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**Effects of Leptin and Tumor Necrosis Factor- $\alpha$  on Degranulation and Superoxide Production of Polymorphonuclear Neutrophils from Holstein Cows**

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**Running head:** EFFECT OF LEPTIN AND TNF- $\alpha$  ON BOVINE NEUTROPHIL

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**Abbreviations used:** ALK-P, alkaline phosphatase; CTAB, hexadecyltrimethyl-ammonium bromide; FBS, fetal bovine serum; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks' balanced salt solution; MPO, myeloperoxidase, Ob-Rb, long isoform of leptin receptor; OZP, opsonized zymosan particles; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PMN, polymorphonuclear neutrophils; PMA, phorbol myristate acetate; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; ZAS, zymosan-activated serum.

## **Abstract**

Leptin, a pleiotropic hormone regulating food intake and energy expenditure, has been shown to directly modulate human polymorphonuclear neutrophil (PMN) functions or indirectly through the action of tumor necrosis factor- $\alpha$ (TNF- $\alpha$ ). Bovine PMN has considerable different characteristics from human PMN. For example, it does not respond to *N*-formyl-Methionyl-Leucyl-phenylalanine, a well known human PMN activator. In the present study, we tested the effects of leptin and TNF- $\alpha$  on superoxide production and degranulation of bovine peripheral PMN, in which both long isoform of leptin receptor (Ob-Rb) and TNF receptor 1 were expressed. Human leptin, human TNF- $\alpha$ , phorbol myristate acetate (PMA) and opsonized zymosan particles (OZP) did not stimulate degranulation responses, while zymosan-activated serum (ZAS) did. Neither leptin nor TNF- $\alpha$  enhanced the ZAS-induced degranulation responses. TNF- $\alpha$ , PMA, OZP and ZAS increased superoxide production in different magnitudes, whereas leptin did not. TNF- $\alpha$ , but not leptin, enhanced OZP- and ZAS-induced superoxide production, possibly, in part due to facilitating translocation of p47<sup>phox</sup>, a component of NADPH oxidase. These results indicate that, unlike in human PMN, leptin does not have any direct effect on degranulation and superoxide production in bovine PMN, although TNF- $\alpha$  influences superoxide production.

**Key words:** degranulation, NADPH oxidase, Ob-Rb, p47<sup>phox</sup>, TNF- $\alpha$ .

## Introduction

Leptin, the *obese* gene product, is an adipose tissue-derived cytokine and acts centrally via its specific receptor in the hypothalamus to regulate the body weight and energy expenditure [1]. It is also known that leptin plays a role in the regulation of immune functions. For example, *ob/ob* mice being deficient in leptin exhibit a number of immune abnormalities associated with an increased susceptibility to infection [8, 19].

Polymorphonuclear neutrophils (PMN) are the front line of the host defense system. PMN move fast toward infectious bacteria, capture and engulf them, and kill them by producing reactive oxygen species and proteolytic enzymes from cytoplasmic granules [18]. An increased susceptibility to infections is exerted by functional abnormalities of PMN. Indeed, PMN from *ob/ob* mice showed impaired ability of phagocytosis that could be normalized by exogenous leptin administration [16]. These findings suggest that leptin is an immunomodulator for PMN function in rodents.

It is demonstrated in human PMN that leptin is capable of enhancing PMA-induced superoxide production and of stimulating chemotaxis and hydrogen peroxide production without modification of phagocytosis [4, 5]. Partially contradictory to these reports, Ottonello et al. [17] showed that leptin is a pure chemoattractant for human PMN, without having secretagogue properties such as superoxide production. However, Zarkesh-Esfahani et al. [23] failed to observe any direct effect of leptin on human PMN activation, such that leptin does not induce the upregulation of CD11b expression, while tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) up-regulates the expression of CD11b.

Similar to human PMN, bovine PMN have primary azurophilic granules containing

myeloperoxidase and secondary (specific) granules containing anti-microbial proteins such as lactoferrin and defensin. However, different from human, bovine PMN include third large or secretory granules containing anti-microbial proteins and alkaline phosphatase, and contain very low concentration of lysozymes, bactericidal components, and of catalase that eliminates hydrogen peroxide [9,18]. Moreover, compared to human PMN, bovine PMN are differently activated by neutrophil activators such as *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol myristate acetate (PMA) [10, 20].

Susceptibility to infectious insults in cows increases at parturition when plasma leptin concentration is decreased [2, 6, 11-14]. As PMN plays a major role in innate immunity against infection, we hypothesized that leptin might also be an immunomodulatory factor of PMN function in dairy cows. In addition, as described above, the reported effects of leptin on human PMN function seem not to be consistent with each other and bovine PMN show some unique functional characteristics. We therefore examined the effects of leptin, and also those of TNF- $\alpha$  as a reference, on superoxide production and degranulation responses of PMN isolated from Holstein cows.

## **Materials and Methods**

### **Materials**

Recombinant human leptin and TNF- $\alpha$  were purchased from PeproTech EC Ltd. (London, UK) and Serotec (Oxford, UK), respectively. Zymosan A, horseradish peroxidase, PMA, *p*-nitrophenyl phosphate, *o*-phenylenediamine dihydrochloride and hexadecyltrimethylammonium bromide (CTAB) were bought from Sigma-Aldrich Co. (St. Louis, MO, USA). Luminol was from Wako Pure Chemicals, Ltd. (Osaka, Japan), while Ficoll-Paque PLUS was from Amersham Biosciences Co. (Piscataway, NJ, USA). Anti-p47<sup>phox</sup> antibody was a generous gift from Dr. B.M. Babior (the Scripps Research Institute, La Jolla, CA, USA). Bovine brain and spleen were obtained from a local slaughterhouse.

### **Preparation of bovine polymorphonuclear neutrophils (PMN) and monocytes/macrophage**

Experimental procedure was in accordance with the guidelines of the animal use and regulations of Hokkaido University. Seven Holstein cows without being milked for more than one year and without having any clinical symptoms of diseases were used for multiple blood sampling. Blood was collected from jugular vein into heparinized tubes and blood cells were suspended in Ca<sup>2+</sup>, Mg<sup>2+</sup>-free Hanks' balanced salt solution (HBSS (-)). PMN were separated by density gradient centrifugation of blood over Ficoll-Paque PLUS as previously described [22]. PMN were then incubated with 0.8% NH<sub>4</sub>Cl for 10 min at 4 °C to remove remaining erythrocytes and finally suspended in HBSS containing

0.5 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub> (HBSS (+)). Viability of PMN judged by trypan blue exclusion test was more than 95%.

