Biochemical and Biophysical Research Communications **270,** 331–335 (2000)
doi:10.1006/bbrc.2000.2416, available online at http://www.idealibrary.com on IDEAL®

Serum Amyloid A Is a Chemotactic Agonist at FPR2, a Low-Affinity *N*-Formylpeptide Receptor on Mouse Neutrophils

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Received February 29, 2000

Serum amyloid A (SAA) is an acute-phase plasma protein and the source of amyloid A, which accumulates in lesions of secondary amyloidosis. SAA can induce phagocyte migration *in vitro* and *in vivo,* and is a specific chemotactic agonist for the human low-affinity *N*-formylpeptide receptor FPRL1R, a G-protein-coupled receptor expressed on phagocytes. Here we show that FPR2, a mouse counterpart of FPRL1R, is also an SAA receptor. SAA selectively induced calcium flux and chemotaxis in mouse PMN, which express FPR2, as well as in HEK 293 cells expressing recombinant FPR2 but not in HEK 293 cells expressing FPR, a closely related high affinity *N*-formylpeptide receptor. Consistent with this, SAA activity on PMN from FPR+/+ and FPR−/− mice was indistinguishable. Moreover, the prototype N-formylpeptide fMLF desensitized SAA-induced calcium flux in a dose-dependent manner in both mouse neutrophils and HEK 293/FPR2 transfectants. Our results suggest that FPR2 specifically mediates mouse neutrophil migration in response to SAA. © 2000 Academic Press

Key Words: **chemoattractant; inflammation; neutrophil; G-protein-coupled receptor; serum amyloid A.**

Acute-Serum Amyloid A (SAA) refers to the products of two highly conserved, coordinately expressed genes hyperinduced during the acute phase inflammatory response (1). As for other acute phase proteins, the liver is a major site of SAA production, and SAA gene expression is strongly upregulated by the pro-inflammatory cytokines IL-1, IL-6 and TNF (2, 3). SAA is released from the liver into the circulation where it is transported to other tissues bound to high-density lipoprotein (HDL) (1).

SAA has been implicated as both a beneficial and harmful factor in inflammatory processes. Potential beneficial roles include reverse transport of cholesterol at sites of inflammation, through its ability to displace cholesterol from HDL (4, 5), and regulation of immune effector responses (1). With respect to harmful roles, SAA has been strongly associated with chronic inflammatory diseases, particularly atherosclerosis and secondary amyloidosis (6, 7). In atherosclerosis, SAA accumulates in macrophages, macrophage-derived "foam cells," adventitial macrophages, adipocytes, endothelial cells and smooth muscle cells in the vascular plaque (6). In contrast, in secondary amyloidosis, SAA does not directly accumulate in the pathologic lesion, but instead undergoes partial proteolysis resulting in the formation of amyloid A (AA), a major constituent of amyloid fibrils (8). Macrophages are often found associated with amyloid deposits and may be responsible for cleavage of SAA to AA (9–11).

Conversely, SAA can also act directly on macrophages. It is a potent chemoattractant for human blood-derived monocytes *in vitro,* and when injected subcutaneously can elicit migration of mouse monocytes (12). SAA also chemoattracts human neutrophils *in vitro* and mouse neutrophils *in vivo* (12). Thus, SAA may be responsible in part for accumulation of phagocytes at sites of chronic inflammation.

SAA-induced phagocyte chemotaxis is pertussis toxin-sensitive, which suggests involvement of a seven transmembrane domain G-protein-coupled receptor (13). We have recently identified the human N-formylpeptide receptor like-1 receptor (FPRL1R) as a candidate for SAA receptor on phagocytes (13). SAA chemoattracts HEK 293 cells transfected with FPRL1R, and FPRL1R is expressed in neutrophils and monocytes (14), FPRL1R agonists also include N-formyl-

