Phosphorylation of the 27-kDa heat shock protein via p38 MAP kinase and MAPKAP kinase in smooth muscle

Larsen, Janice K., Ilia A. Yamboliev, Lee A. Weber, and William T. Gerthoffer. Phosphorylation of the 27-kDa heat shock protein via p38 MAP kinase and MAPKAP kinase in smooth muscle. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L930–L940, 1997.—The 27-kDa heat shock protein (HSP27) is expressed in a variety of tissues in the absence of stress and is thought to regulate actin filament dynamics, possibly by a phosphorylation/dephosphorylation mechanism. HSP27 has also been suggested to be involved in contraction of intestinal smooth muscle. We have investigated phosphorylation of HSP27 in airway smooth muscle in response to the muscarinic agonist carbachol. Carbachol increased 32P incorporation into canine tracheal HSP27 and induced a shift in the distribution of charge isoforms on two-dimensional gels to more acidic, phosphorylated forms. The canine HSP27 amino acid sequence includes three serine residues corresponding to sites in human HSP27 known to be phosphorylated by mitogen-activated protein kinase-activated protein (MAPKAP) kinase-2. To determine whether muscarinic receptors are coupled to a “stress response” pathway in smooth muscle culminating in phosphorylation of HSP27, we assayed MAPKAP kinase-2 activity and tyrosine phosphorylation of p38 mitogen-activated protein (MAP) kinase, the enzyme thought to activate MAPKAP kinase-2. Recombinant canine HSP27 expressed in Escherichia coli was a substrate for MAPKAP kinase-2 in vitro as well as a substrate for endogenous smooth muscle HSP27 kinase, which was activated by carbachol. Carbachol also increased tyrosine phosphorylation of p38 MAP kinase. SB-203580, an inhibitor of p38 MAP kinases, reduced activation of endogenous HSP27 kinase activity and blocked the shift in HSP27 charge isoforms to acidic forms. We suggest that HSP27 in airway smooth muscle, in addition to being a stress response protein, is phosphorylated by a receptor-initiated signaling cascade involving muscarinic receptors, tyrosine phosphorylation of p38 MAP kinase, and activation of MAPKAP kinase-2.

Carbachol; mitogen-activated protein kinase; p38 mitogen-activated protein kinase-activated protein; trachea

MITOGEN-ACTIVATED PROTEIN (MAP) kinases play a central role in intracellular signal transduction pathways initiated by a variety of cellular stimuli. Although activation of MAP kinases often leads to a mitogenic response, there is evidence to support the involvement of MAP kinases in cellular functions in addition to proliferation. For example, in neutrophils, interleukin-8 activation of the p38 MAP kinase pathway may lead to respiratory burst and granule secretion (17). In intact, differentiated smooth muscles, extracellular signal-regulated kinase (ERK)-1 and ERK2 MAP kinases are activated by a variety of stimuli, including neurotransmitters and phorbol esters (1, 10, 16). The actin-binding protein caldesmon is thought to be an important downstream target for the ERK MAP kinases in intact smooth muscle (2). Another downstream target of MAP kinases is mitogen-activated protein kinase-activated protein (MAPKAP) kinase-2 (29). MAPKAP kinase-2 is a Ser/Thr kinase shown to catalyze phosphorylation of 27-kDa heat shock protein (HSP) in non-muscle cells (3, 19, 29, 32). Although many investigators have demonstrated activation of MAPKAP kinase-2 via MAP kinase pathways, the cellular role of MAPKAP kinase-2 and phosphorylation of HSP27 in smooth muscle has not been described.

The mammalian HSP27 is a member of the highly conserved family of HSPs (14) and is expressed in a variety of tissues in the presence and absence of stress. Although HSP27 has been shown to exhibit chaperone activities in vitro (15) and to modulate actin microfilament dynamics (4, 22) and although overexpression of the protein confers increased resistance to heat killing (21), the physiological function of HSP27 in unstimulated cells remains unclear. HSP27 becomes phosphorylated in response to heat shock and to a variety of cytokines and growth factors in cultured endothelial cells (28), fibroblasts (32), and monocytes (3). Landry et al. (19) mapped the phosphorylation sites in human HSP27 and showed that MAPKAP kinase-2 phosphorylates human HSP27 protein on Ser-15, Ser-78, and Ser-82. Ser-82 appears to be the major site of in vivo phosphorylation followed by Ser-78 and Ser-15, the minor sites (19, 29).

Recent studies suggest that p38 MAP kinases are immediately upstream of MAPKAP kinase-2 in the stress response pathway leading to phosphorylation of HSP27. The p38 MAP kinases are homologs of yeast HOG1 MAP kinase, which is important for growth of yeast in high osmolality media as well as for the formation of buds (13). It has been shown that both ERK and p38 MAP kinases can activate MAPKAP kinase-2 in vitro; however, only activation of p38 correlates with MAPKAP kinase-2 activation in vivo (8, 12, 27, 33). Less is known about the molecular details of the p38 signaling pathway compared with the ERK MAP kinases. Many investigators have demonstrated that environmental stresses and proinflammatory cytokines can activate this pathway, possibly via activation of upstream kinases p21-activated kinase 1 (PAK1) and MAP kinase/ERK kinase (MKK3; see Refs. 8, 9, 27, and 33). However, relatively little is known about the activation of p38 MAP kinases through G protein-linked, seven-transmembrane-spanning (STM) receptors. To address this issue, we examined the ability of carbachol, a muscarinic agonist, to increase phosphorylation of HSP27, activate MAPKAP kinase-2, and in-
crease tyrosine phosphorylation of p38 MAP kinase. The results suggest that muscarinic receptors are coupled to the stress response pathway leading to phosphorylation of HSP27 in intact airway smooth muscle.

