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LEPTIN MEDIATES THE LIPOPOLYSACCHARIDE-INCREASED PULMONARY PERMEABILITY IN RATS

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ABSTRACT. The aim of our study was represented by the leptin involvement as a mediator of lipopolysaccharide (LPS)-increased pulmonary permeability in rats. Pretreatment with aminoguanidine (20 mg/kg i.p), 2-aminopurine (20 mg/kg i.p.) and subjecting to food deprivation (48 h, with water ad libitum) reduced the LPS-induced vascular leak, measured as Evans blue extravasation in lung tissue, by 41.70%, 21.39% and, respectively, 52.92%, when compared to LPS (10µg/kg, 100µl aliquots) administered alone. In addition, leptin alone induced Evans blue leakage that represents 74.38% from that induced by LPS. Since the inhibitory effects of 2-aminopurine are with 59.34% higher than that induced by aminoguanidine on leptin, we were able to conclude that leptin is at least partially mediating the LPS-increased pulmonary permeability in rats. Lung concentrations of leptin might increase as an early event of LPS challenge. Furthermore, leptin effects might be partially realized through NO production after leptin receptors activation.

Keywords: leptin, lipopolysaccharide, nitric oxide, pulmonary permeability, rat

INTRODUCTION

Sepsis and septic acute lung injury are among the leading causes for morbidity and mortality of critical illness. Meanwhile, despite the associated morbidity and mortality, underlying mechanisms leading to the development of acute lung injury remain incompletely understood. Acute lung injury (ALI) is characterized by significant pulmonary inflammation and alveolar/vascular barrier dysfunction, i.e. increased pulmonary capillary permeability. Lung edema during sepsis is triggered by formation of gaps between endothelial cells followed by macrophage infiltration. To evaluate such a pathologic entity there might be used the well-described rat model of lipopolysaccharide (LPS)-induced shock. LPS treatment has as effects the increasing of lung injury and inflammatory mediators production, i.e. the amplification of vascular permeability, myeloperoxidase content, bronchoalveolar lavage inflammatory cells (polymorphonuclear leukocytes-neutrophils, macrophages) and inflammatory cytokine/chemokine content (cytokine-induced neutrophil chemoattractant; interleukin-1□; tumor necrosis factor-□), nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) nuclear translocation, as well as interleukin 1 receptor-associated kinase 1 (IRAK-1) and stress-activated protein kinase (SAPK) phosphorylation (Merry et al., 2010).

Adipose tissue is an active endocrine organ that secretes various humoral factors (adipokines), and its shift to production of proinflammatory cytokines in obesity likely contributes to the low-level systemic inflammation that may be present in metabolic syndrome-associated chronic pathologies such as atherosclerosis. Leptin is one of the most important hormones secreted by adipocytes, with a variety of physiological roles related to the control of metabolism and energy homeostasis. One of these functions is the connection between nutritional status and immune competence. The role of leptin in regulating immune response has been assessed in vitro as well as in clinical studies. It has been shown that conditions of reduced leptin production are associated with increased infection susceptibility. Conversely, immune-mediated disorders such as autoimmune diseases are associated with increased secretion of leptin and production of proinflammatory pathogenic cytokines. Thus, leptin is a mediator of the inflammatory response (Fernández-Riejos et al., 2010).

Obesity is an independent risk factor for asthma. Recent studies suggest that obesity is also an independent risk factor for chronic airflow obstruction, as is seen with chronic obstructive pulmonary disease (COPD). The mechanistic basis for these associations in humans is not established, although a possible role for adipokines has been invoked. Leptin, a proinflammatory adipokine, and adiponectin, an anti-inflammatory adipokine, are causally associated with asthma in mice. Although human studies are currently inconclusive, high-serum leptin and low-serum adiponectin concentrations predict asthma, independent of obesity, in select population groups, such as premenopausal women in the United States. In contradistinction, low-serum leptin and high-serum adiponectin concentrations are associated with stable COPD, although these associations are likely confounded by fat mass. Interestingly, leptin may promote systemic and airway inflammation in stable COPD patients. On the other hand, COPD may upregulate systemic and lung adiponectin expression. The precise mechanism and significance of the associations between these adipokines and lung disease

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at the current stage is confusing and frankly paradoxical in places. This area of research needs additional study that may open up novel therapeutic strategies for these lung diseases (Sood, 2010).

