

THE acute phase response is a systemic reaction to inflammatory processes characterized by multiple physiological adaptations, including the hepatic synthesis of acute-phase proteins. In humans, serum amyloid A (SAA) is one of the most prominent of these proteins. Despite the huge increase of serum levels of SAA in inflammation, its biological role remains to be elucidated, even though SAA is undoubtedly active in neutrophils. In a previous study, we reported that SAA induces the release of tumor necrosis factor- α , interleukin (IL)-1 β and IL-8 from human blood neutrophils. Here, we extend our earlier study, focusing on the effect of SAA on neutrophil IL-8 transcription and on the signaling pathways involved. We demonstrate herein that SAA, in relatively low concentrations (0.4–100 $\mu\text{g/ml}$) compared with those found in plasma in inflammatory conditions, induces a dose-dependent release of IL-8 from neutrophils. The p38 mitogen-activated protein kinase inhibitor SB 203580 inhibits the IL-8 mRNA expression and the release of protein from neutrophils. The release of IL-8 from SAA-stimulated neutrophils is strongly suppressed by the addition of *N*-acetyl-L-cysteine, α -mercaptoethanol, glutathione, and dexamethasone. SAA also induces IL-8 expression and release from monocytes. In conclusion, SAA appears to be an important mediator of the inflammatory process, possibly contributing to the pool of IL-8 produced in chronic diseases, which may play a role in degenerative diseases.

Key words: Leukocytes, Chemokines, Cellular activation, Inflammation, Infection

mRNA expression and release of interleukin-8 induced by serum amyloid A in neutrophils and monocytes

Fernanda Pereira Ribeiro¹,
Cristiane Jaciara Furlaneto¹, Elaine Hatanaka¹,
Wesley Bueno Ribeiro², Glaucia Mendes Souza²,
Marco A. Cassatella³ and Ana Campa^{1,CA}

¹Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo CEP 05508-900, SP, Brazil;

²Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, Brazil; and

³Departamento di Patologia, Sezione di Patologia Generale, Università di Verona, Italy

^{CA}Corresponding author

Tel: +55 11 3091 3637

Fax: +55 11 3813 2197

E-mail: anacampa@usp.br

Introduction

Interleukin (IL)-8, the most well-characterized member of the CXC-chemokine subfamily, plays a central role in inflammatory processes.¹ A wide variety of cell types, including neutrophils and cells of monocyte lineage, release IL-8 upon exposure to bacterial products and inflammatory cytokines.^{2–4} The chemotactic and stimulatory activities of IL-8 in blood cells lead to the recruitment of a great number of neutrophils and other immune cells to the inflammatory site. Although cell recruitment is essential in overcoming infectious processes, the uncontrolled release of IL-8, and hence the uncontrolled influx of inflammatory cells, is associated with the pathogenesis of various diseases.^{5,6} IL-8 levels in plasma or biological inflammatory fluids are often correlated with the severity of the disease and the outcomes of the patients. Accordingly, studies identifying stimuli for IL-8 production and the signaling events involved

in this process are of significant interest in gaining a greater understanding of inflammatory diseases.

Serum amyloid A (SAA) is a 12-kDa protein synthesized by the liver during microbial infections and inflammatory diseases.^{7,8} In these conditions, the serum concentration of SAA may reach levels up to 1000-fold greater than those found in the non-inflammatory state. Although the concentration of SAA in serum during inflammations is considerably enhanced, its role is uncertain. In addition to the hepatic synthesis of SAA, macrophages,⁹ smooth muscle cells and endothelial cells¹⁰ can also synthesize SAA. Furthermore, SAA has been found in atherosclerotic lesions¹¹ and in the brain of Alzheimer patients.¹² These findings indicate a localized production and action of SAA, emphasizing the importance of this protein in inflammations.

