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Neopterin inhibits ATP-induced calcium release in alveolar epithelial cells *in vitro*

Georg Hoffmann[1,CA], Frank Gollnick[2] and Rainer Meyer[2]

[1]Department of Physiology I, and [2]Department of Physiology II, University of Bonn, Nussallee 11, 53111 Bonn, Germany

[CA]Corresponding author:
Tel: +49 228 732337
Fax: +49 228 732408
E-mail: umvb01@uni-bonn.de

***Background*: Serum neopterin concentrations rise during activation of the cellular immune system. It is suggested that neopterin interacts with cellular redox mechanisms. This induces oxidative stress, which inhibits intracellular Ca^{2+} transients in various cell types. In type II alveolar epithelial cells, Ca^{2+} increase is considered involved in the exocytosis of surfactants. This exocytosis is disturbed during inflammation.**

***Aims*: To clarify whether neopterin affects adenosine triphosphate (ATP)-induced Ca^{2+} transients in an alveolar epithelial cell line (L2).**

***Methods*: Ca^{2+} transients were detected as fura-2 fluorescence by an image analysis system.**

***Results*: Cells were exposed for 100 sec to ATP (1 μM, repeated four times). The first application of ATP induced an increase of the fluorescence ratio by approximately 100%, while the following stimulations resulted in smaller transients. In a second set of experiments, L2 cells were exposed to ATP or ATP + neopterin (100 nM), alternately. Simultaneous application of neopterin inhibited Ca^{2+} transients almost completely.**

***Conclusions*: Inhibition of Ca^{2+} transients by neopterin may lead to suppressed exocytosis of surfactant proteins in alveolar epithelial cells. This might contribute to the deterioration of pulmonary functions in the course of inflammatory processes.**

Key words: Neopterin, Calcium, Alveolar epithelium, Inflammation

Introduction

Neopterin is a pyrazino pyrimidine compound that is biosynthesized from guanosine triphosphate in the pathway leading to tetrahydrobiopterin. Due to a low constitutive activity of the second enzyme within this pathway, 6-pyrovoyl tetrahydropterin synthase, human monocytes/macrophages produce and release large amounts of neopterin following activation with T-lymphocyte-derived interferon-γ. Thus, serum and urinary neopterin levels serve as an indicator in patients with diseases associated with an increased activity of the cellular immune system (e.g. viral and bacterial infections, acute graft rejections, and acquired immune deficiency syndrome). In the lung, correlations between serum levels of neopterin and the severity of disease have been demonstrated for inflammatory disorders such as sarcoidosis, lung cancer, tuberculosis, and sepsis.[1] Apart from being a clinical marker for these conditions, neopterin seems to exhibit distinct biochemical functions via interactions with cellular redox mechanisms and the promotion of oxidative stress. Therefore, neopterin may play a role as a modulator during host defence reactions. Neopterin was shown to enhance chloramine-T-mediated and H_2O_2-mediated chemiluminescence as well as toxicity against bacteria,[2] to activate the transcripiton factor NF-κB with a subsequent stimulation of inducible nitric oxide synthase gene expression in vascular smooth muscle cells,[3] or to induce apoptosis in different cell lines.[4] Since these events are mediated by oxidative stress, one may speculate that neopterin enhances oxidative mechanisms, either by stressing pro-oxidative mediators or by the consumption of anti-oxidative counter-regulatory means, or both.

Surfactant proteins are stored in and released from intracellular lamellar bodies by alveolar type II epithelial cells. The importance of an increase of cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) for surfactant release is supported by studies using Ca^{2+} ionophores and by the observation that surfactant release by β-adrenergic and purinergic stimulation is accompanied by an elevation in intracellular Ca^{2+}.[5,6] It has been demonstrated that oxidative stress inhibits intracellular Ca^{2+} transients in various cell types; for example, in retinal cells[7] or in ventricular cardiomyocytes.[8] However, the influence of oxidative stress on $[Ca^{2+}]_i$ has not yet been investigated in lung epithelial cells. Disturbances of cellular Ca^{2+} homeostasis by oxidative

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stress in type II alveolar epithelial cells might inhibit surfactant release and may thereby contribute to pulmonary dysfunctions commonly observed in the course of inflammatory diseases.

