

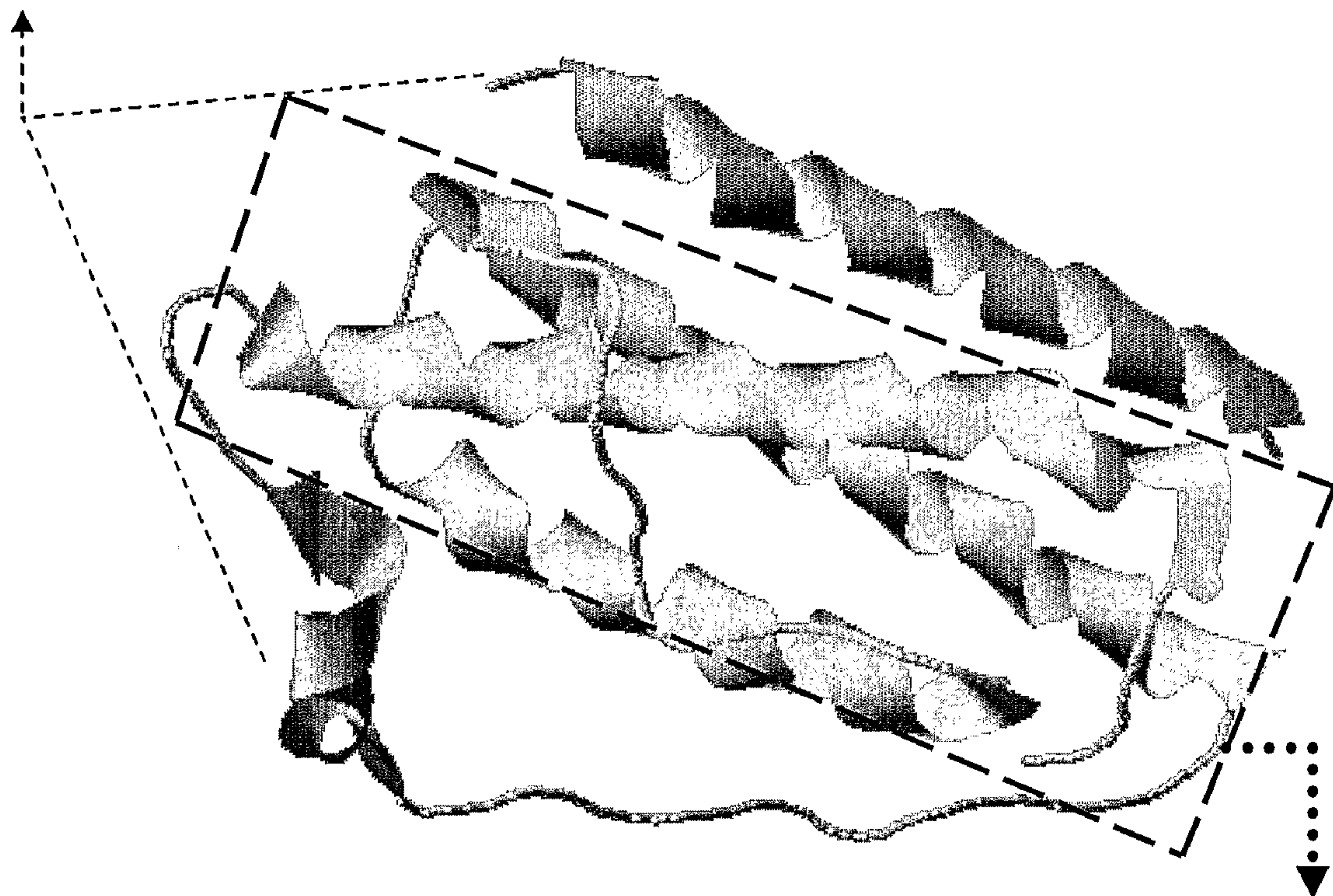


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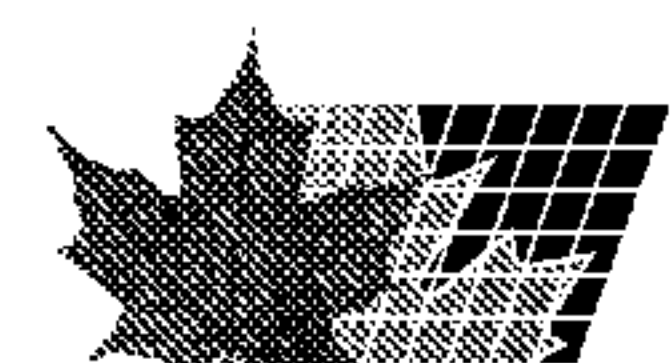
exposed region



core region

(57) **Abrégé/Abstract:**

A polypeptide that contains an amino acid sequence present in human leptin blocks the inhibitory effect of human C-Reactive Protein on human leptin. Accordingly, such a polypeptide is implicated in an approach to treating or preventing conditions associated with the impact of CRP on leptin.



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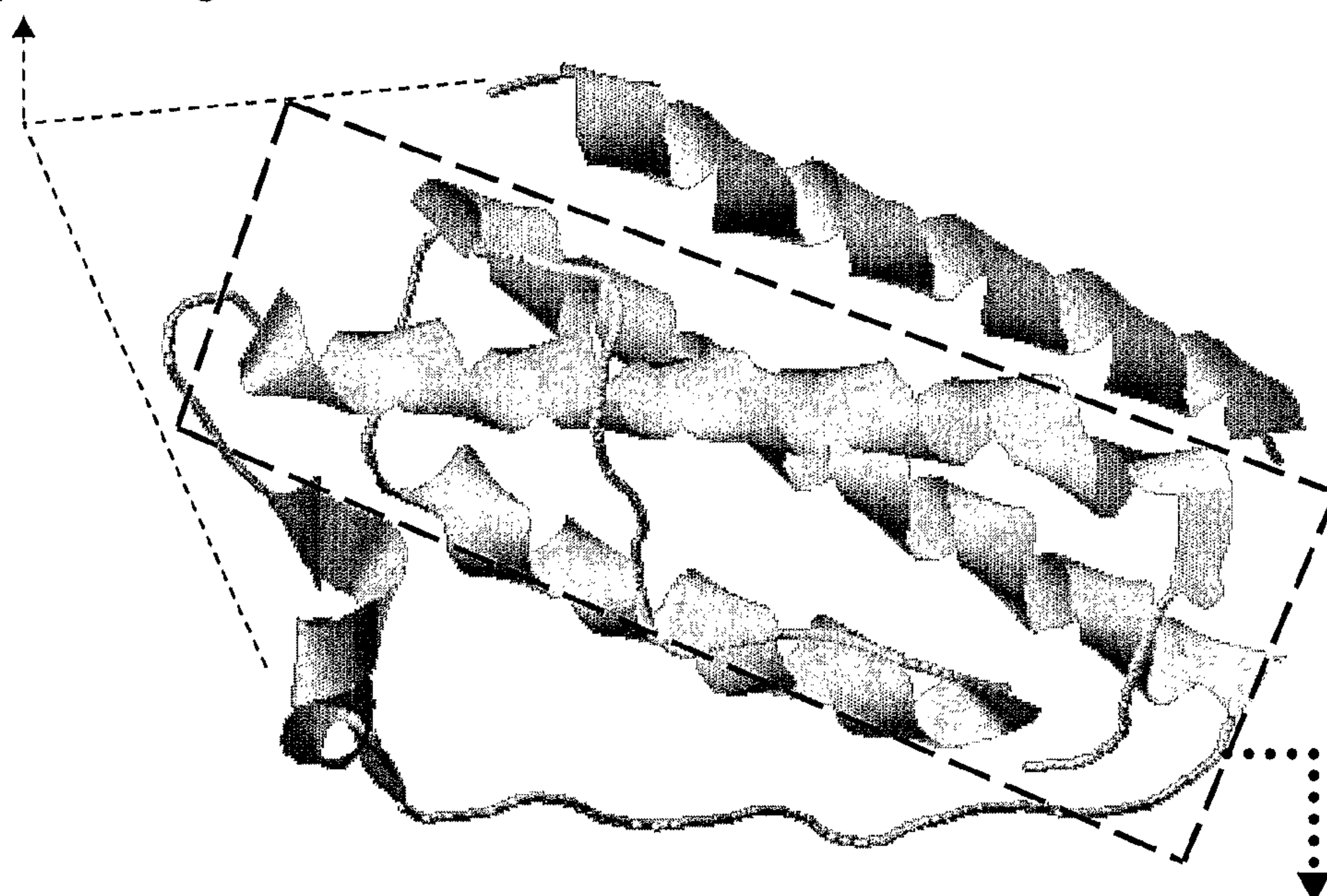
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HUMAN LEPTIN-DERIVED POLYPEPTIDES AND USES THEREOF

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

This application claims the benefit of priority with respect to U.S. provisional patent
5 application No. 60/659,757, filed on March 9, 2005, and to U.S. application No.
11/327,596, filed January 6, 2006.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Work related to the present invention had U.S. government support under Grant No.
10 1RO1DK064383-01, awarded by the National Institutes of Health. The government
has certain rights in this invention.

BACKGROUND OF THE INVENTION

The present invention relates to the prevention and treatment of conditions associated
with C-Reactive Protein (CRP). More specifically, the invention relates to the
15 utilization of human leptin-derived polypeptides for blocking the leptin-inhibitory
effect of human CRP.

Documents cited in this description are denoted numerically, in parenthetical, by
reference to a bibliography below.

Molecular and physiological evidence accumulated in the past decade has firmly
20 established that leptin is a critical adipocyte hormone involved in regulation of energy
intake and expenditure (1,2). Leptin provides a signal to the central nervous system
(CNS) to efficiently maintain a stable body weight, by its binding to the trans-
membrane leptin receptor and subsequent stimulating proteins in the signaling
pathways, such as signal transducers and activators of transcription (STAT), Janus

- kinases (JAK), and phosphatidylinositol-3 kinase (PI3-kinase) (12,13). A null mutation in leptin or a leptin receptor gene can cause hyperphagia, severe obesity, and hyperglcemia (2-4). On the other hand, leptin replacement in leptin-deficient animals and humans can have profound normalizing effects on food intake and body weight.
- 5 Paradoxically, the majority of obese individuals have elevated rather than depressed levels of leptin (5). Therapeutic trials with exogenously administered leptin, which further raise leptin levels, have failed to induce meaningful weight loss (6). These observations present a conundrum, over the apparent ineffectiveness of leptin in preventing obesity, and have spawned the concept of "leptin resistance"(7,8).
- 10 Recent studies have begun to elucidate potential molecular mechanisms. For example, elevation of suppressor of cytokine signaling-3 (SOCS-3), which is induced by leptin, might diminish leptin actions in the CNS and in pancreatic β -cells (8). Nevertheless, it remains unclear whether this mechanism can fully account for all leptin resistance.
- 15 C-reactive protein (CRP) is produced by the liver, in response to stress conditions, such as infection, trauma, and advanced cancer. Serum CRP level is generally elevated in obesity and is a marker of the low-grade inflammatory state associated with obesity and increased cardiovascular risk of obesity (9-11). Elevation of CRP and its relationship to obesity and other disorders have yet to be adequately explained,
- 20 however.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a new approach to treating and preventing conditions associated with CRP, such as obesity, insulin resistance, diabetes, inflammation, metabolic syndromes, atherosclerosis, coronary artery

25 disease, and infertility.

Accordingly, the present invention provides, in one aspect, a method for blocking the inhibitory effect of CRP on leptin to normalize the level of free leptin, by employing

an agent to disrupt formation of CRP/leptin complexes. In one embodiment, the agent is a polypeptide comprised of a segment of 8-40 contiguous amino acids, which segment is present in full-length human leptin (SEQ ID NO: 1), exclusive of the core region and a segment of amino acids from human leptin residue 57 to residue 74:

- 5 VTGLDFIPGLHPILTLISK (SEQ ID NO: 2). Thus, any polypeptide comprised of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 amino acid residues may be used in this context. Preferably, the polypeptide is between 15 to 25 amino acid residues in length.

10 In accordance with another aspect of the present invention, polypeptides are provided that exhibit CRP/leptin complex-disruption activity. In one embodiment, a polypeptide of this category comprises the amino acid sequence of polypeptide B (SEQ ID NO: 3), or a sequence at least 60% identical to that of polypeptide B (SEQ ID NO: 3) with at least one modification. Other embodiments relate to a polypeptide comprising (i) the amino acid sequence of polypeptide C1 (SEQ ID NO: 4) or (ii) the
15 amino acid sequence of polypeptide E (SEQ ID NO: 5), or a sequence at least 60% identical to these, respectively. The modifications contemplated in this regard are illustrated by but not limited to a conservative amino acid substitution, insertion or deletion, a C-terminal truncation, and a N-terminal truncation.

In accordance with a further aspect of the present invention, a method is provided for
20 detecting CRP/leptin complex-disruption activity in an agent, comprising:
(A) contacting the agent with CRP and leptin, in any order; and then (B) measuring an indicator selected from the group of CRP/leptin complex formation, amount of leptin, and amount of CRP, in the absence and presence of the agent, respectively, such that the indicator evidences the CRP/leptin complex disruption activity. In one
25 embodiment, the amount of the CRP/leptin complex or the amount of CRP is measured by means of a leptin affinity column. In another embodiment, the amount of leptin is measured by assessing leptin activity, for example, by monitoring any component of leptin-stimulated signaling pathways, such as JAK2, STAT3, STAT5, STAT6, SH2 domain-containing protein tyrosine phosphatase (SHP-2), growth factor

receptor binding-2 (Grb-2), extracellular-signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK) and PI-3 kinase.

In a related aspect, the invention provides a pharmaceutical composition comprising, with a pharmaceutically acceptable carrier, either a polypeptide with CRP/leptin
5 complex-disruption activity or a nucleic acid encoding such a polypeptide. A pharmaceutical composition of the invention may be used to ameliorate a condition associated with CRP, including but not limited to obesity, inflammation, coronary heart disease, infertility, and diabetes. A composition of the invention also may be used to suppress food intake, to reduce body weight and adiposity, and to alleviate
10 insulin resistance, respectively.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Complete sequence of human leptin protein (SEQ ID NO: 1). Underlined sections represent sequences of polypeptide B (SEQ ID NO: 3), polypeptide C1 (SEQ ID NO: 4) and polypeptide E (SEQ ID NO: 5), from residues 26 to 50, 51 to 75 and
15 111 to 137, respectively.

Figure 2A. Linear diagram of human leptin structure. Highlighted sections represent alpha-helix A from residues 25 to 44, alpha-helix B from residues 72 to 88, alpha-helix C from residues 92 to 115, alpha-helix D from residues 141 to 164, and helix E from residues 127 to 136, respectively.

20 Figure 2B. Amino acid sequence alignment of human and mouse leptin protein. The conserved amino acids are shown as "*" in the sequence of mouse leptin, and the variable residues are shown in bold in both human and mouse leptin sequence. The underlined sequences represent the core region comprised of α -helices B, C and D.

Figure 2C. Three-dimensional view of human leptin structure. The core region,
25 comprised of α -helices B, C and D, is outlined.

Figure 3. Purification of serum leptin interacting proteins (SLIPs) and identification of CRP. Figure 3A shows five major SLIPs eluted of leptin affinity columns from

human serum. From top to bottom on the silver-stained SDS gel, the bands correspond to SLIP-5, SLIP-4, SLIP-3, SLIP-2 and SLIP-1, with apparent molecular weight of 85, 70, 65, 42 and 30 Kd, respectively. Serum leptin is co-eluted with SLIPs, as pointed to by the dashed arrows. Figure 3B confirms that SLIP-1 is human
5 CRP with specific anti-CRP antibody in Western blot assay. Left lane represents the column fraction of SLIP-1, and right lane represents the human CRP standard.

Figure 4A. Interference effect of human CRP on human leptin binding to its receptor. HEK293 cells stably transfected with the long-form human leptin receptor, OB-Rb (14), are used to examine whether human CRP interferes with the ability of human
10 leptin to bind to its receptor. It is found that the K_d for human leptin and human leptin receptor on this cell line is 1.0×10^{-9} M. Pre-incubation of various amount of human CRP with ^{125} I-labeled human leptin reduces leptins' binding to its receptor in a dose-dependent manner. The IC_{50} value is approximately 80 ng/ml of human CRP in the presence of about 2 ng/ml human leptin, which yields a molecular ratio of 5.8:1
15 (CRP:leptin, based on the pentameric structure of CRP). The dashed line at the bottom right of the graph indicates ^{125} I-leptin bound when two thousand fold excess of non-labeled leptin is added.

Figure 4B. Effects of human CRP on mouse leptin. Figure 4B shows that 2 ng/ml ^{125} I-labeled mouse leptin is pre-incubated with various amount of human CRP, before
20 added to the HEK293 cells stably transfected with human OB-Rb. The IC_{50} value is greater than 64 μ g/ml of CRP in the presence of 2 ng/ml mouse leptin, which indicates that human CRP has much lower affinity toward mouse leptin than that toward human leptin. The dashed line at the bottom right of the graph indicates ^{125} I-leptin bound when two thousand fold excess of non-labeled mouse leptin is added.

25 Figure 5. Determination of the effects of purified human or rat CRP on leptin signaling. Figure 5A and 5B illustrate the attenuating effects of human CRP (5A) and rat CRP (5B) on tyrosine phosphorylation of STAT3 induced by human- or murine-leptin, respectively, in a HEK293 cell line stably transfected with OB-Rb. Figure 5C illustrates that human CRP does not have attenuation effect on tyrosin-

phosphorylation of STAT3 induced by murine-leptin. Figure 5D and 5E illustrates the quantitative evaluation of the leptin-stimulated STAT3 activation in the presence of human CRP (5D) or rat CRP (5E) based on analysis of the digitally scanned images (such as those shown in 5A and 5B) with NIH-IMAGE 6.0 software. The data
 5 (relative fold of activation) represents the average of three different sets of experiments for human CRP, and two different sets for rat CRP, respectively. $P < 0.05$ when compared to the leptin-only treated group.

Figure 6. The effects of human CRP on leptin-induced satiety and weight-reducing effects in *ob/ob* mice. Various amount of human CRP is either infused alone or
 10 together with human leptin at 0.3 mg/kg/day into 8-week old male *ob/ob* mice with osmotic pumps. The arrow indicates the day of surgery. Food intake (6A and 6C) and body weight (6B and 6D) are monitored daily throughout the infusion period of 6 days. Human CRP is infused at low dosage of 10 μ g/day (6A & 6B) and high dosage (6C & 6D), respectively. Due to the variations in the body weight of *ob/ob* mice,
 15 body weight is expressed as a percentage of the pre-surgery body weight. In 6A and 6C, $n=4$ for each group except for saline and leptin group. In 6B and 6D, $n=5$ for the saline group, $n=6$ for the CRP- and leptin-infused, and $n=7$ for the group infused with leptin plus CRP. The symbols “*” and “#” indicate $P < 0.01$ and $P < 0.05$, respectively, in a tow-tailed Student’s t-test. In both 6A and 6B, $P < 0.01$ for leptin vs. saline and
 20 CRP from day 2; $P < 0.05$ for leptin plus CRP vs. saline, vs. CRP, and vs. leptin group from day 3. In both 6C and 6D, $P < 0.01$ or $P < 0.05$ for leptin vs. saline, vs. CRP, and Vs. leptin plus CRP group form day 3. In addition, $P < 0.01$ for leptin plus CRP vs. saline and vs. CRP in 6C on day 2.

Figure 7. The effect of human leptin on CRP expression. Human hepatocytes are
 25 treated with physiological concentrations of human leptin for 24 hours before the medium is collected for Western assay of secreted CRP, and the results shown are quantified following the digital scanning of images of two different experiments.

Figure 8. Western blot analysis of STAT3 activation as reflected by its tyrosine phosphorylation. Human CRP suppresses activation of STAT3 by leptin in the

absence of polypeptide B. However, pre-incubation of human CRP with polypeptide B is able to block the inhibitory effect of human CRP on leptin signaling. The control indicates that polypeptide B itself does not have any effect on STAT phosphorylation.

