Mechanism of fluoride-induced MAP kinase activation in pulmonary artery endothelial cells

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Bogatcheva, Natalia V., Peiyi Wang, Anna A. Birukova, Alexander D. Verin, and Joe G. N. Garcia. Mechanism of fluoride-induced MAP kinase activation in pulmonary artery endothelial cells. Am J Physiol Lung Cell Mol Physiol 290: L1139–L1145, 2006. First published January 13, 2006; doi:10.1152/ajplung.00161.2005.—In this study, we demonstrate that challenge of endothelial cells (EC) with NaF, a recognized G protein activator and protein phosphatase inhibitor, leads to a significant Erk activation, with increased phosphorylation of the well-known Erk substrate caldesmon. Inhibition of the Erk MAPK, MEK, by U0126 produces a marked decrease in NaF-induced caldesmon phosphorylation. NaF transiently increases the activity of the MEK kinase known as Raf-1 (~3- to 4-fold increase over basal level), followed by a sustained Raf-1 inhibition (~3- to 4-fold decrease). Selective Raf-1 inhibitors (ZM-336372 and Raf-1 inhibitor 1) significantly attenuate NaF-induced Erk and caldesmon phosphorylation. Because we have previously shown that Ca2+/calmodulin-dependent protein kinase II (CaMKII) participates in Erk activation in throbini-challenged cells, we next explored if CaMKII is involved in NaF-induced EC responses. We found that in NaF-treated EC, CaMKII activity increases in a time-dependent manner with maximal activity at 10 min (~4-fold increase over a basal level). Pretreatment with KN93, a specific CaMKII inhibitor, attenuates NaF-induced barrier dysfunction and Erk phosphorylation. The Raf inhibitor C3 exotoxin completely abolishes NaF-induced CaMKII activation. Collectively, these data suggest that sequential activation of Raf-1, MEK, and Erk is modulated by Raf-dependent CaMKII activation and represents important NaF-induced signaling response. Caldesmon phosphorylation occurring by an Erk-dependent mechanism in NaF-treated pulmonary EC may represent a link between NaF stimulation and contractile responses of endothelium.

Fluoride-induced high-affinity complex between small G protein and GAP limits GAP activity, thereby resulting in small G protein activation (37). Unlike Ras, fluororalanum induces GDP-bound Cdc42 to form stable complexes with effectors as well as GAP (21), suggesting important differences in the fluoride effects downstream of Ras and Rho.

Exposure to fluorides induces inflammatory reactions, cell contractile responses, cell proliferation or cell cycle arrest, and apoptosis, which is dependent on the experimental cellular system used. Both G protein-dependent and -independent pathways are likely involved in the physiological response to fluoride (11, 25). The pattern of G proteins activated by fluoride is also cell specific and determines the nature of cellular response. In osteoblasts, fluoride stimulates pertussis toxin-sensitive G; and pertussis toxin-insensitive G proteins, most likely from G12 class (33), whereas endothelial cells (EC) respond with pertussis toxin-insensitive phosphoinositide hydrolysis and calcium mobilization (13) as well as Rho activation (38).

Fluoride has been reported to activate the stress-response signaling cascade involving MAP kinases (2, 7, 18, 29, 32, 35, 39); however, the molecular events leading to such activation are poorly understood and have not been explored in endothelium. Erk is activated by the Ras/MEKK/MEK-mediated pathway (28). Ras being a direct target for fluoride (24) provides a highly plausible mechanism of NaF-induced Erk activation. Both the PKC-dependent pathway as well as alterations in tyrosine phosphorylation are also likely to be involved in the NaF-induced MAP kinase activation (7, 29, 39).

In the present study, we focused on the mechanism of NaF-induced MAP kinase activation in pulmonary EC. One of the important features of endothelium is the ability to maintain a selective barrier between the inner vascular space and underlying tissues. Our previous findings suggest a primary role for Rho activation rather than calcium influx in NaF-induced endothelial barrier dysfunction (38). However, the precise pathways downstream of Rho activation are not completely explored. In this paper, we examine the link between Rho activation and MAP kinase activation in NaF-stimulated EC and examine the contribution of Ca2+/calmodulin-dependent protein kinase II (CaMKII) activity in these pathways. We characterize the NaF-induced MAPKKK/MAPKK/MAPK cascade and clarify the role of MAP kinase Erk in phosphorylation of caldesmon, an important regulatory cytoskeletal protein known to be involved in vascular barrier regulation.

