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Role of insulin on PGE_2 generation during LPS-induced lung inflammation in rats

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Abstract

Alterations in arachidonic acid (AA) metabolism have been reported to occur in diabetes mellitus. The present study was carried out to verify if these alterations are due to the relative lack of insulin or to high levels of blood glucose. Male Wistar rats were rendered diabetic by alloxan injection (42 mg/kg, i.v.), 10 or 30 days before the experiments. Some diabetic rats received a single dose (4 IU, s.c.) of NPH insulin 2 h before an intratracheal instillation of lipopolysaccharide (LPS, 750 μg) or saline. Six hours after LPS challenge, the following parameters were analysed: blood glucose levels, total and differential leukocyte counts in bronchoalveolar lavage (BAL) fluid; linoleic acid and AA content in blood neutrophils (HPLC), and levels of prostaglandin (PG)E_2 in BAL (ELISA). Relative to controls, a reduced number of neutrophils (18%) and decreased amounts of PGE_2 (40%) were observed in the BAL fluid of diabetic rats in response to LPS. A single dose of insulin was not able to reduce blood sugar levels to normal values, but instead resulted in the normalization of both leukocyte migration to the lungs and levels of PGE_2. Accordingly, these abnormalities might be primarily linked to a continuing insulin deficiency rather than to secondary hyperglycaemia occurring in the diabetic rat. In conclusion, data presented suggest that insulin might regulate neutrophil migration and generation of PGE_2 during the course of acute lung injury induced by LPS.

Keywords: Lipopolysaccharide; Acute lung injury; Neutrophils; Arachidonic acid; Insulin; Diabetes mellitus

Introduction

Evidences have been accumulated that insulin is involved with the inflammatory response (Garcia-Leme, 1989; Garcia-Leme and Farsky, 1993). Alterations that have been reported to occur during inflammation in diabetes mellitus include the following: decreased microvascular responses to inflammatory mediators (Fortes et al., 1984); decreased protein leakage and edema formation (Llorach et al., 1976; Gamsé and Jancsó, 1985; Woie and Reed, 1997); reduced mast cell degranulation (Casacó et al., 1991; Diaz et al., 1996; Cavalher-Machado et al., 2004); decreased leukocyte–endothelial interactions and reduced accumulation of leukocytes in inflammatory lesions (Pereira et al., 1987; Sannomiya et al., 1990, 1997; Fortes et al., 1991; Cruz et al., 2000, 2003; Zanardo et al., 2003); lowered airway inflammation to antigen challenge (Vianna and Garcia-Leme, 1995; Belmonte et al., 1998); reduced superoxide generation and TNF-α release by leukocytes upon exposure to lipopolysaccharise (LPS) (Boichot et al., 1999); increased leukocyte apoptosis after stimulation with endotoxin (Tennenberg et al., 1999), and reduction in lymph node retention capacity (Moriguchi et al., in press). These abnormalities might contribute to the increased susceptibility and severity of infections in diabetic patients.

Alterations in polymorphonuclear leukocyte functions and arachidonic acid (AA) metabolism in diabetes have been also described. These include impaired uptake of AA (Watts et al., 1982), reduced levels of cyclooxygenase (COX)-1 (Fang et al., 1997), and diminished eicosanoid production (Watts et al., 1982; Qvist and Larkins, 1983; Hui et al., 1989; Greco et al., 1991; Nakagawa and Ishii, 1996). In addition, Qvist and Larkins (1983) verified that the production of prostaglandin

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(PG) E_2 and thromboxane (Tx) B_2 is reduced in leukocytes from diabetic patients in response to *Staphylococcus aureus* or zymosan. It remains, however, to be determined if the abnormalities in AA metabolism are due to the relative lack of insulin or are associated to high levels of blood glucose. The present study was carried out to address this point. Rats were injected with alloxan to induce the diabetic state. After 10 or 30 days, some of the diabetic rats were given insulin before responses to LPS were evaluated. The following parameters were examined: cell composition of bronchoalveolar lavage (BAL) fluid, linoleic acid (LA) and arachidonic acid (AA) content in blood neutrophils by using HPLC, and levels of PGE_2 in BAL fluid by ELISA. LA and AA are involved in generation of PGE_2.

