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ORIGINAL ARTICLE

Low-level laser therapy (LLLT) acts as cAMP-elevating agent in acute respiratory distress syndrome

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Abstract The aim of this work was to investigate if the low-level laser therapy (LLLT) on acute lung inflammation (ALI) induced by lipopolysaccharide (LPS) is linked to tumor necrosis factor (TNF) in alveolar macrophages (AM) from bronchoalveolar lavage fluid (BALF) of mice. LLLT has been reported to actuate positively for relieving the late and early symptoms of airway and lung inflammation. It is not known if the increased TNF mRNA expression and dysfunction of cAMP generation observed in ALI can be influenced by LLLT. For in vivo studies, Balb/c mice (n=5 for group) received LPS inhalation or TNF intra nasal instillation and 3 h after LPS or TNF-α, leukocytes in BALF were analyzed. LLLT administered perpendicularly to a point in the middle of the dissected bronchi with a wavelength of 660 nm and a dose of 4.5 J/cm^2. The mice were irradiated 15 min after ALI induction. In vitro AM from mice were cultured for analyses of TNF mRNA expression and protein and adenosine3':5'-cyclic monophosphate (cAMP) levels. One hour after LPS, the TNF and cAMP levels in AM were measured by ELISA. RT-PCR was used to measure TNF mRNA in AM. The LLLT was inefficient in potentiating the rolipram effect in presence of a TNF synthesis inhibitor. LLLT attenuated the neutrophil influx and TNF in BALF. In AM, the laser increased the cAMP and reduced the TNF-α mRNA. LLLT increases indirectly the cAMP in AM by a TNF-dependent mechanism.

Keywords Acute lung inflammation · Alveolar macrophages · Cyclic AMP · TNF · Low-level laser therapy

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Introduction

LPS stimulates macrophages to produce different mediators, particularly tumor necrosis factor-α (TNF-α), which is considered a primary inflammatory mediator [1, 2]. At the lung level, it has been demonstrated that the release of TNF-α favors the sequestration and migration of neutrophils, which play a critical role in the pathogenesis of acute lung inflammation [3, 4]. In pathophysiological conditions, the generation of TNF-α at high levels leads to the development of inflammatory responses that are hallmarks of many diseases. TNF is implicated in asthma, chronic bronchitis, chronic obstructive pulmonary disease, and acute lung injury and acute respiratory distress syndrome

[5]. Using a model to mimic the local inflammation caused by bacterial particles present in inhaled air, Moraes and coworkers [5] observed that the inhalation of aerosols of LPS led to a significant production of TNF-α detected in the bronchoalveolar lavage fluid (BALF), followed by to the infiltration of neutrophils. These results are in accordance with those obtained after aerosolization or intratracheal administration of LPS in rats or guinea-pigs [6–8], showing a neutrophil infiltration into pulmonary spaces probably due to the LPS-induced TNF-α formation.

A number of in vitro studies have shown that adenosine3':5'-cyclic monophosphate (cAMP)-elevating agents, such as prostaglandin E_2 (PGE_2), can also down-regulate the LPS-induced TNF-α synthesis at the transcriptional level [9–13]. In a rat model of pulmonary inflammation[14] or in mice treated with an intraperitoneal injection of LPS [15], it has been demonstrated that rolipram, a selective inhibitor of phosphodiesterase type 4 (PDE 4), the isoform of the enzyme present in lung tissues, inhibited the TNF-α production. Moraes and colleagues [16] observed that local treatment of mice with dibutyryl cyclic AMP or PGE_2 reduced the TNF-α production and neutrophil recruitment induced by inhalation of aerosols of LPS. Moreover, the level of TNF-α mRNA transcript in alveolar macrophages of rats pretreated with rolipram and challenged with LPS was reduced [16]. These results indicated that the local inflammation induced by LPS could be modulated by systemic treatment with drugs capable of increasing the intracellular cyclic AMP concentration.

Despite anti-TNF-α therapeutic strategies represent an important breakthrough in the treatment of inflammatory diseases [17]; some immunological side-effects may compromise the treatment for individuals more sensitive to these side-effects. Thus, the study of alternative therapies and its respective action mechanism became relevant on the context of news therapies that are more effective, non-invasive, and without side-effects.

Therapeutic advantages of low-level laser therapy (LLLT) as anti-inflammatory agent have been shown by several authors in different pathologies [18–23]. Some reports have referred that laser therapy can interfere positively in order to relief the clinical signals and the late and early symptoms of lung inflammation [24–30]. These authors evidenced that patients treated with LLLT presented a significant improve in bronchial asthma course which result of outpatient treatment and rehabilitation, recovers bronchial sensitivity to sympathomimetics and xantine derivatives, reduces glucocorticoids dose and duration of hospital stay and disability in bronchial asthma. Moreover, these authors confirmed that this therapeutic method is supplementary to the conservative drug therapy in the treatment of acute bronchial obstructive in asthmatic children. Concerning to local irradiation is important stand out that all treatments with LLLT on lung inflammation described above were done in a non-invasive form; thus is reasonable suggest that LLLT would can bring beneficial results on non allergic lung inflammation such as that induced by endotoxemia.

There are some reports in which the principal goal is try to understand which the cellular signaling responsible by LLLT anti-inflammatory action in lung and airways. In ALI elicited by immune-complex reaction in rat, the laser irradiation reduced either the MPO activity as TNF-α level in BALF, beside it attenuated the pulmonary hemorrhagic lesion [31, 32]. In ALI induced by exposition of airways smooth muscle to TNF-α, Aimbire and colleagues [33] demonstrated that LLLT restored the relaxation capacity of rat bronchi segments by increasing cAMP. Still about TNF-α-induced ALI, LLLT reduced the bronchial hyperreactivity by a mechanism that involves the reduction either the calcium sensitivity as inositol triphosphate receptor expression [34]. Moreover, in a model of ALI induced by LPS, Mafra de Lima and coworkers [35] reported the efficiency of LLLT in reducing both the cholinergic hyperreactivity and TNF-α mRNA expression by a mechanism dependent of NF-κB in rat bronchi. In the same experimental model, Mafra de Lima and colleagues [36] demonstrated that LLLT reduces in vivo the lung edema and neutrophil influx; in vitro the laser irradiation attenuated the TNF-α-induced disruption of pulmonary endothelial barrier restoring the endothelial integrity. Despite this, the action mechanism of LLLT in conditions as asthma or endotoxemia is still somewhat understood.

In this perspective, the present study was developed to investigate if LLLT can modulate the acute lung inflammation as cAMP-elevating agent in alveolar macrophages from mice submitted to inhalation of LPS.

Material and methods

Animals

All experiments were carried out in accordance with the guidelines of University Camilo Castelo Branco for animal care. The experiments were carried out on male BALB/c mice weighing 25-30 g each, maintained under standard conditions of temperature (22-25°C), relative humidity (40-60%), and light/dark cycle with access to food and water ad libitum. The animals were provided by Central Animal House of the Research and Development Institute of University Camilo Castelo Branco (UNICASTELO). All mice were placed in a common box and divided randomly into groups of five animals each.

Anesthesia

For experiments with LPS, the mice were pre-anaesthetized with butorfanol (acepromazine, 0.1 mg kg^{-1}, i.p.) and anesthetized with zoletil (chloridrate of zolazepam, 0.1 mg kg^{-1}, i.p.+tiletamine chloridrate 0.1 mg kg^{-1}, i.p.). In the assays with TNF-α, the mice were lightly anaesthetized with halothane.