**MATERIALS AND METHODS**

Materials. Adult mongrel dogs of either sex were killed by barbiturate overdose. The trachea was removed and was placed in cold physiological salt solution (PSS) composed of (in mM) 2,3-N-morpholino)propanesulfonic acid (MOPS), pH 7.4, 140 NaCl, 4.7 KCl, 1.2 MgSO4, 2.5 CaCl2, 1.2 Na2HPO4, 0.02 EDTA, and 5.6 D-glucose. Tracheal smooth muscle was dissected free of connective tissue and epithelium. Expression vectors pET3a and pET24a as well as dissected free of connective tissue and epithelium. Expression vectors pET3a and pET24a as well as 32P was purchased from Novagen. Eileen Hickey and Lee Weber (14, 20). Full-length human and canine cDNA with pET24a, whereas truncated canine recombinant proteins were created by fusing human and canine cDNA with pET24a, whereas truncated canine recombinant protein containing the majority of the canine HSP27s sequence was created by fusing a cDNA fragment encoding amino acids 33–209 with the T7 promoter in pET3a. When expressed in BL21(DE3)pLysS cells, all recombinant HSP27 (rHSP27s) comprised ≈40% of the total cell protein 1.5 h after induction. Total soluble protein from each preparation was applied to a 1 × 13-cm DEAE Sephacel Column equilibrated with 20 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.5, 30 mM NaCl, 0.1 M EDTA, 1.0 mM dithiothreitol (DTT), and the rHSP27 eluted at 10 ml/h with a linear NaCl gradient (0.01–0.4 M). Fractions highly enriched in rHSP27 were collected and pooled, and purity was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Purity was >85%.

Preparation of anti-HSP27 antibodies and immunoblot analysis. DEAE column fractions enriched in rHSP27 were further purified by SDS-PAGE (12% acrylamide), and the gel-purified rHSP27 (~100 µg) was then injected into the pectoral muscles and thighs of laying hens. Booster injections were administered every 2 wk, and eggs were collected. Antibodies were isolated from egg yolks by polyethylene glycol precipitation. Polyclonal HSP27 antibodies were affinity purified with a canine rHSP27-Sepharose CL 4B column, and specificity was tested on Western blots of homogenates of canine colon and tracheal smooth muscle. The polyclonal antibody was found to react against truncated canine rHSP27 and a protein of 27 kDa in both canine colon and tracheal smooth muscle extracts. The antibody reacted only very weakly to human rHSP27.

Immunoblots were performed by transferring proteins from SDS-polyacrylamide gels to pure nitrocellulose paper using either a Hoefer TE Transfor electrophoresis unit (90 V, 4–14 h, 15°C) or a Bio-Rad (Hercules, CA) Mini Trans-Blot unit (120 V, 1–2 h, 4°C). Nitrocellulose was blocked with 5% powdered milk in TBS (10 mM Tris·HCl, pH 7.4, and 150 mM NaCl) and was probed with chicken anti-canine HSP27 antibodies (1:150 dilution), and bands were visualized with goat anti-chicken IgG alkaline phosphatase conjugate (1:1,000 dilution). For detection of p38 tyrosine phosphorylation, tracheal smooth muscle strips were treated with 1 µM carbobal and were frozen by immersion in liquid nitrogen, and proteins were extracted in MAP kinase extraction buffer containing 20 mM Tris, pH 7.5, 5 mM ethylene glycol-bis-(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), 1 mM Na3VO4, 20 mM β-glycerophosphate, 10 mM NaF, 1 mM DTT, 1 µg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and were calfied by centrifugation at 10,000 g for 10 min at 4°C. The protein extract was separated on a 12% SDS-polyacrylamide gel and was transferred to nitrocellulose paper. Immunodetection was performed using anti-phospho-tyrosine p38 antibody (1:3,000) followed by goat anti-rabbit alkaline phosphatase secondary antibody (1:5,000).

For identification of contractile proteins on two-dimensional gels, Western blots were probed with anti-calcin polyclonal antibodies (a gift of Dr. Michael Walsh, University of California), anti-actin (A-2547) monoclonal antibody from Sigma (St. Louis, MO), anti-tropomyosin (T-2780) polyclonal antibody from Sigma, and anti-22-kDa smooth muscle protein (SM22) polyclonal antibodies (a gift of Dr. L. B. Smillie, University of Alberta). Affinity-purified anti-myosin light chain (MLC) antibodies were prepared by immunizing rabbits with chicken gizzard 20-kDa MLC. Proteins on Western blots were detected using goat anti-chicken IgG alkaline phosphatase (Southern Biotechnology Associates) or goat anti-rabbit or goat anti-mouse alkaline phosphatase-conjugated secondary antibodies (Promega). HSP27 phosphorylation. Canine tracheal smooth muscle strips were mounted on stainless steel hooks and were incubated for 3 h at 37°C in oxygenated phosphate-free PSS containing 0.2 mM/µl [32P]. After [32P] labeling, tissue strips were allowed to equilibrate in PSS for 30 min. The strips were stimulated two times with 60 mM K+ for 5 min each, followed by a 15-min wash. Tissues were then stimulated with 1 µM carbachol for specified times as described below and were frozen in acetone-5% trichloroacetic acid over dry ice. After warming to room temperature in pure acetone, the muscle strips were vacuum dried and weighed. The tissues were then homogenized in 50 µl of lysis buffer per milligram dry weight. Lysis buffer was composed of 9.5 M urea, 0.5% SDS, 5.0% β-mercaptoethanol, and 0.16% Ampholine, pH 3–10. Proteins were resolved by two-dimensional nonequilibrium pH gel electrophoresis (NEPHGE)-SDS-PAGE (24). The tissue extract was first subjected to NEPHGE in 0.1 × 13-cm tube gels for 4,800 V·h (pH 3–10) followed by separation in the second dimension by SDS-PAGE (12% acrylamide). The slab gels were then stained overnight with 0.4% Coomassie brilliant blue R-250 in 25% isopropanol and 10% acetic acid. Gels were then stained in 25% isopropanol and 10% acetic acid, and the protein bands were quantified by densitometry. The mass of HSP27 was calculated from densitometric scans using 1–8 µg of purified rHSP27 as dye binding standards. HSP27 bands were then cut from the slab gels, and the amount of radioactivity was measured by scintillation counting. Protein content and radioactivity were calculated for each HSP27 isoform (a, b, and c). Phosphorylation was expressed as counts per minute of each isoform per total micrograms of HSP27.

