Tumor Necrosis Factor-α Increases Airway Smooth Muscle Oxidants Production through a NADPH Oxidase-like System to Enhance Myosin Light Chain Phosphorylation and Contractility*

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Asthma is a complex inflammatory disease of the lung whose incidence, morbidity, and mortality have dramatically increased worldwide over the last two decades. Airway inflammation and ASM1 hyperresponsiveness, leading to an increased airway resistance, are characteristic features of asthma (1).

The inflammatory response in the asthmatic lung is characterized by an infiltration of the airway wall by mast cells, lymphocytes, and eosinophils. Activation of these cells results in the release of a plethora of inflammatory mediators that individually or in concert induce changes in the airway wall geometry and produce the symptoms of the disease. There is increasing evidence that one of these mediators, the pro-inflammatory cytokine TNF may be one of the primary components responsible for the ASM hyperresponsiveness observed in asthma (see Ref. 2 for review). However, the mechanism of this TNF-induced ASM hyperresponsiveness remains unclear. TNF may act indirectly, via the release of other inflammatory or bronchoconstricting agents such as leukotrienes, by inflammatory cells (2), or directly on ASM cells that express TNF receptors (3). Indeed, different investigators have shown that a short time incubation of tracheal smooth muscle strips with TNF enhances the contractile response to acetylcholine (4, 5) secondary, at least partially, to an increase in MLC phosphorylation (6).

This direct effect of TNF on ASM contractility could be mediated by ROS synthesized by the muscular cells. At least three lines of evidence support this hypothesis: 1) TNF leads to the generation of ROS in various cell systems (7, 8), 2) incubation of guinea pig tracheal smooth muscle with SOD decreases the contractile response to metacholine (9), suggesting that endogenous ROS can increase ASM contractility, and 3) ROS could increase phosphorylation of the MLC by activating the MLC kinase and/or by inhibiting the MLC phosphatase, as previously described with other kinases/phosphatases systems (10). However, very few studies investigated the capacity of ASM cells to generate ROS and the intracellular source of this generation (11, 12). Furthermore, no study is available in the literature concerning a potential autocrine role for muscular ROS in mediating TNF-induced ASM hyperresponsiveness. Such a role could open new insights in the pathophysiology of asthma.

The aim of this study was therefore to assess in guinea pigs: 1) if ASM produces ROS when stimulated by TNF; 2) the cellular source of ROS production in this condition; 3) the role of TNF-induced ROS production in ASM hyperresponsiveness. The relation between ROS production by ASM, muscular hyperresponsiveness, and the level of phosphorylated MLC was also evaluated to investigate the mechanism linking TNF-induced ROS production in ASM to hypercontractility.

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† The abbreviations used are: ASM, airway smooth muscle; DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichlorofluorescein diacetate; DPI, diphenylene iodonium; T-NOA, t-nitro-l-arginine; MLC, myosin light chain; MLC1, myosin light chain phosphatase; MnTmP-P, Mn(III)tetrakis-(1-methyl-4-pyridyl)porphyrin; PI, propidium iodide; PTP, protein-tyrosine phosphatase 1B; ROS, reactive oxygen species; TNF, tumor necrosis factor; SOD, superoxide dismutase; HBSS, Hank's balanced salt solution; PEG, polyethylene glycol; P-MLC, phosphorylated myosin light chain; TFFA, thenoyltrifluoroacetone.
duced ROS production and muscular hyperresponsiveness. Finally, the clinical relevance of our findings in animals was evaluated by studying human muscle in surgical biopsies.

**Experimental Procedures**

**Animals**

Pathogen-free male Hartley guinea pigs (250–300 g body weight; Charles River, France) were housed in individual cages in climate-controlled animal quarters and were given water and food ad libitum. The experiments conducted in the present study were approved by the local Institutional Animal Care and Use Committee, and the experimental protocol was in agreement with the recommendations related to animal studies of the French Law (Ministère des Affaires Sociales et de la Solidarité Nationale, Paris, France).

**Preparation of Guinea Pig ASM Cell Culture**

Guinea pig ASM cells were isolated from tracheal smooth muscle by enzymatic digestion as previously described by Pyne and Pyne (13). Cells were grown in Ham’s F-12 medium supplemented with 10% fetal calf serum and antimicrobial agents at 37 °C in a humidified incubator under 5% CO2. Upon reaching confluence, cells were passaged by lifting the cells with 0.05% trypsin, 0.5 mM EDTA and resedering them into three new culture plates per confluence. Cells from passage 2 to 4 were used. Prior to performing experiments, cells were grown to 60–70% confluence and then cultured for 24 h with medium containing 1% fetal calf serum.

