

Influence of exogenous leptin on redox homeostasis in neutrophils and lymphocytes cultured in synovial fluid isolated from patients with rheumatoid arthritis

Michał Gajewski¹, Joanna Gajewska², Przemysław Rzdokiewicz^{1,3}, Elżbieta Wojtecka-Łukasik¹

¹Department of Biochemistry and Molecular Biology, National Institute of Geriatrics, Rheumatology and Rehabilitation, Warsaw, Poland

²Screening Department, Institute of Mother and Child, Warsaw, Poland

³Department of General and Experimental Pathology, II Faculty of Medicine, Medical University of Warsaw, Poland

Abstract

Objectives: Leptin is an adipose cells derived hormone that regulates energy homeostasis within the body. Energy metabolism of immune cells influences their activity within numerous pathological states, but the effect of leptin on these cells is unclear. On the one hand, it was observed that leptin induces neutrophils chemotaxis and modulates phagocytosis. On the other hand, neutrophils exposed to leptin did not display detectable Ca^{2+} ions mobilization or β_2 -integrin upregulation. In this study, we investigated the effect of leptin on the redox homeostasis in lymphocytes and neutrophils.

Material and methods: Neutrophils and lymphocytes were isolated by density-gradient centrifugation of blood from healthy volunteers. Cells were cultured with or without leptin (100 ng/ml for lymphocytes and 500 ng/ml for neutrophils) or with or without synovial fluid (85%) for 0–72 h. Culture media were not changed during incubation. Cells were homogenized and homogenate was frozen until laboratory measurements. Redox homeostasis was assessed by the reduced glutathione (GSH) vs. oxidized glutathione (GSSG) ratio and membrane lipid peroxidation evaluation.

Results: Lymphocytes cultured with leptin and synovial fluid showed a significant increase of the GSSG level. The GSSG/GSH ratio increased by $184 \pm 37\%$. In neutrophils incubated in a similar environment, the GSSG/GSH ratio increased by just $21 \pm 7\%$, and the effect was observed irrespectively of whether they were exposed to leptin or synovial fluid or both together. Neither leptin nor synovial fluid influenced lipid peroxidation in neutrophils, but in lymphocytes leptin intensified lipid peroxidation.

Conclusions: Leptin altered the lymphocytes, but not neutrophils redox state. Because firstly neutrophils are anaerobic cells and have just a few mitochondria and secondly lymphocytes have typical aerobic metabolism, the divergence of our data supports the hypothesis that leptin induces oxidative stress by modulation of mitochondria.

Key words: leukocytes, lipid peroxidation, leptin, glutathione.

Introduction

Leptin modulates the development, proliferation, maturation, activation and apoptosis of immune cells [1]. So far, leptin receptors have been found in neutrophils, monocytes, and lymphocytes [1]. The influence of leptin on neutrophils is unclear. On the one hand, leptin promotes neutrophils' chemotaxis and modu-

lates neutrophils' phagocytosis of bacteria [2]. In diabetic patients' neutrophils, it was observed that an increase in serum leptin levels correlates with the degree of CD11b expression on neutrophils [3]. On the other hand, neutrophils exposed to leptin did not display detectable Ca^{2+} ions mobilization or β_2 -integrin upregulation [4].

Address for correspondence:

Michał Gajewski, Department of Biochemistry and Molecular Biology, National Institute of Geriatrics, Rheumatology and Rehabilitation, Spartańska 1, 02-637 Warsaw, Poland, e-mail: michal.gajewski@spartanska.pl

Submitted: 9.06.2016, Accepted: 30.06.2016

Previous studies on the influence of leptin on neutrophils' oxidative activity bring contradictory results. Caldefie-Chezet et al. [5] detected a significant increase in oxidant production by leptin in human neutrophils, but, in contrast, Ottonello et al. [2] noted that neutrophils exposed to leptin did not display oxidant production.

In our previous study [6], we found that leptin does not affect the level of the chemiluminescence (CL) in inactive neutrophils incubated in normal serum, and it does not modulate the level of oxidative activity in resting neutrophils incubated with synovial fluid (SF). However, leptin reduces emission of extracellular reactive oxygen intermediates (ROI) in activated neutrophils, and that effect is dependent on the concentration and duration of exposure to leptin. The role of leptin in modulation of the oxidative activity of neutrophils is still an open question. The influence of leptin on the metabolism of neutrophils infiltrating the joints of patients with rheumatoid arthritis (RA) should be carefully examined. In another study of ours, we suggested that neutrophils' oxidative activity should be investigated in an experimental model simulating conditions similar to those prevailing in the joint – physiological concentrations of SF [7].