In vitro phosphorylation of HSP27. Canine rHSP27 (3.7 µM) was added to rabbit skeletal muscle MAPKAP kinase-2
(1 U/80 μl) in a kinase reaction buffer (in mM: 10 MOPS, pH 7.5, 10 β-glycerolphosphate, 6 MgCl₂, 0.4 EGTA, 0.04 NaF, 0.4 Na₃VO₄, and 1.6 DTT). A mixture of MgATP (0.25 mM) plus [γ-³²P]ATP (2 μCi) was then added to the kinase reaction to total 120 μl. After incubation at 30°C for 0, 5, 10, 30, 60, 90, and 120 min, 15-μl samples were removed. Concentrated SDS-PAGE sample buffer was added to each sample to yield 60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.025% bromphenol blue, and 1 mM DTT. HSP27 was separated from MAPKAP kinase-2 by one-dimensional SDS-PAGE (12% acrylamide), and phosphorylation of canine rHSP27 was visualized using a Bio-Rad model 525 Molecular Imager.

Activation of in vivo HSP27 kinase. Muscle strips (30-40 mg) were stimulated with 1 μM carbachol for 0.5, 1, 2, 5, 15, and 60 min and then were frozen by immersion in liquid nitrogen. Frozen muscle strips were pulverized and homogenized in MAP kinase extraction buffer (see above). The extracts were clarified by centrifugation at 10,000 g for 10 min at 4°C. The kinase reaction mixture contained (in 40 μl) 25 mM Tris, pH 7.0, 0.1 mM EGTA, 0.2 mM Na₃VO₄, 10 mM magnesium acetate, 1 mM DTT, and 3.3 μM human rHSP27 plus clarified tissue extract. The reaction was started by the addition of Na₂ATP to give a 0.25 mM final concentration containing 3-5 μCi [³²P]ATP. After 15 min, the reaction was terminated by addition of SDS sample buffer (see above). Proteins were separated by SDS-PAGE and were stained with Coomassie brilliant blue. Phosphorylation of rHSP27 was detected with a Bio-Rad model 525 Molecular Imager. Background signal due to the phosphorylation of endogenous HSP27 comprised <9% of the total kinase activity and was corrected by subtracting signal from reactions lacking rHSP27 from reactions containing rHSP27.

Mono-Q chromatography. Tracheal tissue strips (50 mg) were frozen by immersion in acetone chilled with crushed dry ice (−80°C). The proteins were extracted in MAP kinase extraction buffer. The extract was clarified by centrifugation at 100,000 g for 10 min at 4°C and then was applied to a Mono-Q HR 5/5 column (Pharmacia Biotech, Piscataway, NJ) equilibrated with 20 mM Tris, pH 7.5, 2 mM EGTA, 1 mM Na₃VO₄, 10 mM β-glycerolphosphate, 1 mM DTT, 1 μg/ml aprotinin, and 0.1 mM PMSF. The column was washed with 10 column volumes of equilibration buffer and was eluted with a 60-ml linear NaCl gradient (0-0.4 M) at a flow rate of 0.5 ml/min. One-milliliter fractions were collected and concentrated, and every other fraction was analyzed by 12% SDS-PAGE. The proteins were transferred to nitrocellulose paper and were probed separately with either anti-ERK1-CT antibody (1:2,000 dilution) or anti-p38 antibody (1:1,000 dilution) as described above.

Treatment of tracheal smooth muscle with SB-203580. Canine tracheal smooth muscle strips (−2 × 10 mm) were mounted on stainless steel hooks and were incubated in oxygenated PSS at 37°C. Muscle strips were stimulated three times for 5 min with 70 mM K⁺ to produce stable, reproducible contractions. The muscles were then stimulated with 1 μM carbachol for 10 min to serve as a control response before the addition of SB-203580. Muscle strips were then incubated with either 0.1% dimethyl sulfoxide (DMSO; vehicle) or 25 μM SB-203580 dissolved in 0.1% DMSO for 1 h. They were then stimulated with 1 μM carbachol, frozen in liquid nitrogen, and homogenized in MAP kinase extraction buffer. The extracts were clarified by centrifugation at 10,000 g for 10 min. The soluble proteins were added to equal volumes of lysis buffer composed of 9.5 M urea, 2.5% Triton X-100, 5% β-mercaptoethanol, and 0.16% Ampholine, pH 3-10 (Pharmacia LKB Biotechnology). Charge isoforms of HSP27 were then resolved by NEPHGE-SDS-PAGE as described above, and proteins were stained with Coomassie brilliant blue as described above. Images of gels scanned with a UMAX Powerlook flatbed scanner were analyzed using the Volume Analyze feature of Molecular Analyst software (Bio-Rad) to determine the mass of HSP27 in each charge isoform. Dye binding standard curves were constructed by densitometry of one-dimensional SDS-polyacrylamide gels containing 1-8 μg rHSP27.

