ENDOTOXIN-INDUCED AIRWAY HYPERRESPONSIVENESS IN RABBITS: CONTRIBUTION OF NEUROPEPTIDES

W. MAREK, M. OZYURT, J. POTTHAST, T. MENSING

Institut für Arbeitsphysiologie an der Augusta-Kranken-Anstalt, Bochum, Germany; Research Institute for Occupational Medicine, Ruhr-University Bochum, Bochum, Germany

Endotoxin (ET) contaminated dusts frequently can be found in the environment. Especially in agriculture, ETs represent one of the main factors in the development of respiratory diseases. In order to investigate the pathomechanisms of ET induced lung injury, the contribution of the NANC-system of vagal C-fibres to increased airway responsiveness (AR) to cholinergic agents was investigated in anesthetized rabbits. In two control groups of 6 anesthetized Wight New Zealand rabbits each, E. coli ET was infused i.v. (0.4µg/kg) (Group 1) or the aerosolized ET was inhaled for 10 min (Group 2). Before and 1 and 3 hours after ET exposure, AR to 0.2 und 2.0% acetylcholine aerosol was measured. The increased AR after ET application was compared with the ET responses in rabbits with neuropeptides depleted by subchronic capsaicin treatment on four consecutive days (Groups 3 and 4). ET-inhalation and ET-infusion both resulted in a significant increase in AR to ACH (P<0.005). The increase in dynamic elastance (E_{dyn}) as a measure of airway resistance to 0.2% ACH after ET in both groups was comparable with the response to 2.0% ACH before exposure. In rabbits with capsaicin treatment, basal ACH-responsiveness was similar to the control group. After ET-Exposure, increase in AR to ACH was significantly (P<0.05) smaller compared with untreated rabbits. In conclusion, depletion of neuropeptides by capsaicin resulted in a significantly decreased ET-induced airway hyperresponsiveness (AHR) in rabbits, indicating the contribution of the NANC-system and their neuromodulators to ET-induced AHR. The results confirm the important role of the NANC-system environmental and occupational agents induced-AHR in rabbits.

Key words: airway hyperresponsiveness, capsaicin, endotoxins, occupational exposure, neuropeptides, rabbits
Besides industrial branches, work in agriculture is associated with exposure to numerous respiratory hazards. The inhaled dusts are a complex mixture; farm workers are exposed to excretions, food, skin components, germs, microbes, insects, storage mites, and mould spores (1). In addition, low molecular gases like ozone, CH$_4$, NH$_3$ and H$_2$S may potentiate the irritant effects of dusts in the airways.

Especially, grain dusts might contain semen, mites, inorganic substances derived from insects, gram-negative-bacteria (e.g., Enterobacter, Pseudomonas, Actinobacter, and Escherichia) and their metabolites (endotoxins) (1). Endotoxins (lipopolysaccharids, LPS) in dusts are present in the outer bacterial cell wall, or fragments of whole bacteria. The endotoxin contaminated dusts can be found in swine and poultry confinement buildings, saw mills, and poultry houses, and in dusts from sewage sludge and compost. Schwartz et al (2) demonstrated that the concentration of endotoxin in the bioaerosol may be particularly important in the development of grain dust-induced lung disease. Moreover, Olenchock et al (3) and Attwood et al (4) presented the problem of endotoxin as a potential health hazard for farmers. Vogelzang et al (5) reported BHR after exposure to ET in pig farmers.

Presumably, endotoxins are involved in the development of obstructive airway diseases in agriculture, causing byssinosis (6), bronchial asthma (7), or pig breeder lung (8). Moreover, chronic bronchitis, hypersensitivity pneumonitis, organic dust toxic syndrome and chronic airflow limitation have been reported in farm workers (9). Additionally, Michel et al (10) demonstrated a dose-response relationship of clinical and inflammatory responses to inhaled endotoxin in normal subjects. In clinical medicine, diseases are correlated to endotoxins. For example, endotoxemia is one of the most important factor in the development of adult respiratory distress syndrome (ARDS) (11). ARDS is accompanied by disturbed pulmonary gas exchange and is related to pulmonary edema, leading to altered pulmonary hemodynamics and reduced lung compliance. The ARDS triggers are transported to the lung via inhalation or on the systemic route.

