Iron Causes Interactions of TAK1, p21ras, and Phosphatidylinositol 3-Kinase in Caveolae to Activate IκB Kinase in Hepatic Macrophages

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We recently discovered a novel signaling phenomenon involving a rapid and transient rise in intracellular low molecular weight iron complex(es) in activation of IκB kinase (IKK) in hepatic macrophages. We also showed direct treatment with ferrous iron substitutes for this event to activate IKK. The present study used this model to identify upstream kinases responsible for IKK activation. IKK activation induced by iron is abrogated by overexpression of a dominant negative mutant (DN) for transforming growth factor β-activated kinase-1 (TAK1), NF-κB-inducing kinase, or phosphatidylinositol 3-kinase (PI3K) and by treatment with the mitogen-activated protein kinase (MAPK) kinase-1 (MEK1) inhibitor. Iron increases AKT phosphorylation that is prevented by DNTAK1 or DNp21ras. Iron causes ERK1/2 phosphorylation that is attenuated by DN-PI3K, prevented by DNp21ras, but unaffected by DNTAK1. Iron-induced TAK1 activity is not affected by the PI3K or MEK1 inhibitor, suggesting TAK1 is upstream of PI3K and MEK1. Iron increases interactions of TAK1 and PI3K with p21ras as demonstrated by co-immunoprecipitation and co-localization of these proteins with caveolin-1 as shown by immunofluorescent microscopy. Finally, filipin III, a caveolae inhibitor, abrogates iron-induced TAK1 and IKK activation. In conclusion, MEK1, TAK1, NF-κB-inducing kinase, and PI3K are required for iron-induced IKK activation in hepatic macrophages and TAK1, PI3K, and p21ras physically interact in caveolae to initiate signal transduction.

Iron accumulation often accompanies chronic inflammatory diseases such as atherosclerosis (1), pulmonary fibrosis (2), Parkinson disease (3), and chronic liver disease (4–6). Mechanisms by which inflammation leads to increased iron storage have been enlightened by the recent revelation of induction of hepcidin by inflammatory cytokines (7) and the ability of this protein to bind to ferroportin for its internalization and degradation and consequently to reduce export of cellular iron (8). Conversely, iron loading causes chronic tissue damage and inflammation. The most prevalent hypothesis for this causal link has been that the iron-catalyzed Fenton pathway generates a hydroxyl radical and causes oxidative tissue injury. However, emerging evidence indicates that the effects of iron may be more complex and intricate than simple oxidant injury and involve molecular mechanisms by which expression of proinflammatory cytokines is induced by enhanced iron-dependent signaling. To this end, we have demonstrated a novel signaling event of a transient rise in intracellular low molecular weight-iron complex(es) in 2 min after the treatment of cultured hepatic macrophages (HM)2 with lipopolysaccharide (LPS) or tumor necrosis factor-α (TNFα) (9). This unique event precedes and is required for activation of IκB kinase (IKK) and NF-κB (9). Furthermore, an increase in intracellular non-heme iron store caused by a mutation in natural resistance-associated macrophage protein-1 results in coordinated accentuation of the low molecular weight-iron complex(es) response, IKK, NF-κB activation, and TNFα release in LPS-stimulated RAW264.7 cells (9). Interestingly, the direct treatment with iron causes within 2 min an immediate and maximal quenching of RAW264.7 cells loaded with an iron-sensitive fluorescent probe, suggesting an immediate uptake of iron by the cells (10). Addition of ferrous sulfate (5–50 μM) to cultured HM activates IKK but not JNK (11). Thus, the low molecular weight-iron complex(es) response induced by a ligand-receptor interaction is substituted for by exogenous ferrous iron in transducing signals to activate IKK in primary cultures of HM.

IKK plays a central role in a classical activation pathway of NF-κB through phosphorylation of IκB for subsequent polyubiquitination and proteasome-mediated degradation (see Ref. 12 for review). Three IKK subunits have been identified, IKKα and IKKB as catalytic subunits and IKKγ as a regulatory unit. The most common form of IKK is composed of a heterodimer of IKKα and IKKB and bound to a dimer of IKKγ. IKK activation

