A paradoxical protective role for the proinflammatory peptide substance P receptor (NK1R) in acute hyperoxic lung injury

Marwan Dib,1,3 Zsuzsanna Zsengeller,1,3 Alex Mitsialis,2,3 Bao Lu,1 Stewart Craig,1 Craig Gerard,1,3 and Norma P. Gerard1,3

1Ina Sue Perlmutter Laboratory, Pulmonary Division and 2Division of Newborn Medicine, Department of Pediatrics, Children’s Hospital, and 3Department of Medicine, Harvard Medical School, Boston, Massachusetts

Submitted 30 September 2008; accepted in final form 20 July 2009

Dib M, Zsengeller Z, Mitsialis A, Lu B, Craig S, Gerard C, Gerard NP. A paradoxical protective role for the proinflammatory peptide substance P receptor (NK1R) in acute hyperoxic lung injury. Am J Physiol Lung Cell Mol Physiol 297: L687–L697, 2009. First published July 24, 2009; doi:10.1152/ajplung.90509.2008.—The neuropeptide substance P manifests its biological functions through ligation of a G protein-coupled receptor, the NK1R. Mice with targeted deletion of this receptor reveal a preponderance of proinflammatory properties resulting from ligand activation, demonstrating a neurogenic component to multiple forms of inflammation and injury. We hypothesized that NK1R deficiency would afford a similar protection from inflammation associated with hyperoxia. Counter to our expectations, however, NK1R−/− animals suffered significantly worse lung injury compared with wild-type mice following exposure to 90% oxygen. Median survival was shortened to 84 h for NK1R−/− mice from 120 h for wild-type animals. Infiltration of inflammatory cells into the lungs was significantly increased; NK1R−/− animals also exhibited increased pulmonary edema, hemorrhage, and bronchovascular lavage fluid protein levels. TdT-mediated dUTP nick end labeling (TUNEL) staining was significantly elevated in NK1R−/− animals following hyperoxia. Furthermore, induction of metallothionein and Na+−K+−ATPase was accelerated in NK1R−/− compared with wild-type mice, consistent with increased oxidative injury and edema. In cultured mouse lung epithelial cells in 95% O2, however, addition of substance P promoted cell death, suggesting the neurogenic component of hyperoxic lung injury is mediated by additional mechanisms in vivo. Release of bioactive constituents including substance P from sensory neurons results from activation of the vanilloid receptor, TRPV1. In mice with targeted deletion of the TRPV1 gene, acute hyperoxic injury is attenuated relative to NK1R−/− animals. Our findings thus reveal a major neurogenic mechanism in acute hyperoxic lung injury and demonstrate concerted actions of sensory neurotransmitters revealing significant protection for NK1R-mediated functions.

tachykinins; neurogenic inflammation; oxidative lung injury; transient receptor potential vanilloid type 1; TRPV1

NEUROGENIC INFLAMMATION is characterized as sensations of pain, increased vascular permeability, increased blood flow, influx of inflammatory cells, and contraction of smooth muscle (9, 25). Perhaps the most well-known mediator associated with this type of inflammation is the tachykinin, substance P. Substance P is a low-molecular weight (~1,000 Da) peptide that is released from sensory neurons and several types of inflammatory cells including macrophages, eosinophils, lymphocytes, and dendritic cells (5, 7, 13, 38, 47). Its functions are mediated via a seven-transmembrane segment (7TMS) G protein-coupled receptor, the neurokinin-1 (NK1) receptor (NK1R), and include increased vascular permeability, contraction of smooth muscle, chemotaxis of inflammatory cells, and transmission of painful sensations, among others (22, 43–45). A second high-affinity ligand for the NK1R has been identified as hemokinin-1, which differs from substance P by a single in amino acid residue and appears to originate primarily from hematopoietic sources (41, 68).

Targeted deletion of the NK1R in mice has revealed a predominance of proinflammatory functions resulting from activation by substance P and/or hemokinin-1. In our initial characterization of the NK1R−/− animals, we (8) observed protection from inflammatory cell influx and edema formation resulting from acute immune complex injury of the lung. Inflammation resulting from immune complex cystitis was similarly absent in NK1R−/− mice compared with wild-type controls (53). Inflammation and airway hyperresponsiveness resulting from sensitization with dinitrofluorobenzene followed by intranasal challenge with dinitrobenzene sulfonic acid were also reduced (62). Additionally, ethanol-induced activation of gastric inflammation was absent in NK1R−/− mice (21).