Materials and methods

Animals

Male Wistar rats weighing 200±20 g (about 2 months of age) at the beginning of the experiments were used. The rats were allowed access to food and water ad libitum and maintained at 23±2 °C under a cycle of 12-h light/12-h darkness. The Animal Ethical Committee of the Institute of Biomedical Sciences of the University of Sao Paulo approved the experimental procedure of this study.

Induction and treatment of diabetes mellitus

Diabetes mellitus was induced by the intravenous injection of alloxan (42 mg/kg) dissolved in physiological saline 3, 10 and 30 days before the experiments. Control rats were injected with physiological saline. The presence of diabetes was verified by blood glucose concentrations >200 mg/dL, determined with the aid of a blood glucose monitor (Advantage®, Eli Lilly, Sao Paulo, SP, Brazil), in samples obtained from the cut tip of the tail. Ten or thirty days after alloxan treatment, some diabetic rats were treated with a single dose (4 IU, s.c.) of neutral protamine Hagedorn (NPH) insulin (Biobrás, Montes Claros, MG, Brazil) 2 h before the intratracheal instillation procedure.

LPS instillation

The animals were anesthetized by an intraperitoneal injection (400 mg/kg) of chloral hydrate (LabSynth, Sao Paulo, SP, Brazil) and the trachea exposed through a midline ventral incision of approximately 0.5 cm length in the neck. Saline (0.4 mL) containing 750 μg of *Escherichia coli* LPS (serotype 055:B5, Sigma Chemical Co., St. Louis, MO, USA) was instilled into the airways. A dose–response curve to the intratracheal instillation of LPS (100 to 1000 μg) was carried out. Control animals received saline only by the same route. The incision was closed with cotton suture. After the initial dose of chloral hydrate and the intratracheal instillation procedure, it takes 60 to 90 min for the animals to re-awaken.

Bronchoalveolar lavage procedure

BAL was performed 6 h afte the intratracheal administration of LPS or saline. The animals were anesthetized as described above and the abdominal cavity was opened for blood collection and exsanguination from the abdominal aorta. The lungs were lavaged by instillation of 5 mL phosphate-buffered saline (PBS) at room temperature through a polyethylene tube (1 mm in diameter) inserted into the trachea. The procedure was repeated 4 times. The BAL fluid was not used if the retrieved volume was less than 85% of the 25 mL instilled. Low recovered volumes indicate that substantial amount of cells still remains in the lung and so it does not reflect the inflammatory process in course. Total cell counts were determined by using an automatic hemacytometer (CC510; CELM, Sao Paulo, SP, Brazil). Differential cell counts were carried out on stained films under oil immersion microscopy. A total of 100 cells were counted and classified as neutrophils, eosinophils or mononuclear cells on the basis of normal morphological criteria.

Neutrophil separation

After 6 h of LPS instillation, neutrophils were isolated from blood collected from the abdominal aorta using syringes containing heparin, and the polymorphonuclear leukocytes (PMN) isolated by Ficoll-dextran sedimentation (Boyum, 1968). Briefly, blood samples diluted (1:1) in sterile PBS were layered on an equal volume of Ficoll-Hystopaque (density 1.077 g/mL). After centrifugation (400×*g*, 45 min, room temperature), the superior mononuclear-rich layer was discarded and red blood cells were separated from the neutrophil-rich pellet by the addition of 2 mL dextran (6%) for 1 h at 37 °C. Cells were then washed and the remaining erythrocytes removed by hypotonic lysis. Neutrophils accounted for 95% of the cells, with 5% of mononuclear cell contamination.

