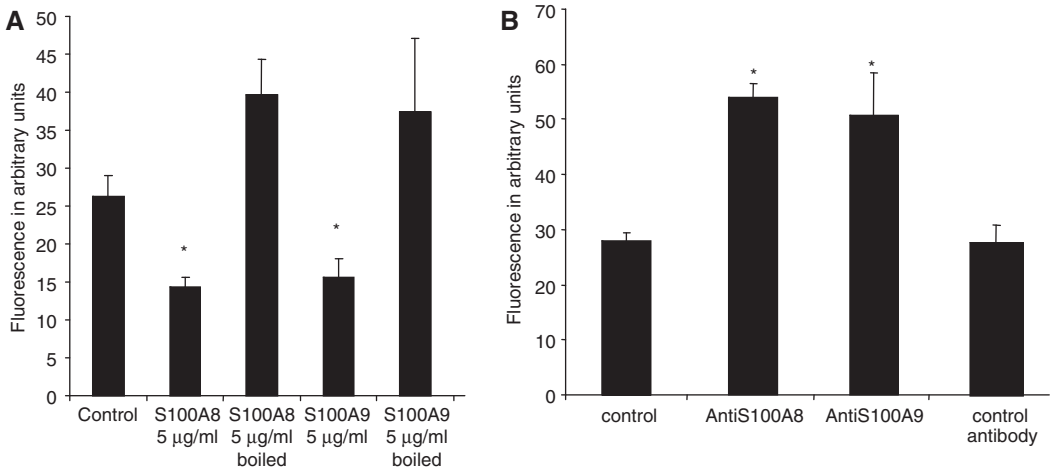

Figure 2. Fluorescence emission of neutrophils incubated with DCFH-DA probe for 3 h in the presence of 5 μg/ml S100A8 or S100A9 which were heat inactivated S100A8 (boiled for 5 min) (A) or antibodies directed against S100A8 or S100A9 (B) at a concentration of 1 μg/ml each. $*p < 0.05$ compared to control. The data represents the mean ± SD. This is a representative experiment of three experiments conducted in triplicate.

inhibited both the PMA induced and the spontaneous activation of neutrophils oxidative metabolism (Figure 3A). This would indicate that the spontaneous activation of neutrophils, similarly to the PMA activation, was dependent on PKC activity, supporting the argument that the spontaneous activation was a genuine activation of neutrophil oxidative mechanism.

S100A8 and S100A9 inhibited the stimulated activation of neutrophils oxidative metabolism

In the next set of experiments, we tested the effect of S100A8 and S100A9 on PMA stimulation of neutrophil oxidative metabolism. The data indicated that S100A8 and S100A9 produced a dose-dependent inhibition of PMA activation of neutrophils (Figures 3B and C)