In more precise mechanistic studies, it was found that the induction of cutaneous inflammation by intradermal injection of carrageenan followed by substance P resulted in neutrophil influx and edema formation in wild-type mice but not NK1R−/− animals (11). Intradermal injection of substance P alone in normal wild-type mice resulted in edema but little or no infiltration of neutrophils. In the absence of the NK1R, intradermal substance P produced neither edema nor neutrophil influx (11, 66). In injury caused by noxious heat, NK1R deficiency resulted in a delay in the formation of edema compared with NK1R sufficient animals but no change in the infiltration of neutrophils (52). Application of the sensory nerve stimulus capsaicin to the mouse ear produced increases in both edema and blood flow in wild-type animals (23). In NK1R−/− mice, the edema response was absent, but blood flow was increased ~2-fold relative to the wild-type response. That this was not a compensatory effect of NK1R deletion was shown by a similar increase in blood flow in animals treated with the NK1R antagonist, SR 104333. The increased blood flow in this model has been attributed to CGRP released from sensory nerve fibers concomitantly with substance P (23). Inhibition of the receptor for CGRP abolished the increase in blood flow in the absence of the NK1R but did not alter the response in wild-type mice, and studies have demonstrated that substance P-NK1R interactions negatively modulate the vasodilatation caused by CGRP. Thus, although distinctions exist in the physiological responses to a variety of inflammatory stim-
Inflammatory injury to the lung caused by hyperoxia is characterized by edema and an abundance of inflammatory cells as well as epithelial and endothelial damage indicative of a combination of apoptosis and necrosis (10, 27). In humans, such pulmonary oxygen toxicity may occur as a result of high inspired oxygen concentrations associated with mechanical ventilation. The initiating factors involved in this type of lung injury include the generation of reactive oxygen species (ROS), including superoxide anion (O$_2^-$), hydroxyl radical (OH$^-$), and hydrogen peroxide (H$_2$O$_2$) (28, 30). These highly oxidative species appear to overwhelm the endogenous lung antioxidants (e.g., glutathione, SOD, and catalase) and produce cellular injury through direct damage to DNA and mitochondria as well as oxidation of lipids and intracellular proteins. Exposure to ROS has also been shown to activate MAPK pathways in various models, leading to activation of the transcription factor, NF-κB (1, 39, 49, 56, 65). This, in turn, results in production of the cytokines, TNFα, IL-1β, IL-6, and IL-8, among others (15, 16, 32, 34). N-acetyl-l-cysteine, a precursor of glutathione, as well as other antioxidants such as SOD, dithiocarbamates, and metal chelators have been shown to inhibit NF-κB activation induced by ROS (30, 54, 55, 57).

Signaling pathways involving CXCR2 have further been demonstrated to contribute to the inflammatory cell influx (58), and constitutively elevated expression of the cytokines IL-6 and IL-11 can serve a protective role against apoptotic processes by upregulating the expression of antiapoptotic Bcl-2 proteins (24, 63, 64).

Thus considerable knowledge is extant relating to oxidative mechanisms of lung injury and the signaling pathways associated with hyperoxia. A clinical study indicates patients exposed to increased oxygen levels for therapeutic purposes exhibit increased bronchoalveolar lavage (BAL) fluid substance P levels (20), however, virtually nothing is known of the potential for contributions of other host defense mechanisms in hyperoxic lung injury including those of neuroendocrine origin. As our previous studies using NK1R$^{-/-}$ mice have uniformly revealed a proinflammatory role for activation of this receptor, we hypothesized that these animals would be at least partially protected from hyperoxic lung injury. Paradoxically, our data reveal a major protective role for activation of this neuropeptide receptor.

**EXPERIMENTAL PROCEDURES**

**Mice.** The generation and phenotypic characterization of NK1R$^{-/-}$ mice has been previously described (6, 8, 11, 21, 23, 52, 53, 62). NK1R-deficient animals backcrossed through at least 12 generations to C57BL/6 and wild-type C57BL/6 mice were used for the studies described here. Mice with targeted deletion of the vanilloid receptor, TRPV1, on the C57BL/6 background were obtained from The Jackson Laboratories (12, 19). All studies were conducted in accordance with the Institutional Animal Care and Use Committee of Children’s Hospital, Boston, Massachusetts.

**Hyperoxia exposure.** Mice of the genotypes described, at 8–12 wk of age, were placed in an Oxycycler chamber (Biospherics) and maintained at atmospheric pressure in 90% O$_2$–10% N$_2$ with food and water ad libitum and a 12:12-h light-dark cycle. Control animals were maintained in room air. To assess sensitivity to the lethal effects of hyperoxia, animals were monitored 2–3 times per day over a period of 7–8 days. Posthyperoxic mortality was determined after 72- or 96-h exposure to 90% O$_2$ followed by return to room air and subsequent monitoring for 7–8 days. For all mechanistic studies, mice were maintained in 90% O$_2$ for 72 h before death. Lungs were obtained for histological or biochemical analyses as described. For some analyses, results from room air-exposed wild-type and NK1R$^{-/-}$ mice were combined in the interest of conserving animals. This modified control is justified by the observation that baseline parameters of inflammation in this mouse line have never been different from those of wild-type animals (6, 8, 11, 17, 23, 52, 53, 62).

**Histopathology.** Following death, the lungs were fixed in situ by intratracheal instillation of 4% paraformaldehyde in PBS at 20 cmH$_2$O pressure for 10 min, subsequently removed, and immersed in additional fixative for 2 h. Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

**Evaluation of edema.** Mice were injected via tail vein with Evans blue dye (0.7% in 0.9% NaCl) 30 min before death as previously described (8). Following euthanasia, lungs were perfused through the right heart ventricle to remove intravascular dye, and extravasated dye was extracted as described previously (48). The Evans blue content was determined by measurement of the A$_{620}$ corrected for the content of heme pigments: A$_{620}$ (corrected) = A$_{620}$ – (1.426 × A$_{740}$ + 0.030). Permeability index was determined from the ratio of Evans blue extracted from the lungs vs. the dye content in plasma.