In a previous study, we reported that SAA induces the release of tumor necrosis factor (TNF)- α , IL-1 β and IL-8 from human blood neutrophils.¹³ Here, we

extend our earlier study, focusing on the effect of SAA on neutrophil IL-8 transcription and on the signaling pathways involved. Furthermore, we studied the effect of SAA on the expression of IL-8 mRNA and on the release of protein in monocytes.

Materials and methods

Bovine fetal serum, dextran, ethylenediamine tetraacetic acid, Hepes, Histopaque®, lipopolysaccharide (LPS) (*Escherichia coli* 026:B6), penicillin, *N*-acetyl-L-cysteine, glutathione, 2-mercaptoethanol, dexamethasone, polymixin-B, pertussis toxin, RPMI 1640 supplemented with L-glutamine, sodium bicarbonate and streptomycin used in this study were supplied by Sigma Chemical Co., St. Louis, MO, USA. The recombinant human SAA utilized came from Peprotech Inc., Rocky Hill, NJ, USA. The SAA was essentially endotoxin free, as indicated by a *Limulus* Amebocyte Lysate test (for the concentrations of SAA used in our experiments, 17 and 100 µg SAA, the level of endotoxin was lower than 4 ng/ml) and by a polymyxin B assay.¹⁴ The drugs PD 98059 and SB 203580 were supplied by Calbiochem Novabiochem Corp., San Diego, CA, USA. Both were solubilized in dimethylsulfoxide (DMSO), resulting in a final concentration of DMSO of less than 0.1% per assay. This amount of DMSO affected neither the mRNA expression nor the release of L-8. All other reagents and media were dissolved in pyrogen-free water for clinical use.

Cell purification and culture

Purified neutrophil preparations (> 98.5%) were isolated, as previously described, from the buffy coat¹⁵ or peripheral blood¹³ of healthy donors, both under endotoxin-free conditions. Monocytes were isolated from the peripheral blood of healthy donors by Percoll gradient.¹⁶

IL-8 protein assay

Neutrophils (2.5×10^6 cells/ml) suspended in RPMI 1640 and supplemented with 0.3 g/l glutamine, 2.32 g/l Hepes, 2 g/l sodium bicarbonate, 100 µg/ml streptomycin, 100 IU/ml penicillin and 10% low endotoxin fetal serum were cultured at 37°C and 5% CO₂, with and without SAA (0.4–100 µg/ml). After 2–24 h, the supernatants were collected and frozen at $\leq -40^\circ\text{C}$ until the IL-8 was determined by enzyme-linked immunosorbent assay (Quantikine, R&D System, Minneapolis, MN, USA).

The experiments with mitogen-activated protein kinase (MAPK) inhibitors included the use of 50 µM PD 98059 [a MAPK extracellular signal-regulated

kinase kinase (MEK-1) inhibitor]^{17,18} or of 10 µM SB 203580 (a p38 MAPK inhibitor).¹⁹ These inhibitors were pre-incubated with neutrophils for 15 min before the addition of SAA and then cultured for 24 h. Neutrophils treated with these concentrations of inhibitors displayed no change in their exclusion of Trypan blue and no leakage of cytosolic lactic acid dehydrogenase. The SB 203580 and PD 98059 were solubilized in DMSO, resulting in a final concentration of DMSO of less than 0.1% in the assay. This amount of DMSO did not affect the mRNA expression or release of L-8.

SAA-activated neutrophils were also incubated with dexamethasone (1 µM), *N*-acetyl-L-cysteine (0.3 mM), glutathione (10 mM) and 2-mercaptoethanol (14 mM). Neutrophils treated with these concentrations of inhibitors showed no change in their exclusion of Trypan blue, leaking no cytosolic lactic acid dehydrogenase.

IL-8 was quantified in the supernatant of monocytes (2.5×10^5 cells/ml), which were kept under the same conditions as those described for neutrophils and cultured for 24 h in the presence of SAA (17 µg/ml) or LPS (1 µg/ml).

