

Super-oxidized solution inhibits IgE-antigen-induced degranulation and cytokine release in mast cells[☆]

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Abstract

Activation of the high affinity IgE receptor (FcεRI) through IgE-antigen complexes induces mast cell degranulation, synthesis of lipid mediators and cytokine production. These effects are involved in Type I hypersensitivity reactions and controlling them has been the main objective of many anti-allergic therapies. Here we report that pretreatment of murine bone marrow derived mast cells (BMMC) with super-oxidized solution (SOS) inhibits FcεRI dependent-β hexosaminidase and cytokine release. This effect is exerted without altering total protein tyrosine phosphorylation, MAPK activation, cytokine mRNA accumulation or calcium mobilization after FcεRI triggering. Our data suggest that this neutral pH-SOS acts like a mast cell-membrane stabilizer inhibiting the cell machinery for granule secretion without altering the signal transduction pathways induced by IgE-antigen receptor crosslinking.

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Keywords: Mast cell; Degranulation; Cytokine production; Allergy; IgE receptor; Super-oxidized solution

1. Introduction

Mast cells (MCs) have long been recognized as effector cells of hypersensitivity Type I reactions and protective responses against parasites [1,2]. These cells begin their differentiation in the bone marrow from CD34⁺ hematopoietic progenitors and migrate to the bloodstream as committed cells. In peripheral tissues [3], they acquire their distinctive phenotype under the influence of stem cell factor (SCF), interleukin (IL)-3 and various other locally-produced mediators [4]. Although MCs do not represent a single homogenous, functional-cell population, there is one characteristic that unites them all with basophils which is the possession of high affinity membrane receptors for immunoglobulin

Abbreviations: SOS, super-oxidized solution; SCF, stem cell factor; MCs, mast cells; BMMCs, bone marrow-derived mast cells; CCL3/MIP1-α, macrophage inhibitory protein 1 alpha; IgE, Immunoglobulin E; FcεRI, High affinity-IgE receptor; NFAT, nuclear factor of activated T cells; 7-AAD, 7 aminoactinomycin; PMA, phorbol 12 myristate 13 acetate; TNF-α, tumor necrosis factor alpha; DNP-HSA, dinitrophenol-human serum albumin.

[☆] AA Gutiérrez conducted the research in Mexico and had become a paid employee of Oculus IS at the time of drafting this paper.

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(Ig) E (Fc ϵ RI). When these receptors are activated by IgE-antigen interactions, the Fc ϵ RI signal transduction pathway can lead to 1) histamine and protease-containing granule release, 2) lipid-derived inflammatory mediator production and 3) cytokine synthesis [5]. The secretion of these mediators is responsible for early and late phases of allergic responses [6]. Depending on the anatomic site where activated mast cells are located, the immediate secreted molecules can play a regulatory role of smooth muscle contraction, immune cell chemotaxis, endothelial cell layer permeability and mucus production. Mast cell activation, therefore, plays an important role in a wide array of inflammatory disorders [7].

Investigation into the mechanisms that control histamine release by mast cells has constituted an important milestone in immunopharmacology [8]. Based on this knowledge, diverse pharmacological agents have been developed to prevent or ameliorate the activation and secretion of mast cells. Among them, β 2 adrenoceptor stimulants, methylxantines, glucocorticoids, antihistamines and mast cell stabilizers (chromones) are the main classes of drugs widely used for allergic diseases [9]. These agents are frequently utilized as tools for basic research too, and have helped to better understand mast cell physiology and the Fc ϵ RI signal transduction system.

Pharmacological stabilization of mast cells is currently used for the therapy of asthma [10,11], rhinitis [12], atopic dermatitis [13], seasonal conjunctivitis [14] and even chronic leg ulcers [15]. This strategy has also been applied to abrogate ocular allergic inflammation [16], prevent adhesion formation after pelvic surgery [17], and diminish inflammation and hyperalgesia induced by nerve injury [18]. Sodium cromoglycate and nedocromyl are the most commonly used mast cell stabilizers for inhibiting cytokine release from bronchial mast cells [19]. When inhaled several times a day, cromoglycate will inhibit both, immediate and late asthmatic responses elicited by antigen or exercise [11]. Given the intimate relationship between mast cells and multiple inflammatory diseases, stabilization of mast cell activity would be expected to be therapeutically effective.

Interestingly, there are some reports showing beneficial effects of super-oxidized solutions in disorders related to mast cell activation, suggesting that these solutions might have a direct modulator activity on this type of cells [20–22].

Super-oxidized solutions (also known as super-oxidized water, SOW) are produced by the electrolysis of purified water and sodium chloride. Water is broken down into oxygen, ozone and other unstable oxidized species. However, the main active chemical species generated during this process are hypochlorite and

hypochlorous species. Despite solutions commercially available are all produced by electrolysis and they differ in the concentration of active components and pH [23]. Therefore, their biocidal activity and toxicity profiles also vary depending on these two characteristics. Microbicidal activity has been shown against bacteria, viruses, fungi and spores in vitro [24,25]. In a rat-burn model, for example, the researchers applied SOS directly to the infected lesion with *Pseudomonas* and were able to efficiently reduce blood levels of endotoxin, systemic effects of septicemia and mortality rates [21]. Similarly in humans, skin infections and ulcers [26], inflammatory skin disorders (e.g. pemphigus, psoriasis) [27], burns [28] and peritonitis [20,29] have all improved their outcomes with the use of SOS treatment. But whether these better outcomes are related to the antiseptic efficacy of the SOS, or to a synergistic anti-inflammatory effect, is unknown [29,30]. To the best of our knowledge, there is no information on the direct effects that a SOS might exert on the inflammatory process itself or in the process of mast cell activation.

It is well established that studies on mast cell physiology are useful to identify possible therapeutic targets for allergic/inflammatory diseases. Murine bone marrow-derived mast cells (BMMCs) are one of the most common cells used for this purpose. These cells are generated from murine bone marrow cultured in the presence of IL-3 (for the generation of mucosal-type mast cells) or IL-3 and SCF (for the development of connective tissue-type mast cells) [31]. Mucosal-type BMMCs are particularly useful to analyze distinct molecular events leading to signal transduction, degranulation, leucotriene production, and cytokine mRNA expression and secretion [2,3].