For determination of lung wet-to-dry weight ratios, following death, the lungs were dissected free from surrounding tissues, blotted dry, weighed, and dried to a constant weight under vacuum at 60°C. The weight ratio of the wet-to-dry lung was calculated to assess tissue edema.

**BAL and protein determination.** BAL was performed twice with 1 ml of PBS and immediately processed for inflammatory cell content. The total cellularity was determined using an automated cell counter. Cell differentials were determined from Wright-Giemsa-stained cytocentrifuge preparations, counting a minimum of 300 cells from each sample. The remaining BAL fluid was stored at −80°C for subsequent analysis. Total protein content was determined by Coomassie blue staining using a standard curve generated with BSA.

**Neutrophil MPO assay.** Tissue homogenates were suspended in 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer, pH 6.0. MPO content was determined by reaction with o-dianisidine hydrochloride in a microplate assay as previously described, and the cell number was calculated based on a standard curve generated from known cell concentrations (8).

**Cytokine assay.** Measurement of TNFα, IL-1β, IFN-γ, and IL-11 in BAL fluid and tissue homogenates was performed using ELISA kits according to the manufacturer’s instructions (BD Pharmingen).

**TUNEL assay.** Labeling of DNA fragments in paraffin-embedded tissue sections was accomplished using the ApopTag Peroxidase in situ apoptosis detection kit (Millipore) using protocols provided by the manufacturer. After staining, 20 fields of alveoli were chosen randomly, and 2,000 nuclei were counted per lung. The apoptotic index was determined by the percentage of labeled cells.

**Protein extraction and Western blotting.** Lung samples were homogenized in lysis buffer containing 25 mM Tris-HCl, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N’,N’-tetraacetic acid, 10% glycerol, and 1% Triton X-100 supplemented with protease inhibitors (Complete Protease Inhibitor Cocktail; Roche) as previously described (67). The concentration of solubilized protein was determined using Coomassie blue protein assay reagent (Pierce). Equal quantities (25 µg per lane) were subjected to SDS-PAGE under reducing conditions followed by immunoblotting to detect Na$^+$/K$^+$-ATPase α1-subunit (cat. no. 464.6; Abcam) or metallothionein (cat. no. 18-0133; Zymed). The relative amounts of each protein were quantitated by densitometry.

AJP-Lung Cell Mol Physiol • VOL 297 • OCTOBER 2009 • www.ajplung.org

Downloaded from http://ajplung.physiology.org/ by 10.20.32.246 on June 20, 2017
Assessment of caspase activity. The enzymatic activities of caspase-3 and caspase-8 were determined colorimetrically using homogenates of lung tissues reacted with N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) or N-acetyl-Ile-Glu-Thr-Asp-p-nitroanilide (Ac-IETD-pNA), respectively (Millipore). Activity was determined by absorbance at 405 nm and expressed as picomoles per minute per milligram protein.

Cell cultures. Murine lung epithelial MLE-12 cells were a generous gift of Dr. Cindy Bachursky, University of Cincinnati College of Medicine. Cells were maintained in DMEM-10% FBS with antibiotics and transfected for stable expression of the human NK1R (Amaxa). Positive cells were cloned by limiting dilution and selected with G418. Cells at ~80% confluence were subjected to 95% O₂-5% CO₂ in the presence or absence of 1 μM substance P for 24 h and assessed for apoptotic changes by staining with Alexa Fluor 488 annexin V and propidium iodide (PI) (Vybrant Apoptosis Assay Kit; Invitrogen) and flow cytometric analysis.

Statistical evaluation. Data were expressed as means ± SE and evaluated for significance by Student’s t-test. Survival curves were evaluated using log rank analysis. Differences were considered significant for \( P \leq 0.05 \) using Prism software (GraphPad).

RESULTS

**NKIR expression promotes survival in hyperoxia.** In initial experiments to examine a role for the NK1R in hyperoxic lung injury, we compared the survival of NK1R−/- mice, fully backcrossed to the C57BL/6 background, with wild-type C57BL/6 controls, with the toxic effects of supranormal oxygen concentrations. Animals were maintained in 90% O₂-10% N₂ under normobaric conditions with food and water ad libitum and a 12:12-h light-dark cycle. Increased sensitivity of the NK1R−/- mice was evident by the incidence of initial mortality at 72 h (Fig. 1A). All animals died within 96 h of hyperoxic exposure. Survival of the NK1R−/- mice was significantly attenuated compared with that of wild-type animals, in which initial deaths were observed at 96 h, and 100% mortality was not reached until 144 h. The median survival of NK1R−/- mice in 90% O₂ was 84 h, compared with wild-type animals with median survival of 120 h (different at \( P < 0.0001; n = 10-16 \) mice per group).

