Novel Insights Into Phosgene-Induced Acute Lung Injury in Rats: Role of Dysregulated Cardiopulmonary Reflexes and Nitric Oxide in Lung Edema Pathogenesis

Wenli Li,*1 Fangfang Liu,†1 Chen Wang,‡ Hubert Truebel,§ and Juergen Pauluhn¶2

*Department of Toxicology, Fourth Military Medical University, Xi’an, 710032, China; †Beijing Institute of Respiratory Medicine, Capital Medical University, Beijing, 100069, China; ‡Beijing Institute of Respiratory Medicine, Beijing Hospital, Ministry of Health, Beijing, 100730, China; §Department of Pharmacology Vascular Diseases, Cardiology & Hematology, Bayer Pharma AG, 42096 Wuppertal, Germany; and ¶Department of Toxicology, Bayer Pharma AG, 42096 Wuppertal, Germany

These authors contributed equally.

2To whom correspondence should be addressed at Bayer Pharma AG, Research Center Wuppertal, Toxicology, Aprather Weg 18a, D-42096 Wuppertal, Germany. Fax: +49 (202) 364589. E-mail: juergen.pauluhn@bayer.com.

Received August 16, 2012; accepted October 30, 2012

Phosgene is a lower respiratory tract irritant. As such, it stimulates nociceptive vagal C-fiber–related reflexes in a dose-rate and concentration × exposure duration (C × t)–dependent manner. In rats, this reflex is characterized by extended apnea time periods, bradycardia, and hypothermia. Although inhalation exposures at nonlethal C × t products show rapid reversibility of reflexively induced changes in respiratory patterns, lethal C × t products seem to cause prolonged stimulation after discontinued exposure to phosgene. This observation has been taken as indirect evidence that phosgene-induced lethal lung edema is likely to be associated with a dysfunctional neurogenic control of cardiopulmonary and microvascular physiology. In order to verify this hypothesis, data from respiratory function measurements during and after the inhalation exposure to phosgene gas were compared with time-course measurements of cardiac function over 20 h post-phosgene exposure. These data were complemented by time-course analyses of nitric oxide (NO·) and carbon dioxide in exhaled breath, including time-dependent changes of extravasated protein in bronchoalveolar lavage fluid and hemoglobin in blood. The nitric oxidase synthetase inhibitors L-NAME and L-NIL were used to further elucidate the role of NO· in this type of acute lung injury and whether its analysis can serve as an early biomarker of pulmonary injury. Collectively, the sequence and time course of pathological events in phosgene-induced lung edema appear to suggest that overstimulated, continued sensorimotor vagal reflexes affect cardiopulmonary hemodynamics. A continued parasympathetic tone appears to be involved in this etiopathology.

Key Words: pulmonary irritation; lung function; exhaled nitric oxide; nitric oxidase synthetase inhibition.

Phosgene (carbonyl chloride) is manufactured from the reaction of carbon monoxide and chlorine gas in the presence of activated charcoal and is an important and indispensable high production volume intermediate in the manufacture of building blocks of various types of plastics and numerous industrial materials, such as polyurethanes, polycarbonates, and in the production of dyestuffs, pharmaceuticals, and agrochemicals. Historically, the respiratory irritants chlorine and phosgene were used during World War I in military conflicts but were superseded by agents, which are orders of magnitude more potent (Kluge and Szinicz, 2005; Szinicz, 2005).

Currently, many drug-related treatment strategies of phosgene-induced lung injury preferentially rely on countermeasures against reactive oxygen species produced secondary to tissue injury and inflammation (Duniho et al., 2002; Ji et al., 2010; Sciuto and Hurt, 2004; Wang et al., 2011). A lesser focus has been directed toward intervention strategies on hemodynamics and vascular control (Sciuto and Hurt, 2004, Sciuto et al., 1997). In spite of the extensive research on pharmacological countermeasures to mitigate phosgene-induced acute lung edemagenesis, most of the approaches taken have shown limited benefits in experimental models simulating clinical usage. By contrast, oxygen treatment and mechanical ventilation received increased attention and proved to be efficient to mitigate the anoxic anoxia as the most pressing sequela of pulmonary edema. It also seems that there is a lack of understanding as to how the cardiopulmonary system and the systemic circulation respond to phosgene-induced pulmonary injury (Gibbon et al., 1948; Grainge and Rice, 2010).

The development of severe pulmonary edema, in the presence of extensive disturbances of the heart rate and blood volume, requires effective adjustments within the cardiopulmonary system to maintain yet physiological intracapillary pressure levels and gradients. Such changes are complex due
to their specialized local and central nervous control. It may be unwise to interfere with this complex interplay of parasympathetic and sympathetic control by drugs designed to modulate one sector of the circulation without considering the compensatory response in the other sector.