Induction of acute lung inflammation

In order to induce lung inflammation, male Balb/c mice received inhalation of lipopolysaccharide from Gram-negative bacteria *Escherichia* coli dissolved at 0.3 mg ml^{-1} in 2 ml saline for 10 min or intranasal instillation of 0.5 μg rmTNF.

LLLT

One group of animals received inhaled aerosol of LPS or intranasal TNF, and in addition, scheduled for LLLT using a InGaAlP laser coupled to a 600-μm-diameter optical fiber with the following parameters: 35 mW cw output power, wavelength of 685 nm, energy density of 4.5 J/cm^2, power density of 17.85 W/cm^2, irradiation time of 252 s, and dose of 8.82 J. All mice were irradiated on the skin over the right upper bronchus 15 min after LPS inhalation or TNF intra nasal instillation.

Bronchoalveolar lavage fluid (BALF)

Three hours after LPS aerosol or TNF instillation, airspace was washed and BALF aliquots were employed for leukocyte analysis. For this procedure, the mice were then tracheostomized with a gauge catheter. The lungs were washed with a sterile Hank's balanced salt solution (HBSS), without Ca^{+2} or Mg^{+2} (GIBCO, Grand Island, NY) to provide 4 ml of BALF. Samples were centrifuged at 200 × *g* for 10 min at 25°C and the red blood cells were lysed from the resulting pellet with distilled water (1 ml for 30 s) before restoring osmolarity by adding 10 ml of HBSS. The samples were centrifuged a second time (200 × *g*, 10 min, 25°C) and the resulting pellet was resuspended in 1 ml of HBSS. The total cell number was determined by Trypan Blue (Sigma Chemical, St. Louis, MO) exclusion from an aliquot of cell suspension using a hemocytometer. The data were expressed as BALF (cells ml^{-1}).

Cell isolation and culture

AM were obtained from male BALB/c mice via ex vivo bronchoalveolar lavage fluid (BALF) 30 min or 1 h after of LPS inhalation and resuspended in RPMI 1640 to a final concentration of 10^6 cells ml^{-1}. Cells were allowed to adhere to plates for 1 h (37°C, 5% CO_2) followed by two washes with warm RPMI 1640, resulting in values higher than 99% of adherent cells identified as AM by use of modified Wright-Giemsa stain. Cells were cultured overnight in RPMI 1640 containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin B before use. After this period, the supernatant was collected to detect the cAMP level or the TNF mRNA expression from these cells.

Measurements of intracellular TNF production

The level of TNF in culture supernatants from AM was determined by enzyme-linked immunoassay (ELISA) technique 1 h after inhalation of LPS. Quantitative expression of the membrane-bound TNF was detected by a sandwich ELISA (R&D Systems, USA) method with a rat TNF-α DuoSet kit (Genzyme). The amount of TNF-α measured was expressed in ng ml^{-1}.

Measurement of intracellular cAMP production

An aliquot of 360 μl of glacial ethanol was added to 40 μl of a suspension of freshly collected AM (2.5 × 10^6 cells) from BALF 30 min after LPS or TNF. The concentration of cAMP of each sample was determined by enzyme-linked immunoassay (ELISA) method accordingly to the manufacturer (R&D Systems, USA) 30 min after LPS inhalation. The amount of cAMP measured was expressed in pg/2.5 × 10^6 cells.

Real-time PCR

AM (10^6 cells) were obtained from male BALB/c mice via ex vivo BALF 1 h after inhalation of LPS. PCR was performed on a 7000 Sequence Detection System (ABI Prism, Applied Biosystems, Foster City, CA) using the SYBRGreen core reaction kit (Applied Biosystems). RT-PCR was performed with specific primers for rat TNF 195-305 (GenBank accession number X66539) forward primer 5'- AAATGGGCTCCCTCTATCAGTTC-3' and reverse primer 5'-TCTGCTTGGTGGTTTGCTACGAC- 3'; rat Exon (GenBank accession number D00475) and rat **β-actin** -3474-3570 (GenBank accession number J00691) forward primer 5'- AAGTCCCTCACCCTCCCAAAAG-3' and reverse primer 5'-AAGCAATGCTCACCTTCCC-3') as control. One microliter of RT reaction was used for real-time PCR. The PCR primer efficiencies were calculated using standard curves, and the relative expression levels of TNF-α in real time were analyzed using the 2^{CT} method, presented as the ratio to the expression of the housekeeping gene actin.

Experimental groups

The experiment consisted of 45 male Balb/c mice divided into nine groups as follows:

Control: Animals that received saline
LLLT: Animals irradiated with laser
LPS: Animals challenge with LPS
Rolipram+LPS: Animals pretreated with a phosphodiesterase inhibitor
LLLT+LPS: Animals challenge with LPS and irradiated with laser
TNF: Animals challenge with TNF
Rolipram+TNF: Animals pretreated with a phosphodiesterase inhibitor
LLLT+TNF: Animals challenge with TNF and irradiated with laser
Rolipram+LLLT: Animals pretreated with rolipram and irradiated with laser

Experimental protocol

For experiments with LPS, mice were treated with saline (vehicle) or 20 mg kg^{-1} of rolipram intraperitoneally (i.p.) 45 min before inhalation of LPS. The inhalation procedure has been done as described elsewhere [5]. Briefly, mice inhaled aerosols of LPS dissolved at 0.3 mg ml^{-1} in 2 ml saline (vehicle) for 10 min. For experiments with TNF-α, animals lightly anaesthetized with halothane received directly into their muzzles 50 μl of a solution containing 0.5 μg of rmTNF-α in 0.2% bovine serum albumin (BSA) or saline (vehicle) or 0.2% BSA or inactive rmTNF-α obtained by boiling the solution for 1 h. Three hours after LPS or TNF-α administration, airspaces was washed with saline in order to provide 4 ml of bronchoalveolar lavage fluid (BALF) and aliquots were used for leukocyte analysis. In the BALF from mice exposed to LPS, the TNF-α concentration was measured by ELISA method. In another series of experiment, AM were obtained from male BALB/c mice via ex vivo BALF 30 min after LPS inhalation and then were cultured to measure the cAMP concentration in supernatant. In another assay, AM were collected 1 h post LPS and then the TNF-α mRNA expression from these cells was quantified with the real-time PCR technique. In order to investigate if the LLLT effect on cAMP level released by AM is dependent of the presence of TNF-α, the mice were pretreated intraperitoneally with 1.25 mg kg^{-1} of chlorpromazine, a known TNF-α synthesis inhibitor, 30 min before LPS aerosol.

Statistics

The data were expressed as mean±SEM. The InStat program (GraphPad Software) was used for the statistical analysis. The data were examined by ANOVA followed by the Tukey post hoc test to determine differences between groups, and the results were considered significant when $p<0.05$.

Results

Effect of LLLT or rolipram on lung neutrophil infiltration induced by LPS or TNF-α

Figure 1 represents the number of leukocytes that migrated into mice lung after stimulation of LPS aerosol (Fig. 1a) or rmTNF-α into muzzles (Fig. 1b). Three hours after the aerosol of LPS or rmTNF-α, around 65–70% of the cells present in the BALF were neutrophils, expressed as cells/ml. The data also show that the pre-treatment with rolipram (27.5±12.1 cells ml^{-1}) was more efficient than LLLT (103.1 ±8.5 cells ml^{-1}) in reducing the inflammatory infiltrated when mice were stimulated with LPS (350.2±10.4 cells ml). Differently, the LLLT (29.30±8.5 cells ml^{-1}) have an effect more pronounced than rolipram (150.2±12.1 cells ml^{-1}) when mice were challenged with TNF-α (270.5±10.4 cells ml^{-1}). It is observed that laser irradiation on animals non-challenged with LPS or TNF-α has no effect on neutrophil influx.