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MATERIALS AND METHODS

Reagents. Unless otherwise specified, reagents were obtained from Sigma Chemical (St. Louis, MO), PBS, medium 199, antibiotic and antimiycotic solution (10,000 U/ml penicillin, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B), and nonessential amino acids were purchased from Gibco (Grand Island, NY). EC growth supplement was purchased from Upstate Biotechnology (Lake Placid, NY). C3 exoenzyme, KN93, Raf-1 kinase inhibitor 1, ZM-336372, and U0126 were purchased from Calbiochem (La Jolla, CA). Protein concentration was determined using BCA protein assay reagent (Pierce, Rockford, IL). Anti-phospho-Erk, anti-phospho-caldesmon, and anti-Erk antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-caldesmon antibody was purchased from BD Biosciences (San Jose, CA). All radioactive reagents were purchased from NEN (Boston, MA).

Cell culture. Bovine pulmonary artery EC (BPAEC) obtained from Clonetics (Walkersville, MD), cultured in EBM-2 medium (Clonetics) with 10% FBS, 1% antibiotic-antimycotic mixture, and 0.1% growth supplement.

Measurement of transendothelial electrical resistance. The cellular barrier properties were measured using the highly sensitive biophysical assay with an electrical cell-substrate impedance sensing system (Applied Biophysics, Troy, NY) as described previously (36, 38). Briefly before experiments, the media in wells were changed to Optimem, and cells were incubated for ~1 h to reach a steady-state resistance. Cells were pretreated with indicated concentrations of inhibitors for ~30 min and stimulated with 20 mM NaF. Transendothelial electrical resistance (TER) was monitored for 4 h. TER values in vehicle-treated and inhibitor-treated cells were compared using one-way ANOVA; differences were considered significant at P < 0.05.

Western immunoblotting. Proteins were extracted from BPAEC monolayers with SDS sample buffer. The extracts were separated by SDS-PAGE, transferred to nitrocellulose (30 V for 18 h or 90 V for 2 h), and incubated with the specific antibodies. Immunoreactive proteins were detected with an enhanced chemiluminescence detection kit, according to the manufacturer’s directions (Amersham, Little Chalfont, UK).

Raf-1 activity assay. Raf-1 kinase activity was assessed as previously described (36) with c-Raf immunoprecipitation kinetics cascade assay kit (Upstate Biotechnology, Lake Placid, NY), according to the manufacturer’s recommendation, with minor modification. Confluent BPAEC grown in 60-mm dishes were serum-starved in medium 199 for 18 h and then treated with 20 mM NaF for different time periods. The cells were lysed in 500 μl of lysis buffer A [50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na3VO4, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, and 1:500 diluted protease inhibitor cocktail (Calbiochem)] for 30 min. Cell debris was removed by a 10-min centrifugation at 16,000 g, and the supernatant was incubated with 4 μg of anti-human c-Raf kinase COOH-terminal antibodies at 4°C for 2 h. After that, 100 μl of a PBS-prewashed protein G Sepharose slurry (containing 30% protein G Sepharose 4 Fast Flow; Amersham Pharmacia Biotech, Piscataway, NJ) were added, and samples were incubated for another 2 h at 4°C with gentle agitation. Immunoprecipitates were washed several times with lysis buffer, and immunoprecipitated active Raf-1 was used to phosphorylate and activate glutathione S-transferase (GST)-MEK, which, in turn, phosphorylates and activates p42 GST-ERK2. Active GST-ERK2 was then used to phosphorylate myelin basic protein (MBP) with [γ-32P]ATP. The radiolabeled substrates were allowed to bind to P81 phosphocelulose liquid paper (Whatman, Clifton, NJ), and the radioactivity was assessed by measurement in a scintillation counter.

CaMKII activity assay. CaMKII activity was assessed as previously described (5) in BPAEC monolayers incubated with 20 mM NaF for indicated times and lysed in 500 μl of lysis buffer containing 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na3VO4, 0.1% 2-mercaptoethanol, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, and 1:500 diluted protease inhibitor cocktail (Calbiochem) for 30 min. Cell debris was removed by 10-min centrifugation at 16,000 g, and the supernatants were incubated with 2.5 μg of CaMKII monoclonal antibodies (BD Biosciences) at 4°C for 1 h, followed by incubation with 100 μl of protein G Sepharose slurry (containing 30% protein G Sepharose 4 fast flow) for 1 h at 4°C with gentle agitation. Immunoprecipitates were washed twice with lysis buffer, and the CaMKII activity was determined by the incorporation of [32P]orthophosphate into its specific substrate peptide using the CaMKII assay kit (Upstate Biotechnology, Lake Placid, NY).