**Measurement of Reactive Oxygen Species Production by ASM Cells**

ROS production was assessed both intra- and extracellularly.

**Intracellular ROS Generation—Intracellular ROS generation in ASM cells was assessed by using 2’,7’-dichlorofluorescein diacetate (DCDFH) (14). ROS in the cells oxidize DCDFH yielding 2’,7’-dichlorofluorescein (DCF). Fluorescence was quantified either by fluorescence microscopy or using a multowell fluorescence plate reader.**

**Fluorescence Microscopy—Cells on coverslips were perfused in a flow-through chamber (Penn Century, Philadelphia, PA) at 37 °C on an inverted microscope. Fluorescence images were acquired with a 12-bit digital camera (excitation 480 ± 20 nm, emission 527 ± 15 nm). Cells were loaded with 5 μM DCDF. Fluorescence was measured every 15 min. After a 1-h stabilization period, cells were treated during 1 h with different concentrations (1 and 10 ng ml−1) of TNF alone, or vehicle, or TNF plus an anti-TNF antibody. Cell viability was evaluated using the fluorescent dye PI at a 5 μM concentration (15), (excitation 515–590 nm, emission 590 nm).

**Quantification of DCF Fluorescence with a Multiwell Fluorescence Plate Reader—Cells were grown in 96-well plates. Immediately before the experiments, cells were washed with HBSS and loaded with 50 μM DCDFH-diacetate dissolved in HBSS for 30 min at 37 °C. They were then incubated 30 min with different inhibitors (see "Results") followed by a 1-h stimulation with 10 ng ml−1 of TNF alone, or vehicle, or TNF plus an anti-TNF antibody. Cell viability was evaluated using the fluorescent dye PI at a 5 μM concentration (15), (excitation 515–590 nm, emission 590 nm).

**Measurement of Superoxide Anion Release by Tracheal Rings**

Guinea pigs were stunned by a blow to the head and exsanguinated. The trachea was removed using open tracheotomy and cut into rings of ~2 mm in width. The epithelial layer was then removed as described previously (20).

**Western Blot Analysis of NADPH Oxidase Subunits and Phosphorylated MLC in ASM Cells and Tissue Homogenate**

To prepare tissue homogenate, tracheal smooth muscle was dissected from guinea pig trachea under a binocular dissecting microscope and cleaned of epithelium, fat, blood, and connective tissue.

**Immunohistochemical Detection of p22phox and p47phox NADPH Oxidase Subunits in Human ASM**

To determine whether human ASM expressed p22phox and p47phox in vivo, 10-μm frozen sections from normal lung biopsies (obtained from patients undergoing surgical lung resection for a localized lung tumor) were stained for p22phox and p47phox as described for ASM cells.
versus per se, but totally abolished the increase in DCFH fluorescence. Anti-TNF IgG antibodies had no effect. Removal of TNF was associated with a progressive decrease in fluorescence. In some experiments (dashed line), TNF was removed from the perfusate after a 30-min period. Mean ± S.E. values for DCF fluorescence are expressed as percentage of initial values, n = 6 independent studies in each group. Significance levels are given for the whole curves: *p < 0.05 versus control; †, p < 0.05 versus TNF 1 ng ml⁻¹.

(Paris, France). Culture media, supplements, and fetal calf serum were from Invitrogen SARL (Cergy Pontoise, France). Tissue culture plasticware was supplied by Costar Corp. (Cambridge, MA). Other reagents were from Sigma.

**Statistical Analysis**

Values are given as mean ± S.E. Dose-response curves of carbamylcholine-induced tracheal rings contraction in the different groups of animals were compared using two-way analysis of variance for repeated measures. The other data were analyzed by one-way analysis of variance; differences between means were analyzed with the Fisher’s protected least-significant difference multiple comparison test. Significance for all statistics was accepted at p < 0.05.

