Effects of leptin on the epithelial cell proliferation from the small intestine and nitric oxide (NO) production in rats

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SUMMARY

Leptin encoded by the obese gene exhibits various functions, especially in the regulation of food intake and energy expenditure. The aims of this study are to investigate some specific intestinal roles of leptin, i.e. the regulation of epithelial cell proliferation and the nitric oxide (NO) production in the small intestine from rats. A total of 32 male, 3 month old, Swiss albino rats were divided into 4 equal groups: animals received a single intraperitoneal injection of the recombinant leptin (200 µg/kg) in the group 1 and of N-nitro-L-arginine methyl ester (L-NAME) (30 mg/kg), a nitric oxide synthase (NOS) inhibitor, in the group 2. Rats of the group 3 were treated by L-NAME (30 mg/kg) 15 minutes before the leptin injection (200 µg/kg) and, in the group 4, rats received saline and served as controls. One hour after the last injection, blood samples were collected for the determination of plasma NO concentrations. After slaughtering, small intestines were harvested and treated for histological observations and immunohistochemistry in order to evaluate NOS expression and cell proliferation via proliferating cell nuclear antigen (PCNA) immunostaining. Significant morphological changes of epithelial cells evidencing by enlargement of cellular height and a marked increase of epithelial cell proliferation compared to the controls were induced by treatment with leptin alone or in combination with L-NAME. Furthermore, in leptin-treated rats, endothelial nitric oxide synthase (eNOS) synthesis was enhanced in goblet cells from the Lieberkühn glands leading to a slight increase of plasma NO concentrations whereas inducible nitric oxide synthase (iNOS) expression remained unchanged. Although L-NAME alone or injected before leptin depressed plasma NO concentrations, modifications of epithelial cell chacteristics, a strong intensity of epithelial cell proliferation, as well an increased eNOS expression were also observed in the groups 2 and 3. These results demonstrate that leptin acts as a mitogene factor on epithelial cells of the small intestine and would have some medical indications. But even if eNOS was up-regulated in parallel, the molecular mechanisms leading to cell proliferation seem to be NO independent.

Keywords : Rat, small intestine, leptin, proliferation, NO, L-NAME, eNOS.

RÉSUMÉ

Effets de la leptine sur la proliferation des cellules épithéliales de l'intestin grêle et sur la production d'oxyde nitrique (NO) chez le rat.

La leptine codée par le gène de l'obésité assure différentes fonctions, en particulier dans le contrôle de la prise alimentaire et des dépenses énergétiques. Les objectifs de cette étude sont d'explorer certains rôles spécifiques de la leptine dans 1'intestin grêle, notamment sur la régulation de la prolifération des cellules épithéliales et sur la production d'oxyde nitrique (NO). Pour cela, 32 rats mâles Suisses albinos âgés de 3 mois ont été répartis en 4 groupes égaux: les animaux ont reçu une seule injection intrapéritonéale de leptine recombinante (200 _g/kg) dans le groupe 1, ou d'un inhibiteur des NO synthases (NOS), le N-nitro-L-arginine méthyl ester (L-NAME) (30 mg/kg) dans le groupe 2. Les rats du groupe 3 ont été traités par le L-NAME (30 mg/kg) 15 minutes avant 1'injection de leptine (200 µg/kg) et ceux du groupe 4 ont reçu du sérum physiologique et ont servi de contrôles. Une heure après, des échantillons sanguins ont été recueillis en vue de la détermination des concentrations plasmatiques de NO et après sacrifice, 1'intestin grêle a été prélevé et analysé histologiquement et par immunohistochimie afin d'évaluer 1'expression des NOS et 1'intensité de la prolifération cellulaire par détection du PCNA (Proliferating Cell Nuclear Antigen). Par rapport aux contrôles, des modifications cytologiques significatives (agrandissement de la hauteur des cellules épithéliales) et une forte augmentation de la prolifération des cellules epitheliales ont été induites par la leptine utilisée seule ou en combinaison avec le L-NAME. De plus, chez les rats traités par la leptine, l'expression de la NOS endothéliale (eNOS) a été accrue dans les cellules caliciformes des glandes de Lieberkühn ce qui a conduit à de faibles augmentations des concentrations plasmatiques de NO, alors que 1'expression de la NOS Pinducible (iNOS) n'a pas été modifiée. Bien que le L-NAME seul ou injecté avant la leptine ait légèrement diminué les concentrations plasmatiques de NO, la cytologie des cellules épithéliales a également varié, de même qu'une importante prolifération cellulaire et une expression augmentée de la eNOS ont été observées chez les rats des groupes 2 et 3. Ces résultats démontrent que la leptine agit comme un facteur mitogène sur les cellules épithéliales de 1'intestin grêle et qu'elle pourrait avoir des indications médicales. Cependant, même si en parallèle, la eNOS est sur-exprimée, les mécanismes moléculaires conduisant à une prolifération cellulaire semblent indépendants du NO.