EC permeabilization. It is well known that Clostridium botulinum C3 exoenzyme does not easily pass through the cell membrane under native conditions. To penetrate cell membrane, BPAEC monolayers (80–100% confluence) grown on 60-mm culture dishes were rinsed with Optimem-I medium, and Lipojontamine reagent (GIBCO) was added at a final concentration of 20 μg/ml for 1 h, followed by the addition of C3 exoenzyme (2.5 μg/ml) for an additional 11 h as we have previously described in detail (4).

RESULTS

Effect of NaF on MAPK activity and caldesmon phosphorylation in bovine endothelium. It has been previously shown that NaF increases Erk, p38, and JNK activity in human lung epithelial cells (29, 35). We examined here the role of Erk activation in NaF-treated EC. Measurements of TER in BPAEC (Fig. 1A) and HPAEC (data not shown) revealed that NaF-induced TER decline was partially attenuated by a potent and specific inhibitor of MEK1 and MEK2, U0126. Western immunoblotting with specific anti-phospho-Erk antibodies showed that treatment with NaF increased Erk phosphorylation level, indicative of the elevated Erk activity (Fig. 1A). Erk p42 activation began as early as 5 min, reached a maximum at 10 min, and persisted for at least 60 min. Erk p44 activation was also maximal at 10 min but declined more rapidly after this point.

To elucidate relevant Erk targets and biochemical pathways involved in NaF-induced EC activation, we next studied the phosphorylation of caldesmon, a regulatory cytoskeletal protein known to be phosphorylated by Erk (17). We employed anti-phospho-caldesmon antibodies specific toward the MAPK phosphorylation site and observed that increased caldesmon phosphorylation occurred in a time-dependent manner (Fig. 1C). The level of caldesmon phosphorylation increased significantly after 10 min of NaF treatment and reached a maximum at 60 min of incubation (Fig. 1C). Because p38 along with Erk is known to phosphorylate caldesmon at MAPK-specific sites, we next examined the effects of MEK inhibition on caldesmon phosphorylation. Cell pretreatment with U0126 completely
abolished NaF-induced Erk activation (Fig. 2A) and significantly decreased NaF-induced caldesmon phosphorylation in BPAEC (Fig. 2B). These data indicate that NaF-induced caldesmon phosphorylation is at least in part Erk dependent.

**Fig. 1.** A: Erk involvement into NaF-induced barrier disruption. Bovine pulmonary artery endothelial cells (BPAEC) pretreated with vehicle (black bars) or the MEK inhibitor U0126 (5 μM; gray bars) were stimulated with 20 mM NaF for the time indicated. Transendothelial electrical resistance (TER) was normalized at the point of NaF addition. Data are means ± SE, n = 3 experiments, *P < 0.05 compared with vehicle-treated controls. B: effect of NaF stimulation on Erk activation in BPAEC. Serum-starved BPAEC monolayers were stimulated with 20 mM NaF for the indicated periods of time. The level of Erk activation was analyzed by Western immunoblotting of endothelial cell homogenates with specific anti-phospho-Erk antibodies. The level of Erk protein expression was analyzed by anti-Erk antibodies. Shown are results from a representative experiment (n = 3). C: effect of NaF stimulation on caldesmon (CaD) phosphorylation in BPAEC. The level of caldesmon phosphorylation was analyzed by Western immunoblotting of endothelial cell homogenates with specific anti-phospho-CaD antibodies. The level of CaD expression was analyzed by pan-CaD antibody. Shown are results from a representative experiment (n = 3).

**Fig. 2.** A: effect of MEK inhibition on NaF-induced Erk activation in bovine endothelium. Serum-starved BPAEC monolayers were pretreated with either vehicle (0.1% DMSO) or 10 μM U0126 for 30 min and then challenged with 20 mM NaF for 10 min. Erk activity was assessed by Western immunoblotting with phospho-specific Erk antibody. Shown are results from a representative experiment (n = 3). B: effect of MEK inhibition on NaF-induced CaD phosphorylation in bovine endothelium. Serum-starved BPAEC monolayers were pretreated with either vehicle (0.1% DMSO or water) or 10 μM U0126 for 30 min and then challenged with 20 mM NaF for 60 min. CaD phosphorylation was assessed by Western immunoblotting with anti-phospho-CaD antibody. Shown are results from a representative experiment (n = 3).