Peripheral blood mononuclear cells (PBMC) were also separated by the density gradient centrifugation and washed twice in phosphate-buffered saline (PBS) containing 1 mM EDTA and resuspended in RPMI1640 medium (Sigma–Aldrich) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin, 2.38 mg/ml HEPES, and 110 µg /ml sodium pyruvate. PBMC were incubated at 37 °C for 2 h on dishes coated with heat-inactivated FBS, and the adherent cells mainly consisting of monocyte/macrophage were washed with PBS and used for RNA extraction.

### **Preparation of opsonized zymosan and serum containing anaphylatoxin**

Zymosan A was incubated with normal bovine serum at a concentration of 3 mg/ml or 10 mg/ml at 37 °C for 30 to 60 min. After centrifugation of the incubation mixture at 2,000 x g for 10 min, the supernatant containing anaphylatoxin such as C3a and C5a was collected and used as zymosan-activated serum (ZAS). The pellet containing opsonized zymosan particles (OZP) was washed twice with HBSS(-) and resuspended at a concentration of 3 mg/ml in HBSS(+) for degranulation assay or 12 mg/ml in HBSS(-) for superoxide production assay. The ZAS and OZP were further treated with heat at 56 °C for 60 min to inactivate endogenous alkaline phosphatase (ALK-P) and remaining complement activating components.

### **Superoxide production assay**

Superoxide production from bovine PMN was examined as described previously [22]. Briefly, PMN ( $3 \times 10^6$  cells/well) were suspended in 315  $\mu$ l of HBSS (+) containing 10  $\mu$ M luminol and 50  $\mu$ g/ml of horseradish peroxidase and incubated for 5 min at 37 °C in a 96-well plate (Greiner bio-one, Frickenhausen, Germany). After adding 35  $\mu$ l of stimulants such as ZAS and OZP, chemiluminescence released from luminol by superoxide produced from cells and peroxidase was measured by luminometer (ATTO Co., Tokyo, Japan) at 37 °C.

### **Degranulation assay**

Degranulation responses of bovine PMN were assessed by ALK-P release from specific granules and myeloperoxidase (MPO) release from azurophilic granules [7]. PMN ( $10^6$  cells) were treated with various stimuli or CTAB as a positive control in a total volume of 300  $\mu$ l of HBSS (+) for 30 min at 37 °C and then centrifuged at  $800 \times g$  for 10 min at 4 °C to precipitate PMN. For assay of ALK-P, an aliquot of the supernatant (100  $\mu$ l) was transferred into 96-well flat bottom plate and incubated with 100  $\mu$ l of *p*-nitrophenyl phosphate solution for 10 min at room temperature. After addition of 100  $\mu$ l of 1.5M NaOH, developed color was measured spectrophotometrically at 405 nm. MPO activity of another aliquot of the supernatant was also measured by spectroscopy at 450 nm with *o*-phenylenediamine dihydrochloride (0.4mg/ml) and 2M H<sub>2</sub>SO<sub>4</sub> as a substrate and stopping solution, respectively.

### **RT-PCR**

Expression of Ob-Rb, a predominant isoform of leptin receptor, and TNF- $\alpha$  receptor 1 (TNF-R1) in bovine PMN and monocyte/macrophage, spleen and brain (hypothalamus) were examined by RT-PCR. Total RNA was extracted from PMN and the other tissues with TRIzol reagent (Invitrogen) and 2  $\mu$ g of RNA were reverse-transcribed with oligo-dT primer and M-MLV reverse transcriptase (Invitrogen). PCR amplification were performed using specific primers for Ob-Rb (GenBank accession number U62385) (forward: 5'-GCCAGCAACTACAGATG-CTCTAC-3'; reverse: 5'-TCCCATGATCTCT-TAGAGGAAG-3'; 288 bp PCR product) and for TNF-R1 (GenBank accession number U90937) (forward: 5'-TGGAGATTTTCGCCTTGTGTA-3'; reverse: 5'-GTAGTGCCTG-GGTCCTGAGA-3'; 295 bp product) with 35 cycles, each of which consisted of denaturation at 94 °C for 30 sec, annealing at 58 °C for 1 min for Ob-Rb and 59 °C for 1 min for TNF-R1 and DNA extension at 72 °C for 1 min. The band was analyzed in 1.5 % agarose gel with ethidium bromide staining. As a reference, glyceraldehydes-3-phosphate dehydrogenase (G3PDH) mRNA expression was detected as a 452 bp band using specific primers designed based on the sequence reported (GenBank accession number U85042)(forward: 5'-ACCACAGTC-CATGCCATCAC-3', reverse: 5'-TCCACCACCCTGTTGCTGTA-3') with 27 cycles, each of which consisted of denaturation at 94 °C for 30 sec, annealing at 59 °C for 1 min and DNA extension at 72 °C for 1 min.

### **p47<sup>phox</sup> translocation**

Translocation of p47<sup>phox</sup> from cytosol to membrane fraction of bovine PMN was examined as previously reported [21]. Briefly, PMN ( $3 \times 10^7$  cells) in 0.1 ml of HBSS (+) were pre-warmed at 37 °C and treated with stimulants in the absence or presence of TNF- $\alpha$  for 6 min. After adding 1 ml ice-cold buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl<sub>2</sub>, 10 mM PIPES (pH 7.4), 1 mM of PMSF, 1.25 mM EDTA and Complete protease inhibitor cocktail (Roche Diagnosis, GmbH, Mannheim, Germany)), the cells were centrifuged at 3,000 x g for 30 sec at 4 °C. The pellets were re-suspended in 500  $\mu$ l of the buffer and then sonicated three times, each consisting 20 sec at maximal setting of sonicator at 4 °C. The solution was centrifuged at 600 x g for 10 min at 4 °C and the supernatant was further centrifuged at 40,000 x g for 30 min at 4 °C. The precipitate (10  $\mu$ g) was dissolved in SDS-buffer containing 50 mM Tris /HCl (pH 7.5), 9% SDS, 15 % glycerol, 6%  $\beta$ -mercaptoethanol, and subjected to SDS-PAGE (10%). The proteins were electro-blotted onto a PVDF membrane (Immobilon, Millipore, Bedford, MA, USA) and the membrane was immersed in 20 mM Tris /HCl (pH 7.5), 0.15 M NaCl, 0.01% tween 20 and 5 % (w/v) skimmed milk, overnight at 4 °C. The membrane was then incubated with anti-p47<sup>phox</sup> antibody for 1 h at room temperature, followed by incubating with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Zymed Lab. Inc., San Francisco, CA, USA). Visualization of p47<sup>phox</sup> was performed using ECL detection system (Amersham Biosciences) according to the manufacturers' instruction. Intensity of the immunoreactive band was analyzed densitometrically using public domain National Institutes of Health (NIH) Image Program (<http://rsb.info.nih.gov/nih-image/>).