In the present study, we investigate the effects of neopterin on adenosine triphosphate (ATP)-induced Ca^{2+} transients in a type II-like alveolar epithelial cell line (L2). Within type II-like cells, ATP is a potent stimulus of $[Ca^{2+}]_i$ due to intracellular Ca^{2+} release.[9,10] Neopterin, the hypothetical inducer of oxidative stress, was added to look for its inhibitory potential on ATP-induced Ca^{2+} transients.

Materials and methods

Culture of L2 cells

The lung epithelial cell line, L2 (adult female Lewis strain rat), was purchased from the American Type Culture Collection, No. CCL-149 (Rockville, MD, USA). Cells were grown in 25 cm^2 culture flasks in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS), L-glutamine (2 mM), and penicillin–streptomycin (100 U/ml–100 µg/ml). Every 5th day, confluent cultures were passaged by incubation (10 min at 37°C) in phosphate-buffered saline solution containing 0.05% trypsin and 0.02% ethylenediamine tetraacetic acid (EDTA). For experiments, cells were seeded (10^4 cells/cm^2) into 24-well plates (diameter, 35 mm/dish; media volume, 1 ml) containing coverslips for fluorescence microscopy and were grown to confluence. Passages 45–55 of subcultured cells were used for the present experiments.

Fura-2 experiments

L2 cells were incubated with 4 µM Fura-2 for 15 min and were mounted in a perfusion chamber that allowed complete changing of the external medium within 1 min, and they were investigated at 37°C in permanent perfusion. Fura-2 fluorescence was recorded using a Zeiss IM 35 microscope with a Zeiss Ultrafluor 100 objective (Carl Zeiss, Jena, Germany). The excitation pathway consisted of a Leitz xenon lamp (150 W) and a filter wheel with two excitation filters (340 nm, 380 nm, 10 nm bandwidth). The emitted light was passed through a filter combination (Zeiss LP 429 plus Schott BG39). TV images were acquired by an intensified video camera, C 2400–77H, with an amplifier unit, C 2400–80 (Hamamatsu Photonics, Hamamatsu City, Japan) and passed to a Hamamatsu Interactive Calcium Monitoring System. Two subsequent TV images were recorded at 340 nm excitation and averaged online. The resulting image was stored in a personal computer. Then the filter was changed to 380 nm within 40 msec and the same procedure as described for 340 nm excitation was repeated. The next image pair was taken after 10 sec. Parameters for image acquisition were 256 × 256 pixels/image with 256 gray levels, and each digitized image was corrected for the camera dark current. Images of cells were discriminated at their borders from the extracellular space by transforming all gray values below a threshold value to zero. In a second step, the gray-value distribution of one selected ratio image was exhibited as a histogram to chose the borders for an interactive gray-value scaling. All ratio images were calculated pixel by pixel according to the chosen gray-value scaling and documented in pseudocolor mode.

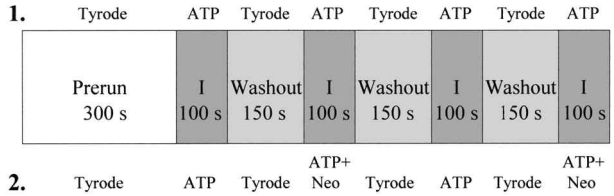

FIG. 1. Schedule for the first and second experimental protocols (I, incubation; Neo, neopterin).

Neopterin excited with UV light acts as a fluorescent dye (excitation maximum, 353 nm; emission, 438 nm). To prevent overlap between Fura-2 and neopterin fluorescence emission, the neopterin concentration was kept below the level of detectable fluorescence.