**Fig. 3.** Effect of NaF on Raf-1 activity. Confluent BPAEC monolayers were serum deprived and then incubated with 20 mM NaF for the indicated periods of time. Cells were lysed, and cell lysates were immunoprecipitated with anti-human c-Raf kinase COOH-terminal antibody. Raf-1 activity was measured with Raf-1 kinase cascade assay kit as described in MATERIALS AND METHODS. Data are means ± SE, n = 4 experiments, *P < 0.05 compared with control.

**Signaling events involved in NaF-induced Erk activation.** To examine the involvement of MEK kinase Raf in NaF-induced EC activation, we measured Raf-1 kinase activity by immunoprecipitation, followed by sequential phosphorylation of MEK, Erk, and MBP. Figure 3 demonstrates that NaF significantly increased Raf-1 activity during first 5 min of NaF stimulation, which correlates with the initiation of Erk activation (Fig. 1B). Prolonged incubation with NaF, however, led to the decline in Raf-1 activity (Fig. 3). To further evaluate the role of Raf-1 in NaF-induced Erk activation, we pretreated BPAEC with specific Raf-1 inhibitors, ZM-336372 and inhibitor 1. Raf-1 inhibition significantly attenuated both NaF- and phorbol myristate ester-induced Erk activation.
acetate-induced Erk activation and caldesmon phosphorylation (Fig. 4). Although the IC\textsubscript{50} for ZM-336372 is eight times higher than that for Raf-1 inhibitor 1, 1 μM ZM-336372 was more effective than 1 μM Raf-1 inhibitor 1 while used to treat EC. The results above indicate that Raf-1, transferring signal to MEK, plays an important role in NaF-induced Erk activation.

Recently, our laboratory has shown that CaMKII inhibition affects thrombin-induced Erk activation and caldesmon phosphorylation in EC (5). We next addressed whether CaMKII activation actually occurs in NaF-stimulated EC and whether CaMKII participates in NaF-induced MAPK activation. We assessed CaMKII activity in NaF-treated cells using CaMKII immunoprecipitation and measurement of the phosphate incorporation into specific CaMKII substrate. We found that 10-min treatment with NaF produces maximal CaMKII activity, followed by a return to the basal level at 30 min (Fig. 5A). Inhibition of CaMKII by KN-93 significantly attenuated NaF-induced Erk phosphorylation (Fig. 5B). These data indicate that NaF-induced Erk activation is dependent, at least in part, on CaMKII activity.

**Signaling pathways leading to CaMKII activation in NaF-treated cells.** NaF is known to increase the level of intracellular Ca\textsuperscript{2+} (13), which in turn stimulates Ca\textsuperscript{2+}/calmodulin-dependent enzymes, including CaMKII. However, as we have shown earlier, the depletion of calcium with BAPTA does not alter significantly TER decline observed in NaF-treated EC. Here, we demonstrate that the CaMKII inhibitor KN-93 partially attenuates NaF-induced barrier disruption (Fig. 6A).

We have previously shown that Rho is critically involved in NaF-induced endothelial cytoskeletal rearrangement and permeability (38). To ascertain whether Rho takes part in CaMKII activation, we assessed CaMKII activity in cells pretreated with the specific Rho inhibitor C3 exotoxin. Such pretreatment completely abolished a NaF-induced increase in CaMKII activity (Fig. 6B), indicating that NaF-induced Erk activation in pulmonary EC involves Rho-dependent activation of CaMKII.

**DISCUSSION**

In the present study, we report that NaF evokes time-dependent Erk activation in pulmonary EC. Erk activation contributes to the NaF-induced EC barrier disruption, presumably via phosphorylation of caldesmon, an actin-binding protein, involved in actomyosin ATPase regulation and actin stabilization (16). Phosphorylated by Erk, caldesmon is proposed to have reduced affinity for actin (8), which could lead to the disinhibition of actomyosin ATPase or to the rearrangement of actin filaments.