**Statistical analysis**

All values are expressed as means  $\pm$  standard error of the mean. Statistical analysis was done using ANOVA and Fischer's test. Difference having the  $p$  value less than 0.05 is considered significant.

## Results

PMN were isolated from blood of Holstein cows and their mRNA expression of the long isoform of leptin receptor, Ob-Rb and TNF- $\alpha$  receptor, TNF-R1, were examined by RT-PCR. As shown in Fig.1a, Ob-Rb mRNA was detected in PMN as well as the bovine hypothalamus, as a 288 bp PCR product. TNF-R1 mRNA was also detected in PMN, like that in the bovine spleen, and monocyte/macrophage isolated from the blood (Fig. 1b).

PMN were treated with various stimulants and their degranulation responses were assessed by two marker enzyme activities in the supernatant. ALK-P and MPO are the markers of specific and azurophilic granules, respectively, and CTAB was used as a chemical inducer of degranulation to determine maximal enzyme release [7]. Among tested, human leptin in three different concentrations, human TNF- $\alpha$  in two different concentrations, PMA and OZP did not stimulate any of the enzyme activities (Fig. 2a). In contrast, ZAS containing anaphylatoxin enhanced basal ALK-P and MPO activities to about half the extent induced by CATB (Fig. 2a). Next, PMN were treated with either leptin or TNF- $\alpha$  in the presence of PMA (Fig. 2b), OZP (Fig. 2c) or ZAS (Fig. 2d). However, neither leptin nor TNF- $\alpha$  enhanced the enzyme activities even in combination with the PMN stimulants (Figs. 2b-d).

Similarly, PMN were treated with various stimulants and their superoxide production was examined. PMA (Fig. 3b), OZP (Fig. 3c), ZAS (Fig. 3d) and TNF- $\alpha$  at a higher concentration (10 ng/ml) (Fig. 3a) increased superoxide production in order of their magnitudes. However, leptin at three different concentrations did not increase superoxide production (Fig. 3a). Once again, PMN were treated with either leptin or TNF- $\alpha$  in the

presence of PMA, OZP and ZAS. Although leptin had no synergistic effect on superoxide production caused by the PMN stimulants, TNF- $\alpha$  at 10 ng/ml enhanced superoxide production induced by OZP (Fig. 3c) and ZAS (Fig. 3d), but not by PMA (Fig. 3b).

To further characterize synergistic effect of TNF- $\alpha$  on superoxide production, PMN were treated with the PMN stimulants either in the absence and presence of TNF- $\alpha$ , and translocation of p47<sup>phox</sup>, an essential component of NADPH oxidase activation, from cytosol to membrane was examined. As shown in Fig. 4, the PMN stimulants and TNF- $\alpha$  significantly increased the membrane-associated p47<sup>phox</sup>, but the extent of translocation by OZP, ZAS and TNF- $\alpha$  seemed to be smaller than that by PMA. In the presence of TNF- $\alpha$ , translocation of p47<sup>phox</sup> by either OZP or ZAS was virtually identical to PMA alone.

## Discussion

In the present study, we demonstrated that human leptin did not induce degranulation or superoxide production of bovine PMN either in the absence or the presence of neutrophil stimulants such as PMA and ZAS, while human TNF- $\alpha$  did stimulate a release of superoxide. There might be a criticism that the unresponsiveness of leptin was due to the species difference of recombinant protein. However, bovine and human leptin shares 87% amino acids in mature form, and human leptin at 1nM, comparable to physiological concentration, could affect bovine lymphocyte and macrophage/monocyte functions (Ahmed et al. unpublished results). Therefore, we suggest unresponsiveness of bovine PMN to leptin was one of their characteristics.

The characteristics were consistent with the results reported by Zarkesh-Esfahani et al. [23] showing that leptin has no direct effect on human PMN activation, and also those by Ottonello et al. [17] demonstrating that leptin shows no secretagogue properties such as superoxide production in human PMN. However, these are rather conflicting with the reports by Caldefie-Chezet et al. [4, 5] who demonstrated that leptin is capable of inducing hydrogen peroxide and enhancing PMA-induced superoxide production in human PMN, although the reasons for such discrepancies currently are unknown. One noticeable difference between human and bovine PMN is that human PMN express only a short isoform of leptin receptor (Ob-Ra) lacking the large part of the intracellular domain [23], whereas bovine PMN expressed a long isoform of leptin receptor (Ob-Rb). We therefore speculate, although leptin had no effect on degranulation and superoxide production, it might influence bovine PMN functions, such as chemotaxis as observed in

human PMN [17].

We demonstrated for the first time that TNF- $\alpha$  alone did stimulate a marginal but significant release of superoxide in bovine PMN accompanying with an increase in the membrane associated p47<sup>phox</sup>, an essential component of NADPH oxidase activation. The ability of TNF- $\alpha$  to mobilize p47<sup>phox</sup> protein from cytosol to membrane may explain the finding that TNF- $\alpha$  triggers superoxide production in adherent bovine PMN to extracellular matrix without any other stimulant [3]. That is, two signals from cell adhesion molecules and TNF- $\alpha$  act synergistically to produce superoxide. It is also known that TNF- $\alpha$  enhances superoxide production of bovine PMN if they are stimulated by OZP [15]. This was confirmed as shown in Figs. 3c and 3d, although OZP and ZAS themselves can induce superoxide production. Actually OZP and ZAS increased the membrane-associated p47<sup>phox</sup>, and inclusion of TNF- $\alpha$  seemed to enhance the translocation of p47<sup>phox</sup> to the levels obtained by PMA. This synergistic effect of TNF- $\alpha$  with OZP or ZAS, but not with PMA, on translocation of p47<sup>phox</sup> might be important for their synergism of superoxide production. However, the quantities of superoxide produced (PMA/TNF ~200 fold) seemed not to be simply reflected by the amounts of the membrane associated of p47<sup>phox</sup> protein (PMA/TNF ~1.5 fold). Further works on translocation and assembly of other cytosolic components of NADPH oxidase may be required to solve these issues.