Predominantly produced by adipose tissue, circulating leptin induces satiety by acting on the hypothalamus. Emerging data are establishing new roles for leptin in the cardiovascular and immune systems; however the role for leptin in the lung is unknown. The nuclear transcription factor peroxisome proliferator-activated receptor-gamma (PPAR-γ) regulates inflammation and lipid metabolism and is constitutively expressed in healthy alveolar macrophages. Obesity is often accompanied by pulmonary inflammatory diseases, including asthma, in which PPAR- γ deficiencies in alveolar macrophages are observed. It was demonstrated that the deletion of PPAR-g in murine alveolar macrophages of conditional knockout mice (PPAR-KO) results in pulmonary inflammation, making this an appropriate model to investigate the relationship of PPAR-γ and leptin to pulmonary inflammation. Reports have indicated that elevated leptin levels result in decreased expression of PPAR- γ and leptin receptors in primary macrophages. Based on these findings, it was hypothesized that the expression of leptin receptors would be reduced in the alveolar macrophages of PPAR-KO. Alveolar macrophages were obtained from C57/Bl6 wild type mice and PPAR-KO by bronchoalveolar lavage. Analysis by real time-PCR showed a 7.5-fold decrease in leptin receptor mRNA expression in the PPAR-KO alveolar macrophages, compared to wild type mice. These results demonstrate the expression of leptin receptors on alveolar macrophages, suggesting an interaction between leptin and PPAR-γ in the lung. Future studies will determine leptin levels in the lungs of wild type and PPAR-KO mice and its effects on pulmonary inflammation (Andrews et al., 2009).

The appetite suppressing hormone leptin has emerged as an important modulator of immune function and is now considered to be a critical link between energy balance and host defense responses to pathogens. These 'adaptive' responses can, in situations of severe and sustained systemic inflammation, lead to adverse effects including brain damage that is partly mediated by neutrophil recruitment into the brain. There was examined the contribution of leptin to this process in leptin-deficient (ob/ob), -resistant (db/db) and wild-type (WT) mice injected intraperitoneally with a septic dose of lipopolysaccharide (LPS). This treatment induced a dramatic increase in the number of neutrophils entering the brain of WT mice, an effect that was almost totally abolished in the mutant mice and correlated with a significant reduction in the mRNA levels of interleukin-1□, intracellular adhesion molecule-1 and neutrophil-specific chemokines. These effects were reversed with leptin replenishment in ob/ob mice leading to recovery of neutrophil recruitment into the brain. Moreover, 48 h food deprivation in WT mice, which decreased circulating leptin levels, attenuated the LPS-induced neutrophil recruitment as did a single injection of an anti-leptin antiserum 4 h before LPS treatment in WT mice. These results provide the first demonstration that leptin has a critical role in leukocyte recruitment to the brain following severe systemic inflammation with possible implications for individuals with altered leptin levels such as during obesity or starvation (Rummel et al., 2009).

The aim of our study was represented by the leptin involvement as a mediator of lipopolysaccharide-increased pulmonary permeability in rats, as well as the modulation of the processes by 2-aminopurine (2-AP), aminoguanidine (AG) and food deprivation (for 48 h, water ad libitum).

MATERIALS AND METHODS

Present studies were carried out in accordance with the "Guide for Care and Use of Animal Experiments" of U.S. National Institutes of Health (NIH), published by the U.S. National Academy in 1996, and approved by the Ethics Committee of the University of Medicine and Pharmacy "Gr. T. Popa" Iaşi.

For the experiments we used 48 Wistar adult male rats (Băneasa source), weighing 150-200 g, divided in series of 6. All rats were housed at room temperature (22-24oC) and constant humidity (50-60%) under a 12-h light-dark (07:00-19:00 and 19:00-07:00) cycle.