**RESULTS**

**TNF induces an intracellular production of ROS in ASM cells**—We first assessed the effect of TNF on ROS production using cultured guinea pig ASM cells. To visualize the cells producing ROS, video fluorescent microscopy was used. ASM cells were exposed to either TNF (1 or 10 ng ml⁻¹) or vehicle for 1 h. Fig. 1A shows a typical photograph of ASM cells 1 h after exposure to 10 ng ml⁻¹ TNF or vehicle. The results of this experiment clearly show that oxidized DCFH fluorescence was increased inside TNF-treated cells, indicating a rise in ROS production by the cells. By contrast, there was no modification for PI fluorescence throughout the study, indicating that TNF did not impair cell viability. Fig. 1B displays the mean results of these experiments (n = 6 experiments). TNF induced a dose-dependent increase in fluorescence after 15 min of perfusion. Removal of TNF was associated with a progressive decrease in fluorescence. Anti-TNF IgG antibodies had no effect per se, but totally abolished the increase in DCFH fluorescence induced by 1 ng ml⁻¹ TNF, showing the specificity of the effect induced by TNF.

**NADPH oxidase is the main source of TNF-induced ROS production by ASM cells**—The source of ROS generation in guinea pig ASM cells in response to TNF stimulation was studied using a multwell fluorescent plate reader. Pretreatment with exogenously added SOD (500 units ml⁻¹) and catalase (1000 units ml⁻¹) slightly reduced TNF-induced ROS production, whereas the combination of cell-permeable PEG-SOD plus the cell-permeable PEG catalase (100 units ml⁻¹), respectively (24) or the cell-permeable SOD-mimetic MnTMPyP (10 μM) (25) significantly reduced TNF-induced ROS production by 70 and 50%, respectively (p < 0.05 in each case, Table I).

TNF-induced ROS production was not significantly affected by pretreatment with the cyclooxygenase inhibitor mfenamic acid (20 μM), the xanthine oxidase inhibitor allopurinol (10 μM), the NO synthase inhibitor L-NNA (1 μM), or the respiratory chain inhibitors (rotenone (50 μM) and TTFA (1 μM), or myxothiazole (1 ng/ml)) (15).

**Table I**

| Pharmacological probes | − TNF | + TNF |
|------------------------|-------|-------|
|                        | % of increase | |
| None                   | 7.6 ± 3.2 | 23.5 ± 3.2* |
| Intracellular antioxidants |       |       |
| PEG-SOD (100 units ml⁻¹) + PEG-catalase (100 units ml⁻¹) | 2.1 ± 2.3 | 7.4 ± 3.3* |
| MnTMPyP (10 μM) | 3.2 ± 2.4 | 12.4 ± 2.3 |
| Extracellular antioxidants |       |       |
| SOD (500 units ml⁻¹) + Catalase (1000 units/ml⁻¹) | 7.3 ± 2.4 | 18.2 ± 2.4 |
| Inhibitors |       |       |
| Rotenone (5 μM) + TTFA (10 μM) | 5.3 ± 1.4 | 22.8 ± 3.4 |
| Myxothiazole (100 ng ml⁻¹) | 5.4 ± 2.7 | 21.2 ± 4.7 |
| Mefenamic acid (20 μM) | 5.9 ± 2.4 | 21.5 ± 3.8 |
| t-NNA (1 mM) | 5.7 ± 1.2 | 19.7 ± 2.7 |
| Allopurinol (10 μM) | 5.3 ± 3.1 | 20.2 ± 2.0 |
| DPI (10 μM) | 2.4 ± 2.0 | 17.2 ± 3.8 |
| Apocynin (10 μM) | 3.1 ± 2.1 | 13.2 ± 2.4 |

| Substrates | NADH (1 mM) | NADPH (1 mM) |
|------------|-------------|--------------|
|            | 7.1 ± 2.4   | 22.4 ± 4.2*  |
|            | 6.9 ± 2.3   | 87.1 ± 10.3* |

*a p < 0.05 versus −TNF.  
b p < 0.05 versus −TNF alone.  
c p < 0.05 versus +TNF alone.