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2 The abbreviations used are: HM, hepatic macrophage; TAK1, transforming growth factor β-activated kinase-1; NIK, NF-κB-inducing kinase; IKK, IκB kinase; TNF, tumor necrosis factor; MAPK, mitogen-activating protein kinase; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; ERK, extracellular signal-regulated kinase; LPS, lipopolysaccharide; DN, dominant negative; m.o.i., multiplicity of infection; JNK, c-Jun N-terminal kinase; GST, glutathione S-transferase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.
occurs when IKKβ is phosphorylated by mechanisms that appear to involve upstream kinases recruited by IKKy. This phosphorylation triggers intra- and intermolecular trans-phosphorylation of IKK subunits. Much research has been done to identify these upstream kinases. NF-κB-inducing kinase (NIK) and mitogen-activated protein/extracellular signal-regulated kinase 1 are shown to directly interact with and activate IKK (13–15). NIK mediates IKK activation by double-stranded RNA-activated serine-threonine protein kinase (16). Other MAP3ks, such as MEKK2 and MEKK3, are also shown to induce IKK activation (17). Survival signaling is mediated by phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) via NF-κB-dependent and -independent mechanisms in many cell types (18), including macrophages (19). On the other hand, conflicting results are available for the role of PI3-kinase pathway in regulation of NF-κB-dependent inflammatory gene expression. Inhibitors for PI3-kinase are shown to enhance (LPS)-induced expression of inducible nitric-oxide synthase (iNOS) in macrophages (20) and activation of PI3K conversely inhibits iNOS expression in glial cells (21). This negative regulation may be because of interference of LPS-induced MAPK pathways such as extracellular signal-regulated kinase-1/2 (ERK1/2), p38 MAPK, and JNK with PI3-kinase pathway (22). In contrast, opposing results are also available. PI3-kinase is shown to phosphorylate and increase transactivation activity of p65 (23). Overexpression of a constitutively active AKT enhances NF-κB-dependent gene expression via IKK activation in fibroblasts (24), and a dominant negative mutant of the p85 regulatory subunit of PI3-kinase blocks TNF-induced NF-κB-dependent gene expression (25).

The present study has investigated upstream kinases that are required for iron-induced IKK activation in HM. The use of dominant negative mutants (DN) and pharmacological inhibitors for potential upstream effector molecules demonstrated that multiple kinases, including MEK1, NIK, TAK1, and PI3K, are required for ferrous iron to activate IKK in HM. Further, TAK1 PI3K and p21ras are shown to physically interact in caveolae to initiate iron-induced signaling.

**EXPERIMENTAL PROCEDURES**

**Materials**—RAW264.7 cells employed in this study were obtained from the American Tissue Culture Collection (Rockville, MD). Fetal bovine serum, ferrous sulfate, LPS (Escherichia coli 055:B5), filipin III, and phosphatase inhibitor mixture were purchased from Sigma. Adenoviral vectors Ad.DNTAK1, Ad.DNNIK, Ad.DNPI3K expressing DN mutants of TAK1 (K63W), NIK, and PI3K (Δp85) were obtained from Dr. David A. Brenner (Columbia University Medical Center, New York), and Ad.DNp21ras was given by Dr. L. F. Parada (University of Taxes Southwestern Medical Center). Antibodies against p-AKT and p-p44/p42 ERK1/2 were obtained from Cell Signaling Technology Inc. (Beverly, MA). Antibodies against p85α, TAK1, AKT, p21ras, and IKKα/β were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A TNFα enzyme-linked immunosorbent assay kit was purchased from R&D Systems (Minneapolis, MN). [γ-32P]ATP was purchased from PerkinElmer Life Sciences. LY294002, U0126, and SB203580 were obtained from Calbiochem. Pronase was obtained from Roche Applied Science. Nitrocellulose membranes were obtained from Bio-Rad. ECL kit was obtained from Pierce Biotechnology, Inc. Iron chelators 1,2-dimethyl-3-hydroxypropyridin-4-one (L1) and N,N′-bis-2-hydrobenzylethlenediamine-N,N′-diacetic acid were generous gifts from Dr. Gary Brittenham (College of Physicians and Surgeons, Columbia University).

**Cell Preparation**—HM were isolated from male Wistar rats by the Non-parenchymal Liver Cell Core of the Research Center for Alcoholic Liver and Pancreatic Diseases as previously published (4, 9, 11). Briefly, the liver was digested in situ by sequential perfusion with pronase and collagenase, and non-parenchymal liver cells were fractionated by discontinuous gradient ultracentrifugation using arabinogalactan. An HM-enriched fraction was further purified by the adherence method to achieve a final purity exceeding 95%. After 3 days of culture, the cells were treated with LPS (100 or 500 ng/ml) or ferrous sulfate (50 μM) in serum-free Dulbecco’s modified Eagle’s medium in the presence or absence of various inhibitors as described below and subsequently subjected to collection of cell lysate for IKK and TAK1 assays and immunoblot analysis. RAW264.7 cells were cultured in Dulbecco’s modified Eagle’s medium with 25 mM glucose and 10% fetal calf serum.