The aim of this study was to analyze the oxidative/antioxidative status in neutrophils and lymphocytes cultured in SF from patients with RA and to correlate obtained redox markers with leptin level. Redox homeostasis was evaluated by the reduced to oxidized glutathione ratio (GSH/GSSG) and lipid peroxidation (LPO) level. The measurement was performed in homogenates from immune cells: neutrophils and lymphocytes cultured in physiological concentrations of SF.

Material and methods

Chemicals

Leptin was obtained from PeproTech. Phosphate buffered saline (PBS) and Gradisol G and L were obtained from Polfa, Poland. The cells were cultured in DMEM from PAA Lab GmbH, Austria. Human (GSH) Elisa Kit and Human (GSSG) Elisa Kit were obtained from DRG Instruments GmbH, Germany.

Preparation of synovial fluid

Synovial fluid samples were obtained from patients with RA, centrifuged at 10,000 g for 30 minutes, divided into 1 ml aliquots and stored at -20°C [6].

Leukocyte isolation

Neutrophils and lymphocytes were isolated by density-gradient centrifugation, from heparinized blood of healthy volunteers by Gradisol G ($d = 1.119 \text{ g/cm}^3$ – neu-

trophils) and Gradisol L ($d = 1.077 \text{ g/cm}^3$ – lymphocytes) after hypotonic shock lysis of contaminating red blood cells. Neutrophils and lymphocytes were morphologically normal, and their viability was $> 90\%$ (trypan blue exclusion test). Cells were used within 3 hours of preparation.

Biochemical assays

Cell cultures with or without leptin (100 ng/ml for lymphocytes and 500 ng/ml for neutrophils) were incubated for 0–72 h. Culture media were not changed during incubation. After the experiment, media were removed after centrifugation. The cell pellet was homogenized and homogenate was frozen until laboratory measurements.

To determine the level of lipid peroxidation, malondialdehyde (MDA) was assayed as in [8] by measuring the amount of thiobarbituric acid (TBA)-reactive materials. Intracellular reduced (GSH) and oxidized (GSSG) glutathione content was quantified according to the manufacturers' protocol.

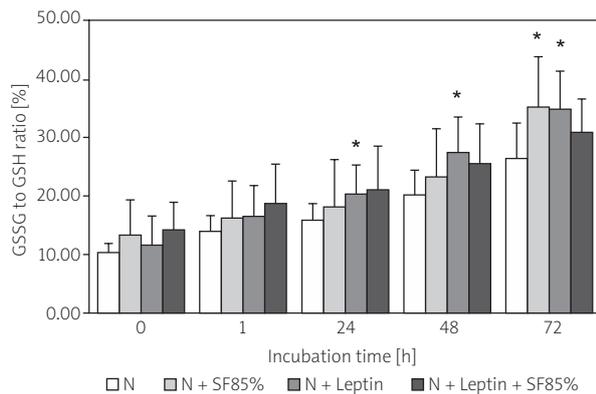
Results

In our preliminary experiments, we tested the influence of leptin concentration on redox homeostasis (GSH/GSSG balance) and lipid peroxidation (LPO) of lymphocytes and neutrophils cell membranes. The minimal recordable effect of leptin on GSSG/GSH ratio in lymphocytes was observed at a concentration of 50 ng/ml, whereas a ten times higher level was required to indicate a response from neutrophils (data not shown). Therefore, for the further experiments, we chose 100 ng/ml and 500 ng/ml concentrations of leptin for lymphocytes and neutrophils respectively.

After 24 hours of incubation, we observed a slight but constant increase of GSSG/GSH levels resulting in redox imbalance. The effect was observed irrespectively of whether it was exposure to leptin or SF or together (Fig. 1). This effect is probably related to maturation and apoptosis of neutrophils, which are short living cells (confirmed by observations after staining cells with trypan blue – data not shown).

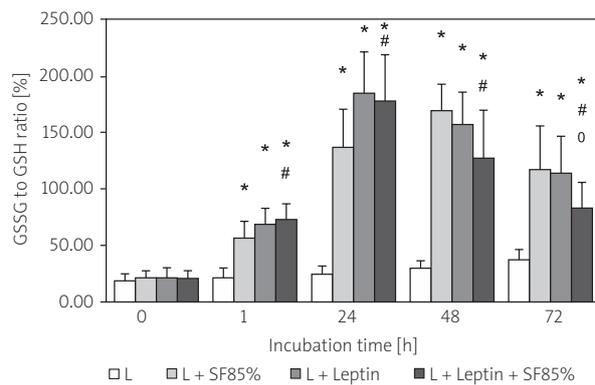
After 24 h of incubation, we observed a significant increase in GSSG level. The GSSG/GSH ratio increased by $184 \pm 37\%$. After the first day of the experiment we found a small decrease of this ratio, but even after 72 hours it was much higher than the output level (Fig. 2). It seems that leptin increase redox imbalance in lymphocytes but with increasing time of exposure the effect is weaker.