Mots-clés : Rat, intestin grêle, leptine, proliferation, NO, L-NAME, eNOS.

Introduction

Leptin first discovered by ZHANG in 1994 [45] is a 16 kDa protein of 167 amino acids encoded by the *obese* (ob) gene [16, 18, 21, 33, 45]. It is produced by adipocytes and is secreted in plasma. Its plasma concentrations strongly correlate with adipose mass. Leptin inhibits food intake, reduces body weight and stimulates energy expenditure by controlling body fat stores [16, 17, 26, 45]. This protein can reach the central nervous system, mainly the hypothalamus, where

it binds to a long-form of leptin receptor and induces the decrease of the production of the neuropeptid Y, a neurotransmitter of food intake [16]. The relationship between temporal profiles of plasma leptin concentrations, body weight and food intake is complex, suggesting that in addition to body weight other factors and organs, particularly the gastrointestinal tract, are implicated in the control of leptin release [16, 19, 33, 35].

Recent studies have shown that leptin plays important regulator roles in most physiological functions, i.e reproduction system, angiogenesis, hematopoiesis, immune system, lipid metabolizm, insulin effect, sympathic activation [3, 8, 10, 13, 16, 18, 19, 22, 24, 25, 27, 31, 33, 35, 43]. Although it is secreted principally by adipocytes, leptin and leptin receptor are expressed in many tissues including hypothalamus, liver, pancreas, lung, kidney, placental trophoblastic cells, haemopoietic cells, gonads, gastric mucosal cells and skeletal muscle [8, 16, 17, 26].

Leptin and its receptors have been detected for the first time in the fundic epithelium of the stomach of rats by BADO *et al*. [2]. The amount of leptin was found to decrease immediately after feeding, and this was accompained by a rise in plasma concentrations of immunoreactive hormone, suggesting that leptin is released into circulation from the gastric mucosa [21]. Leptin can affect the proliferation of T cells [20], murine myelocytic progenitor cells [40] and a mouse embryo cell line *in vitro*, via the mitogen-activated protein kinase (MAPK) cascade [37]. Apparently, it does not stimulate intestinal epithelial cell proliferation, and by contrast present a paradoxical inhibitory action on the caecum and colon [8].

Leptin stimulates also NO release from the hypothalamus, the anterior pituitary gland and the adipocytes [18, 22]. In addition, it significantly increases plasma concentrations of NO metabolites (nitrates+nitrites) in a dose-dependent manner [3, 11, 22]. Leptin and NOS have been shown to be involved in the regulation of various physiological processes and functions: NO is known to be positively correlated with increased food intake and body weight [28, 29] and NO regulates thirst and water intake [6, 7]. FRUCHBECK [10] has demonstrated that the infusion of leptin increases serum NO concentrations and that after the inhibition of NO synthesis, and that acute leptin infusions significantly increased arterial pressure.