HPLC equipment and chromatographic conditions

The HPLC used was purchased from Shimadzu (Kyoto, Japan). The system consists of two pumps (model LC-10AD), auto injector (model SIL-10^A), G-ODS shim-pack pre-column (2 cm × 4.6 mm × 5 μm) and analytical shim-pack column CLC-C8 (25 cm × 4.6 mm × 5 μm). The separation of fatty acids was carried out using an acetonitrile (EM Science-Merck, Darmstadt, Germany) gradient (77–90%) at 1.0 ml/min. The results were processed using a Class LC-10^A software, version 1.4. The fatty acids used as standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA): linoleic (C18:2), arachidonic (C20:4), and margaric (C17:0) acids.

Lipid extraction and saponification

Total lipids of blood neutrophils were extracted using the Folch method (Folch et al., 1957) and saponified afterwards. Margaric acid (5 mg/mL) was added to the samples (250 μg) to estimate the loss during the process. After homogenization for

30 s with chloroform and methanol (2:1 v/v) (EM Science-Merck, Darmstadt, Germany), the samples were centrifuged and filtered twice. The procedure was repeated with aqueous solution, and after the extraction, the aqueous phase was discharged. The organic phase was then evaporated in a speed-vac Sc110 (Savant). The lipids were saponified using 1.4 mL of an alkaline methanol solution (1 mol/mL NaOH in 90% methanol) at 37 °C, for 2 h, in a shaking water bath. Afterwards, the alkaline solution was acidified to pH 3.0 with HCl solution (1 mol/mL). Fatty acids were then extracted twice with 1 mL hexane (EM Science-Merck, Darmstadt, Germany). The samples were dried and kept under a nitrogen atmosphere and protected from light at −20 °C until the fatty acid determination (Beyer and Jensen, 1989; Hamilton et al., 1992).

Extraction and quantification of PGE_2

The cell-free BAL fluid was acidified with HCl 1 N to pH 3.4–3.6 and passed slowly through an octadecylsilyl silica column (Sep Pak C 18 column), pre-washed with 10 mL ethanol and 10 mL water. After washing the column with 10 mL water and 1 mL ethanol (35%), the eicosanoid was eluted