**IKK and TAK1 Assays**—To assay the activity of IKK or TAK1, HM cultured in a 100-mm dish were treated with ferrous sulfate (50 μM) or LPS (500 ng/ml) for 30 min in the presence and absence of the iron chelators or inhibitors, washed once with phosphate-buffered saline, and lysed with a lysis buffer (20 μM Tris-HCl, pH 7.5, 20 mM NaF, 20 mM β-glycerophosphate, 0.5 mM Na3VO4, 2.5 mM metabisulphite, 5 mM benzamidine, 1 mM EDTA, 0.5 mM EGTA, 10% glycerol and protease inhibitors, 300 mM NaCl, and 1.5% Triton X-100). The lysates were immediately frozen in liquid nitrogen and stored at −80°C until assayed. After infection with an adenoviral vector expressing a dominant negative mutant of TAK1, NIK, PI3K, or p21ras, HM were treated with FeSO4 to test their effects on iron-induced IKK and TAK1 activities. IKK activity was determined as previously described (9). Briefly, IKK was immunoprecipitated with IKKα/β antibodies and protein G-Sepharose. The assay was performed at 30°C for 1 h in a buffer containing 20 mM Tris-HCl, pH 7.5, 20 mM MgCl2, 2 mM dithiothreitol, 20 μM ATP, 2 μg of glutathione S-transferase (GST)-IκBα, and [γ-32P]ATP (0.5 μCi). The reaction was stopped by addition of Laemmli buffer and was resolved by 10% SDS-PAGE followed by transfer onto a nitrocellulose membrane. Phosphate incorporated into GST-IκBα was visualized by analyzing the membrane with a PhosphorImager (Amersham Biosciences). For TAK1 assay, the identical procedure was performed except for the use of anti-TAK1 antibody and GST-MKK6 (Chemico) as a substrate. For the treatment with superoxide dismutase, 5 min before iron treatment superoxide dismutase was added to the HM culture at the concentration of 150 μg/ml.

**Immunoblot Analysis, Co-immunoprecipitation, and Enzyme-linked Immunosorbent Assay**—For immunoblot analysis, cytosolic extracts were prepared as previously reported (9, 11). Proteins were resolved on a 10% SDS-polyacrylamide gel (SDS-PAGE), transferred to a nitrocellulose membrane, incubated with different antibodies, and detected by chemiluminescence.
Iron Activation of IKK 1

For co-immunoprecipitation analysis for TAK1, p21ras, and p85α, cell lysate was first immunoprecipitated with antibody against p21ras (or p85α), the immune complex was resolved on a 10% SDS-PAGE and transferred to a membrane, and the membrane was incubated with anti-TAK1 and p85α (or p21ras). The release of TNFα by iron-treated HM was examined by using a commercially available mouse TNFα enzyme-linked immunosorbent assay kit following the manufacturer’s protocol.

**Immunofluorescent Microscopy**—The HM were washed with phosphate-buffered saline and fixed in cold methanol for 20 min at −20 °C after cultivation and iron/L1 treatment. After three washes the cells were permeabilized by incubation with cold 0.1% Triton X-100 in phosphate-buffered saline for 15 min. After treatment with blocking solution containing 5% nonfat milk for 60 min, the staining was performed by incubation of 1:200 dilution of antibody against p21ras or p85α, the nuclei were stained with 4',6-diamidino-2-phenylindole (0.1 μg/ml) for 2 h at room temperature. The nuclei were stained with 4',6-diamidino-2-phenylindole (0.1 μg/ml) for 10 min after three washes. The cells were viewed using a Nikon microscope equipped with a digital camera. For triple immunostaining, mouse anti-p85α antibody (1:200 dilution), goat anti-TAK1 (1:200 dilution), and rabbit anti-caveolin1 (1:100 dilution) (all from Santa Cruz Biotechnology) were used. As the secondary antibodies, AlexaFluor488-labeled anti-rabbit IgG, fluorescein isothiocyanate-labeled anti-goat IgG, and rhodamine-conjugated anti-mouse IgG were used. The immunofluorescence-stained HM were examined by confocal laser scanning microscopy using a Zeiss LSM 510 equipped with lasers of argon, HeNel, HeNe2, and titanium and the appropriate filters for detection of fluorescence from AlexaFluor488, fluorescein isothiocyanate, or rhodamine.