Results of the current studies are insufficient to elucidate proliferative effects of leptin on intestinal epithelial cells. Moreover, the effect of leptin on NO synthesis is still controversial. Association of leptin with cell height has not been confirmed by literature up to date. Consequently, the objectives of the present study are to investigate how exogenous leptin acts on mucosa and NO production in small intestine.

Materials and methods

ANIMALS AND SAMPLING

Three month old male Swiss albino rats weighing 180-250 g were used in this study. They were housed individually in plastic cages in an air-conditioned room (23-25°C) under a 12-h light (07.00-19.00 h)/12-h dark (19.00-07.00 h) cycle. All experimental procedures are confirmed by the Experimental Animals Ethics Commitee (DHEK) Health Guide for the care and use of laboratory animals. The rats were divided into 4 groups of 8 animals which the body weights were recorded. The first group received an intraperitoneous injection of the recombinant rat leptin (200 µg/kg), the second group received 30 mg/kg L-NAME, whereas the third group was treated with L-NAME (30 mg/kg), 15 min before leptin (200 µg/kg) injection. The fourth group was fed ad *libitum* and injected with saline, and served as the control.

One hour after the last injection, blood samples were collected by heart puncture into sterile tubes containing EDTA for the determination of plasma NO concentrations. After centrifugation (1 000g, 4°C, 10 minutes) plasma were carefully harvested and stored at -70°C until analysis.

Small intestines of animals were taken under euthanasia after deep ether anesthesia. Small intestine pieces were fixed in 10% formalin, dehydrated through graded alcohols and cleared in xylene. Tissues were infiltrated and embedded in paraffin. Sections of 4 µm thickness were stained with the Hematoxylin-Eosin (HE) stain and then examined under Image Pro-Plus.

HISTOLOGICAL EXAMINATION

a) Cellular morphology: At least 350 epithelial cells were measured for each experimental group. Morphometric analyses were made by using ocular micrometer and x 40 magnification objective lens.

b) Immunohistochemical staining: Four-micrometer formalin-fixed parafin embedded tissue sections were mounted on poly-L-lysine slides. The slides were air-dried and the tissue deparaffinized. Mounted specimens were washed in 0.01 mol/L phosphate-buffered saline (PBS). After three washes with PBS, an antigen retrival solution (0.01 M citrate buffer, pH 6.0) for 10 min at 100ºC in a microwave oven, endogenous peroxidase were blocked by 3 % hydrogen peroxide in distilled water for 10 min at room temperature. Subsequently, rabbit polyclonal anti-eNOS (endothelial nitric oxide synthase, NeoMarkers, dilution 1:100), rabbit polyclonal anti-iNOS (inducible nitric oxide synthase, NeoMarkers, dilution 1:100) and rabbit polyclonal anti-PCNA (proliferating cell nuclear antigen, Santa Cruz, dilution 1:300) was applied and reacted with tissue specimens at 37 ºC for 45 minutes. The sections were washed three times with PBS, and incubated with biotinylated secondary antibody at room temperature for 30 minute. Finally, immunohistochemical staining was performed using the avidin-biotin-peroxidase complex (DakoCytomation LSAB+ System-HRP kit, Carpinteria, CA, USA). Diaminobenzidine (DAB) was used as a chromogen, and the sections were counterstained with hematoxylin. Specifity of the immunohistochemical staining was checked by using posphate buffer in the same dilutions. Control intestine sections were used as positive control.

c) Intensity of proliferation: The distribution and the intensity of epithelial cell proliferation were recorded by counting

cells in a G1/S phase determined by PCNA immunohistochemistry. PCNA labelling index was calculated as the percentage of positive cells of the total cells by counting 10 different crypts. All cell counts were performed using the x 40 magnification objective lens.

NO DETERMINATION

NO was quantified photometrically in plasma by measuring its oxidation products, nitrite and nitrate, using a colorimetric assay research and development system (R & D System) catalog number DE 1600, USA). As described by the manufacturer, NO was measured by the Griess method [1]. after reduction of nitrate to nitrite with nitrate reductase. It was measured on the basis of its absorbance at 540 nm using a microtiter plate reader.