Experimental data support the view that the low water solubility of phosgene gas favors its retention in the lower lung. In terms of toxicity and chemical mode of action (acylation of nucleophilic moieties), this makes phosgene to a relatively simple toxicant with a clearly defined target localization of the inhaled dose. Consistent with this simple mode of action is that phosgene follows Haber’s rule \((C \times t = \text{constant effect}; n = 1; \text{AEGL}, 2002)\) as long as the exposure concentration \(C\) is low enough not to stimulate vagal C-fibers to an extent that provokes changes in ventilation. First concentration-dependent alterations in respiratory minute ventilation occurred in rats at an exposure concentration of 25 mg/m³ (6 ppm) (Pauluhn, 2006a). Accordingly, \(C \times t\) products of 30 mg/m³ × 30 min and 15 mg/m³ × 60 min (=225 ppm × min) produced similar increases in lung weights and BAL protein, whereas comparable exposure intensities at 354 mg/m³ × 3 min and 44 mg/m³ × 20 min (220–266 ppm × min) produced underproportional effects as a result of a transient depression in ventilation and inhaled dose (Li et al., 2011).

Acute inhalation exposure studies on rats have provided evidence of an association of concentration-dependent reflexively induced changes in breathing patterns. These were suggestive of an involvement typical of vagal C-fiber stimulation causing a depression in ventilation by prolongation of “apnea time periods (AT)” (Li et al., 2011, Pauluhn, 2006a). Although the AT of rats exposed at 25 mg/m³ × 30 min (188 ppm × min) showed evidence of recovery after cessation of exposure to phosgene, \(C \times t\) relationships at and above 48 mg/m³ × 30 min (360 ppm × min) remained elevated. These \(C \times t\) relationships to vagal C-fiber stimulation are in remarkable accord to the time-adjusted median lethal concentration \((\text{LC}_{50})\) and nonlethal threshold concentration \((\text{LC}_{10})\) of 1741 and 1075 mg/m³ × min (435 and 269 ppm × min), respectively (Pauluhn, 2006a). This association appears to portend that a dysregulated, continued neurogenic overstimulation of pulmonary C-fibers is an essential contributing factor for the lethal acute lung edema to occur (Li et al., 2011).

The objective of study was to compare time-course changes of selected cardiopulmonary endpoints with phosgene-induced lung edema, yet bearing in mind the stereotypical physiological changes of the rat following inhalation exposure to pulmonary irritants. Previously published investigations focusing on reflexively related acute changes in breathing patterns during and shortly after exposure to phosgene (Pauluhn, 2006a) were extended by measurements of body temperature, heart rate, and electrocardiography (ECG) over a post-phosgene exposure time period of 20h. These data were supplemented by time-course analyses of pulmonary edema and inflammation by noninvasive analysis of nitric oxide (NO) in exhaled breaths and measurements of blood hemoglobin (Hb) as a marker of redistributed organ blood volumes and ensuing hemococoncentration. Interventions with NOS inhibitors L-NAME and L-NIL were used to further assess whether the hypothesized phosgene-induced dysregulated hemodynamics can be modulated by NOS inhibitors and probed by the analysis of NO in exhaled breath.

**MATERIALS AND METHODS**

**Test materials.** Phosgene (carbonyl chloride), certified gas of 150 ppm in synthetic air contained in 10 l cylinders @150 bar, was from Linde, Germany. The conversion 1 ppm in 4.1 mg/m³ phosgene is based on 25°C and 1 atmosphere. Nitric oxide (nitrogen monoxide, NO), a certified gas standard of 268 mg/m³ (200 ppm) in pure nitrogen, was also from Linde, Germany. Synthetic air (20% oxygen in 80% nitrogen [\(NO_2 \leq 0.1\) ppm specified, NO ~11 ppb measured]) was also from Linde. No-Nitro-L-arginine methyl ester hydrochloride (L-NAME) and N-N-ethylnicotinamide hydrochloride (L-NIL) were from Sigma-Aldrich, Germany.

**Animals, diet, and housing conditions.** Healthy young adult male Wistar rats, strain Hsd Cpb:WU (SPF), from the experimental animal breeder Harlan-Winkelmann GmbH, Horst (The Netherlands), were used. Animals were placed in polycarbonate cages (1 rat per cage), containing bedding material low-dust wood granulate from Lig nomec BK 8–15 (Rettenmaier), and were provided with a standard fixed formula diet (KLBA 3883 = NAFAG 9441) pellets maintenance diet for rats and mice; PROVIMI KLBA SA, 4303 Kaiseraugst, Switzerland) and municipality tap water (drinking bottles). Both food and water were available ad libitum. The pelletized feed was contained in a rack in the stainless steel wire cage cover. At the study start, average body weights were in the range of 215 g. Rats of the weight class used are approximately 2–3 months old and hence fulfill the criterion for young adults. Rats subjected to telemetry were approximately 2–3 weeks older due to the surgical intervention related to the implantation of telemetry transmitters. Animal rooms were maintained at approximately 22°C with relative humidity at 40–60% and a 12-h light cycle beginning at 0600 h. All animal experiments were in accord with contemporary, internationally harmonized testing standards/guidelines (OECD, 2009). The studies were performed in an animal care–approved laboratory in accordance with the German Animal Welfare Act and European Council Directive 86/609 (EEC, 1986) and the adopted 2010/63/EU.