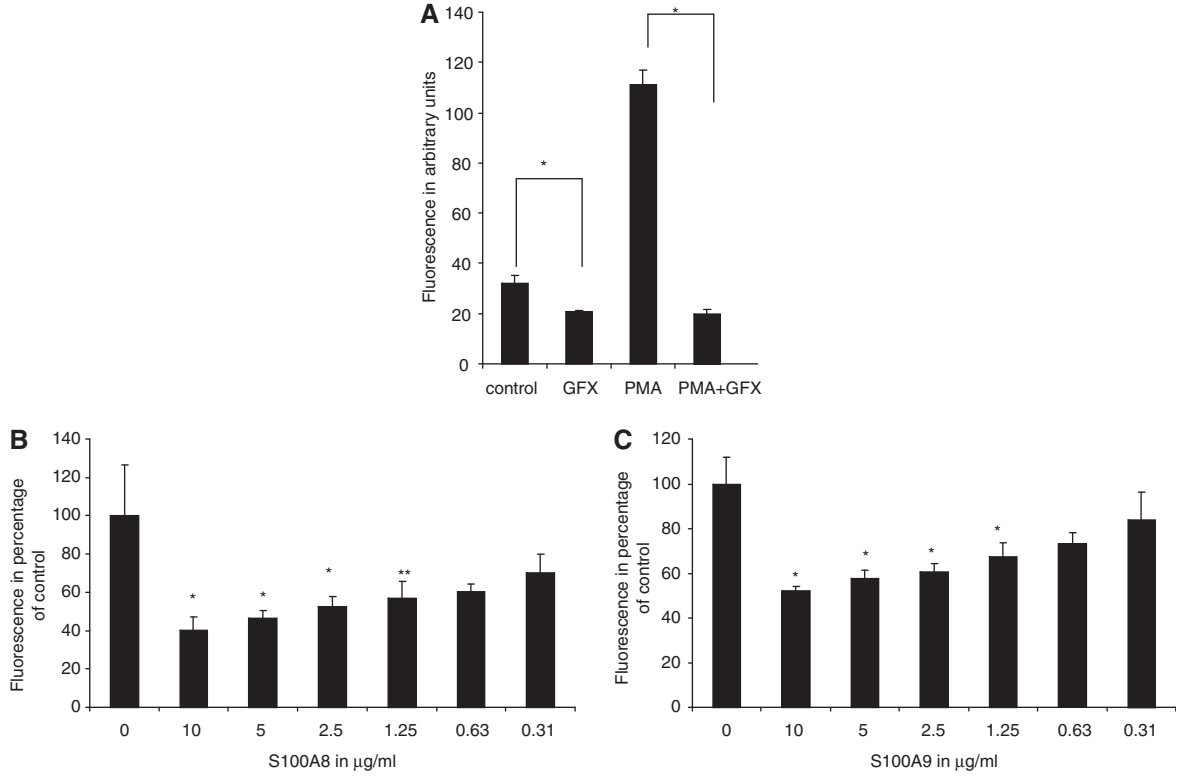

Figure 3. Fluorescence emission of neutrophils incubated with DCFH-DA probe in the presence of 1 μM PMA; 5 μM GFX caused a significant decrease in the spontaneous and the PMA induced oxidation of the DCFH-DA probe (A). S100A8 (B) and S100A9 (C) produce a dose sensitive inhibition of PMA (1 μM) induced oxidative activation. $*p < 0.05$ compared to control. The data represents the mean ± SD. This is a representative experiment of at least three experiments conducted in triplicate or quadruplicate.