Effect of LLLT or rolipram on BALF TNF level from mice inflamed with LPS

An aerosol of 0.3 mg ml^{-1} LPS induced the release of TNF-α (324.0±37.3 pg ml^{-1}) in the BALF of mice 1 h after LPS inhalation. Systemic treatment with 20 mg kg^{-1} rolipram (105.40±12.1 pg ml^{-1}) before LPS inhalation reduced TNF-α levels detected in BALF. Similar to rolipram, the irradiation with low-level laser (100.60 ± 13.5 pg ml^{-1}) also reduced dramatically the TNF-α level in BALF after LPS inhalation. Although in this assay the mice had been inflamed with LPS inhalation, curiously the effect of rolipram on TNF-α level was not better than laser effect when compared with neutrophil infiltration. It is observed that laser irradiation on animals non-challenged with LPS has not effect on TNF-α level in BALF. The results are summarized in Fig. 2.

Effect of LLLT on LPS-induced alveolar macrophages TNF production

Figure 3 represents the effect of rolipram or LLLT on AM from mice stimulated by LPS. Inhalation of LPS caused a marked increase on the levels of TNF-α (1.32±0.15 ng ml^{-1}) detected in culture supernatant after 1 h when compared to control unstimulated cells (0.25±0.11 ng ml^{-1}). The pretreatment with rolipram (0.62±0.17 ng ml^{-1}) or low-level laser

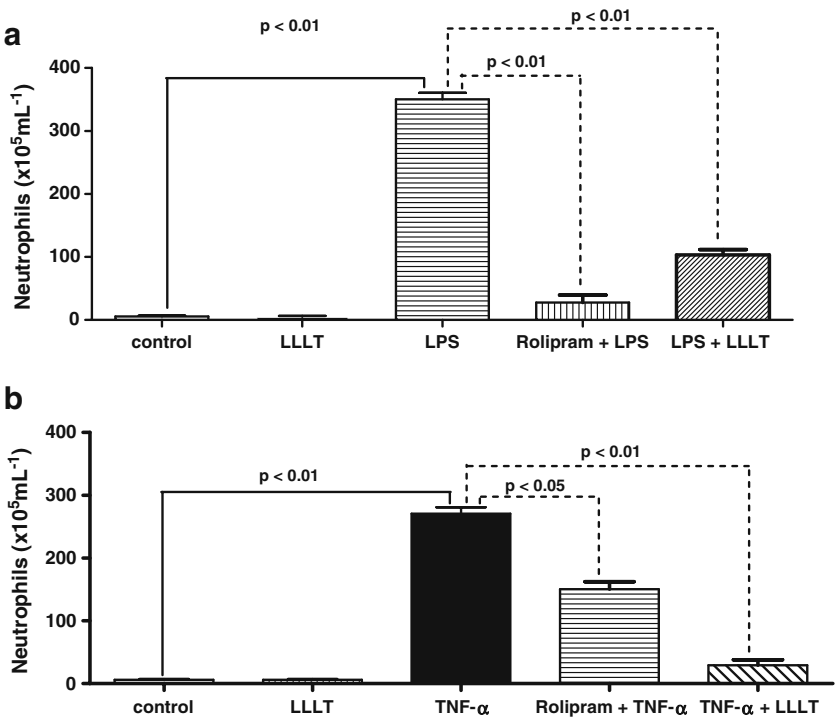

Fig. 1 Effect of LLLT or rolipram on the neutrophil infiltration 3 h after inhalation of LPS (**a**) or instillation of TNF (**b**). Each mouse received saline or 20 mg kg^{-1} rolipram 45 min before inhalation of 0.3 mg ml^{-1} LPS or intra nasal instillation of 0.5 μg of rmTNF. Fifteen minutes after LPS or TNF, the mice were irradiated with 4.5 J/cm^2 for 252 s. BALF were collected 3 h after LPS or TNF and then neutrophils were counted. The results were considered significant when $p<0.05$

(0.60±0.12 ng ml^{-1}) brought levels of TNF-α back to basal values. Surprisingly, the LLLT presented the same level of efficacy in reducing the TNF-α level in AM when compared to rolipram. It is observed that laser irradiation on animals non-challenged with LPS has not effect on TNF-α production by alveolar macrophages.

Effect of LLLT on alveolar macrophages cAMP concentration from mice exposure to LPS or TNF

Figure 4 represents the cAMP concentration measured in freshly collected AM from mice inflamed with LPS or TNF-α treated with rolipram or LLLT. A significant reduction of cAMP amount in AM was observed in mice that received either LPS (210.0±51.2 ng ml^{-1}) or TNF-α (243.0 ± 50.2 ng ml^{-1}) when compared to respective saline groups (385.8±50.2 ng ml^{-1} and 350.2±50.4 ng ml^{-1}). As shown in Fig. 4a, there was a significant increase in the cAMP inside AM from rolipram (710.2 ± 52.1 ng ml^{-1}) or LLLT (532.5±53.5 ng ml^{-1})- treated mice inflamed with LPS when compared to untreated mice (385.8±50.2 ng ml). In the same way, Fig. 4b showed that rolipram (710.2± 52.1 ng ml) or LLLT (681.5±51.5 ng ml^{-1}) also increased the cAMP concentration in mice challenged with TNF-α in comparison to mice inflamed with TNF-α but treated neither rolipram nor LLLT. In mice inflamed with TNF-α, the LLLT effect was very similar to rolipram. It is observed that laser irradiation on animals non-challenged with LPS

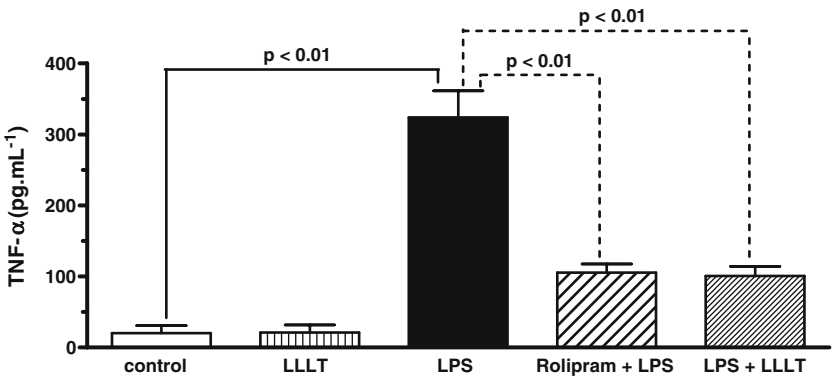

Fig. 2 Effect of LLLT or rolipram on the TNF production in BALF 3 h after inhalation of LPS. Mice were pretreated with saline or 20 mg kg^{-1} rolipram 45 min before inhalation of 0.3 mg ml^{-1} LPS. Fifteen minutes after LPS or TNF the mice were irradiated with 4.5 J/cm^2 for 252 s on the skin over the upper bronchus. BALF were collected 3 h after LPS and then TNF production measured by ELISA method. The results were considered significant $p<0.05$