The present results indicates that leptin may not be a direct immunomodulatory factor of PMN function in dairy cows, at least in terms of degranulation responses and superoxide production. However, leptin inhibits mitogen-induced proliferation of

peripheral T lymphocytes from Holstein cows, accompanying an alteration of cytokine profile (Ahmed et al. unpublished results). It is worth noting that plasma leptin is greatly decreased after parturition if it was determined by a specific enzyme immuno assay [2, 6, 11, 14] and that susceptibility to infectious insults in cows increases during peri-parturition period [12, 13]. Thus, the decrease in plasma leptin levels during the period may influence immunocompetence to infectious insults in cows, by predominantly affecting acquired immunity rather than innate immunity.

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## Figure Legends

**Fig. 1. Expression of long-isoform of leptin receptor and TNF receptor 1 on bovine peripheral polymorphonuclear neutrophils.** Total RNA was extracted from bovine PMN and subjected to RT-PCR analyses to detect mRNA expression of long-isoform of leptin receptor (Ob-Rb, a) and TNF- $\alpha$  receptor 1 (TNF-R1, b). As a reference, mRNA expression of G3PDH was also determined. B, RNA from the bovine brain (hypothalamus); C, cloned Ob-Rb cDNA as control; NC, negative control; S, RNA from the bovine spleen; M: RNA from bovine monocytes and macrophages. Results of PMN from four cows (1, 2, 3, 4) are shown.

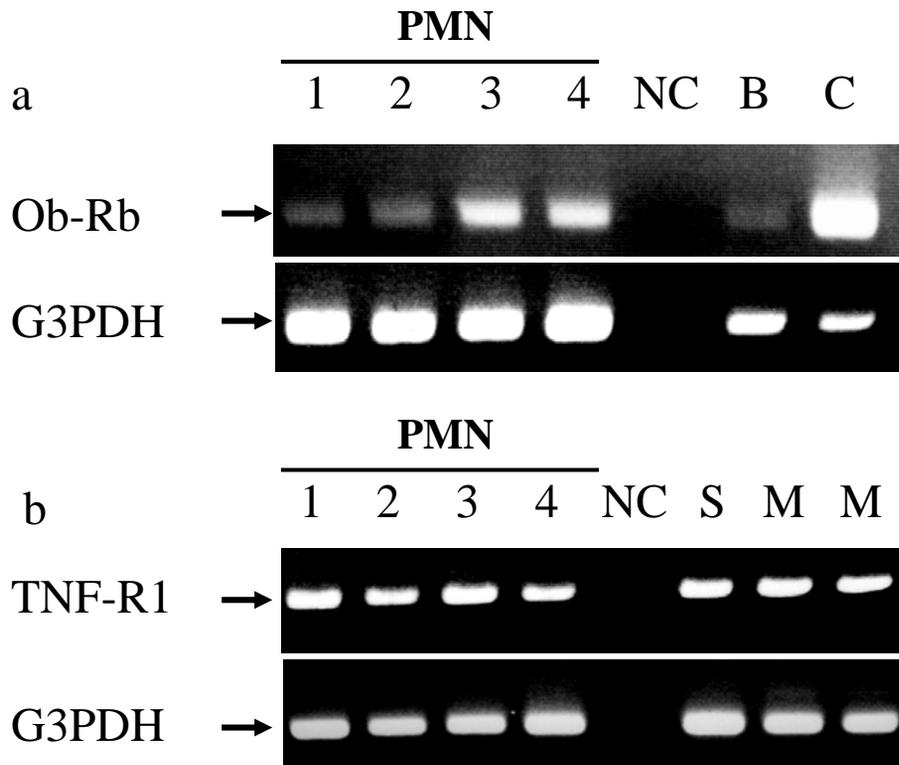
**Fig. 2. Effect of leptin and TNF- $\alpha$  on degranulation of bovine peripheral polymorphonuclear neutrophils.** PMN were treated with either leptin (0.1-10nM) or TNF- $\alpha$  (1-10 ng/ml) in the absence (a) and the presence of phorbol myristate acetate (PMA 1  $\mu$ M, b), opsonized zymosan particle (OZP 1mg/ml, c) and zymosan-activated serum (ZAS, d). Alkaline phosphatase (ALK-P) and myeloperoxidase (MPO) activities in these supernatants were determined. CTAB was used as a positive control of degranulation. The results are from 4 separate experiments. C in Fig. 2a is a control supernatant from PMN without any stimulant. Asterisk indicates statistical significant difference from the control in Fig. 2a and control supernatant from PMN treated with respective stimulants (Figs. 2b-d).

**Fig. 3. Effect of leptin and TNF- $\alpha$  on superoxide production of bovine peripheral**

**polymorphonuclear neutrophils.** PMN were treated with either leptin (0.1-10nM) or TNF- $\alpha$  (1-10 ng/ml) in the absence (a) and the presence of phorbol myristate acetate (PMA 1  $\mu$ M, b), opsonized zymosan particle (OZP 1mg/ml, c) and zymosan-activated serum (ZAS, d). Superoxide production from PMN was determined and total superoxide production (integrated chemiluminescence) of PMN is expressed. The results are from 4 to 7 separate experiments. Asterisk indicates statistical significant difference from the control value obtained from PMN without any stimulants.

**Fig. 4. Effect of TNF- $\alpha$  on translocation of p47<sup>phox</sup> in bovine peripheral polymorphonuclear neutrophils.** PMN were treated with phorbol myristate acetate (PMA 1  $\mu$ M), opsonized zymosan particle (OZP 1mg/ml) and zymosan-activated serum (ZAS) either in the absence or presence of TNF- $\alpha$  (10 ng/ml). Translocation of p47<sup>phox</sup> from cytosol to membrane was determined. Shown are representative results and densitometric analyses of 5 separate experiments. Asterisk indicates statistical significant difference from the control value (-) obtained from PMN without any stimulants.