We therefore explored the effects of a pH neutral-SOS on the activation and secretion processes of mucosal-type BMMCs after Fc ϵ RI triggering using IgE-antigen. For this purpose, cells were incubated in serial dilutions of a pH neutral-SOS for a short period of time before being activated. The SOS treatment was not toxic but blocked both, IgE/antigen- and calcium ionophore-induced mast cell degranulation in a concentration dependent manner. SOS pretreatment also significantly inhibited the release of TNF- α , CCL-3, IL-13 and IL-6 from activated mast cells. Yet, the treatment did not interfere with early stages of the signal transduction mechanism of the Fc ϵ RI receptor, since protein tyrosine phosphorylation, MAPK activation, calcium mobilization and cytokine mRNA production were observed after receptor crosslinking. Altogether these data suggest that SOS pretreatment is able to partially inhibit the secretion of mediators of both, acute and late induced responses in mast cells. The exact targets of SOS in mast cells are unknown but clearly deserve further evaluation.

2. Materials and methods

Culture media, bovine fetal serum, essential amino acids, and sodium pyruvate solutions for cell growth and differentiation were from Invitrogen (Carlsbad, California). Antibodies used for western blot analysis included anti-p38 (Cat. # SC-535), ERK2 (Cat. # SC-154), and p-ERK2 (Cat. # 7976) from Santa Cruz Biotechnology (California, USA), p-p38 (Cat. # 9211S) from Cell Signaling (Beverly, MA), and anti-p-Tyr, clone 4G10, (Cat. # 05-321) from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-DNP IgE (clone SPE-7), calcium ionophore A23187, PMA and reagents for buffer preparation, electrophoresis and calcium mobilization assays were purchased from Sigma Chemical Co. (St. Louis MO). Antigen stimulation of the cells was performed adding distinct amounts of dinitrophenol-human serum albumin (DNP-HSA, Sigma Chemical Co. Cat # A-6661) to overnight IgE sensitized BMMC. The SOS used for this study was a stable, neutral pH preparation commercially available in Mexico (Microcyn®, Oculus Innovative Sciences, CA, USA).

2.1. Mice and BMMC culture

Bone marrow derived mast cells (BMMC) were isolated from the tibia of 4–8 weeks old mice (strain 129S1/SvImJ), from Jackson Laboratories (Maine, USA, stock # 002448). Briefly, total bone marrow was isolated and cultured in RPMI media containing IL-3 (20 ng/mL) and 10% Fetal Bovine Serum (FBS) in order to differentiate mast cells, as previously described [31]. After 4–6 weeks of culture, between 97 to 99% of the cells were positive to FcεRI staining as measured by a standard flow cytometry assay [32].

2.2. SOS treatments

A pH neutral, super-oxidized solution (Microcyn®) that contains a small amount of free available chlorine (<80 ppm) in the form of hypochlorous acid, sodium hypochlorite and sodium chloride was used for this study. This solution is

produced through the electrolysis of purified water containing limited amounts of chloride ion in a unique multi-chamber cell (Oculus Innovative Sciences). This stable, super-oxidized solution was mixed with concentrated Tyrode's/BSA buffer (see degranulation assay section) in order to obtain solutions with different percentages of SOS (v/v) without significantly altering the osmolality or pH of Tyrode's/BSA buffer. Sensitized cells (see Degranulation section) were preincubated with Tyrode's/BSA buffer alone, or with 10% to 50% SOS containing-Tyrode's/BSA buffers, at 37 °C for distinct periods of time to test viability or during 15 min to test physiological activation. After SOS pretreatments, cells were collected and resuspended in fresh SOS free-Tyrode's/BSA in order to be stimulated with antigen or with a mixture of calcium ionophore and PMA, as a positive control for cell activation.

2.3. Viability assays

Cell viability analysis of SOS exposed mast cells was carried out by three independent methodologies. Propidium iodide incorporation, annexin V exposition and 7AAD positive cells were measured as follows.

Mature BMMCs (5×10^5) were centrifuged at room temperature during 5 min at 800 $\times g$. After removal of the supernatant, cells were carefully resuspended in 0.5 mL of the different SOS dilutions in Tyrode's/BSA (see previous section) and kept at 37 °C, 5% CO₂. After different times of exposure, treatment was stopped by addition of 0.5 mL of the complete cell culture media. Cells were then pooled by centrifugation during 5 min at 800 $\times g$, the resultant pellets were disaggregated and cells were resuspended and stained with 7-aminoactinomycin D (7-AAD) according to the manufacturer's instructions (7-AAD; BD., Palo Alto, CA, USA). For long-term cytotoxicity showed in Table 1, SOS dilutions were made in complete RPMI media (see cell culture section). Osmolality was not significantly affected by dilution process, since mean osmolality of complete medium containing 50% SOS of three different lots was 284.9 ± 1.05 , 296.34 ± 0.78 , 317.82 ± 0.48 , determined using an Advanced 3D3 Osmometer

Table 1
Effect of 50% SOS or 0.0075% sodium hypochlorite on mast cell viability depending on exposure time

Cell viability determination method	Treatment	t=0	t=5 min	t=15 min	t=30 min	t=60 min	t=120 min	t=4 h
7AAD	Buffer	99±1%	99±2%	99±1%	99±4%	99±5%	99±5%	99±6%
7AAD	50% SOW	99±2%	99±2%	99±3%	99±5%	97±5%	95±4% *	95±11% *
7AAD	0.0075% sodium hypochlorite	99±5%	99±8%	99±6%	95±9%	90±12% *	85±12% *	66±12% *
Propidium iodide	Buffer	99±0.6%	99±1.4%	99±0.3%	98±1.4%	98±0.5%	98±0.7%	96±2% *
Propidium iodide	50% SOW	98±1.4%	99±2.0%	99±0.5%	97±2.1%	96±0.5% *	95±0.7% *	93±4% *
Propidium iodide	0.0075% sodium hypochlorite	99±2.3%	99±3.2%	98±0.3%	88±2.6% *	86±4% *	89±6% *	54±19% *
Annexin C	Buffer	99±0.03%	99±0.01%	99±0.07%	99±0.01%	99±0.02%	99±0.03%	99±0.10%
Annexin C	50% SOW	99±0.08%	99±0.05%	99±0.09%	98±0.03%	98±0.03%	98±0.09%	99±0.07%
Annexin C	0.0075% sodium hypochlorite	99±0.16%	99±0.12%	95±1.5% *	86±2.6% *	87±3.6% *	80±14% *	47±16% *

Time-dependent cytotoxicity of 50% SOS or 0.0075% sodium hypochlorite on mature BMMC's. Cell viability was determined using three different reagents as described in Materials and methods section. Results are expressed as percentage of viable cells in each condition. No difference was noticed between buffer treated or non-treated BMMCs. * denote significant differences from cells exposed to Tyrode's BSA/culture media mixture.

from John Morris Scientific. As a control for long-term cytotoxicity, different dilutions of a Voluette Analytical Chlorine Standard (HACH Company, Loveland, CO, USA) were prepared in complete media and cells were pretreated during distinct periods of time in order to test viability. Given the presence of NaCl in complete media, after chlorine addition, sodium hypochlorite (NaOCl) was produced and the amount of NaOCl in the preparation was measured by using a molar extinction coefficient of $350 \text{ M}^{-1} \text{ cm}^{-1}$ at 290 nm at pH 12 [33].