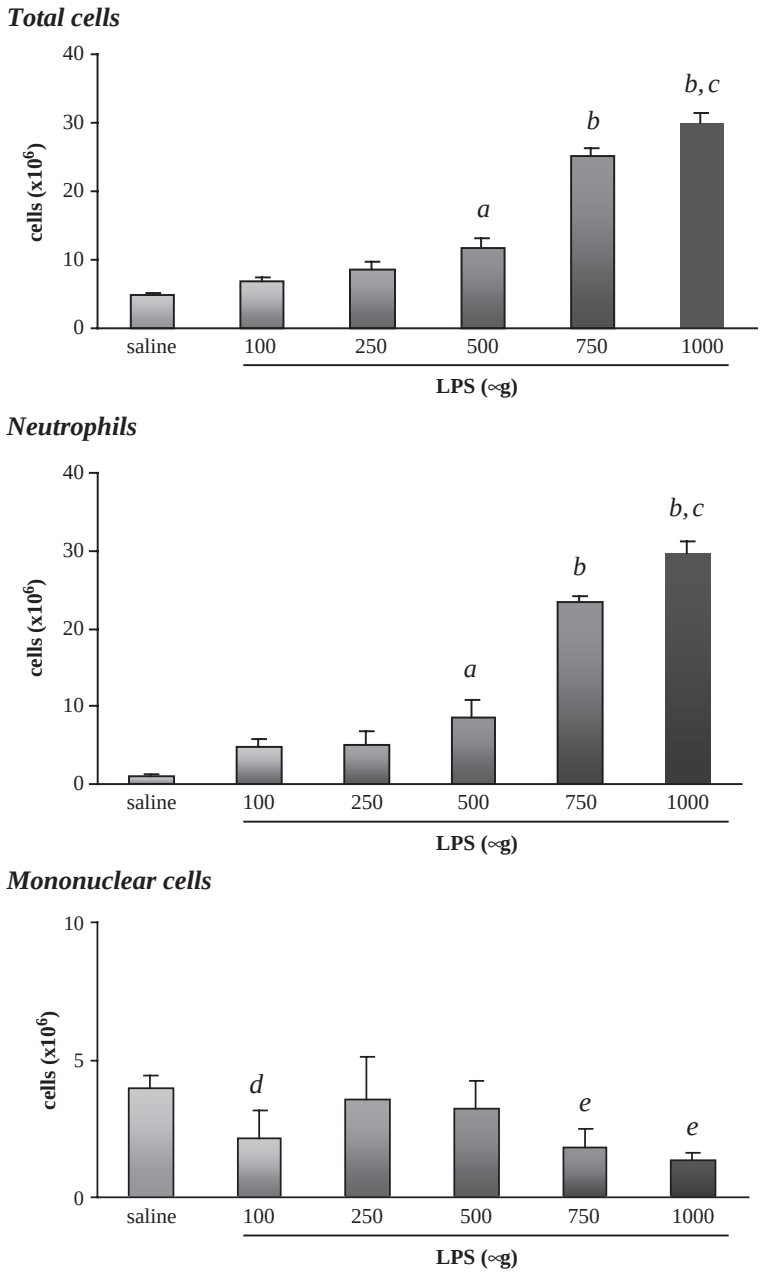

Fig. 1. Cell composition of bronchoalveolar lavage fluid in response to increasing doses (100–1000 μg) of *E. coli* lipopolyssacharide (LPS) dissolved in saline (400 μL), or the equivalent volume of saline, instilled into the airways of control non-diabetic rats. The bronchoalveolar lavage was carried out 6 h after LPS instillation. Values are presented as means ± S.E.M. from 5 to 8 animals in each group. $^{a}p<0.001$ vs. saline, $^{b}p<0.001$ vs. saline, 100, 250 and 500 μg, $^{c}p<0.01$ vs. 750 μg, $^{d}p<0.05$, $^{e}p<0.01$ vs. saline.

from the column with 2 mL absolute ethanol and the samples dried under a stream of nitrogen. PGE_2 concentration was determined by using ELISA kit (Cayman Chemical Co., Ann Arbor, MI, USA). The sensitivity of the assay was 5 pg/mL.

Statistical analysis

Data are expressed as means ± S.E.M. Statistical comparisons were made by the Student's *t*-test or one-way analysis of variance followed by the Tukey–Kramer multiple comparisons test. Data were considered statistically significant if p values were lower than 0.05.

Results

Results illustrated in Fig. 1 showed that there were significant increases in the number of leukocytes harvested from the BAL fluid of the animals instilled with 500 μg up to 1000 μg of LPS 6 h after instillation as compared with saline instilled rats. Neutrophils accounted for more than 90% of the cells migrated into the airways, the remaining cells were mononuclears. In the present study, the dose of LPS selected to perform the following experiments was 750 μg.

In order to study the role of insulin on acute lung injury induced by LPS, rats were rendered diabetic by the injection of alloxan and evaluated 10 and 30 days thereafter. Characteristics of the groups studied are summarized in Table 1. Relative to matching controls, 10- and 30-day diabetic rats exhibited a significant reduction in body weight gain during the experimental period. Blood glucose levels were significantly elevated compared with matched control values. The administration of a single dose (4 IU) of NPH insulin, 2 h before the intratracheal instillation procedure, significantly reduced blood glucose levels but it was not sufficient to reduce glycemia to control values.

The intratracheal instillation of LPS significantly increased the cell number in BAL fluid of control rats (Table 2). Neutrophil counts were elevated, the total cell number being approximately 6 times the values attained in saline instilled rats. The number of mononuclear cells did not change and eosinophils were absent. In contrast, leukocyte counts in the BAL fluid of 10-day diabetic rats after LPS instillation was markedly reduced. Treatment with a single dose (4 IU) of NPH insulin before LPS instillation restored the capacity of diabetic animals to respond to LPS. Moreover, the number of neutrophils recovered from the BAL fluid increased by 65% over values attained in control rats treated intratracheally with LPS (Table 2). Similar results were observed in rats rendered diabetic by the injection of alloxan 30 days before the experiments (Table 2). Impaired responses to LPS is an early event in diabetes mellitus as indicated by experiments performed 3 days after alloxan injection. There was a decrease in the number of neutrophils harvested from the BAL fluid of these animals. Values (mean ± S.E.M.) were as follows: 67.8 ± 6.8 cells $\times 10^6$ for control ($n = 4$), and 27.8 ± 4.4 cells $\times 10^6$ for diabetic rats, both groups exposed to LPS instillation.