STATISTICAL ANALYSIS:

All results were expressed as mean ± standard errors (SE). Comparisons between groups were performed by Kruskal-Wallis variance analysis. Level of $p<0.05$ was assumed to be statistically significant.

Results

CELL SIZE AND ENTEROCYTE MORPHOLOGY

In the small intestine sections of the control group, the epithelial cells, their nuclei, and the connective tissue presented a normal appearance (figure 1a). Following leptin treatment, disruption of the striated border was observed and the epithelial cell nuclei were flattened and fusiform compared to those in the control group (figure 1b). In addition, leptin caused a significant increase of the cellular height ($p<0.001$) (figure 2). Under treatment with L-NAME, the striated border in apical areas (figure 1c) was preserved, but a marked increase of cellular height was also induced ($p<0.001$) (figure 2). Moreover, the nuclei of epithelial cells were also flattened. L-NAME pretreatment plus leptin injection also significantly modified the height of epithelial cells compared to the control group ($p<0.001$) (figure 1d) but, this effect was not as much intense as the one induced by L-NAME alone ($p<0.005$) (figure 2). Furthermore, the striated border of epithelial cells was not distrupted (figure 1d).

NOS ACTIVITY AND PLASMA NO CONCENTRATIONS

Increases of the eNOS activity compared to basal activity in the controls (figure 3a) were evidenced in the duedonal epithelium mainly in the goblet cells from the Lieberkühn glands in all experimental groups [leptin (figure 3b), L-NAME (figure 3c), leptin+L-NAME (figure 3d)]. The iNOS expression was also evidenced in intestinal connective tissue from the controls and from all the treated rats (data not shown), but this expression was weak compared to the eNOS synthesis. Plasma NO concentrations were slightly but not significantly increased in the leptin group compared to the control group, whereas in rats treated with L-NAME alone or before leptin injection, they seemed to be decreased but the differences were not significant (figure 4).

PROLIFERATION INTENSITY

The immunostaining of PCNA positive cells was shown as brown deposited in the nuclei. The PCNA positive cells in rat duodenum were mainly distributed in duodonal cript and in the *lamina propria*. As shown in Table I, the intensity of the epithelial cell proliferation was markedly enhanced in all experimental groups compared to the control group ($p<0.05$). Besides, the proliferative cell indexes were similar whatever the treatment.

Discussion

CHAUDHARY *et al.* [8] reported that starvation led to a 20% decrease of the inbody weight, and of the intestine weights. They have also shown that starvation markedly inhibited intestinal epithelial cell-proliferation, and treatment with leptin has little effect for stimulating proliferation. By contrast, leptin injection (200 µg/kg) has induced marked changes of epithelial cell morphology (increase of height) and has also significantly stimulated proliferation of small intestine epithelial cells in the present study. The intestinal

Groups	n	Proliferative cell index
Leptin (group 1)	5	32.80 ± 2.37^{b}
L-NAME (group 2)	5	34.80 ± 3.69^{b}
L-NAME + Leptin (group 3)	5	31.20 ± 3.02^{b}
Control (group 4)	5	18.20 ± 2.59^{a}

n = Number of mice.
Means with different superscript are significantly different ($p<0.05$).

TABLE I : The proliferation intensity of small intestine epithelial cells evidencing by PCNA immunostaining in the rats of the control, leptin, L-NAME and Leptin + LNAME groups. Results are expressed as means ± standard deviations.