**Fig. 1**



**Fig. 2 a**

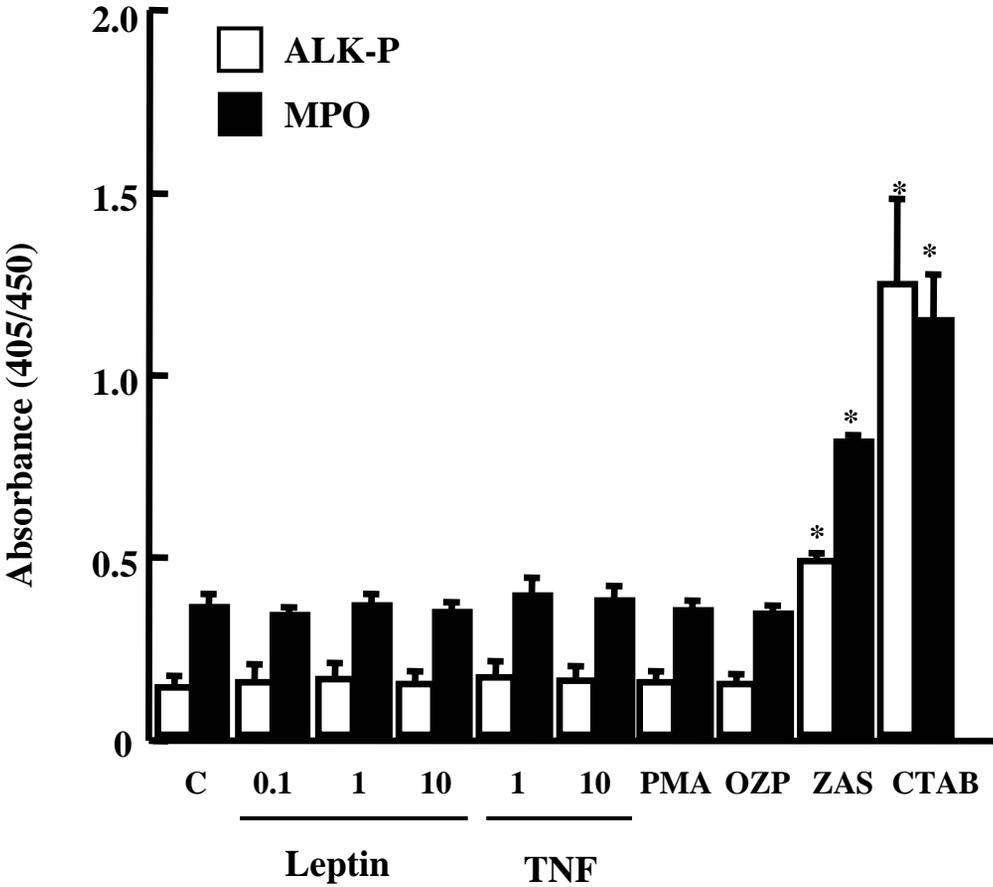
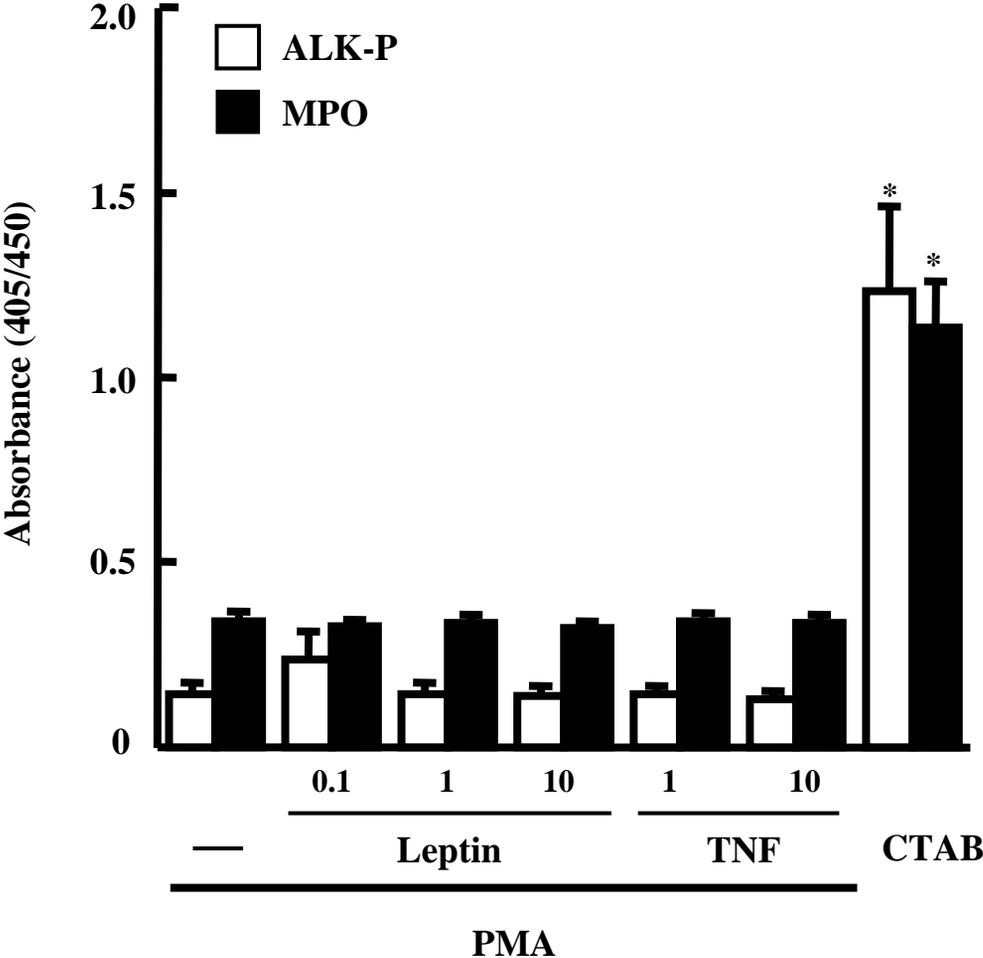