**Fig. 1. Effect of TNF on intracellular ROS production by ASM cells, evaluated by fluorescent videomicroscopy.** Cells were perfused with Krebs solution containing 5 μM DCFH diacetate or 5 μM PI for 1 h. Then, different concentrations of TNF were added to the perfusion, and fluorescence was recorded every 15 min during a 1-h period. A, typical image of fluorescent videomicroscopy of cells 1 h after exposition to 10 ng ml⁻¹ TNF or vehicle; B, mean ± S.E. values for DCF fluorescence. ○, control; ·, TNF 1 ng ml⁻¹; ▽, TNF 10 ng ml⁻¹; ■, TNF 1 ng ml⁻¹ plus antibody. In some experiments (dashed line), TNF was removed from the perfusate after a 30-min period. Mean ± S.E. values for DCF fluorescence were from 6 independent studies in each group. Significance levels are given for the whole curves: *p < 0.05 versus control; †, p < 0.05 versus TNF 1 ng ml⁻¹.
However, the TNF-induced ROS production was significantly reduced by pretreatment with DPI (10 μM) an inhibitor of flavin-containing enzymes such as NADPH oxidase (26) or by the NADPH oxidase inhibitor apocynin (10 μM) (27, 28). Furthermore, in separate experiments, we confirmed the effect of DPI by adding this agent 30 min after TNF. This addition resulted in a progressive decrease of DCFH fluorescence, which returned to basal levels 60 min after the beginning of TNF application (data not shown). Finally, we tested the effect of NADH and NADPH substrates on this activity. NADPH, but not NADH, increased TNF-induced DCFH fluorescence about 3-fold (Table I).

TNF induces extracellular superoxide anion production by ASM cells via NADPH oxidase. — TNF induced an extracellular production of superoxide anion by ASM cells as determined by SOD-inhibitable reduction of cytochrome c at 10 ng ml⁻¹ of TNF, ASM cells produced 3.66 nmol of superoxide anion 10⁶ cells⁻¹ h⁻¹. This release was significantly reduced by DPI and apocynin (Table II).

### Table II

| Pharmacological probes   | − TNF | + TNF |
|-------------------------|-------|-------|
| None                    | 1.30 ± 0.12 | 3.66 ± 0.31a |
| DPI (10 μM)             | 0.66 ± 0.06b | 1.35 ± 0.01b |
| Apocynin (10 μM)        | 0.66 ± 0.05b | 1.32 ± 0.01b |

* a p < 0.05 versus −TNF.
* b p < 0.05 versus −TNF alone.
* p < 0.05 versus +TNF alone.

NADPH Oxidase Subunits p22phox and p47phox Are Expressed in Guinea Pig ASM Cells—To assess the presence of NADPH oxidase in ASM cells, Western blot analysis with anti-p22phox or anti-p47phox antibodies were performed (29). Whole ASM cell homogenates showed expression of p22phox and p47phox with the same molecular weight as those found in human neutrophils (Fig. 2A). Expression of these proteins was also detected by immunohistochemistry (Fig. 2B). We further confirmed the presence of the cytochrome b₅₅₈, the membrane component of the NADPH oxidase system, by spectral analysis of ASM cells membranes (Fig. 2C). The reduced spectrum from ASM cells showed absorption at two main wavelengths: 426 and 558 nm, identical to previous reports in human neutrophils (17, 30). Indeed, the peak at 558 nm is characteristic of cytochrome b₅₅₈ present in phagocyes.

Transfection with a p22phox Antisense Oligonucleotide Impaired TNF-induced ROS Production by ASM Cells—TNF-induced ROS formation was significantly reduced in cells transfected with a p22phox antisense oligonucleotide as compared with cells transfected with a control sense oligonucleotide (Fig. 3A). Western blot analysis demonstrated that p22phox protein expression was substantially reduced in ASM cells transfected with the antisense-p22phox oligonucleotide as compared with the control sense oligonucleotide (Fig. 3, B and C).

NADPH Oxidase-derived ROS Are Involved in TNF-induced Muscular Hyperresponsiveness—As in cultured cells, Western blot analysis of ASM tissue homogenate allowed us to identify NADPH oxidase subunits p22phox and p47phox with a similar molecular weight as those observed in a lysate of human neutrophils (Fig. 2A). TNF induced the release of superoxide anion by epithelium denuded tracheal rings (Table III). This release was significantly reduced by pre-incubation with DPI and apocynin (Table III).