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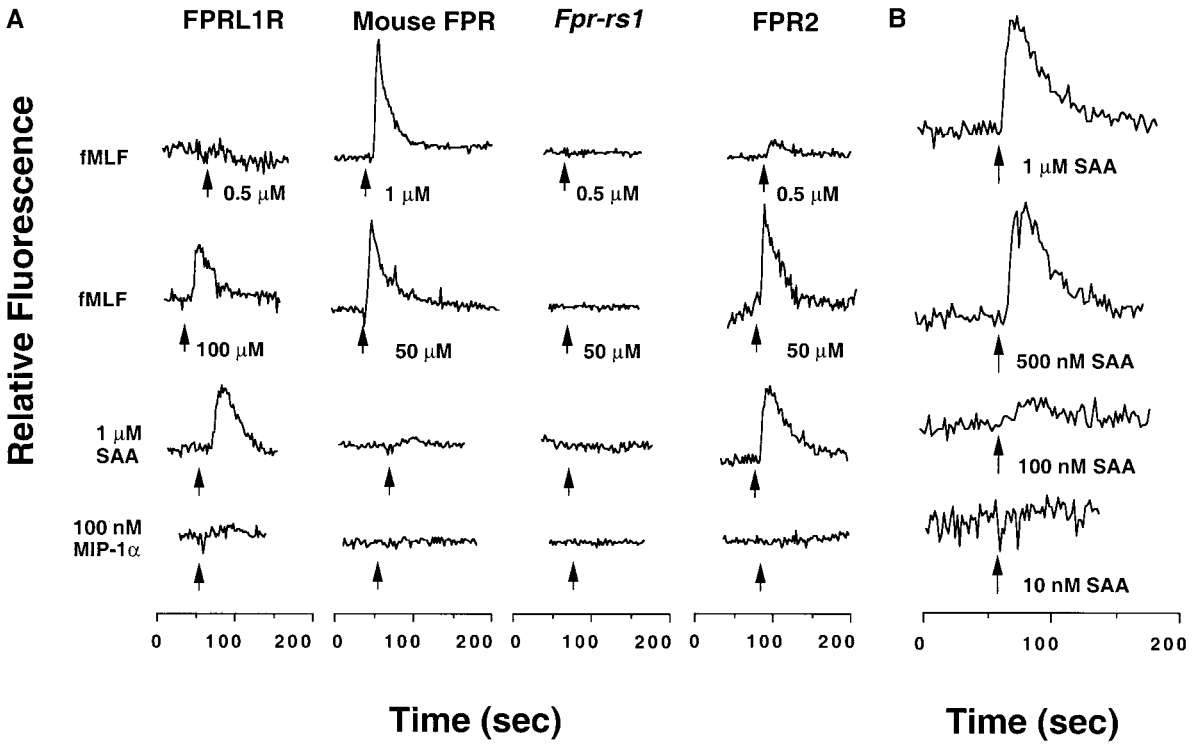

FIG. 1. FPR2 is a specific receptor for SAA. (A) Calcium mobilization in HEK 293 cells transfected with human FPRL1R, mouse *Fpr-rs1*, mouse FPR, and mouse FPR2. Agonists and concentrations corresponding to each tracing are indicated to the left of the row in which it is found, unless otherwise indicated. Each tracing corresponds to the receptor indicated at the top of the column in which it is found. Agonists were added at the time indicated by arrows. (B) HEK 293/FPR2 cells were stimulated with SAA at various concentrations between 10 nM and 1 μM in a calcium flux assay. The ratio of fluorescence at 340 and 380 nm wavelengths were recorded in real time. Data are from a single representative experiment repeated at least three times.

peptides, which derive from bacterial and mitochondrial proteins, as well as the lipid lipoxin A4, and several HIV envelope glycoprotein-derived peptides (15–17).

FPRL1R binds N-formylpeptides with low affinity and is structurally related to FPR, a high affinity N-formylpeptide receptor, as well as to a third orphan receptor named FPRL2 (14, 17, 18). The genes for these receptors are clustered on human chromosome 19 (18). In mouse, the corresponding locus on chromosome 17 contains six related genes, including a functional counterpart of FPR (19). Two of the other genes, *Fpr-rs1* and *Fpr-rs2*, encode receptors which are each ~65% identical to FPRL1R, indicating lineage specific gene replication after divergence of mouse and human. *Fpr-rs1* encodes a lipoxin A4 receptor designated LXA4R (20), whereas *Fpr-rs2* encodes a low affinity receptor for N-formylpeptides designated FPR2 (21), suggesting that in mouse the N-formylpeptide and lipoxin A4 signaling functions of FPRL1R may be carried out by these separate receptors (21). This may provide an advantage for analyzing the biological importance of SAA-specific chemotactic signaling by a gene knockout experiment applied to mouse models of SAA-associated disease. However, it introduces the problem of first identifying which mouse receptor actually binds SAA. Here we identify FPR2 as a specific mouse SAA receptor.