Neither leptin nor SF influenced lipid peroxidation in neutrophils, but in lymphocytes leptin intensified LPO (Figs. 3 and 4). An interesting synergistic effect was observed on lymphocytes incubated with both SF and leptin, because SF itself did not modulate LPO detectably (Fig. 4).



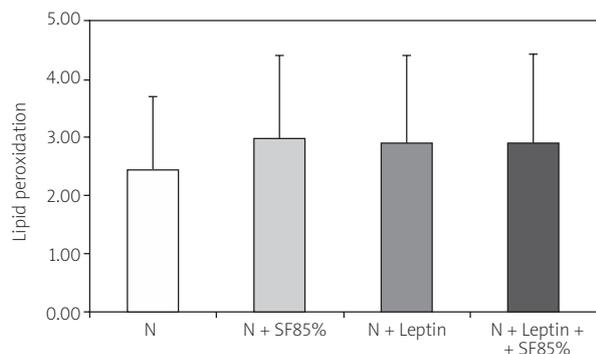
N – neutrophils; SF85% – synovial fluid 85%. Differences between groups were tested with Mann-Whitney U test with a level of significance $\alpha = 0.05$. * – compared to N group.

Fig. 1. GSSG to GSH ratio in neutrophils incubated with 500 ng/ml leptin. Data are presented as mean \pm SD for 12 patients.



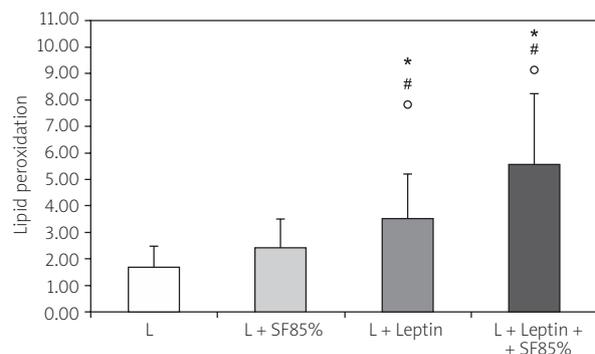
L – lymphocytes; SF85% – synovial fluid 85%. Differences between groups were tested with Mann-Whitney U test with a level of significance $\alpha = 0.05$. * – compared to N group; # – compared to L + SF85% group; o – compared to L + Leptin group.

Fig. 2. GSSG to GSH ratio in lymphocytes incubated with 100 ng/ml leptin. Data are presented as mean \pm SD for 14 patients.



N – control neutrophils cells, DMEM for 24 h; N + SF85% – neutrophils cultured in 85% synovial fluid (SF) for 24 h; N + leptin – neutrophils treated by 500 ng/ml leptin for 24 h; N + leptin + SF85% – neutrophils treated by 500 ng/ml leptin and cultured for 24 h in SF85%. Differences between groups were tested with Mann-Whitney U test with a level of significance $\alpha = 0.05$.

Fig. 3. Effect of leptin on the lipid peroxidation (LPO) level in neutrophils (ng/mg protein).



L – control lymphocytes cells, DMEM for 24 h. L + SF85% – lymphocytes cultured in 85% synovial fluid (SF) for 24 h; L + leptin – lymphocytes treated by 100 ng/ml leptin for 24 h; L + leptin + SF85% – lymphocytes treated by 100 ng/ml leptin and cultured in SF85% for 24 h. Differences between groups were tested with Mann-Whitney U test with a level of significance $\alpha = 0.05$; * – compared to N group; # – compared to L + SF85% group; o – compared to L + Leptin group.

Fig. 4. Effect of leptin on lipid peroxidation (LPO) level in human lymphocytes/monocytes (ng/mg protein).