Statistical methods. Results are presented as means ± SE. Hypothesis testing was performed using SigmaStat, version 1.0 (Jandel Scientific, San Rafael, CA). Differences between treatment means were evaluated by Student’s t-test for unpaired data as appropriate. Multiple comparisons among mean protein densities of HSP27 charge isoforms were made using the Bonferroni t-test. A probability of P < 0.05 was accepted as a significant difference.

RESULTS

Identification of canine smooth muscle HSP27 by Western blotting. To study HSP27 in canine airway smooth muscle, we developed a canine specific antibody. Despite high homology among eukaryotic HSPs, antibodies raised against human and rodent HSP27 do not cross-react with canine HSP27. Therefore, we isolated, cloned, and sequenced cDNAs encoding canine HSP27 from a canine colonic smooth muscle cDNA library using a human HSP27 cDNA probe (20). The deduced amino acid sequence showed a high degree of identity to other mammalian species (Fig. 1). The canine HSP27 sequence also has three potential MAPKAP kinase-2 phosphorylation sites at Ser-15, Ser-82, and Ser-86 that correspond to known phosphorylation sites in human HSP27 (boxed sequences in Fig. 1). The canine HSP27 cDNA was then subcloned into an expression vector and rHSP27 expressed in E. coli. rHSP27 was purified by DEAE Sephacel chromatography and SDS-PAGE. We then immunized chickens with a truncated form of canine rHSP27 (amino acids 33-209) and prepared affinity-purified polyclonal anti-HSP antibodies that were used to identify HSP27 in homogenates of canine tracheal smooth muscle.

SDS extracts of canine tracheal smooth muscle were subjected to NEPHGE-SDS-PAGE as shown in Fig. 2. Proteins identified by Western blotting of similar gels include actins, tropomyosins, calponin, SM22, and the 20-kDa MLC. The identity of HSP27 was determined using anti-HSP antibodies that were used to identify HSP27 in homogenates of canine tracheal smooth muscle.

Phosphorylation of canine HSP27 in tissue. To investigate the function of HSP27 in mammalian smooth muscle, we tested the notion that it is phosphorylated in intact smooth muscle in response to stimulation with a muscarinic agonist. Carbachol was used to stimulate strips of canine tracheal smooth muscle that had been metabolically labeled with [³²P]. Muscle strips were frozen 0, 1, 2, and 60 min after treatment with carbachol, and proteins were resolved by NEPHGE-SDS-PAGE. Protein bands were visualized in slab gels stained with Coomassie brilliant blue. In a single
representative experiment, phosphoproteins were visualized with a Phosphorimager as shown in Fig. 4. The phosphorimages show that the a spot does not contain any radioactivity. Both b and c spots contain measurable radioactivity in the unstimulated muscle and incorporate increased $^{32}$P after stimulation (Fig. 4, bottom).

We measured the effect of carbachol stimulation on the level of radioactivity in each isoform and on the

![Fig. 2](image2.png)

Fig. 2. Separation of HSP27 charge isoforms by 2-dimensional nonequilibrium pH gel electrophoresis (NEpHGE)-SDS-polyacrylamide gel electrophoresis (PAGE) of canine tracheal protein extracts. Tissue protein extracts were subjected to NEpHGE in the first dimension and then were separated by molecular weight (MW) by SDS-PAGE in the second dimension. + and −, polarity of pH gradient in the first dimension of the separation. Known proteins clearly separated by this process and included actin, tropomyosin (TM), myosin light chain (MLC), 22-kDa smooth muscle protein (SM22), calponin (CP), and HSP27. HSP27 resolved into a, b, and c spots as identified by Western blotting (Fig. 3).

![Fig. 3](image3.png)

Fig. 3. A: section of a NEpHGE-SDS-PAGE Coomassie brilliant blue-stained gel as shown in Fig. 2 demonstrating HSP27 spots a, b, and c. B: corresponding Western blot of gel in A probed with affinity-purified chicken polyclonal anti-HSP27. Anti-HSP27 antibodies recognize all 3 HSP27 charge isoforms.
distribution of protein in each isoform. Gels were scanned, and protein content was determined by densitometry using purified rHSP27 as a dye binding standard. Radioactivity incorporated in each spot was determined by scintillation counting. Figure 5A shows that stimulation with 1 µM carbachol caused the tissue to contract tonically. Figure 5B and C, shows the relative increase in radioactivity in isoforms b and c after 1, 2, and 60 min normalized to basal levels (0 min). We observed a 1.6-fold increase in phosphorylation of the b isoform and a 1.7- to 1.8-fold increase in phosphorylation of the c isoform. Both increases were statistically significant at all time points compared with 0 min. The distribution of protein among the charge isoforms also changes significantly upon stimulation. The unphosphorylated a form decreased significantly from 41 ± 1% of total HSP27 to 27 ± 5% after 60 min (P < 0.05, Bonferroni t-test). This was accompanied by a corresponding increase in the protein content of the more acidic, phosphorylated b and c isoforms. The b isoform increased from 36 ± 4% of total HSP27 to 39 ± 6%. The c isoform increased from 23 ± 5 to 34 ± 3% of total HSP27 after 60 min. This suggests that the charge isoforms are produced by changes in the incorporation of phosphorus where the a spot is unphosphorylated and both b and c spots are phosphorylated.