**Study rationale and experimental protocols.** Based on the objective of study, all phosgene exposures were targeted on a \(C \times t\) product of approximately 1050 mg/m³ × min (=35 mg/m³ × 30 min), which is in the range of the nonlethal threshold dose \((\text{LC}_{10})\) of rats. During the exposure of phosgene gas, rats elabo rate instant changes in breathing patterns typical of pulmonary (alveolar) vagal C-fiber stimulation characterized by concentration-dependent increased apnea time (pause between breaths). Following exposure to phosgene up to 25 mg/m³ (6 ppm), changes in breathing patterns diminished already during the exposure period, whereas concentrations at and above 48 mg/m³ elicited a sustained increase in apnea time (Pauluhn, 2006a). Accounting for the exposure duration of 30 min, the estimated \(C \times t\) equivalent for nonreversibility within a postexposure period of 30 min was 1440 mg/m³ × min (360 ppm × min). The coincidence in the \(C \times t\) relationship that causes persistent vagal C-fiber stimulation with that \(C \times t\) causing mortality \((\text{LC}_{50})\ to \text{LC}_{100}\ range: 269 to 435 ppm × min; Pauluhn, 2006a) appears to support a hypothesis linking any mismatch in neurogenic control with dysregulated cardiopulmonary and/or cardiovascular etiopathologic factors causing acute pulmonary edema. This coincidence prompted a first “proof-of-principle” study in rats with 20h measurements of breathing patterns (Penh, enhanced pause, a lung function parameter highly dependent...
on the apnea time period) postexposure to phosgene (Li et al., 2011). These authors showed that Pehn increased instantly and concentration dependently. Despite progressive pulmonary edema, Pehn remained remarkably unaffected during the data collection period. These findings triggered the study described in this article.

The endpoints of this study were chosen based on the following rationale: Body temperature, heart rate, and ECG were recorded for approximately 20 h post-phosgene exposure at a C x t, which showed a definite and sustained raise in Pehn and evidence of lung edema (Li et al., 2011). Sympathetic vasoconstriction of the systemic and pulmonary vessels can cause shifts of blood volume from the splanchnic vascular beds to the lung with hemodynamic changes leading to systemic/pulmonary hypertension and lung edema. Shift in plasma volume indicative of hemococoncentration was addressed by measurements of Hb in blood. Measurements were supplemented by endpoints suitable to probe for pulmonary inflammation and edema by bronchoalveolar lavage and whether their time course is mirrored by proportionally increased NO in exhaled breath. Phosgene-induced lung edema and mortality were partially attenuated by the NOS inhibitor L-NAME (Torkunov and Shabanov, 2009). It has been shown to attenuate plasma extravasation in the lung (Grant et al., 2002). However, these authors surmise this was most likely to be a side effect of its vasoconstrictor activity reducing the blood supply to the lung rather than direct inhibition of the plasma extravasation. To further elucidate NO-related pathways potentially involved in phosgene-induced lung edema, the NOS inhibitors L-NAME and L-NIL, L-NAME were therapeutically administered post-phosgene exposure. L-NAME, after hydrolysis of the methyl ester by cellular esterases, exhibits some selectivity for inhibition of neuronal and endothelial isoforms of NOS. L-NIL is a selective inducible NOS inhibitor (Moore et al., 1994). Published evidence suggests that pulmonary vasoconstriction caused by parasympathetic pathways (acetylcholine) and tachykininogenic pathways (bradykinin) was attenuated by L-NIL but not L-NAME (Fischer et al., 1999). Within the lung, a potential target of phosgene, the alveolar macrophages, is also a major source of the inducible isoform of NOS (Chen, 2009).

The following protocols were executed:

(1) L-NAME NOS inhibitor prestudy: Rats from all groups consisting of eight male rats each were exposed for 30 min to 35 mg phosgene/m³ (1050 mg/m³ x min). These rats were therapeutically dosed with the NOS inhibitor L-NAME at 0 (vehicle control: saline), 2, 10, 25, 50, and 100 mg L-NAME/kg bw. Administration was within 5 min postexposure to phosgene by ip injection. Efficacy was assessed by changes in lung weights relative to the phosgene-exposed vehicle control 1 day postexposure to phosgene. This dose selection was based on the range of doses published (Fischer et al., 1999; Li et al., 2011; Torkunov and Shabanov, 2009).

(2) NO in exhaled breath, lung edema, and hemoconcentration: Exhaled breath was analyzed for NO and carbon dioxide (CO₂) after 5 and 24 h postexposure to phosgene (exposure for 30 min to 35 mg phosgene/m³, 1050 mg/m³ x min). Measurements of the breathing frequency were included in case exhaled NO shows any dependence on breathing frequency. Data were collected consequently in three rats/group/time point. Additional groups of animals (three rats/group/interim sacrifice) were examined 0.5, 2.5, 5.5, and 24 h postexposure for changes in lung weights, total protein in bronchoalveolar lavage fluid (BALF), and Hb in blood collected from the left ventricle. The exhaled breath procedures applied were validated prior to this study using a rat lipopolysaccharide (LPS) intratracheal instillation model. Reference data of this prestudy were also used in this study.