RNA isolation and northern blot analysis

Neutrophils (1.0×10^7 cells/ml) and monocytes (5.0×10^6 cells/ml) were cultured in the presence of SAA (40 µg/ml) for different lengths of time (2, 6 and 21 h). Cell-free supernatants were discarded and total RNA was extracted from the pellets, using the guanidinium isothiocyanate method, and processed for northern blot analysis, as previously described.¹⁵ Probes were prepared using the Random Primer Labeling Kit (Gibco BRL, Life Technologies, Gaithersburg, MO, USA). The hybridization signals were estimated by scan and quantification, using the levels of actin for normalization. The absence of monocyte mRNA contamination in neutrophils was confirmed by the lack of hybridization with an IL-6 cDNA probe, as recommended by Wang *et al.*²⁰

The statistical analysis consisted of one-way analysis of variance with the Student–Newman–Keuls Multiple Comparisons test.

Results

Neutrophil viability, tested by the Trypan blue exclusion assay, was found to be unaltered in 24 h cultures in the presence of SAA. Human neutrophils incubated with SAA released considerable amounts of IL-8 in 24 h cultures (Fig. 1). The amount of IL-8 released depended on the concentration of SAA. Although significant variations are expected in IL-8 release, depending on the neutrophil preparation, a

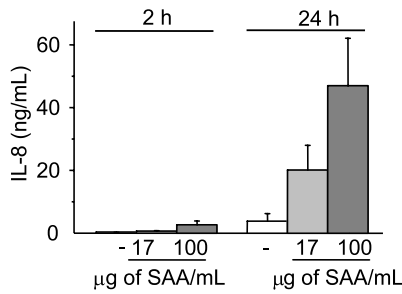


FIG. 1. IL-8 protein levels found in the supernatant of 2 and 24 h cultures of neutrophils (2.5×10^6 cells/ml) in the absence and with the presence of SAA (17 and 100 $\mu\text{g/ml}$ SAA). The data represent the average \pm standard deviation of at least five experiments.

concentration of SAA as low as 0.4 $\mu\text{g/ml}$ induced IL-8 release and saturation was reached at close to 40 $\mu\text{g/ml}$ SAA (Fig. 2). It is important to note that, in the range assayed here (0.4–100 $\mu\text{g/ml}$), the concentrations of SAA were much lower than those present in serum in acute phase conditions (up to 400 $\mu\text{g/ml}$).²¹

Neutrophils release IL-8 upon exposure to different classes of stimuli (e.g. bacterial products such as bacterial LPS). The presence of two or more stimuli may induce synergic or opposing effects that are important *in vivo*. Because serum SAA has been found to increase during bacterial infection, we evaluated the combined effect of SAA and LPS on the release of IL-8 by neutrophils. Mutual interference between the effects of LPS and SAA was not observed, since the resulting amount of IL-8 release in cells treated with them was apparently additive (Fig. 3).

To determine the IL-8 mRNA expression levels, northern blots were performed using neutrophils stimulated with SAA. Although no IL-8 protein was detected in the supernatant of SAA-stimulated neutrophils after 2 h of incubation, the levels of IL-8 mRNA were higher than that found in the control cell (Fig. 4). IL-8 mRNA levels are kept high for prolonged exposure to SAA. This temporal pattern of IL-8 expression and release resembles that described for LPS-stimulated neutrophils and differs considerably

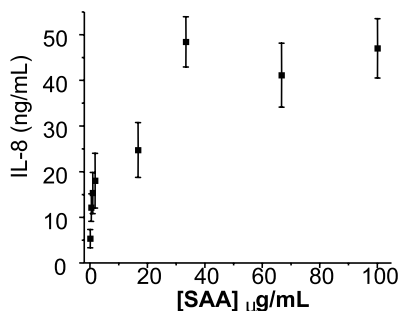


FIG. 2. IL-8 protein levels found in the supernatant of 24 h cultured neutrophils (2.5×10^6 cells/ml) incubated with different concentrations of SAA. The data represent the average \pm standard deviation of at least five experiments.