Experimental set-up

As shown in Figure 1, each experiment started with a pre-run phase by perfusing the cells for 5 min with standard Tyrode's solution (composed of NaCl, 135 mM; KCl, 4 mM; $CaCl_2$, 1.8 mM; $MgCl_2$, 1 mM; glucose, 11 mM; and Hepes, 2 mM; adjusted to pH 7.2 with NaOH). In control experiments, cells were exposed at regular intervals (every 250 sec, for 100 sec) to Tyrode's solution containing ATP (1 µM, repeated four times). Between the ATP incubations, cells were perfused with Tyrode's solution. In test experiments, the L2 cells were superfused alternately with Tyrode's solution containing ATP (1 µM) or with Tyrode's solution containing a combination of ATP (1 µM) with neopterin (100 nM).

Reagents

DMEM with and without phenol red, FCS, L-glutamine, penicillin–streptomycin, and trypsin–EDTA was purchased from Gibco Life Technology (Eggenstein, Germany). Neopterin was purchased from Schircks Lab. (Jona, Switzerland). All other chemicals were from Sigma Chemicals (Deisenhofen, Germany).

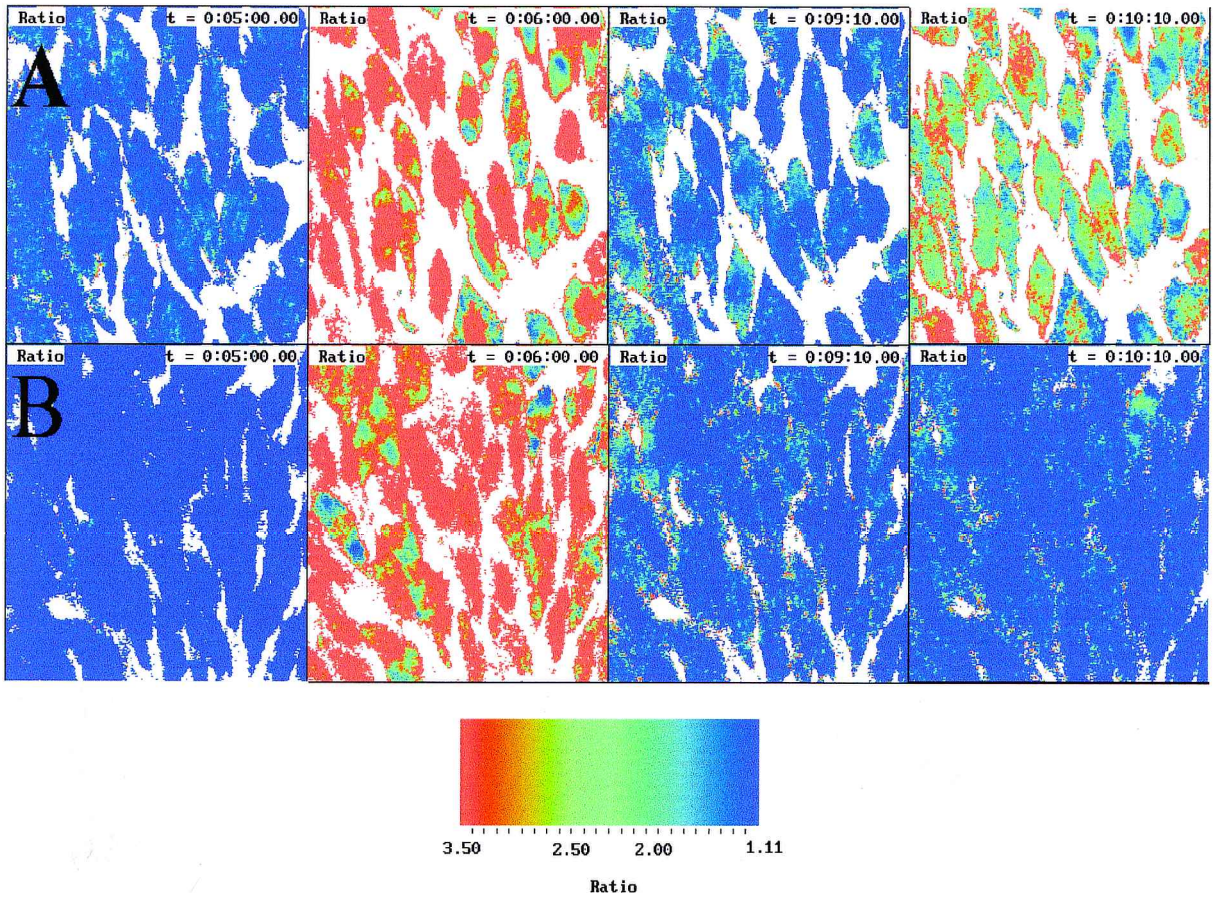

FIG. 2. Pseudocolor images of the Ca^{2+} distribution in L2 cells at different stages of experiments. (a) The upper row of images was taken during an experiment with two consecutive ATP stimulations. In Tyrode's solution, $[Ca^{2+}]_i$ in the cells is low (t = 00:05:00). After a stimulation with ATP, $[Ca^{2+}]_i$ rises in nearly all cells (t = 00:06:00). During the washout, $[Ca^{2+}]_i$ returns to baseline level (t = 00:09:10). A second stimulation with ATP elicits an increase in $[Ca^{2+}]_i$ again (t = 00:10:10). (b) In the lower row of images, an experiment is exhibited during which the cells were stimulated with ATP or with ATP and neopterin, alternately. After the expected increase in $[Ca^{2+}]_i$ following a stimulation with ATP (t = 00:06:00), the simultaneous application of ATP and neopterin did not induce a change in $[Ca^{2+}]_i$ (t = 00:10:10).

Statistical analysis