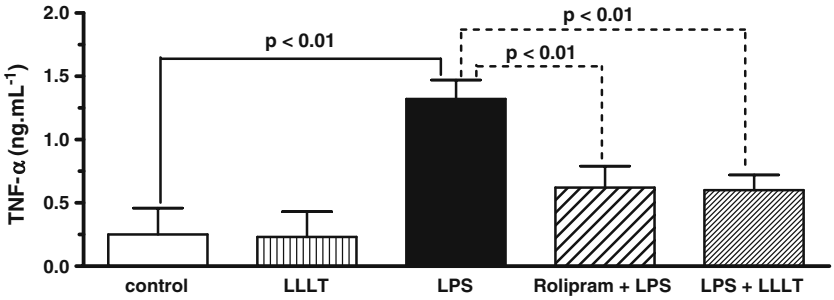

Fig. 3 Effect of LLLT or rolipram on production of TNF by alveolar macrophages from mice challenged by LPS. Mice were pretreated with saline or 20 mg kg^{-1} rolipram 45 min before inhalation of 0.3 mg ml^{-1} LPS. Fifteen minutes after LPS, the mice were irradiated with 4.5 J/cm^2 for 252 s on the skin over the upper bronchus. AM were collected 1 h after instillation of LPS and then the TNF production was measured by ELISA method. The results were considered significant when $p<0.05$

or TNF-α has no effect on cAMP concentration from alveolar macrophages.

Effect of LLLT plus rolipram on alveolar macrophages cAMP concentration from mice exposure to LPS or TNF-α

To investigate if laser anti-inflammatory effect has synergism with rolipram effect in increasing of cAMP production in AM from mice after LPS or TNF-α, the mice were treated with a combination of rolipram and laser. As shown in Fig. 5, the cAMP level into AM from mice inflamed with LPS (277.3±51.2 pg/ 2.5 × 10^5 cells) or TNF-α (210.0± 51.2 pg/ 2.5 × 10^5 cells) was reduced significantly when compared with respective groups of mice not inflamed (385.8±50.4 pg/ 2.5 × 10^5 cells and 390.5±50.5 pg/ 2.5 × 10^5 cells). Figure 5a illustrates that the combination of rolipram and laser potentiated the increase of cAMP (730.4± 50.5 pg/ 2.5 × 10^5 cells) inside AM from mice inflamed with LPS as compared to each isolate therapy. The same beneficial response was obtained in Fig. 5b when the combined therapy with rolipram and laser was applied to increase the cAMP inside AM from mice inflamed by TNF-α (865.8±50.5 pg/ 2.5 × 10^5 cells) compared to mice inflamed but not treated with the combined therapy (210.0± 51.2 pg/ 2.5 × 10^5 cells). These results showed that the phototherapy potentiates the effect of rolipram on cAMP level in AM from mice inflamed.

Effect of LLLT on alveolar macrophage cAMP concentration from mice pretreated in vivo with TNF inhibitor

Figure 6 represents a series of experiments that was set up in order to investigate if laser acts on TNF-α axis and thus indirectly permits the increase of cAMP production by AM. Therefore, we pretreated the mice with chlorpromazine (1.25 mg kg^{-1}, i.p.), an inhibitor of TNF-α synthesis, 30 min before LPS inhalation. As shown in Fig. 6, the additional effect of laser on increase of cAMP concentration induced by rolipram in mice inflamed with LPS was abolished when the mice were pretreated with chlorpromazine.

Effect of LLLT on LPS-induced alveolar macrophages TNF-α mRNA expression

Figure 7 represents that AM collected 1 h after inhalation of LPS from mice pretreated or not with chlorpromazine. This figure indicates high levels of TNF-α mRNA expression (10.40±1.40) differently from AM obtained from mice that had inhaled saline (0.98±0.2). The pre-treatment of mice with rolipram (2.15±0.80) led to a suppression of LPS-induced expression of TNF-α mRNA when compared to the LPS group. The most effective result was obtained with LLLT (1.95 ± 0.85) 15 min after LPS aerosol in comparison with the LPS group not irradiated. Under our experimental conditions, GAPDH mRNA accumulation was not affected by LPS and/or rolipram treatment and/or LLLT, demonstrating that these treatments did not specifically affect the global mRNA production. It is observed that laser irradiation on animals non-challenged with LPS has no effect on TNF-α mRNA expression in alveolar macrophages.

Discussion

The conception of the present manuscript was based on results reported by Moraes and coworkers [16] in which these authors described that the systemic treatment with cAMP-elevating agents can act by down-regulating some essential functions of alveolar macrophages, showing a potential therapeutic advantage to limit inflammation caused by bacterial LPS. Herein we tested if LLLT, an alternative therapy largely used in several pathologies, mainly in skeletal-muscle disorders but that come increasing in treatment of pulmonary inflammation, such as bronchial asthma and pneumonia, could modulate the infiltration of neutrophils and TNF level in a similar form to rolipram.

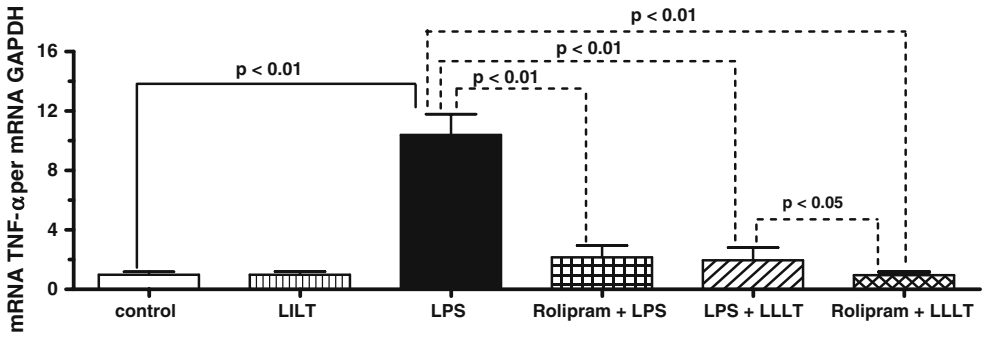

Fig. 4 Effect of LLLT or rolipram on cAMP concentration of alveolar macrophages from mice exposure to LPS (**a**) or TNF (**b**). Each mouse received saline (control) or 20 mg kg^{-1} rolipram 45 min before inhalation of 0.3 mg ml^{-1} LPS or intra nasal instillation of 0.5 μg of rmTNF. Fifteen minutes after LPS, the mice were irradiated with 4.5 J/cm^2 for 252 s on the skin over the upper bronchus. AM were collected 30 min after LPS or TNF and then cAMP concentration was measured by ELISA method. The results were considered significant when $p<0.05$

Actually, the major studies that investigate the clinical efficacy of LLLT on inflammatory diseases, such as asthma, do not evaluate by which mechanism of cellular signaling the laser irradiation is able to attenuate the important features of lung inflammation, such as infiltration of inflammatory cells and airway hyperreactivity. Because of this fact, the investigation of the mechanism of action of LLLT on inflammatory diseases that compromised the respiratory tract became important. Moreover, LLLT is not an invasive therapy, differently of traditional pharmacological therapies, in which the side-effects are a serious problem, especially in individuals with cardiac diseases or treated chronically with corticoids.

To reach the best efficiency of LLLT, it is necessary a better comprehension focused on its cellular signaling as well as in the anti-inflammatory activity of this therapy. One of the questions that preclude the wide employment of the laser therapy in several pathologies is related to the adequate dosimetry to reach the maximal efficiency of this methodology, since the determination of this optimum value is not a trivial procedure. In many cases, the same wavelength, energy density, and time of irradiation of LLLT may be efficient for a determined inflammatory process and inefficient to other.