Discussion

Leptin acts on energy metabolism and plays a role in the modulation of cellular redox balance, but the latest research on the regulation of oxidative metabolism of various cells by leptin has brought contradictory data. Caldefie-Chezet et al. [5] showed for the first time that leptin may influence redox homeostasis. The researchers detected the leptin receptor on neutrophils and demonstrated that leptin increases ROI production in stimulated neutrophils [5]. These observations were confirmed by Fernandez-Riejos et al. [1], who showed that leptin is an essential activator of the immune sys-

tem and inflammatory mediator that affects neutrophils and other immunological cells. Opposite findings were presented by Kamp et al. [9], who observed that leptin in a physiological concentration (250 ng/ml) does not influence on neutrophils at all. Only in extremely high concentrations (25 000 ng/ml) does leptin induce some changes in the proteome of neutrophils, but this effect is most likely mediated by indirect effect consisting of a protective action on acceleration, activation and cell survival [9]. Moreover, there was no influence on polymorphonuclear leukocyte chemotaxis [9]. Recently there has been no clear consensus on leptin's influence on neutrophils.

It is commonly known that ROI have an impact on oxidative status and proliferation of various cells. It is believed that leptin might also regulate these processes, but the type of this action is unclear. Some reports have suggested that leptin may increase antioxidant enzymes activity by regulation of membrane lipids peroxidation (LPO). In the acute response to renal ischemia-reperfusion in rats, it was observed that leptin decreases the level of the oxidative stress marker MDA and increases the activity of antioxidant enzyme scavengers [10]. Equivalent results were obtained in studies by Zwirska-Korczała et al. [11] and Schling and Löffler [12], who observed that leptin decreased MDA levels in a dose-dependent manner in an in vitro study of preadipocyte cells. Zheng et al. [13] demonstrated that leptin abolishes augmented LPO in cardiomyocytes by restoring the activity of antioxidant enzymes. Contrary to that, Bouloumie et al. [14] found that in cultured endothelial cells leptin-induced ROI production. It was suggested that leptin increases mitochondrial electron transport chain activity and in turn increases mitochondrial production of ROI [14]. Similar results were obtained by Kutlu et al. [15], who observed that leptin increases peroxidation of lipids in the mouse brain. Alteration of the cellular redox state was also observed by Savini et al. [16] who found that in keratinocytes cultured with supplementation of leptin an increased GSH/GSSG ratio was observed. This effect was blocked by inhibitors of electron transport and uncouplers of oxidative phosphorylation. These data suggested that leptin induces adaptation to oxidative stress by modulation of mitochondrial homeostasis [16]. Leptin seems to exert a different effect on different cells, sometimes increasing, sometimes decreasing oxidative status.

In a recent study, we examined the influence of leptin on oxidative metabolism of neutrophils and other leukocytes in physiological concentrations of SF. Our study, on the one hand, showed that neither leptin nor synovial fluid influences the GSSG/GSH ratio or lipid peroxidation in neutrophils. On the other hand, we observed that both SF and leptin increase redox imbalance, and we observed a synergistic effect between SF and leptin on the LPO level in lymphocytes. Thus we found a differential effect of leptin on various immune cells. Similar results were obtained in our previous study, where SF did not have any impact on glutathione homeostasis but, depending on the concentration of leptin, increased extracellular and decreased general ROI production by neutrophils [6]. This observation suggests that small changes in neutrophils are purely related to a mechanical shift. Growth in lipid peroxidation in lymphocytes confirms that not the physical (pressure) but the biochemical mechanism is responsible for the redox imbalance in these cells.

In neutrophils energy metabolism is limited to glucose. The few mitochondria that are present in the cells play a role in apoptosis only. In contrast to neutrophils, lymphocytes in a resting state primarily uses mitochondria to derive energy from the process of oxidative phosphorylation [17, 18]. Only after activation in the course of inflammation do they switch their metabolism to anaerobic glycolysis. This phenomenon is related to reduced oxygen concentration in inflamed tissue and is essential for several immunological functions of these cells such as clonal expansion and the production of antibodies and cytokines [19, 20].

Resting lymphocytes produce 88% of ATP in the process of oxidative phosphorylation while activated or proliferating cells produce 86% from anaerobic glycolysis [17]. ROI are a byproduct of the energy-generating process in mitochondria. The intensity of oxidative stress in activated lymphocytes is related to oxidative phosphorylation process. Minimization of oxidative stress, by decreasing ROI production during cellular activation, is a cell-protecting strategy, but the result is their longer life at the expense of reduced ability to regulate the inflammatory process [18].

Conclusion

Because neutrophils are anaerobic cells and have just a few mitochondria and lymphocytes have typical aerobic metabolism, the divergence of our data supports the hypothesis that leptin induces oxidative stress by modulation of mitochondria.

Immune cells differentially regulate redox balance depending on their metabolism [20, 21]. In neutrophils, anaerobic metabolism dominates, while in lymphocytes and monocytes aerobic metabolism shifts to anaerobic glycolysis in the course of inflammation. The possibility of influencing those processes may be an alternative method of treatment of inflammatory diseases.