(3) ECG by telemetry: This part of study was designed as “proof-of-principle” study and is limited to the analysis of relaxation-related changes in cardiac function and body temperature. Due to the surgical procedures necessary to implant calibrated transmitters, three groups (I–III) of four rats were sequentially subjected to the following activities: (Ia) collection of pretreatment control data. (Ib) 2 days later these rats were treated with L-NAME (50 mg/kg bw, ip), (Ic) after additional 7 days of recovery these rats were exposed to phosgene for 30 min at 35 mg phosgene/ m³ (1050 mg/m³ x min). (Ia) Exposure to phosgene as in group Ic and treatment with L-NAME shortly thereafter. (Ila) Injection of saline (vehicle control), (Ilb) followed by injection of L-NIL (10 mg/kg bw, ip) the next day, (Ilc) after one additional day of recovery these rats were exposed to phosgene as in group Ic and treated with L-NIL shortly thereafter as in (Ilb). After each dosing activity, the rats were followed up by telemetry from noon to next day morning as detailed in the section below.

**Experimental procedures.** At the end of the at least 5-day acclimatization period, rats were randomly assigned to the respective exposure groups. Rats were exposed nose only by inhalation at a single exposure period of 30 min as described elsewhere (Li et al., 2011; Pauluhn, 2006a). In brief, a controlled flow of phosgene was discharged from the cylinder into a continuous flow of conditioned dry air and then entrained into the plenum of a directed-flow nose-only inhalation chamber (for details see Pauluhn, 2006a; Pauluhn and Thiel, 2007). Temperature and humidity measurements in the inhalation chamber were performed by a computerized Data Acquisition Control System using HC-S3 sensors (Rotronic, http://www.rotronic-usa.com/prod_oem/hc2%20probes/hc2_main.htm). Air flows were calibrated using a Bio Dynamic Defender 510 (http://www.smglink.com/bios/drycalc Defender. html). The total chamber airflow was 15 l/min in order to maintain an air flow rate of 0.75 l/min per exposure port. Nominal and actual concentrations were calculated and measured, respectively. For the latter, a calibrated Gasmet Dx-4000 FT-IR (Fourier Transform Infrared spectroscopy) gas analysis system (http://lato.poutapilvi.fi/p4_gasmet/products/ftir_gas_analysers/on_line_ (continuous)/gasmet_xx4000) was used to quantify phosgene in the exposure atmosphere and to monitor the temporal stability of exposure atmospheres. The FT-IR was calibrated with phosgene span gas and additional analytical verification using the OSHA method no. 61 (http://www.osha-slc.gov/dts/lct/ methods/organic/org061/org061.html). Body weights and clinical signs were recorded systematically. The OECD Guidance Documents on Human Endpoint (OECD, 2000, 2009) and the legal requirements for housing experimental animals (Directive 86/609 EEC), including the EU adopted Directive 2010/63/EU, were followed.

At necropsy, lungs were excised following exsanguination, and wet weights were determined. Then the lungs were lavaged via a tracheal cannula with two volumes of 5 ml of physiological saline (Pauluhn, 2006b). Total protein in BALF was analyzed according to Layne (1957). Blood was collected from the left ventricle and was analyzed for Hb using the Hematology System ADVIA 120, Siemens Healthcare Diagnostics GmbH, Eschborn, Germany. All rats were sacrificed using sodium pentobarbital (Narcoren; 120mg/kg bw, ip injection). Complete exsanguination was achieved by severing the aorta abdominalis. Further experimental details similar to those used in this study have previously been published (Li et al., 2011; Pauluhn, 2006b).

**Measurement of NO and CO₂ in exhaled breath.** NO was analyzed with a chemiluminescence analyzer (Sievers 280B NDA; Sievers Instrument, Inc., Denver, CO). Synthetic air was used during measurements of exhaled NO. The actual concentration of exhaled NO (NO) was not accounted for the residual background level of NO. Measurements were made in two-compartment head-out volume displacement plethysmographs (in-house design). Data were recorded sequentially every 10 s for 10 min approximately 5 and 24 h after exposure to phosgene. The “head compartment” of the plethysmograph (volume: ≈95 ml) was dynamically flushed with synthetic air (0.75 l/min). This flow rate is about three times higher than the respiratory minute volume of the rats used (≈0.25 l/min rat; Pauluhn and Thiel, 2007) and ≈10 times higher than the total dead-space volume (head displacement volume of rat subtracted). Under the conditions chosen, the exhaled concentration of CO₂ in controls was in the range of 0.9–1% (≈9500 ppm). Within the calibration range of 3,000–13,000 ppm CO₂, rats did not experience any CO₂-dependent difference in respiratory rate during the data collection period. The exhaust flow from the head compartment was split via a manifold allowing for a similar detection of NO and CO₂. Real-time CO₂ measurements utilized nondispersive infrared spectroscopy (Leybold-Heraeus-UM-EF-621). All gas
measuring devices were regularly checked and calibrated using span gases of certified composition in synthetic air. Reference gases were diluted to attain concentrations in that range measured in exhaled breath. Pressure fluctuations were measured in the volume displacement body compartment of the plethysmograph equipped with a wire-mesh style pneumotachograph and differential pressure transducers (MP 45 ± 2 cm H2O, Validyne) fitted shortly onto the plethysmograph. The head and body compartments were separated using a double-layer latex neck seal. Precautions were taken to avoid artifacts due to restraint and tight fitting seals around the neck. During all measurements, rats were spontaneously breathing and conscious. Similar to the NO measurements, pressure signals were averaged for 10 s. The PO-NE-MAH/PLUGSYS Data Acquisition, Analysis & Archive System was used for data collection and analysis.