Figure 9. Western blot analysis of STAT3 activation as reflected by its tyrosine phosphorylation. Human CRP suppresses activation of STAT3 by leptin in the absence of polypeptide C1. However, pre-incubation of human CRP with polypeptide C1 is able to block the inhibitory effect of human CRP on leptin signaling. The control indicates that polypeptide C1 itself does not have any effect on STAT phosphorylation. As shown in Figure 9A, the maximal effect of polypeptide C1 can be reached at a concentration as low as 50 nM, while in Figure 9B, higher concentration of polypeptide C1 does not further improve its effect.

Figure 10. Western blot analysis of STAT3 activation as reflected by its tyrosine phosphorylation. Human CRP suppresses activation of STAT3 by leptin in the absence of polypeptide E. However, pre-incubation of human CRP with polypeptide E is able to block the inhibitory effect of human CRP on leptin signaling. The control indicates that polypeptide E itself does not have any effect on STAT phosphorylation.

Figure 11. Co-fusion of polypeptide E with human CRP and leptin through mini-osmotic pumps into the *ob/ob* mice restored the physiological effects of human leptin. Figures 11A and 11B illustrate the experimental data in the absence of polypeptide E, while 11C and 11D illustrate the data in the presence of polypeptide E. Infusion dosages for human CRP and leptin were 48 µg/day/mouse and 12 µg/day/mouse, respectively. The infusion rate for polypeptide E (indicated as "P" in the figure) was at a medium dosage of 24 µg/day/mouse, (indicated as "Pm" in the figure) and at a high dosage of 48 µg/day/mouse (indicated as "Ph" in the figure), respectively. Food-intake (11A and 11C) and body weight (11B and 11D) were monitored daily throughout the infusion. The arrows in A-D indicate the day of surgery. At the end of infusion, blood samples were collected for the measurement of blood-glucose, human CRP, and human leptin. The serum concentrations of human CRP and human leptin were 0.85 ± 0.1 µg/ml and $18 \text{ ng} \pm 2 \text{ ng/ml}$, respectively. Figure 11E shows the

blood glucose concentrations from the group of mice infused the high dosage of polypeptide E. Also shown is glucose concentration from mice infused with human CRP and leptin but not with polypeptide E.

Figure 12. Western blot analysis of STAT3 activation measured by the degree of its tyrosine phosphorylation. As we have shown previously, co-incubation of human CRP with human leptin caused the reduction of leptin-stimulated STAT3 activation. However, the hexamer of U.S. Patent No. 6,777,388 (L-form, NH₂-S-C-H-L-P-W-COOH (SEQ ID NO: 18); D-form, NH₂-W-P-L-H-C-S-COOH (SEQ ID NO: 19)), when pre-incubated with human CRP and human leptin, failed to restore leptin-stimulated STAT3 activation.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention is rooted in the inventor's discovery that leptin interacts with proteins in mammalian serum, so-called "Serum Leptin Interaction Proteins," enumerated 1 through 5 (SLIPs 1-5). The inventor further determined that SLIP-1 is C-reactive protein (CRP).

The identification of CRP as a SLIP is remarkable in several respects. First, CRP is an inflammatory marker and generally increases in obesity (9,15). An increase of CRP in association with obesity is consistent with the fact that inflammation is a metabolic complication of obesity, closely linked to central patterns of fat deposition and hepatic steatosis. Furthermore, elevated CRP in obesity and in insulin resistance is predictive of high risk for cardiovascular disease (11,16).

The inventor has found that the physiological roles of CRP in energy balance are dependent on the presence of leptin. CRP alone does not influence food intake and body weight in the *ob/ob* mice, for instance.

Conversely, the inventor has discovered that CRP acts to abrogate leptin actions by directly binding to leptin. The formation of CRP/leptin complex impedes binding of leptin to its receptor and thereby interferes with leptin-mediated signal transduction.

Consequently, the formation *in vivo* of CRP/leptin complex contributes to attenuation of the physiological effects of leptin, as manifested particularly in leptin resistance. The complex formation also underscores a mechanism by which CRP is understood to contribute directly to the pathogenesis of obesity and to its metabolic and
5 cardiovascular complications.

It also has been found that the role of CRP in leptin physiology is species-dependent. Figure 6, for example, shows not only that human leptin binds to human CRP much more efficiently than to mouse leptin but also that human CRP has a more pronounced inhibitory effect on the signaling capacity of human leptin than mouse leptin. A
10 sequence alignment of human and mouse leptins (Figure 2B) establishes that the flexible domains of leptins, which domains are free of the helix structures, are less conserved between the species.

These findings prompted the inventor's insight that a correlation exists between the leptin structure and its binding to CRP. Thus, a key aspect of the present invention
15 relates to a correlation between the structure of human leptin and its formation of an *in vivo* complex with CRP.

More specifically, the inventor has shown that CRP interacts with critical domains of leptin that are required for binding to its receptor. From this perspective, leptin is seen to have (i) a core region of α -helices B, C, D, which is relatively inaccessible of
20 the surface of the leptin molecule and (ii) α -helix A and other flexible domains that are exposed on the surface of the molecule (see Figure 2C). Based on the understanding that the exposed region (ii) of leptin plays an important role in CRP binding, the present invention provides polypeptides of variable length (a) that have an amino acid sequence that corresponds to or is comprised of a sequence found in the
25 exposed region of leptin and (b) that exhibit CRP/leptin complex-disruption activity, by competitively binding to the CRP.

Thus, the primary structure of a polypeptide of the present invention has a counterpart in those portions of the leptin molecule that do not include its core region (see Figure 2C) and a N-terminal, 21-residue leader segment that is absent from the mature

protein. As noted previously, one embodiment of the invention excludes from this genus of inventive polypeptides a polypeptide with an amino acid sequence consisting of those present in a segment from human leptin residue 57 to residue 74 (SEQ ID NO: 2).

- 5 Notwithstanding the above-stated qualifications, a polypeptide of the invention may embody, in its amino acid sequence, some overlap with the core region or the leader segment, so long as the presence of such portion of the polypeptide as corresponds to the overlap, for example, of 5 to 10 residues in either C- or N-terminal direction, does not substantially abrogate the CRP/leptin complex-disrupting activity of the
10 polypeptide.

Illustrative of these polypeptides of the invention are three, designated B, C1, and E, the amino acid sequences of which appear below.

Polypeptide B: KVQDDTKTLIKTIVTRINDISHTQS (SEQ ID NO: 3)

Polypeptide C1: VSSKQKVTGLDFIPGLHPILTL SKM (SEQ ID NO: 4)

- 15 Polypeptide E: LAFSKSCHLPWASGLETLD SLGGVLEA (SEQ ID NO: 5).

Like other polypeptides of the invention, these are readily synthesized chemically and, when tested, display CRP/leptin complex-disruption activity.

- This activity recommends the inventive polypeptides not only as drug candidates themselves but also as objects for conventional optimization and rational drug design,
20 thereby to obtain drugs that block the inhibitory effect of CRP on leptin *in vivo*. Variants on these polypeptides should be effective to this end, for example.

- The “variants” category in this context includes, *inter alia*, any polypeptide that displays both CRP/leptin complex-disruption activity and substantial homology to a polypeptide that corresponds to or comprises the sequence of a portion of the exposed
25 region of leptin, as described above. In this regard, “substantial” denotes a homology at the amino acid level of at least about 60%, preferably about 70%, more preferably about 80%, and especially in the range between about 90% and 95%, as determined through a sequence-alignment comparison.

- For such comparisons, various techniques are available for aligning sequences. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman (33), by the homology alignment algorithm of Needleman and Wunsch (34), and by the search for similarity method of
- 5 Pearson and Lipman (35), for instance. Computerized implementations of these algorithms include but not limited to: CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, California; GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (Madison, Wisconsin, USA), and the CLUSTAL program (36-40). See also 41-43.
- 10 A polypeptide that displays such substantial homology may be characterized by at least one conservative amino acid modification. A "conservative modification" to an amino acid sequence is a substitution, insertion, or deletion that does not eliminate the CRP/leptin complex-disruption activity that emblematic of a compound within the present invention.
- 15 It is known that conservative amino acid substitution may be made with a residue in the same class. In this regard, naturally occurring residues may be divided into classes, based on common side chain properties: (1) hydrophobic - Met, Ala, Val, Leu, Ile; (2) neutral hydrophilic - Cys, Ser, Thr; (3) acidic - Asp, Glu; (4) basic - Asn, Gln, His, Lys, Arg; (5) residues that influence chain orientation - Gly, Pro; and (6)
- 20 aromatic - Trp, Tyr, Phe. Accordingly, one kind of conservative substitution replaces an amino acid at a given position with another within the same class.

In making conservative modifications, the hydropathic index of amino acids may be considered as well. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics. The hydropathic indices are:

25 isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known that certain amino acids may be substituted for other amino acids that have a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ± 2 is preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

It is likewise understood that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. The following hydrophilicity values have been assigned to these amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0. $\pm$.1); glutamate (+3.0. $\pm$.1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5. $\pm$.1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); and tryptophan (-3.4). In making changes based upon similar hydrophilicity values, substitution is preferred of amino acids that have hydrophilicity values that are within ± 2 ; those that are within ± 1 are particularly preferred, and those within ± 0.5 are especially preferred.

The following table enumerates exemplary conservative amino acid substitutions, in accordance with the principles discussed above.

Table I: Amino Acid Substitutions

<u>Original Residues</u>	<u>Exemplary Substitutions</u>	<u>Preferred Substitutions</u>
Ala	Val, Leu, Ile	Val
Arg	Lys, Gln, Asn	Lys
Asn	Gln	Gln
Asp	Glu	Glu
Cys	Ser, Ala	Ser
Gln	Asn	Asn
Glu	Asp	Asp
Gly	Pro, Ala	Ala
His	Asn, Gln, Lys, Arg	Arg
Ile	Heu, Val, Met, Ala, Phe,	Leu

	Norleucine	
Leu	Norleucine, Ile, Val, Met, Ala, Phe	Ile
Lys	Arg, 1,4 Diaminobutyric acid, Gln, Asn	Arg
Met	Leu, Phe, Ile	Leu
Phe	Leu, Val, Ile, Ala, Tyr	Leu
Pro	Ala	Gly
Ser	Thr, Ala, Cys	Thr
Thr	Ser	Ser
Trp	Tyr, Phe	Tyr
Tyr	Trp, Phe, Thr, Ser	Phe
Val	Ile, Met, Leu, Phe, Ala, Norleucine	Phe

Thus, a conservative modification could entail the substitution of a native amino acid residue with a normative residue, such that there is little or no effect on the polarity or charge of the amino acid residue at that position. Alternatively, the involved substitution could introduce one or more non-naturally occurring amino acid residues, e.g., by solid-phase peptide synthesis or by means of genetic programming of a translational system (30).

By these and other approaches, which are known or which will be developed in the future, one can obtain variants that are peptidomimetic oligomers (31), which may display improved stability and/or bioavailability and, hence, represent better candidates for pharmaceutical purposes. In addition to those mentioned already, some modifications for this purpose (32) are: formation of cyclic variants, as by adding a C-terminal and an N-terminal cysteine with disulfide bond formation; amide nitrogen alkylation, e.g., for generation of N-methylated variants where one or more of the amide linkages in the peptide have a structure of C—N(CH₃)—CO—C; side-chain modifications, e.g., methylation or halogenation of selected phenylalanine residues;

substitution of methylalanine for alanine at selected sites; chirality modification, *e.g.*, replacement of selected L-amino acids with D-amino acids; forming retro-inverse peptides, *i.e.*, peptides with inverted sequence and opposite chirality (D instead of L); forming peptides with amide bond surrogates, *e.g.*, selective reduction of —CO—
5 NH—linkages to CH₂—NH- linkages; and replacing one or more alpha-carbons in the chain with nitrogen atoms, providing an azapeptide analogues.

Like other polypeptides within the invention, any given variant can be screened for CRP/leptin complex -disruption activity, as described below. Information gleaned in this fashion can be used in the design of other suitable variants. For example, if one
10 discovered that a change to a particular amino acid residue resulted in destroyed, undesirably reduced, or unsuitable activity, variants with such a change would be avoided. Based on information gathered from such routine experiments and based on the information provided, in other words, one can readily determine the amino acids where further substitutions should be avoided, either singly or in combination with
15 other changes.

Inventive polypeptides and variants can be “PEGylated,” a term that connotes derivatization by covalently binding of one or more molecules of polyethylene glycol (PEG). In various media, particularly aqueous media, the long, chain-like PEG molecule is hydrated and in rapid motion. The motion of the PEG substituent sweeps
20 out a large volume and prevents the approach and interference of other molecules. The PEG polymer chain can protect the molecule from immune responses and other clearance mechanisms, thereby prolonging and preserving bioavailability of the molecule. By the same token, biological absorption, distribution, and clearance can be modified by altering the size, weight, shape, and linkage used to connect the PEG
25 strand to the molecule in question (29).

In accordance with this invention, disruption of the formation of CRP-leptin complex is evaluated as a therapeutic tool for treating or preventing conditions associated with a dysfunction in a leptin-affected pathway. Pharmacological intervention influencing the association between human CRP and leptin, as described above, could be used to

block the inhibitory effects of human CRP on leptin, and could lead to suppression of food intake and reduction of body weight and adiposity, as well as alleviation of obesity, metabolic syndromes, inflammation, atherosclerosis, infertility, insulin resistance, and type II diabetes. In addition, one could alter the interaction between
5 human CRP and leptin, pursuant to the invention, to increase transport of leptin into the central nervous system (CNS), thus enhancing leptin-induced effects in the CNS.

Recent studies have indicated a positive role of circulating leptin in regulating CRP production (15, 26-28), and CRP in turn can bind leptin and dampen the action of leptin in the CNS and periphery. The inventor has discovered that, although human
10 leptin, within the physiological range, produces a dose-dependent positive effect on CRP secretion, sustained increase of leptin does not result in higher production of CRP (see Fig. 7). Thus, polypeptides of the present invention may be used as a therapeutic agent to liberate leptin from CRP/leptin complex formation, consequently restoring the physiological functions of leptin.

15 Accordingly, this invention also relates to a pharmaceutical composition comprised of the polypeptide with CRP/leptin complex-disruption activity or the nucleic acid encoding the polypeptide, plus a pharmaceutically acceptable carrier therefor, as well as to administering the composition to a subject in an amount effective to counter the CRP-mediated diminution of leptin that is available to induce its normal signaling
20 pathways ("free leptin").

In an embodiment that involves the use of a polypeptide-encoding nucleic acid, considerations that pertain to the field of gene therapy become relevant. For example, these considerations would inform (i) constructing a mammalian expression vector, encoding a polypeptide as described here, and (ii) introducing the vector in the
25 context of a therapeutic protocol, according to the invention. Vector transfer technologies for use in this regard are illustrated by: (a) direct DNA microinjection into cells, *in vivo* or *ex vivo*; (b) ballistic gold particle delivery; (c) liposome-mediated transfection; (d) receptor-mediated gene transfer; and (e) the use of DNA transposons (44-46). In addition, the present invention comprehends the use of an adenovirus-

based gene therapy system for delivery of a polypeptide-encoding nucleotide, as mentioned. The construction and packaging of adenoviral vectors are well known techniques, and potentially useful adenoviral vectors are described, for example, in U.S. patent No. 5,707,618 and in cited publications (47-49).

- 5 As mentioned above, a pharmaceutical composition of the invention could be used to treat a subject with any condition associated with the impact of CRP on leptin. Consequently, the modes of administration for a pharmaceutical composition of the invention are those suitable for administering a therapeutic polypeptide or protein.

Thus, a pharmaceutical composition of the invention could be formulated for oral,
10 ocular, nasal, nasolacrimal, topical (e.g., pulmonary, sublingual, buccal), or intrathecal (cerebrospinal fluid) delivery, in accordance with accepted medical practice. By the same token, formulation of a composition of the invention would accommodate the mode of administration, among other considerations, to include in the pharmaceutically acceptable carrier, as appropriate: excipients, such as starch,
15 lactose, crystalline cellulose, calcium lactate, magnesium aluminometasilicate, and anhydrous silicate); disintegrators, e.g., carboxymethylcellulose and calcium carboxymethylcellulose; lubricants, illustrated by magnesium stearate and talc; coating agents such as, hydroxyethylcellulose; and flavoring agents of the sort used for oral and mucosal formulations. For external agents would be used, in
20 conventional manner, solubilizers and auxiliary solubilizers capable of forming aqueous injections (e.g., distilled water for injection, physiological saline, and propylene glycol), suspending agents (e.g., surfactant such as polysorbate 80), pH regulators (e.g., organic acid and metal salt thereof) and stabilizers are used for injections; and aqueous or oily solubilizers and auxiliary solubilizers (e.g., alcohols
25 and fatty acid esters), tackifiers (e.g., carboxy vinyl polymer and polysaccharides) and emulsifiers.

Another aspect of the invention provides a method for detecting CRP/leptin complex-disruption activity in an agent, such as a polypeptide or variant as described above. In a preferred embodiment, the inventive methodology comprises (A) contacting the

agent with CRP and leptin, in any order; and then (B) measuring an indicator selected from the group of CRP/leptin complex formation, amount of leptin, and amount of CRP, in the absence and presence of the agent, respectively, such that the indicator evidences the CRP/leptin complex disruption activity.

- 5 Agents can be screened by various means. The screening can be performed by incubating leptin, concurrently or sequentially, with CRP and a putative agent, to be tested for complex-disruption activity. This may be effected by loading a leptin affinity column with a fixed amount of CRP, in the presence or absence of an agent with potential complex-disruption activity, and then identifying those that interact
10 with CRP in such a way that they preclude or diminish CRP/leptin complex formation. An agent with complex-disruption activity should increase the amount of CRP washed through the column, relative to an equivalent run in the absence of the agent.

- Alternatively, a potential active agent could be identified based on interaction with
15 CRP to inhibit or preclude CRP/leptin complex formation. This might involve first contacting CRP and the agent with potential complex-disruption activity to form CRP-agent complex, removing unbound agent, contacting the resulting CRP with leptin, measuring CRP-leptin complex formation, for example, by immunoprecipitation and radio-labeling, and comparing that value with CRP leptin
20 complex formation in the absence of the agent.

- An agent with complex-disruption activity also could be identified by screening for an ability to inhibit the signaling capabilities of leptin in cultured cells. For example, a potential agent can be co-incubated with leptin and CRP before the mixture is incubated with the cultured cells containing leptin receptors. Subsequently, leptin-
25 induced activity of STAT3 or PI3-kinase, *etc.* can be assayed. The results would be compared to those obtained in the absence of the agent. An active agent should revitalize the ability of leptin to signal in the cultured cells. In this context, the assay could involve monitoring any component of leptin-stimulated signaling pathways, such as JAK2, STAT3, STAT5, STAT6, SH2 domain-containing protein tyrosine

phosphatase (SHP-2), growth factor receptor binding-2 (Grb-2), extracellular-signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK), and PI-3 kinase.

Pursuant to the invention, screening for an agent with complex-disruption activity could be effected on a high-throughput basis. To this end, for example, the HEK293
5 cells over-expressing OB-Rb may be grown in a 96-well plate. The HEK293 cells also could carry a leptin-responsive reporter gene, such as a leptin-inducible promoter operatively linked to a luciferase reporter. The responsiveness of the cells to leptin exposure can induce phosphorylation of STAT3, which is detectable by fluorescence-labeled antibody or by an increase in luciferase activity in a fluorescent plate reader
10 described in previous publications. Pre-incubation of CRP with leptin would negate the effects of leptin, while a polypeptide with CRP/leptin complex-disruption activity would restore the effects of leptin.

The detailed description of the present invention continues by reference to the following examples, which are illustrative only and not limiting of the invention.

15 **Example 1. Purification and identification of serum leptin-interacting proteins**

A. Purification of SLIPs

Mouse or human recombinant leptin (from AF Parlow of NHPP, Torrance, CA) was covalently linked to Sepharose-beads with an Amino-Link kit (Pierce Biotechnology, Rockford, IL). Rat or human serum (1.5 ml) was loaded onto the affinity column,
20 allowed to pass through the resin, and the column was then washed with 15-volumes of PBS-0.5% Tween-20 (for rat samples), or a Ca^{2+} -containing buffer (0.1M Tris-Cl, 0.1M NaCl, 2mM CaCl_2) (for human serum samples). Retained material was eluted with an acidic glycine solution, and the eluate was immediately neutralized in a Tris-buffer (50mM, pH=9.5).