In one series of experiments lung tissues were prepared from rats challenged with LPS (from Escherichia coli 055:B5). LPS dissolved in physiological saline, 10 µg/kg, was administered in aliquots of 100 µl to rats anesthetized with diethyl ether by intranasal instillation, delivered with a blunted hypodermic 25 G needle gauge attached to a 1-ml syringe, whereas the control animals were given the vehicle alone. For a second series, beside and 30 minutes before LPS, rats received aminoguanidine (AG, 20 mk/kg i.p. in 1 ml saline) and the third series 2-aminopurine (2-AP, 20 mk/kg i.p. in 1 ml saline) in the same conditions. The fourth series was subjected to 48 h food deprivation, with water ad libitum, before LPS administration. Leptin (LEPT) was solved in physiological saline and was administered in 100 µl aliquots (10µg/kg) to rats from the fifth series, anesthetized with diethyl ether, by intranasal instillation, delivered with a blunted hypodermic 25 G needle gauge attached to a 1-ml syringe. The rats from the sixth series received 2-aminopurine (2-AP, 20 mg/kg i.p. in 1 ml saline), beside and 30 minutes before leptin (LEPT), and those from the seventh series aminoguanidine hydrochloride (AG, 20 mk/kg i.p. in 1 ml saline) in the same conditions.

After the LPS or leptin instillations the animals were observed for 24 h and, after that, Evans blue (20 mg/kg in saline) was administered i.p. to rats 30 min before they were sacrificed. The rats were sacrificed by bleeding under diethyl ether anesthesia and the tissue was harvested and then introduced, after weighing and mincing, in formamide for 48 hours to extract the

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Evans blue. Evans blue was spectrophotometrically quantified at 620 nm using a UV-VIS diode-array spectrophotometer (HP 8453) and Chemstation software (Hewlett-Packard). Evans blue values were expressed in µM/g tissue. Quantification was done using a standard curve made with 2 concentrations of Evans blue (0.1 µM and 10 µM), while knowing that Evans blue absorbance is linear with concentration in these areas. Concentration calibration equation was as follows: concentration = 12.79100µM/ml x absorbance (A).

All reagents were purchased from Sigma (St. Louis, MO), unless otherwise specified. When needed, dimethyl sulfoxide (DMSO) was used at a final concentration of 0.01% as solvent, having no biological effects.

The statistical significance of test results was highlighted using the Variance One-Way ANOVA (Student-Newman-Keuls method) and Student t-test and the results were expressed as mean ± S.E.M (n = 6). Value of $p<0.05$ was always considered statistically significant.

RESULTS AND DISCUSSIONS

Since the underlying mechanisms leading to the development of acute lung injury remain incompletely understood for the evaluation of such a pathologic entity we used the well-described rat model of LPS-induced shock. When the cells in lung are exposed to LPS, the nuclear factor (NF)-κB is activated. NF-κB is a protein transcription factor that functions to enhance the transcription of a variety of genes, including cytokines and growth factors, adhesion molecules, immunoreceptors, and acute-phase proteins. Upon activation by LPS, NF-κB is required for maximal transcription of many cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8, which are thought to be important in the generation of ALI. These cytokines and chemokines contribute to the vigorous recruitment of neutrophils in lung. Therefore, ALI is substantially caused by excessive neutrophil- and cytokine-mediated inflammation (McIntyre et al., 2000).

The appetite suppressing hormone leptin has emerged as an important modulator of immune function and is now considered to be a critical link between energy balance and host defense responses to pathogens. These 'adaptive' responses can, in situations of severe and sustained systemic inflammation, lead to adverse effects including brain damage that is partly mediated by neutrophil recruitment into the brain (Rummel et al., 2009). Accordingly, we hypothesized that LPS-induced changes in rat lung vascular leak would be mediated by leptin, as measured by lung Evans blue extravasation from the vascular space into surrounding lung tissue.

In our experiments, rats receiving LPS demonstrated significantly higher concentrations of Evans blue leakage than vehicle (saline)-treated rats (figure 1), findings that were commensurate with lung injury. The possible minimum saline-induced Evans blue leakage was always subtracted from the obtained data in the presence of LPS. Pretreatment with aminoguanidine (AG, 20 mg/kg i.p), 2-aminopurine (2-AP, 20 mg/kg i.p.) and subjecting to food deprivation (FD, for 48 h, with water ad libitum) reduced the LPS-induced vascular leak, measured as Evans blue extravasation in lung tissue, by 41.70%, 21.39% and, respectively, 52.92% as means, when compared to LPS (10µg/kg, 100µl aliquots) administered alone (figure 1). There are significant differences ($P<0.05$) either between AG and 2-AP on one hand, and AG and FD on the other hand, as well as between 2-AP and FD pretreatments.