Compared to saline instilled rats, blood neutrophils from control rats exposed to LPS exhibited 50% reduction in the content of AA and 30% reduction in the content of LA (Fig. 2A and B). Similar changes to LPS was observed in the content of LA in blood neutrophils from diabetic rats. The content of AA was 30% reduced in neutrophils from diabetic rats instilled with saline, with no change after LPS stimulation. Treatment of diabetic animals with a single dose (4 IU) of NPH insulin increased the content of both fatty acids, LA and AA, to the levels found in neutrophils from control animals instilled with saline (Fig. 2A and B).

Evaluation of PGE_2 concentration in the BAL fluid of the animals showed that whereas in control rats there was a remarkable increase in PGE_2 levels after instillation with LPS, in diabetic rats levels of PGE_2 did not change after LPS stimulation (Fig. 3). Treatment of diabetic rats with insulin

Table 1
Body weight gain and blood glucose levels of the groups studied

Groups	Body weight gain (g)	Blood glucose (mg/dL) before/after insulin treatment
Control (10 days)	49 ± 3 (14)	87 ± 1 (14)
Diabetic (10 days)	2 ± 5[a] (14)	416 ± 8[a] (11)
+Insulin		512 ± 42[a]/330 ± 118[b] (3)
Control (30 days)	109 ± 6 (28)	90 ± 5 (28)
Diabetic (30 days)	−6 ± 7[a] (36)	474 ± 25[a] (24)
+Insulin		473 ± 29[a]/318 ± 40[c] (12)

Rats were rendered diabetic by the injection of alloxan (42 mg/kg, i.v.) and the measurements were carried out 10 and 30 days afterwards. Insulin (NPH, 4 IU/rat, s.c.) was administered 2 h before the intratracheal instillation procedure. Values are presented as means ± S.E.M. Figures in parentheses indicate the number of rats used in each group.

[a] $p < 0.001$ vs. control.
[b] $p < 0.05$ vs. values obtained before insulin treatment.
[c] $p < 0.01$ vs. values obtained before insulin treatment.

Table 2
Cell composition of bronchoalveolar lavage fluid

Groups	Treatment	n	Number of cells ($\times 10^6$)		
			Total	Mononuclear cells	Neutrophils
Control (10 days)	Saline	6	4.5 ± 0.2	4.1 ± 0.4	0.4 ± 0.3
	LPS	8	29.6 ± 2.5[a]	2.1 ± 0.5	27.5 ± 2.5[a]
Diabetic (10 days)	Saline	5	4.6 ± 0.5	4.0 ± 0.5	0.6 ± 0.2
	LPS	6	9.9 ± 1.4[b]	4.3 ± 0.9	5.6 ± 2.1[b]
+Insulin	LPS	3	49.1 ± 12.7[cc]	3.4 ± 0.8	45.7 ± 12.1[d,e]
Control (30 days)	Saline	5	6.2 ± 0.3	5.7 ± 0.6	0.5 ± 0.6
	LPS	7	33.3 ± 4.0[a]	1.8 ± 0.1	31.5 ± 4.4[a]
Diabetic (30 days)	Saline	3	5.5 ± 1.1	4.0 ± 0.5	1.5 ± 0.6
	LPS	3	7.8 ± 1.3[c]	3.1 ± 1.5	4.7 ± 2.8[c]
+Insulin	LPS	4	50.8 ± 11.5[c,e]	3.3 ± 1.3	47.5 ± 10.4[c,e]

Rats were rendered diabetic by the injection of alloxan (42 mg/kg, i.v.) and the measurements were carried out 10 and 30 days afterwards. Insulin (NPH, 4 IU/rat, s.c.) was administered 2 h before the intratracheal instillation procedure. Values are presented as means ± S.E.M. n indicates the number of rats used in each group. Eosinophils were not detected.