To evaluate the effects of NK1R expression on the recovery from oxygen toxicity, we determined the posthyperoxia mortality by exposing animals to 90% O₂ for 72 h followed by return to room air. Under this condition all the NK1R−/- mice died during the subsequent 96 h of observation, whereas wild-type animals were completely resistant (Fig. 1B; \( P < 0.005; n = 10 \) mice per group). However, when wild-type mice were maintained in 90% O₂ for 96 h before returning to room air, they exhibited a similar mortality as NK1R−/- animals during the subsequent 72–96 h (different from 72-h hyperoxia at \( P < 0.01; n = 10 \) mice per group; data not shown).

Wild-type mice treated with the NK1R antagonist, CP-96345, before hyperoxia exposure exhibited a similar increase in mortality indicating the differences were not due to genetic compensation resulting from NK1R deletion (data not shown).

**NKIR expression protects airway epithelium from hyperoxic damage.** Our initial characterization of the phenotype of NK1R−/- mice revealed no overt morphological differences in the lungs compared with wild-type mice housed in room air (8). Following 72-h exposure to 90% O₂, a striking difference was evident in the histological appearance of the lungs of NK1R−/- and wild-type mice (Fig. 2). The airway epithelium of NK1R−/- mice exhibited considerably greater damage compared with wild-type animals. NK1R−/- mouses lungs also revealed increased numbers of inflammatory cells, entrapped red blood cells, and thickened alveolar membranes compared with wild-type animals.

**NKIR expression is protective against hyperoxia-induced pulmonary edema.** To begin to address the mechanistic changes responsible for the increased sensitivity of NK1R−/- mice to hyperoxic lung injury, we evaluated the resulting pulmonary edema developed by the two mouse strains. Assessment of tissue wet-to-dry weights following 72-h 90% O₂ revealed elevations for both NK1R−/- and wild-type animals compared with mice housed in room air (Fig. 3A; \( P < 0.0001 \) for room air vs. hyperoxia, NK1R−/- or wild-type mice; \( n = 10 \) mice per group). In NK1R−/- animals, this increase was significantly greater than in wild-type mice (\( P < 0.001 \) for hyperoxia-exposed NK1R−/- vs. wild-type mice; \( n = 10 \) mice per group). Evaluation of the changes in vascular permeability by measurement of the extravasation of Evans blue dye revealed an elevation of ~4-fold for NK1R−/- mice compared with wild-type animals (Fig. 3B; \( P < 0.0005 \) for hyperoxia, NK1R−/- vs. wild-type mice; \( n = 5-7 \) animals per group).
Consistent with increased vascular leak in the NK1R−/− mice, the protein content of BAL fluid was significantly elevated following hyperoxia relative to wild-type animals \((P < 0.001 \text{ vs. wild-type mice exposed to } 90\% \text{ O}_2 \text{ for } 72 \text{ h})\). The epithelial injury \((*)\), inflammatory cell infiltrate \((\triangle)\), increased alveolar thickness \((\downarrow)\), and entrapped red blood cells \((\downarrow)\) characteristic of hyperoxic injury are all significantly more pronounced in NK1R−/− mice compared with wild-type animals. Representative of 6–8 mice per group. Hematoxylin and eosin (H&E) stain, magnification ×400.

Another hallmark of acute hyperoxic lung injury is tissue engorgement of red blood cells, reflected as increased hemoglobin content of tissue homogenates \((14, 46)\). In line with this observation, lung homogenates from both NK1R−/− and wild-type mice exposed to hyperoxia also exhibited increased hemoglobin contents compared with room air-exposed animals \((P < 0.0001 \text{ vs. room air vs. hyperoxia-exposed wild-type mice; } n = 6–8 \text{ mice per group per lane} \text{ vs. Fig. } 4C)\).

**Inflammatory cell influx in hyperoxia-exposed NK1R−/− mice.** Previous studies have demonstrated increased pulmonary influx of inflammatory cells following exposure to hyperoxia \((14, 59)\). Consistent with these reports, the BAL fluid content of neutrophils in hyperoxia-exposed wild-type mice was significantly increased over room air-exposed animals \((1.5 \pm 0.5 \times 10^5 \text{ cells for hyperoxia-exposed wild-type mice vs. } 1.0 \pm 0.7 \times 10^5 \text{ cells for room air-exposed animals; } P < 0.002; n = 11–12 \text{ mice per group})\). In contrast, BAL fluid from mice deficient in the NK1R did not exhibit increased neutrophils following 72-h exposure to 90% \text{ O}_2 \((0.9 \pm 0.2 \times 10^5 \text{ cells for hyperoxia-exposed NK1R−/− mice, } n = 11–12 \text{ mice per group})\).

As this result was inconsistent with the histological findings, we also assessed homogenates of lung tissue for neutrophil content based on MPO activity. Reflecting the increase in inflammatory cell content of BAL fluid from hyperoxia-exposed wild-type mice, the MPO activity in lung homogenates was also increased relative to room air-exposed control animals \((P < 0.01 \text{ for hyperoxia vs. room air, wild-type mice; } n = 6–12 \text{ mice per group; Fig. } 4B)\). In striking contrast, the MPO level of hyperoxia-exposed NK1R−/− mice was increased \(~5\)-fold relative to wild-type animals \((P < 0.0001 \text{ for hyperoxia-exposed NK1R−/− vs. wild-type mice; } n = 6–12 \text{ mice per group})\). This suggests the deficiency in NK1R does not block neutrophil recruitment to the lungs but results in defective cellular transmigration into the airways. It further distinguishes the mechanisms involved in cellular transmigration from increases in microvascular permeability.