**Body temperature, heart rate, and ECG.** Body temperature, heart rate, and intervals and amplitudes of heart beats were measured simultaneously on four male rats per group. Measurements were made for approximately 20 h (noon-to-morning next day). During this period, data were averaged over periods of 3 min duration in intervals of 15 min. Calibrated CTA-F40 radiotelemetry transmitters (Data Sciences) were used as sensors, which were placed in a single lead II configuration. DSI PhysioTel Receivers Model RLA1020 was used for data transmission. Although dependent on the brief invasive procedure of transmitter implantation, data collection was made from stress-free, conscious, and freely roaming rats in normal home cages (food/water available ad libitum) after sufficient time of recovery. Some aberrant signals occurred due to feeding/drinking activities during the data collection period. These were averaged post data collection by a digital low-pass filter analysis. Data acquisition and analysis utilized a CED 1401 computer using Spike 2 (vers. 4.7) for ECG-pattern recognition. Transmitters were calibrated as defined by the user manual. Due to the surgical ip implantation of the telemetry transmitters and recovery of animals, the average weight of rats used for telemetry was 320 g.

**Data analysis.** Lung weights, Hb, and BAL protein were analyzed by a one-way ANOVA followed by a Tukey-Kramer post hoc test. Body weight gains/losses and treatment effects were analyzed utilizing a one-way ANOVA with comparison against the control or phosgene exposure group (Dunnett method). Telemetry and NO in exhaled breath data were analyzed by a repeated measurement one-way ANOVA with pairwise multiple comparison procedures (Holm-Sidak method). In case, normality tests failed (Shapiro-Wilk), comparisons were made on ranks. Statistical analyses utilized Sigma Plot 12 (Software, Systat, Point Richmond, CA). For all tests, the criterion for statistical significance was set at $p \leq 0.05$.

**RESULTS**

**Dose-Range Finding Study with the NOS Inhibitor L-NAME**

Rats exposed to 1050 mg phosgene/m³ × min survived in concordance with the hypothesis and rationale of dose selection. All groups shown in Figure 1 were exposed to phosgene and received either saline (control, ip) or L-NAME (ip) shortly thereafter. L-NAME was well tolerated at all dose levels. Consistent, dose-dependent differences in body weight gains or lung weights relative to phosgene-exposed rats sacrificed on the first postexposure day were not observed. Lung weights from nonexposed rats are shown in Figure 12.

**Time-Course Changes in Body Temperatures**

Body temperatures were continuously recorded by telemetry transmitters for approximately 20 h following exposure to air, vehicle (saline, ip), 50 mg L-NAME/kg bw, and 10 mg L-NIL/kg bw either alone or following exposure to 1050 mg phosgene/m³ × min. Control rats displayed night activity–related changes in body temperature typical of rats (Fig. 2). By contrast, rats exposed to phosgene elaborated a statistically significant, persistent hypothermia, which was aggravated further by the treatment with L-NAME but not L-NIL. Of note is the time dependence of interanimal variability that decreased within the first 7 h of data collection which reversed thereafter. Rats dosed with the NOS inhibitors alone did not show any evidence of hypothermia.

**Exhaled Nitric Oxide**

Rats exposed to either air (control) or 1050 mg phosgene/m³ × min were analyzed for NOx and CO2 in exhaled air approximately 5 and 24 h post-phosgene exposure. These measurements were complemented by plethysmographic analysis of the respiratory rate concurrent to the analyses in exhaled breath. The results depicted in Figure 3 demonstrate a minimal although significant increase of NOx 1 day postexposure. NOx was markedly, but not clearly time dependent, increased when normalizing NOx to the average level of exhaled CO2 of the control (Fig. 4). The concentration of exhaled CO2 was equally decreased relative to the nonexposed sham controls at both time points. The mild depression in respiratory rate is pathognostically unremarkable and likely related to the different exposure history of sham controls (no prior inhalation exposure).

**ECG—Telemetry**

Typical ECG tracings from rats made during the stable postexposure period are given in Figure 5. Each cycle was established on its own reference level due to the lack of a common isoelectric baseline. One of the unique electrophysiological characteristics of the rat ECG, *inter alia*, is the absence of a Q wave and the lack of an isoelectric ST segment. Consequently, there is an absence of clear separation between the QRS complex and the T wave. The derived indices of cardiac changes over a postexposure period of approximately 20 h are shown for the heart rate, QT and QP intervals, and P, S, and T amplitudes in Figures 6–11. PR intervals were also recorded but changes were not different from the controls (sham and saline). The most salient changes attributed to phosgene exposure were manifested by sinus bradycardia, which attained a stable plateau within 1–2 h post-phosgene exposure (Fig. 6). Similarly, a shortening of the QT interval (Fig. 7) and lengthening of the QP interval (Fig. 8) occurred. Although the P and S amplitudes showed a minimal decrease relative to the controls (Figs. 9 and 10), T amplitudes increased (Fig. 11). Such PQR interval changes can be attributed to atrioventricular conduction disturbances related to lung edema and/or circulatory changes. At the 50 mg/kg bw dose level chosen for L-NAME, a further aggravation in bradycardia, amplitudes, and segments was apparent. Overall, the phosgene-induced bradycardia was unresponsive to any darkness-related rhythms. Treatments with L-NIL, at the chosen dose levels and time of administration, did
not show any mitigating effects on cardiac function, whereas L-NAME resulted in a further aggravation of phosgene-induced pathophysiology. Amplitudes showed a markedly higher inter-animal variability than intervals. Although L-NAME alone was tolerated without marked cardiac changes, the combination of phosgene and L-NAME resulted in high fluctuations of the P amplitude.