**RESULTS**

**Iron Chelators Block Iron-induced IKK Activity**—We first validated iron-induced IKK activation in cultured rat HM. As shown in Fig. 1, the treatment with iron sulfate (50 μM) clearly increases IKK activity at 30 min (lane 2). This effect is completely abrogated by pretreatment of the cells with L1 or N,N′-bis-2-hydroxybenzylidenediamine-N,N′-diacetic acid, a bidentate or hexadendate iron chelator following immunoprecipitation of the IKK complex. The lower panel shows equal loading of IKKα by immunoblot analysis.

**Iron-induced IKK activity and this induction is blocked by iron chelators.** Addition of iron sulfate (50 μM) to primary cultures of rat HM induces IKK activity at 30 min (lane 2) as previously shown. The pretreatment of HM with L1 (100 μM) or N,N′-bis-2-hydroxybenzylidenediamine-N,N′-diacetic acid (100 μM) (HBED), a bidentate or hexadendate iron chelator (added 5 min before iron addition), completely prevents iron-induced IKK activity (lanes 3 and 4). IKK activity was assessed by phosphorylation of GSK3 (p-IκBα) following immunoprecipitation of the IKK complex. The lower panel shows equal loading of IKKα by immunoblot analysis.
Iron Induces Phosphorylation of ERK and PI3K—We next examined the relationship between MEK1 and PI3K in iron-induced signaling. Addition of ferrous sulfate induces AKT phosphorylation as effectively as the treatment with LPS, demonstrating PI3K activation by iron (Fig. 3A, lanes 2 and 4). Iron chelator L1 largely blocks this effect (lane 3). AKT phosphorylation is, as expected, prevented with LY294002 (lane 5) but not with U0126 (lane 6). SB203580 has no effect on AKT phosphorylation (lane 7). We also examined ERK1/2 phosphorylation as a downstream target of MEK1. Iron clearly induces ERK1/2 phosphorylation, and this is blocked by the iron chelator. As expected, U0126 blocks ERK phosphorylation (lane 5). LY294002 mildly suppresses iron-induced ERK phosphorylation (lane 5), whereas SB203580 enhances it (lane 7). These results suggest that MEK1 is not involved in iron-induced PI3K activity in HM. They also suggest that iron-induced ERK phosphorylation is enhanced by PI3K but inhibited by p38 MAPK. The latter corroborates the observed enhancement of IKK activity by SB203580 and again may be caused by MEK1 dephosphorylation by p38 MAPK. 

**TAK1 and p21ras Are Involved in Iron-induced PI3K Activity in HM**—NIK has been shown to be downstream of TAK1 in interleukin 1-induced signaling (28). However, the relationship between PI3K and TAK1 in IKK activation is not fully known. Thus, we examined whether PI3K activation is dependent on TAK1 activity in our model of iron-induced IKK activation. To this end, we assessed iron-induced AKT phosphorylation without or with transduction of DNTAK1. AKT phosphorylation is induced by iron in the cells infected with the green fluorescent protein control vector (Fig. 3B, lane 2). Transduction of DNTAK1 prevents this effect as effectively as DNP13K expression (Fig. 3B, lanes 3–6). This result suggests that TAK1 is involved in iron-induced PI3K activity. We also examined the effect of DNP21ras on AKT phosphorylation and IKK activation because this GTPase was previously shown to be activated by reactive free radicals (28–30) and to physically interact with PI3K (31). Infection with a viral vector expressing DNP21ras also blocks AKT phosphorylation (Fig. 3B, lanes 7 and 8) and IKK activation in iron-treated HM (Fig. 3C, lane 4). These data demonstrate that DNp21ras prevents iron-induced PI3K activity as DNPI3K and DNTAK1 do. The PI3K (LY294002) and MEK1 (U0126) inhibitors have no effects on iron-induced TAK1 activity, whereas they abrogate AKT and ERK phosphorylation, respectively. E, addition of superoxide dismutase (150 μg/ml) attenuates iron-induced TAK1 and IKK activities and ERK and AKT phosphorylation.
Iron Activation of IKK 1

