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Leptin enhances peroxide production in human umbilical cord leucocytes

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Abstract: We have used flow cytometry to assess the effect of human recombinant leptin on peroxide production by human umbilical leucocytes in cord blood from 12 normal newborns (seven females and five males). Peroxide production was determined by the oxidation of 2′, 7′ dichlorofluorescein diacetate (DCFH-DA) in the three cell populations studied. The cells were stimulated with an optimal and physiological concentration of leptin (10 ng/ml), which induced a constant and significant production of peroxide as compared to non-stimulated cells. There was a significant two-fold increase ($P < 0.005$) in peroxide formation at time points 10, 20 and 30 min for polymorphonuclear cells and lymphocytes. Monocytes, in very low number as compared to the other cell populations, were also activated by leptin. However, the effect on peroxide production was far below the amount observed when PMA was used as a stimulus ($P < 0.00001$). There was no significant correlation between the weight or sex of the newborn and the effect of leptin. Future studies should ascertain the role of this cytokine in cord blood cells.
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Keywords: leptin, newborns, peroxide production, polymorphonuclear cells, umbilical cord leucocytes

Introduction: Leptin is a hormone, encoded by the *ob* gene, whose major source is adipose tissue [1]. Its levels increase at night and circulating concentrations reflect body fat stores [2,3]. The hormone has been found in the plasma and serum of newborns [4,5] and its levels correlated with birth weight [4].

Nevertheless, Yura *et al.* [6] found that the level of leptin was significantly lower in umbilical veins as compared to umbilical arteries, suggesting that the placenta is one of its major sources in the fetal circulation [6]. At birth, levels decline rapidly to a mean of 3 ng/ml [6] and leptin is probably still supplied by the mother since it is secreted in the colostrum and/or breast milk [7]. Thus leptin may be an important modulator of substrate supply in newborns.

In adults, Bornstein and coworkers [8] have shown that levels of plasma leptin are raised in survivors of acute sepsis. A tentative hypothesis is that leptin concentration, along with proteins of the acute phase response, may be important in determining survival. In light of this hypothe-

Materials and methods: Whole blood was obtained from 12 umbilical cords of normal at term neonates (38–40 weeks of gestation). There were seven females and five males (mean weight = 3.1 ± 0.1 kg), delivered by cesarean section in the morning (8–10 am). Written consent from the mothers was obtained prior to the cesarean and the protocol was approved by the Ethical Committee of the Institute.

The blood was collected in sterile Falcon tubes containing EDTA as anticoagulant. The erythrocytes were lysed using buffered ammonium chloride, then the leucocytes were washed and resuspended in PBS-gel (2 mM EDTA, 5 mM glucose, 0.1% gelatin), as described previously [9].

Peroxide production was determined by flow cytometry using the intracellular oxidation of 2′, 7′ dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, OR, USA) to generate 2′, 7′ dichlorofluorescein (DCF) as described previously [9,10].

The cells were labelled for 15 min at 37°C with 1 µl of 20 mM DCFH-DA. Then they were stimulated either with leptin (human recombinant, endotoxin < 100 pg/µg, Calbiochem, La Jolla, CA, USA) or 150×10^{-9} M of phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Company, St. Louis, MO, USA) and a time kinetic assay was performed. The reactions were stopped by placing the tubes on ice. Mean channel green fluorescence intensity (which represents fluorescence intensity in logarithmic units) was reported as DCF production.

Three bitmaps on a forward light scatter (FALS) versus side scatter (FALS vs SS) cytogram were used to define cell populations by granularity and antigen expression. The lymphocyte map contained 66.1 ± 8.3% CD3+, 15.6 ± 3.4% CD19+, 5.5 ± 3.2% CD56% cells, the monocyte map contained 95 ± 2.8% CD14+ cells and the polymorphonuclear cell map contained 94.5 ± 3.6% CD16b+ cells.

The results are expressed by the means ± SD. Student's *t*-test was used for the analysis and P values < 0.05 were considered significant.

Results: Using the three electronic maps, based on FALS vs SS cytograms, circumscribing the specific populations

Table 1. Effect of PMA on peroxide production (means ± SD of the ratios recorded for the 12 umbilical cord leucocytes studied)

Cell type	Time	Ratio PMA/control
Lymphocyte	10 min	5.3 ± 0.5
	20 min	8.4 ± 0.7
	30 min	8.5 ± 0.8
Monocyte	10 min	5.6 ± 0.6
	20 min	7.2 ± 0.5
	30 min	7.4 ± 0.6
[illegible]	10 min	8.4 ± 0.8