In the rat, plasma leakage in various vascular beds, including the whole lung, occurs after administration of LPS. LPS-induced microvascular plasma leakage in many organs is usually associated with an enhanced formation of nitric oxide (NO) after the induction of iNOS. Aminoguanidine is a relatively selective inhibitor of inducible nitric oxide synthase (iNOS). The important reduction of LPS-induced Evans blue leakage by aminoguanidine (AG, 20 mg/kg i.p) in our experiments is in high accordance with the previous experiments, involving the inhibition of NO production (Tulić et al., 2000).

Besides aminoguanidine, a relatively slight but significant inhibition of LPS-induced Evans blue leakage in rats was achieved by 2-aminopurine (2-AP, 20 mg/kg i.p.) pretreatment. As known, 2-AP is widely used as a specific inhibitor for double stranded-RNA dependent protein kinase (PKR).

One of the mechanisms of 2-aminopurine action in our experiments might be attributed to the inhibition of NO production in macrophages, as previously described. Thus, it was reported that 2-AP can inhibit LPS-stimulated NO production through the prevention of interferon (IFN)-β production. 2-AP significantly inhibited NO production in LPS-stimulated RAW 264 murine macrophage cells. 2-AP also reduced the expression of IFN-β and IFN-inducible genes, such as IFN-β-inducible protein (IP)-10 and immune-responsive gene (IRG)-1, and the inducible type of NO synthase (iNOS) mRNA in response to LPS. The addition of exogenous IFN-β restored 2-AP-inhibited NO production in response to LPS. On the other hand, there was only partial inhibition by 2-AP of NF-κB activation, IL-6 mRNA expression and tumor necrosis factor (TNF)-α production. These results suggested that 2-AP inhibited LPS-induced IFN-β production by preventing Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β (TRIF)-dependent signaling rather than myeloid differentiation factor (MyD) 88-dependent signaling, resulting in the inhibition of NO production (Sugiyama et al., 2004).

Another mechanism of 2-aminopurine action in our experiments might be represented by the inhibition of leptin receptor signal transduction. Leptin is an important circulating signal for the regulation of food intake and body weight. The effect of 2-AP on leptin

signal transduction was recently investigated. 2-AP dose-dependently inhibited the leptin-induced phosphorylation of signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) in HEK293 cells stably transfected with the Ob-Rb leptin receptor. The investigators observed only slight inhibition of leptin-induced STAT3 activation by purine treatment, indicating that the inhibitory effect would be dramatically enhanced in the presence of an amino group. 2-AP did not inhibit PMA-induced ERK activation, indicating that the effect may be leptin-signal specific. The inhibitory effect of 2-AP was not mediated by newly synthesized protein because the inhibitory effect of 2-AP on leptin-induced STAT3 activation was not abrogated in the presence of the protein synthesis inhibitor cycloheximide. Interestingly, leptin did not induce PKR activation, suggesting that the effect of 2-AP on the leptin signal may be independent of PKR. Finally, 2-AP inhibited leptin-induced phosphorylation of the Ob-Rb leptin receptor. These results provide evidence of a novel action of 2-AP, i.e., inhibition of the activation of leptin signal transduction at the level of the Ob-Rb leptin receptor (Hosoi et al., 2006).