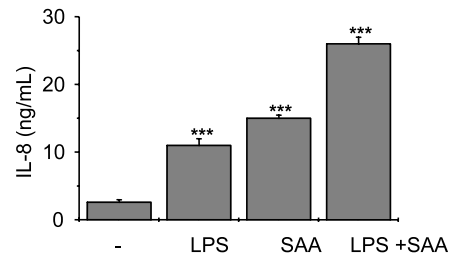


FIG. 3. Effect of the combined addition of SAA (17 $\mu\text{g/ml}$) plus LPS (1 $\mu\text{g/ml}$) on the release of IL-8 in the supernatant of 24 h cultured neutrophils (2.5×10^6 cells/ml). The data represent the average \pm standard deviation of at three experiments (***) $p < 0.001$.

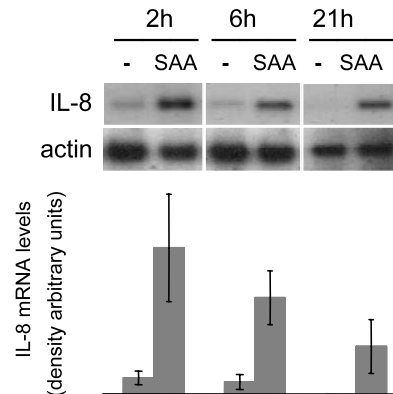


FIG. 4. IL-8 mRNA levels determined by northern blots of 2.0×10^7 neutrophils stimulated for 2, 6 and 21 h with SAA (40 $\mu\text{g/ml}$). Lower part: average \pm standard deviation of the normalized data using the corresponding actin signals of three experiments.

from that induced by formyl-methionyl-leucyl-phenylalanine (fMLP).²²

Members of the MAPK family become phosphorylated in response to diverse extracellular stimuli²³ and may be involved in the signaling that regulates IL-8 expression.²⁴ However, it is also generally accepted that distinct stimuli able to carry the same response may use different intermediate pathways. To evaluate the effect of two specific MAPK inhibitors on SAA-stimulated IL-8 expression and release, we added the inhibitors PD 98059 and SB 203580, which target the upstream effector of extracellular signal-regulated protein kinase (MEK-1)^{17,18} and the p38 kinase,¹⁹ respectively. At concentrations previously used to inhibit cytokine expression and release provoked by other stimuli,²⁴ SB 203580 and PD 98059 (to a lesser degree) decreased IL-8 mRNA levels (Fig. 5). Both these inhibitors had an inhibitory effect on the release of IL-8 protein (Fig. 6). These results indicate the activation of MAPK pathways by SAA, as demonstrated with other stimuli in neutrophils.^{23–25}

The effect of the antioxidants *N*-acetyl-L-cysteine, 2-mercaptoethanol and glutathione, and the anti-inflammatory dexamethasone on SAA-stimulated IL-8 release was investigated. Although these com-

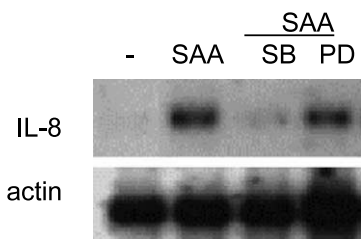


FIG. 5. Effect of SB 203580 (10 μ M) and PD 98059 (50 μ M) on IL-8 mRNA levels determined by northern blots of 2.0×10^7 neutrophils stimulated for 2 h with SAA (40 μ g/ml). The neutrophils were pre-incubated with the inhibitors 15 min before the addition of SAA. The data are representative of three experiments.