**Activation of hyperoxic-sensitive genes.** Gene profiling studies of hyperoxic lung injury in mice reveal, among other changes, marked upregulation in the gene for the metal-binding protein metallothionein \((50, 51)\). Although the mechanism of action of this protein as an antioxidant is not entirely clear, it likely serves to scavenge ROS \((3)\). To determine whether NK1R−/− mice are deficient in regulation of hyperoxia-responsive genes, we evaluated the lung metallothionein content as a function of exposure to 90% \text{ O}_2 \(\text{(44) by } 10.2 \pm 0.3 \text{ mg per lane were subjected to Western blot analyses as shown in Fig. } 5A\). Results reveal significant increases in lung metallothionein content for both mouse strains compared with mice housed in room air. In NK1R−/− mice, induction of this enzyme is evident earlier than in wild-type animals. The metallothionein level in NK1R−/− mouse lungs following 24-h exposure to 90% \text{ O}_2 \(\text{was } \sim 60\% \text{ greater than for wild-type mice; } (P < 0.0001 \text{ for NK1R−/− vs. wild-type animals at } 24 \text{ h; } n = 5 \text{ mice per group})\). At 48- and 72-h hyperoxia, elevated metallothionein levels persisted for both mouse strains compared with room air controls but were not markedly different from one another.

**Expression of Na\(^+\)-K\(^+-\)ATPase is associated with clearance of edema following injury to the lungs \((42)\). Since NK1R−/− mice develop greater pulmonary edema following hyperoxia exposure compared with wild-type animals, we considered the possibility of a deficiency in the induction of this enzyme. Western blot analyses \((25 \mu g \text{ protein per lane})\) shown in Fig. 5B reveal elevated Na\(^+\)-K\(^+-\)ATPase in the lungs of both...
NK1R−/− and wild-type mice following 72-h hyperoxia compared with animals housed in room air (P < 0.05; n = 5 mice per group). Similar to metallothionein induction, Na+K+-ATPase expression was increased earlier in NK1R−/− mice compared with wild-type animals (P < 0.0001 for NK1R−/− vs. wild-type mice at 48 h; n = 5 mice per group). By 72 h, the Na+K+-ATPase levels were elevated 2-fold for both mouse strains compared with room air-exposed animals but not significantly different from one another.

Gene-profiling analyses suggest little change in the expression of several proinflammatory cytokines, however, mice transgenic for the anti-inflammatory cytokines IL-6 or IL-11 exhibit dramatic protection against acute hyperoxic injury (24, 63, 64). Both NK1R−/− and wild-type animals exhibit reduced levels of TNFα, IFN-γ, and IL-1β compared with room air-treated animals following 72-h hyperoxia (Fig. 6). No change was observed in the levels of IL-11 (data not shown).

NK1R expression is protective against hyperoxia-induced apoptosis in vivo. Acute hyperoxic lung injury is associated with cell death caused by a combination of apoptotic and necrotic processes (10, 27, 28, 31). The representative TdT-mediated dUTP nick end labeling (TUNEL) staining shown in Fig. 7A reveals a dramatic increase in the number of TUNEL-positive airway cells NK1R−/− mice compared with wild-type animals after 72-h hyperoxia. Enumeration of these cells indicates an increase of >2-fold for hyperoxia-exposed NK1R−/− mice at 72 h compared with animals exposed to room air and ~80% more than for hyperoxia-exposed wild-type mice (P = 0.04 for wild-type mice, 72-h hyperoxia vs. room air; P < 0.0001 for hyperoxia-exposed NK1R−/− mice vs. wild-type mice at 72 h 90% O2; n = 3 animals per group).

As a further measure of apoptotic changes, we assessed the levels of lung caspase activity following 72-h hyperoxia. Both NK1R−/− and wild-type mice exhibited increases in caspase-3 activity compared with room air-exposed animals, but no significant difference was observed between the two strains following hyperoxia (Fig. 7B; P < 0.0001 for hyperoxia vs. room air, wild-type mice; **P < 0.001 for hyperoxia-exposed NK1R−/− mice vs. wild-type; n = 6–12 mice per group). No difference was observed for caspase-8 (data not shown).

NK1R activation in mouse lung epithelial cells is toxic under conditions of hyperoxia. The data presented above reveal a protective role for expression of the NK1R in hyperoxia-induced acute lung injury. To determine whether this result is mediated by direct action of substance P on the airway epithelium, we examined the response of mouse lung epithelial MLE-12 cells to supranormal oxygen concentrations follow-
ing transfection with the NK1R. Exposure of NK1R-expressing MLE-12 cells to exogenous substance P in room air containing 5% CO2 had no apparent effect on induction of apoptosis based on staining with annexin V and PI (Fig. 8).