[a] $p < 0.001$ vs. control saline.
[b] $p < 0.001$ vs. control LPS.
[c] $p < 0.01$ vs. control LPS.
[d] $p < 0.05$ vs. control LPS.
[e] $p < 0.001$ vs. diabetic saline and LPS.

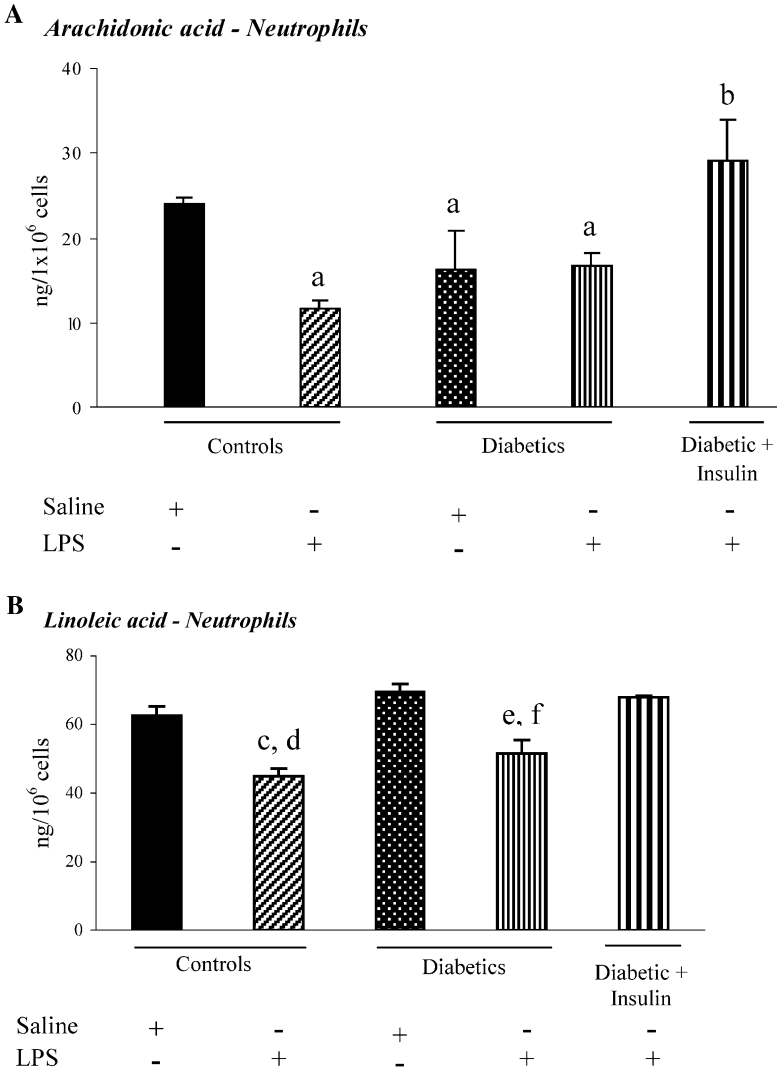

Fig. 2. Arachidonic acid (A) and linoleic acid (B) content in blood neutrophils obtained 6 h after instillation of the rats with LPS (750 μg/400 μL) or with the equivalent volume of saline. Fatty acids were extracted, saponified, and values determined by HPLC. Diabetes mellitus was induced by the injection of alloxan (42 mg/kg, i.v.) 30 days before. Insulin (NPH, 4 IU/rat, s.c.) was administered 2 h before the intratracheal instillation procedure. Values are presented as means ± S.E.M. from 4 animals in each group. $^{a}p<0.05$ vs. other groups, $^{b}p<0.05$ vs. other groups, except for control saline, $^{c}p<0.05$ vs. control saline, $^{d}p<0.01$ vs. diabetic saline and insulin treated diabetic, $^{e}p<0.01$ vs. diabetic saline and $^{f}p<0.01$ vs. insulin treated diabetic.

before LPS resulted in values similar to those in control rats receiving LPS (Fig. 3).