The present study demonstrates that the local acute inflammation induced by LPS or TNF into mice lungs can be attenuated by LLLT in vivo similarly to systemic treatment with cAMP-elevating agents, such as rolipram, an inhibitor of PDE 4. Furthermore, it indicates that this occurs via suppression of in vivo increase of TNF-α mRNA expression in alveolar macrophages.

The respiratory tract is continuously exposed to organisms and particles from inhaled air. In the distal part of the

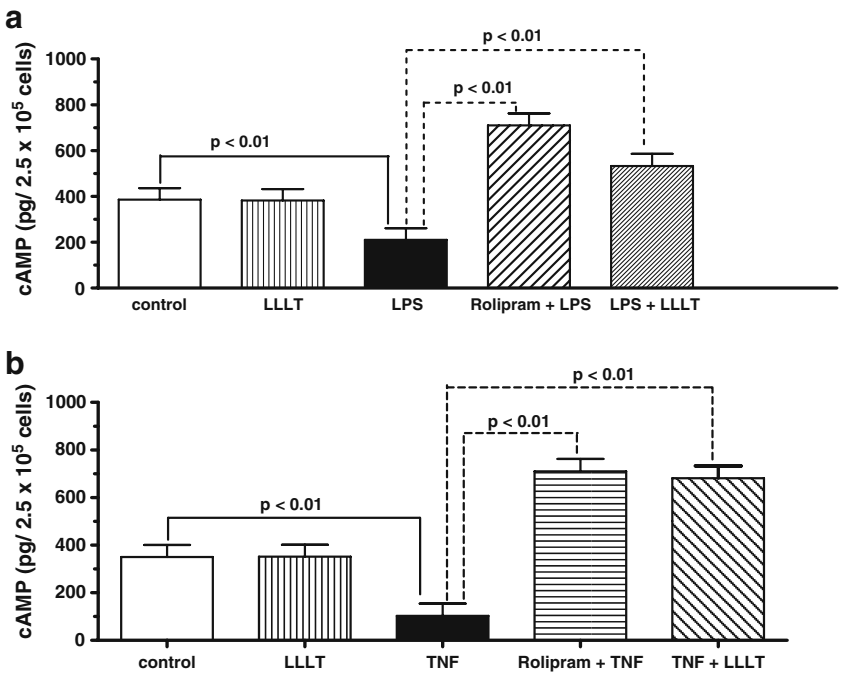

Fig. 5 Effect of LLLT plus rolipram on cAMP concentrations of alveolar macrophages from mice exposure to LPS (**a**) or TNF (**b**). In this series of experiments, the mice were treated with rolipram plus laser. Each mouse received saline (control) or 20 mg kg^{-1} rolipram 45 min before inhalation of 0.3 mg ml^{-1} LPS or intra nasal instillation of 0.5 μg of rmTNF. Fifteen minutes after LPS or TNF, the mice were irradiated with 4.5 J/cm^2 for 252 s on the skin over the upper bronchus. AM were collected 30 min after LPS or TNF and then the cAMP level was measured by ELISA method. The results were considered significant when $p<0.05$

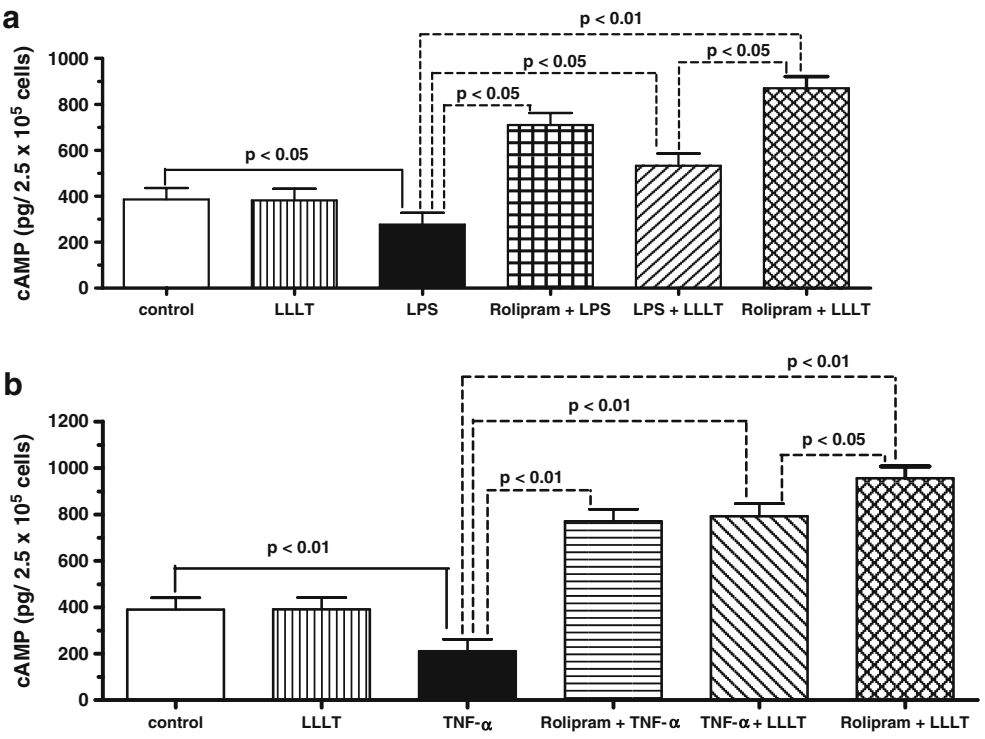

Fig. 6 Effect of LLLT or rolipram on cAMP concentrations of alveolar macrophages from mice pretreated in vivo with TNF inhibitor. In these experiments, each mouse was pretreated with chlorpromazine (1.25 mg/kg, i.p.) 30 min before LPS inhalation. Fifteen minutes after LPS, the mice were irradiated with 4.5 J/cm^2 for 252 s on the skin over the upper bronchus. AM were collected 30 min after LPS and then the cAMP level was measured by the ELISA method. The results were considered significant when $p<0.05$

airways, alveolar macrophages and neutrophils play a pivotal role in the neutralization and clearance of particles or microorganisms that have not been retained in the upper respiratory tract. The strategic position of alveolar macrophages at the air–tissue interface suggests that these cells are implicated in the control of the pulmonary responses to injuries processes [30–32]. Herein we used an animal model that mimics the environmental conditions in which the respiratory tract is continuously exposed to aerosols of LPS [16]. Moreover, with this method is possible minimizes the toxic effects of LPS administered through a systemic route, particularly on endothelial cells[33], and provides a model to study the acute respiratory distress syndrome (ARDS) caused by a direct pulmonary insult.

Despite the major therapies that attenuate significantly the acute lung inflammation to be based on pharmacological strategies, together with pharmacotherapy come the complexity of side-effects. Considering that ARDS and acute lung injury (ALI) are diseases closely related to septic shock and endotoxemia, the treatment of both situations is frequently the administration of drugs that although relieve the symptoms, also caused serious side-effects. For this reason, alternative therapies like low-level laser therapy have been proposed for the treatment of different inflammatory process by fact that the LLLT is cheap and do not present side-effects that would compromise other systems.