Exposure of MLE-12 cells to 95% O2-5% CO2 for 24 h increased annexin V staining but did not substantially alter the proportion of PI-positive cells and was not effected by NK1R expression. Following culture in hyperoxia in the presence of exogenous substance P for 24 h, NK1R-transfected MLE-12 cells exhibited a reduction in the population of cells positive for both annexin V and PI by ~50% and a commensurate increase in PI-positive but annexin V-negative cells. This result indicates increased toxicity to cells exposed to substance P in the presence of elevated oxygen concentrations and suggests a more complex relationship between released substance P and the physiological changes observed in hyperoxia in vivo.

**Hyperoxic activation of TRPV1.** The primary source of substance P is sensory neurons, which release their neurotransmitter components on stimulation of the vanilloid receptor, TRPV1 (12). Alterations in physiological responses resulting from genetic deficiency of this receptor or from neonatal depletion of neurotransmitters is a well-established criterion for invoking release of substance P. TRPV1, characteristically responsive to the pungent agent of hot peppers, capsaicin, is also activated by elevation in temperature, decrease in pH, and exposure to ROS, conditions consistent with inflammation (60). The striking increase in injury and inflammation observed following acute hyperoxic exposure in NK1R−/− mice, coupled with the toxicity of substance P on NK1R-transfected lung epithelial cells in culture, led us to consider the possibility of a more complex mechanism reflecting an interplay of substance P with other constituents of sensory neurons released in vivo. When TRPV1−/− mice were subjected to 90% O2, their survival was significantly prolonged relative to NK1R−/− mice but not different from wild-type animals (Fig. 9). Historical evaluation revealed similar changes in wild-type and TRPV1−/− mouse lungs (data not shown). Thus activation of the NK1R by substance P, in combination with the actions of additional neurotransmitters released on exposure to hyper-

---

**Fig. 4.** Inflammatory cell influx and hemoglobin content of NK1R−/− and wild-type mouse lungs following hyperoxia. A: the content of neutrophils in BAL fluid is elevated for wild-type but not NK1R−/− mice after exposure to hyperoxia (*P < 0.002 for wild-type mice, hyperoxia vs. room air; **no significant difference (NS) for NK1R−/− mice, hyperoxia vs. room air; n = 11–12 mice per group). B: lung content of neutrophils determined from the tissue content of MPO (*P < 0.01 for wild-type mice, hyperoxia vs. room air; n = 6–12 mice per group; **P < 0.0001 for hyperoxia-exposed NK1R−/− vs. wild-type mice; n = 11–12 mice per group). C: lung content of hemoglobin (*P < 0.001 for hyperoxia vs. room air; n = 6–8 mice per group; **P < 0.0005 for NK1R−/− vs. wild-type mice exposed to hyperoxia; n = 6–12 mice per group).
oxia, serves an overall protective role in this form of lung inflammation and injury.

DISCUSSION

Release of the neuropeptide substance P and activation of its selective receptor, the NK1R, are associated with inflammation and pain (9, 11, 25, 44). The primary source of this peptide is the peripheral nervous system, and detection of its contribution to various physiological responses has been used to define a component of neurogenic activation. Previous studies using mice with targeted deletion of the NK1R gene have revealed a predominantly proinflammatory role for receptor activation in hyperoxia, which supports the protective role of NK1R in hyperoxic lung injury.

Fig. 5. Hyperoxia-mediated induction of metallothionein and Na⁺-K⁺-ATPase is accelerated in NK1R−/− mice compared with wild-type. A: densitometric quantitation of Western blots for metallothionein in the lungs of wild-type and NK1R−/− mice as a function of exposure to 90% O₂. Enzyme levels were increased relative to room air-exposed mice as early as 24 h after initiation of hyperoxic conditions for NK1R−/− animals (*P < 0.0001 for NK1R−/− vs. wild-type mice at 24 h; n = 6–8 mice per group). After 48- and 72-h hyperoxia exposure, both NK1R−/− and wild-type mice exhibited elevated metallothionein levels relative to room air, but the levels for NK1R−/− and wild-type animals were not distinguishable (**P < 0.0005 at 24 h, ***P < 0.001 at 72 h, hyperoxia vs. room air, NS for NK1R−/− vs. wild-type mice in hyperoxia; n = 6–8 mice per group). Data are expressed as the percent ± SE of room air-exposed animals. B: representative Western blot of metallothionein extracted from mouse lungs as a function of exposure to hyperoxia. Lung homogenate protein, 25 μg per lane, was separated by SDS-PAGE and Western blotted as described in EXPERIMENTAL PROCEDURES. The enzyme appears as the monomer and β-ME-resistant oligomers. C: Na⁺-K⁺-ATPase levels in lung homogenates were determined by Western blotting and quantitated by densitometry. Values represent the mean percent ± SE relative to room air-exposed mice (*P < 0.0001 for NK1R−/− vs. wild-type mice at 48-h hyperoxia; **P < 0.05 for wild-type mice at 72-h vs. 48-h hyperoxia; n = 5–8 mice per group). D: representative Western blot of Na⁺-K⁺-ATPase extracted from mouse lungs as a function of exposure to hyperoxia. Lung homogenate protein, 25 μg per lane, was separated by SDS-PAGE and Western blotted as described in EXPERIMENTAL PROCEDURES.