The ratio of necrosis and apoptosis were determined simultaneously in the cells using annexin V-FITC and propidium iodide according to the manufacturer's protocol (Annexin-V-Fluos Staining kit, ROCHE, NJ, USA). Positive stain cells were analyzed on a FACScalibur flow cytometer (BD., Palo Alto, CA, USA). All experiments were conducted in triplicate.

2.4. Statistical analysis

Statistical analysis was performed by the *t*-test for Dependent Samples. $p < 0.05$ was considered significant. The Tukey honest significant difference test was used for the analysis of variance (ANOVA) between groups. The Statistica™ version 6.00 software was used for these analyses.

2.5. Degranulation assays

Mature BMMCs were sensitized by overnight incubation in media containing 300 ng/mL of monoclonal anti-DNP IgE (clone SPE7). The following morning, cells were washed and resuspended in SOS-free or SOS-containing Tyrode's/BSA buffer (20 mM HEPES pH7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose, and 0.05% bovine serum albumin) and kept at 37 °C during 15 min. Two million cells in 1 mL buffer were stimulated with different concentrations of DNP-HSA (antigen) or calcium ionophore/PMA at 37 °C during 30 min for degranulation, or during 1 h for total RNA isolation. After the indicated times, cells were collected by centrifugation and supernatants were used for β -hexosaminidase activity determination and pellets for total RNA isolation, as previously described [34].

2.6. RNA isolation, RT-PCR and RPA assays

Pellets from non-stimulated and stimulated cells were used for total RNA isolation. Briefly, the cell pellet obtained in degranulation assays was resuspended in 1 mL of Trizol following manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNA concentration was calculated by absorbance at 260 nm and cDNA synthesis was performed using a first strand DNA synthesis kit (Invitrogen, cat # 12371-019). PCR amplification of cDNAs was conducted in the presence of specific primers for distinct cytokines or GAPDH genes, following the manufacturer's instructions (Mouse Inflammatory Cytokine Set2ms, BioSource™; Invitrogen Carlsbad, CA, USA). RT-PCR products were separated in TBE-prepared 2% agarose gels and analyzed in a UVP image analysis system (Upland, CA, USA). Ribonuclease I Protection Assays (RPA)

were conducted using specific multiprobe RPA sets labeled with ^{33}P , following the instructions provided in the kits (In vitro transcription and RPA kits, BD Pharmingen, cat. # 556850 and # 556134, respectively). Protected fragments were separated on denaturing polyacrylamide gels, which were dried and exposed to Kodak Biomax film at -80°C . Representative results of at least four independent experiments are shown.

2.7. Western blotting

Ten million sensitized BMMCs were pretreated with SOS free-or SOS containing-Tyrode's/BSA buffers, during 15 min at 37°. After this time, cells were collected and resuspended in 250 μL of fresh Tyrode's/BSA buffer. BMMCs were then stimulated by the addition of 30 ng/mL DNP-HSA antigen for 10 min at 37 °C and the stimulus further stopped by adding 250 μL of 2 $\times$ Laemmli buffer to each tube. Samples were boiled during 5 min and 30 μL of the sample was separated on 10% Tris-glycine-SDS polyacrylamide gels. Proteins were transferred to PVDF membranes using standard procedures. Membranes were blocked 1 h at room temperature in 4% non-fat milk. First antibody incubation was performed overnight at 4 °C using recommended dilutions. The following day, antibody excess was eliminated by washing the membrane several times in TTBS buffer (50 mM Tris; 150 mM NaCl; 0.1% Tween 20). Secondary antibody incubations were performed 1 h at RT using 1:10,000 dilutions of HRP-conjugated specific antibodies. Chemiluminescence was measured with a SuperSignal detection kit (Roche, NJ, USA).

2.8. Cytokine secretion

The concentration of murine cytokines in supernatants from treated and non-treated, stimulated and naive cells, was evaluated by standard ELISA kits as recommended by the manufacturer (Biosource-Invitrogen, Carlsbad, CA). Evaluated cytokines included $\text{TNF}\alpha$ (cat. KMC3011), CCL-3 (MIP1 α) (cat. KMC2201), IL-6 (cat. KMC0061) and IL-3 (cat. KMC0031). Supernatants were obtained as follows: 40 million of sensitized BMMC were preincubated with SOS free-or SOS containing-Tyrode's/BSA buffers for 15 min at 37 °C. After this time, cells were collected and resuspended in 10 mL of fresh cell culture media supplemented with a protease inhibitor cocktail (Roche, NJ, USA). Cells were stimulated with 30 ng/ml of DNP-HSA at 37 °C for different periods of time, at the end of which 1 mL of media was collected from the supernatant and frozen down at -80°C for cytokine determination. Supernatants of non-stimulated cells were used as negative controls. Between 20 to 60 μL of the supernatant was used for ELISA cytokine detection, depending on the specific cytokine tested.

2.9. Calcium mobilization and NFAT transcription factor activity

Calcium mobilization was measured as described elsewhere [35]. In brief, sensitized BMMC were loaded with

5 mM Fura-2AM dissolved in Tyrode's/BSA at 37 °C for 30 min. After loading, cells were pre-incubated with SOS free or SOS containing Tyrode's/BSA buffers, during 15 min at 37°. Cells were collected and resuspended in cold Tyrode's/BSA until use. For stimulation, 4 million cells were placed in a 2 mL cuvette of a Jobin Yvon-Fluorolog Spectrofluorometer and the ratio of fluorescence at 340/380 nm was recorded during 100 s before adding antigen (30 ng/mL) or Calcium ionophore/PMA (100 nM/1 μ M) to the cells. Ratio of fluorescence was recorded for an additional 300 s and calibration was performed by the addition of Triton X-100 (for maximal fluorescence) and 10 mM EGTA (for minimal fluorescence) to the cuvette. NFAT transcription factor activity was determined using a commercial TransAM Kit for NFATc1 activation, from Active Motif (Cat # 40269). Briefly, 10 million mast cells were sensitized and pre-incubated with buffer, 25% SOS or 50% SOS during 15 min. After this time, cells were washed once and resuspended in Tyrode's/BSA buffer, prior to being activated during 15 min using 9 or 27 ng/ml of DNP-HSA at 37 °C. Total nuclear extracts were performed and NFAT activity was tested following instructions provided by the manufacturer. Data are presented as optical density values at 450 nm in non-treated and treated BMMCs.