MATERIALS AND METHODS

Mouse leukocyte purification. Methods for isolation of mouse neutrophils have been previously described (22). Mice used in this study were from FPR +/− × FPR +/− matings of an F1 backcross of FPR +/− 129/Sv with wild type C57B1/6 mice. Leukocytes were harvested from the peritoneal cavity following thioglycollate irritation, as previously described (22). After 3 h, >90% of cells isolated were neutrophils, as confirmed by morphological appearance of Diff-Quick-stained preparation.

Cell lines. HEK 293 cells expressing human FPRL1R, mouse FPR, and mouse FPR2 were generated previously (13, 21) and maintained in DMEM with 10% FBS and 2 g/L G-418 (GIBCO-BRL, Gaithersburg, MD) in an incubator at 37°C, 5% CO_2 and 100% humidity.

Intracellular [Ca^{2+}] measurements. Cells were grown to confluence in 170 cm^2 flasks, washed once with PBS, then incubated with 10 ml of PBS and 2.5 μM Fura-2AM (Molecular Probes, Eugene, OR) for 1 h at 37°C in the dark. Cells were harvested and washed twice with HBSS. Then 4×10^6 cells in 2 ml HBSS were placed in a continuously stirred cuvette at 37°C in a fluorimeter (Photon Technology Inc., South Brunswick, NJ). Data were recorded every 200 ms as the relative ratio of fluorescence emitted at 510 nm after sequential excitation at 340 and 380 nm. Recombinant human SAA was

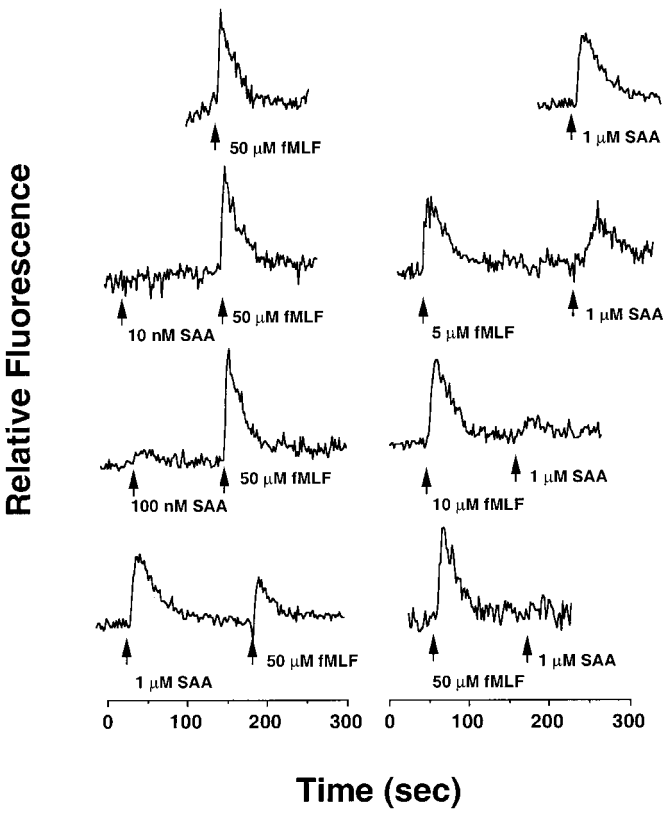

FIG. 2. SAA and fMLF signal through a common pathway: Cross desensitization at FPR2. Sequential stimulation of HEK 293/FPR2 transfectants with SAA and fMLF and vice versa analyzed by calcium flux. Agonists and concentrations are indicated to the right of each arrow, which marks the time of addition. The ratio of fluorescence at 340 and 380 nm wavelengths were recorded in real time. Data for each curve are from a single representative experiment repeated at least twice.

obtained from Peprotech (Rocky Hill, NJ) and fMLF was obtained from Sigma (St. Louis, MO).