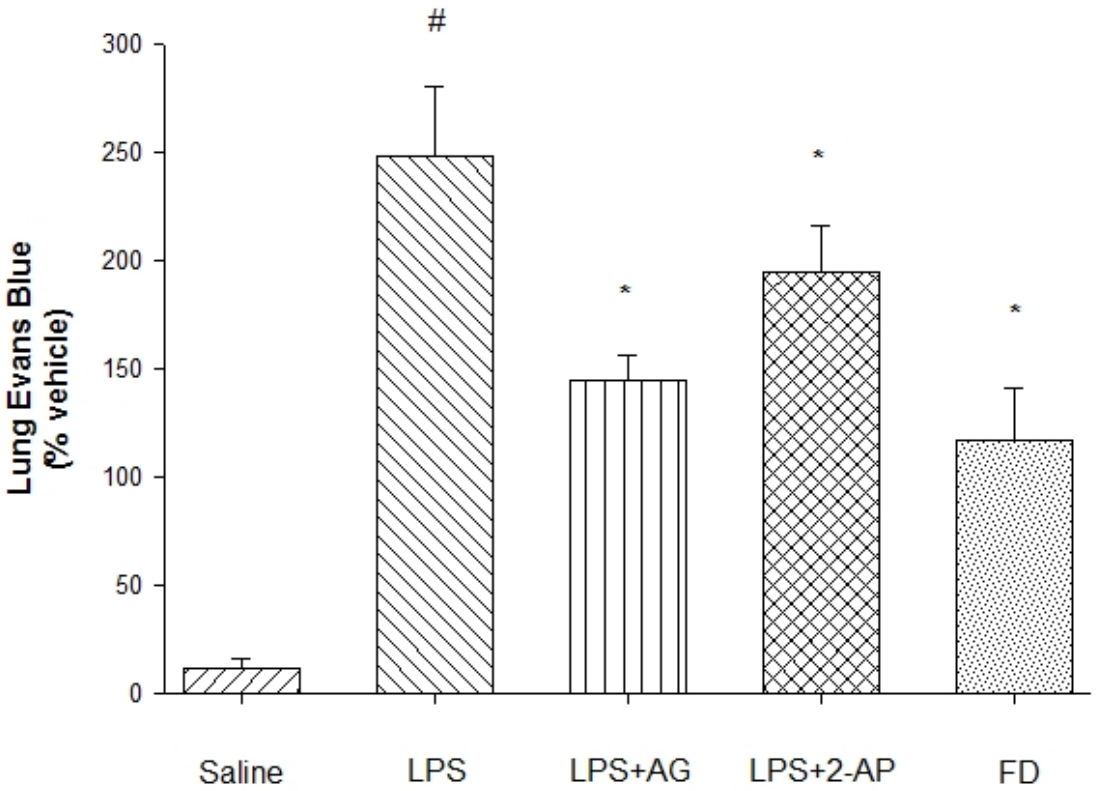

Fig. 1 Effects of aminoguanidine (AG), 2-aminopurine (2-AP) and food deprivation for 48 h (FD, water ad libitum) on increased lung vascular leak induced by LPS. Evans blue dye (20 mg/kg) was injected i.p. 30 min before rats were sacrificed, and its subsequent concentrations in lung tissues were assessed. Whereas Evans blue concentrations were markedly increased in response to LPS (#P<0.001 relative to vehicle control), significant reductions were observed with AG, 2-AP and FD treatments (*P<0.05 relative to LPS alone, n=6 in each group)

To further demonstrate the involvement of leptin in LPS-induced Evans blue leakage, in one series of experiments the rats were subjected to food deprivation for 48 h, having water ad libitum. This was done in agreement with previous experiments in which was examined the contribution of leptin to the process of neutrophils recruitment into the brain in leptin-deficient (ob/ob), -resistant (db/db) and wild-type (WT) mice injected intraperitoneally with a septic dose of LPS. This treatment induced a dramatic increase in the number of neutrophils entering the brain of WT mice, an effect that was almost totally abolished in the mutant mice and correlated with a significant reduction in the mRNA levels of interleukin-1□, intracellular adhesion molecule-1 (ICAM-1) and neutrophil-specific chemokines. These effects were reversed with leptin replenishment in ob/ob mice leading to recovery of neutrophil recruitment into the brain. Moreover, 48 h food deprivation in WT mice, which decreased circulating leptin levels, attenuated the LPS-induced neutrophil recruitment as did a single injection of an anti-leptin antiserum 4 h before LPS treatment in WT mice. These results provided the first demonstration that leptin has a critical role in leukocyte recruitment to

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the brain following severe systemic inflammation with possible implications for individuals with altered leptin levels such as during obesity or starvation (Rummel et al., 2009). In our experiments, food deprivation for 48 h (that is equivalent to leptin plasma concentrations high decrease) largely reduced the LPS-induced Evans blue leakage in lungs of treated rats. Thus, we can conclude that leptin is at least partially mediating the LPS-increased pulmonary permeability in rats.

The role of the adipose tissue in immunity has recently emerged, and there is now ample evidence that this role is elucidated by a number of cytokine-like hormones produced by adipocytes-called adipokines. The most relevant adipokines are leptin, adiponectin and visfatin, and all have marked effects on metabolic and immune function (Matarese et al., 2007). The adipocyte-derived hormone leptin has been shown to regulate the immune response, innate and adaptive response, both in normal and pathological conditions. The role of leptin in regulating immune response has been assessed in vitro as well as in clinical studies. It has been shown that conditions of reduced leptin production are associated with increased infection susceptibility. Conversely, immune-mediated disorders such as autoimmune diseases are associated with increased secretion of leptin and production of proinflammatory pathogenic cytokines. Thus, leptin is a mediator of the inflammatory response (Fernández-Riejos et al., 2010).