25 Five major SLIPs were identified from the elute of human leptin-affinity columns on a silver-stained SDS gel with apparent molecular weight of 30-, 42-, 65-, 70-, and 85-Kd, correspondingly designated as the human SLIP-1, 2, 3, 4, and 5. Serum leptin

could also be co-eluted with SLIPs. All five human SLIPs have rat counterparts since passage of rat serum through the mouse leptin-affinity column yielded proteins of very similar molecular weights.

B. Identification of SLIP-1

5 Rat SLIP-1 was excised from an SDS-PAGE gel and subjected to MALDI-TOF assay following tryptic digestion. This analysis identified rat SLIP-1 as rat C-reactive protein (CRP). Similarly, human SLIP-1 was also identified as human CRP in a mass-spec analysis (Nano-LC-MS/MS in-gel protein identification). The mass-spec assay was performed on Thermo Finnigan LTQ mass spectrometer by CTO-BIO
10 Services (Rockville, MD). Data analysis was carried out with the Turbo-Sequest software. A filtered score of > 2.33 for a doubly charged peptide ("z") indicates a significant match with human CRP. SLIP-1 is further confirmed as CRP with polyclonal antibodies that specifically recognize human or rat CRP in Western blot assays.

15 **Example 2. Demonstration of direct binding of CRP and leptin by immuno-precipitation assay**

To explore a physical interaction between CRP and leptin, the direct binding of these proteins was determined by an immuno-precipitation assay. Rat CRP was purified from fresh rat serum to $>95\%$ purity employing a previously established affinity-
20 purification protocol (20). This degree of purity was comparable to that of a human CRP preparation from a commercial source. The purity was further confirmed by mass spectrometry. The purified human- and rat-CRP proteins were pre-mixed with recombinant human and mouse leptin, respectively, before addition of antibodies specific for human- and mouse-leptin. The concentrations of CRP and leptin in the
25 precipitation reaction were all within the physiological ranges that have been observed in humans or rats (11 & 15). In parallel experiments, recombinant leptin also was pre-mixed with human- or rat-serum to allow for direct interaction prior to immuno-precipitation. The protein precipitates obtained were subjected to Western

blot assays using specific anti-CRP antibodies. Immuno-precipitation using anti-human leptin could pull down human CRP from both the leptin/CRP mixture and from human serum. Similarly, immuno-precipitation using anti mouse-leptin could bring down rat CRP from the leptin/CRP mixture as well as from rat serum. Direct
5 interaction of CRP and leptin is further confirmed, where immuno-precipitation was performed using anti human-CRP and rat CRP-antibodies and was found to pull down leptin proteins.

Example 3. Demonstration of CRP interference on leptin binding to its receptor

To examine if CRP binding interferes with the stability of human leptin to bind to its
10 receptors, a HEK293 cell line stably transfected with the long-form human leptin receptor, OB-Rb, was used (17). Human leptin was iodinated with Na¹²⁵I using the Iodogen method. Briefly, 15 µg of recombinant human leptin in 100 mM Phosphate buffer pH 7.5 were incubated with 1 mCi of carrier-free Na¹²⁵I (2200 Ci/mmol) in a glass tube containing 50 µg Iodogen. After 10-minute incubation at room
15 temperature, the reaction was stopped with 100 µl 0.1% trifluoroacetic acid (TFA). The reaction mixture was immediately purified by reverse-phase HPLC. The separation employed a 5-minute isocratic step at 20% eluant B in A, followed by two consecutive 30 min linear gradients from 20 to 50%, then from 50 to 60% eluant B in A (where eluant A is water containing 0.1% TFA and eluant B is acetonitrile
20 containing 0.1% TFA) at a flow rate of 1.5 ml/min. The ¹²⁵I-labeled leptin (~2ng/ml) was pre-incubated with various concentrations of human CRP in 1 ml of µ-MEM for 1 hour before added to the OBR-expressing HEK293 cells. The incubation would last for 3 hour at 4°C before the media was aspirated and the cells were dissolved in 0.1N NaOH for scintillation counting.

25 It was found that the K_d for leptin and its receptors on this cell line was 1.0x10⁻⁹ M. As shown in Figure 4A, pre-incubation of human CRP with ¹²⁵I-labeled human leptin reduced leptin's binding to its receptors in a dose-dependent manner. The IC₅₀ value was approximately 80 ng/ml of CRP in the presence of about 2 ng/ml human leptin, which yielded a molecular ratio of 5.8:1 (CRP:leptin, based on the pentameric

structure of CRP). On the other hand, human CRP displayed lower affinity towards mouse leptin (IC_{50} greater than 64 $\mu\text{g/ml}$ in the presence of about 2 ng/ml mouse leptin, as shown in Figure 4B).

Example 4. Signaling studies

5 To determine if the interaction between CRP and leptin dampens the cellular actions of leptin, the ability of leptin to stimulate tyrosine phosphorylation of STAT3 and PI3-kinase activity was assessed in the presence of CRP *in vitro*. HEK293 cells over-expressing OB-Rb were serum starved for 2 hours before addition of leptin and CRP (CHEMICON or EMD Biosciences). Leptin, in the presence or absence of CRP, was
10 pre-incubated in $\mu\text{-MEM}$ (with additional 1mM Ca^{2+}) for 30 minutes at 37°C before being applied to the cells. After a 30-minute incubation with leptin, the cells were harvested in lysis buffer (18). The resultant protein extract was subjected to a Western blot analysis using an anti-phospho-STAT3 specific antibody (Cell Signaling, Beverly, MA). For studies involving primary hypothalamic neurons, the
15 hypothalamus from rats was surgically isolated immediately after euthanasia, and placed in the DMEM for incubation with leptin in the presence or absence of CRP.

Both human CRP and rat CRP were found to attenuate the tyrosine-phosphorylation of STAT3 induced by human- or murine- leptin, respectively (Fig. 5A & 5B). The concentrations of human CRP and rat CRP required to block leptin-induced STAT3
20 phosphorylation are within the ranges observed in the human- and rat- plasma (11, 21 & 22), although higher amounts of rat- versus human-CRP were required to achieve equivalent effects. Consistent with the observation of low affinity between human CRP and mouse leptin, high concentrations of human CRP were unable to block mouse leptin-induced STAT3 activation (Fig. 5C).

25 Further studies revealed that the attenuation of leptin signaling by human CRP requires the presence of calcium ions, since addition of excess EGTA to the medium blocked the inhibitory effects of human CRP on leptin signaling.

More phosphor-STAT3 and PI3-kinase assays have demonstrated that human CRP specifically inhibited leptin signaling in primary hypothalamic neurons, since parallel assays show that human CRP, at an even higher concentration, did not suppress IL-6 induced activation of STAT3 in human primary hepatocytes. Similarly, rat CRP did not block insulin-stimulated PI3-kinase activity in 3T3-L1 adipocytes.

Human hepatocytes were isolated from donor livers. Briefly, the livers were perfused in a three-step process. A calcium-free buffer supplemented with EGTA is used to break intracellular junctions, which is followed by the same perfusion buffer without EGTA. Finally, a calcium-containing buffer supplemented with digestive enzymes (collagenase and DNase) was used. Following digestion, parenchymal hepatocytes were isolated by three steps of low-speed centrifugation. Cells with a viability of at least 85% were plated and cultured in hepatocytes maintenance medium (HMM) (Cambrex BioScience, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS), 0.1 μ M dexamethasone, 0.1 μ M insulin, and 50 μ g/ml gentamicin on collagen-coated tissue culture dishes for 2-4 hours. The cells were subsequently cultured in the above medium without FBS.

Approximately 24 hours after seeded, the hepatocytes were washed with serum-free HMM supplemented with 0.1 μ M dexamethasone and 0.1 μ M insulin, and then cultured in the same medium for another 48 hours. Subsequently, the cells were switched to serum-free HMM supplemented with 0.1 μ M dexamethasone before stimulated with leptin (NHPP) or rhIL-6 (R&D Systems, Minneapolis, MN) for 24 hours or 48 hours. For experiments involving PI3-kinase inhibitors, hepatocytes were pretreated with LY294002 (EMD Biosciences, CA) for 1 hour before hormonal stimulation.

In a separate control, it has been found that human serum albumin, even at concentrations of several hundred-fold higher than human CRP, had no appreciable effect on leptin-induced STAT3 phosphorylation. Similarly, human serum amyloid P component (SAP), circulating at much higher concentrations than CRP, also did not influence leptin signaling when co-incubated in cultured cells.

Example 5. Assessment of CRP functions and monitoring physiological indicators

The functional effects of human CRP were evaluated to see whether elevated circulating CRP via infusion would be able to negate or attenuate the suppression of food intake and loss of weight that are normally evoked by leptin. *Ob/ob* mouse, in lieu of rat model, was adopted in the experiments due to its minimum basal plasma level of CRP (23)

Micro osmotic pumps (#1007D, DURECT, Cupertino, CA) were subcutaneously implanted into *ob/ob* or wild-type mice according to the instructions of the manufacturer. The osmotic pumps were pre-filled with saline, CRP, leptin or leptin plus CRP. Food intake (24-hour), body weight, and body temperature were monitored daily post-surgery. Blood glucose concentrations were measured using tail-vein blood samples using a Precision Plus® glucose meter, product of Medisense (Abbot Park, IL).

Human- and mouse- leptin concentrations were measured using ELISA kits from CHEMICON (Temecular, CA) and R&D Systems (Minneapolis, MN), respectively, accordingly to the instructions of the manufacturers. Serum mouse insulin and human CRP were determined with ELISA kits from LINCO Research (St. Charles, MO) and HELICA (Fullerton, CA), respectively. Serum- and tissue- triglycerides were determined with the protocols described previously (19).

Various amounts of human CRP, either alone or with human leptin, were administered via micro-osmotic pumps implanted *s.c.* into 8-week old *ob/ob* mice. With an ELISA assay that can detect both free and bound forms of leptin, the serum leptin concentrations were found to be almost identical in the leptin- and leptin/CRP-infused animals. During the 6-day continuous infusion period, human leptin produced the expected reduction in food-intake and body weight in the *ob/ob* mice, as shown in Fig. 6A-6D. Although co-infusion of human CRP at a low infusion dosage at 10 µg/day only partially attenuated these effects of human leptin, at a higher

concentration of 40 µg/day, human CRP was able to completely block the actions of leptin to restrain appetite and induce weight loss.

Consistent with these observations, there was also attenuation of leptin-induced energy expenditure as gauged by recordings of body temperature. Human CRP, at the
5 high infusion dosage of 40 µg/day, completely blocked the increase of rectal temperature induced by leptin.

Importantly, the serum concentrations of human CRP attained in these mice are similar to the physiological range observed in human plasma from ostensibly healthy donors.

10 Although leptin administration to *ob/ob* mice alleviated diabetes, including the lowering of blood glucose, plasma insulin, and serum- and hepatic triglyceride, co-infusion of human CRP has reverted these effects. As a further confirmation of the negative effects of CRP on leptin actions, activation of STAT3 in the hypothalamic tissues of *ob/ob* mice was also inhibited by co-infusion of CRP with leptin. By
15 contrast, administration of human CRP alone did not affect food intake or body weight. Thus, the impact of CRP is dependent upon the presence of leptin.

The above *in vivo* data were harvested from mice experiencing infusions of a mixture of CRP and leptin stored in a single mini-osmotic pump. Human CRP and leptin were also infused in separate osmotic pumps. The total human leptin concentrations in the
20 sera of all groups infused with leptin (leptin only or leptin plus CRP) were very comparable to each other (15-20 ng/ml). For reasons not yet clear, with the separate pump approach, higher infusion dosages of human CRP were required to achieve serum concentrations of human CRP in *ob/ob* mice comparable to those realized with the single pump approach. Also compared to infusion from a single pump, detectable
25 effects of human CRP on leptin were realized later, likely due to the time required to achieve diffusion and binding equilibrium. Nevertheless, at serum concentrations that matched those achieved with a single osmotic pump, human CRP was again found to attenuate the physiological actions of human leptin, including reductions of food intake and body weight as well as correction of blood glucose and serum insulin.

To further complement the in vivo studies, the satiety and weight-reducing functions of human leptin were evaluated in mice producing transgenically expressed human CRP (24). The average baseline serum concentration of human CRP in these mice was approximately 15 µg/ml. Despite such high basal levels, human CRP alone did not meaningfully affect the food intake and body weight of the transgenic mice, consistent with the low affinity of human CRP towards mouse leptin. To evaluate the effects of human leptin in the transgenic mice, a dose of 0.6 mg/kg/day, which has been known to maximally but only temporarily affected energy balance in wild-type mice (25), was infused. Infusion of human leptin into wild-type littermates of CRP transgenics produced the expected reductions in food intake, body weight, and epididymal fat-pad weight. Nevertheless, these physiological effects of human leptin were completely blunted in the CRP-transgenic mice. At the end of infusion, the total human leptin concentration in the sera of transgenic mice averaged at 7.6 ng/ml, which was higher than that in the wild-type littermates at 3.2 ng/ml. These results indicate a negative effect of human CRP on the physiological actions of leptin.

Example 6. Assessment of leptin's effect on CRP production

In order to test if leptin itself might stimulate the expression of CRP in hepatocytes, CRP secretion and expression were analyzed.

Hepatocytes were isolated and treated as described in Example 4. Culture medium was centrifuged to remove detached cells and then heated at 95°C for 5 minutes in SDS-sample buffer (Boston Bioproducts, MA). After separation on 10% SDS polyacrylamide gels, proteins were electro-transferred overnight to nitrocellulose membranes. Pre-blocked using 5% non-fat milk in PBS with 0.5% Tween 20, the membranes were incubated with appropriate primary antibody for 2 hours at room temperature, washed and incubated for 1 hour with a corresponding peroxidase-conjugated anti-CRP antibody at room temperature. To detect proteins, the membranes were incubated with ECL reagent and the blots were exposed to X-Omat film (Kodak). Two kinds of CRP antibodies were used: anti-human CRP (EMD Biosciences, CA) and anti-rat CRP (Alpha Diagnostic Intl., San Antonio, TX).

CRP mRNA expression was measured by quantitative real-time RT-PCR. In brief, total hepatocyte RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA), treated with DNase I (Ambion, Austin, TX), and reverse-transcribed using a Superscript kit (Invitrogen, Carlsbad, CA). The real-time PCR reaction was
 5 performed with a Taqman machine (ABI7700) as follows: 50°C, 2 minutes, 1 cycle; 95°C, 10 minutes, 1 cycle; 95°C, 15 seconds, 60°C, 1 minute, 40 cycles. The sequences of probes and primers are as follows:

Human CRP probe: 5'-/6 FAM/TGCAAGGCGAAGTGTTACCAAACC/BHQ_A/-
 3' (SEQ ID NO: 6); Human CRP 5' primer: 5'-GGCGGGCACTGAAGTATGAA-
 10 3' (SEQ ID NO: 7); Human CRP 3' primer: 5'-GCCTCAGGGCCACAGCT-3'
 (SEQ ID NO: 8); Human 18s rRNA probe: 5'-/6-
 FAM/CGCGCAAATTACCCACTCCCGA/BHQ_A/-3' (SEQ ID NO: 9); Human
 18s rRNA 5' primer: 5'-ACATCCAAGGAAGGCAGCAG-3' (SEQ ID NO: 10);
 Human 18s rRNA 3' primer: 5'-TCGTCACCTACCTCCCCGG-3' (SEQ ID NO: 11).
 15 Rat 18s rRNA probe: 5'-/6-FAM/CGCGCAAATTACCCACTCCCGA/BHQ_A/-3'
 (SEQ ID NO: 12); Rat 18s rRNA 5' primer: 5'-GCACGAGGCGAGAAAGGA-3'
 (SEQ ID NO: 13); Rat 18s rRNA 3' primer: 5'-TTCGTCACCTACCTCCCCGG-3'
 (SEQ ID NO: 14); Rat CRP probe: 5'-/6-
 FAM/CCTTCTTGGGACTGATGCTGGTGACA/BHQ_A/-3' (SEQ ID NO: 15); Rat
 20 CRP 5' primer: 5'-TGTGCCACCTGGGAGTCTG-3' (SEQ ID NO: 16); Rat CRP 3'
 primer: 5'-TTCCGCACCCTGGGTTT-3' (SEQ ID NO: 17).

Although a short treatment of 6-8 hours did not influence CRP gene expression, incubation of human hepatocytes with human leptin at physiological concentrations for 24 hours produced a dose-dependent positive effect on secreted CRP in the culture
 25 medium (Fig. 7). A parallel examination of CRP gene expression, using real-time PCR assay, also showed a dosage-dependent effect on human CRP mRNA expression. Pre-incubation of human primary hepatocytes with a specific PI3-kinase inhibitor, LY294002, completely blocked the effect of leptin on CRP expression, suggesting that leptin-induced hepatic production of CRP is a PI3-kinase dependent
 30 process.

Example 7. Assessment of polypeptides

To determine the domains that are relevant to the interaction between human leptin and human C-reactive protein, a series of polypeptides (average length of 25 amino acids) were chemically synthesized. Polypeptide B (SEQ ID NO: 3), polypeptide C1
5 (SEQ ID NO: 4) and polypeptide E (SEQ ID NO: 5) are representatives of such peptides.

In the *in vitro* experiment, each of these polypeptide was pre-incubated with human CRP for 30 minutes before being mixed with human leptin for an additional 45 minutes. The resultant mixture was then added to the HEK-293 cells over-expressing
10 the long form leptin receptors, OB-Rb. After a 30-minute incubation, the cells were harvested in a protein extraction buffer (18). The signaling of leptin in the cells, measured by its ability to stimulate tyrosine phosphorylation on STAT3 in a western blot assay, was evaluated (see Figures 8, 9, 10 for polypeptides B, C1 and E, respectively). Human CRP suppressed the activation of STAT3 by leptin, but pre-
15 incubation of each of the polypeptide with human CRP was able to block the inhibitory effect of human CRP and to restore leptin signaling. Each of the polypeptide by itself did not induce any changes in STAT phosphorylation. It was also observed that polypeptide C1 is more effective at a lower concentration in blocking the CRP activity on leptin.

20 In the *in vivo* experiment, polypeptide E was co-infused through mini-osmotic pumps with human CRP and leptin into the *ob/ob* mice. Food intake in the period of 24 hours and body weight were followed during the infusion period (Figures 11A-11D). Although in the absence of the polypeptide, human CRP was able to block the satiety and weight-reducing effects of human leptin, co-fusion of polypeptide E with human
25 CRP and leptin was able to restore both of these functions of human leptin. At the end of infusion, it was found that polypeptide E was able to restore the beneficial effect of human leptin on blood glucose (Figure 11E).

Example 8. Comparison of polypeptides with the hexamer of U.S. Patent No. 6,777,388

Pursuant to an *in vitro* protocol similar to that described in Example 6, a heximer peptide with a sequence of NH₂-S-C-H-L-P-W-COOH (SEQ ID NO:18), derived
5 from a peptide disclosed in U.S. patent No. 6,777,388, was pre-incubated with human CRP (8 µg/ml) and human leptin (2 nM) for 1 hour. The resultant mixture then was added to HEK-293 cells that over-expressed the long form leptin receptor, OB-Rb. After a 30-minute incubation, the cells were harvested in a protein extraction buffer. To evaluate signaling of human leptin in the cells, tyrosine phosphorylation on
10 STAT3 was measured in a western blot assay.

The results (Figure 12) show that the hexamer of the '388 patent, at concentrations as high as 0.8 µM (or 800 nM), was unable to block the negative effect of human CRP on the signaling capability of human leptin. In the same set of experiments, polypeptide E was able to restore the signaling capability of human leptin (figure 10).