collectively suggest that TAK1 and p21ras are involved in iron-induced PI3K activity. Using the same blot, we assessed ERK phosphorylation. As shown in the two lower panels of Fig. 3B, iron-induced ERK phosphorylation is ameliorated by DNPI3K much like the effect of LY294002 (Fig. 3A). DNTAK1 has no effect on iron-induced ERK phosphorylation, but DNp21ras prevents this signaling event. These results suggest that iron-induced MAPK signaling via MEK1/ERK requires p21ras, but not TAK1, and is positively regulated by PI3K. Next, we examined whether iron-induced TAK1 activity is affected by the PI3K or MEK inhibitor. As shown in Fig. 3D, iron induces TAK1 activity as determined by phosphorylation of GST-MMK6 but this induction is not influenced by either LY294002 or U0126, suggesting TAK1 activity is upstream of PI3K and MEK1.

We also tested whether iron-induced TAK and IKK activation and ERK and AKT phosphorylation are dependent on generation of superoxide anion. For this, we added superoxide dismutase to the HM culture. This treatment indeed attenuates iron-induced TAK1 and IKK activities and ERK and AKT phosphorylation (Fig. 3E).

**TAK1 and p21ras Physically Interact with PI3K in Iron-stimulated HM**—In light of the previous report on a physical interaction between p21ras and PI3K (31), our observation of the involvement of TAK1 and p21ras in PI3K activation suggests that TAK1 may also interact with PI3K along with p21ras in iron-stimulated HM. This notion was tested by co-immunoprecipitation. Cell lysate from iron-treated HM was subjected to immunoprecipitation with an anti-p21ras antibody, and the immune complex was resolved on SDS-PAGE and immunoblotted with an antibody against TAK1 or the p85 regulatory subunit of PI3K. As shown in Fig. 4A, iron treatment increases TAK1 association with p21ras (upper panel) and p85α behaves similarly (lower panel). Conversely, immunoprecipitation was performed with an antibody against p85α regulatory subunit and the complex was immunoblotted with an anti-TAK1 or anti-p21ras antibody. Iron indeed increases interactions of TAK1 and p21ras to the p85α regulatory subunit (Fig. 4B). Iron treatment does not alter the level of p21ras or p85α (Fig. 4, A and B, lowest panels). These results demonstrate both TAK1 and p21ras physically interact with PI3K in iron-stimulated HM.

To demonstrate co-localization of p21ras and p85α in iron-treated HM, the cells were immunostained with the respective antibodies for p21ras and PI3K and examined under fluorescence microscopy. As shown in Fig. 5A, p85α and p21ras begin to co-localize in small punctate structures at 5 min after addition of iron. At 15 min, this process is clearly induced and numerous co-localization sites are observed. At 30 min, co-localization staining becomes larger and globular. Pretreatment with L1 abolishes this co-localization phenomenon (data not shown). We then performed triple immunostaining for TAK1, p21ras, and caveolin-1, a marker for caveolae, and examined it under confocal microscopy. This staining indeed reveals co-localization of three proteins following iron treatment (Fig. 5B). These results support physical interactions between TAK1, PI3K, and p21ras and co-localization of these three effectors in caveolae.

**Caveolae Inhibitor Blocks Iron-induced IKK and TAK1 Activities**—Finally, we examined whether inhibition of caveolae formation, which would theoretically prevent recruitment and interactions of TAK1, PI3K, and p21ras, prevents iron-induced TAK1 and IKK activation. For this experiment, we used filipin III, which selectively binds cholesterol and inhibits caveolae formation (32). Indeed, this treatment completely abrogates iron-induced IKK and TAK1 activity (Fig. 6, A and B), suggesting that caveolae facilitate the formation of the functional signaling complex of TAK, p21ras, and PI3K for iron-induced IKK activation. Indeed, iron-induced AKT phosphorylation is also suppressed by filipin III but ERK phosphorylation is only partially inhibited.

