Effect of Human Defensins on the Cytoplasmic Ca^{2+} Content in Platelets

S. B. Tkachenko, V. N. Kokryakov, I. P. Ashmarin, S. V. Grachev, and A. A. Kubatiev

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The effect of the total fraction of human defensins (HNP-1, HNP-2, and HNP-3) on the cytoplasmic Ca^{2+} content ($[Ca^{2+}]_i$) in the platelets of healthy donors was studied. At concentrations of 0.1-40 μg/ml and an incubation time of 10 min defensins have no effect on $[Ca^{2+}]_i$ in platelets labeled with Fura-2AM. However, at higher concentrations (100 μg/ml) they increased platelet $[Ca^{2+}]_i$. In addition, defensins (40 μg/ml) inhibited the Ca^{2+} increase in platelets induced by thrombin, adenosine diphosphate, and the lipopolysaccharide of *S. typhimurium* endotoxin. The most pronounced inhibitory effect was observed in a suspension of thrombin-stimulated platelets. It is shown that the effect of human defensins on the functional activity of platelets is due to the alterations in the intracellular Ca^{2+}.

Key Words: *defensins; platelets; Ca^{2+}*

The protection of the organism against bacterial and other infections depends largely on the secretion of a vast array of antimicrobial proteins by activated neutrophils. The cationic proteins defensins (HNP), which are among the least investigated of these, have not only antimicrobial but also other biological properties [6,9]. Previously, we demonstrated that along with known substances released by activated polymorphonuclear leukocytes (cathepsin G, elastase, nitrogen oxide, and arachidonic acid metabolites) defensins can help regulate the functional activity of the platelets [2,10]. Human defensins induced by thrombin, collagen, or adenosin diphosphate (ADP) inhibit platelet aggregation and the secretion of adenosine triphosphate by platelets. Further studies of the biological effects of defensins prompted us to investigate the effect of the proteins on the concentration of cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$), which is a key regulator of platelet aggregation.

Department of General Pathology and Pathological Physiology, Russian Medical Academy for Postgraduate Education, Moscow; Department of Biochemistry, Moscow State University; Department of Pathophysiology, Institute of Experimental Medicine, St. Petersburg

MATERIALS AND METHODS

Venous blood of healthy donors was collected in 3.8% sodium citrate (1:9). Platelets were isolated as described [1]. The cytoplasmic Ca^{2+} in platelets (both basal and agonist-induced) was determined fluorimetrically [11] using the fluorescent calcium probe Fura-2AM (Calbiochem). Fluorescence was measured in a Hitachi-3000 spectrophluorimeter in a thermostatically controlled cuvette at 37°C with constant stirring. The excitation and fluorescence wavelengths were 350 and 500 nm, respectively. The intracellular calcium concentration was calculated from the following formula [12]:

$$[Ca^{2+}]_i = K_d(F-F_{min}) / (F_{max}-F).$$

where F is the recorded fluorescence intensity, F_{max} is the fluorescence intensity after the cells have been

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TABLE 1. Effect of Human Defensins on the Cytoplasmic Ca^{2+} Content (nM) in Platelets ($M \pm m$)

Group	Defensin concentration, μg/ml			
	0.1	1.0	40	100
Defensins ($n=16$)	125±13	119±8	131±10	195±21*
Control ($n=24$)	108±9	132±11	115±12	136±11

Note. These values were obtained in the 5th min of incubation of platelets with defensins. Here and in Table 2: n indicates the number of experiments and an asterisk indicates $p<0.05$.

destroyed with digitonin (50 μM), F_{min} is the fluorescence intensity in the presence of EGTA (2 mM) and Tris (40 mM), and K_d-224 nm is the dissociation constant for the Fura-Ca^{2+} complex.

The total defensin fraction isolated from human peripheral blood neutrophils was used in the study [5]. Acid-soluble proteins were extracted with 10% acetic acid from a leukocyte suspension enriched with neutrophil granulocytes (85-90%). The extract was lyophilized and subjected to gel-filtration on an Acrylex P-10 column (Reanal) equilibrated with 5% acetic acid. The protein fraction eluted from the column after lysozyme (M<15,000 D) was analyzed for defensins by analytical electrophoresis in polyacrylamide gel. This analysis showed that the total defensin fractions consisted of two components which included the main defensins HNP-1, HNP-2, and HNP-3.

For the assessment of the agonist-dependent increase in platelet calcium we used thrombin (0.5 U/ml, Chrono-Log), ADP (5 μM, Sigma), and lipopolysaccharide (LPS) of the *S. typhimurium* endotoxin (200 μ/ml, Sigma). The agonists were added to the platelet suspension in the 2nd and 5th min of incubation.

The data were statistically analyzed using Student's t test.