Chemotaxis. HEK 293 cell lines at 75% confluence were washed once with PBS, incubated for 1 min in 0.05% trypsin/EDTA (Quality Biological, Gaithersburg, MD), resuspended in DMEM with 10% FBS, pelleted, and resuspended at 1 million cells/ml in chemotaxis buffer (RPMI supplemented with 1% BSA and 20 mM HEPES). SAA and fMLF were resuspended in chemotaxis buffer and approximately 27 μl was loaded into the lower chamber of a 48-well microchemotaxis apparatus (NeuroProbe, Cabin John, MD). The chamber had been previously soaked in 1% SDS overnight, rinsed with distilled water several times, and dried. A polyvinylpyrrolidone-free polycarbonate filter (10 and 3 μm pores for HEK 293 cells and neutrophils, respectively) was used. The filters used for HEK 293 cells had been previously soaked on both sides overnight at 4°C in RPMI 1640 with 0.05 mg/ml rat tail collagen and 70 mM HEPES and air dried. The filter was placed between the upper and lower compartments of the chemotaxis chamber, covering all wells filled with ligand. The upper chamber was loaded with 50 μl of cells containing 10^6 HEK 293 cells/ml or 1.5×10^6 neutrophils/ml of chemotaxis buffer. The chemotaxis chamber was incubated at 37°C, 100% humidity and 5% CO_2, for 1 h for neutrophils and 5 h for HEK 293 cells. The filter was then removed, washed, fixed, and stained using Diff-Quick. Cells that migrated through the filter were counted in a high power field (hpf) in triplicate. Data are presented as the mean ± SEM number of cells/hpf.

RESULTS AND DISCUSSION

FPR2 is a mouse receptor for SAA. Previously, we reported that SAA is a chemotactic agonist at FPRL1R but not at FPR. To determine whether there is a functional counterpart in the murine family of FPR-related receptors, we first tested HEK 293 cells expressing the three candidate mouse receptors: FPR, a polymorphic variant of LXA4R (referred to in this paper as *Fpr-rs1*); and FPR2. As previously reported, HEK 293/FPRL1R transfectants exhibited calcium flux responses to both fMLF and SAA, whereas mouse FPR and FPR2, but not *Fpr-rs1*, responded to fMLF (15, 21). The concentration dependence of receptor activation was the same as previously reported. HEK 293/FPR2 but not mouse FPR or *Fpr-rs1* transfectants also responded to SAA (Fig. 1A). The response of HEK 293/FPR2 cells to SAA was dose-dependent, with a threshold ~100 nM, an EC_{50} ~300 nM and saturation at ~1 μM (Fig. 1B). None of the cell lines studied responded to the chemokine MIP-1α. We do not yet have a way to monitor surface expression of *Fpr-rs1* protein, therefore we cannot draw conclusions about its specificity for fMLF or SAA.

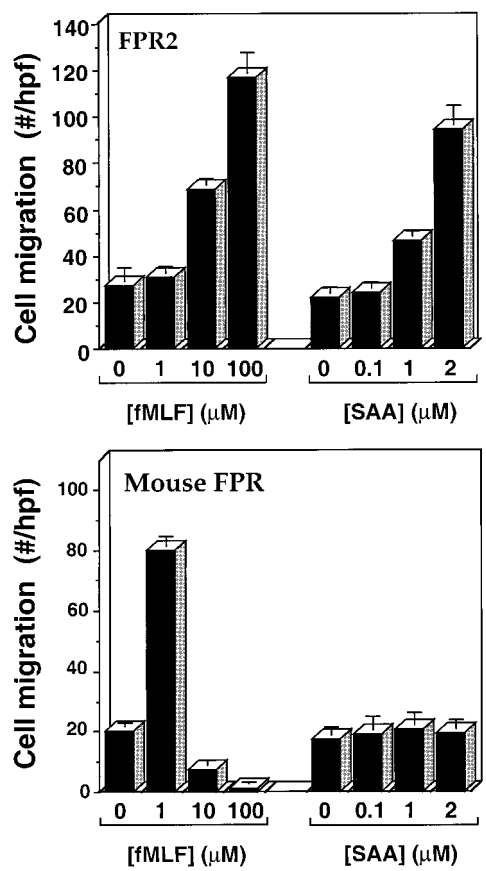

FIG. 3. FPR2 is a functional chemoattractant receptor for SAA. Chemotactic activity of fMLF and SAA for HEK 293 cells expressing FPR2 (top) and mouse FPR (bottom). Data are from a single experiment representative of two experiments.