For further analyses, those cells that exhibited sufficient fluorescence at the end of the experiment were selected. On each of these cells, an average ratio value was calculated in a representative part. This was plotted as a function of time. For standardization, the first 10 ratio values were averaged and set to 100%. All ratio values were brought into relation with this reference value. By this procedure, ratio changes are transferred to percent changes. Thus, under control conditions, each cell starts with values near 100%. At each time point, the percent values of all cells were averaged and their standard deviations (SD) were calculated. Significance of differences was calculated by analysis of variance followed by Tukey's post test. $p < 0.05$ was considered significant.

Results

L2 cells stimulated with 1 μM of ATP develop $[Ca^{2+}]_i$ transients (i.e. $[Ca^{2+}]_i$ increases and returns to control levels in the presence of ATP after about 3 min) (Fig. 2a), as indicated by the change in color. During the washout phase in Tyrode's solution, $[Ca^{2+}]_i$ returned to baseline values. This procedure was repeated three times with ATP causing a rise in $[Ca^{2+}]_i$ each time; however, with a decreasing amplitude. This incubation protocol has been applied to a total of approximately 200 different cells in nine independent runs.

Figure 2b shows a representative result of the corresponding incubations according to the second experimental protocol where cells were exposed to either ATP or ATP with neopterin alternately. In comparison with the first protocol, there was a pronounced increase in $[Ca^{2+}]_i$ after the first stimulation with ATP. The second ATP pulse was performed in the presence of neopterin (100 nM). This time, a change in fluorescence ratio could not be detected, indicating a stable $[Ca^{2+}]_i$. However, after a washout phase, cells reacted again to stimulation with ATP. As a repetition of the second incubation, the fourth incubation was performed with ATP (1 μM) and neopterin (100 nM). Again, $[Ca^{2+}]_i$ did not respond to