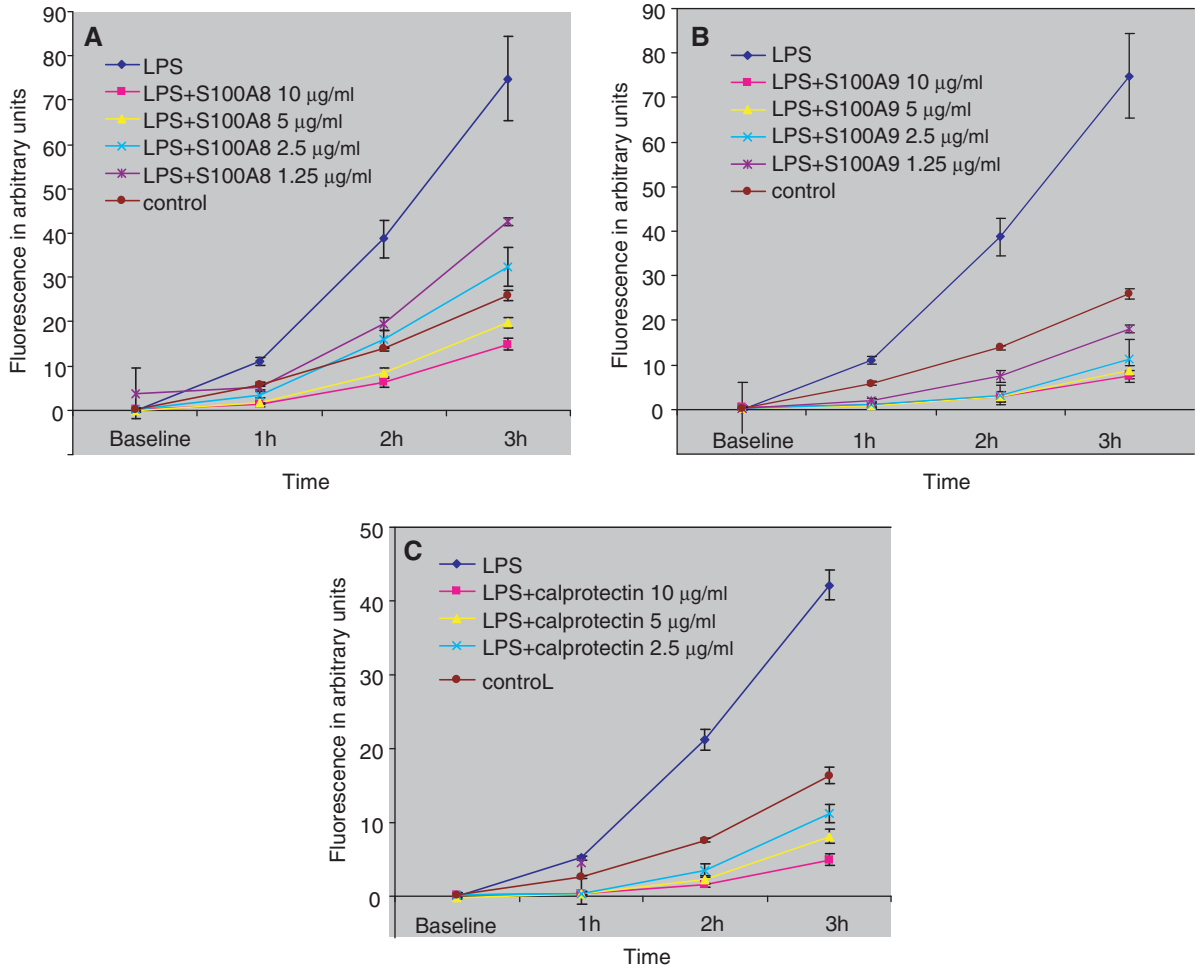

Figure 4. Time course of fluorescence emission of neutrophils incubated with DCFH-DA probe and 10 μg/ml LPS alone or with various concentrations of S100A8 (A), S100A9 (B) and calprotectin (C). Each point is the mean ± SD. S100A8, S100A9 and calprotectin produced a significant reduction to LPS response at all time points and all concentrations ≥ 2.5 μg/ml with a $p < 0.05$ when compared to LPS control. Each point is the mean + SD. This is a representative experiment of at least three experiments conducted in quadruplicate.

similar to what was observed above with the inhibition of the spontaneous oxidative activation.

The effect of S100A8 and S100A9 together and each alone was further tested on the rate at which LPS stimulated neutrophil. The data indicated that at concentrations ranging from 2.5–10 μg/ml of protein S100A8 and S100A9 strongly inhibited LPS stimulation of neutrophil oxidative activity (Figures 4A–C), further indicating that S100A8 and S100A9 anti-oxidative effects were not restricted to the spontaneous activation of neutrophil but were also relevant to controlled stimulated oxidative activation.

S100A8/A9 anti-oxidative effect is mediated by adenosine metabolites

To test whether S100A8/A9 activity was mediated through adenosine metabolites, we next examined their effect on PMN oxidative metabolism in the presence of adenosine deaminase (ADA1), an enzyme which deaminates adenosine to inosine and inhibits its anti-oxidative functions in humans [26]. The data indicated that whereas S100A8 and S100A9 inhibited both the spontaneous and LPS stimulated oxidative metabolism at concentration ranging from 2.5–10 μg/ml (Figures 1 and 4), this effect was partially or fully abrogated in the presence of ADA1 (Figures 5A and B). PMNs express several purinergic receptors including A2A and A3 receptors [22]. Those two receptors have been implicated in the inhibition leukocytes oxidative metabolism [20,21,27]. Accordingly, we next tested the inhibition of A2A and A3 adenosine receptors on S100A8/A9 anti-oxidative effect. The data indicated that inhibitors of A3 receptors abrogated the anti-oxidative effects of S100A8 and S100A9 (Figures 6A and B) on LPS stimulated and on the constitutive oxidative metabolism of neutrophils. Inhibitors of A2A had a more modest effect, whereas inhibitors of A1 and A2B P1 adenosine or P2 purinergic receptors had no measurable effect (data not shown)