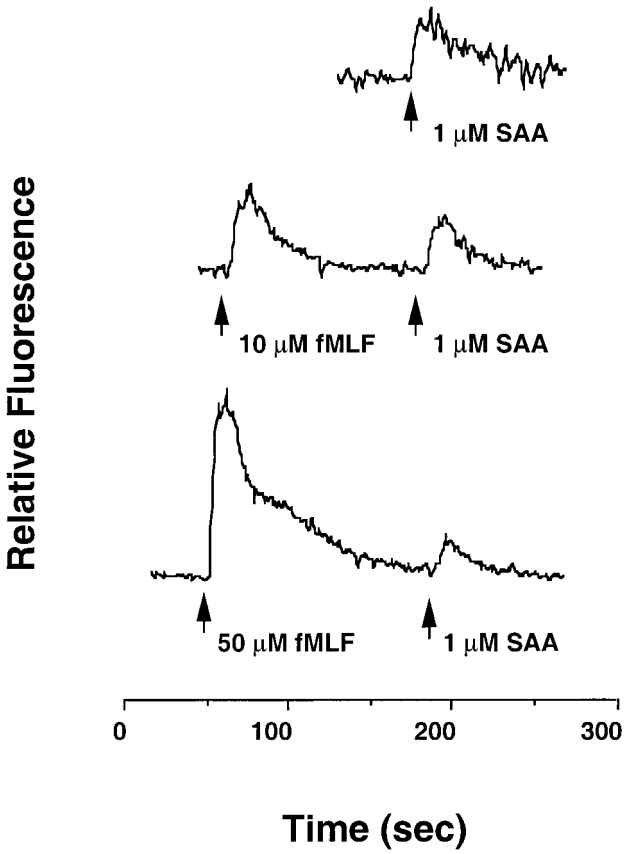

FIG. 4. SAA activates mouse neutrophils by calcium flux through an fMLF signaling pathway. Calcium mobilization by SAA and heterologous cross-desensitization between sequential stimulations of fMLF and SAA on mouse neutrophils. Agonists were added at the time indicated by arrows with the concentration on the right. The cells used were thioglycollate-elicited peritoneal cells (>90% PMN) from FPR−/− mice.

To support the notion that SAA and fMLF use the same receptor, we performed cross-densensitization experiments, a widely used method to infer shared usage of a receptor by two agonists. This is accomplished by testing whether stimulation of calcium flux in real time by one agonist can reduce the response to a second agonist. When the HEK 293/FPR2 cells were sequentially stimulated with SAA and fMLF, the response to 50 μM fMLF given second was reduced in a dose-dependent manner when preceded by stimulation with SAA (10 nM to 1 μM), indicating cross-desensitization. Likewise, stimulation with increasing concentration of fMLF (5–50 μM) reduced the responsiveness of cells to SAA given second. When preceded by 50 μM fMLF, a 1 μM SAA stimulus was completely desensitized (Fig. 2). These results are consistent with usage by fMLF and SAA of the same receptor, FPR2.

SAA is a chemotactic agonist at FPR2. We next tested the ability of SAA to elicit a chemotactic response via FPR2. HEK 293/FPR2 cells migrated specifically in response to SAA in a dose-dependent manner (Fig. 3). The threshold was between 100 and 1000 nM. HEK 293/mouse FPR cells migrated in response to fMLF but not SAA, which is consistent with the inability of SAA to induce calcium signaling in these cells (Fig. 3). Therefore, SAA can induce both calcium flux and chemotaxis of cells expressing FPR2.