The prevalence of obesity has increased dramatically worldwide, predisposing individuals to an increased risk of morbidity and mortality due to cardiovascular disease and type 2 diabetes. Less recognized is the fact that obesity may play a significant role in the pathogenesis of pulmonary diseases through mechanisms that may involve proinflammatory mediators produced in adipose tissue that contribute to a low-grade state of systemic inflammation. In animal models, inflammatory responses in the lung have been shown to influence the production of the adipocytokines, leptin and adiponectin, cytokines, acute phase proteins, and other mediators produced by adipose tissue that may participate in immune responses of the lung. An increased adipose tissue mass may also influence susceptibility to pulmonary infections, enhance pulmonary inflammation associated with environmental exposures, and exacerbate airway obstruction in preexisting lung disease. An increased understanding of the mechanisms by which obesity influences pulmonary inflammation may facilitate the development of novel therapeutic interventions for the treatment of lung disease (Mancuso, 2010).

Male Wistar rats were treated with saline or recombinant rat leptin via the tail vein. Leukocyte trafficking in mesenteric venules was quantified by intravital microscopy. Treatment with leptin resulted in 3- and 5-fold increases in rolling and firm adhesion, respectively. Compared to vehicle controls, leptin enhanced mRNA levels of IL-6 (8-fold) and macrophage chemoattractant protein (MCP-1, 5-fold) in mesenteric adipose tissue (MAT). Similar increases in these markers were observed in mesenteric venules and in liver. Finally, the direct effect of leptin was assessed in C3A hepatocytes treated with leptin for 24 hours. Consistent with observations in vivo, production of ICAM-1, MCP-1, and IL-6 by hepatocytes was increased significantly. These findings support the hypothesis that leptin directly initiates inflammation in the local environment of mesenteric adipose tissue as well as systemically (Allman et al., 2009).

To verify the possibility for leptin to mediate the LPS-increased Evans blue leakage in rat lung, in one series of experiments we administered leptin in 100µl aliquots (10µg/kg) by intranasal instillation. As in the case of LPS, the possible minimum saline (vehicle)-induced Evans blue leakage was always subtracted from the obtained data in the presence of leptin. As seen in figure 2 leptin induced Evans blue leakage in rat lung that represents 74.38% as mean when compared to that induced by LPS. When we administered both LPS and leptin by intranasal instillation (10µg/kg, 100µl aliquots each) the obtained effect wasn't significantly different from that induced by LPS alone (personal observation, data not shown).

Since we didn't measure the leptin plasmatic levels induced by LPS challenge we were not able to entirely correlate LPS- and leptin-induced Evans blue leakage in rat lungs. Furthermore, the pretreatment with 2-AP (20 mg/kg i.p.) reduced the leptin-induced pulmonary permeability by 76.40% as mean value. The 2-AP effects might be firstly attributed to the inhibition of leptin receptor signal transduction (Hosoi et al., 2006) and secondly to the inhibition of NO production in rat lung (Sugiyama et al., 2004). This last mechanism is supported by the reduction of leptin-induced Evans blue leakage (41.95% as mean), obtained through the administration in pretreatment of aminoguanidine (AG, 20 mg/kg i.p.), a relatively selective inhibitor of iNOS. Since the inhibitory effects of 2-AP are with 59.34% as mean higher than that induced by AG we were able to conclude that leptin is at least partially mediating the LPS-increased pulmonary permeability in rats. Leptin lung concentrations might thus increase as an early event of LPS challenge. Furthermore, leptin-increased pulmonary permeability in rats is partially achieved through NO production.

CONCLUSIONS

The appetite suppressing hormone leptin has emerged as an important modulator of immune function and is now considered to be a critical link between energy balance and host defense responses to pathogens.