3. Results

3.1. SOS pretreatment does not affect BMMC viability

The neutral SOS herein tested is a solution with broad antimicrobial activity commonly used in wound care treatment. Yet, its direct effects on cells mediating allergic or inflammatory processes, like mast cells, had never been explored. Thus, the cytotoxicity of this SOS was first evaluated in IgE-sensitized BMMCs treated with Tyrode's/BSA buffer containing distinct concentrations of SOS (i.e. 1% to 50%, v/v). After incubation for 15 min at 37 °C, cells were processed to simultaneously evaluate cell viability, necrosis and apoptosis by flow cytometry.

Fig. 1 shows the concentration-response curves of BMMCs cultures exposed to different dilutions of SOS. Cell viability was analyzed by exclusion of 7AAD. Of all SOS dilutions tested, only 50%-SOS induced a statistically significant reduction in BMMCs' viability in comparison to the control group ($p < 0.05$). Despite this, the cell viability of BMMCs pretreated with 50% SOS was $\geq 95\%$.

Apparently, 50% SOS induced cell death through necrosis because $\geq 3\%$ of the cells incorporated propidium iodide in the flow cytometry analysis (Fig. 1B). This percentage, although low, was also significantly higher than the rest of the groups ($p < 0.05$). Apoptosis, on the contrary, does not seem to be the mechanism by which 50% SOS induces cell death. In fact, the percentages of cells exposing Annexin-V in the cellular surface ranged from 0.4% to 0.9% in all groups (Fig. 1C). These results show that mild cytotoxicity in BMMCs is only induced by 50%-SOS in vitro. Instead, lower SOS concentrations (i.e. 1% to 25%-SOS) do not seem to affect BMMCs cell membrane permeability as measured in these assays.

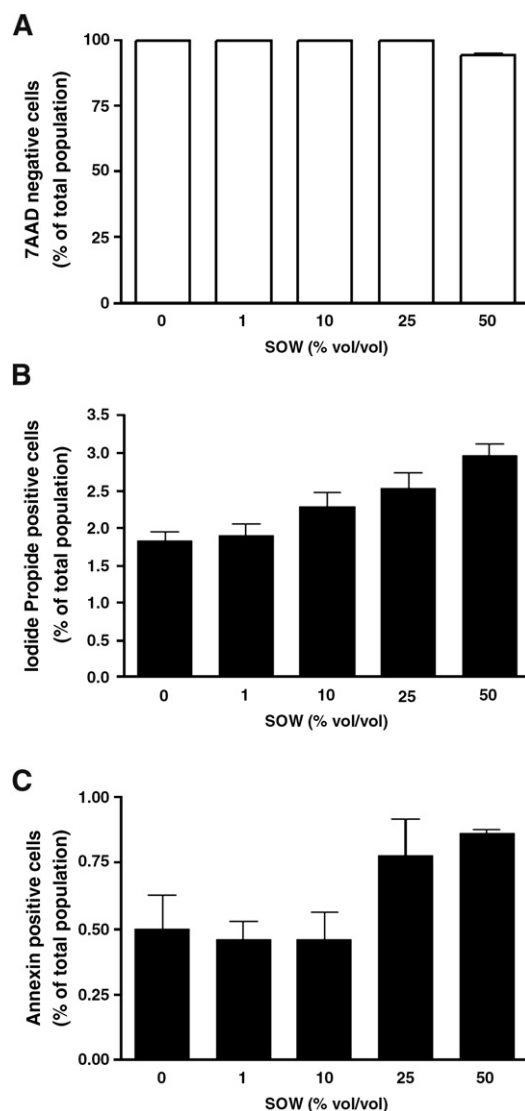


Fig. 1. Mast cell treatment with SOS (SOW) does not affect cellular viability. BMMCs (5×10^5) were incubated at 37 °C for 15 min in Tyrode's/BSA alone, or in the same buffer containing different dilutions of neutral pH SOS (v/v). After this time, cells were analyzed by flow cytometry looking for 7AAD (A), propidium iodide (B) and Annexin (C) positive cells. SOS solutions were prepared as described in the Materials and methods section. Results are expressed as the mean from three independent experiments $\pm$ SD.

To establish the sustained effect of SOS on mast cell-cytokine release, a time-course experiment was first conducted to show that cell viability was not significantly modified for several hours after a 15 min exposure to 50% SOS. As described in previous sections, cells were collected and incubated for 15 min in complete media containing Tyrode's/BSA buffer, 50% SOS or 0.0075% sodium hypochlorite. Care was taken to keep the osmolarity of the media in the range of 275 to 317 mOsm/L in all testing conditions. At the end of each