RESULTS

There were no statistically significant differences between the cytoplasmic Ca^{2+} in platelets incubated with 0.1, 1.0, 10, and 40 μg/ml defensin and the baseline Ca^{2+} (123±23 nM). The incubation time varied from 2 to 10 min. When the defensin concentration was increased to 100 μg/ml, $[Ca^{2+}]_i$ increased, the mean increase by the 5th min of incubation being 43% ($p<0.05$) compared with the initial values (Table 1). During subsequent incubation of platelets with defensins in most experiments (90%) the cytoplasmic Ca^{2+} concentration either fell, approaching the baseline level, or remained at the plateau. A further increase in this parameter was observed only in 10% of observations.

Consequently, at 0.1-40 μg/ml human defensins had no effect on the Ca^{2+} level in the platelet cytoplasm. However, higher concentrations of defensins induced an increase in $[Ca^{2+}]_i$, which was reversible in most experiments.

Defensins markedly inhibited the agonist-induced $[Ca^{2+}]_i$ rise in platelets. The peptides were the most active at a concentration of 1-40 μg/ml. It can be seen from Table 2 that the thrombin-induced increase in $[Ca^{2+}]_i$ against the background of defensins (40 μg/ml) was 45% ($p<0.01$) less pronounced than in the control (without defensins). Prolonging the incubation time from 2 to 5 min had no effect on the inhibition of the thrombin-induced $[Ca^{2+}]_i$ rise. When the defensin concentration was increased to 100 μg/ml or decreased to 0.1 μg/ml, the inhibitory effect of the thrombin-induced $[Ca^{2+}]_i$ rise was not observed. The Ca^{2+} concentration in ADP-stimulated platelets incubated with defensins (40 μg/ml) increased to a lesser degree (21%) than in the absence of defensins. It should noted that the ADP-induced increase in platelet cytoplasmic Ca^{2+} in most experiments (70%) had a two-wave character. The effect of defensins on the ADP-induced

TABLE 2. Changes in the Agonist–Induced $[Ca^{2+}]_i$ Rise in Platelets against the Background of Human Defensins (40 μg/ml) ($M \pm m$)

Parameter	Agonist		
	thrombin, 0.5 U/ml	ADP, 5 μM	LPS, 200 μg/ml
$\frac{[Ca^{2+}]_i \text{ (agonist)}}{[Ca^{2+}]_i \text{ (basal)}}$ ($n=15$)	3.6±0.5	1.7±0.2	2.1±0.1
$\frac{[Ca^{2+}]_i \text{ (agonist)}}{[Ca^{2+}]_i \text{ (defensins)}}$ ($n=15$)	1.9±0.1*	1.3±0.2	1.7±0.1*

Note. Agonists were added in the 2nd min of incubation of platelets with defensins.

$[Ca^{2+}]_i$ rise manifested itself mainly in the suppression of the second wave of fluorescence by 41% ($p<0.05$). We could not find any dependence between the inhibitory effect of defensins on the ADP-induced $[Ca^{2+}]_i$ rise and the period of incubation of platelets with defensins. Defensins also inhibited the increase in the Ca^{2+} content of platelets treated with LPS of *S. typhimurium* endotoxin. After incubation with defensins (40 μg/ml) for 2 min the $[Ca^{2+}]_i$ rise induced by subsequent stimulation of platelets with LPS was 19% lower ($p<0.05$) than in the absence of the peptides. However, when the incubation time was increased to 5 min, the inhibitory effect of defensins on the LPS-induced $[Ca^{2+}]_i$ rise diminished to 11%.

Hence, at a concentration of 40 μg/ml human defensins inhibited the thrombin-, ADP-, or LPS-induced $[Ca^{2+}]_i$ rise. The effect of defensins was the most pronounced against the background of thrombin.

As is well known, Ca^{2+} is an important second messenger which, upon being released from the depots or entering the cytoplasm from the extracellular medium, participates in the regulation of the key enzymes in the cell and eventually determines the changes in platelet shape and aggregation and the secretion of intracellular granules [8]. The present study has shown that, in addition to the known antimicrobial activity, defensins isolated from human neutrophils elicit a direct effect on the mobilization of intracellular Ca^{2+}. Depending on their concentration, defensins induced contralateral shifts in the platelet cytoplasm Ca^{2+}. A dose-dependent effect of HNP-1 on blood cells *in vitro* has been demonstrated for human monocytes [3]. It was found that at high concentrations (400 μg/ml) the defensin elicited a cytotoxic effect, inducing death of cultured cells, at medium concentrations (0.4-40 μg/ml) it inhibited the production of tumor necrosis factor α. At lower concentrations (40 ng/ml), acting together with the activating signal, this defensin stimulated the production of the factor by monocytes. In our experiments, low concentrations of defensins (1-40 μg/ml) inhibited the agonist-induced $[Ca^{2+}]_i$ rise in platelets. These findings agree with the data obtained in our previous experiments demonstrating that the same concentrations of defensins prevent platelet aggregation and platelet secretion of ATP induced by thrombin, collagen, or ADP [2,10]. These findings indicate that the inhibitory effect of defensins on platelet aggregating and secretory activities is realized via alterations in the free intracellular Ca^{2+}.