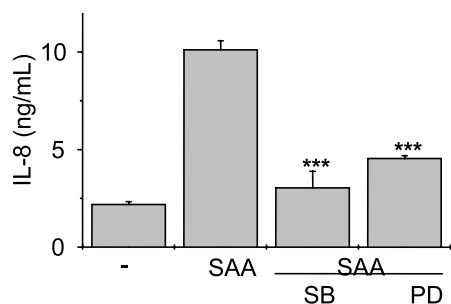


FIG. 6. Effect of SB 203580 (10 μ M) and PD 98059 (50 μ M) on SAA-induced IL-8 release in the supernatants of 24 h neutrophil cultures (2.5×10^6 cells/ml). The neutrophils were preincubated with the inhibitors 15 min before the addition of SAA (17 μ g/ml). The data represent the average \pm standard deviation of five experiments (***) $p < 0.001$.

pounds produce innumerable biological effects, they share an inhibitory effect on the nuclear transcription factor nuclear factor- κ B.^{26–29} These four compounds caused a strong reduction in the amount of IL-8 released from SAA-stimulated neutrophils (Fig. 7).

Finally, although it is clear from this study that SAA induces the expression and release of IL-8 from human blood neutrophils, it was important to determine whether this effect was restricted to neutrophils or whether it might extend to other cellular types. We observed that monocytes also respond to SAA

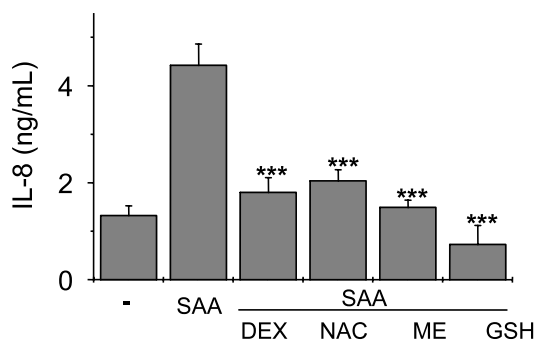


FIG. 7. Effect of dexamethasone (DEX) (1 μ M), *N*-acetyl-L-cysteine (NAC) (0.3 mM), 2-mercaptoethanol (ME) (14 mM) and glutathione (GSH) (10 mM) on SAA-induced IL-8 release in the supernatants of 24 h neutrophil cultures (2.5×10^6 cells/ml). The neutrophils were preincubated with these compounds 15 min before the addition of SAA (17 μ g/ml). The data represent the average \pm standard deviation of five experiments (***) $p < 0.001$.

increasing IL-8 expression and release. Figs. 8 and 9 illustrate the effect of SAA on monocytes in comparison with LPS.

Discussion

The acute phase response is a systemic reaction to inflammatory processes characterized by multiple physiological adaptations, including the hepatic synthesis of acute-phase proteins.³⁰ In humans, SAA is one of the most prominent of these proteins.^{30–33} Despite the huge increase of serum levels of SAA in inflammation, its biological role remains to be elucidated, even though SAA is undoubtedly active in neutrophils and monocytes. SAA affects neutrophil degranulation, phagocytosis, killing activity,³⁴ and neutrophil and monocyte cytokine release (see reference 13 and the present study).

In neutrophils, SAA binds to the G-protein-coupled receptor FPRL 1.³⁵ The responses mediated by G-protein-coupled receptors involve both the stimulation of conventional second-messenger-generating systems, and the functional integration of the intracellular signaling network.³⁶ The association of SAA with the FPRL 1 receptor is probably involved in the intracellular Ca^{2+} mobilization and produces some of the biological effects described for SAA. In a recent study simultaneous to our own, He *et al.*³⁷ showed that the production of IL-8 induced by SAA in neutrophils is also triggered by this receptor. As described for a variety of agonists acting on G-

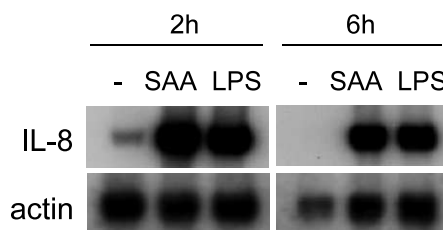


FIG. 8. IL-8 mRNA levels determined by northern blots of 5.0×10^6 monocytes stimulated for 2 and 6 h with SAA (40 μ g/ml) or LPS (100 ng/ml). The data are representative of three experiments.