Phosphorylation of canine rHSP27 by MAPKAP kinase-2. Stokoe et al. (29) showed that small-molecular-weight HSPs are phosphorylated by MAPKAP kinase-2. To determine if canine HSP27 is a substrate for MAPKAP kinase-2, we incubated full-length canine rHSP27 with purified skeletal muscle MAPKAP kinase-2 in vitro. Phosphorylation of canine rHSP27 by MAPKAP kinase-2 was initiated by the addition of MgATP (2 µCi [γ-32P]ATP) and was allowed to proceed for 120 min. Aliquots were removed at the times shown in Fig. 6, and rHSP27 was isolated by SDS-PAGE. Phosphorylation of rHSP27 occurred within 5 min and reached a maximum in this reaction by 120 min. This result clearly demonstrates that canine rHSP27 is a substrate for MAPKAP kinase-2.

Fig. 4. Top: HSP27 isolated from canine tracheal smooth muscle tissue strips under control conditions (0 min) and after stimulation with carbachol for 2 and 60 min. Extracted proteins were resolved by NEPHGE-SDS-PAGE and were stained with Coomassie brilliant blue. Bottom: corresponding gels from top imaged with a Bio-Rad Molecular Imager to detect changes in radioisotope labeling. HSP27 b and c spots incorporated additional 32P within 2 min after stimulation with carbachol. In addition, phosphorylation of the b and c spots was maintained for 60 min. MLC, used as an internal control, was also phosphorylated at 2 min but decreased somewhat at later time points. HSP27 a spot did not contain any radioactivity.

Fig. 5. Changes in phosphorylation of HSP27 charge isoforms in tracheal muscle strips stimulated with 1 µM carbachol. A: force developed by an intact strip of tracheal smooth muscle stimulated with 1 µM carbachol (Carb). B: relative phosphorylation of the HSP27 b isoform was determined by scintillation counting of the b spots cut from 2-dimensional gels prepared as in Fig. 3. Radioactivity [counts/min (cpm)] was corrected for protein content to give cpm/total µg HSP27 protein, which was then normalized to phosphorylation at 0 min. Relative phosphorylation of the b spot at 1, 2, and 60 min was significantly greater than at 0 min. C: phosphorylated c isoform of HSP27 showed a >1.7-fold increase over basal levels at 1, 2, and 60 min after carbachol treatment. *Significant difference from 0 min, P < 0.05, Student's t-test; n = 4 (1 min) and 5 (2 and 60 min).

Fig. 6. In vitro phosphorylation of canine recombinant HSP27 (rHSP27) by MAPKAP kinase-2. Kinase reaction, in 120-µl total volume, was started with [γ-32P]ATP and was stopped at the times indicated by removing 15-µl samples and mixing with concentrated SDS-PAGE sample buffer (see MATERIALS AND METHODS). Phosphorylated HSP27 was isolated by SDS-PAGE (12% acrylamide). Phosphorimage of 1-dimensional polyacrylamide gel illustrates that canine rHSP27 (27 kDa) is a substrate phosphorylated by MAPKAP kinase-2.
HSP27 kinase activity in canine trachea. Because canine rHSP27 was a good substrate for MAPKAP kinase-2 in vitro, we tested for endogenous HSP27 kinase activity in smooth muscle tissue. Muscle strips were stimulated with carbachol for times varying between 30 s and 1 h and then were frozen in liquid nitrogen. The homogenates were assayed for in vivo HSP27 kinase activity using human rHSP27 as a substrate. As shown in Fig. 7, rHSP27 was rapidly phosphorylated by an in vivo kinase. This HSP27 kinase activity reached maximum by 5 min and was sustained for 60 min. The time course of activation of endogenous HSP27 kinase activity (Fig. 7) correlated well with phosphorylation of HSP27 observed in tissue strips after carbachol treatment (Fig. 5); that is, we observed a 1.6-fold increase in HSP27 phosphorylation at 1 and 2 min (Fig. 5) and a 1.1- to 1.4-fold increase in the in vivo kinase at 30 s and 2 min (Fig. 7) after carbachol treatment. Moreover, both HSP27 phosphorylation and HSP27 kinase activity remain elevated at 60 min after stimulation.

From the deduced amino acid sequence, we know that HSP27 contains four different kinase recognition motifs. These include consensus phosphorylation sites for MAPKAP kinase-2 (HXRXXS, where H is hydrophobic amino acid and X is any amino acid), protein kinase C (S/TXR/K), protein kinase A (R/KXXS/T), and tyrosine kinase (R/KXXD/EXXY). Therefore, a crude tissue homogenate could contain multiple kinase activities that might phosphorylate HSP727 in our assay. To further define the endogenous HSP27 kinase, we added chelerythrine to the kinase extract to inhibit any protein kinase C activity. We observed no changes in the in vivo kinase activity with the addition of 10 mM chelerythrine (data not shown).