15

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WHAT IS CLAIMED IS:

1. A polypeptide comprising a segment of 8-40 amino acid residues that are present, as a contiguous sequence, in the human leptin protein sequence (SEQ ID NO: 1) exclusive of a core consisting of α -helices B, C and D, wherein (i) said polypeptide disrupts formation of CRP/leptin complex and (ii) the amino acid sequence of said polypeptide is exclusive of SEQ ID NO: 2.
2. The polypeptide according to claim 1, wherein said segment is present in an exposed surface region of said human leptin protein.
3. The polypeptide according to claim 1, comprising a segment of 15-25 amino acid residues.
4. The polypeptide according to claim 2, comprising:
 - (A) the amino acid sequence of SEQ ID NO: 3; or
 - (B) the amino acid sequence of SEQ ID NO: 3, with at least one modification that is (i) a conservative amino acid substitution, insertion, or deletion, (ii) a C-terminal truncation, or (iii) a N-terminal truncation, wherein the polypeptide is at least 60% identical to the amino acid sequence of SEQ ID NO: 3.
5. The polypeptide according to claim 2, comprising:
 - (A) the amino acid sequence of SEQ ID NO: 4; or
 - (B) the amino acid sequence of SEQ ID NO: 4, with at least one modification that is (i) a conservative amino acid substitution, insertion, or deletion, (ii) a C-terminal truncation, or (iii) a N-terminal truncation, wherein the polypeptide is at least 60% identical to the amino acid sequence of SEQ ID NO: 4.
6. The polypeptide according to claim 2, comprising:
 - (A) the amino acid sequence of SEQ ID NO: 5; or
 - (B) the amino acid sequence of SEQ ID NO: 5, with at least one modification that is (i) a conservative amino acid substitution, insertion, or deletion,

(ii) a C-terminal truncation, or (iii) a N-terminal truncation, wherein the polypeptide is at least 60% identical to the amino acid sequence of SEQ ID NO: 5.

7. A pharmaceutical composition comprising (i) the polypeptide of claim 1 or the nucleic acid encoding said polypeptide and (ii) a pharmaceutically acceptable carrier therefor.

8. A method for detecting CRP/leptin complex disruption activity in an agent, comprising:

(A) contacting said agent with CRP and leptin, in any order; and then

(B) measuring an indicator selected from the group of CRP/leptin complex formation, amount of leptin, and amount of CRP, in the absence and presence of said agent, respectively,

wherein said indicator evidences said CRP/leptin complex disruption activity.

9. The method according to claim 8, wherein step (B) comprising loading a leptin affinity column with said CRP in the absence and presence of said agent, respectively, and then measuring the amount of CRP washed through.

10. The method according to claim 8, wherein step (B) comprising measuring leptin activity by STAT3 and/or PI3-kinase assay.

11. A method for normalizing the level of free leptin in a subject, comprising administering to said subject a pharmacologically effective amount of a pharmaceutical composition of claim 7.

12. A method according to claim 11, wherein said subject suffers from or is at risk of a condition selected from the group consisting of obesity and type II diabetes.

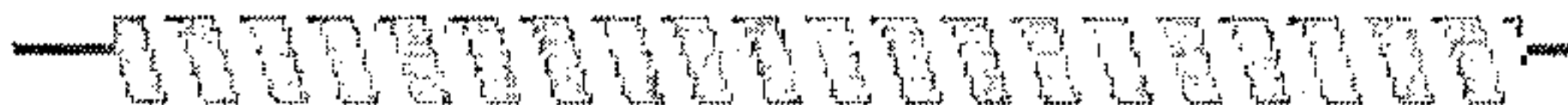
13. A method according to claim 11, wherein said subject suffers from or is at risk of a condition selected from the group consisting metabolic syndromes, inflammation, atherosclerosis, and infertility.

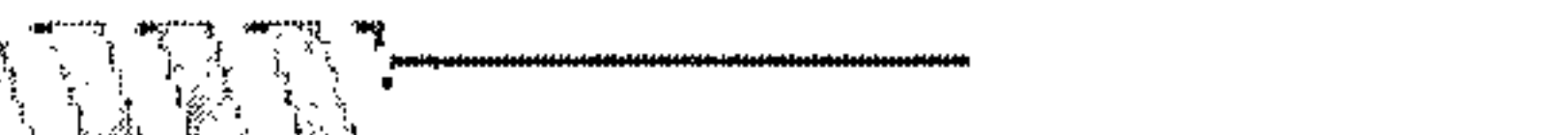
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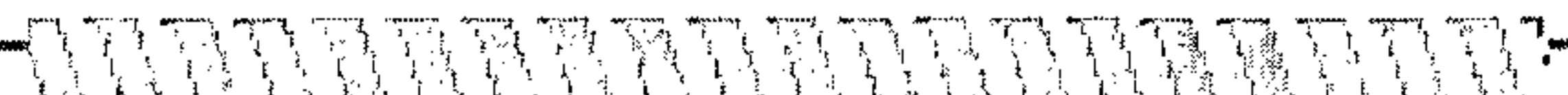
Figure 1

1	MHWGTL	CGFL	WLWPYLFYVQ	AVPIQKVQDD	TKT	LIKTI	VT	40
41	RINDISHTQS	VSSKQKVTGL	DFIPGLHPIL	TL	SKMDQ	TLA	80	
81	VYQQILT	SMP	SRNVIQISND	LENLRDLLHV	LAF	SKSCHLP	120	
121	WASGLE	TLD	SLGGVLEASGY	STEVVALSRL	QGS	LQDMLWQ	160	
161	LDL	SPGC						

Figure 2A

——
--IQKVQDDTKT**LIK**TIVTRINDI-----LDFIPGLHPILT
| | | | |
22 30 40 50 60 70

———
LSKMDQTLAVYQQILTSMPSRNVIQISNDLENLRDLLHVLA**F**SKSCHLPW
| | | | |
80 90 100 110 120

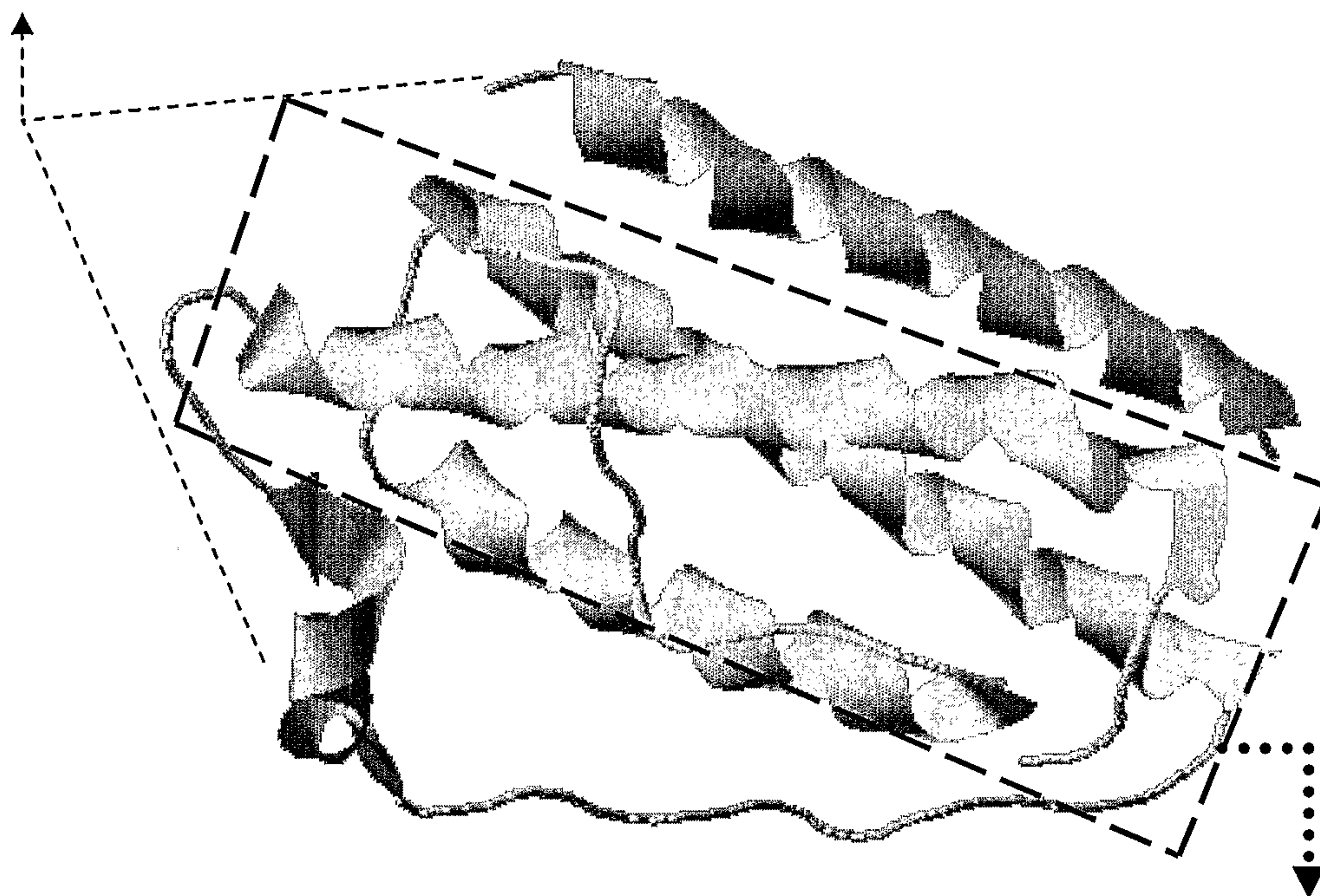
———
ASGLETLDSLGGVLEASGYSTEVVALSRLQGS**L**QDMLWQ**LDL**SPGC
| | | |
130 140 150 160

Human LDLSPGC
 Mouse ****V******E***

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Figure 2C

exposed region



core region

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Figure 3A

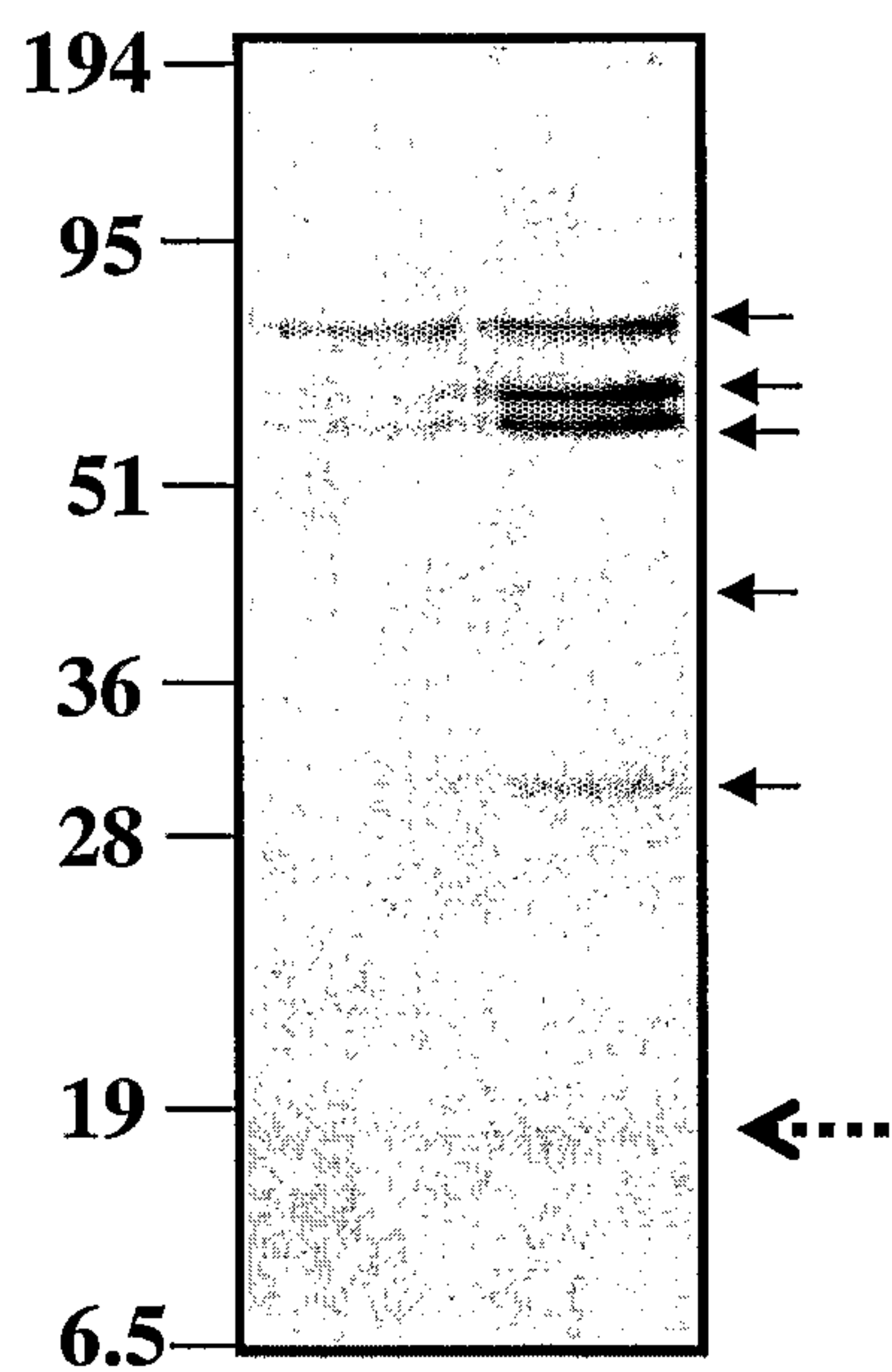


Figure 3B

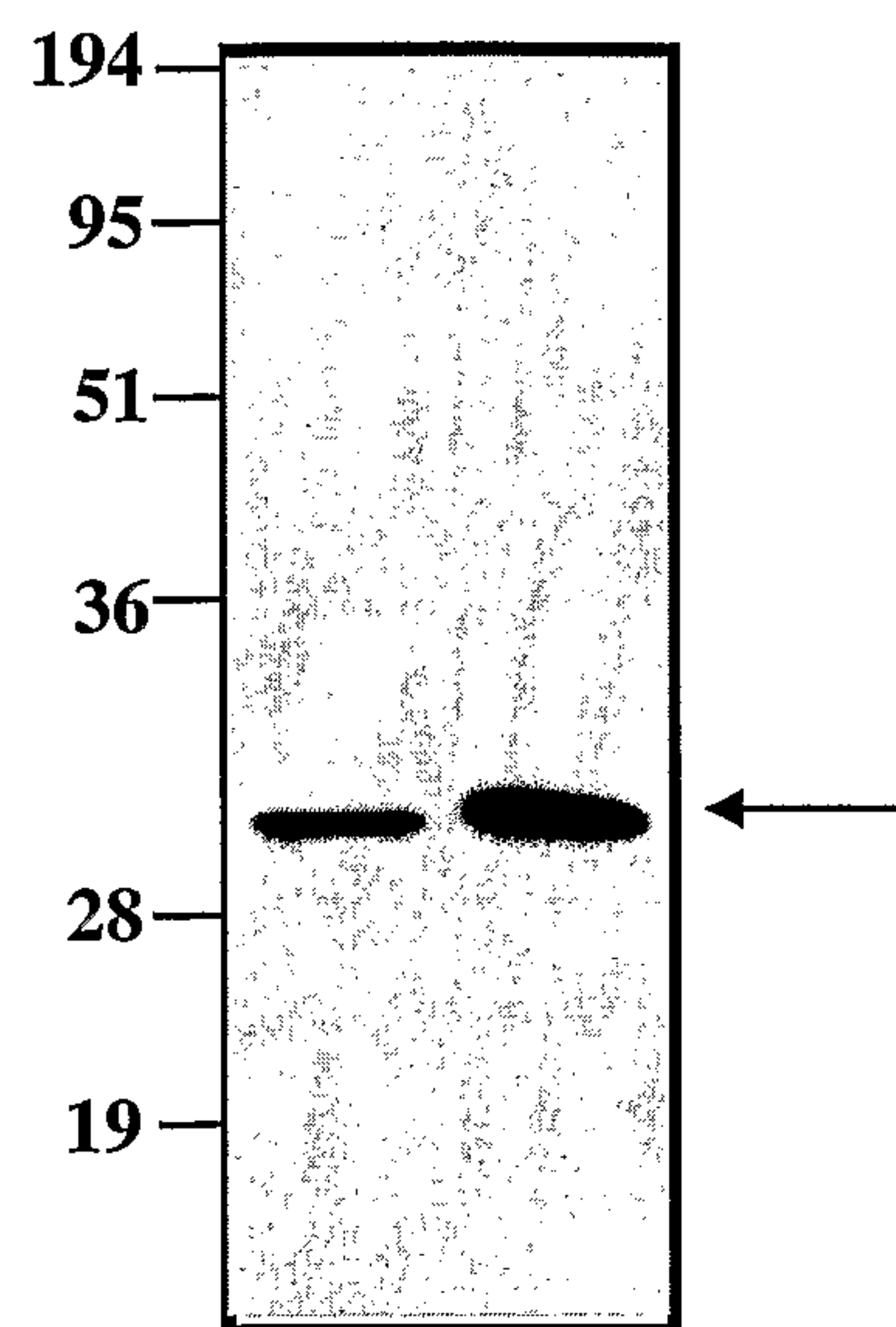


Figure 4A

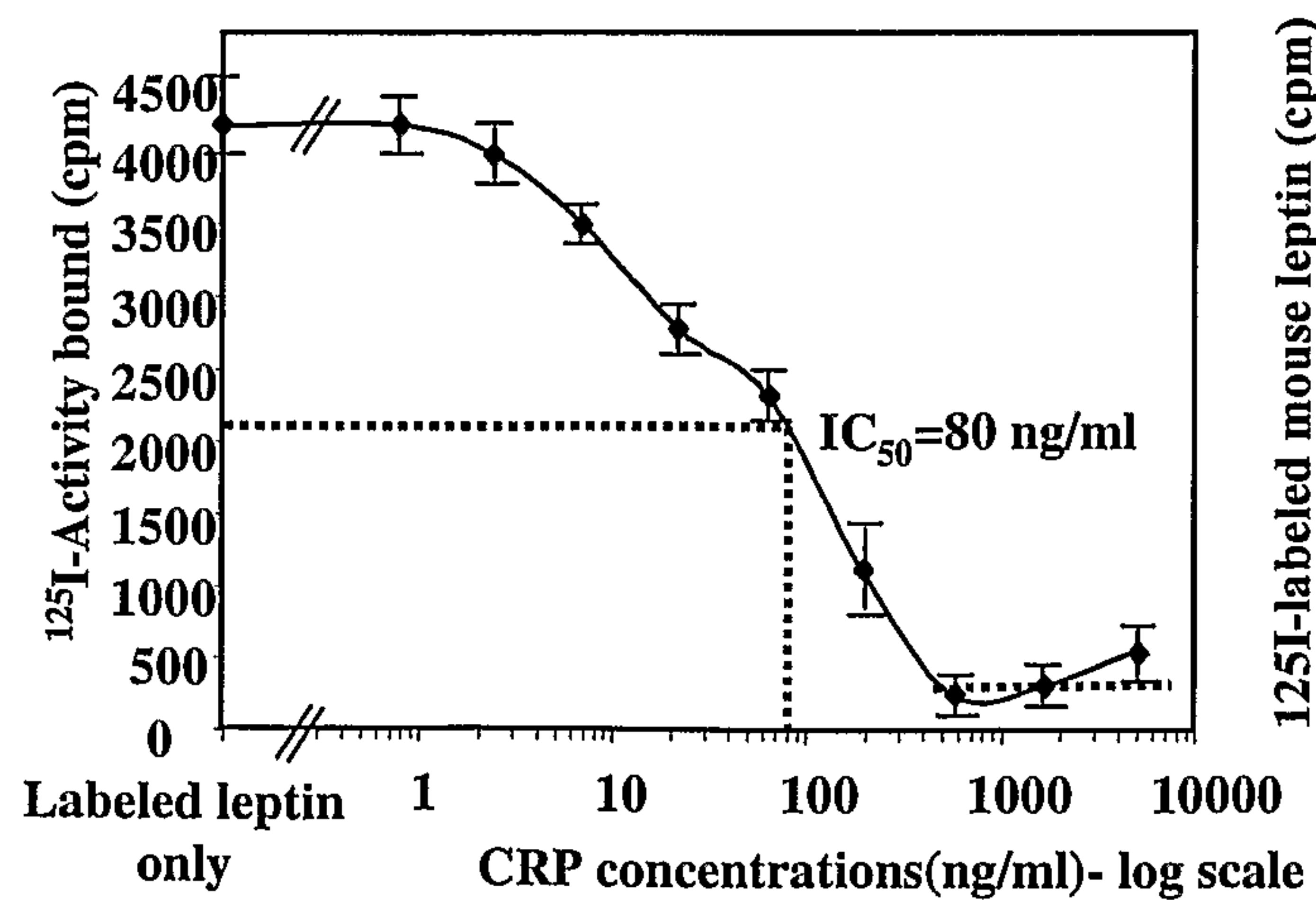
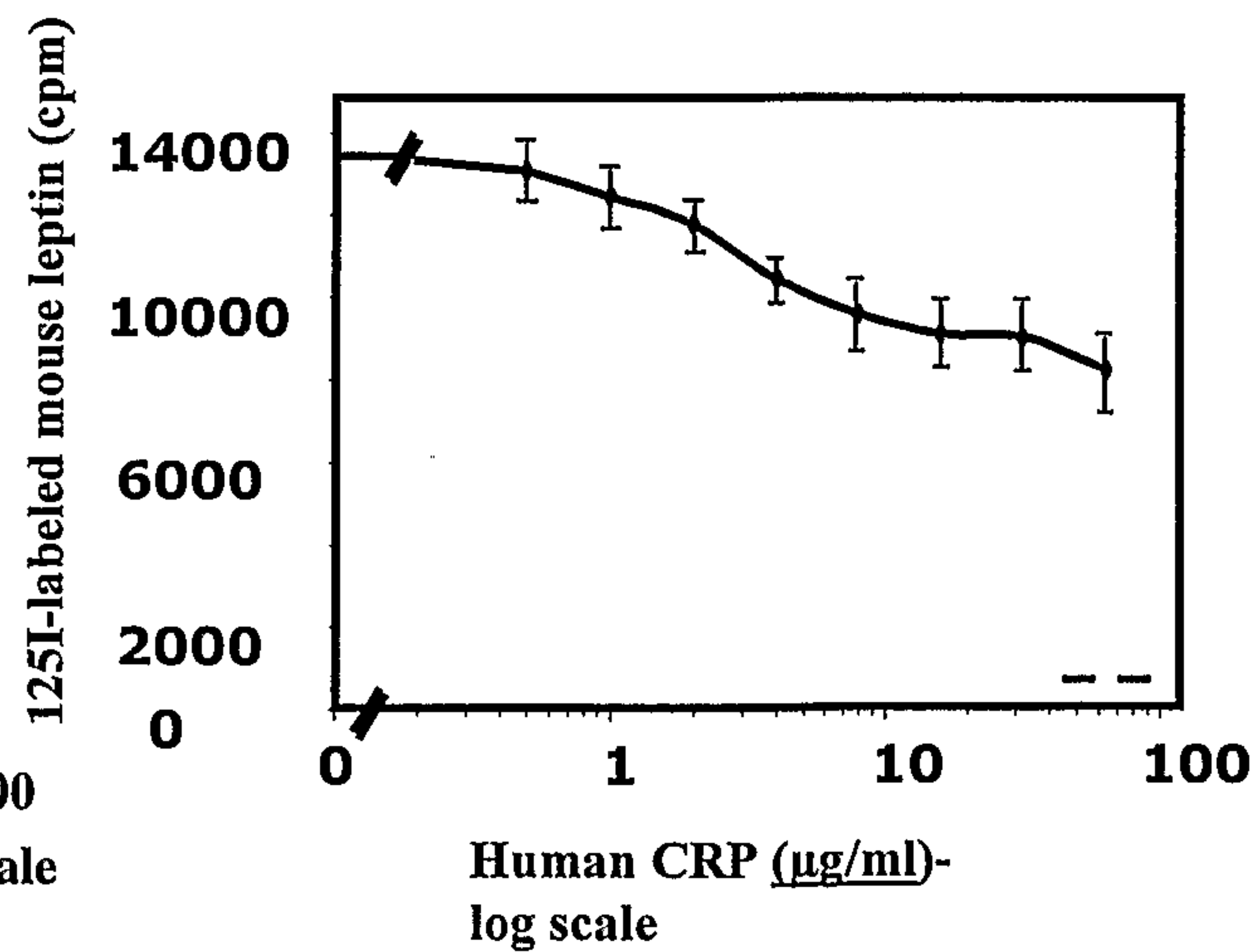