We focused our study on the elucidation of events leading to Erk activation in NaF-stimulated cells. Erk dual phosphorylation on a Thr-X-Tyr motive is catalyzed by specific MAPK kinases, which in turn are activated by MAPKK kinases (20). Recently, we have shown the sequential activation of the MAPKK kinase Raf-1, MAPK kinase MEK, and MAP kinase Erk in response to protein kinase C activator phorbol myristate acetate (36). To evaluate signaling events in NaF-treated endothelium, we examined the effect of MEK and Raf inhibitors on NaF-induced Erk phosphorylation. Our data demonstrate that MAPK kinases MEK1 and MEK2 as well as MAPKK
kinase cRaf are involved in the NaF-induced Erk activation, consistent with the rapid cRaf-1 activation by NaF. It has been previously shown that the small G protein Ras recruits Raf-1 to the membrane and results in its activation after growth factor receptor stimulation (23). With Ras effectively binding fluoride (24), Ras/Raf-1/MEK/Erk cascade may represent the direct target for fluoride action in endothelium.

Fluoride may also evoke additional mechanisms of MAPK activation (Fig. 7). Previously we have shown that the challenge of human endothelium by NaF led to phosphoinositide hydrolysis, generation of inositol phosphates, and mobilization of calcium (13). It is well known that calcium influx or the release of internally stored calcium can lead to Ras and MAP kinase activation (10). One Ca^{2+}-dependent mechanism of Ras activation involves the proline-rich tyrosine kinase 2 (Pyk2)-mediated regulation of the RasGEF, Sos. Another RasGEF, GRF, is also regulated by calcium, although the exact molecular mechanism by which this occurs remains unclear (10). Accumulating evidences support a role for Ca^{2+}/calmodulin-dependent kinases in MAP kinase cascade activation. Thus CaMKII was shown to mediate Erk activation in smooth muscle, EC, and neurons stimulated with a wide range of agonists (1, 5, 9, 22, 26, 40), whereas CaMKIV stimulates JNK and p38 pathways in neuroblastoma/glioma cells (12). Our data indicated that NaF-induced activation of Erk in endothelium is in a large extent mediated by CaMKII, consistent with significant increase of CaMKII activity in NaF-treated BPAEC.

The interconnecting pathway between CaMKII and Erk in EC was not examined in this study, and the mode of MAP kinase cascade activation by CaMK is currently unknown. In fibroblasts, CaMKII phosphorylates and facilitates membrane translocation of Tiam1, the GEF for Rac1 small G protein, acting upstream of JNK cascade (6). Novel neuronal RasGAP SynGAP was shown to be phosphorylated and potently inhibited by CaMKII (30). In smooth muscle cells, two mechanisms of MAP kinase activation were suggested: one involving CaMKII-dependent activation of Pyk2 and the subsequent transactivation of the EGF receptor (14), another implicating arachidonic acid generated by CaMKII-stimulated cytosolic phospholipase A2 (27).

The biochemical events linking NaF and CaMKII activation are not confined to the Ca^{2+}/calmodulin-dependent pathway. We previously demonstrated that Rho/Rho kinase is critical for NaF-induced EC barrier dysfunction (38) and importantly now show that NaF-induced increase in CaMKII activity is also regulated by Rho. To the best of our knowledge, Rho-dependent activation of CaMKII has not been reported. The mechanism of Rho-induced CaMKII activation is yet to be elucidated. Rho-kinase-mediated phosphatase inactivation could be responsible for the maintenance of high CaMKII autophosphorylation level; however, NaF-induced CaMKII activity reported in this study precedes myosin phosphatase targeting subunit phosphorylation and phosphatase inactivation shown in NaF-treated endothelium (38).

In summary, biochemical data provided in this report characterize MAPK activation pathway induced by NaF in endothelium.

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**Fig. 6.** A: calcium and CaMKII involvement into NaF-induced barrier disruption. BPAEC pretreated with vehicle (open bars), 5 μM BAPTA (gray bars), or 5 μM KN-93 (black bars) were stimulated with 20 mM NaF for the times indicated. TER was normalized at the point of NaF addition. Data are means ± SE, n = 3 experiments, *P < 0.05 compared with vehicle-treated controls. B: effect of Rho inhibition on NaF-induced CaMKII activation. BPAEC were pretreated with C3 exoenzyme (2.5 μg/ml for 10 h) in the presence of Lipofectamine and then stimulated for 10 min with 20 mM NaF followed by CaMKII immunoprecipitation and CaMKII activity analysis as described in MATERIALS AND METHODS. Data are means ± SE, n = 3 experiments. *P < 0.05 compared with vehicle.

**Fig. 7.** Schematic representation of the intracellular signaling involved in NaF-induced Erk activation and CaD phosphorylation. Our data confirm the pathways indicated by light-colored arrows. Data of literature suggest other possible pathways indicated by dark-colored arrows.
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