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# Impaired D-*myo*-Inositol 1,4,5-Triphosphate Generation from Cord Blood Polymorphonuclear Leukocytes

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## ABSTRACT

D-*myo*-Inositol 1,4,5-triphosphate ($IP_3$) is a key second messenger in many cells, including macrophages, T and B cells, and neutrophils, in which it regulates free intracellular calcium ion levels. In human polymorphonuclear leukocytes the rise of intracellular $[Ca^{2+}]$ is the signal that activates a number of functions such as adherence, aggregation, chemotaxis, and degranulation, which are typically depressed in newborn infants. $IP_3$ generation can be stimulated by *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) tripeptide, which mimics the naturally occurring bacterial oligopeptides. In this study both neonatal and adult polymorphonuclear leukocytes were stimulated by fMLP ($1 \times 10^{-6}$ M) and the levels of $IP_3$ were assayed by a specific radiometric method. The time course of $IP_3$ generation was studied for up to 60 s in a total of 10 samples. The response appeared reduced in cord blood samples. To confirm this observation, we extended our study to a larger number of samples, quantitating $[IP_3]$ at the time peak of 10 s. As expected $IP_3$ generation was significantly (*F* test, $p < 0.0001$, $n = 39$) lower in newborns than in adults (means ± SD = 0.64 ± 0.25; 1.26 ± 0.36, ng/$10^6$ cells, respectively). Besides soluble stimulus, neutrophils were treated with a particulate stimulus, namely serum-treated zymosan, which is also able to stimulate $IP_3$ synthesis from polymorphonuclear leukocytes. Serum-treated zymosan produced a prolonged elevation in the level of $IP_3$, reaching a plateau within 120 s in both cord blood and in control samples. At the 120-s time point significantly (*F* test, $p < 0.002$, $n = 10$) lower amounts of $IP_3$ were found in newborn samples than in adult preparations (mean ± SD = 1.09 ± 0.45; 2.54 ± 0.55, ng/$10^6$ cells, respectively). These data suggest that an impaired synthesis of $IP_3$ is involved in the defective signal transduction of neonatal polymorphonuclear leukocytes and could represent an important biochemical mechanism behind the defective functions of neonatal neutrophils. (***Pediatr Res* 38: 564–567, 1995**)

**Abbreviations**

**$IP_3$,** D-*myo*-inositol 1,4,5-triphosphate
**$PIP_2$,** phosphatidylinositol 4,5-biphosphate
**DAG,** 1,2-diacylglycerol
**PMN,** polymorphonuclear leukocyte(s)
**fMLP,** formyl-methionyl-leucyl-phenylalanine
**TCA,** trichloroacetic acid
**$[Ca^{2+}]_i$,** free intracellular calcium ion concentration
**STZ,** serum-treated zymosan
**PLD,** phospholipase D
**$PLA_2$,** phospholipase $A_2$
**ARF,** ADP-ribosilation factor
**PA,** phosphatidic acid

Human PMN are the first line of defense against nonviral infections. In response to an appropriate stimulus, they are able to diapedese through the endothelium and vessel wall in to the inflammatory focus killing and/or inactivating the invading organisms.

Newborn infants show an increased susceptibility to serious and overwhelming bacterial and fungal infections. This leads to increased morbidity and mortality, in spite of advances in antibiotic therapy. Although abnormalities have been described in all compartments of the neonatal host defense system (1), altered PMN functions are considered to be one of the main factors involved in susceptibility to infection (2).

FMLP is a synthetic tripeptide with structural and chemotactic characteristics similar to the naturally occurring bacterial oligopeptides (3). It is able to stimulate chemotaxis by binding to specific plasma membrane receptors of polymorphonuclear cells (4). The signal is transduced by the dissociation of the pertussis toxin-sensitive α-GTP complex from the heterotrimeric G protein and the consequent activation of a phospholipase C (5, 6). This, in turn, hydrolyzes PIP2 into two second messengers, namely DAG and $IP_3$. The simultaneous generation of these two second messengers is responsible for activating a cascade of events that results in the cellular response. In particular, $IP_3$ is able to mobilize $Ca^{2+}$ from the intracellular

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stores through interaction with specific receptors (5, 7–9). The increase in free intracellular $Ca^{2+}$ concentration is the signal activating many PMN functions such as diapedesis, chemotaxis, and phagocytosis (10, 11). These functions are typically depressed in newborns (12–14). In particular, both the chemotaxis response and the increase in $[Ca^{2+}]_i$ triggered by fMLP are deficient in newborns (15). In this study we assessed the production of $IP_3$ in response to either fMLP or STZ, which is also able to stimulate the synthesis of $IP_3$ from PMN. The aim of this research is to investigate whether reduced synthesis of $IP_3$ could play a role in impaired signal transduction in neonatal PMN.