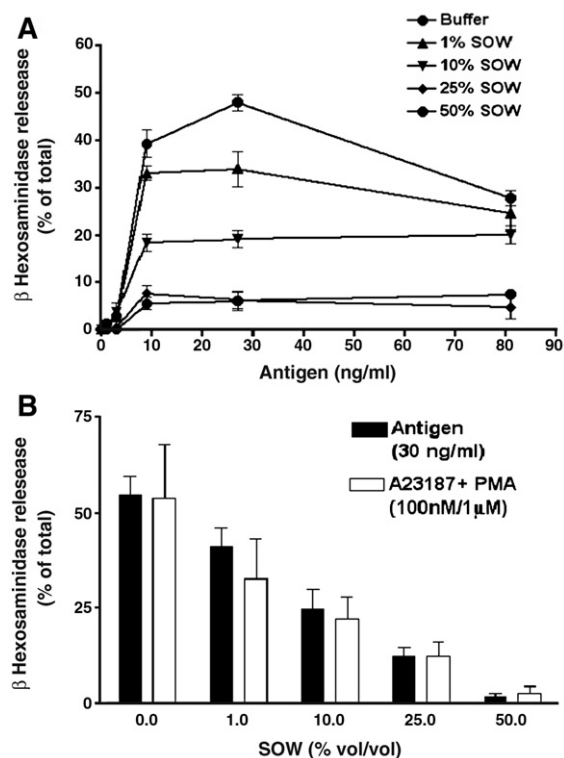


Fig. 2. SOS (SOW) pretreatment of mast cells inhibits antigen and calcium ionophore/PMA-induced degranulation. (A) Sensitized mature BMMCs were collected from culture media and resuspended in normal Tyrode's/BSA buffer or in the same buffer containing different dilutions of neutral pH SOS (v/v) at 37 °C for 15 min. After this time, cells were collected, washed once and resuspended in SOS-free Tyrode's/BSA (37°). Then they were stimulated with different concentrations of DNP-HSA (antigen) during 30 min. After this time, cells were collected and β -hexosaminidase activity was determined in the supernatant. A pellet of non-stimulated cells was lysed with 0.5% Triton and used as the positive control (i.e. 100% enzymatic activity). Results are expressed as percent of the total β -hexosaminidase activity. (B) Degranulation stimulated by antigen or A23187/PMA was inhibited by SOS containing Tyrode's/BSA. Cells were treated as in panel A but β -hexosaminidase release was measured after antigen (30 ng/ml) or calcium ionophore/PMA (100 nM/1 μ M) stimulation. The graph shows the mean degranulation values obtained from 5 independent experiments $\pm$ SE.

incubation time, new fresh RPMI media was added to each tube and cell viability was determined. Propidium iodide (PI) incorporation, annexin V exposition and 7AAD staining at different times of incubation at 37 °C were the techniques used for cell viability determination. As shown in Table 1, viability in 50%SOS-treated cells dropped to 95% after 2 and 4 h post exposure as evaluated by 7AAD. Instead, 0.0075% hypochlorite significantly reduced mast cell viability down to 90%, 85% and 66% at 1, 2 and 4 h post exposure, respectively. Another difference was that hypochlorite induced cell death through both, necrosis and apoptosis, as demonstrated by increasing PI incorporation and annexin V exposition from minute 30 post-

exposure. These experiments show that hypochlorite is far more toxic than SOS to mast cells, even when both solutions contained a similar concentration of free available chlorine.

3.2. SOS pretreatment of BMMC inhibits Fc ϵ RI-induced degranulation

Mast cell degranulation initiates the early phase of allergic responses. In order to test the effect of SOS treatment on the degranulation of mast cells, IgE-antigen-induced β -hexosaminidase release was measured in BMMCs pretreated with either, SOS free-or SOS containing-Tyrode's/BSA buffer for 15 min at 37°. Pretreatment of the cells with 25% and 50% SOS inhibited antigen-induced mast cell degranulation by 75–80% (Fig. 2A). This effect was also observed when cell preincubation

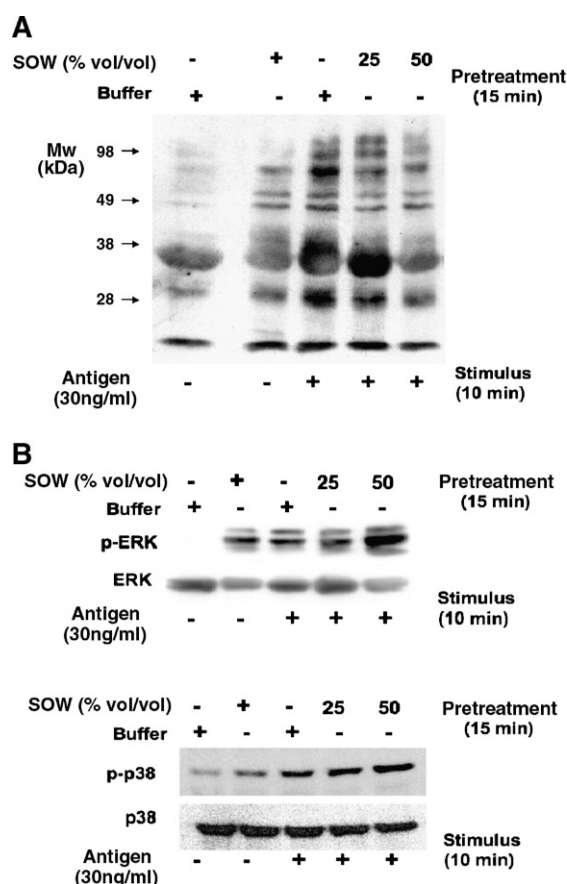


Fig. 3. SOS (SOW) pretreatment of mast cells does not prevent antigen-dependent protein tyrosine phosphorylation and MAPK activation. Mature, sensitized BMMCs were collected from culture media and resuspended in SOS free- or SOS containing-Tyrode's/BSA buffers at 37 °C during 15 min. After this time, cells were washed once in SOS-free Tyrode's/BSA (37 °C) and stimulated with 30 ng/ml DNP-HSA during 3 min. Reaction was stopped by addition of boiling 2 $\times$ Laemmli buffer and total proteins were analyzed using phospho-tyrosine (A), or phospho ERK and phospho p38 (B) specific antibodies. A representative image of three independent experiments is shown. When SOW was added alone, the final concentration was 50%.