In measurements of total dust concentrations in 213 crop and farming environments by personal sampling, values of up to 7.01 mg/m$^3$ were found (1). Median airborne ET concentrations ranged between 0.36 ng/m$^3$ in Spanish green houses to and 257.6 ng/m$^3$ in Swiss poultry houses (1). In German swine confinement buildings, endotoxin concentrations between 0.01 and 2090 ng/m$^3$, median 76.3 ng/m$^3$, were measured and similar ET concentrations (1.3 to 1101 ng/m$^3$, median 58 ng/m$^3$) were reported from Denmark (1).

There is evidence for interactions between non-myelinated vagal C-fibres and their neuromodulators, the neuropeptides, contributing as an important mechanism to the development of airway hyperresponsiveness (12, 13). The role of neuropeptides in a number of physiologic responses has been studied by comparing responses of control animals with those treated with the neurotoxin capsaicin (CAPS), a regimen designed to deplete endogenous neuropeptides (14).
After chronic CAPS treatment, complete neuropeptide depletion can be obtained. In this way, CAPS treatment can be used as a tool to investigate the contribution of neuropeptides in C-fibre afferent nerves to airway responses. The release of neuropeptides, especially the tachykinins substance P (SP) and neurokinin A (NKA), are discussed as an important pathway in lung injury. Because SP und NKA are assumed to be important mediators of inflammation and can affect airway smooth muscles (12), they might play a role in the acute increase in airway response after exposure to endotoxin. Jarreau et al (15) and Loeflter et al (16) demonstrated that in the guinea pig, capsaicin-sensitive nerves are involved in LPS-induced airway hyperresponsiveness and inflammation.

In previous studies, application was performed via intratracheal LPS instillation or in inhalation studies contribution of inflammatory mediators was examined (17, 18). However, there is less information about the influence of neuropeptide contribution in aerosolized LPS inhalation induced AHR. In the present study we wanted to evaluate if endotoxin inhalation or i.v. infusion may induce airway hyperresponsiveness in a rabbit model under participation of neuropeptide release. For that reason, endotoxin was applied without and after capsaicin induced neuropeptide depletion. Respiratory and cardiovascular parameters were measured during both LPS-application and airway challenge tests with acetylcholine.

MATERIAL AND METHODS

All experiments were performed with written consent of an institutional Ethics Committee for animal experiments. White New-Zealand rabbits (3.5-4.0 kg body weight) from the same breed and similar age were used in the present study. Under 0.1 mg/kg xylazain (Rompun®, Bayer, Leverkusen, Germany) and 50 mg/kg ketamine hydrochloride (Ketanest 50®, Parke-Davis, Berlin, Germany) anesthesia, the animals were placed in the supine position; a polyethylene catheter was inserted into the right femoral vein, and a thiopental-sodium (Trapanal®, Byk Gulden, Konstanz, Germany) solution was slowly infused (20-30 mg/kg). The level of anaesthesia was kept constant by continuous infusion of 0.2 mg/kg/h thiopental-sodium using a perfusor. Rectal temperature was measured with a thermistor and was maintained at 38.5°C by means of a heating pad. Two additional polyethylene catheters were placed into the right femoral artery and the left femoral vein to record heart rate (f_C), systemic systolic and diastolic arterial blood pressure (P_a,syst, P_a,diast), for sampling of arterial blood for subsequent measurements of pH, bicarbonate (HCO3-) and blood gases (P_O2, P_CO2), and for i.v. infusion of endotoxin solution. Systemic arterial blood pressure was monitored with miniaturized pressure transducers (Statham Pb23Db, Hato Rey, Puerto Rico). The parameters were recorded on a multichannel pen recorder (Graphtec Linearcoreder WR 3310, Japan) and on a personal computer after A/D conversion.