**Biomarkers of Pulmonary Irritation and Associated Changes in Blood Hb**

Time-course analyses of Hb in blood, extravasated protein in BALF, and lung weights from rats exposed to either air (control) or 1050 mg phosgene/m³ × min were determined as presented in Figure 12. For all endpoints examined, a coherent increase occurred 5–6 h post-phosgene exposure with maximum effects.

**FIG. 1.** Body and lung weight changes of rats exposed to 1050 mg phosgene/m³ × min and treated with variable doses of the NOS inhibitor L-NAME by ip injection shortly after the inhalation exposure to phosgene (day 0). Rats of the control (C) were exposed to phosgene only and were given saline under otherwise identical conditions. Rats were necropsied approximately 20h postexposure to phosgene (day 1). Bars represent group means ± SD (n = 8). The statistical analysis of data did not reveal differences between the groups.
24 h postexposure. The pathodiagnostic sensitivity of BALF-protein was approximately two orders of magnitude higher than that of wet lung weights. In concert, all three endpoints provided unequivocal evidence of redistributed plasma fluid and proteins from the peripheral circulation to the lung. Exhaled NO was determined prior to the 5 and 24 h sacrifices; the results are presented in Figure 3.

DISCUSSION

The current treatments of phosgene-induced acute lung injury (ALI) are mainly focused on alleviating hypoxemia by administration of supplemental oxygen when patients develop acute symptoms of respiratory distress as a result of lung edema. At stages where the deterioration of alveolar epithelia...
FIG. 3. Dynamic measurement of exhaled NO, CO₂, and respiratory rate from rats in volume displacement plethysmographs 5 and 24 h post-phosgene exposure (1050 mg/m³ × min). Similarly, examined sham control rats served as concurrent control. The respiratory rate was analyzed by the measurement of the pressure fluctuations in the thoracic compartment. Exhaled NO, CO₂, and respiratory rate were digitally recorded every 10 s over a time period of 10 min. Bars represent means ± SD of three rats/group/time point.
and vascular endothelia has progressed to frank injury and apoptosis (Li et al., 2005, 2006), the only resources left are oxygen and mechanical ventilation (Grainge and Rice, 2010). Apart from these physical mitigation measures, any generally accepted rationalized and empirically verified intervention principle has not yet been established for this particular type of ALI. One major obstacle of reaching this objective is the lack of a clear understanding of the involved mechanisms, including their time course of phenotypic manifestation. Dose-response and time-course analyses of protein, collagen, and neutrophils in BALF showed reversibility within 2 weeks postexposure in rats exposed nose only to 1008 mg/m³ × min (250 ppm × min) (Pauluhn 2006b). Of note is that the BALF protein–based no-observed adverse effect $C \times t$ (NOAEL) from this single exposure study matches the borderline histology-based NOAEL of the 12-week repeated exposure rat inhalation study from Kodavanti et al. (1997). This appears to suggest that low dose

![Fig. 4. Exhaled NO data normalized to the concentration of exhaled CO$_2$ of the control. For further details see legend of Figure 3.](image)

**FIG. 4.** Exhaled NO data normalized to the concentration of exhaled CO$_2$ of the control. For further details see legend of Figure 3.

![Fig. 5. Typical electrocardiograms (ECG) recorded by telemetry of rats from the nonexposed sham control group, the 1050 mg phosgene/m³ × min exposure group, and rats exposed both to phosgene and dosed additionally by with L-NAME (50 mg/kg bw, ip injection) or L-NIL (10 mg/kg bw, ip injection). The most salient change in the ECG was likely be related to the phosgene-induced bradycardia (x-axis: s, y-axis: mV).](image)

**FIG. 5.** Typical electrocardiograms (ECG) recorded by telemetry of rats from the nonexposed sham control group, the 1050 mg phosgene/m³ × min exposure group, and rats exposed both to phosgene and dosed additionally by with L-NAME (50 mg/kg bw, ip injection) or L-NIL (10 mg/kg bw, ip injection). The most salient change in the ECG was likely be related to the phosgene-induced bradycardia (x-axis: s, y-axis: mV).
rates of phosgene are “buffered away” and the NOAEL based on pulmonary irritation is essentially independent on intermittent reexposures as long as the NOAEL $C \times t$ per day is not exceeded (Pauluhn et al., 2007).