Fig. 2 b



**Fig. 2 c**

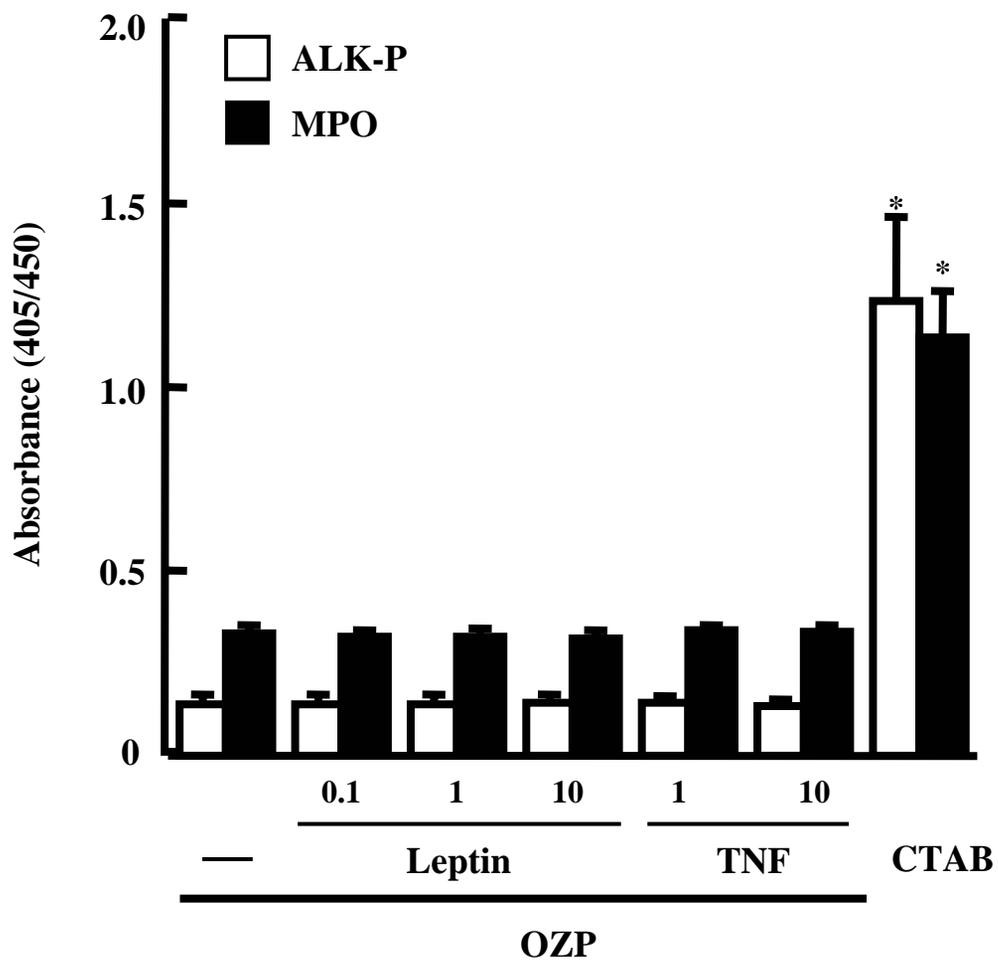
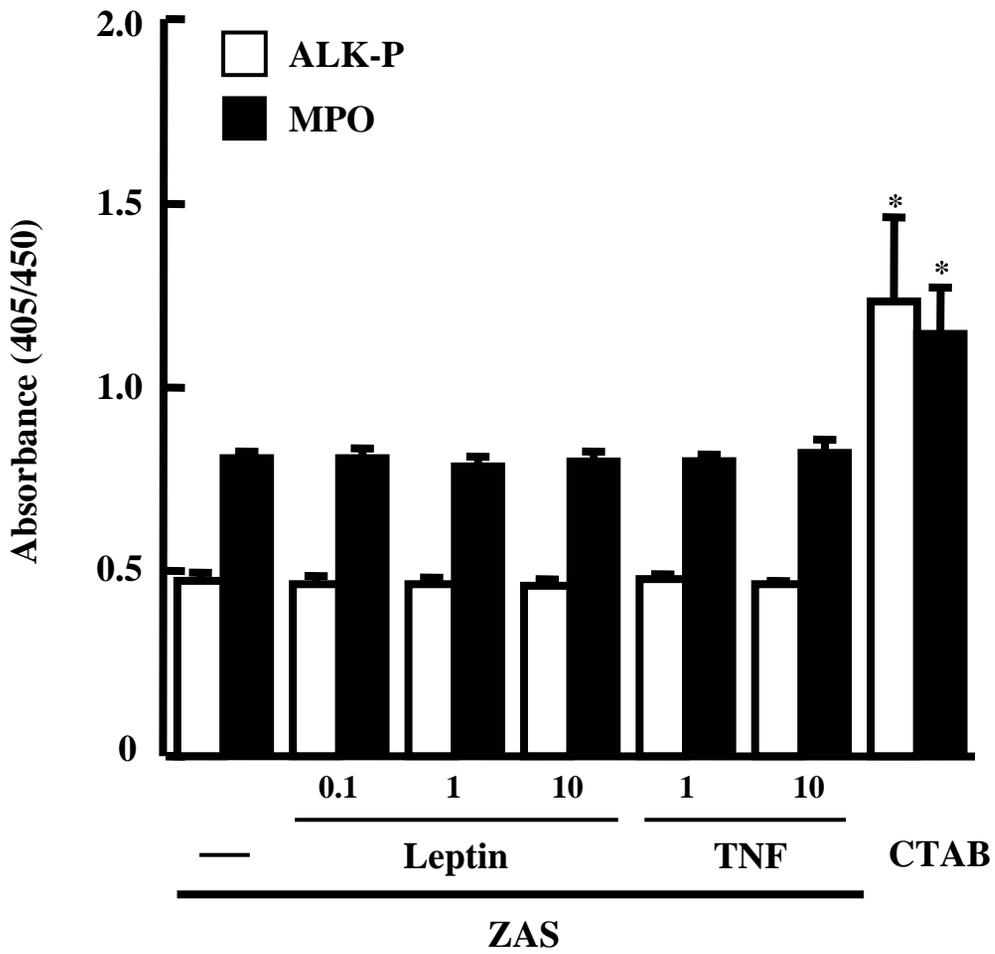
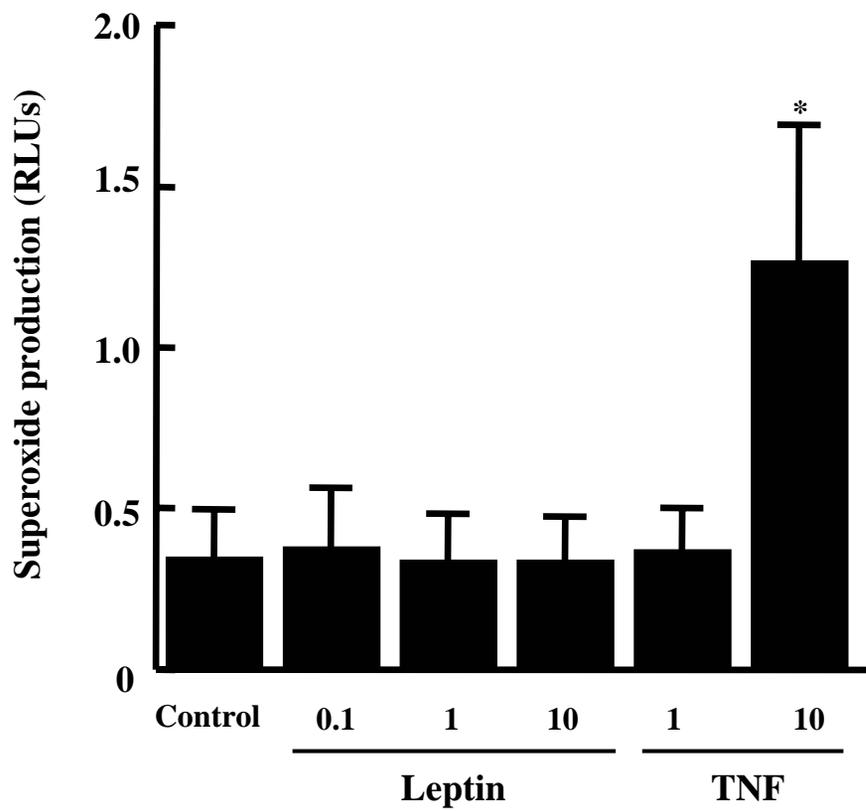