Recording of respiratory and cardiovascular parameters

After intubation (Mallinckroth Athlone, Ireland, 3.0 mm ID), we recorded the respiratory airflow (V') by means of a Fleisch head (No. 00) connected to a pneumotachograph. Tidal volume (V_T) was measured by electrical integration of the inspiratory flow signal, minute volume (V') was obtained from V_T and frequency of breathing (f_R). We calculated the airway resistance (R_L) at mid-inspiratory flow from the resistive part of esophageal pressure (∆P_res) and flow signal (V'_max,I).
compensating of the elastance component of pressure (ΔP_{compl}). Dynamic elastance (E_{dyn}) was calculated from ΔP_{es} and V_t along with the slope of inspiratory pressure (ΔP_{es}/t). The respiratory and cardiovascular parameters were recorded on a polygraph while a computer performed continuous measurements and calculated mean values over sample periods of 10 s. Arterial blood samples (0.3 ml) were collected at specified times to measure P_{aCO_2}, P_{aO_2}, and pH using the Astrup method (AVL 947). A detailed description of the rabbit model and the contribution of the vagal NANC-system can be found in previous papers (19, 20-22).

Airway challenges with ACH aerosols

We generated the acetylcholine aerosols (ACH, Sigma Chemie, Deisenhofen, Germany) by a commercial jet nebulizer (Pari, Provocationstest I, Medanz, Starnberg, Germany) nebulizing 0.2 ml ACH solution in saline per min in 4.5 l of room air (20°C, 50-60% relative humidity). The animals breathed spontaneously and inhaled 1.1 ±0.3 l of the aerosol per min (range 0.8-1.4 l). This dose is equivalent to 0.98 ±0.26 mg ACH, a dose commonly used in bronchial challenge tests in humans for detection of an increased AR. 96% of the particles were <5.0 µm and 56% <2.0 µm. Previous detailed investigations demonstrated aerosols from 0.2% ACH not to be effective in healthy rabbits, whereas aerosols from 2% ACH resulted in an increase of E_{dyn} by nearly 100% of the basic value, which is a common criterion for a significant obstructive response (19). In previous studies the ACH challenge tests were reproducible over several hours, so we used a sensitive tool to examine changes in airway responsiveness to ACH aerosols (19, 21, 22).

Experimental Protocol

Application of endotoxin was performed via inhalation or i.v. infusion of lipopolysaccharid (LPS) solutions. Before and after LPS application, changes in AR to ACH were determined by challenges with 0.2% and 2.0% ACH aerosols in saline for 1 min each at 20 min intervals.

Group 1 (Airway responsiveness to ACH after LPS infusion)

After evaluation of the baseline airway responsiveness in the ACH challenge test, LPS infusion was performed in a group of six rabbits. The LPS was extracted from Escherichia coli, serotype 0111:B4 (Sigma Chemie, Deisenhofen, Germany). The LPS stock solution (1600 µg/100 ml saline) was diluted 1 to 50 (0.1 ml into 4.9 ml saline). The infusion was performed via the femoral vein catheter in a concentration of 0.4 µg/kg body weight for 10 min (0.5 ml/min). Respiratory and cardiovascular parameters were measured during and after the application period. One and three hours after the LPS application, ACH challenge tests were repeated.

Group 2 (Airway responsiveness to ACH after LPS inhalation)

After evaluation of the baseline AR in the ACH challenge test, aerosolized LPS inhalation was performed for 10 min in 6 rabbits. The LPS stock solution was diluted (1 ml in 4 ml saline) and aerosolized with the same nebulizer used for the ACH aerosol generation. Respiratory and cardiovascular parameters were measured continuously during and after the LPS exposure. One and three hours after the LPS application started, the ACH challenge tests were repeated.