Fig. 6. Cytokine levels are decreased for NK1R−/− mice compared with wild-type animals following 72-h hyperoxia. ELISA assays of TNFα, IFN-γ, and IL-1β in homogenates of NK1R−/− and wild-type mouse lungs housed in room air or 90% O₂ for 72 h (TNFα, *P < 0.05 for NK1R−/− vs. wild-type mice exposed to hyperoxia; IFN-γ, *P < 0.0001 for both strains, hyperoxia vs. room air, **P < 0.005 for NK1R−/− vs. wild-type mice exposed to hyperoxia; IL-1β, *P < 0.0001 for both strains, room air vs. hyperoxia, **P < 0.0001 for NK1R−/− vs. wild-type mice exposed to hyperoxia; n = 8–15 mice per group).
multiple experimental models. Deficiency of the NK1R in the mouse afforded protection from edema and inflammatory cell influx associated with passive immune complex injury in the lung (8). In allergic airway inflammation and cystitis induced by antigen sensitization and challenge, NK1R animals exhibited significantly increased mast cell numbers following sensitization, and histological evidence of degranulation was apparent following antigen challenge (17, 53). Thus substance P-NK1R interactions contribute to the pathophysiology of allergic inflammatory reactions.

Fig. 7. NK1R mice exhibit greater evidence of apoptosis compared with wild-type animals after exposure to hyperoxia. A: representative TdT-mediated dUTP nick end labeling (TUNEL) staining of sections of lung tissues from wild-type and NK1R mice housed in room air or 90% O2 for 72 h. Cobalt-enhanced peroxidase staining (black) indicates apoptotic cells (representative of 3 animals per group). Sections were counterstained with nuclear fast red, magnification ×400. B: TUNEL-positive nuclei were enumerated from 20 randomly chosen fields, and 2,000 nuclei per lung were counted. Data are presented as the percent positive cells (*P < 0.05 for wild-type mice, hyperoxia vs. room air; **P < 0.0001 for NK1R vs. wild-type following 72-h hyperoxia; n = 3 mice per group). C: enzymatic activity of caspase-3 in lung tissues from mice housed in room air or 90% O2 for 72 h. Equal amounts of protein were assessed colorimetrically for caspase-3 activity by reacting with N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) as described in EXPERIMENTAL PROCEDURES. Data are expressed as the means ± SE picomoles per minute per milligram protein (*P < 0.0001 for both strains, hyperoxia vs. room air; **NS between hyperoxia-exposed NK1R and wild-type mice; n = 4–14 mice per group).

Fig. 8. Cytotoxicity of substance P in NK1R-transfected mouse lung epithelial cells cultured in 95% O2. Murine lung epithelial MLE-12 cells stably transfected with the human NK1R were grown in room air with 5% CO2 or 95% O2-5% CO2 for 24 h in the presence or absence of 1 μM substance P (SP). Cells were harvested, stained with Alexa Fluor 488 annexin V and propidium iodide, and analyzed by flow cytometry. The percent of gated cells in each quadrant is indicated. Representative of 3 independent experiments.
treated with an NK1R antagonist. The increase in blood flow induced by capsaicin, however, was significantly potentiated in NK1R−/− or antagonist-treated mice compared with wild-type animals. Subsequent studies showed this component of the response was mediated, at least in part, by CGRP released with and acting in concert with substance P. In wild-type mice treated with capsaicin, a CGRP receptor antagonist did not alter blood flow but in NK1R−/− animals abolished the increased blood flow (23). Following cutaneous administration of the mast cell degranulating agent, compound 48/80, NK1R−/− mice developed much more edema compared with wild-type animals (11). This response was associated with vasodilatation caused by bradykinin. Thus these studies highlight some of the complexities in neurogenic inflammatory reactions in which substance P release may either exacerbate or temper aspects of the injury identified.

Acute hyperoxic lung injury is characterized by airway epithelial damage resulting from exposure to reactive oxygen products that overwhelm the lung’s endogenous armament of antioxidants (2, 27, 31, 33). Mechanistically, the injury is associated with a combination of cellular apoptosis and necrosis followed by an influx of inflammatory cells (31, 36, 37). To determine the possibility that a neurogenic component contributes to acute hyperoxic lung injury, we examined mice with a targeted deletion of the NK1R for differences in responses from wild-type animals after exposure to 90% oxygen. Our data provide positive evidence for NK1R activation in acute hyperoxia. However, in contrast to the proinflammatory role evident for substance P-NK1R interactions in other models, animals deficient in the NK1R exhibited much more severe injury compared with receptor-sufficient mice. Survival was significantly shortened in NK1R−/− animals, and other markers of injury including pulmonary edema and tissue influx of inflammatory cells were dramatically elevated in NK1R−/− mice compared with wild-type animals. Similarly enhanced responses were observed in animals treated with an antagonist of the NK1R. Apoptosis/necrosis of airway cells and induction of enzymes with homeostatic functions including metallothionein and Na+/K+-ATPase were also more pronounced in NK1R−/− mice, suggesting exacerbation of all aspects of this model.