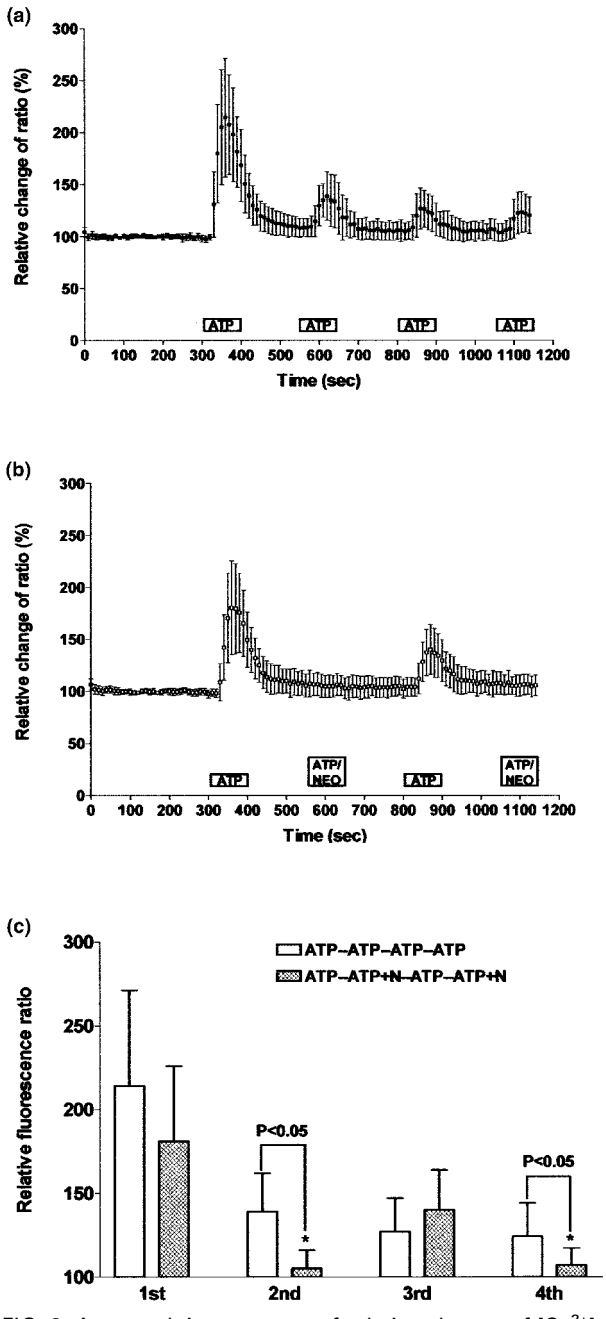

FIG. 3. Averaged time courses of relative change of $[Ca^{2+}]_i$. (a) Each of four consecutive applications of ATP induces an increase in $[Ca^{2+}]_i$. Data are presented as mean ± SD of cells. (b) Application of ATP induces an increase in $[Ca^{2+}]_i$, whereas simultaneous application of ATP and neopterin (NEO) fails to change $[Ca^{2+}]_i$. Data are presented as mean ± SD of 200 cells. (c) Comparison of maximal changes in fluorescence ratio for both the first (open bars) and the second (hatched bars) experimental protocols. 1st, ATP versus ATP; 2nd, ATP versus ATP + neopterin (N); 3rd, ATP versus ATP; 4th, ATP versus ATP + neopterin. * Significant differences.

the stimulation. Similar to the first experimental protocol (Fig. 2a), a total of nine different cell layers with approximately 200 individual cells were investigated.

Figure 3 summarizes the results of the statistical analyses performed with the digitized fluorescence ratio images. Figure 3a shows the changes in relative fluorescence ratio as observed in L2 cells subjected to the first experimental protocol. All four applications of ATP led to a significant increase in fluorescence ratio, and thus a rise in $[Ca^{2+}]_i$. In Fig. 3b, the results of the second experimental protocol are demonstrated. Again, the addition of ATP resulted in an increased $[Ca^{2+}]_i$. There is no detectable change of fluorescence ratio when cells were exposed to the combination of ATP and neopterin for the first time. This was not due to an inability of the cells to increase $[Ca^{2+}]_i$ because a consecutive application of ATP alone led to an increase in fluorescence ratio again.

The maximal changes in relative fluorescence ratio of the first incubation procedure were compared with the corresponding data from the second experimental protocol (Fig. 3c). Both ATP-induced Ca^{2+} transients observed in the first incubation procedure (first step, 214 ± 57%; third step, 127 ± 20%) did not differ significantly from the respective ratios of the second set-up (first step, 181 ± 45%; third step, 140 ± 24%). However, addition of neopterin to ATP inhibited the intracellular Ca^{2+} transients significantly (in the second step, from 139 ± 23% to 105 ± 11%, $p < 0.001$; in the fourth step, from 124 ± 20% to 107 ± 10%, $p < 0.001$).