Thus it is important to investigate the action mechanism of LLLT in a similar situation with clinical treatment, the present manuscript studied the LLLT effect applied in an in

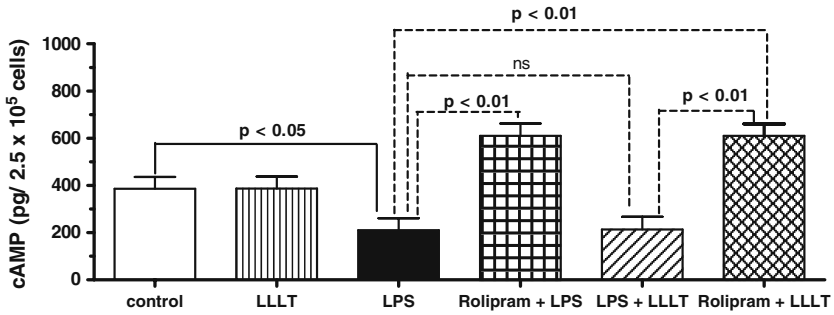

Fig. 7 Effect of LLLT or rolipram on LPS-induced on TNF mRNA expression in alveolar macrophages from mice. Mice were pretreated with saline or 20 mg kg^{-1} rolipram 45 min before inhalation of 0.3 mg ml^{-1} LPS. Fifteen minutes after LPS, the mice were irradiated with 4.5 J/cm^2 for 252 s on the skin over the upper bronchus. AM were collected 1 h after LPS and then the TNF mRNA expression was quantified by RT-PCR. The data represents one of three experiments. The results were considered significant when $p<0.05$

vivo experimental model of lung inflammation. It is important stand out that the LLLT has not effect on mice not submitted to LPS inhalation or any other inflammatory stimuli. In fact, if the biological system is not perturbed, the energy gives to cell or tissue by low-intensity laser do not cause alteration in its metabolism [34].

Previously, we have demonstrated that laser therapy is able to reduce the airway hyperreactivity and inflammation pulmonary earlier and later after intravenous injection or instillation intratracheal of LPS by a mechanism that involves the participation of eicosanoids, such as prostaglandin-E_2 (PGE_2) and thromboxane (TXA_2) [35]. Some authors have shown the LLLT effect on inflammatory mediators in different models of inflammation [36]. In another study, we demonstrated that phototherapy reduces the hemorrhagic lesion and the myeloperoxidase activity in lungs of rat inflamed by reaction of immune-complex [37]. In another condition, the LLLT was also able to increase the levels of cAMP in trachea segments cultured and bathed with TNF [29]. Regarding the effect of LLLT on mRNA expression, we previously demonstrated that LLLT reduces the mRNA expression of anti-apoptotic factors from mice lung neutrophils after LPS injection [38]. Still on this work, interesting data is that the LLLT effect on anti-apoptotic factors was abolished when animals were pretreated with NF-κB inhibitor (BMS 205820). Still regarding the fact that LLLT can interfere with mRNA expression for inflammatory mediators, we demonstrated that this therapy reduced the pulmonary edema and the IL-1β mRNA expression in rat trachea segments after LPS injection [39]. Recently, we reported that cholinergic hyperreactivity, the β_2-adrenergic hyporesponsiveness, and the TNF mRNA expression from rat bronchi segments are attenuated by LLLT by a mechanism that seems to involve the participation of NF-κB, since when this nuclear factor was inhibited, the beneficial effect of LLLT was reduced dramatically [40].

Although the action mechanism of LLLT still was not elucidated, the results described above pointing for a possible LLLT effect on lung inflammation and the alterations of airway reactivity observed in situations such as ARDS. In the majority of cases, where the study about cellular signaling of laser therapy was the priority, we have found the participation of cyclooxygenase-2 (COX-2) metabolites and TNF and IL-1β pro-inflammatory cytokines with involvement of nuclear factor-κB (NF-κB). Naturally, we cannot discard that these other inflammatory mediators can be a trigger for LLLT such as nitric oxide and chemokines. Moreover, LLLT can affect calcium (Ca^{+2}) metabolism, which is an indispensable mediator of diverse cellular functions by activating various mechanisms of signaling. Recently, we reported that the hypersensitivity of bronchi segments to Ca^{+2} after TNF-α exposure, which determine hyperreactivity of this segments, can be modulated by LLLT through the reduction of mRNA expression for inositol triphosphate, an important second messenger in signaling that determines the bronchoconstriction to cholinergic agonist [41]. Therefore, the ability of LLLT in reducing the ALI seems to be closely linked to the axis of TNF action.

In the present study, we evaluated the laser effect on ALI in two different situations: in one of them the mice were stimulated by inhalation of LPS and in another assay the mice received an instillation of TNF. In both experimental assays, the effect of rolipram, a selective inhibitor of PDE4, was compared to laser effect in order to know if laser therapy acts similarly to this inhibitor. Rolipram was chosen according to the study reported by Moraes and colleagues [16] in which it reduced either the infiltration of neutrophils into lung as the levels of TNF in bronchoalveolar lavage fluid from mice inflamed with LPS or TNF.

Herein, an interesting result was observed when the LLLT and rolipram were tested on neutrophil recruitment induced by LPS or TNF. Although both rolipram and laser treatment were effective in reducing the amount of neutrophils that migrated into mice lung, the treatment with rolipram was more effective than LLLT in animals challenged with LPS. On the contrary, LLLT was more effective than rolipram in reducing neutrophil migration stimulated by TNF, which suggests that although LLLT can reduce the lung inflammation triggered by LPS, the direct effect of TNF permits laser therapy to be more functional. Rolipram was much less effective in inhibiting the neutrophil recruitment directly induced by TNF, suggesting that the neutrophil infiltration can be controlled by modulation of cAMP concentration at a stage leading to the production of TNF. The results obtained with rolipram are in accordance with those from others authors, indicating the essential role of cAMP for TNF suppression. The treatment with rolipram inhibited almost completely the LPS-induced neutrophil influx, whereas the treatment with anti-TNF antibodies reduced it by 70% [16]. This difference may be due to an effect of rolipram at different sites, such as on the PDE 4 isoenzymes ubiquitously distributed in the airways, on the expression of endothelial adhesion molecules, or on the release of corticosteroids [42]. Regarding the concentration of TNF in BALF after aerosol of LPS, the treatment with either rolipram or LLLT presented similar efficacy.

Considering that the cellular response to LPS have more impact on lung than TNF alone, an LLLT effect more pronounced on inflammation induced by TNF in this case makes sense. Still in this context but now based on other viewpoint is reasonable hypothesizes that the LLLT beneficial effect on neutrophils migrated into lung would occur in a manner more efficacious than laser effect on the TNF level into BALF. Maybe that reducing the leukocytes

number into lung the laser therapy justifies the efficacy on inflammation. Evidently that further studies are warranted to conclude it, but considering it as a possibility reasonable, the LLLT could be modulating the lung inflammation through of a way linked to the process of cellular migration.

Despite the results presented herein, we cannot let to consider that phototherapy was efficient in reducing TNF concentration produced by AM from mice challenged with LPS. The same effect was observed with rolipram. From these results, the perspective is that inhibiting the production of TNF in AM the LLLT can modulate at least partially the leukocyte infiltration since the TNF produced by AM after LPS inhalation attracts more leukocytes into the lung. Considering that TNF released from AM has a pivotal role in the process of cellular migration maybe that the laser anti-inflammatory effect would be explained by it.