Expression of MAP kinases in canine trachea. MAPKAP kinase-2 is known to be a substrate for ERK1 and ERK2 MAP kinases in vitro (29) and p38 MAP kinase in vivo (8). To investigate the role of these MAP kinase signaling pathways in phosphorylation of HSP27, we tested for the expression of these MAP kinases in tracheal tissue. Tracheal protein extracts were separated by Mono-Q chromatography and were analyzed for expression of ERK1, ERK2, and p38 MAP kinases with isoform-selective antibodies. As shown in Fig. 8, all three MAP kinases are expressed in canine tracheal smooth muscle. These MAP kinases eluted at different salt concentrations, with p38 MAP kinase eluting later (0.32–0.35 M NaCl) than the ERK MAP kinases (0.24–0.29 M NaCl). The order of elution is consistent with differences in isoelectric points (pI) calculated from deduced amino acid sequences of human MAP kinases (ERK2, pI 6.72; ERK1, pI 6.29; p38, pI 5.41).

Activation of p38 MAP kinase in tracheal smooth muscle. ERK MAP kinases as well as p38 MAP kinases are potential upstream activators of MAPKAP kinase-2, and, as shown in Fig. 8, canine tracheal smooth muscle express ERK1, ERK2, and p38 MAP kinases. Cuenda et al. (8) suggest that p38 MAP kinase is...
upstream of MAPKAP kinase-2 in PC-12 and KB cells because the p38 MAP kinase inhibitor SB-203580 blocks MAPKAP kinase-2 activity and HSP27 phosphorylation, whereas ERK MAP kinase activity remains unaffected. We examined the question of p38 MAP kinase activation by assaying tyrosine phosphorylation of p38 MAP kinase in response to carbachol stimulation. Tissue strips were stimulated with carbachol for 10 min and then were frozen in liquid nitrogen. Protein extracts from unstimulated and stimulated tissue were processed by SDS-PAGE, transferred to nitrocellulose, and probed with anti-phospho-p38 MAP kinase antibodies that recognize only tyrosine-phosphorylated p38 MAP kinase (Fig. 9). Figure 9 shows that tyrosine phosphorylation of p38 is increased after 10 min of carbachol treatment. These results show that tyrosine phosphorylation of p38 MAP kinase is an early event after muscarinic receptor activation.

Phosphorylation of HSP27 through p38 MAP kinase pathway. Because p38 MAP kinase is expressed in canine tracheal smooth muscle and it becomes tyrosine phosphorylated upon carbachol stimulation, we used SB-203580 to test the notion that p38 MAP kinase is upstream of MAPKAP kinase and phosphorylation of HSP27. Tracheal tissue strips were stimulated with 1 µM carbachol for 10 min in the absence or presence of 25 µM SB-203580, frozen in liquid nitrogen, and homogenized. HSP27 kinase activity was assayed as described above (Fig. 7). Stimulation with carbachol in the absence of SB-203580 induced a 3.6-fold increase in kinase activity (Fig. 10). Preincubation of muscle strips for 60 min with 25 µM SB-20580 reduced carbachol-stimulated kinase activity to a 1.9-fold increase above basal activity. SB-203580 alone had no significant effect on basal kinase activity. The selectivity of SB-203580 was confirmed by assaying activation of ERK MAP kinases using the same homogenates that were assayed for HSP27 kinase activity. An in-gel kinase assay was used as described previously (10, 11). Figure 11A shows phosphorylation of myelin basic protein by ERK1 and ERK2 in tracheal smooth muscle homogenates. Figure 11B shows that there was no effect of pretreatment with SB-203580 on the mean activation of ERK1 or ERK2 produced by 1 µM carbachol. We also assayed tyrosine phosphorylation of p38 MAP kinase in the absence and presence of SB-203580 to determine whether upstream steps in activation of p38 MAP kinase were sensitive to the antagonist. Figure 11C shows an example of a Western blot probed with antibody selective for tyrosine-phosphorylated p38 MAP kinase. Densitometry of blots from five experiments (Fig. 11D) demonstrated that phosphorylation of p38 MAP kinase was not affected by SB-203580. The results suggest that treatment of intact tracheal smooth muscle with SB-203580 inhibits activation of MAPKAP kinase with no effect on activation of the ERK MAP kinases or tyrosine phosphorylation of p38 MAP kinase.

Inhibition of MAPKAP kinase activity by SB-203580 also resulted in inhibition of HSP27 phosphorylation, as demonstrated by separation of charge isoforms of HSP27 by NEphGE-SDS-PAGE. Figure 12A shows a cropped image of a Coomassie blue-stained gel and a bar graph illustrating the distribution of HSP27 charge isoforms in unstimulated muscle (basal). About one-half of the HSP27 is in the unphosphorylated a spot. As shown in Fig. 12B, 10 min of stimulation with 1 µM carbachol induces a redistribution of charge isoforms with a significant reduction in the unphosphorylated a spot and a significant increase in the phosphorylated c spot. Figure 12C and D shows that pretreatment for 60 min with 25 µM SB-203580 blocks the agonist-

Fig. 9. Tyrosine phosphorylation of p38 MAP kinase in canine tracheal smooth muscle. Two muscle strips from a single animal were isolated, and 1 muscle strip was stimulated with 1 µM carbachol for 10 min. Total proteins were extracted in SDS-PAGE sample buffer (see MATERIALS AND METHODS) and were resolved by SDS-PAGE. Tyrosine phosphorylation of p38 MAP kinase was detected by Western blotting with anti-phospho-p38 MAP kinase antibody. Tyrosine phosphorylation increased by ~2-fold after 10 min of stimulation with carbachol.