Group 3 and group 4 (Systemic capsaicin treatment)

We treated two groups of 6 anesthetized rabbits with subcutaneous injections of capsaicin (Sigma Chemie, Deisenhofen, Germany) in increasing doses (50, 100, 150 mg/kg), dissolved in 1% ethanol
and 9% Tween 80 in saline over 4 days, after recovery for 5 days, the ET exposure was performed. During capsaicin application rabbits were anesthetized with ketamine hydrochloride (25 mg/kg, i.m.) and 0.05 mg/kg body weight fentanyl dihydrogencitrat (Fentanyl®, Janssen, Neuss, Germany). On the eighth day after the first capsaicin treatment, the absence of the corneal reflex as a result of neuropeptide depletion was confirmed by instillation of one drop of 1% capsaicin-solution into one conjunctival sack. Two groups of animals in which the corneal reflex was eliminated were treated with LPS inhalation or infusion, as described above for Group 1 and Group 2, respectively.

**Examinations of the tracheal smooth muscle responsiveness in the organ bath**

At the end of the experiments, we examined isometric pressure in the isolated trachea in an organ bath after instilling 2 ml of capsaicin (4 µg/ml) for 5 min. The costal ribs were cut laterally, uncovering the thoracic viscera. After ligating the left principal bronchus, we removed the lung lobes and cut the trachea transversally at the laryngeal end. Mounted on a holder the trachea was transferred to into an organ bath containing oxygenated Ringer solution at 37°C, and connected to a Statham P23 DC pressure transducer. Changes in isometric intraluminal pressure were recorded on a polygraph (Graphitec Linearorder, WR3310, Japan). A detailed description of the method is given in previous papers (23, 24).

**RESULTS**

**Airway responsiveness to ACH after LPS infusion (Group 1)**

Infusion of LPS solution performed for 10 min resulted in an increased airway responsiveness to 2% ACH aerosol (Fig. 1) compared with the baseline responses before endotoxin infusion. Application of LPS alone did not initiate any significant airway smooth muscle constriction or cardiovascular changes. However, in the second ACH challenge 2 h after LPS infusion, constrictive responses to ACH were significantly enlarged. Inhalation of 0.2% ACH caused an increased response of $E_{\text{dyn}}$ ($P<0.005$), compared with the baseline response, along with rapid shallow breathing. 2% ACH challenge increased $E_{\text{dyn}}$ significantly ($P<0.05$) compared with the baseline response from 21.8 ±5.5 to 127.1 ±51.1 mmHg/dlV$_T$. Before the third 2% ACH challenge, the baseline value of $E_{\text{dyn}}$ was slightly but significantly enlarged ($P<0.05$) and the constrictive responses to the ACH aerosols were further increased.

**Airway responsiveness to ACH after LPS inhalation (Group 2)**

Inhalation of aerosolized LPS solution performed for 10 min resulted in a significant increase in dynamic elastance during inhalation period and an increase in airway responsiveness to ACH aerosols (Fig. 2). Application of LPS alone initiated transient airway muscle constriction according to a significant increase in $E_{\text{dyn}}$ ($P<0.05$). In the second ACH challenge, constrictive responses to acetylcholine were significantly enlarged. Inhalation of 0.2% ACH caused an increased response of $E_{\text{dyn}}$ ($P<0.05$) compared with the baseline response, due to an exaggerated rapid shallow breathing. 2% ACH challenge increased $E_{\text{dyn}}$ significantly ($P<0.05$)
Fig. 1. Time course of the experiment. Changes in the responses of $E_{\text{dyn}}$ to ACH and to LPS infusion over 10 min. LPS did not alter the baseline values. After the infusion responses of $E_{\text{dyn}}$ are significantly enlarged in the ACH challenge tests compared with the baseline responses (means ±SD, *P<0.05, **P<0.005, and ***P<0.0005).

Fig. 2. Time course of the experiment. Changes in the responses of $E_{\text{dyn}}$ to ACH and to LPS inhalation over 10 min. LPS inhalation caused a significant increase in $E_{\text{dyn}}$. After the LPS inhalation, responses of $E_{\text{dyn}}$ to ACH were significantly increased compared with the baseline response (means ±SD, *P<0.05, **P<0.005, and ***P<0.0005).
compared with the baseline response from 21.2 ± 2.8 to 102.3 ± 39.0 mmHg/dlV₆. However, before the last ACH challenge test, the baseline value of Edyn was marginally enlarged (P<0.05). After inhalation of 0.2% and 2% ACH, Edyn increased to 71.3 ± 27.4 and 116.0 ± 37.1 mmHg/dlV₆, respectively. 

