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# Chitosan-induced phospholipase $A_2$ activation and arachidonic acid mobilization in $P388D_1$ macrophages

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**Abstract We have found that chitosan, a polysaccharide present in fungal cell walls, is able to activate macrophages for enhanced mobilization of arachidonic acid in a dose- and time-dependent manner. Studies aimed at identifying the intracellular effector(s) implicated in chitosan-induced arachidonate release revealed the involvement of the cytosolic Group IV phospholipase $A_2$ ($PLA_2$), as judged by the inhibitory effect of methyl arachidonoyl fluorophosphonate but not of bromoenol lactone. Interestingly, priming of the macrophages with lipopolysaccharide renders the cells more sensitive to a subsequent stimulation with chitosan, and this enhancement is totally blocked by the secretory $PLA_2$ inhibitor 3-(3-acetamide)-1-benzyl-2-ethylindolyl-5-oxy-propanesulfonic acid (LY311727). Collectively, the results of this work establish chitosan as a novel macrophage-activating factor that elicits AA mobilization in $P388D_1$ macrophages by a mechanism involving the participation of two distinct phospholipases $A_2$.**



*Key words:* Chitosan; Phospholipase $A_2$; Arachidonic acid; Macrophages

## 1. Introduction

Chitosan, a polysaccharide comprising copolymers of glucosamine and *N*-acetyl-glucosamine, is naturally present as an essential component of fungal cell walls [1]. It can be obtained by partial deacetylation of chitin (poly *N*-acetyl-D-glucosamine) from crustacean shells. Naturally chitosan is formed by the action of chitin deacetylases, enzymes that have been involved either in the formation of the cell wall or in the deacetylation of chitin oligosaccharides following the action of an endochitinase on cell walls during autolysis. The formation of chitosan by chitin deacetylases has also been proposed to play a role in plant–pathogen interactions [2].

As chitin and chitosan are essential components of fungal cell walls, both have been the targets of antifungal drugs [3]. Because some of their derivatives are essentially non-toxic, biodegradable and biocompatible, prostheses produced from chitin derivatives have been developed [1]. Chitin and chitosan have been observed to accelerate wound healing properties and the attainment of an attractive skin surface. It has been suggested that the mechanism by which chitin and chitosan act at a wound site involves activation of neutrophils and macrophages [4]. Histological findings suggest that these compounds stimulate the migration of polymorphonuclear and mononuclear cells, and accelerate the reformation of connective tissue and angiogenesis [5].

Some chitin/chitosan derivatives have been shown to upregulate the production of tumor necrosis factor, interleukin-1 and colony-stimulating factor by macrophages and to induce immunologic adjuvant effects [1,6–8]. Therefore, it was of interest to examine the capacity of chitosan to mediate macrophage activation through the release of arachidonic acid (AA). The results reported here demonstrate that chitosan can act as a macrophage activator through the mobilization of AA and have explored the molecular effectors involved in this event.

## 2. Materials and methods

Low molecular weight (LMW) chitosan (molecular weight distribution 0.5–15 kDa), having a degree of deacetylation of at least 85%, was obtained from Aldrich (Milwaukee, WI, USA). It was solubilized in 1% acetic acid, centrifuged to remove insoluble material and purified by precipitation at alkaline pH by the addition of 1 M NaOH until a $pH > 9$ was reached. The pellet was centrifuged at 5000 rpm for 10 min and washed several times with water. Three cycles of solubilization at acidic pH in 1% acetic acid and precipitation at alkaline pH were performed in order to remove any contaminating material from the commercial samples of LMW chitosan. After the third precipitation at alkaline pH, the pellet was washed with water until neutral pH was reached. The neutralized chitosan preparation was then washed several times with ethanol, dried and stored at 4°C. Stock solutions of chitosan were prepared in 0.1 M HCl and diluted with serum-free medium to the appropriate concentration for the experiments.

$P388D_1$ cells (MAB clone) [9,10] were maintained at 37°C in a humidified atmosphere at 90% air and 10% $CO_2$ in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. $P388D_1$ cells were plated at $10^6$ per well, allowed to adhere overnight and used for experiments the following day. All experiments were conducted in serum-free Iscove's modified Dulbecco's medium. Radiolabeling of the $P388D_1$ cells with [$^3$H]AA (specific activity 100 Ci/mmol; New England Nuclear, Boston, MA, USA) was achieved by including 0.5 μCi/ml [$^3$H]AA during the overnight adherence period (20 h). Labeled AA that had not been incorporated into cellular lipids was removed by washing the cells four times with serum-free medium containing 0.5 mg/ml albumin.