In NK1R−/− mice exposed to hyperoxia, the inflammatory cell content of BAL fluid was significantly reduced compared with wild-type animals, whereas the MPO content of the lung tissues was ∼5-fold greater (Fig. 4, A and B). This suggests the mechanism of cellular chemotaxis is intact, but deficiency of the NK1R impairs the ability of cells to traverse the endothelium. This result is somewhat reminiscent of our findings with CCR3-deficient mice, in which eosinophils accumulated in the subendothelial space following antigen sensitization and challenge but were blocked from traversing into the air spaces (26). In the case of CCR3 deficiency, the defect in migration was likely a reflection of eosinophil function, whereas in NK1R deficiency, receptor distribution makes the mechanism more difficult to predict. The defect in NK1R-deficient animals is not a direct reflection of the vascular permeability enhancing property of substance P since edema formation determined either by Evans blue extravasation or tissue wet-to-dry weight ratios was greater in the absence of the receptor (Fig. 3, A and B).

Studies of the response to hyperoxia in mice with targeted deletion of CXCR2 revealed complete protection from neutrophil influx based on the tissue MPO levels in lung homogenates, indicating ligands of CXCR2, CXCL1, and CXCL2/3 are at least partially responsible for the neutrophil influx associated with hyperoxia. In addition, the edema response (lung wet/dry weight or Evans blue extravasation) was absent following exposure to hyperoxia (58), suggesting the release of substance P is downstream from chemokine production.

Gene profiling studies of the response to hyperoxia in C57BL/6 mice indicated little or no change in the majority of “classic” antioxidant enzymes, including catalase, MnSOD, Cu/Zn SOD, glutathione peroxidase, glutathione S-transferase, and heme oxygenase-1 (50). An exception was the heavy metal binding enzyme metallothionein, which showed a dramatic increase following hyperoxia. We also observed increased metallothionein protein expression following hyperoxia in both wild-type and NK1R−/− mice (Fig. 5, A and B). This increase was accelerated in NK1R−/− mice relative to wild-type controls, revealing significant induction at 24 h but no difference from wild-type animals at later time points.

Clearance of pulmonary edema is dependent on active transport of sodium across the alveolar epithelium mediated in part by the activity of Na+/K+-ATPase (35, 42). Although changes in gene expression of this enzyme were not identified in hyperoxic lung injury, multiple reports indicate its essential function (35, 69). Consistent with the increased pulmonary edema observed in response to hyperoxic exposure in both NK1R−/− and wild-type mice (Fig. 3, A and B), we also observed increases in Na+/K+-ATPase immunoreactivity (Fig. 5, C and D). In NK1R−/− animals, induction of Na+/K+-ATPase was detectable in advance of that in wild-type mice, likely reflecting the relative increase in edema formation in this strain.

ROS-mediated activation of NF-κB is a generally accepted mechanism for regulation of transcription of multiple proinflammatory products (39). Jensen et al. (29) reported that mRNAs levels for proinflammatory products downstream from NF-κB activation, including TNFα, IL-1β, and IL-6, were upregulated in C57BL/6 mice after 3 days of hyperoxia. Perkowski et al. (50), however, failed to identify increases in expression of these cytokines. Indeed, TNFα expression was reduced to ∼35% of room air after 24-h hyperoxia. In the studies reported here, we observed significant reductions of

Fig. 9. Survival of transient receptor potential vanilloid type 1 (TRPV1) −/− mice in hyperoxia is increased relative to NK1R−/− animals and indistinguishable from wild-type mice. Mice of the genotypes indicated were placed in 90% O2-10% N2 and monitored for mortality. Survival of TRPV1 mice was significantly increased compared with NK1R−/− animals (P < 0.0001; n = 12 mice per group) but not different from wild-type mice (P = NS; n = 12 mice per group).
TNFα for NK1R−/− mice but not wild-type animals following 72-h hyperoxia (Fig. 6). IFN-γ was significantly reduced by hyperoxia in both mouse strains, and NK1R−/− animals showed a greater reduction than wild-type mice. Levels of IL-1β were increased for both strains following hyperoxia compared with room air but less so for NK1R−/− animals. The significance of these findings is not clear, especially given the discordance in literature reports of cytokine production in hyperoxia.

Another hallmark of acute hyperoxic lung injury is apoptosis and/or necrosis of airway cells (2, 4, 18, 31, 36, 37). Reflecting the discordance in literature reports of cytokine production in hyperoxia.

The significance of these findings is not clear, especially given the discordance in literature reports of cytokine production in hyperoxia.

The significance of these findings is not clear, especially given the discordance in literature reports of cytokine production in hyperoxia.

The significance of these findings is not clear, especially given the discordance in literature reports of cytokine production in hyperoxia.

The significance of these findings is not clear, especially given the discordance in literature reports of cytokine production in hyperoxia.

The significance of these findings is not clear, especially given the discordance in literature reports of cytokine production in hyperoxia.

The significance of these findings is not clear, especially given the discordance in literature reports of cytokine production in hyperoxia.

The significance of these findings is not clear, especially given the discordance in literature reports of cytokine production in hyperoxia.
42. Rawlingson A, Gerard NP, Brain SD. Interactive contribution of NK1(1) and kinin receptors to the acute inflammatory oedema observed in response to noxious heat stimulation: studies in NK1(1) receptor knockout mice. Br J Pharmacol 134: 1805–1813, 2001.