Effects of systemic capsaicin treatment before LPS infusion (Group 3)

Systemic treatment with capsaicin did not alter basic respiratory (Edyn, Rl, ΔPes/tl), cardiovascular (Pa,sys, Pa,diast, fC) and blood gas parameters (PaO₂, PaCO₂). capsaicin application reduced the endotoxin-induced increase in AR, although the development of AHR could not be completely abolished, thus the responses to 2.0% ACH were still significantly enlarged compared with the baseline responses (P<0.05). However, after LPS-infusion, responses of Edyn to 0.2% ACH challenges were reduced (ns). Accordingly, responses to 2.0% ACH were significantly reduced to almost 50% compared with the values without capsaicin treatment (P<0.05; Fig. 3).

Effects of systemic capsaicin treatment before LPS inhalation (Group 4)

Systemic treatment with capsaicin did not alter basic respiratory (Edyn, Rl, ΔPes/tl), cardiovascular (Pa,sys, Pa,diast, fC) and blood gas parameters (PaO₂, PaCO₂).
Fig. 4. Responses of $E_{dyn}$ in ACH challenge tests and to LPS inhalation. The values of depleted and non-depleted animals are both given. After LPS inhalation, airway responsiveness was significantly enhanced in the non-depleted animals. However, after capsaicin -treatment, responses to ACH were significantly reduced (means ± SD, *P<0.05).

Fig. 5. Isometric responses of tracheal smooth muscles to capsaicin-instillation in control animals (Caps cont.), left column, and in capsaicin-treated rabbits (Caps) and to neurokinin A and ACH, measured 1 min (left columns) and 2 min (right columns) after instillation in the organ bath.
capsaicin application reduced the airway constrictive response to LPS inhalation slightly (ns). The endotoxin-induced increase in AR was diminished compared with the untreated control group, although development of AHR could not be completely abolished. Thus, the responses to 2.0% ACH are still significantly enlarged compared with the baseline responses (P<0.05). However, after LPS-infusion, responses of $E_{dyn}$ to 0.2% ACH challenges were reduced significantly (P<0.05) in the third provocation test. Accordingly, responses to 2.0% ACH were significantly reduced compared with the values without capsaicin treatment in the third ACH challenge test (P<0.05; Fig. 4).

**Responses of tracheal smooth muscles to CAPS in the organ bath**

The effects of subchronic capsaicin -treatment could be demonstrated by the lack of corneal reflexes to substance P, and on the isolated trachea. In the organ bath, there was only a minor increase in isometric pressure 1 and 2 min after instillation of capsaicin ($10^{-5}$ mol/l) into the isolated trachea in capsaicin -pre-treated animals (0.90 ±0.03 cmH$_2$O), whereas in non- capsaicin-pre-treated animals, we could detect an effective increase in isometric pressure in the isolated organ after 2 min to 12.2 ±2.3 cmH$_2$O (P<0.0005), comparable with the ACH ($10^{-4}$ mol/l)-induced tracheal muscle constriction. In capsaicin pre-treated rabbits, tracheal muscle responses to NKA ($10^{-5}$ mol/l) were still present, 6.0 ±1.6 cmH$_2$O, or nearly 50% of the responses to ACH 12.2 ±1.3 cmH$_2$O).

**DISCUSSION**

Depletion of neuropeptides by repeated capsaicin treatment effectively reduced LPS-induced increases in the AR response to ACH in our rabbits, as a model for occupational lung disease. Respiratory and cardiovascular alterations caused by LPS have not yet been sufficiently characterized. There is evidence for participation of neuropeptides in the development of airway hyperresponsiveness (12) in general and after endotoxin treatment (15) in guinea pigs. Several studies in different species have shown that non-adrenergic, non-cholinergic vagal C-fibres (NANC) effects are mediated by the release of tachykinins, a subgroup of neuropeptides (12, 25-30). Several neuropeptides have been localized in the lungs of humans and other mammals (12, 31, 32). In our experiments using anesthetized rabbits, application of LPS for 10 min led to a significant increase in the AR response to ACH, whereas systemic pre-treatment with capsaicin over 4 days diminished the increased ACH response after LPS (Fig. 3 and Fig. 4). The results suggest that capsaicin -sensitive, neuropeptide-containing vagal afferent nerves might also play an important role in LPS-induced airway hyperresponsiveness in rabbits and confirm the findings in guinea pigs (15).