**DISCUSSION**

Redox-mediated signal transduction and the roles of free radicals as intracellular messengers have long been recognized (see Ref. 33 for review). One of the most classical examples of the involvement of reactive oxygen or nitrogen species is signaling induced by growth factors. Upon binding of growth factors such as platelet-derived growth factor, epidermal growth factor, and insulin to respective receptors, their intrinsic tyrosine kinase activity increases and recruits via SH-2 containing docking proteins (e.g. Shc), adaptor molecules such as GRB2 pre-associated with a guanine nucleotide exchange factor, SOS. This results in activation of p21ras and the well known Ras-Raf-MAPK pathway. The tyrosine kinase receptor also binds the SH-2 domain in the p85 regulatory subunit of PI3K and activates the PI3K via p21ras. Thus, p21ras serves as the convergent point for the Ras-Raf-MAPK and PI3K-AKT pathways (31). It is this convergent point that reactive oxygen species or reactive nitrogen species were suggested to act to trigger signals. Indeed, our results on iron-induced signaling demonstrate activation of both Ras-MEK1-ERK1/2 and Ras-PI3-AKT pathways with p21ras as a convergent point (Fig. 3A). Further, our study reveals the first evidence for the interactions by TAK1 with...
p21ras and PI3K (Fig. 4, A and B). This physical association is considered to be important because TAK1 plays a role in iron-induced AKT phosphorylation (Fig. 3B) as previously observed for LPS-induced protein kinase B/AKT activation (34). However, TAK1 association with p21ras appears to have no significance in the Ras-MAPK pathway because TAK1 has no role in iron-induced ERK phosphorylation (Fig. 3B) as also shown in LPS-stimulated myeloid cells (34). Although iron-induced AKT phosphorylation is dependent on TAK1 activity (Fig. 3B), TAK1 activity is not affected by PI3K inhibition (Fig. 3D). These results suggest that TAK1 is upstream of PI3K. Our results also demonstrate the involvement of both TAK1 and NIK in iron-induced activation. TAK1 was previously shown to phosphorylate and activate NIK in interleukin 1-induced IKK activation (28). Whether the same signaling cascade takes place in iron-induced IKK activation needs to be addressed in our future study.

Reactive free radicals activate p21ras (29, 30–32), and Cys-118 that is located within the highly conserved GTP-binding site is believed to be a critical site for this redox regulation (35). S-nitrosylation of Cys-118 represents a well known post-translational modification that causes guanine nucleotide exchange and consequent activation of p21ras (29) and recruitment of PI3K (31). S-nitrosylation involves transfer of nitrosonium (NO\(^+\)) to a SH group, and iron catalyzes conversion of NO to NO\(^+\) (36). Because signaling induced by addition of ferrous sulfate is immediate (5–15 min) as judged by co-localization of the effector proteins (Fig. 5A) and activation of IKK (11), NO must be constitutively released to participate in this proposed mechanism. Use of the cells transduced with a C118S p21ras mutant should allow testing the role of Cys-118. Our results also demonstrate the role of superoxide anion (O\(_2^•\)) as superoxide dismutase attenuates iron-induced TAK1 and IKK activities. It is yet to be known whether O\(_2^•\) is a direct effector reactive oxygen species or works with reactive nitrogen species to initiate iron-induced signaling.

Our results also highlight the importance of caveolae as a site of iron-induced signal transduction involving the p21ras/PI3K/TAK1 interactions. Caveolin-1, the marker for caveolae, co-localizes with TAK1 and p85\(_\alpha\) subunit of PI3K (Fig. 5B), and filipin III, an inhibitor for caveolae formation, completely abrogates iron-induced TAK1 and IKK activation (Fig. 6). Caveolae are plasma membrane microdomains that play pivotal roles in cellular signal transduction besides clathrin-independent endocytosis, cholesterol transport, and regulation of calcium flux to name a few (see Ref. 37 for review). This multifunctional organelle is known to house many signaling components, including tyrosine kinase recep-
Iron Activation of IKK 1

A  B

Filipin III (2 μg/ml)  0  Fe (50 μM)

IKK assay:

IB: IKKα

p-IRBβx

IKKα

Filipin III (2 μg/ml)
p-MKK6
IB: TAK1
p-ERK1/2
ERK1/2
p-AKT

AKT

FIGURE 6. Caveolae inhibitor filipin III blocks iron-induced IKK activity. A, filipin III (2 μg/ml, added 5 min before iron addition) blocks iron-induced IKK activity (top panel). Relatively equal levels of IKKα are shown by immunoblot analysis (bottom panel). B, filipin III addition abrogates iron-induced TAK1 activity and AKT phosphorylation and partially suppresses ERK phosphorylation.

In summary, we demonstrate that ionic iron activates IKK in hepatic macrophages through mechanisms involving collective actions of p21ras, PI3K, TAK1, NIK, and MEK1. Iron stimulates interactions of p21ras with PI3K and TAK in caveolae to initiate some of these signals, and p21ras appears to serve as a convergent point for iron-induced activation of both MAPK and PI3K pathways.

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