Figure 4B



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Figure 5A

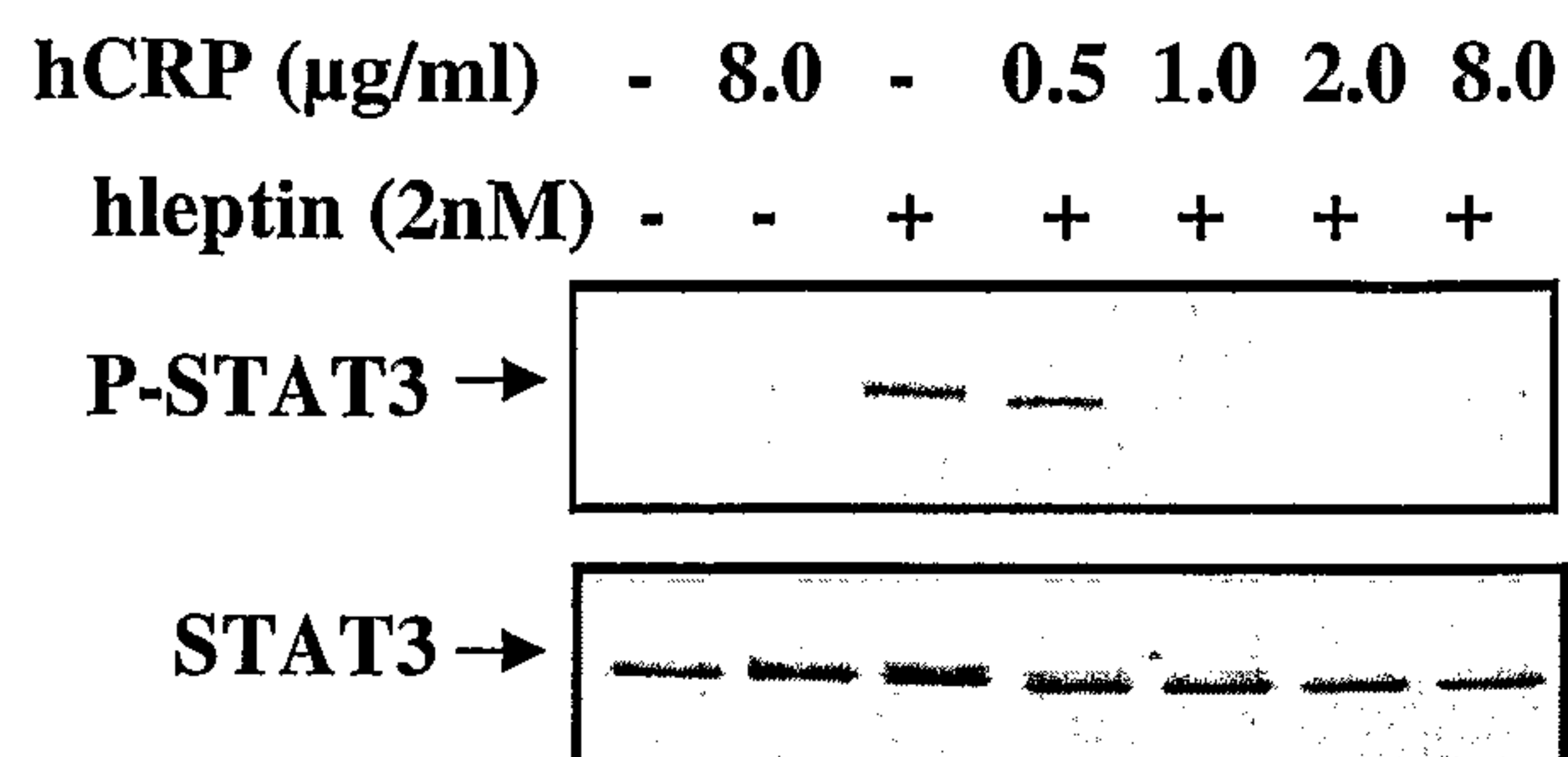


Figure 5B

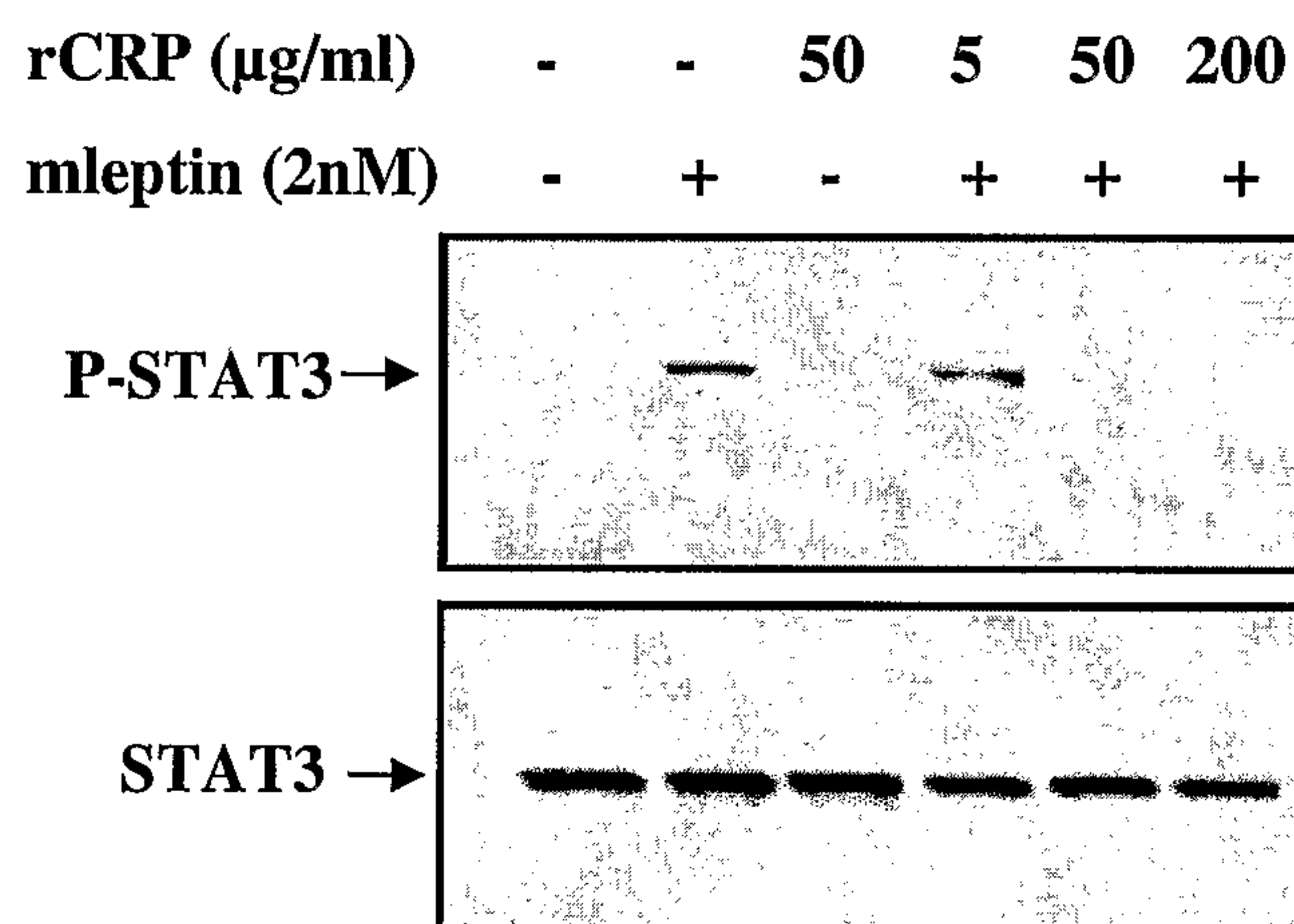
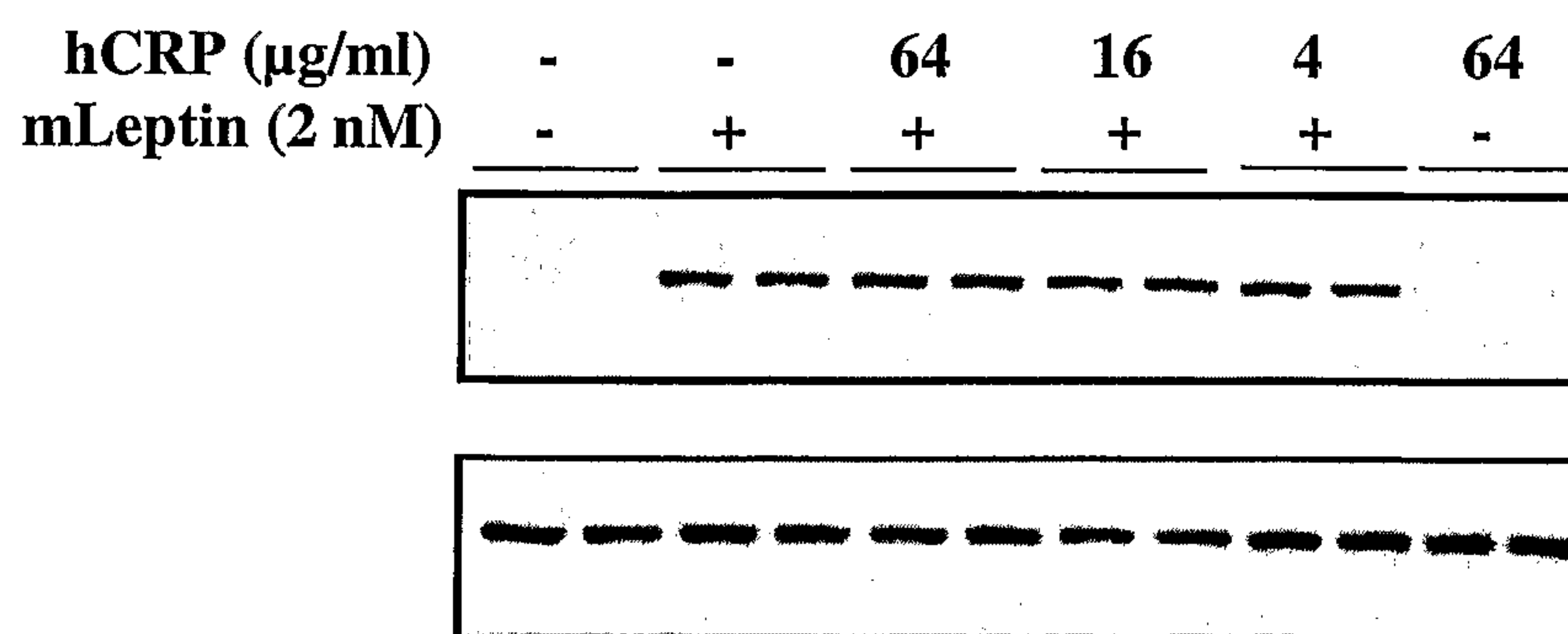


Figure 5C



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Figure 5D

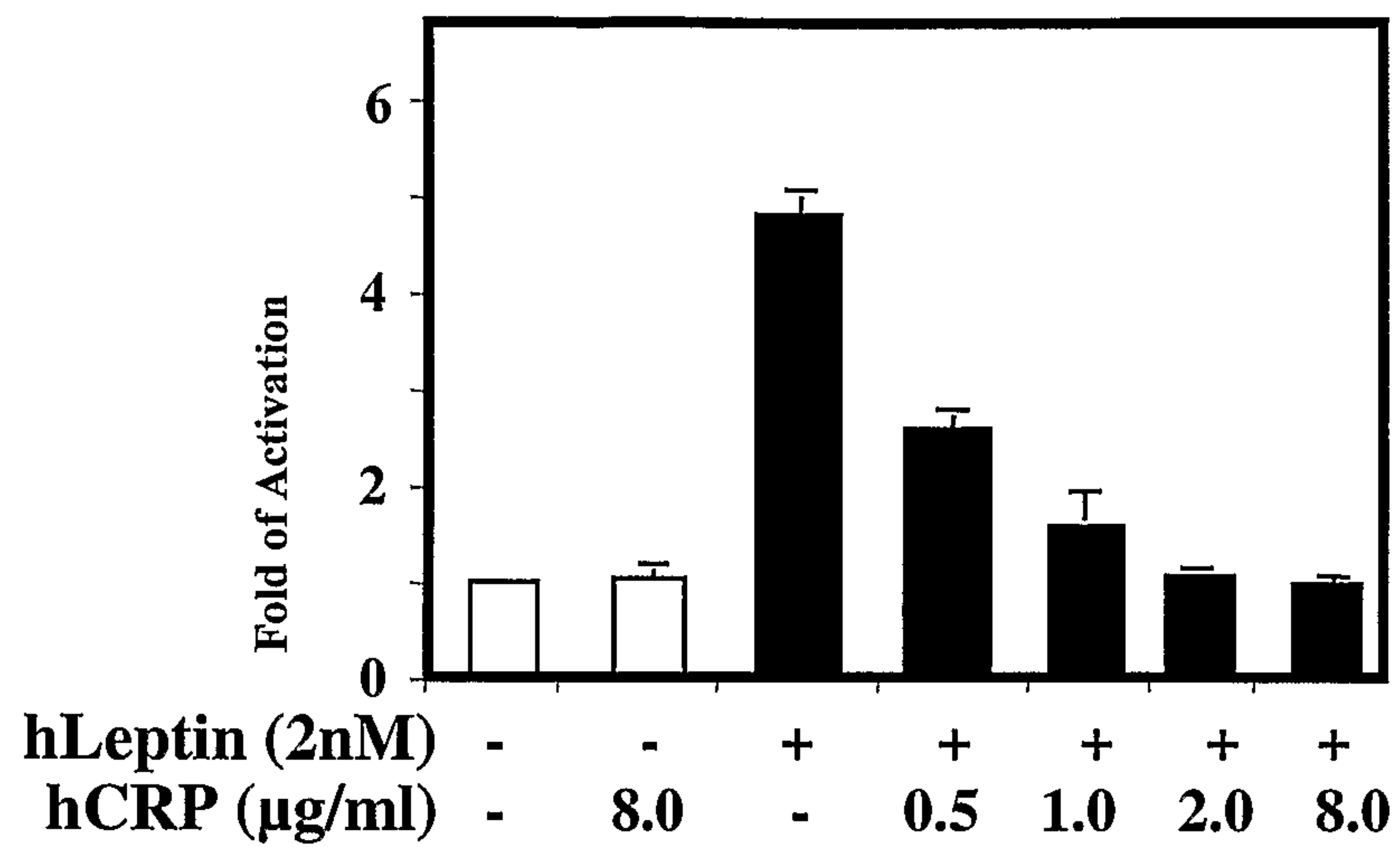
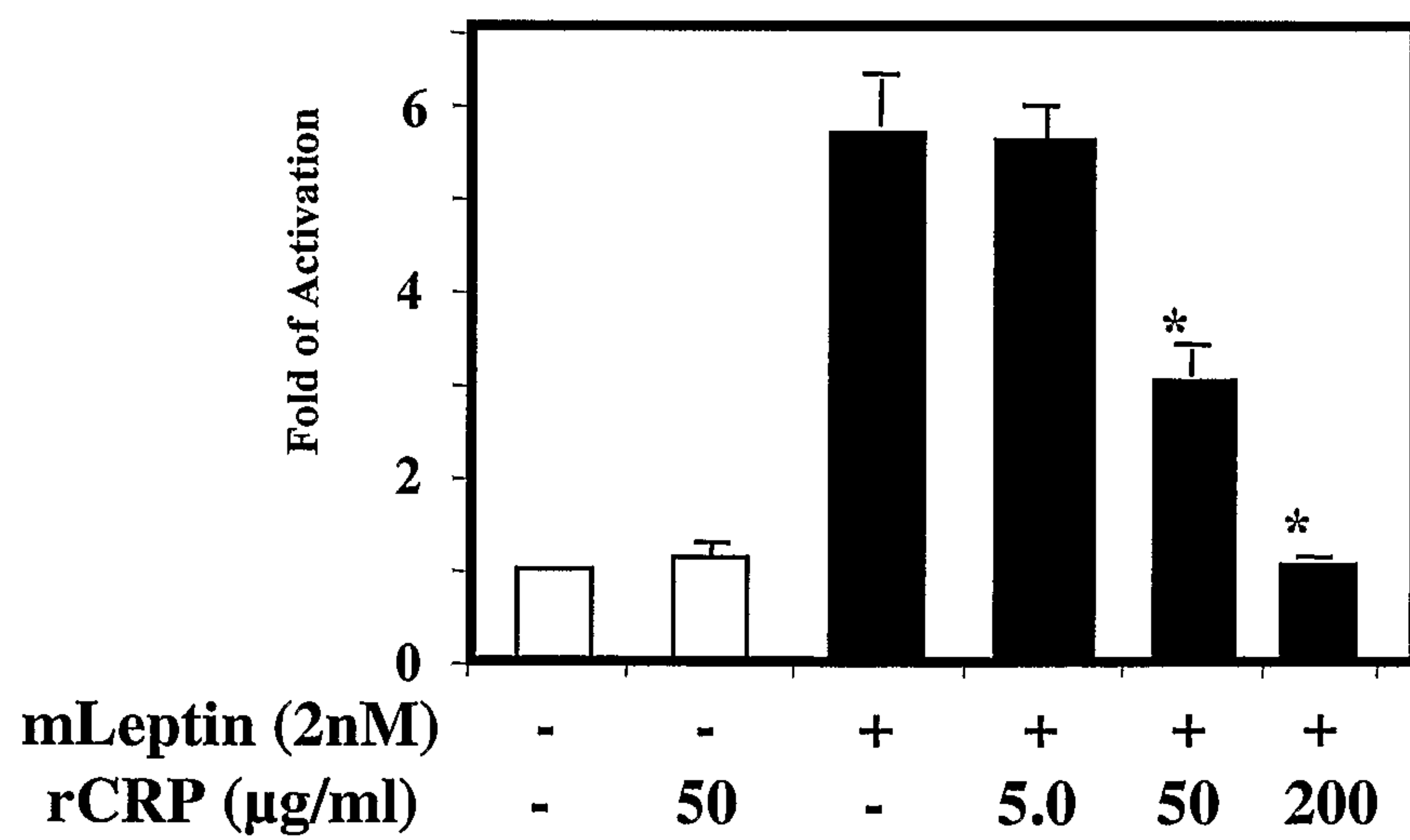