The role of NADPH oxidase-derived ROS in ASM hyperre-

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**Fig. 2.** Expression of NADPH oxidase components p22phox and p47phox in airway smooth muscle. A, Western blot analysis of p47phox and p22phox proteins expression. Whole-cell proteins were extracted from human neutrophils (HN) and guinea pigs ASM cells (ASM) and tracheal smooth muscle (TSM) and subjected to 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Immunoblot analysis with a polyclonal antibody against p47phox and p22phox was performed as described under “Experimental Procedures”; B, immunohistochemical analysis of p47phox and p22phox in ASM cells. Cells were cultured in a chamber slide, fixed with 3.5% paraformaldehyde in phosphate-buffered saline, and stained by immunoreaction with polyclonal antibodies against p47phox and p22phox; C, reduced minus oxidized difference spectrum of flavocytochrome b₅₅₈. Cells (10⁷ ml⁻¹) were lysed in phosphate-buffered saline + 2% Triton X-100 at 4°C for 10 min. Dithionite-reduced minus oxidized difference spectrum of the sample was analyzed as described under “Experimental Procedures.”
sponsiveness induced by TNF was investigated using epithelium-denuded tracheal guinea pig rings both in the absence and presence of either TNF alone, TNF with DPI, or PEG-SOD plus PEG-Catalase. As shown in Fig. 4A, 1-h incubation of tracheal rings with 10 ng ml⁻¹ TNF induced an increased response to carbachol (p < 0.05). This hyperspensiveness was blocked by preincubation of the rings with DPI or the cell-permeable antioxidants PEG-SOD plus PEG-catalase (100 units ml⁻¹, respectively) (Fig. 4A). Interestingly, both DPI and PEG-SOD plus PEG-catalase slightly decreased the contractile response in control rings (Fig. 4B), suggesting a role of NADPH oxidase-derived intracellular ROS in the modulation of ASM contractility in basal conditions.

TNF-induced Phosphorylation of the MLC Is Regulated by NADPH Oxidase-derived ROS—Finally, we evaluated the role of NADPH oxidase derived ROS on TNF-induced phosphorylation of MLC in ASM cells and in tissue homogenates. Fig. 5 shows that TNF increased phosphorylation of MLC both in ASM cells and in tissue homogenates. This phenomenon was prevented by preincubation of the samples with DPI or PEG-SOD plus PEG-catalase (p < 0.05 in each case). Interestingly, independent incubation of the samples with either PEG-SOD or PEG-catalase significantly decreased phosphorylation of MLC, but less than both compounds together. These results clearly show an additive effect of superoxide anion and hydrogen peroxide on MLC phosphorylation.

NADPH Oxidase Subunits p22phox and p47phox Are Expressed in Human ASM—Immunohistochemistry analysis of a human lung specimen showed positive staining for both p22phox and p47phox in airway smooth muscle (Fig. 6). No tissue section showed positive immunostaining when the antibodies were replaced by a control serum, and no staining was observed when the antibodies were omitted (data not shown). Analysis of the other lung specimen showed identical results (data not shown).

**DISCUSSION**

This study shows, to the best of our knowledge for the first time, that NADPH oxidase proteins p22phox and p47phox are expressed in guinea pig ASM cells and tissue homogenates and that NADPH oxidase-derived ROS play a critical autocrine role
in TNF-induced muscular hyperresponsiveness and MLC phosphorylation. These data could be relevant to ASM hyperresponsiveness observed in asthmatic patients, since 1) we used TNF concentrations close to that found in bronchoalveolar lavage of asthmatic patients (31) and 2) we detected p22phox and p47phox proteins in human ASM muscle.

Very scarce data concerning ROS production by ASM are available in the literature (11, 12). These studies suggested indirectly that a NADPH oxidase-like system is present in ASM cells. However, whether components of this system are expressed by ASM and the functional characteristics of this enzyme in these cells were unknown.

The NADPH oxidase of neutrophils and other phagocytic cells is composed of a membrane-bound low potential cytochrome b558 that consists of a small subunit (p22phox) and a 90–110-kDa glycoprotein subunit (gp91phox). In addition to the cytochrome b558 heterodimer, four cytosolic factors (p47phox, p67phox, and p40phox and the small GTPase protein Rac1/2) are required for activity (29, 32). Our results present evidence of the expression of p47phox and p22phox proteins in ASM cells, along with the membrane-bound low potential cytochrome b558. Furthermore, we demonstrate that the NADPH oxidase complex was the main source of ROS in ASM cells under TNF stimulation. Indeed, incubation of ASM cells with two structurally different pharmacological inhibitors of NADPH oxidase (DPI (26) and apocynin (27, 28)) significantly reduced TNF-induced ROS production, while the substrate NADPH significantly increased it. Moreover, inhibitors of mitochondrial respiratory chain, NO synthase, cyclooxygenase, and xanthine oxidase had no effect on TNF-induced ROS production. It must be noted, however, that definitive conclusions from drug-based experiments should be drawn very cautiously, since many com-

FIG. 5. Expression of phosphorylated myosin light chain (P-MLC). A, Western blot analysis of P-MLC in ASM cells. Immunoblot analysis with antibodies against P-MLC, and the housekeeping protein β-actin was performed as described under “Experimental Procedures.” Densitometric analysis of the P-MLC/β-actin ratio in ASM cells and TSM is shown in B and C, respectively (n = 5 in each group).