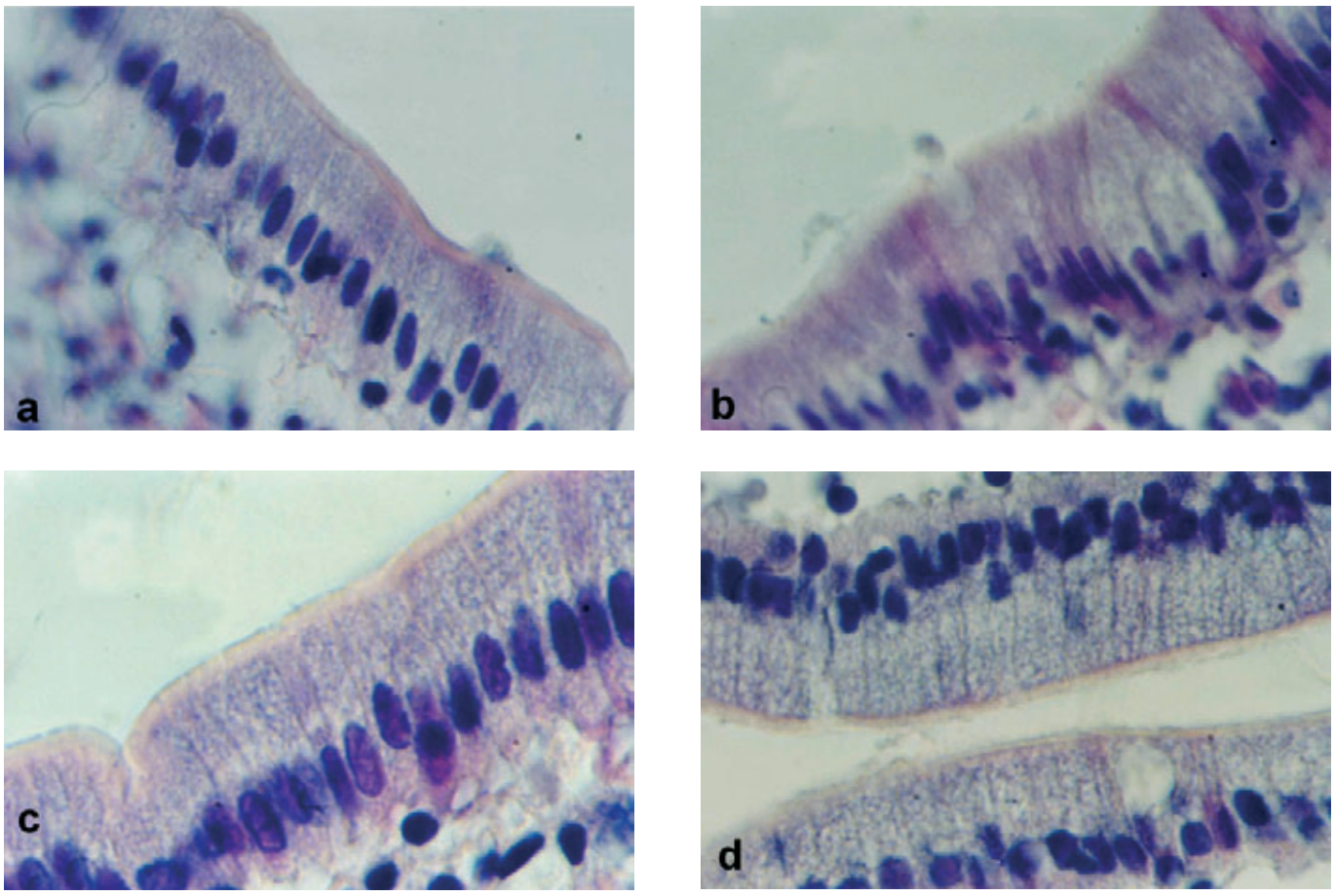
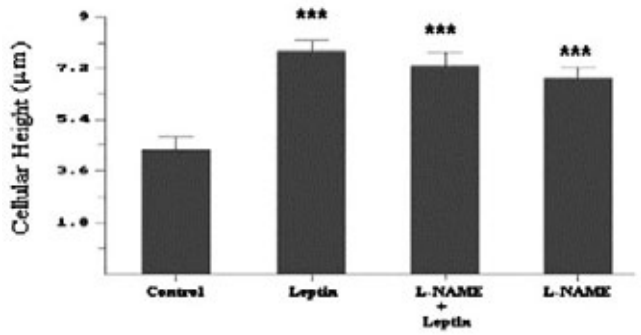


Figure 1: Increase of cell height in the small intestine epithelial cells of rats of the Control (**a**), Leptin (**b**), L-NAME (N-nitro-L-arginine methyl ester) (**c**), Leptin + L-NAME (**d**) groups. Hematoxyline+Eosine, Original magnification, x 1444.