Fig. 10. Inhibition of in vivo activation of HSP27 kinase by SB-203580. Tracheal smooth muscle strips were treated with 25 µM SB-203580–0.1% dimethyl sulfoxide (DMSO; first 2 bars) or with 0.1% DMSO (last 2 bars). Two muscle strips were frozen before stimulation, and two were stimulated with 1 µM carbachol for 10 min. Strips were frozen, and homogenates were assayed for HSP27 kinase activity using human rHSP27 as substrate. Inset shows a phosphorimage of 32P incorporation into HSP27 under basal (open bars) and stimulated (filled bar and gray bar) conditions. Kinase activities were determined by densitometry of the phosphorylated HSP27 bands corrected for total protein loaded in each lane and normalized to basal kinase activity in the absence of carbachol (n = 7).
induced shift in isoform distribution. In unstimulated muscles treated with SB-203580, 78 ± 10% of HSP27 was in the unphosphorylated a isoform (Fig. 12C), and there was no significant change in isoform distribution in response to carbachol (Fig. 12D). The results show that the p38 MAP kinase antagonist SB-203580 inhibits the shift of HSP27 to more acidic, phosphorylated isoforms. This is consistent with the hypothesis that muscarinic receptors are coupled to the activation of p38 MAP kinase and MAPKAP kinase-2 that ultimately leads to phosphorylation of HSP27.

DISCUSSION

We have evaluated the effects of carbachol stimulation on the phosphorylation of HSP27 in isolated canine tracheal smooth muscle strips. In response to stimulation with carbachol, the distribution of HSP27 charge isoforms was shifted to more acidic, phosphorylated forms (Figs. 4 and 5), which is consistent with previous studies of HSP27 phosphorylation in response to heat stress and chemical stressors (3, 4, 19, 28, 32) in nonmuscle cells. Studies of nonmuscle cells have identified MAPKAP kinase-2 as the enzyme most likely to phosphorylate HSP27 in vivo (19, 29). The canine HSP27 sequence contains phosphorylation sites identical to known MAPKAP kinase-2 phosphorylation sites in the human HSP27 protein (Fig. 1). In vitro phosphorylation of canine rHSP27 by purified skeletal muscle MAPKAP kinase-2 (Fig. 6) confirmed that HSP27 is a substrate for this kinase. In addition, activation of endogenous HSP27 kinase by carbachol stimulation is rapid, as is HSP27 phosphorylation, and both are sustained for at least 60 min (Fig. 7). Finally, canine rHSP27 is a substrate for endogenous HSP27 kinase, which was not inhibited by chelerythrine. Moreover, studies by Stokoe et al. (29) demonstrated that MAPKAP kinase-1, calmodulin-dependent protein kinase-II, adenosine 3',5'-cyclic monophosphate-dependent protein kinase, protein kinase C, and ribosomal protein S6 kinase II are not directly involved in the phosphorylation of HSP27. Therefore, in vivo HSP27 kinase activity stimulated by carbachol in tracheal smooth muscle is most likely MAPKAP kinase-2, or possibly MAPKAP kinase-3, which has similar substrate selectivity and sensitivity to inhibition by SB-203580 (7).

MAPKAP kinase-2 can be activated in vitro by both ERK MAP kinases (3, 29) and p38 MAP kinase (8). Activation of either pathway in vivo might lead to phosphorylation of HSP27. We have shown that the ERK MAP kinases are activated after muscarinic stimulation of canine colonic and airway smooth muscle (10, 11), raising the formal possibility that activation of ERK MAP kinases leads to activation of MAPKAP kinase-2 and phosphorylation of HSP27. However, the alternative hypothesis that phosphorylation of HSP27 occurs via a stress response pathway involving the p38 MAP kinases seems more likely. Cuenda et al. (8) showed that the p38 MAP kinase inhibitor SB-203580 completely blocked HSP27 phosphorylation in KB cells treated with arsenite, sorbitol, and interleukin-1. The question of whether muscarinic receptors in smooth muscle are coupled to a stress response pathway was addressed by demonstrating that the p38 MAP kinase is expressed in tracheal smooth muscle (Fig. 8) and that stimulation with carbachol increased tyrosine phosphorylation of the p38 MAP kinase (Fig. 9). Consistent with our results, Kramer et al. (18) showed that p38 MAP kinase is activated in platelets by thrombin, which acts via G protein-coupled STM receptors. We also found that the agonist-induced shift in HSP27 charge isoforms to the more acidic, phosphorylated forms was blocked by SB-203580 (Fig. 12). As shown in Figs. 4 and 10, muscarinic activation results in HSP27 shifting...
from the unphosphorylated state to more acidic forms, which probably represent the mono- and diphosphorylated states (Fig. 12). These results suggest a signaling cascade in which HSP27 is phosphorylated by activation of the p38 MAP kinase and activation of MAPKAP kinase-2. More direct support of this hypothesis was provided by the partial inhibition of HSP27 kinase activity by SB-203580 and no inhibition of ERK MAP kinases or tyrosine phosphorylation of p38 MAP kinase (Fig. 11).

Activation of muscarinic receptors in airway smooth muscle is a normal physiological event in vivo and would not ordinarily be considered a "stress" stimulus in contrast to common experimental stressors such as heat stress, ultraviolet radiation, arsenite, or anisomycin. This raises the interesting question of what the role of the p38 MAP kinase is in the normal physiology of airway smooth muscle and how the extracellular signals are transduced to kinase activation and substrate phosphorylation. A model based on what is known about MAP kinase signaling in both muscle and non-muscle cells is illustrated in Fig. 13. Agonists, including carbachol and interleukin-8, activate ERK1 and ERK2 via an STM receptor (17, 30), whereas stress stimuli, such as sorbitol and anisomycin or ultraviolet light, activate two other parallel MAP kinase stress pathways: MKK3 leading to p38 activation and MKK4 activating jun-NH2-terminal kinase (JNK)/stress-activated protein kinase (SAPK). MKK4 can activate both JNK and p38 MAP kinases. Activation of ERK1/ERK2 by carbachol and interleukin-8 normally leads to nonproliferative events, whereas activation of the JNK/SAPK pathway leads to proliferation. MEK, MAP kinase; MEKK, MAP kinase kinase; UV, ultraviolet.

