Original paper

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The effect of leptin on the respiratory burst of human neutrophils cultured in synovial fluid

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Abstract

Objectives: Leptin is a hormone responsible for nutritional status and immune competence coordination. In rheumatoid arthritis (RA) increased leptin levels were observed in both serum and synovial fluid. Its influence on development of the disease still remains unclear. So far, research on leptin's influence on the emission of reactive oxygen intermediates (ROI) measured with chemiluminescence (CL) has provided unclear and contradictory results. In this study, we evaluated the influence of leptin on oxidative activity of neutrophils isolated from blood of healthy volunteers and cultured in different amounts of synovial fluid (SF) from patients with RA.

Material and methods: Neutrophils' oxidative metabolism was measured by two types of CL. The first one, luminol-dependent CL (CL-lum), allows one to determine phagocytic activity and the level of ROI generated in a myeloperoxidase-dependent manner. The second method used was lucigenin-dependent CL (CL-luc), which monitors ROI production dependent on the NADPH oxidase enzyme complex located in the cell membranes of neutrophils and enables one to determine the scope of extracellular ROI emission.

Results: Neutrophils stimulated by opsonized zymosan show a decrease in the level of CL-lum, proportional to the increasing concentration of both SF and serum collected from healthy donors. The observed effect of decreased CL-lum may, therefore, be dependent on the physical conditions (viscosity of fluids used). None of these experiments showed any effect of leptin on the level of CL-lum.

Conclusions: The present study showed that leptin does not affect the level of any of the CL types in inactive neutrophils incubated in normal serum, and it does not affect the level of oxidative activity in resting neutrophils incubated with SF. However, leptin influences extracellular ROI emission (measured by CL-luc). Leptin reduces extracellular emission of ROI, and this effect is dependent on concentration and duration of exposure to leptin.

Key words: leptin, respiratory burst, neutrophils.

Introduction

Leptin is a very important hormone. It controls the connection between nutritional status and immune competence. Increased secretion of leptin has been observed in several immune-mediated disorders [1].

Leptin receptors are expressed on neutrophils, monocytes, and lymphocytes. The leptin receptor belongs to the family of class I cytokine receptors. Modulation of the immune system by leptin is exerted at the proliferation and activation levels [2].

There are a few contradictory reports on the influence of leptin on leucocytes' oxidative activity. Caldefie-Chezet et al. [3] detected a significant increase in chemiluminescence (CL)-luminol-dependent in human PMA-stimulated neutrophils by leptin in experiments *in*

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vitro. In contrast, neutrophils exposed to leptin did not display oxidant production in the study performed by Ottonello et al. [4]. On the other hand, neutrophils from healthy subjects and haemodialysis patients with end-stage renal disease (ESRD) responded differently in the study of Cohen et al. [5]. Addition of leptin to the blood of healthy subjects leads to diminished activation of oxidative activity by PMA but not in blood from haemodialysis patients. Similar effects of leptin were observed by Sanchez-Pozo et al. [6] on monocytes from HIV-infected patients: leptin stimulates the oxidative activity in monocytes isolated from healthy volunteers, but attenuates the oxidative burst from HIV-infected patients [6].

These contradictory data suggest that the role of leptin in modulating oxidative activity of neutrophils infiltrating the joints of patients with rheumatoid arthritis (RA) should be examined in more detail in our experimental model (physiological concentrations of synovial fluid (SF) [7]).

Material and methods

The study was conducted on a group of 15 outpatients in the Institute of Rheumatology (Warsaw, Poland). All patients had definite seropositive RA according to the American College of Rheumatology criteria. The average age of patients was 52 ±9 years. None had received steroids during the 6 months prior to enrolment.

The study protocol was approved by the local ethics committee.

Chemicals

Zymosan A, albumin, lucigenin and luminol were obtained from Sigma Chemical Co, St Louis, USA. Leptin was obtained from PeproTech. Phosphate buffered saline (PBS), Gradisol G and glucose were obtained from Polfa, Poland.