Multiple hypotheses have been articulated regarding the putative modes of action of phosgene-induced edema formation (Diller, 1980, 1985; Ghio and Hatch, 1996; Hatch et al., 2001; Pauluhn and Hai, 2011; Pauluhn et al., 2007). Most of them focused on an irritant/inflammatory etiopathology. Rothlin (1941), for the first time, coined the hypothesis of a vagolytic-like neurogenic etiopathology of edemagenesis and suggested that the phosgene-induced pulmonary edema is brought about by a reflexively mediated vascular response. Vagal C-fibers represent the majority of vagal afferents innervating the lower airways. The afferent activity arising from these fibers also plays an important role in regulating the cardiopulmonary function under both normal and abnormal physiological conditions (Lee, 2009). Inhalation exposures to phosgene have been associated...
with findings typical of stimulation of pulmonary vagal C-fiber reflexes, such as apnea, bradycardia, and cholinergic symptoms (Bruner et al., 1948a; Diller, 1985; Li et al., 2011; Pauluhn, 2006a). Prolonged apnea periods between breaths are believed to be the hallmarks of this type of parasympathetic stimulation (Paintal, 1969, 1981; Pauluhn, 2006a). Their nociceptive role in detecting the onset of pathophysiological conditions implies that C-fiber nerve endings may possibly be dosed more intensively than any other tissue.

Although vagotomy and parasympatholytic drugs (atropine) prevented or abolished the neurogenic etiopathology of phosgene, they did not affect pulmonary edemagenesis (Bruner et al., 1948a, b). This comes as no surprise because these authors exposed dogs to a $C \times t$ of 15,000 mg/m³ × min, which is 15 times higher than used in this rat and previous dog inhalation studies (Pauluhn, 2006c; Pauluhn et al., 2007). There is reason to believe that yet functional pulmonary endothelium and epithelium are among the most essential prerequisites for any beneficial drug therapy (Berthiaume and Matthay, 2007; Berthiaume et al., 1999, 2002). Bilateral vagotomy does not interrupt all neural connection between parasympathetic ganglia and smooth muscles in lung vessels and airways. Also noncholinergic mechanisms (e.g., tachykinergic activity) can exert multiple actions that can elicit changes in the fluid dynamics in the lungs (Hong et al., 1995).

![Graph showing QT interval analysis](https://example.com/fig7.png)

**Fig. 7.** Time-course analysis of the QT interval by telemetry in rats exposed to 1050 mg phosgene/m³ × min. For further details see legend of Figure 6.
The focus of this “proof-of-principle” study was to further elucidate the plausibility of any involvement of neurogenic factors leading to a dysfunctional cardiopulmonary vascular control and eventually lung edema. At an exposure, \( C \times t \) of phosgene triggering a persistent reflex stimulation resulted in frank edemagenesis and associated lethality (Li et al., 2011; Pauluhn, 2006a, b, c). Continued stimulation of vagal C-fibers appears to make them unresponsive to overriding autonomic control. Consequently, sympathetic nervous failure and vascular dynamic disturbances both in the lung and systemic circulation are a likely outcome. Possibly, this leads to a response-to-injury–related imbalance of factors controlling the systemic and pulmonary vascular dynamics with ensuing lung edema and hypoxemia. In case this hypothesis can be further substantiated, this may call for modified treatment strategies at the incipient yet clinically asymptomatic stages of injury.

The combination of prolonged changes in breathing patterns suggestive of vagal involvement, persistent sinus bradycardia after cessation of exposure to phosgene (Fig. 6), and hypothermia (Fig. 2) may be considered as pathognomonic for heightened vagal activity. This arrangement of alterations is not peculiar to phosgene poisoning because similar findings have been noted following exposure to other lung irritants (Bruner et al., 1948a; Gordon et al., 2008; Pauluhn, 2004). Hypothermia and bradycardia were remarkably stable and nonresponsive to the progressive changes in hemodynamics...
(hemoconcentration, Fig. 12) and increased lung water content as evidenced by increased lung weights and protein in BALF (Fig. 12) over a post-phosgene exposure period of approximately 20 h. Within the time periods of heart rate measurement, heart rate variability, i.e., the degree of difference in the interbeat intervals of successive heartbeats, which is an indicator of the balance between the sympathetic and parasympathetic arms of the autonomic nervous system (Farraj et al., 2011), was not observed. In agreement with the sequence of pathophysiological events discussed above, also the heart rate is controlled by parasympathetic mechanisms. Any stimulation of this system essentially “prolongs” the time to the next heartbeat, thus slowing the pulse (Huston and Tracey, 2011).