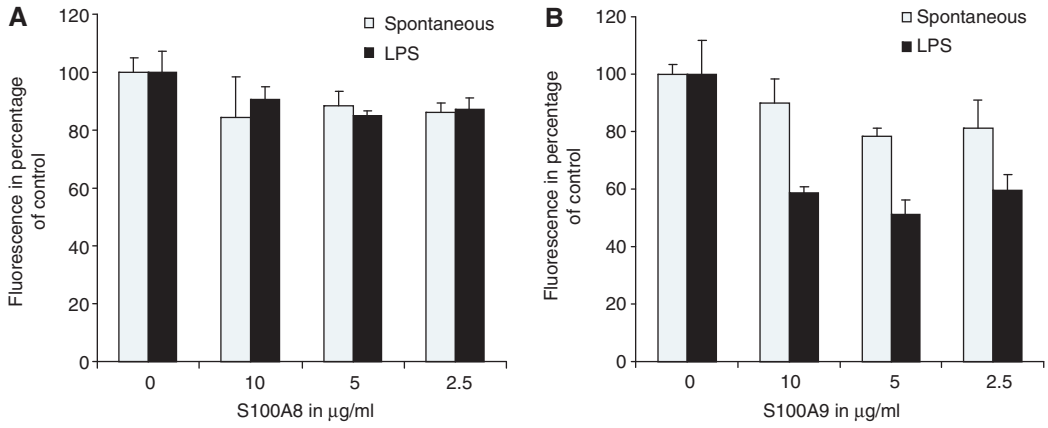

Figure 5. Fluorescence emission of neutrophils incubated with DCFH-DA probe during 1 h alone (spontaneous) or in the presence of LPS 10 µg/ml. The addition of 0.01 mU/ml of human adenosine deaminase 1 (ADA1) caused a substantial reduction in S100A8 (A) and S100A9 (B) anti-oxidative effect when compared to data presented earlier in Figures 1 and 4. Each point is the mean ± SD. This is a representative experiment of at least three experiments conducted in quadruplicate.

Discussion

One of the most important functions of neutrophils is the production of oxidative metabolites in order to kill invading micro-organisms. The same oxidative metabolites may cause serious injury to host tissues. Their production must therefore be tightly regulated. The data included in this report indicate that S100A8 and S100A9 inhibit the spontaneous and stimulated oxidative metabolism of neutrophils *in-vitro*.