The authors declare no conflict of interest.

References

1. Fernandez-Riejos P, Najib S, Santos-Alvarez J. Role of leptin in the activation of immune cells. *Mediators Inflamm* 2010; 2010: 568343; doi: 10.1155/2010/568343.
2. Ottonello L, Gnerre P, Bertolotto M, et al. Leptin as a uremic toxin interferes with neutrophil chemotaxis. *J Am Soc Nephrol* 2004; 15: 2366-2372.
3. Mastej K, Adamiec R. Neutrophil surface expression of adhesion molecule CD11b in patients with type 2 diabetes. *Przegl Lek* 2009; 66: 228-232.
4. Montecucco F, Bianchi G, Gnerre P, et al. Induction of neutrophil chemotaxis by leptin: crucial role for p38 and Src kinases. *Ann N Y Acad Sci* 2006; 1069: 463-471.

5. Caldefie-Chezet F, Poulin A, Tridon A, et al. Leptin: a potential regulator of polymorphonuclear bactericidal action? *J Leukoc Biol* 2001; 69: 414-418.
6. Gajewski M, Rzdokiewicz P, Gajewska J, Wojtecka-Łukasik E. The effect of leptin on the respiratory burst of human neutrophils cultured in synovial fluid. *Reumatologia* 2015; 53: 21-25.
7. Gajewski M, Szczepanik S, Wysocki Ł, et al. the effect of increasing amounts of synovial fluid isolated from patients with rheumatoid arthritis on the respiratory burst of human neutrophils. *Reumatologia* 2009; 47: 273-281.
8. Kramer K, Rademaker B, Rozendal WH, et al. Influence of lipid peroxidation on B-adrenoceptors. *FEBS Lett* 1986; 198: 80-86.
9. Kamp VM, Langereis JD, Aalst van CW, et al. Physiological concentrations of leptin do not affect human neutrophils. *PLoS ONE* 2013; 8: e73170; doi: 10.1371/journal.pone.0073170.
10. Erkasap S, Erkasap N, Koken T, et al. Effect of leptin on renal ischemia-reperfusion damage in rats. *J Physiol Biochem* 2004; 60: 79-84.
11. Zwirska-Korczała K, Adamczyk-Sowa M, Sowa P, et al. Role of leptin, ghrelin, angiotensin II and orexins in 3T3 L1 preadipocyte cells proliferation and oxidative metabolism. *J Physiol Pharmacol* 2007; 58 (suppl. 1): 53-64.
12. Schling P, Löffler G. Effects of angiotensin II on adipose conversion and expression of genes of the renin-angiotensin system in human preadipocytes. *Hormon Metab Res* 2001; 33: 189-195.
13. Zheng J, Fang J, Yin YJ, et al. Leptin protects cardiomyocytes from serum-deprivation-induced apoptosis by increasing anti-oxidant defence. *Clin Exp Pharmacol Physiol* 2010; 37: 955-962.
14. Bouloumie A, Marumo T, Lafontan M, Busse R. Leptin induces oxidative stress in human endothelial cells. *FASEB J* 1999; 13: 1231-1238.
15. Kutlu S, Canpolat S, Aydin M, et al. Exogenous leptin increases lipid peroxidation in the mouse brain. *Tohoku J Exp Med* 2005; 206: 233-236.
16. Savini I, Catani MV, Rossi A, et al. Vitamin C recycling is enhanced in the adaptive response to leptin-induced oxidative stress in keratinocytes. *J Invest Dermatol* 2003; 121: 786-793.
17. Brand KA, Hermfisse U. Aerobic glycolysis by proliferating cells: a protective strategy against oxygen species. *FASEB J* 1997; 11: 388-395.
18. Kramer PA, Ravi S, Chacko B, et al. A review of the mitochondrial and glycolytic metabolism in human platelets and leukocytes: Implications for their use as bioenergetic biomarkers. *Redox Biol* 2014; 2: 206-210.
19. Krauss S, Brand MD, Buttgerit F. Signaling takes a breath—new quantitative perspectives on bioenergetics and signal transduction. *Immunity* 2001; 15: 497-502.
20. Pearce EL, Poffenberger MC, Chang CH, Jones RG. Fueling immunity: insights into metabolism and lymphocyte function. *Science* 2013; 342 (6155): 1242-1245.
21. Gajewski M, Rzdokiewicz P, Wojtecka-Łukasik E. The role of physiological elements in the future therapies of rheumatoid arthritis. II. The relevance of energy redistribution in the process of chronic inflammation. *Reumatologia* 2015; 53: 1-6.