Original Article

Essential Role for Membrane Lipid Rafts in Interleukin-1β–Induced Nitric Oxide Release From Insulin-Secreting Cells

Potential Regulation by Caveolin-1⁺

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We recently reported that the activation of H-Ras represents one of the signaling steps underlying the interleukin-1β (IL-1β)–mediated metabolic dysfunction of the islet β-cell. In the present study, we examined potential contributory roles of membrane-associated, cholesterol-enriched lipid rafts/caveolae and their constituent proteins (e.g., caveolin-1 [Cav-1]) as potential sites for IL-1β–induced nitric oxide (NO) release in the isolated β-cell. Disruption of lipid rafts (e.g., with cyclodextrin) markedly reduced IL-1β–induced gene expression of inducible NO synthase (iNOS) and NO release from β-cells. Immunologic and confocal microscopic evidence also suggested a transient but significant stimulation of tyrosine phosphorylation of Cav-1 in β-cells briefly (for 15 min) exposed to IL-1β that was markedly attenuated by three structurally distinct inhibitors of protein tyrosine phosphorylation. Overexpression of an inactive mutant of Cav-1 lacking the tyrosine phosphorylation site (Y14F) or an siRNA-mediated Cav-1 knock down also resulted in marked attenuation of IL-1β–induced iNOS gene expression and NO release from these cells, thus further implicating Cav-1 in this signaling cascade. IL-1β treatment also increased (within 20 min) the translocation of H-Ras into lipid rafts. Here we provide the first evidence to suggest that tyrosine phosphorylation of Cav-1 and subsequent interaction among members of the Ras signaling pathway within the membrane lipid microdomains represent early signaling mechanisms of IL-1β in β-cells. ***Diabetes*** **54:2576–2585, 2005**

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Cav-1, caveolin-1; FITC, fluorescein isothiocyanate; β-Gal, β-galactosidase; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; MCD, methyl-β-cyclodextrin; Mes, morpholine-ethanesulfonic acid.

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It is well established that inflammatory cytokines such as interleukin-1β (IL-1β) play a major regulatory role in the selective destruction of insulin-producing β-cells, resulting in the onset of type 1 diabetes (1–3). IL-1β–induced pancreatic β-cell demise is attributed to a large degree to the intracellular generation of nitric oxide (NO), which, in turn, initiates a series of poorly understood signaling steps leading to cell death (4–6). Although such cytotoxic effects of IL-1β have been demonstrated to occur in clonal β-cells, normal rat islets, and human islets, the precise signaling mechanisms involved in IL-1β–induced gene expression of inducible NO synthase (iNOS) and NO release remain unclear.

Along these lines, recent data from our laboratory have suggested potential contributory roles for the activation of H-Ras, a small G-protein, in IL-1β–mediated effects on isolated β-cells. For example, using specific *Clostridial* toxins that specifically monoglucosylate and inactivate the Ras superfamily of GTPases, we demonstrated a marked reduction in IL-1β–induced NO release from β-cells (7). These data were further confirmed through the use of inhibitors of requisite posttranslational farnesylation (i.e., 3-allyl and 3-vinyl farnesols) and palmitoylation (i.e., cerulenin) of Ras (8,9). Additional supporting evidence for the involvement of Ras in IL-1β–induced NO release was obtained through transfection approaches in which we demonstrated that overexpression of the dominant negative mutant of H-Ras (N-17 Ras) in clonal β-cells markedly reduced IL-1β–induced NO release from these cells (8). Taken together, our findings suggest key regulatory roles for H-Ras in the IL-1β signaling cascade, specifically at the level of iNOS gene expression and NO release.

As a logical extension to the above studies, we recently reported evidence to suggest that membrane-associated, cholesterol-enriched caveolae and their key constituent proteins (i.e., caveolin-1 [Cav-1]) may play significant regulatory roles in the IL-1β signaling pathway in isolated β-cells (9). Further, our original findings on the localiza-

tion of Cav-1 in β-cells (9,10) were recently confirmed by Xia et al. (11). The current study further examined the potential roles of membrane lipid rafts, and specifically Cav-1, in IL-1β–induced NO release from isolated β-cells.

Several lines of evidence indicate that Cav-1 undergoes posttranslational modifications, such as tyrosine phosphorylation (at Tyr-14) and fatty acylation (at specific cysteine residues); such modification steps have been suggested to control functional properties of the protein (12–14). Extant data also suggest that the tyrosine phosphorylation of Cav-1 is induced by a variety of stress inducers in NIH 3T3 cells (15) and by insulin in adipocytes (16). It is interesting that the Cav-1 phosphorylation appears to be downstream of mitogen-activated protein kinase and *src* kinase activation in NIH 3T3 cells (15) in contrast to adipocytes, in which the insulin receptor has been shown to directly catalyze the phosphorylation of Cav-1 (16). Published evidence also indicates a potential interplay between Cav-1 and the members of the Ras signaling pathway in membrane-associated, cholesterol-enriched lipid rafts (17). Based on these data supporting potential cross-talk between Cav-1 and Ras proteins in other cells and our published evidence for a role of H-Ras in IL-1β–induced NO release in the β-cell (7–10), we hypothesized that the IL-1β−mediated signaling pathway in β-cells involves tyrosine phosphorylation of Cav-1 and subsequent interaction between Cav-1 and Ras signaling proteins, such as Raf-1 (7). Our hypothesis is also based on data from earlier studies by Corbett and colleagues (18,19) indicating a marked inhibition by tyrosine kinase blockers of IL-1β–induced iNOS gene expression and subsequent NO release in isolated islets (18,19).