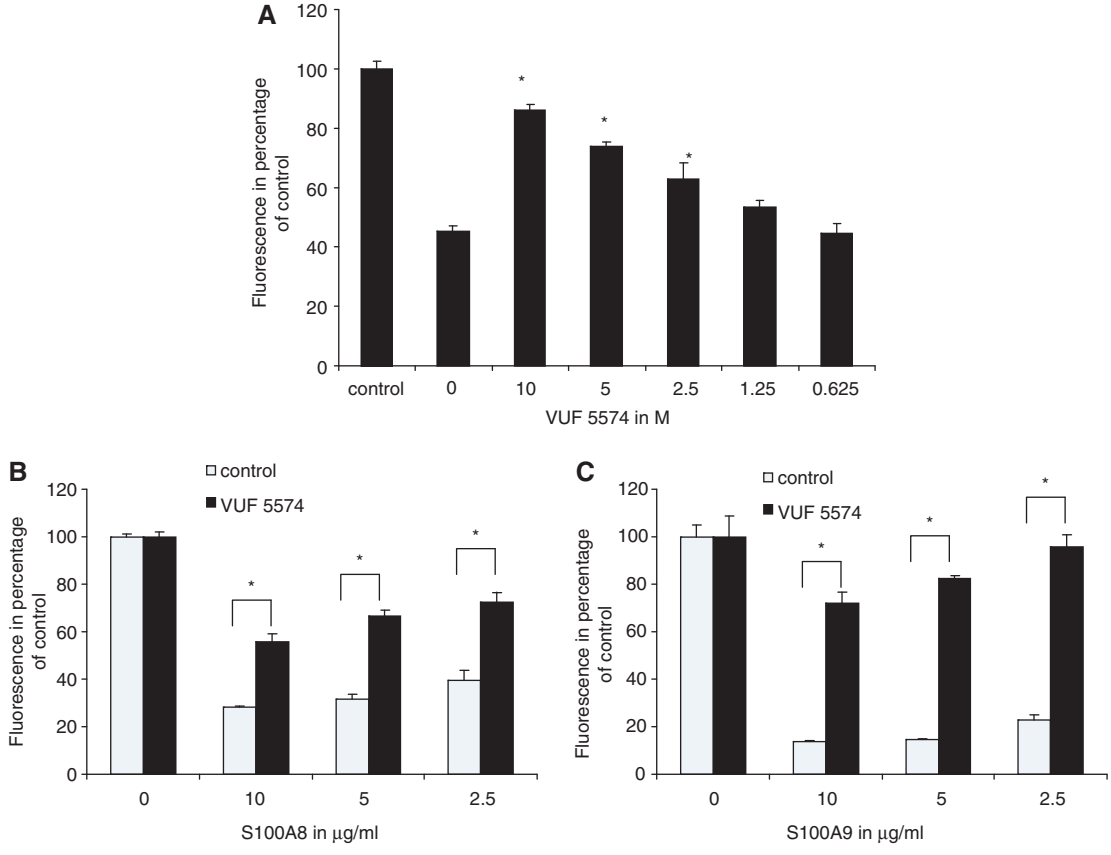

Figure 6. Fluorescence emission of neutrophils incubated with DCFH-DA probe during 1 h alone, LPS, S100A8, S100A9 and/or VUF5574 (an A3 adenosine antagonist). (A) Neutrophils were treated with 2.5 µg/ml S100A8 except for control. VUF 5574 produces a dose-sensitive inhibition the anti-oxidative effect of S100A8 on neutrophil spontaneous oxidative metabolism. S100A8 (B) and S100A9 (C) inhibited the oxidative metabolism of neutrophils exposed to 10 µg/ml LPS. This anti-oxidative effect was countered by 10 µM, VUF 5574. $*p < 0.05$ compared to control treated with S100A8 alone (A) or as indicated. Each point is the mean ± SD. This is a representative experiment of at least three experiments conducted in quadruplicate.

In further support for the anti-oxidative effect of S100A8 and S100A9, the data indicated that blocking the intrinsic S100A8 or S100A9 with specific monoclonal antibodies resulted in an acceleration of the rate of DCFH-DA oxidation, an effect opposite to what was observed with the addition of extrinsic S100 proteins. This finding indicates that S100A8/A9 may play a biologically relevant anti-oxidative function regulating neutrophil activation.

While we have referred to the oxidative activity of neutrophils as spontaneous, it is important to note that this activity is likely the result of cell manipulation and derived from the experimental procedures. Nevertheless, the data showed that spontaneous oxidative metabolism of neutrophils had the hallmarks of a genuine controlled activation of neutrophil oxidative burst. The spontaneous activation was dependent on PKC activity [19], as demonstrated by the effect of GFX. Consequently, whereas it may be difficult to interpret the biological relevancy of the so-called spontaneous activation of neutrophils, it presents the signalling hallmarks of a genuine neutrophil oxidative activation suitable for mechanistic signalling studies.

Beyond the spontaneous activation, the data clearly indicated that S100A8 and S100A9 inhibited the PMA and the biologically relevant LPS stimulation of neutrophil oxidative metabolism. We therefore contend that increased plasma, salivary or faecal levels of S100A8/A9 observed in association with inflammation [28–30] are aimed in part at diminishing the oxidative activation of neutrophils and protect the organism against exaggerated immune response.

DCFH-DA is cleaved by intracellular esterases to the non-fluorescent DCF-DA. It is then believed to be trapped in the cell due to its polarity [15]. The measurements made with the method used in this study are therefore that of intracellular oxidation which likely may also reflect an extracellular oxidative state.

The concentrations at which S100A8 and S100A9 inhibited neutrophil oxidative metabolism are within the range of concentrations recorded during acute inflammation [31–33] and in a syndrome of hypercalprotectinaemia [7] associated with opportunistic infections and auto-immune conditions. It is therefore possible that S100A8/A9 may reach biologically relevant anti-oxidative concentration *in-vivo*.

Whereas the exact mechanism(s) by which S100A8 and S100A9 exert their anti-oxidative effect remains elusive, the data indicated that adenosine metabolites were implicated. Deamination of adenosine resulted in a loss of S100A8/A9 activity. Moreover, pharmaceutical agents targeting adenosine receptors also partially or fully reversed the anti-oxidative effect of the S100A8 and S100A9. The contribution of adenosine metabolites in the regulation of neutrophils or monocytes oxidative metabolism as well as their association with redox-dysregulation associated conditions has been widely reported [20,21,34]. Targeting adenosine signalling to reduce oxidation related damage has also been successfully pursued [35–37]. The implication of adenosine metabolites in S100A8/A9 produced anti-oxidative effect is, however, novel and potentially valuable in the pursuit of adenosine-related therapeutic interventions in conditions associated with deleterious inflammation and redox imbalance.

In conclusion, the anti-oxidative and anti-inflammatory effect of S100A8/A9 may represent a threshold to overcome in the induction of acute inflammation. This model is supported by our previous work in which we presented data indicating that S100A8 and S100A9 repel neutrophils and inhibit their chemotaxis toward pro-inflammatory molecules [10,11]. In this previous work, we reported that the oxidation of S100A8 and S100A9 regulated its anti-chemotactic effect. The complex interactions between neutrophil oxidative metabolism and S100A8/A9 may therefore represent one more layer of regulation by which feedbacks mechanisms cross-regulate the redox state of S100A8/A9 and neutrophil recruitment and activation.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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