Preparation of synovial fluid

Synovial fluid samples obtained from RA patients were centrifuged at 10,000 g for 30 minutes, divided into 1 ml aliquots and stored at –20°C.

Preparation of human blood neutrophils

Neutrophils were isolated from heparinized blood of healthy volunteers by Gradisol G sedimentation followed by centrifugation and hypotonic lysis of contaminating red blood cells. Neutrophils were morphologically normal and their viability was > 90% as judged by the trypan blue exclusion test. Cells were used within 3 h of preparation.

Neutrophil stimulating agents

Opsonized zymosan (OZ) was prepared from pooled human serum. OZ particles (a stimulator of oxidative and phagocytosis activity) were prepared as described previously [7].

Chemiluminescence assay

The generation of reactive oxidative intermediates (ROI) was detected by CL assay. Luminol-dependent CL measures both intracellular and extracellular activity of the myeloperoxidase-hydrogen peroxide system – as a marker of total oxidative and phagocytic activity. Lucigenin-dependent CL measures the NADPH oxidase-dependent extracellular ROI secretion.

The CL response was measured with a BioOrbit 1251 Luminometer at 37°C using a water jacket sample holder. Neutrophils (2×10^{-5}) in 100 µl of PBS were kept at 37°C for 10 min and mixed with 100 µl of OZ (final concentration 1 mg/ml) and with 15 µl of luminol solution (final concentration 150 µM) or 20 µl of lucigenin (final concentration 200 µM). The samples were adjusted to 1 ml of PBS with glucose and albumin (final concentration 1 g/1000 ml) [7]. The neutrophils from healthy subjects were incubated with 0–80% SF or human serum at 37°C and after exposure to leptin (60 or 180 min), in different concentrations of leptin (5, 50, 100, 250 ng/ml), were stimulated by OZ and finally the CL was measured.

After 60–180 min of incubation with leptin, oxidative species produced by stimulated neutrophils were explored by CL assay by recording the maximum CL peak at 10–15 min (mV).

Results

The present study examines the effect of leptin on CL measurements of neutrophils isolated from the blood of healthy subjects in cell culture medium supported by 10% SF or human serum, and at 80% SF or human serum.

Stimulation of neutrophils in the presence of increasing SF concentration led to a decrease in luminol CL and an increase of lucigenin CL, as shown in Tables I and II.

Luminol-dependent CL of neutrophils cultured with SF temporarily increased and next decreased. Similar results were observed previously [7]. Leptin does not influence this effect. Increasing concentrations of serum influence CL similarly to SF. It is probable that a simple effect of viscosity is responsible for the differential effect on total oxidative metabolism (luminol-dependent CL) vs. extracellular ROI emission (lucigenin-dependent CL) observed in our experiments (Table I).

Lucigenin-dependent CL massively increased with increasing concentrations of SF. Similar results were observed in serum. Permanent and proportional growth of lucigenin-dependent CL in SF was strongly limited with increasing concentration of leptin. This effect was proportional to leptin concentration and exposure time.

Table I. Chemiluminescence assays: variations of levels of reactive oxygen intermediates (ROI) produced by stimulated neutrophils in presence of different concentrations of SF or human serum and leptin detected by luminol-dependent chemiluminescence (CL) (total phagocytosis; as percentage). Chemiluminescence assays, including neutrophils, were incubated for 60 or 180 min with increasing concentrations of leptin: 10, 50, 100 and 250 ng/ml. Chemiluminescence was monitored at the maximal light emission, measured in mV. **A.** 60 min incubation with leptin and increasing concentrations of synovial fluid (SF), **B.** 60 min incubation with leptin and increasing concentrations of human serum (HS), **C.** 180 min incubation with leptin and increasing concentrations of SF, **D.** 180 min incubation with leptin and increasing concentrations of HS. Data are presented as percentages from one of the representative experiments from 7 performed