*** p< 0.001 compared to the control group.

Figure 2: Comparison of the height of small intestine epithelial cells in rats of the control, leptin, L-NAME and Leptin + L-NAME groups.

cell proliferation was evaluated through PCNA immunostaining. PCNA is a 36 kDa protein which acts as an effector of DNA polymerase δ and it is markedly expressed in the nuclei of proliferating cells. Actually, PCNA is considered as the best marker for cellular proliferation [9, 12, 14, 36, 42, 44] and the intensity of staining is proportional to its nuclear expression: strongly positive cells present brown-yellow nuclei and moderately positive cells yellow nuclei, whereas weakly positive cells are characterized by buff nuclei. In the present study, proliferating PCNA positive cells were mainly localized in duodenal crypt recesses and the differentiation of young cells on the top of villi was submitted to "ladder-like" movement [12, 14, 42, 44]. These results suggest an intestinal role for leptin. Recent experiments in rodents have suggested that leptin could be gastro-protective against stress-induced or bacterial gastric lesions [16]. Our findings in term of proliferation support these previous results. But, on the other hand, under leptin treatment, distruption of the striated border of the intestinal epithelial cells were appeared and could be due to the interaction between endogenous leptin and leptin receptors that located in the *lamina propria* [21], leading to the shortening or the disappearance of microvilli.

The iNOS is mainly expressed by intestinal smooth muscle cells [41], whereas the eNOS is synthesized by intestinal epithelial cells [38] and this last enzyme is involved in water absorbtion, release of electrolytes and in mucus production [39]. The leptin administration has induced increases of eNOS synthesis in goblet cells of the Lieberkühn glands while the iNOS expression was not modified compared to the controls, suggesting that this small hormone would specifically regulate eNOS expression. Consequently, plasma NO concentrations were slightly increased in the leptin-treated group, but the effect was not statistically significiant compared to the controls. However, leptin was reported to stimulate systemic NO production [3, 18, 22] in a dose-dependent manner [10]. This discrepancy would be due to the different dosages and duration of treatments used. Nevertheless, as iNOS is increased in mostly pathological conditions [4], higher dosages or chronic administration of leptin would