Figure 5E



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Figure 6A

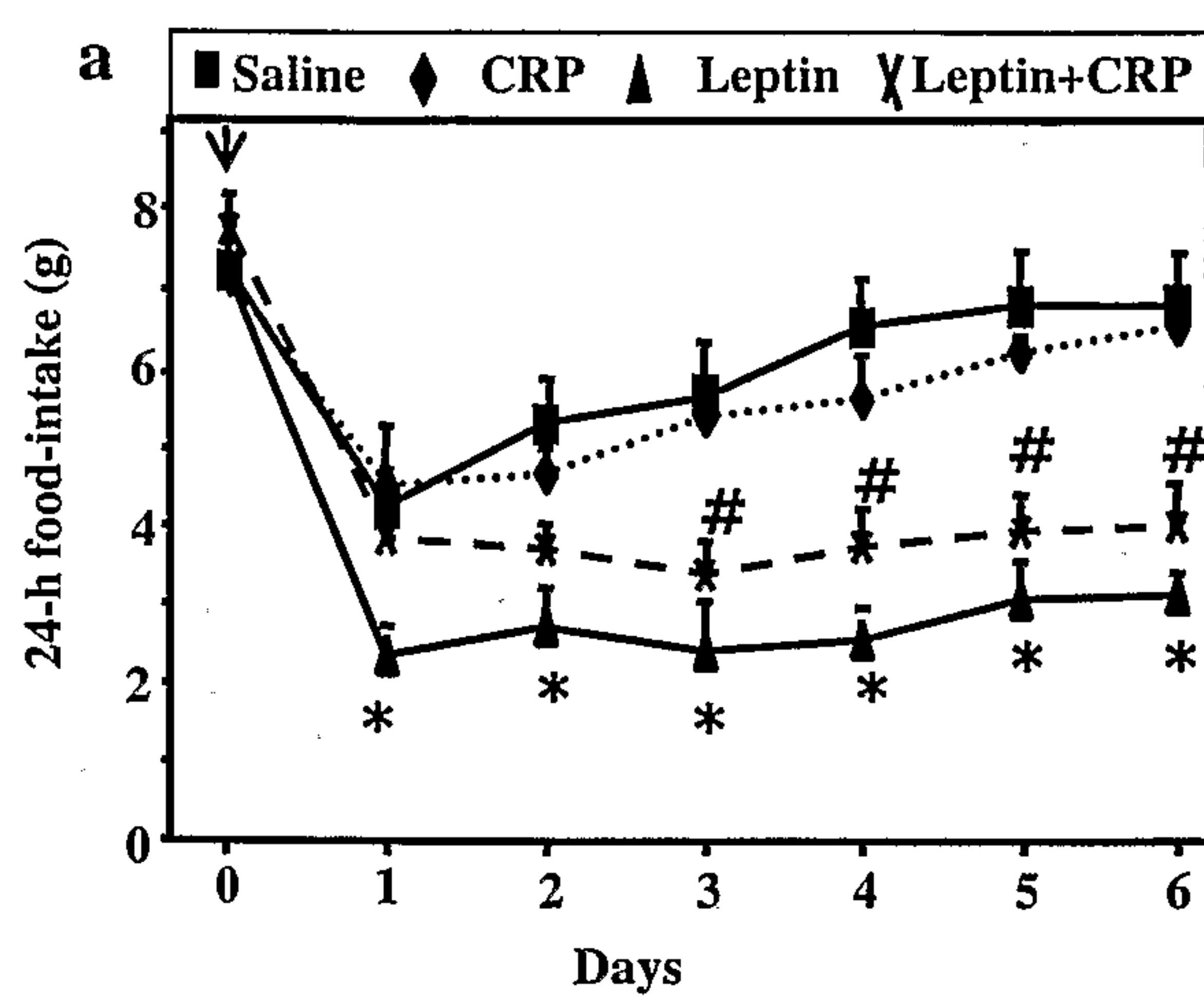


Figure 6B

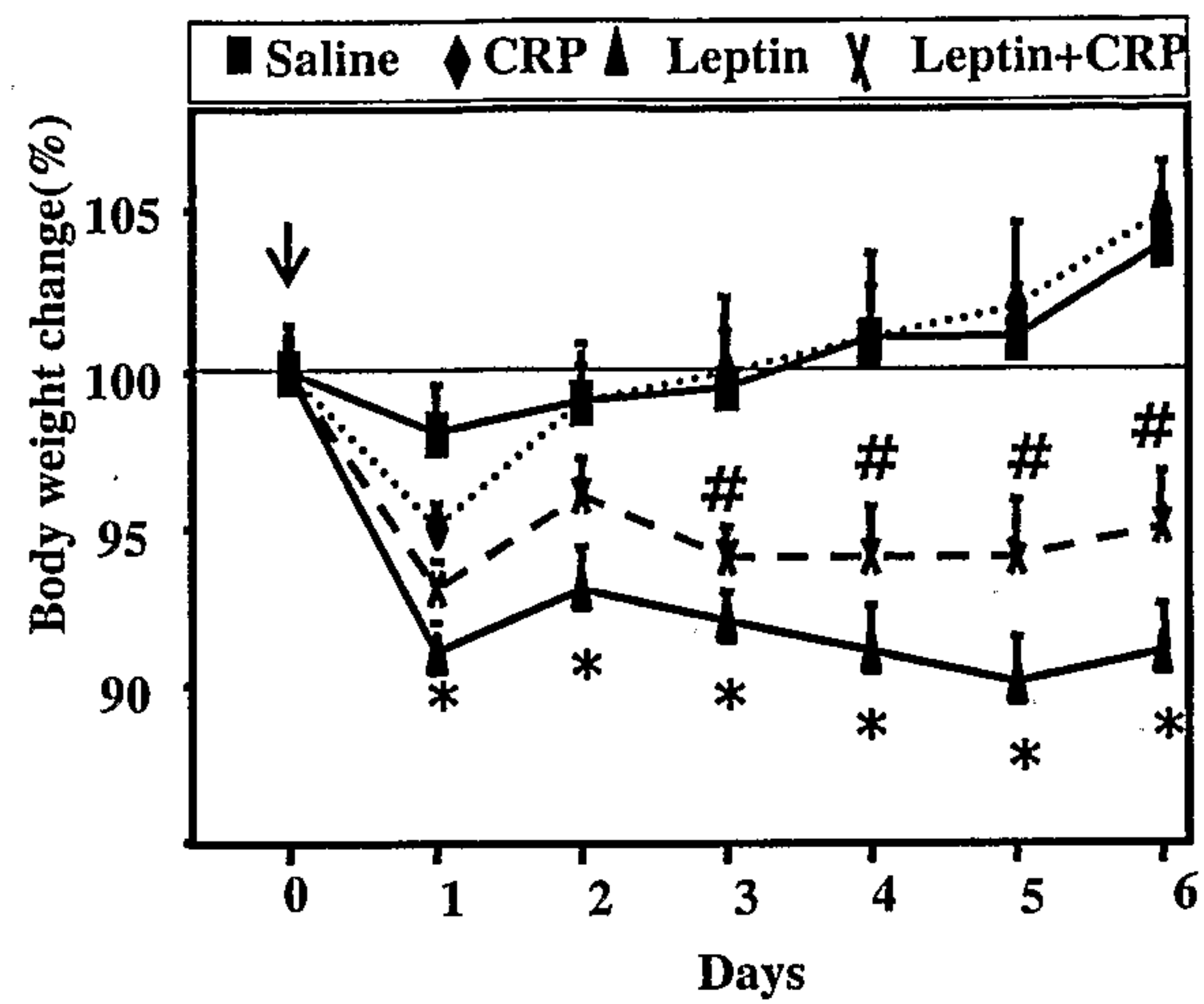


Figure 6C

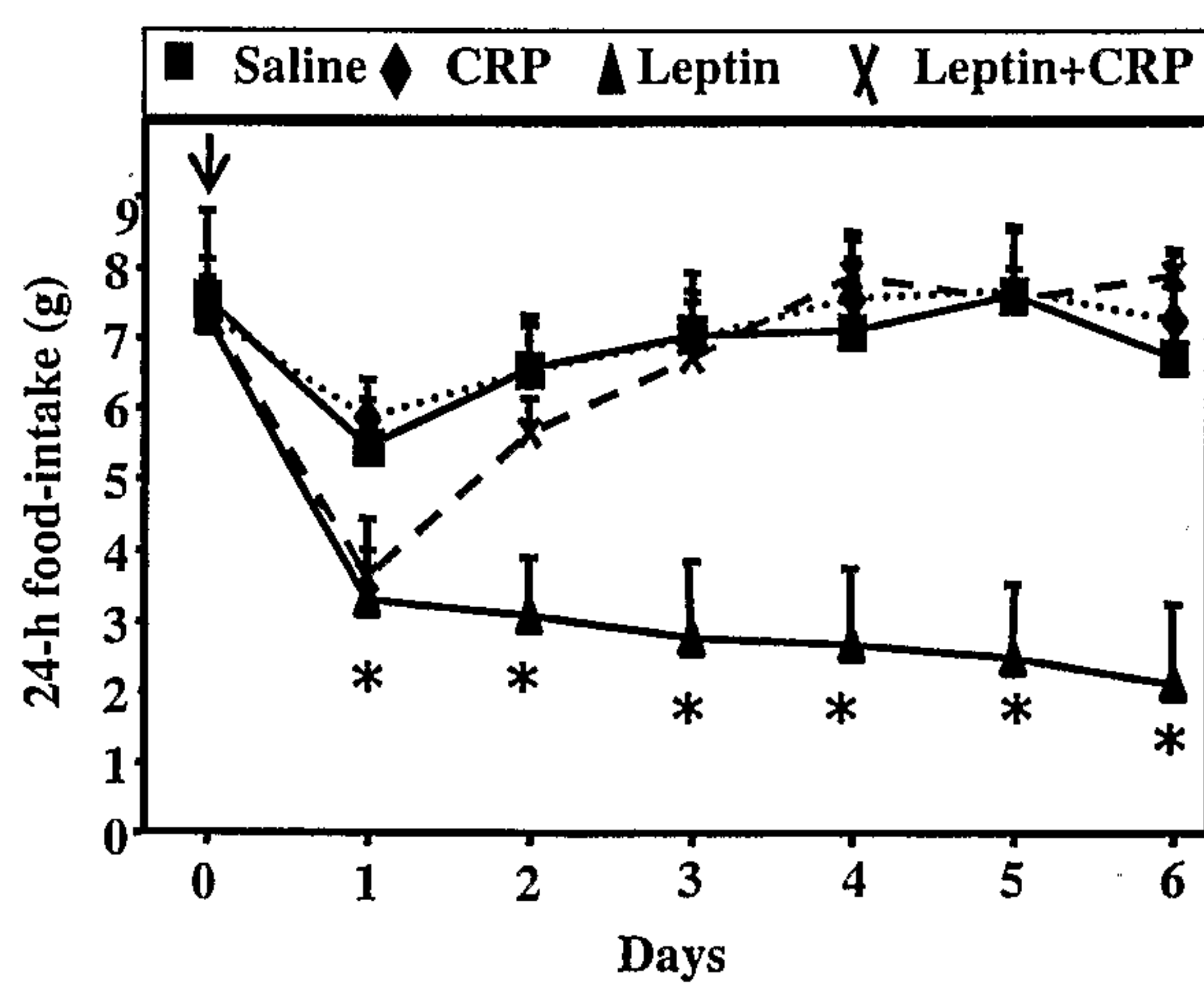
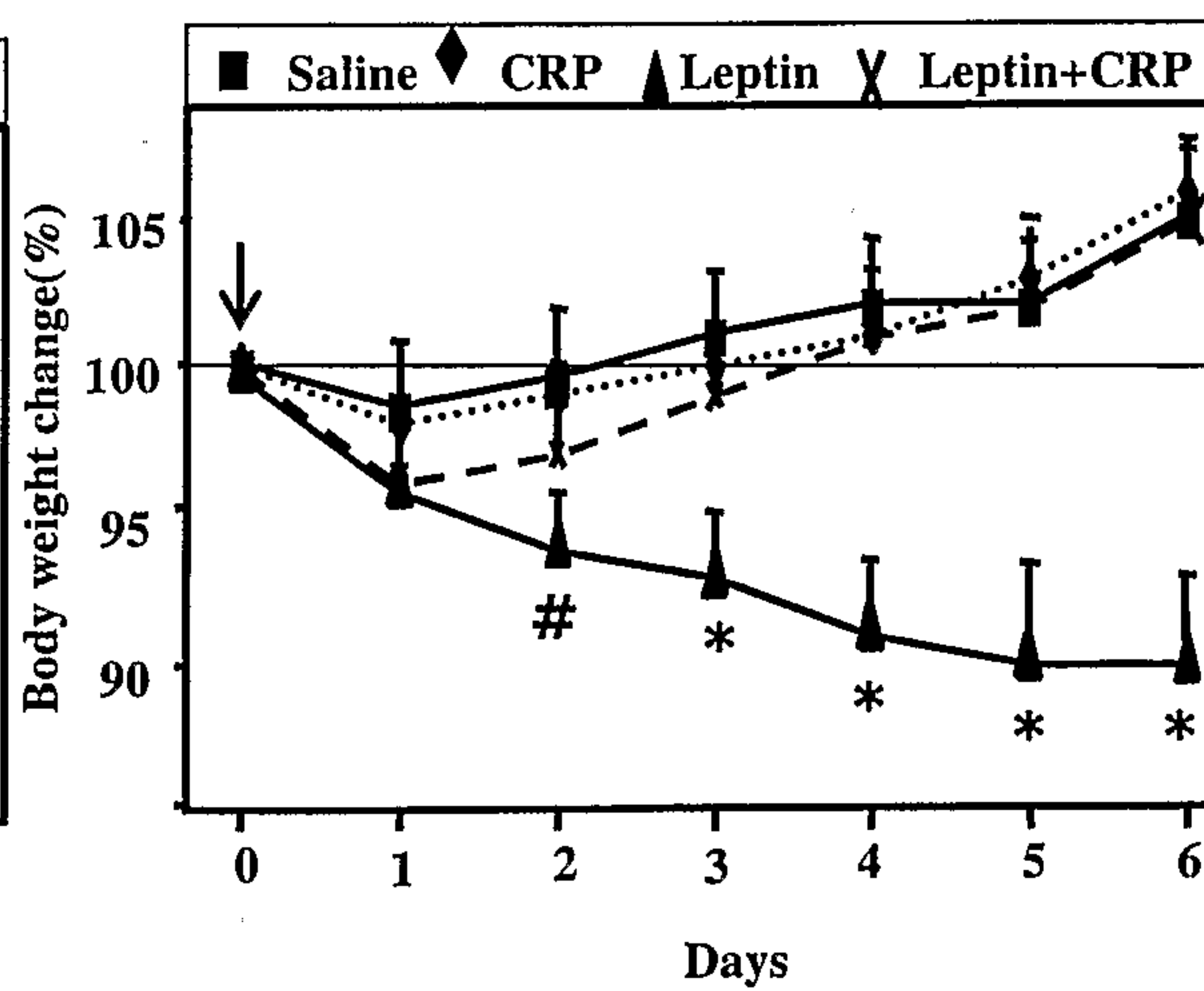


Figure 6D



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Figure 7

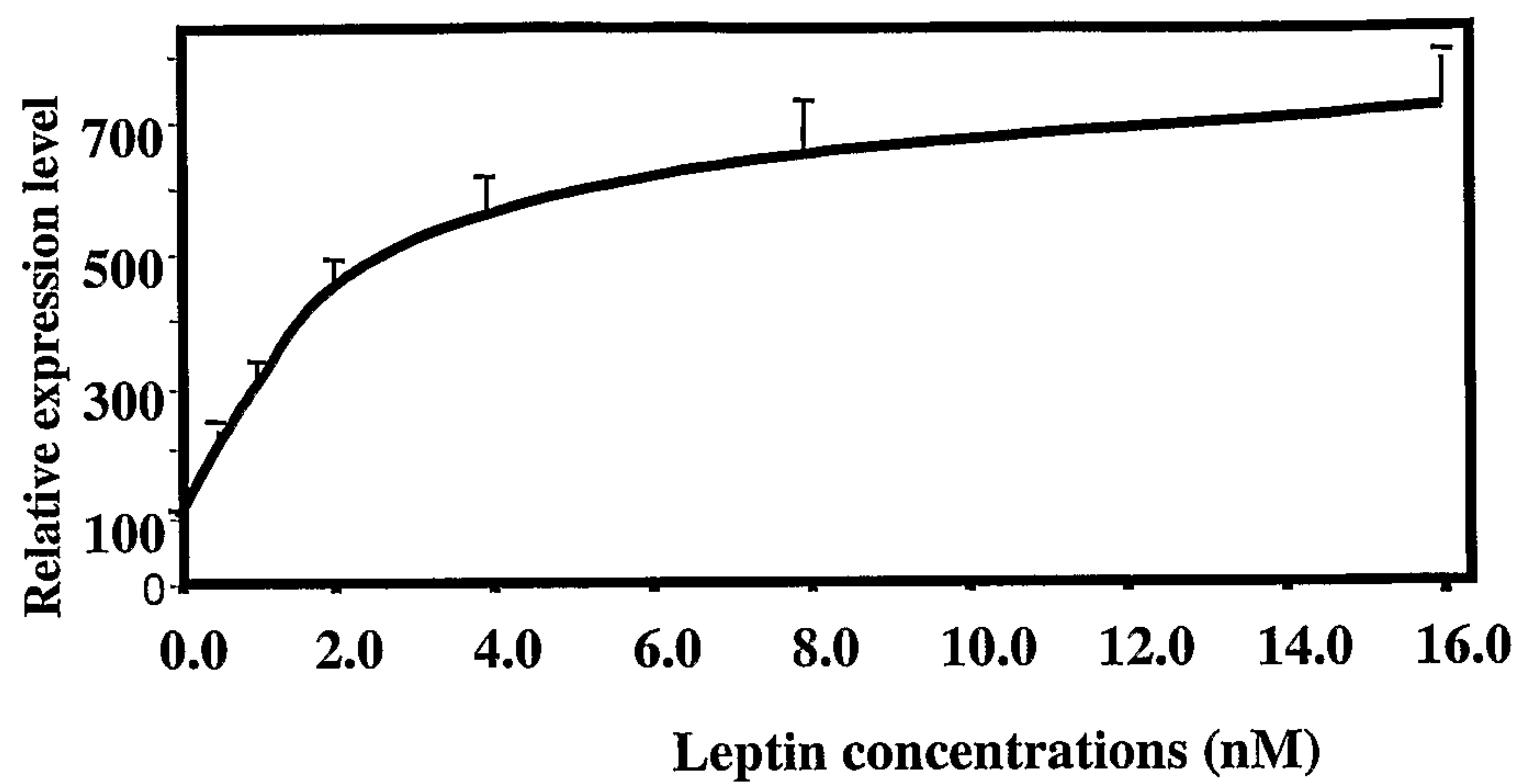
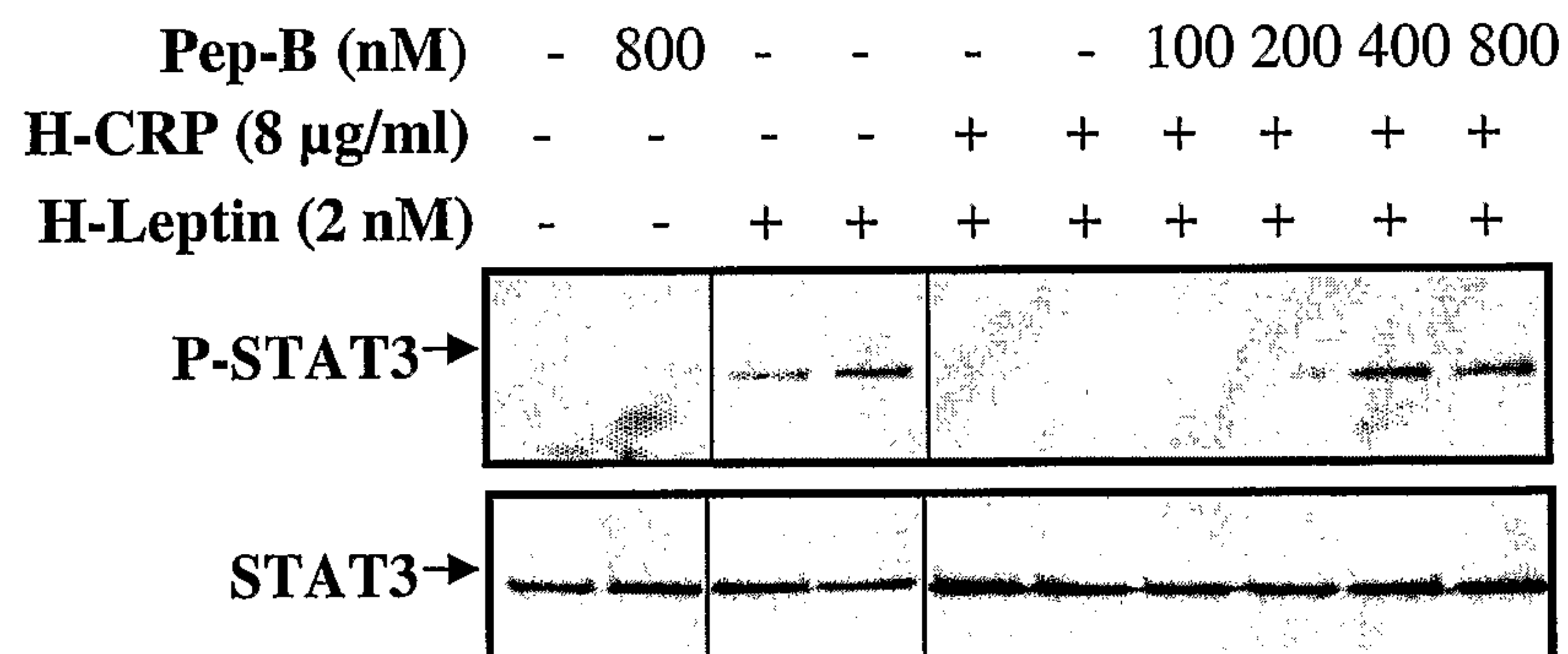