To this end, using immunologic as well as confocal and electron microscopic approaches, we have verified the presence of Cav-1 in insulin-secreting cell lines and rat pancreatic islets. In the current study, we demonstrated the significant reduction by tyrosine kinase inhibitors of IL-1β–induced tyrosine phosphorylation of Cav-1 and NO release in cognate cellular preparations. We further verified potential roles of Cav-1 in IL-1β–induced iNOS gene expression and NO release via transfection protocols and siRNA-mediated knock down of Cav-1. Finally, we demonstrated potential targeting of H-Ras into membrane lipid rafts in isolated β-cells by IL-1β under conditions in which it stimulated Cav-1 phosphorylation. Thus, we present experimental evidence of the potential contributory roles of membrane lipid rafts, and specifically Cav-1, in IL-1β signaling steps leading to NO release from the isolated β-cell.

RESEARCH DESIGN AND METHODS

Human recombinant IL-1β was obtained from R&D (Minneapolis, MN). Genistein, herbimycin-A, anti-mouse IgG-fluorescein isothiocyanate (FITC), Griess reagent, morpholine-ethanesulfonic acid (Mes), cholera toxin B subunit, filipin complex, collagenase, Histopaque-1077, and methyl-β-cyclodextrin (MCD) were obtained from Sigma (St. Louis, MO). Tyrphostin and mouse monoclonal α-tubulin antibody were purchased from Calbiochem (La Jolla, CA). Mouse monoclonal phospho-Cav-1 antibody (which recognizes the phosphorylated Tyr-14 site), mouse monoclonal Cav-2 antibody, mouse monoclonal iNOS antibody, and mouse monoclonal Cav-3 antibody were purchased from Transduction (Lexington, KY). Rabbit polyclonal antibodies directed against H-Ras, Cav-1, iNOS, and mouse monoclonal C-myc antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence plus reagent and hyperfilm were obtained from Amersham Pharmacia (Piscataway, NJ). Goat anti-mouse conjugated to rhodamine and goat anti-rabbit conjugated to fluorescein were purchased from Invitrogen (Carlsbad, CA).

Cell culture. HIT-T15 and RIN5F cells were purchased from American Type Culture Collection (Manassas, VA). INS-1 cells were kindly provided by Dr. Chris Newgard (Duke University Medical Center, Durham, NC). HIT, RIN, and INS cells were cultured as previously described (20,21).

Pancreatic islet isolation. Pancreatic islets were isolated from normal male SD rats (200–250 g body wt) by the collagenase digestion method, as previously described (22).

Quantitation of nitrite release. Clonal β-cells (INS or HIT) were incubated in the absence or presence of IL-1β and/or inhibitors and released NO was quantitated as previously reported (7,9).

Detection of native or phosphorylated Cav-1 by confocal immunofluorescence microscopy. HIT-T15 cells plated on microscopic glass coverslips for 48 h were treated in the absence or presence of genistein (100 μmol/l) for 1 h before being stimulated with IL-1β (600 pmol/l) for 15 min. Cells were then washed with PBS, fixed with ice-cold methanol for 20 min at −20°C, and washed three times with PBS. Cells were blocked by incubation with 10% heat-inactivated horse serum for 20 min. They were then incubated with phospho-Cav-1 antibody (1:200) for 1 h at 37°C and subsequently washed with 20 mmol/l Tris-HCl (pH 7.6), 150 mmol/l NaCl, and 0.1% Tween. The final incubation was carried out in a medium consisting of secondary antibody, anti-mouse IgG-FITC (1:200) for 1 h at 37°C. Cells were then washed and visualized under a confocal scanning laser microscope (Zeiss LSM 510), as previously described (9).