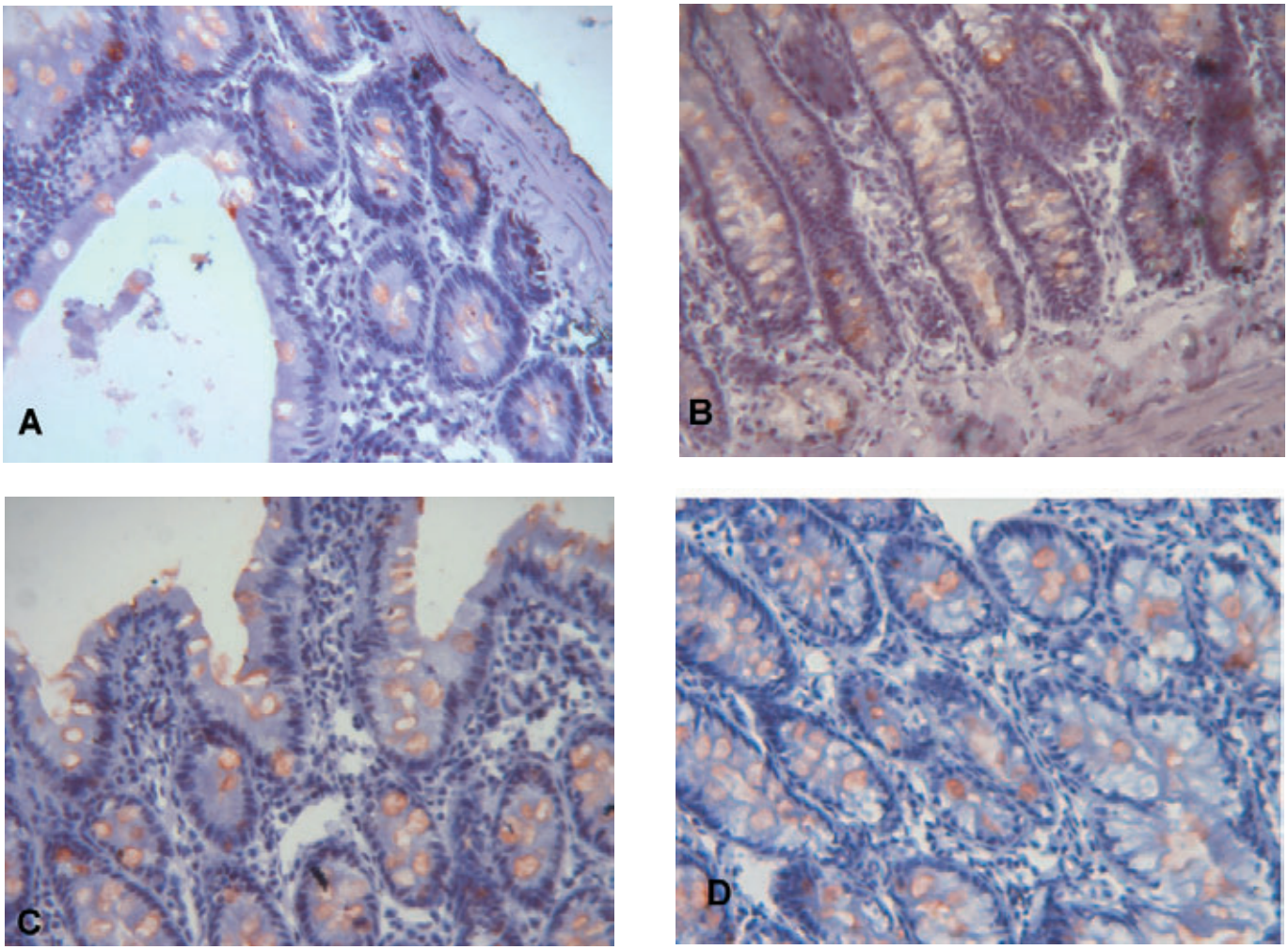


Figure 3: eNOS activity in the goblet cells from the Lieberkühn glands of rats of the Control (**a**), Leptin (**b**), L-NAME (N-nitro-L-arginine methyl ester) (**c**), Leptin+L-NAME (**d**) groups.

probably also enhance iNOS expression and contribute to increase plasma NO concentrations.

Membrane leptin receptors (Ob-R) have been identified in immune cells from the *lamina propria* by immunohistochemistry [21], but the presence of the Ob-R on the other intestinal cells (epithelial and goblet cells) was not still demonstrated. However, if these receptors were expressed by epithelial cells, leptin could directly act as a mitogene factor on these cells. In the other case, the stimulation of the immune cells of the *lamina propria* by leptin could lead to the release of cytokines (IL1β, IL6, LIF) [30] or other chemical mediators (NO, CD+8, IFδ) [34] which, in turn, stimulate epithelial cell proliferation. Moreover, as leptin shares homology with the TNFα [23], it would be possible that this protein binds to TNFα-receptor expressed by epithelial cells and/or the immune cells and mimics the mitogene effects of this cytokine. Additional factors, such IL6, may also lead to cell proliferation via the leptin-Ob-R pathway. Indeed, the receptors of leptin and IL6 present homology [19] and the binding of IL6 by the Ob-R on the epithelial and/or immune cells may also induce cell proliferation directly and indirectly.