## METHODS

***Sampling of blood.*** Heparin at a concentration of 20 U/mL was used as an anticoagulant. Venous blood was collected from the placenta vessels of full-term infants who were delivered vaginally. Newborns were the product of uncomplicated pregnancies and deliveries; neither general nor local anesthesia was administered to the mothers. No perinatal complications occurred in any of the babies studied, and they all had an Apgar score at 1 and 5 min > 8. Control cells were obtained from healthy adult donors.

The following steps of leukocytes separation started within 2 h of the blood collection.

***Preparation of PMN suspensions.*** Neutrophils (95–97% pure and 97–100% viable) were prepared and suspended in RPMI/FCS at a final concentration of $20–25 \times 10^6$ cells/mL as previously described (16). All culture media were purchased as sterile and endotoxin-tested solutions from Sigma Chemical Co., St. Louis, MO.

***fMLP stimulation.*** The synthetic tripeptide was purchased from Sigma Chemical Co. The PMN preparations were stimulated at 37°C at a final fMLP concentration of $1 \times 10^{-6}$ M.

To study the kinetic of $IP_3$ production, 0.5 mL of fMLP was added to 2.5 mL of the cell suspension. Aliquots of 0.6 mL were pipetted from the incubation mixture and immediately mixed with an equal volume of ice-cold 15% TCA at the fixed time of 5, 10, 20, 30, and 60 s. The samples were then kept on ice for 20 min.

The stimulations at the peak value were performed by pipetting ice-cold TCA directly into the cell suspensions after exactly 10 s.

***STZ stimulation.*** Zymosan A was also from Sigma Chemical Co. STZ was prepared as previously described (16).

PMN preparations were stimulated at 37°C at a final concentration of 10 mg/mL.

The kinetics of $IP_3$ production were performed similarly to fMLP stimulation. The incubation was stopped at the fixed times of 10, 60, 120, 150, 180, and 350 s.

To reduce the variance *within* the groups in one way analysis of variance, we performed the stimulations at the time point of 120 s processing adult and cord blood samples simultaneously and in duplicate. We used the same pool of sera for the opsonization of zymosan A.

***$IP_3$ assay.*** $IP_3$ was extracted as previously described (16) and quantitated by a specific RIA (Amersham International plc, UK) (17).

***Statistical analysis.*** The significance of differences between the groups was tested by one way analysis of variance.

## RESULTS

$IP_3$ generation in response to fMLP was assayed for up to 60 s in five samples of cord blood and five samples of adult PMN (Fig. 1*A*). fMLP produced a rapid increase in the level of $IP_3$, which peaked at 10 s in all samples. The response appeared reduced in cord blood preparations. To confirm this observation, we extended our study to a larger number of samples (21 cord blood samples and 18 control adults), quantitating the $IP_3$ generation at the time peak of 10 s. Results are depicted in Figure 1*B*. Consistently, the response was significantly reduced in neonates compared with adults (means ± SD = 0.64 ± 0.25; 1.26 ± 0.36, ng/$10^6$ cells, respectively, Fig. 1*B*). One-way analysis of variance showed an $F$ value of 39.14, $p < 0.0001$.

Although fMLP stimulation resulted in a transient increase in $[IP_3]$, STZ produced a prolonged increase in $[IP_3]$, reaching a plateau within 120 s (Fig. 2*A*). These results agree with the findings of Burnham *et al.* (18). Similarly to fMLP, the response appeared lower in newborns than in adults. We performed additional experiments to confirm this finding, quantitating $IP_3$ levels at 120 s in a total of 10 samples. As shown in Figure 2*B*, the response was significantly lower in cord than in adult PMN (mean ± SD = 1.09 ± 0.45; 2.54 ± 0.55, ng/$10^6$ cells, respectively). One-way analysis of variance showed an $F$ value of 20.33, $p < 0.002$.

## DISCUSSION

An increase in free intracellular $Ca^{2+}$ concentration is an important signal activating several PMN functions, including diapedesis, chemotaxis, and phagocytosis (10, 11, 19, 20).

Although the mechanism involved in the regulation of $[Ca^{2+}]_i$ is far from clear, it is known that its release from

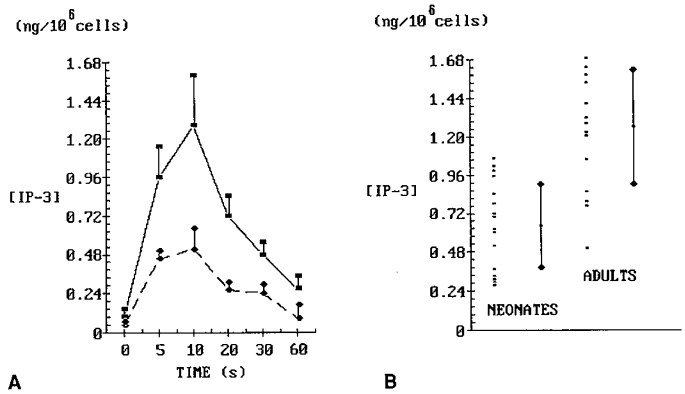


**Figure 1.** (*A*) Time course of fMLP-stimulated $IP_3$ production by adult and cord blood PMN. (*Solid line*) $IP_3$ production from adult neutrophils; (*dashed line*) $IP_3$ production from neonate neutrophils. (*B*) Peak $IP_3$ production from cord blood and adult PMN in response to fMLP. *Vertical bars* represent the SD.