FIG. 6. Immunohistochemical analysis of p47phox and p22phox expression in human ASM. Samples from a macroscopically tumor-free lung specimen were stained by immunoreaction with polyclonal antibodies against p47phox and p22phox or non-immune serum as described under “Experimental Procedures.”
pounds often displays additional biological activities other than those for which they have been selected and utilized. This is particularly true for experiments with DPI, since this compound has been shown to inhibit NO synthases (33) or mitochondrial respiratory chain (34). However, the lack of effect of a NO synthase inhibitor (L-NNA) and of several mitochondrial respiratory chain inhibitors (myxothiazole, rotenone, TTFA) advocate for a selective inhibition of NADPH oxidase by DPI in this study. Furthermore, the reduction of TNF-induced ROS production by a specific inhibitor of NADPH oxidase (apocynin) and by transfecting ASM cells with a p22phox antisense oligonucleotide confirm the role for a NADPH oxidase system in our ASM cells.

The function of NADPH oxidase in our ASM cells presents some particularities: 1) the amount of ROS produced by ASM under TNF stimulation seems lower than that observed with phagocytic cells. Indeed, for example, Teshima and associates (18) have shown that guinea pig neutrophils can release up to 9 nmol·10⁶ cells⁻¹·min⁻¹ superoxide anion upon stimulation with phorbol 12-myristate 13-acetate, whereas our cells release 0.06 nmol·10⁶ cells⁻¹·min⁻¹ when maximally stimulated with TNF, a value that is close to that of guinea pig gastric mucosal fibroblasts (18); 2) there was a basal ROS production in non-stimulated cells and TNF increased this production probably by promoting the membrane assembling of NADPH oxidase subunits, the critical step for enzyme activity (29); 3) ROS production was directed both intra- and extracellularly. Indeed, 10 ng·ml⁻¹ TNF induced a similar increase in both intracellular and extracellular ROS production (three times the control values). Collectively, these results, which are in line with data concerning the function of NADPH oxidases in non-phagocytic cells (29), suggest that ROS produced by muscular NADPH oxidase might fulfill a subtle task in acting as signaling molecules both intra- and extracellularly. These findings stress the role of ASM not only as a determinant of airways tone, but also as an important contributor to the cellular environment in bronchial wall (35).

Having demonstrated that NADPH oxidase is the main source of ROS in ASM cells in culture, we examined the functional relevance of these findings in guinea pig ASM tissue. We therefore utilized either isolated tracheal smooth muscle or epithelium-denuded tracheal rings. Tracheal smooth muscle was used to examine p22phox and p47phox protein expression. Rings were used to study ASM contractility and ROS production, because both techniques have been previously employed in different studies (20, 21). To avoid interference with the detection of ROS production by smooth muscle, care was taken to completely remove the epithelial and the mucosal layers, in which cells producing ROS such as epithelial cells (36) and inflammatory cells are present. Furthermore, removing the epithelium allowed us to study the direct role of TNF on muscle contractility, without the interference of bronchoconstrictant molecules synthetized by the epithelium such as nitric oxide (37).

In a first set of experiments, we found similar results concerning p22phox and p47phox expression and extracellular superoxide anion production in ASM tissue as compared with ASM cells in culture, thus ensuing the in vivo relevance of the cellular data. In a second set of experiments we evaluated the effect of TNF on ASM contractility. These experiments showed that TNF induced ASM hyperresponsiveness to carbachol, as demonstrated previously by different authors (4–6). Parris and co-workers (6) demonstrated that the cellular basis of this effect is an enhanced MLC phosphorylation. Indeed, reversible phosphorylation of MLC is the main mechanism that regulates smooth muscle contraction by modifying the conformation of myosin. However, the mechanisms leading to the increase in TNF-induced MLC phosphorylation in ASM are unknown. In this study, we found that both NADPH oxidase inhibitors and cell-permeable antioxidants prevented both muscular hyperresponsiveness and the increase in MLC phosphorylation induced by TNF. These findings demonstrate that NADPH oxidase-derived ROS are involved in MLC phosphorylation induced by TNF and support the functional relevance of this phenomenon in terms of muscular contraction. This is the first demonstration of a direct role of NADPH oxidase in a step of muscular excitation-contraction. These results agree with recent data published by Lopez-Ongil and co-workers (38) showing that exogenously added hydrogen peroxide increased the amount of phosphorylated MLC and increased contraction of endothelial cells.