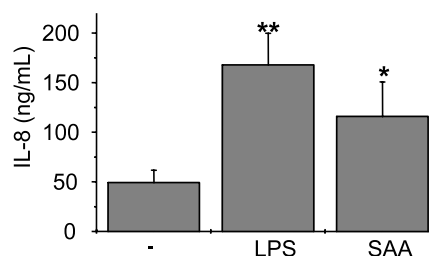


FIG. 9. IL-8 protein levels found in the supernatant of 24 h cultured monocytes (2.5×10^5 cells/ml) incubated with SAA (17 μ g/ml) or LPS (1 μ g/ml). The data represent the average \pm standard deviation of at least five experiments (* $p < 0.05$ and ** $p < 0.01$).

protein-coupled receptors,^{38,39} the activation of MAPK pathways could be operative from the FRLP 1 receptor. Here, we observed that SAA in relatively low concentrations compared with the plasma SAA concentration found in inflammatory conditions is able to induce the release of IL-8, and that MAPK pathways apparently play an important role in this process. A clear effect of the p38 MAPK inhibitor SB 203580 is observed in both neutrophil IL-8 mRNA expression and protein release promoted by SAA. The inhibitory effect promoted by PD 98059 was much more evident at the protein level than at the mRNA level, which seems to be characteristic of this compound, since the same behavior was observed in the induction of TNF- α promoted by LPS in monocytes.⁴⁰

The inhibitory effect of the antioxidants and dexamethasone observed on SAA-induced IL-8 release from neutrophils is consistent with the general mechanism of nuclear factor- κ B activation. This finding may be relevant in the future to support the therapeutic use of antioxidants in chronic diseases in which serum SAA is higher than under normal physiological conditions.

All normal immune responses, including innate and adaptive immunity, are self-limited. For instance, in most acute inflammations, the hepatic synthesis of SAA wanes over time. In a broad sense, the return to homeostasis is essential to prevent uncontrolled tissue damage. It is therefore difficult to interpret the biological significance and impact on the progression of disease of persistently high levels of SAA in some chronic diseases, such as diabetes,⁴¹ cancer,⁴² atherosclerosis⁴³ and rheumatic diseases.⁴⁴ The intriguing positive correlation existing between high SAA serum levels and coronary artery disease⁴³ and the presence of SAA in human atherosclerotic lesions¹¹ and in the brain of Alzheimer patients¹² suggests that SAA plays a role in the genesis and progression of chronic diseases. IL-8 seems to be closely involved in angiogenesis⁴⁵ and atheroma plaque formation.⁴⁶ The SAA induction of IL-8 may, for instance, provide a link between the rise in SAA serum levels and the development of coronary disease. The same rationale could be used for long-term conditions that give rise to basal increasingly higher SAA serum levels than those found in healthy individuals, where a constant stimulus for the release of proinflammatory cytokines by responsive cells may be provided.

In conclusion, SAA appears to be an important mediator of the inflammatory process and may contribute to the pool of IL-8 produced in chronic diseases, which may play a role in degenerative diseases. As is the case with IL-8, the serum level of SAA is associated with the severity of the disease.²¹ In our hypothesis, the deleterious effect of SAA is partially mediated by the release of IL-8 and other

inflammatory cytokines. It is conceivable that some of the activities described for SAA are mediated by these cytokines rather than being a direct effect of SAA.

ACKNOWLEDGEMENTS. The authors are indebted to the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for their financial support of this work. The authors also thank Ms Béatrice Allain for proofreading the English version of this paper.

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Received 1 April 2003
Accepted 18 April 2003