Leptin (ng/ml)	A			B		
	0% SF	10% SF	80% SF	0% HS	10% HS	80% HS
0	100	145	45	100	156	45
10	100	154	54	100	147	54
50	100	162	65	100	176	76
100	100	174	87	100	165	67
250	100	158	59	100	159	57
Leptin (ng/ml)	C			D		
	0% SF	10% SF	80% SF	0% HS	10% HS	80% HS
0	100	145	45	100	158	52
10	100	167	65	100	162	68
50	100	152	75	100	175	72
100	100	189	81	100	135	58
250	100	128	49	100	165	51

Table II. Chemiluminescence assays: variations of levels of reactive oxygen intermediates (ROI) produced by stimulated neutrophils in presence of different concentrations of SF or human serum and leptin detected by lucigenin-dependent chemiluminescence (CL) (extracellular ROI emission) (as percentage). Chemiluminescence assays, including neutrophils, were incubated for 60 or 180 min with increasing concentrations of leptin: 10, 50, 100 and 250 ng/ml. Chemiluminescence was monitored at the maximal light emission, measured in mV. **A.** 60 min incubation with leptin and increasing concentrations of synovial fluid (SF), **B.** 60 min incubation with leptin and increasing concentrations of human serum (HS), **C.** 180 min incubation with leptin and increasing concentrations of SF, **D.** 180 min incubation with leptin and increasing concentrations of HS. Data are presented as percentages from one of the representative experiments from 11 performed

Leptin (ng/ml)	A			B		
	0% SF	10% SF	80% SF	0% HS	10% HS	80% HS
0	100	800	1858	100	800	1858
10	100	799	1705	100	825	1725
50	100	725	1150	100	805	1699
100	100	690	725	100	715	1720
250	100	650	675	100	699	1729
Leptin (ng/ml)	C			D		
	0% SF	10% SF	80% SF	0% HS	10% HS	80% HS
0	100	800	1858	100	800	1858
10	100	725	1350	100	875	1722
50	100	599	850	100	792	1612
100	100	550	425	100	777	1708
250	100	540	315	100	725	1688

After 180 minutes of exposure to leptin, the CL-limiting effect is twice increased to 60-minute exposure. Leptin does not modulate the stimulatory effect of the serum, which also suggests that in serum we are dealing with a purely "natural" phenomenon. The effect of leptin was detectable only when the experiment was conducted on activated neutrophils (OZ). For unstimulated neutrophils exposed to 10% and 80% SF, for 60 and 180 minutes, no effect of leptin was observed (Table II).

Discussion

Only a few studies have described the effects of leptin on oxidative metabolism of neutrophils, and the results were contradictory. Most of the researchers measured CL with spectrophotometric methods or with flow cytometry [4–6]. Only Caldelfie-Chezet et al. [3] evaluated the influence of leptin on luminol-dependent CL, and observed increased neutrophil CL. According to our knowledge, the influence of leptin on lucigenin-dependent CL has never been evaluated. There are no results evaluating the simultaneous influence of leptin and synovial fluid on neutrophil activity either.

For this reason we decided to reappraise the issue of leptin oxidative responsiveness of human neutrophil co-cultures of these cells in physiological concentration of SF, i.e. 80%. Experiments were performed according to our protocol described previously [7]. The phenomenon of CL generated by neutrophils is associated with the formation of ROI by activated neutrophils, during the respiratory burst (RB). This activation can be demonstrated by measuring the energy released as light (CL) [7].

Luminol-dependent CL is capable of monitoring both the intracellular and the extracellular (total) oxidant generation, as luminol freely penetrates these cells. Lucigenin-dependent CL only measures the rate of extracellular oxidant secretion, because lucigenin did not penetrate neutrophils [8, 9].

Results presented in this paper confirm our results obtained in previous studies [7]. The increased concentrations of SF resulted in a reduction of the luminol-dependent CL response, and an increase of lucigenin-dependent CL was recorded. This indicates that the increase of SF concentration results in higher extracellular ROI secretion and in lower phagocytosis activity [7]. Our previous study examined the effect of higher concentrations of SF (up to 80%) on the RB of neutrophils isolated from the blood of RA patients and from healthy subjects [7]. We believe that the concentrations of SF used in our experiments better reflect the real conditions of the inflamed joint (in vivo-like conditions). Oxidant production by neutrophils transforms toward extracellular emission of ROI parallel to increase of SF concentration [7].