On the other hand, it has been reported that the NOS inhibitor, L-NAME, induced depression of cell proliferation and may be an effective chemopreventive agent against colon carcinogenesis [5, 15, 32], suggesting that NO would be one

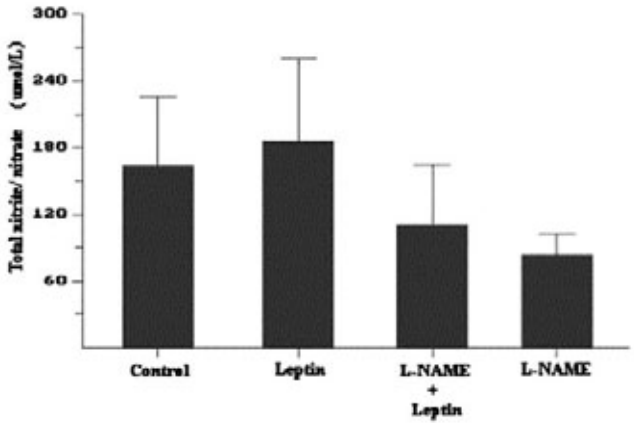


Figure 4: Comparison of plasma NO concentrations (total nitrite + nitrate values) in rats of the control, leptin, L-NAME and Leptin + L-NAME groups.

of the mitogene factors involved in the intestinal cell proliferation. However, the results of the present study are in disagreement with that. Indeed, the treatment of rats with L-NAME alone has also induced significant changes of cellular morphology (increase of the height) and a significant increase of intestinal epithelial cell proliferation compared to the controls. Although the induced cellular modifications

were less pronounced than with leptin, the proliferation intensities were quite identical with the 2 treatments. Moreover, similar morphological changes were also noticed when rats were co-treated with L-NAME and leptin. Consequently, if the cellular effects were only due to the local NO production, L-NAME alone or in combination with leptin would not have induced such cellular changes. These results suggest that NO was not a potent mitogene in small intestine than it was in colon and/or that the L-NAME would have some unknown adverse effects, and that leptin cellular actions would partially be mediated by NO independent biochemical pathways. Because no additive or synergic effects were encountered with the co-treatment, it would be probable that L-NAME and leptin would induce the same reaction cascades leading to cell multiplication. More surprisingly, enhancement of eNOS expression was also observed in rats treated by L-NAME alone or in association with leptin. Several not mutually exclusive hypotheses could be proposed: firstly, the eNOS induction would be simply a consequence of proliferating actions of L-NAME and leptin. Secondly, the decrease of local NO content due to the direct pharmological effects of L-NAME could indirectly lead to the up-regulation of the eNOS gene, whereas leptin would directly induce eNOS synthesis by unknown mechanisms.

On the other hand, the employed dosage of L-NAME would be insufficent for blocking NO production since the decreases of plasma NO concentrations induced by L-NAME treatments (alone or in combination with leptin) have not been significant compared to the untreated controls. Consequently, the pharmological inhibitor did not succeed in effectively counteracting the leptin effects mediated by NO dependent pathways. Such a situation may explain why no additive effect with co-treatment was observed. Further investigations with higher L-NAME dosages are needed for evaluating the NO involvement in leptin molecular mechanisms and the putative persistence of intrinsic L-NAME proliferating effects.

As a conclusion, our results show for the first time direct proliferating effects of leptin on intestinal epithelial cells and suggest that they could be mediated at once by NO dependent and independent pathways, but further experiments are required for elucidating leptin molecular mechanisms on intestinal epithelial cells and its preventive actions against intestinal damage in order to propose therapeutic indications for this hormone.

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2015	637	0.319	0.291	0.374	0.062	48	100.00	17.754
2014	631	0.323	0.296	0.351	0.080	50	100.00	17.669
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