For the [$^3$H]AA release measurements, the cells were placed in serum-free medium for 30 min before the addition of chitosan for different periods of time in the presence of 0.5 mg/ml bovine serum albumin. The supernatants were removed, cleared of detached cells by centrifugation and assayed for radioactivity by liquid scintillation counting. When inhibitors were used, they were added to the cells

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30 min prior to the addition of the stimulus. The $sPLA_2$ (phospholipase $A_2$) inhibitor LY311727 was a generous gift from Dr. Edward Mihelich (Lilly Research laboratories, Indianapolis, IN, USA). The cellular $PLA_2$ ($cPLA_2$) inhibitor methyl arachidonyl fluorophosphonate (MAFP) was purchased from Biomol (Plymouth Meeting, PA, USA).

## 3. Results

We began the current study by determining whether LMW chitosan was able to cause the extracellular release of AA from murine $P388D_1$ macrophages. To this end, cells labeled with [$^3$H]AA were exposed to different concentrations of chitosan for various periods of time. As shown in Fig. 1, chitosan induced AA mobilization in a dose- (Fig. 1A) and time- (Fig. 1B) dependent manner. No AA was released when the concentration of chitosan was below 0.01% (w/v) and the plateau was reached at 0.05% (w/v) (Fig. 1A). AA release proceeded linearly from 5 to 30 min, after which it began to plateau (Fig. 1B). Interestingly, when the cells were first primed with bacterial lipopolysaccharide (LPS) for 1 h and then exposed to chitosan, the AA release response was markedly enhanced. This is essentially the same phenomenon that we have previously documented with platelet-activating factor (PAF) as a triggering agent for AA release [11–14].

Our previous investigations into the molecular mechanisms of PAF receptor-mediated AA mobilization have highlighted the involvement of two distinct $Ca^{2+}$-dependent $PLA_2$ enzymes in this process, namely the Group IV cytosolic $PLA_2$ and the Group V secretory $PLA_2$ [13–16]. A direct approach to study the putative involvement of these $PLA_2$s in chitosan-induced AA mobilization is the use of selective inhibitors of each of these enzymes. Involvement of $cPLA_2$ was assessed using MAFP, an irreversible inhibitor of $cPLA_2$ that has no effect on $sPLA_2$s and other enzymes that could be involved in AA mobilization (arachidonoyl-CoA synthetase, lysophosphatidylcholine:arachidonoyl-CoA acyl transferase or CoA-independent transacylases) [17]. More than 70% of the AA release induced by 0.06% (w/v) LMW chitosan was blocked by MAFP (25 μM) in both the unprimed and the LPS-primed cells (Fig. 2), suggesting the involvement of $cPLA_2$ in chitosan-induced macrophage activation.

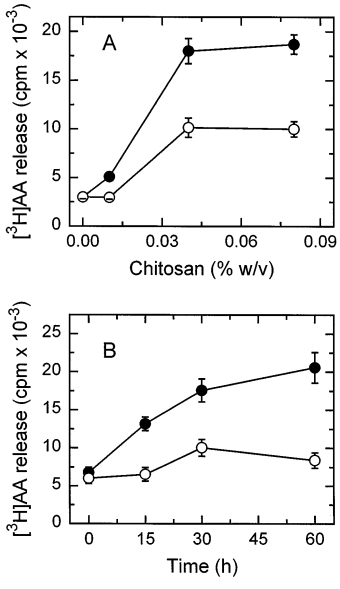


Fig. 1. Chitosan-stimulated [$^3$H]AA mobilization in $P388D_1$ macrophages. A: Dose response of chitosan activation. The cells were exposed to either chitosan alone for 1 h (open circles) or to 100 ng/ml LPS for 1 h followed by chitosan for 1 h (closed circles); B: Time-course of [$^3$H]AA release by 0.06% (w/v) chitosan alone (open circles) or by 100 ng/ml LPS plus 0.06% chitosan (closed circles).

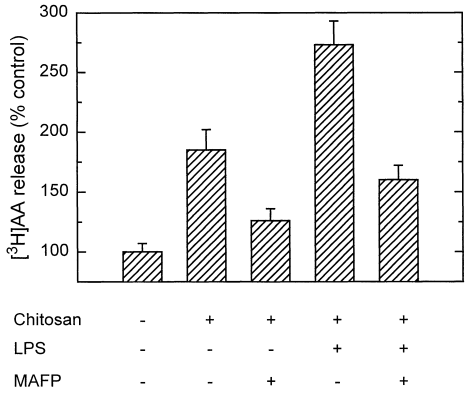


Fig. 2. $cPLA_2$ involvement in chitosan stimulation. Effect of MAFP on chitosan- or LPS/chitosan-induced AA release. [$^3$H]AA-labeled cells were treated for 1 h with (+) or without (−) 100 ng/ml LPS. Afterwards, the cells were incubated for 30 min without (−) or with (+) 25 μM MAFP as indicated. Subsequently, the cells were either untreated (−) or treated (+) with 0.06% (w/v) chitosan as indicated, and AA release was determined after 1 h.