Discussion

Data presented showed that a decreased response to LPS in alloxan-induced diabetic rats is associated with changes in AA metabolism. The suggestion is supported by the following observations: (i) relative to controls, rats rendered diabetic 10 and 30 days before exhibited a sharply reduction, 81% and 84%, respectively, in the number of neutrophils harvested from the BAL fluid after LPS instillation; (ii) in contrast to a remarkable increase in the levels of AA-derived PGE_2 in controls, there were no changes in the content of AA and in PGE_2 levels in diabetic rats instilled with LPS; (iii) the above referred abnormalities were corrected after treatment of diabetic rats with insulin.

LPS induces a local inflammatory response characterized by a massive and acute neutrophil accumulation (Issekutz et al., 1987; Ulich et al., 1991; Alba-Loureiro et al., 2004), followed by a late mononuclear cell and eosinophil influx (Bozza et al., 1991, 1993). After arriving in the lungs these activated leukocytes release cytotoxic substances, including reactive oxygen species, proteolytic enzymes, cytokines, chemokines and eicosanoids, which in turn initiate a chain of events leading to acute inflammation (Lee and Downey, 2001). Neutrophils, the first inflammatory cells recruited in large numbers to the lungs, are capable of synthesizing new enzymes and other proteins. In vitro studies have shown that LPS induces the expression of cyclooxygenase-2 (COX-2) and synthesis of PGE_2 in human neutrophils (Niiro et al., 1997; Pouliot et al., 1998; Maloney et al., 1998). A number of inflammatory agents, including LPS, opsonized bacteria and zymosan, tumor necrosis factor-α, and granulocyte-macrophage colony-stimu-

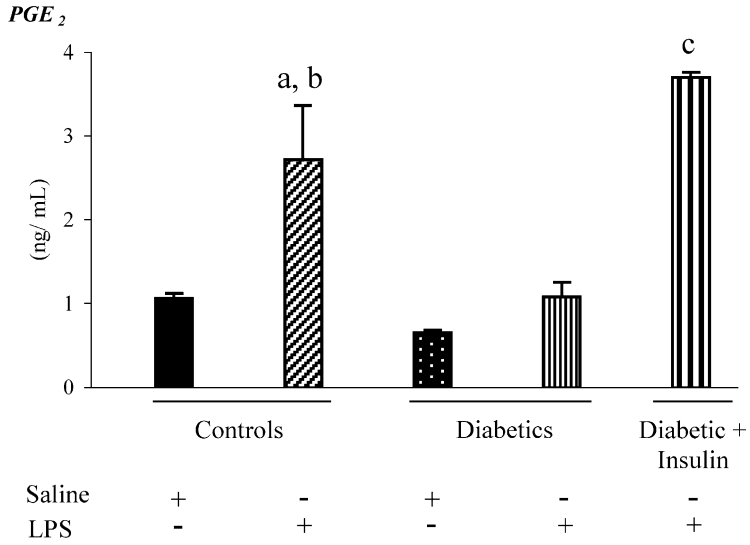

Fig. 3. Levels of PGE_2 in the bronchoalveolar lavage fluid obtained 6 h after instillation of the rats with LPS (750 μg/400 μL) or with the equivalent volume of saline. Diabetes mellitus was induced by the injection of alloxan (42 mg/kg, i.v.) 30 days before. Insulin (NPH, 4 IU/rat, s.c.) was administered 2 h before the intratracheal instillation procedure. Values are presented as means ± S.E.M. from 4 animals in each group. $^{a}p<0.05$ vs. control saline and diabetic LPS; $^{b}p<0.01$ vs. diabetic saline; $^{c}p<0.001$ vs. other groups except control LPS.

lating factor, induce COX-2 protein expression in human neutrophils, through signaling pathways involving transcription and translational events (Pouliot et al., 1998). COX-2 expression and PGE_2 production are also observed after LPS stimulation of peritoneal neutrophils harvested from healthy mice (He et al., 2001). When exposed in vitro to LPS rat whole blood produces significant amounts of PGE_2 through both the induction of COX-2 in blood leukocytes and via COX-1, enzyme constitutively present in platelets (Giuliano and Warner, 2002). In addition, in the rat pleurisy model of acute inflammation, COX-2 protein exists mainly within the infiltrating neutrophils at inflamed lesions (Tomlinson et al., 1994).