The cellular mechanism by which rolipram induces the increase of cAMP trough phosphodiesterase inhibition is very well established. Although our results demonstrate that LLLT is able to increase the content of cAMP into AM from mice with ALI, its effect seems do not occur necessarily due to phosphodiesterase inhibition like rolipram. Interestingly, as in neutrophils migration, the LLLT was more efficient in increasing the content of cAMP into AM when mice were exposed to TNF instillation in comparison to AM from mice inflamed by LPS. Thus, while the rolipram works better when mice are stimulated by LPS, the beneficial effect of phototherapy seems to be linked particularly to the presence of and to the effects of TNF-α. Curiously, the effect of LLLT on cAMP concentration was similar to rolipram in AM from mice challenged with TNF. We cannot discard the idea that the LLLT effect in increasing of cAMP level into AM after exposure of both LPS and TNF would be a consequence of TNF inhibition by LLLT. Considering that the action mechanism of LLLT is an important focus, is possible to suggest that the low-intensity laser acts in increasing of cAMP like an anti-inflammatory drug and not exactly as a agent able to elevate the cAMP level by itself as occurs with phosphodiesterase inhibitors. Curiously, the laser radiation decreases the TNF expression and it also increases the cAMP concentration in AM. With the exception of phosphodiesterase inhibitors or some β_2 adrenergic agonists, there are no drugs that exert an anti-inflammatory effect by increasing cAMP levels.

In a parallel series of experimental assays, we tested if laser therapy could potentiate the effect of rolipram on cAMP concentration in AM from mice challenged with LPS or TNF. In both situations, either in challenge with LPS as TNF, the combination of rolipram with laser resulted in augmentation of cAMP level when compared to each one alone. These results do not discard the possibility that LLLT effect on AM would be modulated at least partially by inhibition of TNF but without a direct action on the cAMP system. This hypothesis was reinforced by response of LLLT when the mice were pretreated with chlorpromazine, a synthesis inhibitor of TNF. In this experiment we blocked the participation of TNF; in this situation although stimulated with LPS, the AM are not able to produce great amounts of TNF. Interestingly, the laser effect was abolished when mice were pre-treated with chlorpromazine. This supports the idea that the LLLT-induced cAMP level increase can be modulated indirectly through inhibition of TNF and not necessarily by signal transduction of cAMP.

Moraes and colleagues [16] indicated that neutrophil infiltration into mice airways after inhalation of LPS aerosols was dependent on protein synthesis. These authors revealed that the systemic treatment of mice with two different anti-TNF antibodies reduced by approximately 70% the neutrophil number in the alveolar spaces, indicating that this cytokine was the main factor involved in the neutrophil recruitment triggered by aerosols of LPS. Our data are in agreement with these findings. Regarding the expression of mRNA to TNF in AM, we demonstrated that laser reduces the TNF mRNA expression in AM from mice that inhaled LPS. Although rolipram shows ability either in increasing the cAMP as in reducing the expression of TNF mRNA in AM stimulated by LPS, it is probable that the synergism between rolipram and LLLT on cAMP level occurs by independent mechanisms, where the rolipram acts by elevating the cAMP level through PDE4 inhibition and the LLLT acts as an anti-inflammatory inhibiting the TNF mRNA expression, and thus increasing the cAMP level in AM.

Regarding the LLLT effect, one interesting point in the present study was that for the first time we demonstrated the beneficial effects of LLLT on acute lung inflammation in animals irradiated shortly after LPS inhalation. Herein each mouse received the irradiation 30 min after inhalation of LPS or intra nasal instillation with TNF. In most of our previous studies, due to the more extended period for analyzing of lung inflammation, the beneficial effects of LLLT were observed in a latter period. Curiously, it does not seem to interfere negatively with LLLT effect, at least in the ALI model used herein.

Further studies are necessary in order to interconnect the cellular signaling responsible by anti-inflammatory action of phototherapy with adequate wavelength. The comprehension that the LLLT can act through of cellular mechanism similar to signaling induced by conventional pharmacological therapies still is poorly studied. Moreover, there is a problem in identifying which chromophore is responsible by laser effect when it is used in non-invasive therapy. Therefore, the conclusion of the present study is that LLLT can act as a non-invasive coadjuvant therapy in

treatment of pulmonary inflammation, principally those where the participation of TNF plays an important role.

Conclusions

Taken together, the results obtained in the present manuscript evidenced that LLLT potentiates the effect of rolipram on ALI by a mechanism that, at least initially, is not directly linked to generation of cAMP but involves the reduction of TNF-α mRNA expression in AM from mice in an experimental model of LPS aerosol-induced ALI.