Fig. 12. Inhibition of HSP27 phosphorylation by SB-203580. Muscle strips were incubated for 60 min with 0.1% DMSO (A and B) or 25 µM SB-203580 and 0.1% DMSO (C and D). Muscle strips were stimulated with 1 µM carbachol for 10 min and then stimulated with 1 µM carbachol for 10 min. C: unstimulated muscles (basal) pretreated with 25 µM SB-203580–0.1% DMSO. D: muscles pretreated with SB-203580–0.1% DMSO and then stimulated with 1 µM carbachol for 10 min. * and ** Significant differences from basal, DMSO-treated samples, P < 0.05, Student's t-test; n = 5.

Fig. 13. Possible parallel arrangement of MAP kinase cascades in mammalian cells. Carbachol and interleukin-8 activate ERK1/ERK2 pathway via 7-transmembrane receptor (STMR) while cellular stresses activate MKK3 and MKK4 pathways. In addition, MAP kinase pathways converge and activate parallel cascades. For example, sorbitol activates MAP kinase/ERK (MKK) 3 as well as ERK1/ERK2 and jun-NH2-terminal kinase (JNK)/stress-activated protein kinase (SAPK). MKK4 can activate both JNK and p38 MAP kinases. Activation of ERK1/ERK2 by carbachol and interleukin-8 normally leads to nonproliferative events, whereas activation of the JNK/SAPK pathway leads to proliferation. MEK, MAP kinase; MEKK, MAP kinase kinase; UV, ultraviolet.
three MAP kinase homologs, ERK1/ERK2 (6), p38 (8), and JNK (6). The cross talk between MAP kinase pathways can occur at several levels, including the dual-specificity protein kinases that activate the MAP kinases. A relevant example is MKK4, which activates JNK/SAPK as well as the p38 MAP kinase in cultured fibroblasts (23).

Our studies suggest that similar activation of parallel MAP kinase pathways occurs in airway smooth muscle. Several agonists acting via STM receptors activate ERK MAP kinases in canine smooth muscles (10, 11). These include carbachol, neurokinin A, and histamine. In the present study, we have shown that HSP27 is phosphorylated after activation of muscarinic receptors, and the signaling pathway probably includes p38 MAP kinase. Until recently, phosphorylation of HSP27 has been thought to be coupled primarily to the stress response pathway (Fig. 11), and there have been few reports that link activation of p38 MAP kinase with STM receptors. Our results suggest that G protein-linked muscarinic receptors activate the p38 MAP kinase pathway in airway smooth muscle and that one result of activating the p38 MAP kinase pathway is phosphorylation of HSP27.

Although the physiological function of HSP27 is unknown, several reports suggest that HSP27 may modulate actin filament dynamics in vivo. This modulation of actin dynamics may be regulated by phosphorylation, since rHSP27 mutated at the phosphorylation sites is unable to promote F-actin polymerization normally seen in control cells transfected with wild-type rHSP27 (22). Moreover, Benndorf et al. (4) observed that nonphosphorylated HSP25 isolated from Ehrlich ascites tumor cells inhibited actin polymerization in vitro, whereas phosphorylated HSP25 failed to exhibit any inhibiting activity. Using isolated, permeabilized rectosigmoid smooth muscle cells, Bittar et al. (5) found that contraction induced by bombesin was blocked by a monoclonal antibody against HSP27. Therefore, phosphorylation and activation of HSP27 may be linked to several cellular functions, including proliferation, locomotion, and contraction via actin remodeling. Control of these cellular responses in rectosigmoid smooth muscle was suggested to be through MAP kinase cascades in part because ERK MAP kinases and HSP27 codistribute in resting and stimulated cells (31), and HSP27 phosphorylation is coupled to MAP kinase cascades in nonmuscle cells (3, 8, 19, 31).

Activation of MAP kinases occurs in response to stimuli that promote actin remodeling in other nonproliferative cells. For example, interleukin-8 activates ERK MAP kinases (17) and induces migration of neutrophils, which is a process involving changes in filamentous actin content. However, the identity and regulation of proteins upstream of p38 MAP kinases in regulating cytoskeletal and contractile proteins is unclear. The protein kinases PAK1 and MKK3 are thought to be upstream of p38 MAP kinase, but details of coupling to STM receptors are not yet defined. In addition, activation of rho and rac are also thought to be important for formation of actin stress fibers, focal contacts, and membrane ruffling in nonmuscle cells (25, 26). If actin filament structure is regulated by MAP kinases in smooth muscle cells, then it may be that HSP27 modulates actin filament dynamics after activation of G protein-coupled STM receptors linked sequentially to ras, rho, rac, p38 MAP kinase, and MAPKAP kinase-2, leading to the phosphorylation of HSP27 and regulation of the actin cytoskeleton.

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Present address of J. K. Larsen: Dept. of Molecular and Integrative Physiology, University of Illinois, 524 Burrill Hall, 407 S. Goodwin Ave., Urbana, IL 61801.

Address for reprint requests: W. T. Gerthoffer, Dept. of Pharmacology/Toxicology, University of Nevada School of Medicine, Reno, NV 89557-0946.

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