Figure 8



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Figure 9A

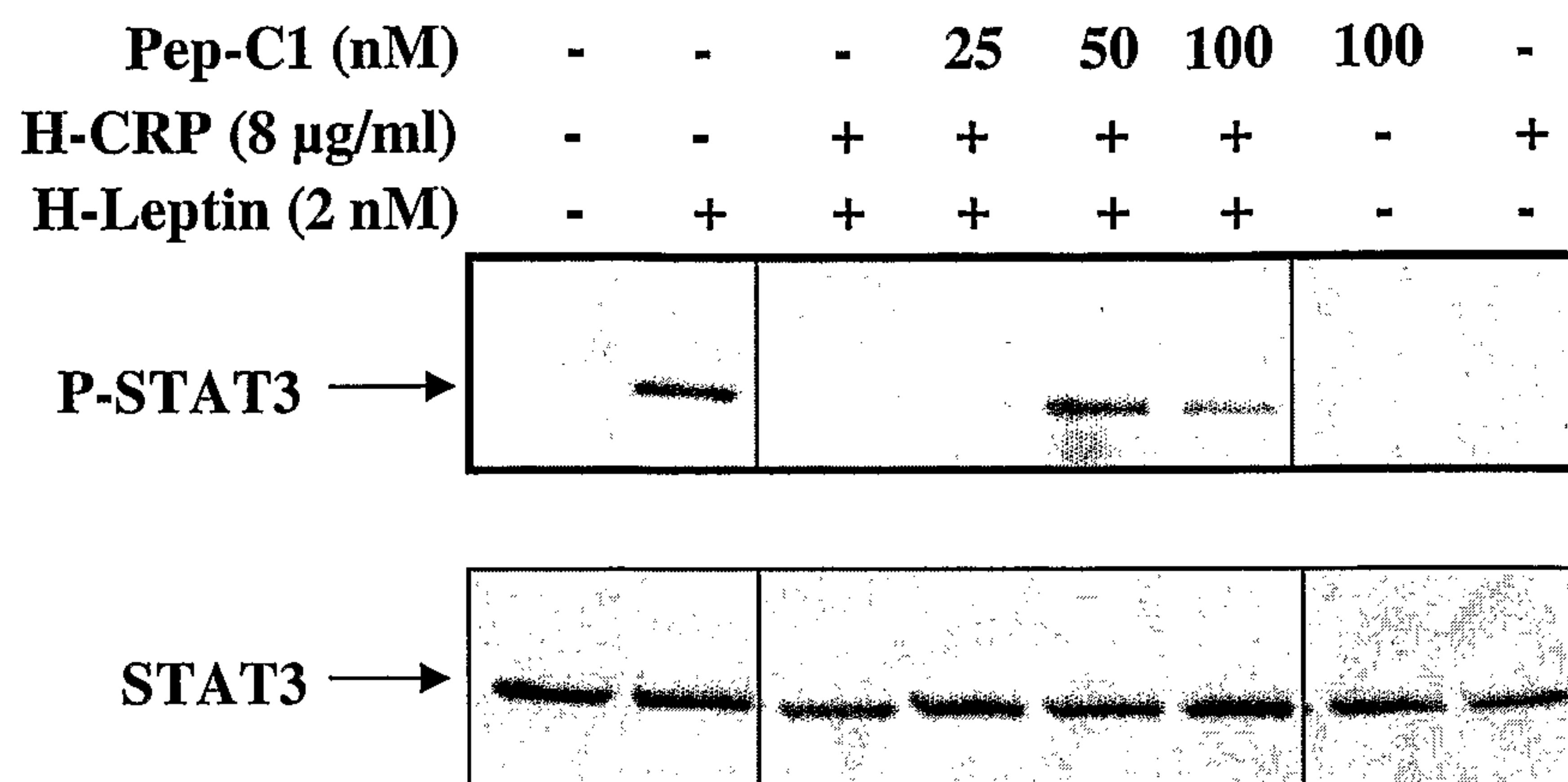


Figure 9B

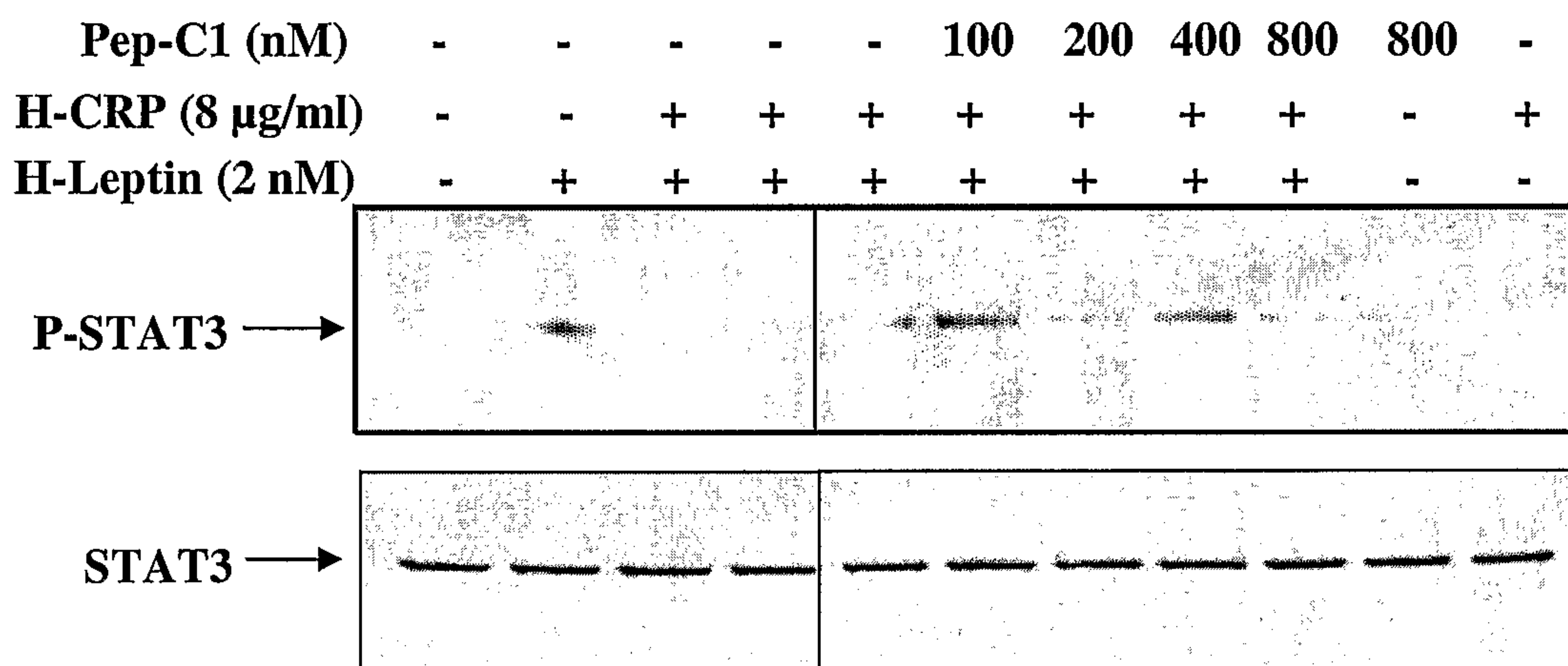


Figure 10

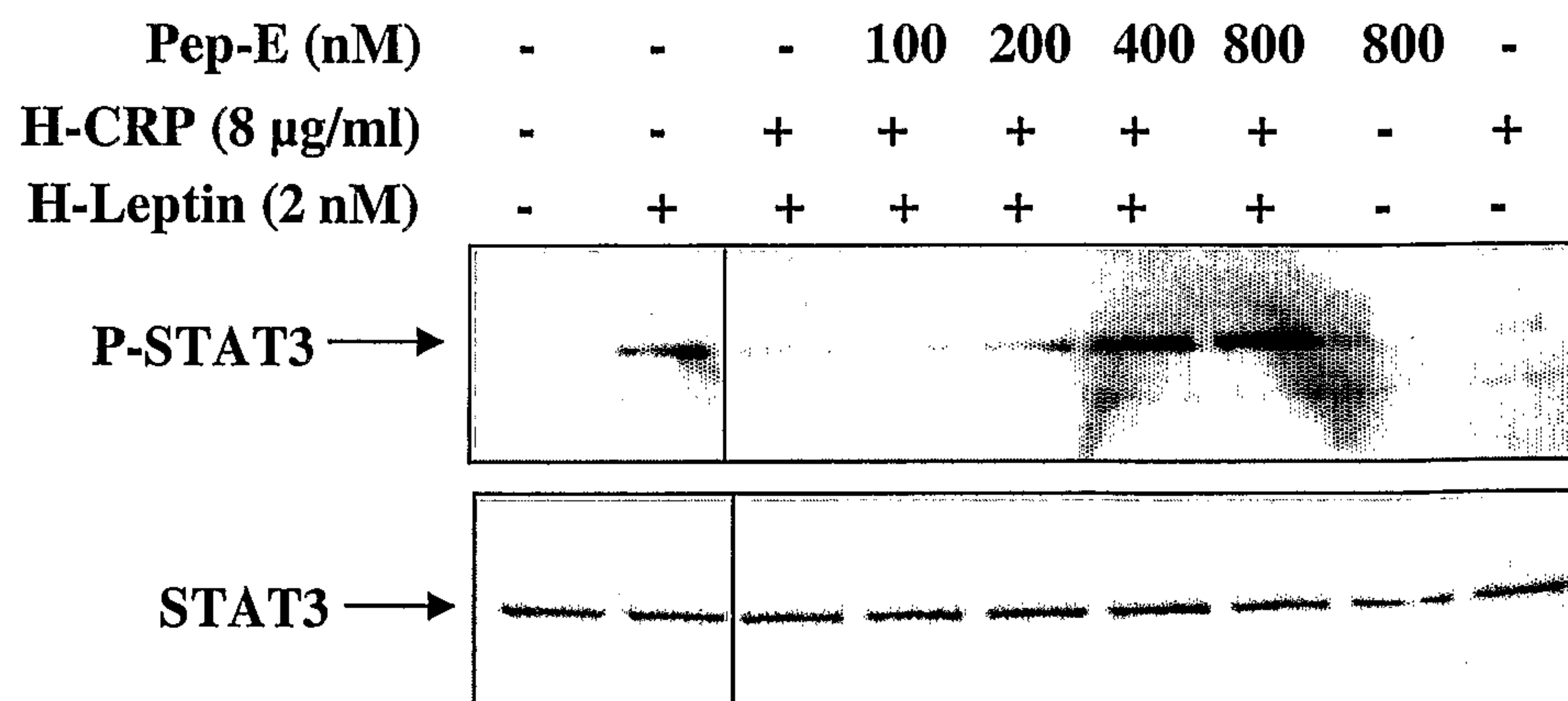


Figure 11A

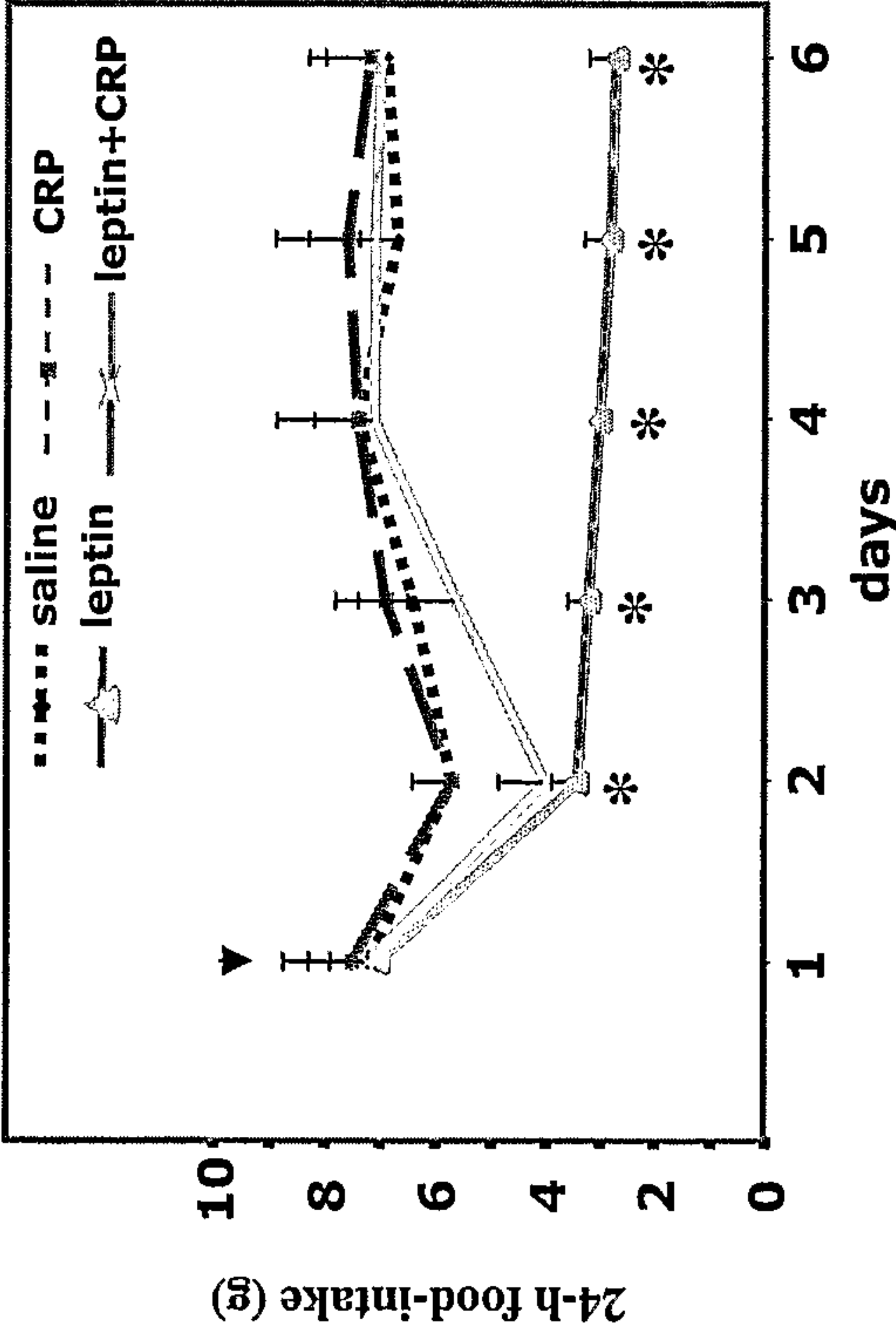


Figure 11B

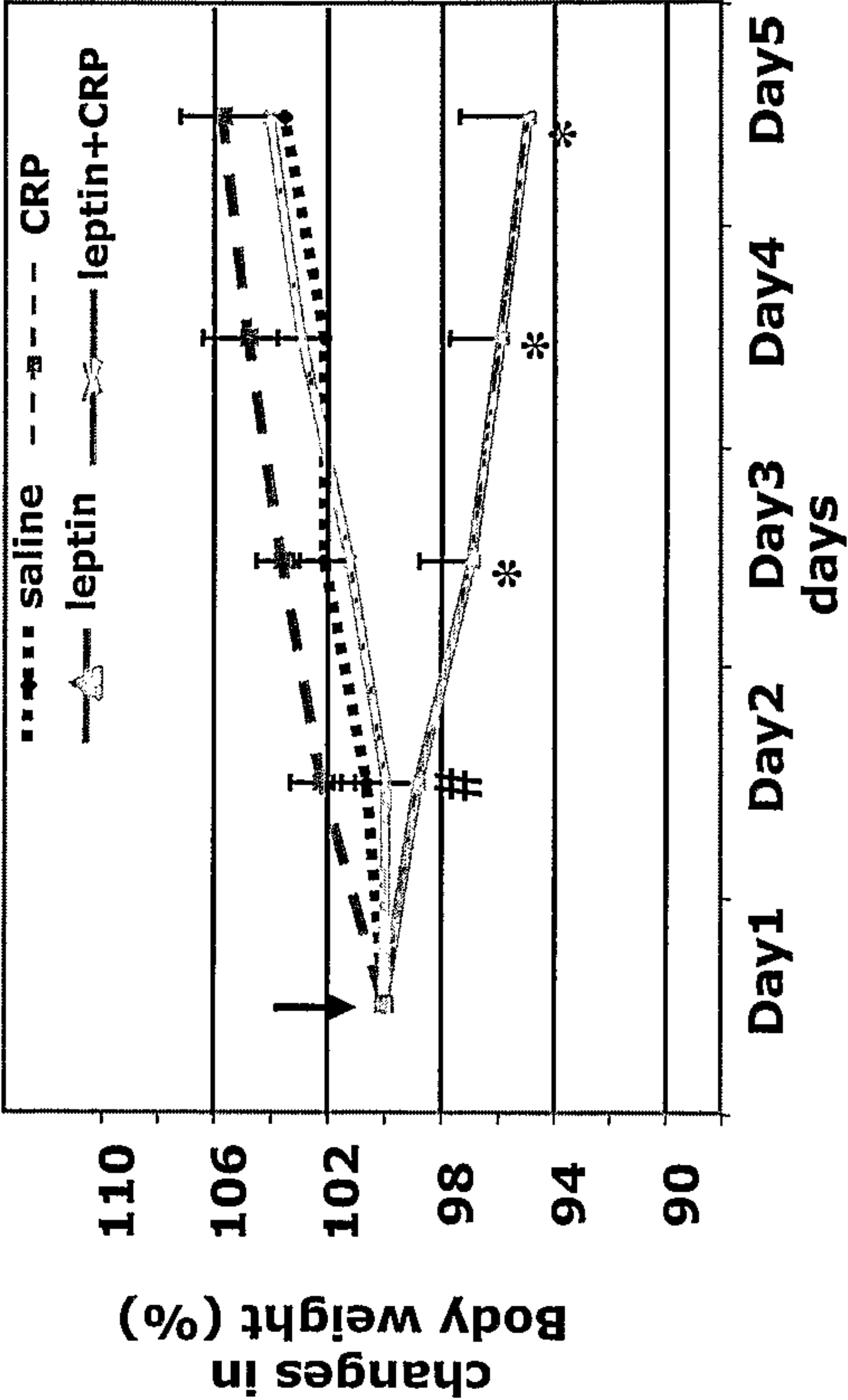