Inhibition of the agonist-induced Ca^{2+} in the platelet cytoplasm occurred irrespective of the type of inductor (thrombin, ADP, LPS of the *S. typhimurium* endotoxin). Consequently, it can be assumed that, on the one hand, when different inducers are used against the background of defensins, the general mechanism of Ca^{2+} suppression is realized and, on the other, the inhibitory effect of the peptides on the studied parameters of platelet activity is realized via several pathways. According to published data, defensins (by virtue of their polycationic properties, peculiarities of chemical structure, and a relatively low molecular weight of 3500-4000 D) can penetrate the plasma membrane lipid bilayer [7]. Our results show that defensins had no effect on the first wave of the ADP-induced Ca^{2+} rise in platelets and inhibited the second wave, which may indicate that these peptides do not affect the release of membrane-bound Ca^{2+}. These results may contradict the notion that defensins possess membrane-protective properties [2]. In addition, it was found that human defensins are potent and specific inhibitors of protein kinase C (PKC) in neutrophils [4]. According to current concepts, PKC regulates Ca^{2+} mobilization and arachidonic acid secretion in agonist-activated platelets [1,8]. On the basis of these findings it can be postulated that the inhibition of PKC is the major mechanism involved in the inhibition of the $[Ca^{2+}]_i$ rise in activated platelets.

In our experiments, high concentrations (100 μg/ml) of defensins induced an increase in platelet $[Ca^{2+}]_i$. This was probably due to the development of platelet aggregation in the presence of high concentrations of defensins [2]. The mechanisms underlying the development of the defensin-induced $[Ca^{2+}]_i$ rise in platelets remain unclear and require further investigation.

Thus, this study has shown that human defensins produce a direct effect on the functional activity of platelets by regulating the mobilization of intracellular Ca^{2+}. At low concentrations defensins inhibit the agonist-induced increase in the cytoplasmic Ca^{2+} in platelets whereas at high concentrations they stimulate it.

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Rat Model of Acute Tetracycline Hepatosis and Its Dynamic Predictors

T. N. Makarenko, A. M. Dudchenko, and L. D. Luk'yanova

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It is demonstrated that intraperitoneal administration of tetracycline hydrochloride in a dose of 125 mg/kg leads to the development of acute fatty hepatosis in rats within a 24-h period, by which time the maximum accumulation of lipids and triacylglycerides is observed in the liver. In addition, a direct dependence is established between the severity of fatty hepatosis and a decrease in the cytochrome P-450 content. The cytochrome P-450 content is a dynamic predictor of tetracycline fatty hepatosis.

Key Words: *tetracycline fatty hepatosis; triacylglycerides; cytochrome P-450*

The model of drug-induced fatty hepatosis obtained by intraperitoneal administration of tetracycline hydrochloride (TC) has found wide application in experimental studies [2,5,6]. However, published data indicate that the blood content of TC varies considerably; consequently, there are considerable individual variations of lipid accumulation in the liver due to the different absorbance of TC from the alimentary canal. The induction of fatty hepatosis by intravenous administration of TC solution has been described [8,9]. Prognostic criteria of the development of this pathology have not been established.

The aim of this study was to develop a model of acute tetracycline fatty hepatosis induced by intraperitoneal administration of the preparation and to define the prognostic criteria for assessing the severity and dynamics of the pathology. Lipid metabolism and microsomal oxidation at different times after administration of various TC doses were studied.

Laboratory of Bioenergetics, Institute of Pharmacology, Russian Academy of Medical Sciences, Moscow

MATERIALS AND METHODS

Experiments were performed on outbred male rats weighing 180-220 g maintained on the standard vivarium diet. Fatty hepatosis was induced by intraperitoneal administration of 4 ml/100 g TC (Serva). The antibiotic was dissolved in normal saline (pH 8.9) and injected in doses of 50, 125, 250, and 330 mg/kg. Biochemical studies were performed 1, 2, 3, 6, 12, 24, 48, 72, and 144 h after TC administration. Control animals received equal volumes of normal saline. The liver was perfused with normal saline under ether anesthesia, and homogenate in normal saline (1:10) was then prepared.

The degree of fatty hepatosis was assessed by the content of total lipids and triacylglycerides in the liver homogenate and blood plasma with the use of standard Lachema kits.

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