The mechanism(s) involved in the modulation of MLC phosphorylation by NADPH oxidase derived ROS is (are) unknown. MLC phosphorylation results from the net effect between the action of the myosin light chain kinase and a type 1 myosin phosphatase (MLCP) (39). Myosin light chain kinase is activated by Ca²⁺ and calmodulin and phosphorylates MLC predominantly at serine 19 (40); MLCP is therefore a serine/threonine phosphatase. To date, most of the agonists that have been shown to increase MLC phosphorylation act via the inhibition of MLCP (41). ROS could also inhibit MLCP. Theoretically, this can be performed directly, by an effect of ROS on MLCP protein itself, or indirectly by an effect of ROS on the different pathways that modulate MLCP activity. To date, the only well established serine/threonine phosphatase that can be regulated directly by ROS is calcineurin (42). Calcineurin is mainly inhibited by superoxide anion under physiological conditions, since its inhibition by hydrogen peroxide requires a relatively high concentration (42), which is not likely to be reached by TNF treatment. Therefore, a direct inhibition of MLCP by superoxide anion and hydrogen peroxide produced by TNF-activated smooth muscle NADPH oxidase is unlikely. Alternatively, ROS modulation of pathways that regulate MLCP activity can also explain the increase in MLC phosphorylation observed in the present study. These effects can result from the well known redox modulation of tyrosine phosphorylation (43–45). MLCP can be inhibited by different proteins, such as the Rho A/Rho kinase system and the CPI-17 protein, a smooth muscle-specific protein inhibitor of MLCP (40, 46). Inhibition of MLCP by CPI-17 is strongly enhanced by a protein kinase C-catalyzed phosphorylation (46). One of the protein kinase C isoforms is activated by tyrosine phosphorylation (47), which can be regulated by protein-tyrosine phosphatase 1B (PTP). Interestingly, PTP is effectively inhibited by both superoxide anion and hydrogen peroxide (44). Therefore, inhibition of PTP by NADPH oxidase-derived ROS can lead to a protein kinase C/CPI-17-mediated inhibition of MLCP, thus resulting in the increased MLC serine phosphorylation induced by TNF in the present study. This pathway could represent a mechanism linking redox regulation of tyrosine phosphorylation to serine phosphorylation of MLC.

In conclusion, the results of this study are consistent with a pathogenic mechanism that, to the best of our knowledge has not been described before, in which TNF-induced ASM hyperresponsiveness is likely mediated by NADPH oxidase-derived ROS via an increase in MLC phosphorylation. Accordingly, the muscular NADPH oxidase pathway may represent a primary step in the pathophysiology of the bronchoconstriction associated with asthma, since in this condition high levels of TNF are synthetized by inflammatory cells close to ASM (31). Furthermore, detection of NADPH oxidase proteins p22phox and p47phox in human ASM muscle strongly suggest that the results obtained in guinea pigs are clinically relevant.
REFERENCES

1. Brar, S. S., Kennedy, T. P., Whorton, A. R., Murphy, T. M., Chitano, P., and Page, K., Li, J., Hodge, J. A., Liu, P. T., Vanden Hoek, T. L., Becker, L. B., Sundaresan, M., Yu, Z. X., Ferrans, V. J., Irani, K., and Finkel, T. (1995) Biochem. Pharmacol 61, 749–759.

2. Pennings, H. J., Kramer, K., Bast, A., Buurman, W. A., and Wouters, E. (1995) Eur. Respir. J. 12, 45–49.

3. Nakatani, Y., Nishiuma, T., Maeda, H., and Yokoyama, M. (1999) Eur. J. Pharmacol. 392, 175–182.

4. Parris, J. R., Cobban, H. J., Littlejohn, A. F., MacEwan, D. J., and Nixon, G. F. (2001) Biochem. Pharmacol 61, 149–154.

5. Williams, R. (1991) J. Biol. Chem. 266, 11233–11237.
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