Fig. 2 d



**Fig. 3 a**



**Fig. 3 b**

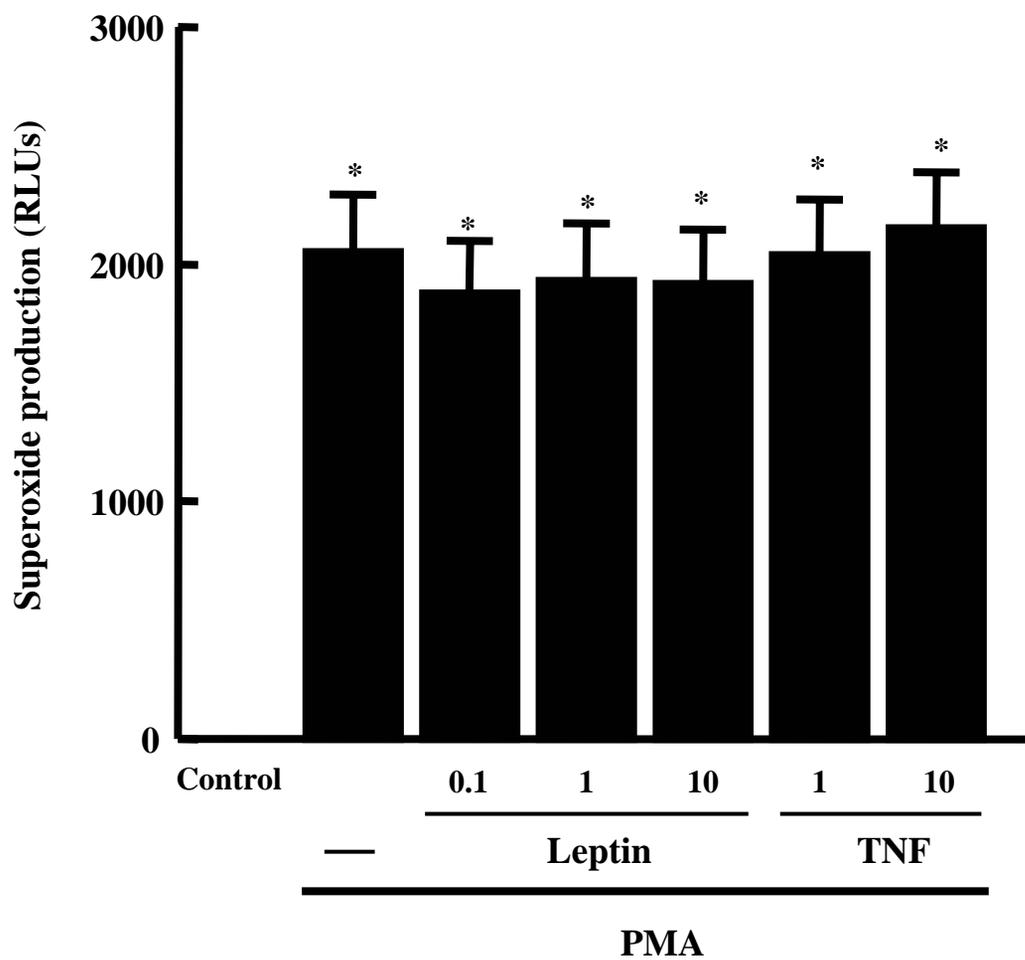
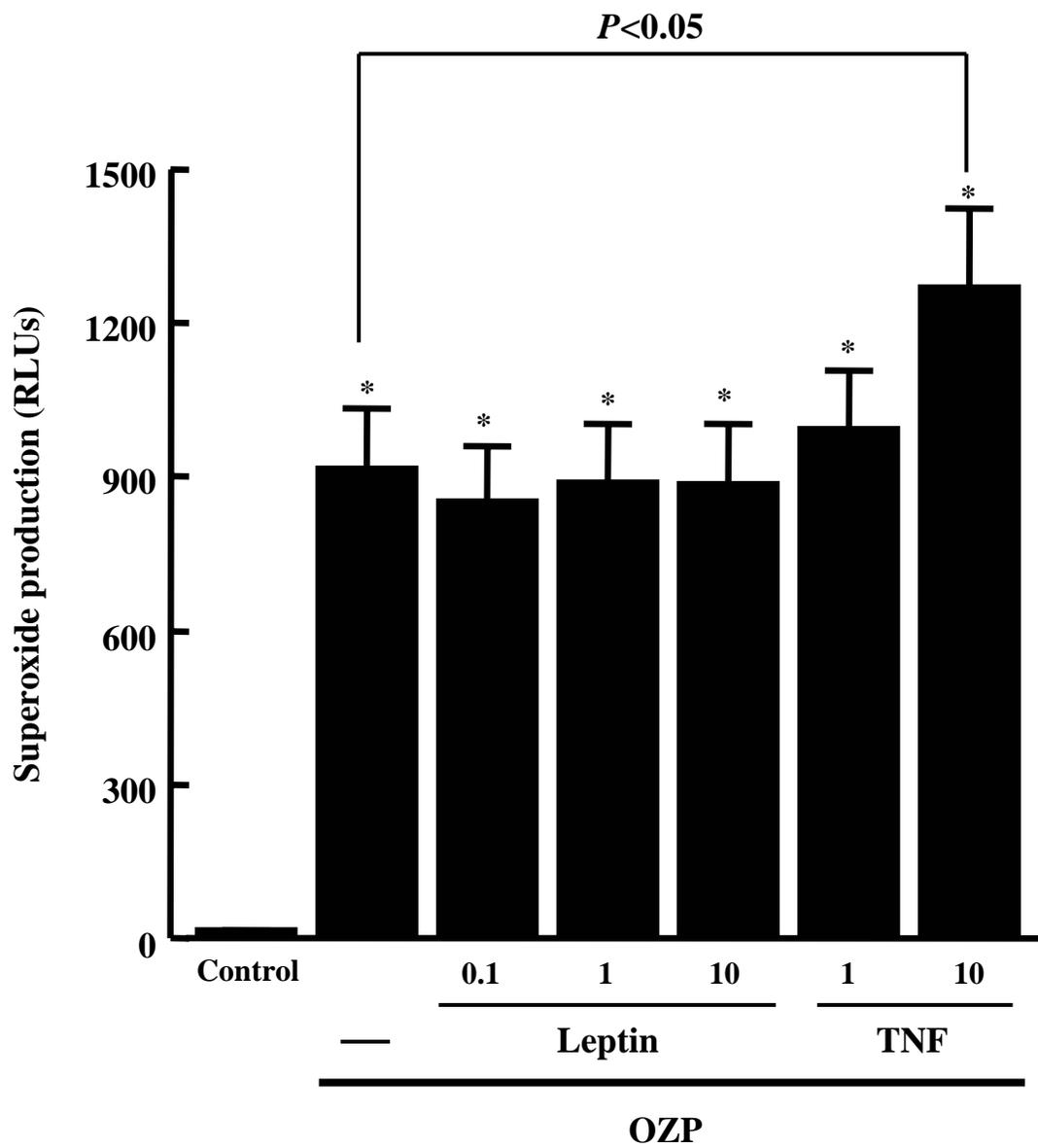