In our experiments, pretreatment with aminoguanidine, 2-aminopurine and subjecting to food deprivation for 48 h reduced the LPS-induced vascular leak, measured as Evans blue extravasation in lung tissue, when compared to LPS administered alone. The important reduction of LPS-induced Evans blue

leakage by aminoguanidine in our experiments is in accordance with the others previous experiments, involving the inhibition of NO production. One of the mechanisms of 2-aminopurine action might be attributed to the inhibition of NO production in macrophages. Another mechanism of 2-aminopurine action in our experiments might be represented by the inhibition of leptin receptor signal transduction.

Food deprivation for 48 h (that is equivalent to leptin plasma concentrations highly decreasing) largely reduced the LPS-induced Evans blue leakage in lungs of treated rats. Leptin itself induced an important Evans blue leakage in rat lung when was administered by intranasal instillation. These leptin effects were largely reduced by aminoguanidine and, especially, 2-aminopurine. Thus, leptin is responsible for the LPS-increased pulmonary permeability in rats through the activation of leptin receptors.

Finally, we can conclude that leptin is at least partially mediating the LPS-increased pulmonary permeability in rats. Leptin lung concentrations might increase as an early event of LPS challenge. Furthermore, leptin effects might be partially realized through NO production after leptin receptors activation.

All the above mentioned effects and mechanisms might be further explored using a specific antagonist for leptin receptors when will be available.

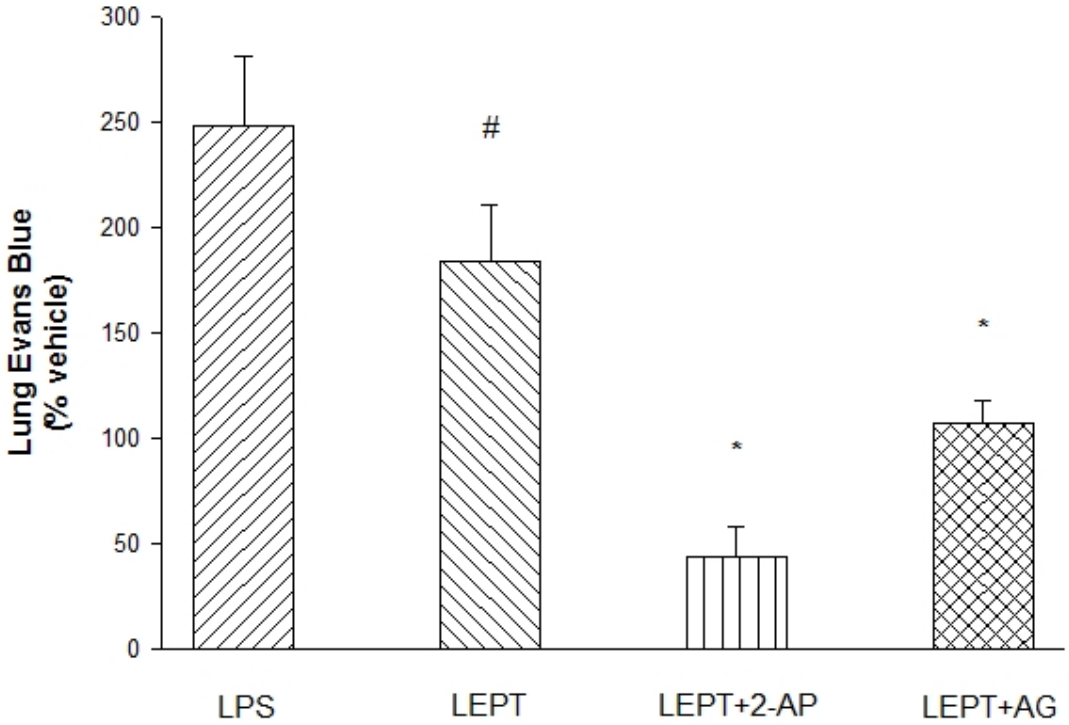

Fig. 2: Significant effects of 2-aminopurine (2-AP) and aminoguanidine (AG) on increased lung vascular leak induced by leptin (LEPT). Evans blue dye (20 mg/kg) was injected i.p. 30 min before rats were sacrificed, and its subsequent concentrations in lung tissues were assessed (*P<0.05 relative to leptin alone, n=6 in each group). On the other hand, Evans blue concentrations were markedly increased in response to leptin treatment (P<0.001 relative to vehicle control), although its effects are lower as compared to LPS administration (#P<0.05 relative to LPS alone, n=6 in each group).

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