Figure 11C

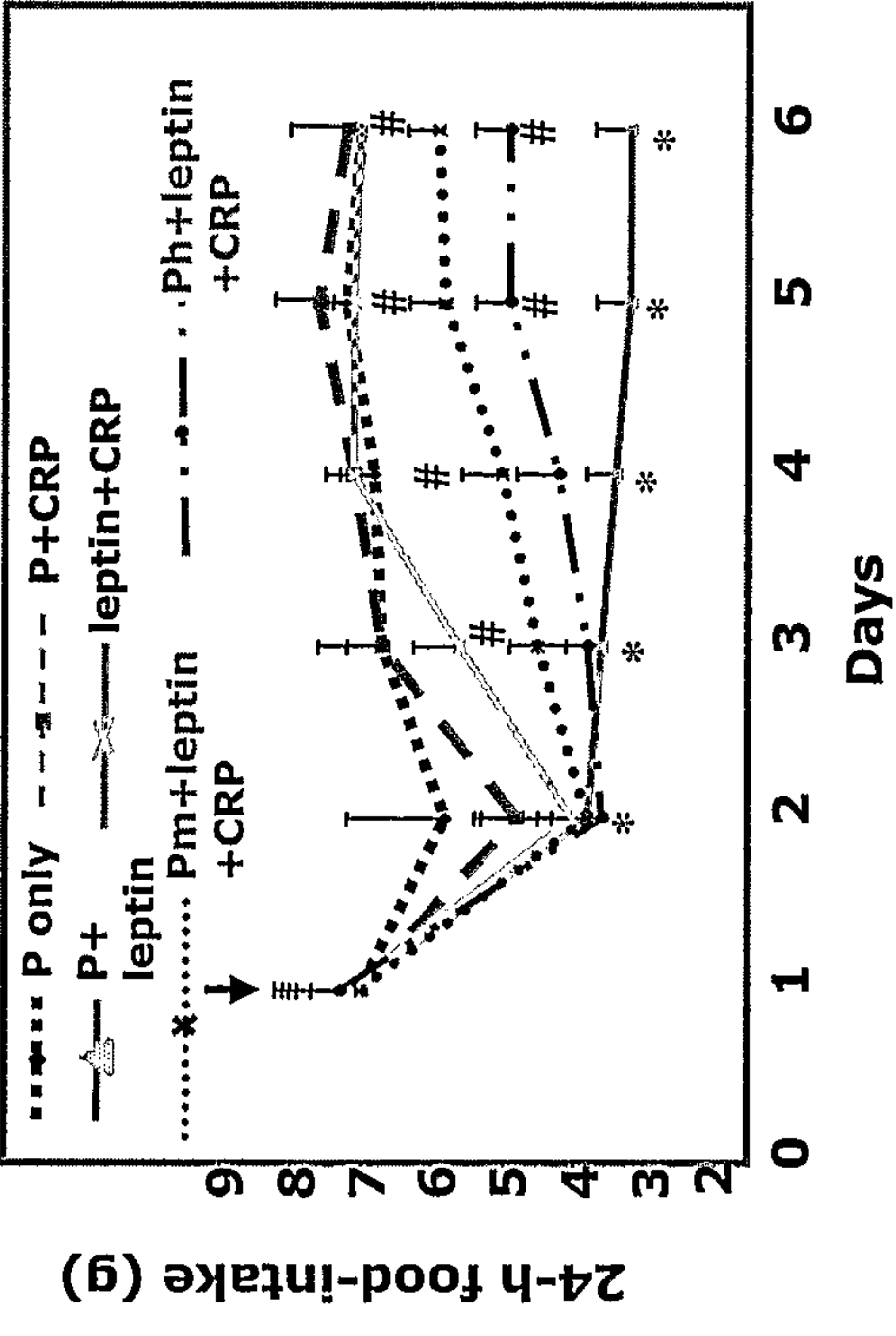
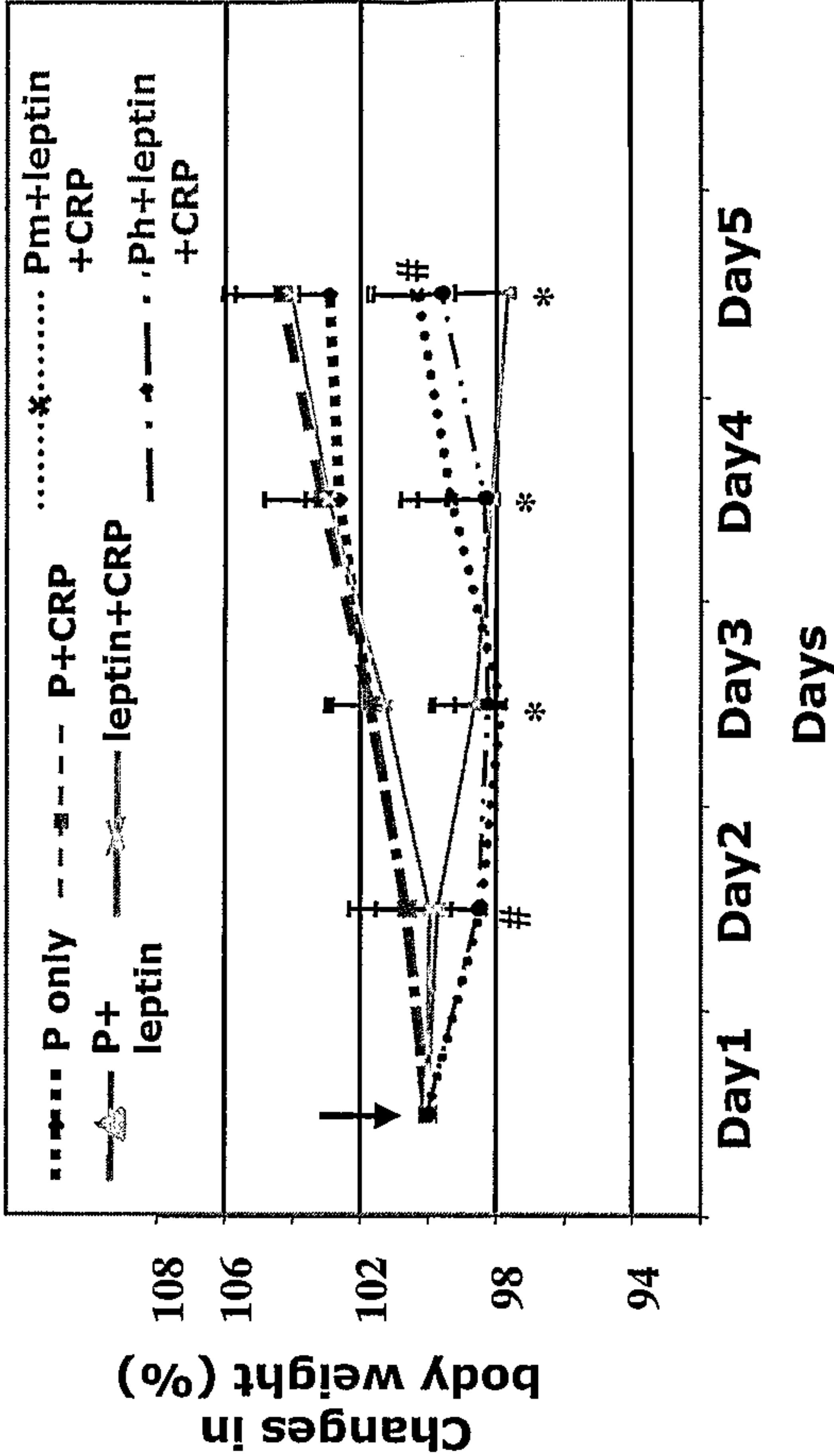


Figure 11D



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Figure 11E

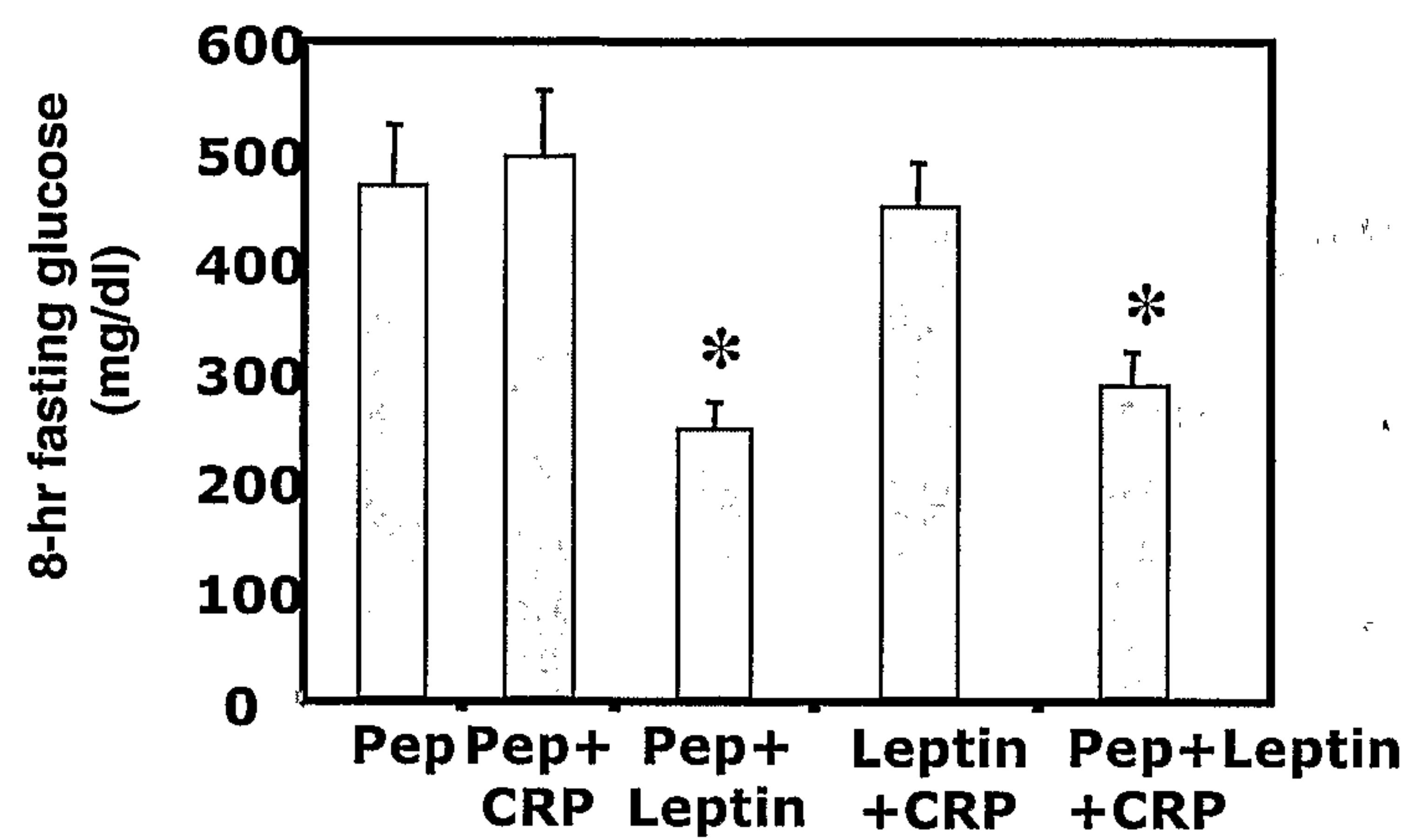
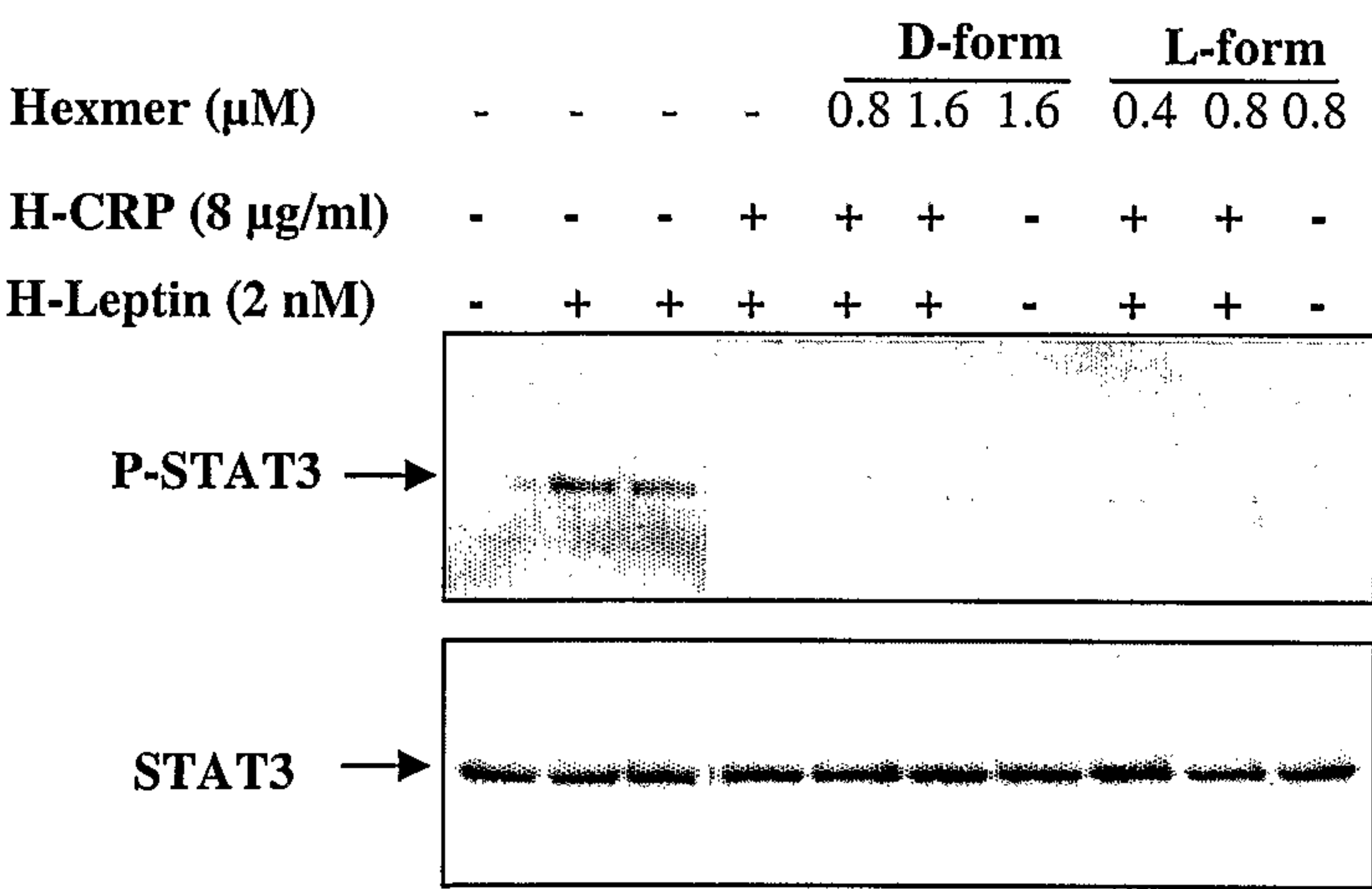
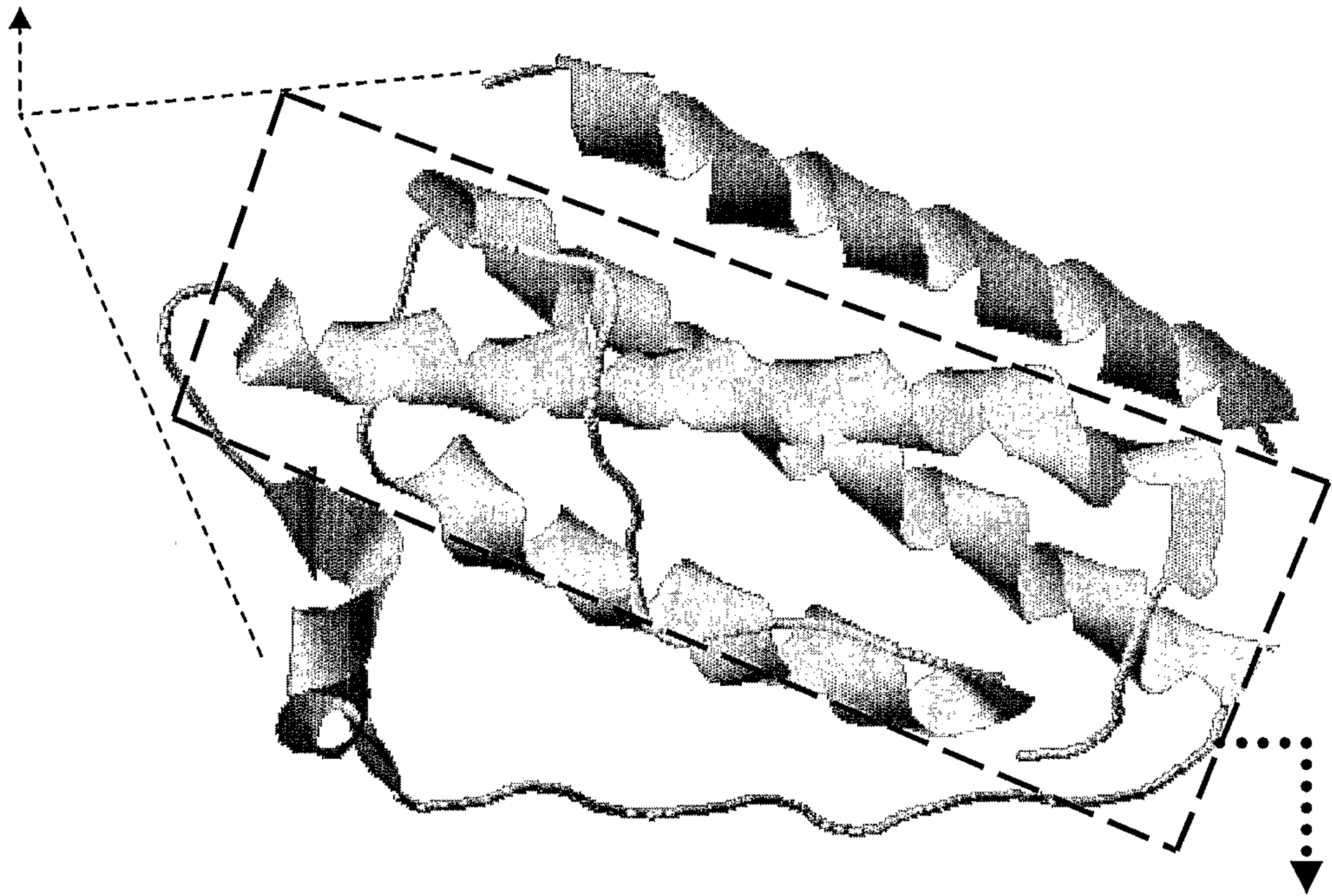


Figure 12



exposed region



core region