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References

1. Beutler B, Milsarek I, Cerami A (1985) Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science 229:869–871
2. Ulich T, Watson L, Yin S, Guo K, Del Castillo J (1991) The intratracheal administration of endotoxin and cytokines. I: Characterization of LPS-induced TNF-α and IL-1β mRNA expression and the LPS, TNF-α, and IL-1β-induced inflammatory infiltrate. Am J Pathol 138:1485–1496
3. Ulich T, Yin S, Remick D, Russell D, Eisenberg S, Kohno T (1993) Intratracheal administration of endotoxin and cytokines. IV. The soluble TNF receptor type 1 inhibits acute inflammation. Am J Pathol 142:1335–1338
4. Denis M, Guojian L, Widmer M, Canin A (1994) A mouse model of lung injury induced by microbial products: implications of tumor necrosis factor. Am J Respir Cell Mol Biol 10:658–664
5. Moraes V, Vargaftig B, Lefort J, Meager A, Chignard M (1996) Effect of cyclooxygenase inhibitors and modulators of cyclic AMP formation on lipopolysaccharide induced neutrophil infiltration in mouse lung. Br J Pharmacol 117:1792–1796
6. Kips J, Joos G, Peleman R, Pauwels R (1993) The effect of zardaverine, an inhibitor of phosphodiesterase isoenzymes III and IV, on endotoxin-induced airway changes in rat. Clin Exp Allergy 23:518–523
7. Tang W, Yi E, Remick D, Wittwer A, Yin S, Qi M, Ulich T (1995) Intratracheal injection of endotoxin and cytokines. IX. Contribution of CD11a/ICAM-1 to neutrophil emigration. Am J Physiol 269:653-669
8. Howell R, Jenkins L, Howell D (1995) Inhibition of lipopolysaccharide-induced pulmonary edema by isoenzymes-selective phosphodiesterase inhibitors in guinea pigs. J Pharmacol Exp Ther 275:703–709
9. Spengler R, Spengler M, Lincoln P, Remick D, Strieter R, Kunkel S (1989) Dynamics of dibutyryl cyclic AMP- and prostaglandin E_2-mediated suppression of lipopolysaccharide-induced tumor necrosis factor alpha gene expression. Infect Immun 57:2837–2841
10. Tannenbaun C, Hamilton T (1989) Lipopolysaccharide-induced gene expression in murine peritoneal macrophages is selectively suppressed by agents that elevate intracellular cAMP. J Immunol 142:1274–1280
11. Prabhakar U, Lipshutz D, Bartus J, Slivjak M, Smith E III, Lee J, Esser K (1994) Characterization of cAMP-dependent inhibition of LPS-induced TNF-α production by rolipram, a specific phosphodiesterase IV (PDE IV) inhibitor. Int J Immunol 16:805–816
12. Verghese M, McConnell R, Strickland A, Gooding R, Stimpson S, Yarnall D, Taylor D, Furdon P (1995) Differential regulation of human monocyte-derived TNF-α and IL-1β by type IV cAMP-phosphodiesterase (cAMP-PDE) inhibitors. J Pharmacol Exp Ther 272:1313–1320
13. Zhong W, Burke P, Drotar M, Chavali S, Forse R (1995) Effects of prostaglandin E_2, cholera toxin and 8-bromo-cyclic AMP on lipopolysaccharide-induce gene expression of cytokines in human macrophages. Immunol 84:446–452
14. Turner C, Esser K, Wheeldon E (1993) Therapeutic intervention in a rat model of ARDS: IV. Phosphodiesterase IV inhibition. Cir Shock 39:237–245
15. Pettipher E, Labasi J, Salter E, Stam E, Cheng J, Griffiths R (1996) Regulation of tumor necrosis factor production by adrenal hormones in vivo: insights into the anti-inflammatory activity of rolipram. Br J Pharmacol 117:1530–1534
16. Moraes V, Singer M, Vargaftig B, Chignard M (1998) Effects of rolipram on cyclic AMP levels in alveolar macrophages and lipopolysaccharide-induced inflammation in mouse lung. Brit J Pharmacol 123:631–636
17. Mukhopadhyay S, Hoidal J, Mukherjee T (2006) Role of TNF-alpha in pulmonary pathophysiology. Respir Res 7:1–9
18. Bjordal J, Bogen B, Lopes-Martins R, Klovning A (2005) Can Cochrane Reviews in controversial areas be biased? A sensitivity analysis based on the protocol of a Systemic Cochrane Review on low-level laser therapy in osteoarthritis. Photomed Laser Surg 23:453–458
19. Sakurai Y, Yamaguchi M, Abiko Y (2000) Inhibitory effect of low-level laser irradiation on LPS-stimulated prostaglandin E2 production and cycloxygenase-2 in human gingival fibroblasts. Eur J Oral Sci 108:29–34
20. Campana V, Moya M, Gavotto A, Juri H, Palma J (1999) The relative effects of He-Ne laser and meloxicam on experimentally induced inflammation. Laser Ther 11:36–41
21. Albertini R, Aimbire F, Correa F, Ribeiro W, Cogo J, Antunes E, Teixeira A, de Nucci G, Castro-Faria-Neto H, Zângaro R, Lopes-Martins R (2004) Effects of different protocol doses of low-power gallium-aluminum-arsenate (Ga-Al-As) laser radiation (650 nm) on carrageenan induced rat paw oedema. J Photochem Photobiol 74:101–107
22. Rizzi C, Mauriz J, Freitas-Correa D, Moreira A, Zettler C, Filippin L, Marroni N, González-Gallego J (2006) Effects of low-level laser therapy (LLLT) on the nuclear factor (NF)-kappaB signaling pathway in traumatized muscles. Lasers Surg Med 38:704–713
23. Franco W, Leite R, Parizotto N (2002) Effects of low-intensity infrared laser irradiation on the water transport in the isolated toad urinary bladder. Lasers Surg Med 32:299–304
24. Kuo Y, Wu W, Wang F (2007) Flashlamp pulsed-dye laser suppressed TGF-beta1 expression and proliferation in cultured keloid fibroblasts in mediated by MAPK pathway. Lasers Surg Med 39:358–364
25. Albertini R, Aimbire F, Villaverde A, Silva J, Costa M (2007) COX-2 mRNA expression decreases in the subplantar muscle of rat paw subjected to carrageenan-induced inflammation after low-level laser therapy. Inflam Res 56:228–229
26. Milojevic M, Kuruc V (2003) Laser biostimulation in the treatment of pleurisy. Med Pregl 56:516–520
27. Landyshev I, Avdeeva N, Goborov N, Krasavina N, Tikhonova G, Tkacheva S (2002) Efficacy of low-intensity irradiation and sodium nedocromil in the complex treatment of patients with bronchial asthma. Ter Arkh 74:25–28

28. Aimbire F, Albertini R, Pacheco M, Castro-Faria-Neto H, Leonardo P, Iversen V, Lopes-Martins R, Bjordal J (2006) Low-level laser therapy induces dose-dependent reduction of TNF-α level in acute inflammation. Photomed and Laser Surg 24:33–37
29. Aimbire F, Bjordal J, Iversen V, Albertini R, Frigo L, Pacheco M, Castro-Faria-Neto H, Chavantes M, Labat R, Lopes-Martins R (2006) Low-level laser therapy partially restores trachea muscle relaxation response in rats with tumor necrosis factor-alpha mediated smooth airway muscle dysfunction. Lasers Surg Med 38:773–778
30. Fels A, Cohn Z (1986) The alveolar macrophage. J Appl Physiol 60:353-369
31. Thepen T, Van Roojien N, Kraal G (1989) Alveolar macrophage elimination in vivo is associated with an increase in pulmonary immune response in mice. J Exp Med 170:499–509
32. Sibille Y, Reynolds H (1990) State of the art. Macrophages and polymorphonuclear neutrophils in lung defense and injury. Am Rev J Respir Dis 141:471–481
33. Meyrick B (1987) Endotoxin-mediated pulmonary endothelial cell injury. Fed Proc 45:19–24
34. Karu T (1999) Primary and secondary mechanisms of action of visible to near-IR radiation on cells. J Photochem Photobiol 49:1–17
35. Aimbire F, Albertine R, de Magalhães R, Lopes-Martins R, Castro-Faria-Neto H, Zângaro R, Chavantes M, Pacheco M (2005) Effect of LLLT Ga–Al–As (685 nm) on LPS-induced inflammation of the airway and lung in the rat. Lasers Med Sci 20:11–20
36. Bjordal J, Johnson M, Iversen V, Aimbire F, Lopes-Martins R (2006) Low-level laser therapy (LLLT) in acute pain. A systematic review of possible mechanism of action and clinical effects in randomized placebo-controlled trials. Photomed Laser Surg 24:158–169
37. Aimbire F, Lopes-Martins R, Albertini R, Pacheco M, Castro-Faria-Neto H, Leonardo P, Bjordal J (2007) Effect of low-level laser therapy on hemorrhagic lesions induced by immune-complex in rat lungs. Photomed Laser Surg 25:112–117
38. Aimbire F, Santos F, Albertini R, Castro-Faria-Neto H, Mittmann J, Pacheco-Soares C (2008) Low-level laser therapy decreases levels of lung neutrophils anti-apoptotic factors by a NF-kappaB dependent mechanism. Int Immunopharmacol 8:603–605
39. Aimbire F, Ligeiro de Oliveira A, Albertini R, Corrêa J, Ladeira de Campos C, Lyon J, Silva J Jr, Costa M (2008) Low- level laser therapy decreases pulmonary microvascular leakage, neutrophil influx and IL-1β levels in airway and lung from rat subjected to LPS-induced inflammation. Inflammation 31:189–197
40. Mafra de Lima F, Costa M, Albertini R, Silva J Jr, Aimbire F (2009) Low-Level Laser Therapy (LLLT): attenuation of cholinergic hyperreactivity, β_2-adrenergic hyporesponsiveness and TNF-alpha mRNA expression in rat bronchi segments in *E. coli* lipopolysaccharide-induced airway inflammation by a NF-kB-dependent mechanism. Lasers Surg Med 4:68–74
41. Aimbire F, de Lima F, Costa M, Albertini R, Corrêa J, Iversen V, Bjordal J (2009) Effect of low-level laser therapy on bronchial hyper-responsiveness. Lasers Med Sci 24(4):567–576
42. Gozzard N, Herd C, Blake S, Holbrook M, Hughes B, Higgs G, Page C (1996) Effects of theophyline and rolipram on antigen-induced airway responses in neonatally immunized rabbits. Br J Pharmacol 117:1405–1412