Vagus nerve afferent activation, originating peripherally, can also modulate efferent sympathetic and parasympathetic function centrally and at the level of the baroreceptor. Parasympathetic stimulation will inhibit tonic sympathetic activation; this means any elevated sympathetic tone is overridden by intense vagus nerve discharge. Elimination of the lung’s sympathetic tone results in a generalized pulmonary vasoconstriction and pulmonary shutdown as already suggested by Ivanhoe and Meyers (1964). This interrelationship is further
complicated by baro- and chemoreceptor activation and vagus-mediated neural mechanisms that inhibit proinflammatory cytokine release via the “cholinergic anti-inflammatory pathway” (Bernik et al., 2002; Borovikova et al., 2000). Ivanhoe and Meyers (1964) conclude that there is vasomotor preponderance of the venous side over the arterial site of the pulmonary circulation and propose that the pulmonary vasoconstriction is a likely cause of the phosgene-induced acute pulmonary edema. This vasoconstriction can further be exacerbated by pulmonary hypoxic vasoconstriction as occurring during life-threatening pulmonary edema. The decrease in heart rate (cardiac output) and systemic vasoconstriction may redistribute the plasma volume into the lung, which aggravates the acute edema and anoxic anoxia in a hemodynamic state of accompanying hemocoagulation and raising blood viscosity. All of these factors seriously impede further with gas exchange and imbalance of the fluid dynamics of the lung.

These processes may also activate NOS through guanylate cyclase signalling cascades. L-NAME has been shown to be effective of mitigating the phosgene-induced lung edema in mice after curative administration of 50 mg/kg bw (Torkunov and Shabanov, 2009). A similar curative dosing regimen was used in this study. However, the examined dose range from 2 to 100 mg L-NAME/kg bw had no effect on body weights or changes in lung weights within a postexposure period shown to attain maximum lung edema (Figs. 1 and 12; see also Pauluhn...
2006b). Similarly, apart from aggravating the bradycardia (Fig. 6) and other ECG indices (see Figs. 7–11), neither NOS inhibitor elicited any beneficial effect on cardiac function or pulmonary edema. Moreover, L-NAME had a deleterious impact on the cardiac endpoints measured but the inducible NOS inhibitor L-NIL had no effect.

Due to the small thermal inertia, unlike humans, small laboratory rodents undergo a hypothermic response when exposed to respiratory tract irritants as shown in Figure 2. This reduction in core body temperature occurred concurrent with a reduction in reflexively related ventilation, heart rate, and metabolism, which may generally confer to rodents a higher resistance to toxic effects (Gordon, 1990, 1993; Gordon et al., 1988, 2008; Watkinson and Gordon, 1993). These species-specific differences need to be appreciated when using small-rodent animal models; otherwise physiological changes secondary to hypothermia are inappropriately attributed as adverse. Phosgene-induced hypothermia is considered to be the cause of reduced CO$_2$ in exhaled breath (Fig. 3). With reference to prestudy validation activities from rats receiving saline (control) or LPS by intratracheal instillation, the NO$_x$ concentrations measured 1 day postinstillation were 15 and 29 ppb, respectively (data not shown). To the contrary, following exposure to phosgene, NO concentrations in exhaled breath of 15.3 ± 0.4 and 17.5 ± 1.5

**FIG. 11.** Time-course analysis of the T amplitude by telemetry in rats exposed to 1050 mg phosgene/m$^3$ × min. For further details see legend of Figure 6.
FIG. 12. Time-course analyses of Hb in blood, concentrations of total protein in BALF, and wet lung weights of non-exposed rats (sham control, C) or rats exposed to 1050 mg phosgene/m³ × min. Examinations were made 0.5, 2.5, 5.5, and 24 h postexposure. Results of rats examined for NO in exhaled breath at the 5- and 24-h time points are shown in Figure 3. Bars represent group means ± SD (n = 3). Asterisks denote statistical significance relative to the concurrent control (*p < 0.05, **p < 0.01).
ppb were measured after 5 and 24 h, respectively. These findings are difficult to put into any pathodiagnostic perspective as normothermic control rats are compared with the hypothermic phosgene-exposed rats. Nonetheless, this comparison shows that in phosgene-exposed rats NO production seems to be less than in septic LPS models of ALI. These findings warrant further study whether NOS inhibitors are useful for edema attenuation or not before arriving at any definite conclusion.

Of note is that the approximately 20% time-related increase in blood Hb appears to match the relative increase in lung weights as shown in Figure 12. Admittedly, shifts in plasma volume from the intravascular space to splanchic organs cannot be confirmed by the method chosen. Likewise, it cannot be excluded that increased Hb in circulating blood is secondary to distress- and/or hypoxia-related autotransfusion of red blood cells from the spleen. Nonetheless, assuming a whole blood volume of 6.4% of the rat’s body weight (Krinke, 2000), a 20% increased Hb would translate to approximately 2.5–3 ml shift of intravascular fluids to the rats’ lung in case systemic vasoconstriction had been the driving force. Interestingly, the observed increase in wet lung weight shown in Figure 12 is not at variance with this crude estimation of blood plasma redistribution into the lung.

In summary, it is concluded that an overstimulated sensorimotor vagal reflex involved in the control of the cardiopulmonary hemodynamics may eventually lead to a self-perpetuating and self-amplifying acute lung edema within 24 h post-phosgene exposure. As the acute pulmonary edema progresses, the significantly depressed cardiac output compromises further the oxygen delivery to tissues. Continued bradycardia after exposure to phosgene and other signs typical of excessive parasympathetic tone have also been observed in man (Bruner et al., 1948b). These characteristics may call for redirection of treatment strategies from anti-inflammatory to reactivation of the mechanisms involved in cardiopulmonary autonomic control and vascular hemodynamics that lead to pulmonary hypertension and edema.

REFERENCES