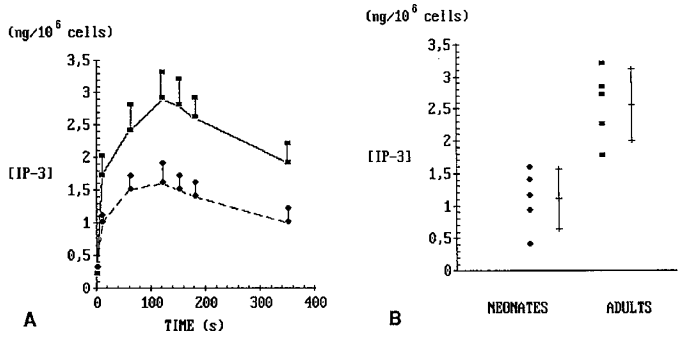


**Figure 2.** (*A*) Time course of STZ-stimulated $IP_3$ production by adult and cord blood PMN. (*Solid line*) $IP_3$ production from adult neutrophils; (*dashed line*) $IP_3$ production from neonate neutrophils. Values are mean ± SD of three experiments. (*B*) $IP_3$ production at 120 s by neonatal and adult PMN in response to STZ. *Vertical bars* represent the SD.

intracellular stores may be directly stimulated by $IP_3$ through the interaction with a specific intracellular receptor localized on the membrane of at least one specialized compartment of the endoplasmic reticulum (5, 7–9, 20, 21).

fMLP induces an impaired release of $[Ca^{2+}]_i$ in neonatal PMN (15), although previous studies reported a normal number of receptors for tritiated fMLP and that the affinity of the binding and dissociation is equivalent to that of PMN from adults (22, 23). We may conclude that a defect may occur in a specific step that follows the ligand-receptor interaction. Our results support the hypothesis that the low levels of $[Ca^{2+}]_i$, generated by neonatal PMN in response to the chemotactic peptide, could be related to an impaired generation of $IP_3$.

Along with fMLP, we studied the $IP_3$ generation in response to a phagocytic stimulus. STZ is made up of zymosan particles, derived from yeast polysaccharide membranes, on which complement proteins and Ig are adsorbed. Interaction of opsonized zymosan with human PMN is prevalently mediated by receptors for C3 fragments (25).

The uptake of opsonized matter is an event that may occur independently of $IP_3$ and $[Ca^{2+}]_i$ (24, 26, 27). However, during phagocytosis the uptake of particles is directly followed by other cellular events, such as the intracellular killing of microorganisms, the digestion of the ingested material, and the fusion of the granule membranes with the membrane of the phagosome. These events are related to $IP_3$ levels and calcium metabolism: the fusion between different granules and the plasma membrane is $Ca^{2+}$-dependent (28, 29); in $Ca^{2+}$-depleted neutrophils the respiratory burst during phagocytosis of yeast-C3b/bi was found to be depressed by approximately 70% and the generation of inositol phosphate, DAG, PA, and arachidonate do not occur (27).

Reduced synthesis of $IP_3$ in neonatal PMN may result in an impaired activation of the two $[Ca^{2+}]_i$ dependent phospholipases: $PLA_2$ and PLD. The latter is a downstream effector of ARF1 and ARF3 (30). ARF belong to the Ras superfamily that participates in the process of intracellular vesicular transport, in endocytosis and they may also function in endosome-endosome fusion (30, 31). The former directly cleaves arachidonic acid from membrane bound phospholipids and converts it, by the 5-lipoxygenase pathway into leukotrienes. We have previously reported that an impaired release of $LTB_4$, which has an important role in initiating and amplifying the inflammatory response of human PMN, may be involved in the biochemical and functional abnormalities of PMN in the neonate (16).

The low levels of $IP_3$ we found in neonatal PMN after fMLP or STZ stimulation could be due to either increased metabolism of the molecule or reduced synthesis and/or hydrolysis of $PIP_2$. However, because of kinetic studies show that neonatal PMN metabolize $IP_3$ similarly to that of the adult PMN, we hypothesize that the impaired signal transduction of neonatal PMN is due to a defect at a stage between the ligand-receptor interaction and $PIP_2$ breakdown. If this is true, the consequence is a low generation of both the second messengers $IP_3$ and DAG, which are simultaneously generated by the $PIP_2$ hydrolysis (9).

In conclusion our study supports the hypothesis that low levels of $IP_3$ could play a role in the impaired response of neonatal PMN to an adequate stimulus and that there is the possibility that concomitant low levels of DAG may ulteriorly impair the capacity of activation of these cells.

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