Discussion

In summary, the present study demonstrates that neopterin inhibits ATP-induced Ca^{2+} transients in the type II-like alveolar epithelial cell line L2. ATP has been shown to be a potent stimulus for surfactant release in type II cells.[11] A role of Ca^{2+} in the secretion of surfactant proteins was suggested by the use of Ca^{2+} ionophores.[5] Considering the fact that ATP evoked a high Ca^{2+} release in many studies,[9,10] one may speculate that $[Ca^{2+}]_i$ is a supportive step for ATP-induced surfactant release. Among the potential mechanisms mediating the effects of ATP on intracellular Ca^{2+} is the activation of L-type voltage-dependent Ca^{2+} channels, as reported for L2 cells by Dietl *et al.*[6] In addition, Ca^{2+} oscillations in primary cultures of airway epithelial cells promoted by ATP have been demonstrated to be due to P2U purinoceptor stimulation and to the continuous production of inositol-1,4,5-triphosphate (IP_3).[12] The inhibitory capacity of oxidative stress on the ion permeability of voltage-sensitive Ca^{2+} channels has been demonstrated.[7,8] In various other cell lines, oxidative stress suppressed the activation of IP_3 induced by both hormonal and non-hormonal stimuli.[12,13] Concerning L2 cells, exogenous nitric oxide donors, which represent another source of reactive oxygen species (ROS), have been shown to inhibit spontaneous depolarizations by L-type Ca^{2+} currents.[14] It has been proposed that pteridine compounds act by the modulation of oxygen

radical-mediated processes (e.g. by activating the ROS-sensitive transcription factor NF-κB).[3] The activation of NF-κB could be prevented by application of anti-oxidants. This may provide an explanation for the stimulatory effects of neopterin on gene expression of inducible nitric oxide synthase or tumor necrosis factor-α.[3,15] Taken together, these data imply that neopterin may not only affect Ca^{2+} transients in alveolar epithelial cells via short-term mediated oxidative stress. Furthermore, long-term interactions between neopterin and various other mediators of the inflammatory system (e.g. activation of nitric oxide synthases with the subsequent production of nitric oxide) may result in prolonged suppression of Ca^{2+}-dependent processes.

According to our knowledge, only one investigation has been performed to date dealing with the effects of neopterin on intracellular Ca^{2+}.[16] Most interestingly, the data of Wöll *et al.* are contradictory to our observations, since they found an increase in $[Ca^{2+}]_i$ in the human myelomonocytic cell line THP-1 in the presence of neopterin. These results suggest that neopterin interferes with $[Ca^{2+}]_i$ in a cell type-specific manner. In the case of monocytes, Ca^{2+} increases have been shown to play an important role in the activation of these cells, with the stimulation of NADPH oxidase being, at least in part, dependent on an elevation of $[Ca^{2+}]_i$.[17] It is tempting to speculate that neopterin-induced Ca^{2+} transients in immune cells contributes to the process of respiratory burst, while in non-immune cells (e.g. alveolar epithelium) neopterin exerts an inhibitory effect on intracellular Ca^{2+}.

Considering inflammatory disorders within the pulmonary system (e.g. sarcoidosis, lung cancer, tuberculosis, adult respiratory distress syndrome, ARDS), the severity as well as the outcome of the disease was found to be correlated with an increase in serum neopterin levels up to ~ 150 nM.[1] Thus, the neopterin concentration of 100 nM applied in this study is representative for inflammatory diseases of the lung. A decreased production and release of surfactant proteins is observed frequently in these situations. By inhibition of Ca^{2+} transients in type II alveolar epithelial cells, neopterin may contribute to this detrimental process, thereby representing an important mediator of the damage and dysfunction of the airway epithelium during lung inflammation.

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