MAFP has been found to also inhibit another $cPLA_2$, the Group VI $Ca^{2+}$-independent $PLA_2$ or $iPLA_2$ [17]. Therefore, at least part of the MAFP-sensitive AA mobilization could be mediated by the $iPLA_2$ in addition to the $cPLA_2$. Group VI $iPLA_2$ is potently and irreversibly inhibited by the mechanism-based inhibitor bromoenol lactone (BEL), a compound that manifests over a 1000-fold selectivity for inhibition of the $iPLA_2$ versus the $Ca^{2+}$-dependent $PLA_2$s [18]. BEL at concentrations that completely inhibit the $iPLA_2$ from macrophage cell homogenates (5–25 μM) [19] had no measurable inhibitory effect on chitosan-induced AA release from either unprimed or LPS-primed cells (data not shown), thus ruling

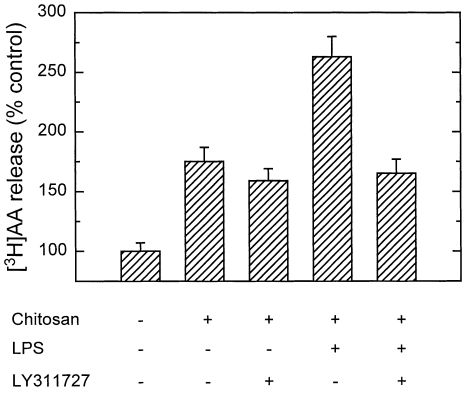


Fig. 3. $sPLA_2$ involvement in chitosan stimulation. Effect of LY311727 on chitosan- or LPS/chitosan-induced AA release. [$^3$H]AA-labeled cells were treated for 1 h with (+) or without (−) 100 ng/ml LPS. Afterwards, the cells were incubated for 30 min without (−) or with (+) 25 μM LY311727 as indicated. Subsequently, the cells were either untreated (−) or treated (+) with 0.06% w/v chitosan as indicated, and AA release was determined after 1 h.

out a role for the $iPLA_2$ in this process. In turn, these results indicate that the MAFP effects described above are all attributable to the inhibition of the $cPLA_2$.

Involvement of the $sPLA_2$ in chitosan-induced AA release was assessed by using the indole derivative LY311727, a potent and selective $sPLA_2$ inhibitor [18]. LY311727 markedly decreased the extracellular release of [$^3$H]AA from LPS-treated cells (Fig. 3), which is again reminiscent of our previous results suggesting the involvement of this enzyme in AA release from LPS-primed $P388D_1$ macrophages stimulated with PAF [14,16]. Interestingly, LY311727 showed little or no effect on chitosan-induced AA release from the unprimed cells (Fig. 3), which is consistent with our previous finding that unprimed $P388D_1$ cells contain low amounts of $sPLA_2$ [15].

## 4. Discussion

LMW chitosan particles have previously been shown to bind to macrophage mannose receptors, which mediate the internalization of the chitosan particles [5]. Once inside the macrophage, chitosan is eventually degraded by hydrolytic enzymes present in the phagosome such as lysozyme and *N*-acetyl-β-D-glucosaminidase [5]. In the current study, we demonstrate that LMW chitosan particles are able to activate the macrophages for an enhanced AA release response. Interestingly, in addition to direct macrophage activation by chitosan, our current study has led to the unexpected discovery that if the cells are pretreated with LPS at non-stimulatory conditions (100 ng/ml, 1 h pretreatment), a high level of priming is induced. This results in an enhanced AA release response to chitosan. Therefore, chitosan behaves in this respect much like the pro-inflammatory lipid mediator PAF [11–14].

Examination of the effectors responsible for chitosan-induced AA mobilization in unprimed and LPS-primed cells showed again a remarkable similarity with the LPS/PAF situation [14,16], in that Group IV $cPLA_2$ and Group V $sPLA_2$ but not Group VI $iPLA_2$ were found to be involved in the response. Chitosan appears to induce AA release in the unprimed cells by activating the Group IV $cPLA_2$. In contrast, in the LPS-primed cells, not only the Group IV $cPLA_2$ but also the Group V $sPLA_2$ appear to be involved. This results in an enhanced AA release response. That the $sPLA_2$ does not appreciably participate in the AA release response to chitosan of the unprimed cells was not unexpected, since unprimed cells have been found to possess little $sPLA_2$ [15], and our previous data have indicated that one main mechanism for the LPS to prime the cells for an enhanced AA release involves the increased expression of Group V $sPLA_2$ [15]. As a consequence of such an increase in $sPLA_2$ levels in the LPS-primed cells, the cells are now better equipped to release higher amounts of AA in response to a subsequent chitosan stimulation.

In summary, the data presented in this study indicate that chitosan possesses pro-inflammatory properties similar to those of PAF. In accordance with our data, it has recently been reported that chitosan particles are also able to stimulate canine neutrophils to synthesize leukotriene $B_4$ [20].

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