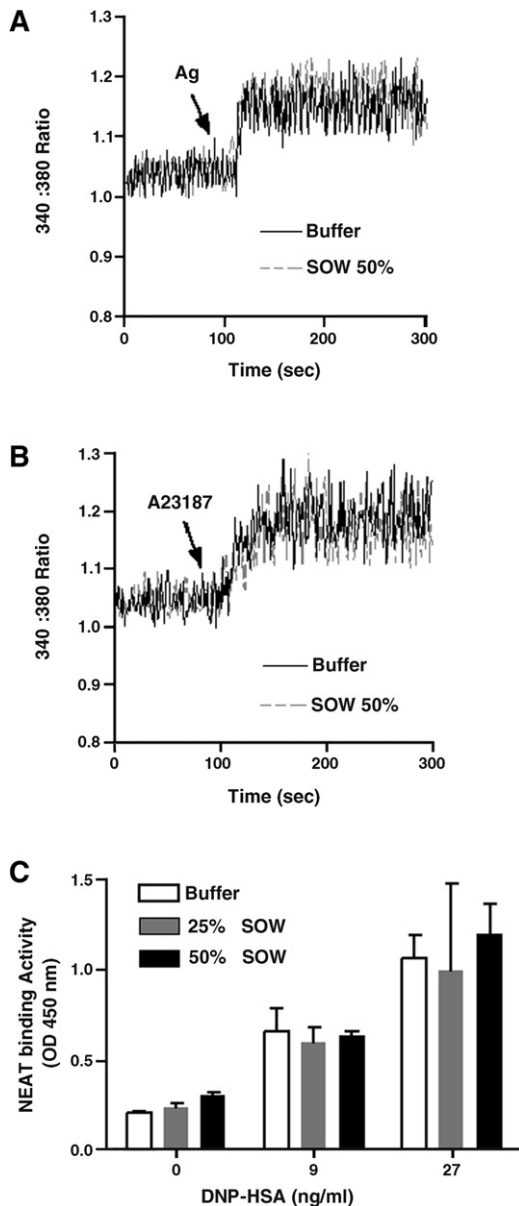


Fig. 4. SOS (SOW) pretreatment of mast cells does not inhibit calcium mobilization after antigen or calcium ionophore stimulation. Sensitized, mature BMMCs were collected from culture media and resuspended in FURA-2 AM containing Tyrode's/BSA buffer at 37 °C during 30 min. Then, cells were washed once and resuspended in normal Tyrode's/BSA buffer or in 50% SOS-containing buffer at 37 °C during 15 min. After this incubation, cells were washed again and resuspended in SOS-free Tyrode's/BSA buffer before to be transferred to a spectrofluorometer cuvette. Fluorescence 340:380 ratio was recorded during 100 s and after this time (A) 30 ng/ml of DNP-HSA (Antigen, Ag) or (B) 100 nM calcium ionophore (A23187) was added to the cells. Traces were followed during 200 s after stimuli addition. Traces shown are representative from three independent experiments with similar results.

was conducted in 1% SOS. Of note, the inhibition of antigen-induced mast cell degranulation was not avoided by addition of increasing antigen doses. Furthermore, the blockage of β -hexosaminidase release induced by other stimuli, like calcium ionophore and PMA, was also inhibited by $90 \pm 10\%$ in cells pretreated with 50% SOS (Fig. 2B). These results suggest that SOS blockage of β hexosaminidase release is a non-specific effect on the secretion process.

3.3. SOS pretreatment does not affect Fc ϵ RI-induced tyrosine phosphorylation and MAPK activation

Fc ϵ RI receptor crosslinking rapidly induces tyrosine phosphorylation of a variety of proteins inside mast cells leading to degranulation and cytokine production. Since some tyrosine kinase-inhibitors are able to halt β -hexosaminidase release [35], we tested the effect of SOS pretreatment on antigen induced-tyrosine phosphorylation of proteins in mast cells. Neither of the two SOS dilutions tested (i.e. 25–50%) prevented total tyrosine phosphorylation after Fc ϵ RI activation, as judged by western blot using an antiphosphotyrosine

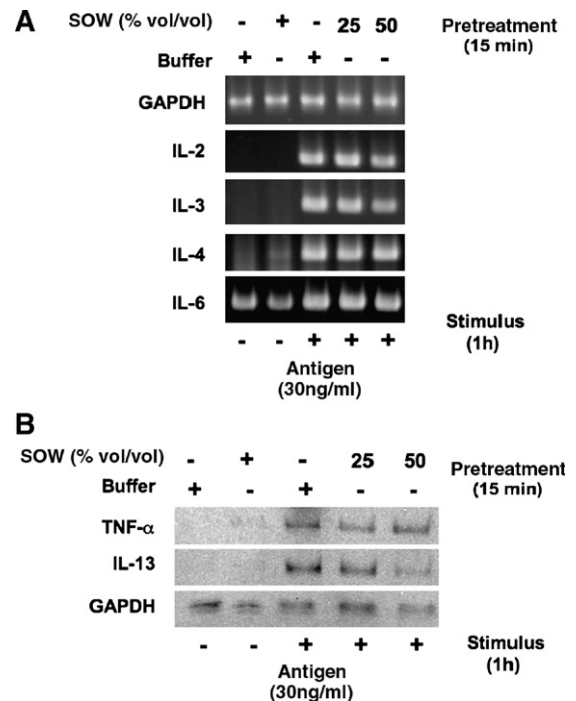
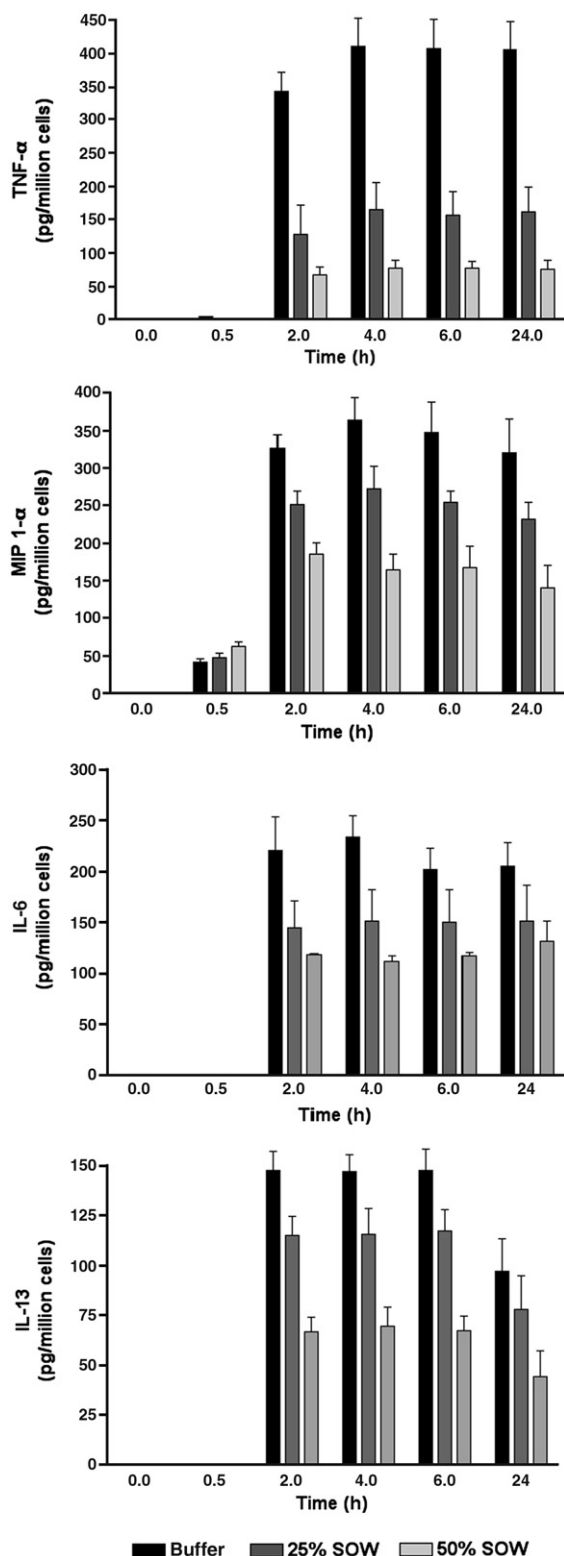


Fig. 5. SOS (SOW) pretreatment of mast cells does not significantly inhibit antigen-dependent cytokine mRNA accumulation. Mature, sensitized BMMCs were collected from culture media and resuspended in normal Tyrode's/BSA buffer or in SOS-containing buffer at 37 °C during 15 min. After this time, cells were washed once in SOS-free Tyrode's/BSA (37 °C) and stimulated with 30 ng/ml DNP-HSA during 1 h. Total RNA isolation, RT-PCR or RPA was performed as described in the Materials and methods section. mRNA accumulation of GAPDH, IL-2, IL-3, IL-4 and IL-6 was determined by RT-PCR (A), and GAPDH, TNF- α and IL-13 mRNA accumulation was determined by RPA (B). When SOW was added alone, the final concentration was 50%.