Using the CL assay, in standard in vitro conditions, Caldefie-Chezet et al. [3, 10] demonstrated that leptin enhanced oxidative species production by OZ-stimulated neutrophils, isolated from healthy volunteers. They found that a functional leptin receptor is present on neutrophils and may be able to stimulate intracellular hydrogen peroxide production, without modification of phagocytosis [3, 10]. Zarkesh-Esfahani et al. [11] confirmed that leptin activated neutrophils, collected from healthy adult male volunteers, but found that it does so indirectly via tumor necrosis factor α (TNF-α) release from monocytes.

In contrast, neutrophils exposed to leptin did not display detectable Ca^{2+} immobilization or oxidant production, in standard *in vitro* conditions, as was shown by Ottonello [4] and Montecucco [12]. Similar results were obtained by Kamp et al. [13]; in similar in vitro conditions of experiments, at physiological concentrations, leptin does not affect oxidative metabolism of neutrophils isolated from human blood of healthy volunteers.

Additional problems were described by Cohen et al. [5] and Sanchez-Pozo et al. [6]. In the study of Cohen et al., in the presence of leptin, neutrophils from healthy patients and haemodialysed patients responded differently to stimuli. Sanchez-Pozo et al. [6] found that leptin significantly increases ROI production by human monocytes; this effect is dependent on the dose and maximal response is achieved at 10 nM leptin. On the other hand, it was found that leptin reduced ROI production in monocytes from subjects infected with HIV, which spontaneously produces an increased ROI level. This reduction was maximal at the same leptin concentration, i.e. 10 nM [6]. It is extremely difficult to conclude what the influence of leptin on neutrophils is. These results are contradictory.

The problem of leptin concentrations in different tissues or diseases was summarized by Zarkesh-Esfahani et al. [14, 15]. Their experiments were performed with a leptin dose of 250 ng/ml. This dose was chosen because their dose-response studies clearly indicated that this high dose could activate neutrophils in whole blood (*in vivo*-like conditions). Similar, and in some cases higher, serum leptin levels are found in some non-physiological conditions. For example, leptin levels up to 400 ng/ml have been reported in children with chronic renal failure and also in individuals undergoing leptin treatment [15]. Treatment of a patient with genetic leptin deficiency with low levels of leptin (0.028 mg/kg lean body mass) increased the serum leptin up to 107 ng/ml. In obese, but otherwise healthy subjects treated with leptin (1 mg/kg/day) serum leptin levels up to 736 ng/ml have been reported [16].

Thus the high leptin levels that we used in our study were similar to those found in some non-physiological

conditions. However, Bokarewa et al. [17] found that in synovial fluid and the serum the leptin concentration was respectively 5.4 and 6.2 ng/ml, but in the study of Wislowska et al. [18] the serum level of leptin in RA patients ranged from 1.8 to 81.1 ng/ml. It can be assumed that the level of leptin between 100 and 250 ng/ml, which presented maximal effects in our study, may be reached in SF RA. Moreover, the serum leptin level and synovial/serum leptin ratio are significantly correlated with the RA duration, according to Olama et al. [18]. Contrary to that, there was no significant correlation between the disease activity and plasma leptin in the study of Kopec-Medrek et al. [20]. On the other hand, the existence of some regulatory influence of leptin concentration on expression of the leptin receptor on leukocytes was observed in experiments performed by Gorska and Wasik [21].

Our results show that leptin influences the CL level but only when experiments are conducted in high SF concentrations (80%) – similar to physiological ones. The effect of leptin on neutrophils increases with duration of exposure to the protein. Leptin within concentrations that can be observed in SF reduced extracellular ROI production six-fold.

It seems that our preliminary study concerning the role of leptin in modulating the oxidative metabolism of neutrophils penetrating inflamed SF provided a promising result and should be continued.

The authors declare no conflict of interest.

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