In previously published studies a prevalence of linoleic acid, and other fatty acids like palmitic, oleic, and stearic acids in glycogen-elicited peritoneal rat neutrophils (Lopes et al., 1999) has been shown, as well as higher levels of linoleic acid, compared to other fatty acids, in blood neutrophils derived either from LPS or saline instilled rats (Alba-Loureiro et al., 2004). The contents of LA and AA were reduced in blood neutrophils after instillation of the animals with LPS. In this condition, control rats presented a significant increase in the levels of PGE_2 in the BAL fluid, which is compatible with the consumption of LA and AA noted in blood neutrophils. In diabetic rats, the number of neutrophils migrated to the lungs during the course of LPS-induced inflammation was remarkably reduced to approximately 20% of the values attained in controls. Levels of PGE_2 in the BAL supernatant as well as the content of AA in blood neutrophils of diabetic rats did not change after LPS challenge.

The availability of AA in blood neutrophils was already reduced before challenge of diabetic rats with LPS. A substantial decrease in the relative amount of AA in peritoneal neutrophils (Nakagawa and Ishii, 1996) and in platelet phospholipids (Takahashi et al., 1986) was reported to occur in streptozotocin-induced diabetic rats. Alterations in the membrane lipid pattern of a variety of cells and tissues in diabetes affect the generation of AA-derived metabolites and this may contribute to the pathogenesis of some diabetic complications. Examples include the suppression of leukocyte aggregation as a result of decreased production of leukotriene B_4 by calcium ionophore-stimulated neutrophils from diabetic rats (Nakagawa and Ishii, 1996), decreased production of prostacyclin (PGI_2) in vascular tissues of streptozotocin-induced diabetic rats (Gerrard et al., 1980) and an enhancement of glomerular synthesis of vasodilatory prostaglandins (PGE_2 and PGI_2) with concurrent smaller increases in thromboxane A_2 associated with glomerular hyperfiltration characteristic of early diabetes (Craven et al., 1987; DeRubertis and Craven, 1993).

Results presented herein showed that treatment of diabetic animals with insulin increases the content of LA and AA in blood neutrophils and restores the levels of PGE_2 in the BAL fluid during inflammation induced by LPS. A single dose of insulin was not able to reduce blood sugar levels to control values, but instead resulted in the normalization of both generation of PGE_2 and leukocyte migration to the lungs. Therefore, these latter changes might be primarily linked to a continuing insulin deficiency rather than to secondary hyperglycaemia occurring in the diabetic rat. Adequate concentrations of insulin seem to be required for the normal function of endothelial cells and neutrophils during the course of the inflammatory process. The local exudative reaction in an inflammatory lesion, including carrageenin-induced injury (Pereira et al., 1987; Sannomiya et al., 1997; Fortes et al., 1991), allergic pleurisy (Diaz et al., 1996), allergic airway inflammation (Cavalher-Machado et al., 2004; Vianna and Garcia-Leme, 1995), as well as the uptake of macromolecules into lymph nodes and lymphocyte recirculation (Moriguchi et al., in press) depend on the availability of insulin. It is well

established that certain infections occur almost exclusively in diabetic patients, and that many diabetics have a worse prognosis once infection is established (Garcia-Leme, 1989; Leibovici et al., 1991; Geerlings and Hoepelman, 1999; Akbar, 2001). Whether these observations are results of a direct or indirect effect of insulin remain to be determined.

Conclusion

In conclusion, results presented suggest that the reduction in neutrophil migration and in the generation of PGE2, in response to LPS, might represent an aggravating factor for the defence of the diabetic host against infections. Accordingly, effective control with insulin treatment is likely to be relevant during infection in diabetic patients.

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