(4G10) antibody (Fig. 3A). Yet, total tyrosine-phosphorylated proteins decreased after antigen stimulation if mast cells were pretreated with 25% or 50% SOS. Since IgE receptor crosslinking is able to induce MAPK activation, we also tested the effect of the same SOS dilutions on p38 and ERK2 MAP kinase phosphorylation levels in mast cells (Fig. 3B). In the absence of antigen-induced stimulation, the sole exposure of BMMCs to 50% SOS induced ERK2 and p38 phosphorylation. Antigen stimulation for 10 min further elevated SOS induced-MAPK activation in BMMCs. According to these results, it is possible that SOS pretreatment can activate MAPK without impairing the capacity of the FcεRI receptor to further stimulate MAPK activity.

3.4. SOS pretreatment does not inhibit FcεRI-induced calcium mobilization or NFAT-transcription factor activation in mast cells

Mast cell degranulation and the synthesis of some cytokines, are absolutely dependent on calcium mobilization [36,37]. Given the fact that SOS pretreatment inhibits the degranulation capabilities of mast cells, we examined the rise of intracellular calcium in 50% SOS pretreated-mast cells during IgE-antigen or calcium ionophore stimulation. Under these experimental conditions, calcium mobilization was not affected in stimulated mast cells with either agent if they were pretreated in 50% SOS for 15 min (Fig. 4A,B). Since NFAT-transcription factor is activated after IgE-antigen stimulation and calcium rise in mast cells, we also investigated NFAT binding activity in 50% SOS-treated cells. As it can be observed in Fig. 4C, no statistical difference on NFAT activation was observed between SOS treated- and non-treated-BMMCs after antigen stimulation. Thus, SOS does not seem to interfere with calcium mobilization or calcium activated-effectors in mast cells.

3.5. SOS pretreatment does not prevent IgE-antigen-induced cytokine mRNA accumulation in mast cells

Murine BMMCs, through activation of distinct Src family kinases, are able to synthesize many different cytokines and chemotactic factors in response to antigen stimulation, including: IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNF-α, TGF-β, GM-CSF, IFN-γ, CCL2, CCL3 and CCL4, among others [38]. It is known that FcεRI crosslinking

Fig. 6. SOS (SOW) pretreatment of mast cells significantly inhibits antigen-induced cytokine secretion. A total of 40 million mature, sensitized BMMCs were collected from culture media and resuspended in normal Tyrode's/BSA buffer or in SOS-containing buffer at 37 °C during 15 min. After this time, cells were washed, resuspended in 10 ml of SOS-free, complete media at 37 °C and stimulated with 30 ng/ml DNP-HSA (antigen). Samples of the supernatant were taken at indicated times and cytokine content was determined by cytokine-specific ELISA kits. Results of three independent experiments ±SE are presented.

activates a signal transduction system that modulates the activity of a number of different transcription factors (i.e. NFAT, NF κ B, AP-1). These, in turn, are responsible for de novo synthesis of cytokine mRNA [39]. Once secreted, cytokines induce recruitment and proliferation of T cells, macrophages, eosinophils and other cellular types that initiate the inflammatory response of late phase-allergic reactions. We, therefore, tested the effect of 25% and 50% SOS-preincubation on cytokine mRNA accumulation in BMMCs. For this purpose, RT-PCR or RPA assays were used to measure IL-2, IL-3, IL-4, IL-6, TNF- α and IL-13 specific transcripts in mast cells pretreated with SOS free-and SOS-containing Tyrode's BSA for 15 min at 37 °C (Fig. 5). In the absence of antigenic stimulation, the sole exposure of mast cells to 50% SOS did not induce detectable cytokine mRNA synthesis. Pretreatment of mast cells with 25% or 50% SOS, however, did not prevent Fc ϵ RI-induced cytokine mRNA synthesis.

3.6. SOS pretreatment diminishes cytokine secretion in mast cells.

Since SOS pretreatment of BMMC had inhibited Fc ϵ RI-induced degranulation but not Fc ϵ RI-induced cytokine mRNA synthesis, we further evaluated cytokine release in control and SOS-preincubated cells by ELISA. For these experiments, IgE-sensitized cells were preincubated in SOS free- or SOS containing-Tyrode's/BSA buffer for 15 min at 37 °C. After this time, cells were washed and resuspended in SOS-free culture media containing 30 ng/ml DNP-HSA, as described in "Materials and methods". Cells were then incubated at 37 °C and samples taken from the supernatant at different time points to determine the amount of cytokine release after IgE-antigen stimulation. Cytokines detected included TNF α , CCL3 (MIP-1 α), IL-6 and IL-13 (Fig. 6). The extent of inhibition of cytokine release in SOS-pretreated cells was found to be concentration dependent and molecule specific. In general, 50%-SOS was more effective than 25% SOS pretreatment in the inhibition of antigen-induced cytokine release, except for IL-6 secretion. After pretreatment of mast cells with 50% SOS, the inhibitory effect was more noticeable in TNF- α release ($83 \pm 10\%$ max. inhibition), followed by IL-13 and MIP-1 α ($50 \pm 7\%$) and IL-6 ($40 \pm 6\%$). Interestingly, the inhibitory effect of SOS-pretreatment was sustained from 2 to 24 h and there were not significant changes in the concentration of all four cytokines tested in the supernatant of BMMCs' cultures, during this period of time.

4. Discussion

Inhibition of degranulation of mast cells has been proposed as a feasible therapeutic target for allergic and inflammatory diseases [8,9]. In this study we report that pretreatment of a model of mucosal-type mast cells (BMMCs) with different dilutions of a neutral pH super-oxidized solution (SOS), does neither significantly reduce the viability of the cells nor interfere on early

Fc ϵ RI signal transduction processes. Yet, SOS pretreatment importantly inhibits the early secretion of granule content and the late secretion of at least four regulatory and chemoattractant cytokines. These results are intriguing and suggest that SOSs might be potent stabilizers of mast cell activity with potential therapeutic applications.