The airways of rodents are innervated by sensory C-fibres, synthesizing and storing neuropeptides in dense core vesicles (28). Systemic capsaicin treatment
can result in the depletion of neuropeptides. This depletion is a result of toxic effects of capsaicin on non-myelinated afferent nerve fibres (33). Capsaicin triggers the antidromic release of neuropeptides from non-myelinated sensory C-fibres via local axon reflex mechanisms (33). In the present study, rabbits were treated with capsaicin in doses previously reported to deplete neuropeptides in guinea pigs (14, 34), rats (35) and rabbits (21). Further studies demonstrated that the loss of neuro-secretory granules in the C-fibres in capsaicin treated animals correlated with the loss of AHR during and after exposure to TDI (36). In addition, after capsaicin treatment, the isolated rabbit tracheae showed a largely diminished response during instillation of capsaicin in the organ bath, whereas in untreated preparations, a significant increase of isomeric pressure during capsaicin instillation was observed, which can be compared with ACH responses. It is obvious that our capsaicin application selectively impaired the function of sensory C-fibres and led to a complete or, at least, to a functional depletion of neuropeptides.

After treatment with capsaicin, the baseline of $E_{dy}$ and $R_l$ remained unaltered and there was a decrease in the response to ACH after LPS application. This result reveals that neuropeptides are involved in controlling airway smooth muscle tone in rabbits. Apparently, capsaicin treatment diminished the LPS-induced AHR response to ACH by depletion of neuropeptides from vagal, non-myelinated, capsaicin-sensitive nerve endings. It is probable that inhalation or infusion of LPS stimulates the release of neuropeptides from neuropeptide-containing nerves in the airways via toxic effects, or in a similar manner, but less intensively, than capsaicin does. Because the method we used is not specific to a single neuropeptide, it is not possible to conclude from the results of capsaicin treatment which neuropeptide is responsible for the induction of AHR. However, in a previous study (21), it was demonstrated that airway hyperresponsiveness is mediated by release of neuropeptides, especially of the tachykinins SP and NKA. Particularly, the neuropeptides SP and NKA act via their receptors on the airway smooth muscles. It is to assume that these neuropeptides are involved in the development of LPS-induced AHR.

In contrast to a study on the TDI-induced AHR, capsaicin treatment did not abolish the LPS-induced AHR completely. This result suggests that further mechanisms also contribute to the LPS-induced enlarged ACH responses. From studies on guinea-pigs, there is evidence that leukocyte infiltration after LPS inhalation contributes to the development of AHR (37). Tachykinins has been demonstrated to initiate a cascade of events that result in the release of inflammatory mediators inducing airway hyperresponsiveness (12). In this way, participation of inflammatory mediators has to be regarded as a further part in the development of the LPS-induced AHR.

In conclusion, in the present study we demonstrated the LPS-induced airway hyperresponsiveness under the participation of non-adrenergic and non-cholinergic NANC-system of vagal C-fibres and the release of neuropeptides.
Further studies should include examinations of the specific antagonists of receptors, mediating the neuropeptide signals and the contribution of inflammatory mechanisms.

Conflicts of interest: No conflicts of interest were reported in relation to this article.

REFERENCES


Received: May 31, 2008

Accepted: August 17, 2008

Author’s address: W. Marek, Institut für Arbeitsphysiologie an der Augusta-Kranken-Anstalt, Bergstr. 26, D-44971 Bochum, Germany; phone: +49 234 517 2474, fax: +49 234 517 2463; e-mail: wolfgang.marek@ruhr-uni-bochum.de