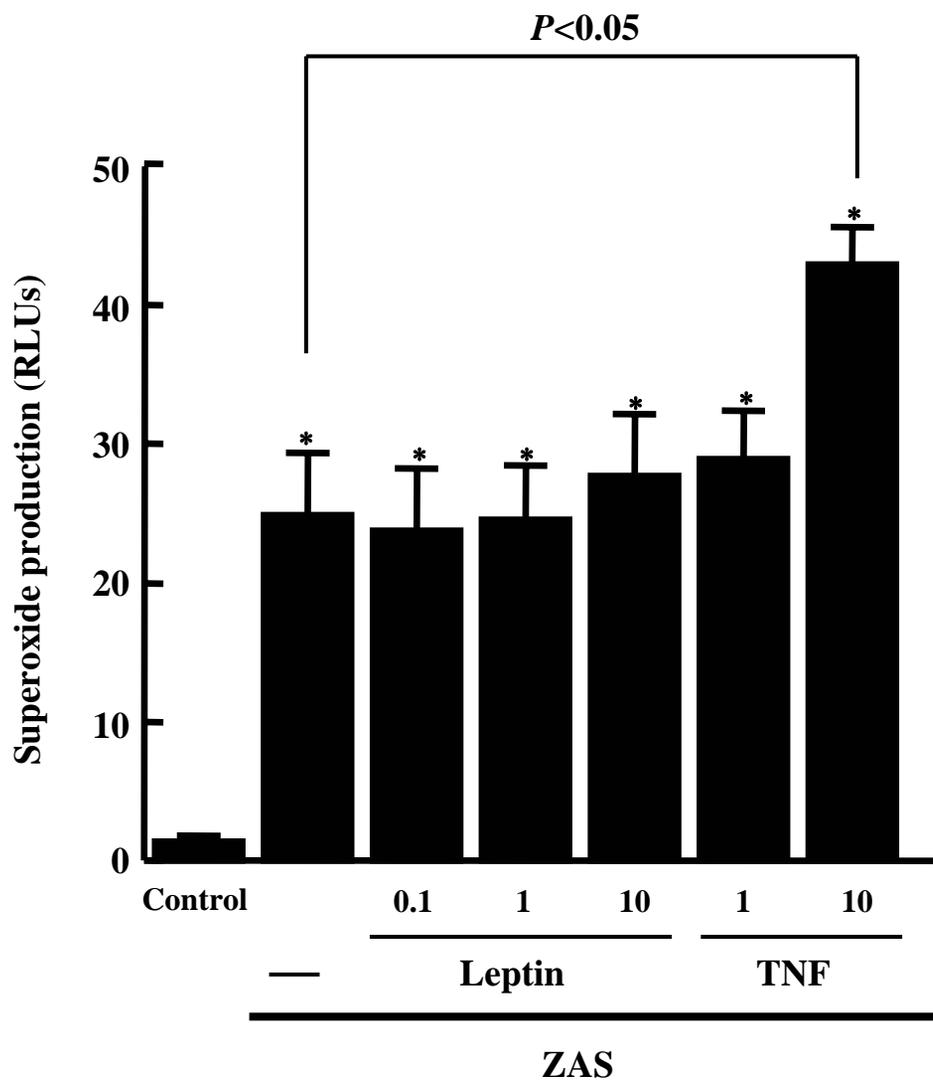


Fig. 3 c



**Fig. 3 d**



**Fig. 4**