We first evaluated the toxicity of SOS on mast cells. As expected, the treatment of BMMCs with different SOS dilutions did not significantly affect cell viability as assayed by three independent methods (i.e. 7AAD and propidium iodide exclusion and annexin V detection) (Fig. 1, Table 1). These results are in accordance with the lack of toxicity in primary irritancy and sensitization studies conducted with this same SOS in skin and mucosas, as well as in *in vitro* experiments (i.e. agarose overlay using fibroblasts) [30]. SOS pretreatment, however, was able to block the degranulation of mast cells induced by IgE-antigen crosslinking of the Fc ϵ RI receptor. This effect was concentration dependent, with reductions of allergen induced-histamine release ranging from $86 \pm 8\%$ and $27 \pm 5\%$ in pretreated cells with 50% and 1% SOS, respectively. Since calcium ionophore and PMA-induced degranulation were affected to the same extent, we concluded that the effects of SOS pretreatment on histamine release in mast cells are receptor-independent (Fig. 2A).

In order to further explore the potential mechanism of action of SOS on degranulation, we evaluated the total tyrosine phosphorylation and MAPK phosphorylation in antigen activated-BMMCs (Fig. 3). It is known that molecular crosslinking of the Fc ϵ RI receptor activates a complex series of biochemical reactions involving the activation of distinct Src-family kinases (Fyn and Lyn) [38], activation of Syk kinase, phosphorylation of adapter proteins (like LAT, and Gab2), activation of Phospholipase C and PI3K enzymes and intracellular calcium mobilization [5]. This complex transduction system also involves the activation of MAP kinases, phosphorylation of transcription factors, and synthesis of new cytokine mRNA [5]. We therefore evaluated the global tyrosine phosphorylation level after Fc ϵ RI activation, as judged by western blot (using 4G10 antibody) and the phosphorylation levels of ERK2 and p38 MAP kinases. Total tyrosine phosphorylation after Fc ϵ RI triggering seems to decrease in mast cells pretreated with 25% or 50% SOS. In contrast, the sole exposure of the cells to 25% or 50% SOS-containing buffers elicited the phosphorylation of ERK2 and p38 proteins (Fig. 2B). These phenomena could be due to the induction of a mild cellular stress by the low concentration of chlorine species found in this SOS

(<80 ppm). These species are not themselves free radicals but these oxygen-containing molecules can facilitate free-radical formation [43]. In fact, oxidative damage is known to induce ERK and p38 activation, and even cell death, depending on the time of exposure and concentration of the oxidizing agent [40]. Due to the low concentration of chlorine species found in this particular SOS, the mast cells could have been stressed without compromising the viability. Taken together, these results suggest that SOS pretreatment does not significantly affect the signaling machinery depending on tyrosine phosphorylation of the FcεRI receptor.

Another required condition for IgE-antigen induced-mast cell degranulation is intracellular calcium mobilization. Unexpectedly, cells pretreated with 50% SOS did not show inhibition of calcium mobilization nor in NFAT activation after allergen-or calcium ionophore-stimulation (Fig. 4). This result supports the hypothesis that SOS pretreatment does not interfere with the signal transduction process required for calcium mobilization and it does not seem to modify the membrane capacity of calcium influx. However, it does suggest that SOS pretreatment can induce uncoupling of calcium flux and granule secretion in this cells, since it has been widely understood that calcium mobilization is an essential requisite for IgE-antigen induced degranulation [41].

Cytokine mRNA expression was also determined in control and SOS-treated cells after 1 h of IgE-antigen stimulation. As measured by RT-PCR and RPA, we did not detect a significant effect of 50% SOS pretreatment on IL-2, IL-3, IL-4, IL-6, TNF-α and IL-13 mRNA accumulation either in basal or IgE-antigen induced conditions (Fig. 5). In contrast, cytokine secretion was severely inhibited by SOS pretreatment in a concentration dependent and molecule specific way. TNF-α secretion, for example, was inhibited by 80%, whereas CCL3 and IL-13 release were blocked by 50%, and IL-6 by only 40%. According to these results, SOS pretreatment seems to be able to inhibit the cytokine secretory pathway(s) in mast cells, without altering the transcription machinery.

Altogether, our study suggests that this particular neutral pH super-oxidized solution induces most of the effects of a classical mast cell stabilizer. Cromolyn sodium and nedocromil sodium are typical membrane stabilizers because they produce blockage on mast cell degranulation without affecting other parameters of cell activation [42]. Similarly, the SOS herein used was able to strongly inhibit both antigen and calcium ionophore-induced mast cell degranulation without affecting cell viability or even FcεRI signal transduc-

tion processes. The precise mechanism(s) by which this SOS inhibits degranulation without disturbing RNA synthesis and early signal transduction is unknown. It is possible that the components of this SOS partially modify cytoskeleton or plasma membrane targets that uncouple calcium rise from granule secretion. Instead, this SOS does not appear to inhibit intracellular signaling processes depending on tyrosine phosphorylation.

At this point, however, it is not possible to make a generalization about the effects of SOS in mast cells because not all super-oxidized solutions have the same properties [23]. The more acidic or alkaline the solutions are, the more corrosive and toxic they become [43, 44]. Another major problem with previous SOSs formulations, had been the lack of stability for long periods of time. Basically, most of SOSs available today are stable for only few hours or days. But, apparently, neither one of these two problems seem to be of major concern with the neutral pH-SOS herein tested. In aging studies, for example, it was demonstrated that this SOS was stable for up to 2 years. Preclinical data also showed that this SOS does not irritate or sensitize skin and mucosas (e.g. ocular, peritoneal, nasal, oral) [30]. Finally, pilot human studies have been successfully conducted in various clinical conditions, including those associated with mast cell activation [25–28]. The clinical outcomes in cases of non-infected-bullous (pemphigus) and hyperkeratotic (psoriasis) lesions, for example, have suggested that this SOS could exert a direct anti-inflammatory effect [27]. However, the use of neutral pH super-oxidized solution in any allergic and inflammatory condition will have to be evaluated in proper controlled studies.

In summary, our data suggest that SOS is able to inhibit the cell machinery for granule secretion without altering the main signal transduction pathway induced by IgE-antigen receptor crosslinking. This is a relevant finding because the identification of the mechanism(s) of action of SOSs on mast cells could help to identify novel targets in the intricate pathways that control allergic and inflammatory reactions.

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