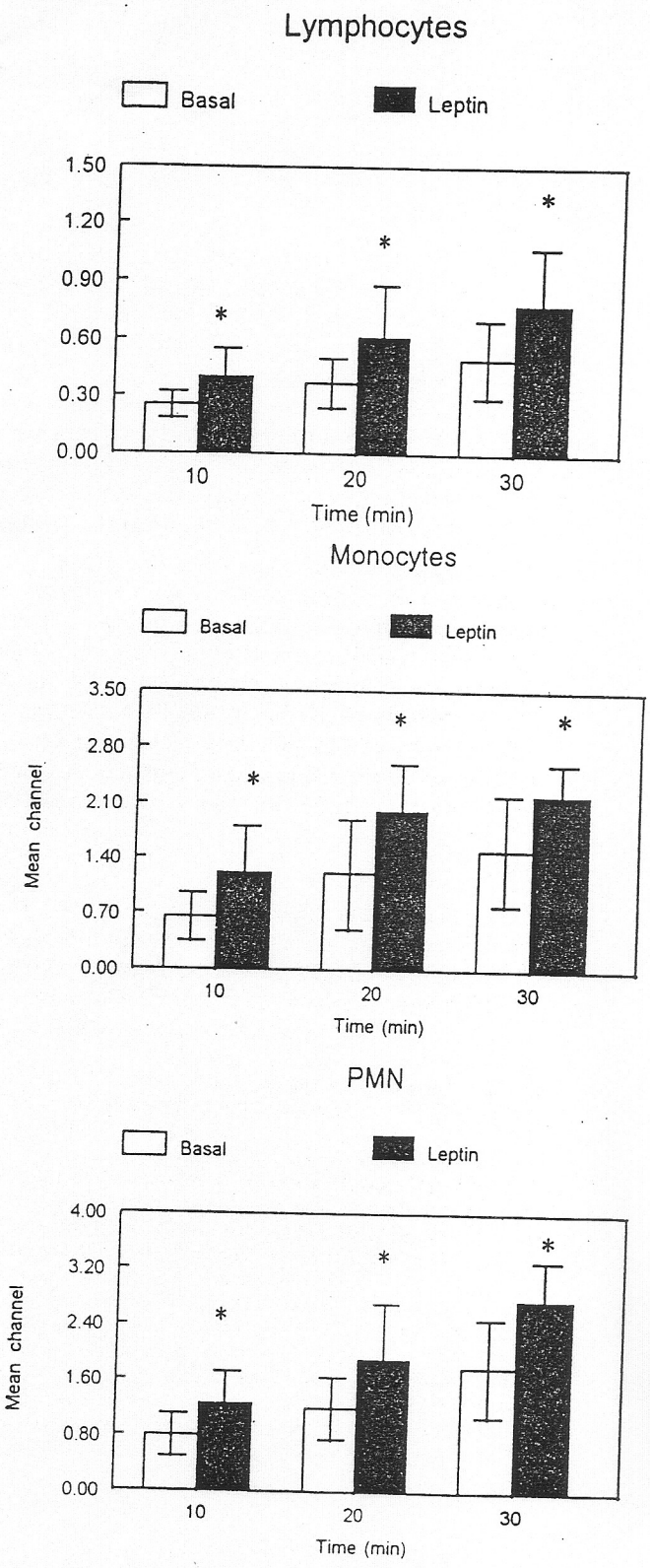

*Figure 1. Effect of leptin on cord blood leucocyte peroxide production (means ± SD). The mean fluorescent intensity in logarithmic units (proportional to peroxide production) of the three different cell populations (n = 12) is shown. *As compared with basal. P < 0.005 (Student's t-test).*

(lymphocytes, monocytes and polymorphonuclear cells), the formation of DCF was assessed by flow cytometry. There was a significant increase in green light fluorescence intensity (mean channel fluorescence in logarithmic units), proportional to peroxide production in the three cell populations stimulated with leptin (Figure 1).

This rise in peroxide production was significant for the three cell populations ($P < 0.005$) and was independent of sex and weight of the neonate. The effect on peroxide production occurred in every neonate studied and was significantly lower than the amount observed when PMA (Table 1) was used as a stimulus.

Discussion: Leptin receptors belong to the superfamily of cytokine receptors [11]. Several types have been reported: a long form (OBRI), 302 cytoplasmic residues, and several short forms (OBRs), including one that has 34 cytoplasmic residues, that it is widely expressed. Bjorbaek *et al.* [12] have shown that in response to leptin, OBRI, and to a less extent OBRs, underwent tyrosine phosphorylation by JAK2 activation. Since JAK2 activation is one of the common events of cytokine stimulation [13], it is not surprising that leptin can prime or activate neonatal leucocytes.

The effect of leptin on peroxide formation was similar in the three cell populations tested. Experiments ($n = 6$) performed with a wide range of leptin concentrations, from 1 pg/ml up to 0.1 µg/ml, revealed that, in most samples, the optimal effect occurred at 10 ng/ml. These results suggest the expression of functional OBR receptors in every cell population studied, which may be important in the neonatal immune response. Further studies should ascertain the role of leptin receptors and the effect of leptin in neonatal immune cells.

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