SAA is a chemotactic agonist for mouse neutrophils. Since FPR2 is expressed in mouse neutrophils, we next tested the biological relevance of SAA action at this receptor by analyzing SAA-induced responses of thioglycollate-elicited primary mouse peritoneal cells (>90% PMN). SAA (1 μM) induced calcium flux in cells from FPR −/− mice, which could be desensitized in a dose-dependent manner by prior stimulation with fMLF (Fig. 4). Ten μM fMLF, which is close to the saturating concentration for induction of calcium flux via mouse FPR, caused only about 10% reduction of the 1 μM SAA-induced signal, whereas 50 μM fMLF, which is close to the EC_{50} concentration for FPR2, was much more effective (Fig. 4). This experiment suggests that mouse neutrophils respond to SAA via FPR2.

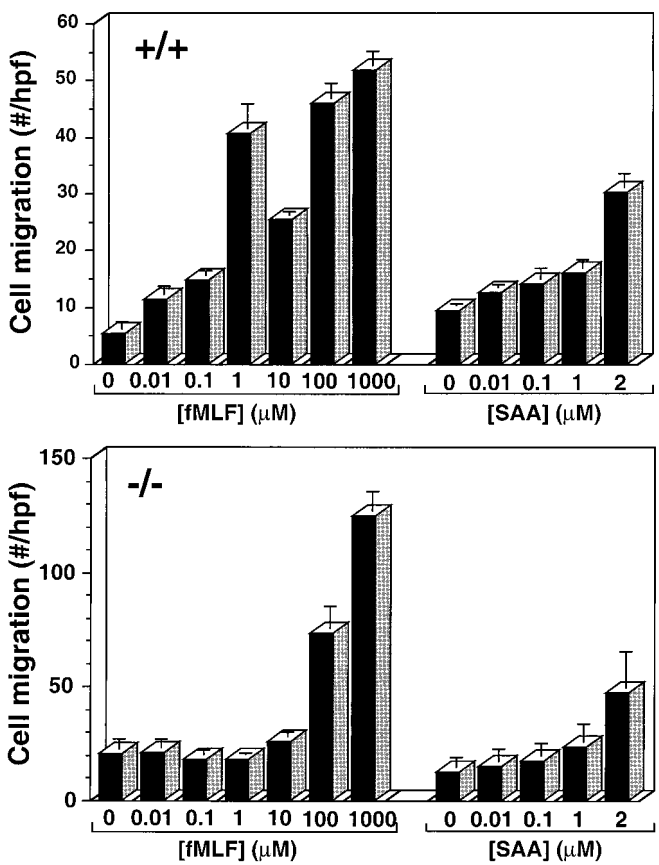

FIG. 5. SAA is a chemotactic agonist for mouse neutrophils. Cell migration response to fMLF and SAA on thioglycollate-elicited primary mouse peritoneal cells (>90% PMN) from FPR +/+ (top) and FPR −/− (bottom) litter mates. Data are from a single representative experiment repeated at least three times.

Mouse neutrophils were also able to migrate in response to increasing concentrations of SAA (Fig. 5), with a threshold concentration between 100 and 1000 nM. Responses of neutrophils from FPR +/+ and −/− mice to SAA were indistinguishable (Fig. 5), further supporting a specificity of SAA on FPR2. As described previously, fMLF induced neutrophil chemotaxis through FPR at low concentrations and FPR2 at high concentrations (Fig. 5, ref. 21).

In summary we have shown that SAA is a chemotactic agonist for mouse thioglycollate-elicited peritoneal neutrophils and HEK/293 cells expressing the low affinity N-formylpeptide receptor FPR2. These results suggest that SAA may chemoattract neutrophils by specifically activating FPRs, and suggest a specific molecular mechanism for SAA induction of neutrophil migration *in vivo* (12). This work extends our previous report that SAA is a chemotactic agonist at the FPR2 human counterpart FPRL1R (13).

FPRL1R and FPR2 are both expressed in neutrophils (14, 21). FPRL1R is also expressed in human monocytes (14) whereas FPR2 expression in thioglycollate elicited peritoneal macrophages is very low, as assessed by RNA analysis and selective agonists (21). Additional studies will be needed to assess expression of FPR2 in other types of mouse macrophages and the specific interaction of mouse SAA with FPR2. Nevertheless, our current results validate creation of an FPR2 knockout mouse, as a way to test the role of chemotactic signaling by SAA in mouse models of inflammation.

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