Transfection experiments using Cav-1 constructs. Mammalian expression vectors containing full-length cDNA for C-myc−tagged canine Cav-1 (wild type) were generated as previously described (23). An additional Cav-1 mutant (Y14F) was constructed using the Stratagene QuickChange mutagenesis kit in accordance with the manufacturer's instructions. A control siRNA (pSilencer-Control) was generated as a control for vector expression with a nonmammalian 19-nt sequence (5′-GCGCGCTTTGTAGGATTCG-3′). The pSilencer Cav-1 construct was generated by inserting annealed complementary double-strand oligonucleotides encoding 19 nt (5′-GCCCAACAACAAG GCCATG-3′) of canine Cav-1, followed by a loop region (TTCAAGAGA) and then the antisense of the 19 nt. Oligonucleotides were engineered with *Apa* I and *Eco*RI sites at the 5′ and 3′ ends for insertion into the pSilencer 1.0 vector (Ambion, Austin TX). INS-1 cells were subcultured at 70–80% confluence and transfected using Effectene (Qiagen, Valencia, CA), with a maximum 0.8 μg of plasmid DNA constructs (wild-type Cav-1 or Y14F) per well of six-well dishes and 0.4 μg of siRNA (control or Cav-1 siRNA). To estimate the efficiency of transfection, cells were cotransfected with Cav-1 and β-galactosidase (β-Gal) constructs; 24 h later, the transfection level of β-galactosidase expression was detected using the β-Gal staining kit (Invitrogen), according to the manufacturer's recommendations. The total number of cells and cells stained in blue were counted to determine the efficiency of transfection (data not shown). To confirm the expression of the wild-type and mutant forms of Cav-1, lysate proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with Cav-1, C-myc, or α-tubulin antibodies.

Detection of iNOS expression in cells transfected with Cav-1 mutants by confocal immunofluorescence microscopy. INS-1 cells plated on microscopic glass coverslips for 48 h were transfected with 0.8 μg of plasmid C-myc−tagged DNA (wild type or Y14F) per well. Then 24 h after the transfection, cells were treated with diluent alone or IL-1β (600 pmol/l) for an additional 24 h. After this incubation, cells were washed once with PBS fixed with ice-cold methanol for 20 min at −20°C and then washed three times with PBS. The cells were blocked by being incubated for 30 min with 3% heat-inactivated horse serum. They were incubated further with rabbit anti-iNOS polyclonal antibody (1:300) for 1 h, washed, and incubated with mouse anti−C-myc monoclonal antibody (1:500) for an 1 h. After being washed several times with PBS, the cells were incubated for 1 h with an anti-rabbit serum conjugated to FITC to detect iNOS. To detect the Cav-1 mutants, the cells were incubated with anti-mouse conjugated to rhodamine. The incubated cells were then washed and visualized under a confocal scanning laser microscope (Zeiss LSM 510), as previously described (9).

Isolation of lipid rafts from β-cells. Clonal β-cells were washed in PBS and spun at 1,000*g* for 5 min to remove the culture medium. The cellular pellets were then homogenized in Mes-buffered saline (25 mmol/l Mes [pH 6.5], 0.15 mol/l NaCl) containing Triton X-100 and protease inhibitor cocktail. They were then sonicated at 4°C for 20 s in a bath sonicator (3×), mixed with an equal volume of 80% sucrose, and placed into thin-walled centrifuge tubes, layered successively with a 5–30% linear sucrose gradient. The gradients were then subjected to centrifugation at 248,000*g* for 16 h in a Beckman TL-100 ultracentrifuge. The 14 fractions from the top of the gradient (195 μl each) were removed into separate tubes, and protein concentrations were measured using 10 μl from each fraction. Next, 1.2 ml of Mes-buffered saline (25 mmol/l

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Mes [pH 6.5], 150 mmol/l NaCl) was added to the remaining fraction, which was vortexed to dissolve the sucrose and centrifuged for an additional 30 min at 105,000*g*. After being centrifuged, the supernatant was removed and the pellet was reconstituted in 60 μl of Laemmli sample buffer (2×) and analyzed for Cav-1 and H-Ras by Western analysis, as previously described (24). The purity of lipid rafts isolated by sucrose density gradient centrifugation was determined by quantitative measurements by Western blot of relative de-enrichment of Na^+/K^+ ATPase, an integral plasma membrane marker, in the lipid raft fractions.

Protein assay. Protein concentrations were determined by Bradford's dye-binding method (25) using BSA as the standard.

Statistical analysis. The statistical significance of differences between control and experimental groups was determined by Student's *t* test and ANOVA. $P < 0.05$ was considered significant.

RESULTS

Disruption of membrane-associated, cholesterol-enriched lipid rafts results in inhibition of IL-1β–induced iNOS gene expression and NO release in insulin-secreting cells. Cholesterol constitutes a key component of lipid rafts and caveolar structures, and depletion of membrane-associated cholesterol with MCD or filipin results in the disruption of both lipid rafts and caveolae (26,27). Cholera toxin B, on the other hand, selectively binds to ganglioside GM1 within the lipid rafts and disrupts caveolar ultrastructure (28). The data depicted in Fig. 1*A* suggest that exposure of INS-1 cells to MCD, filipin, or cholera toxin B results in complete inhibition of IL-1β–induced NO release. These agents, however, had no significant effect on basal NO release (data not shown). Furthermore, we observed a significant inhibition of IL-1β–induced iNOS gene expression in the INS-1 cells in which lipid rafts were disrupted using MCD (Fig. 1*B*); these data, which are congruous with those in Fig. 1*A*, suggest that the integrity of lipid rafts may be necessary for IL-1β–induced iNOS gene expression and NO release.

Immunologic and microscopic localization of Cav-1 in clonal β-cells and normal rat islets. We next examined the potential role of membrane-associated caveolar structures and their key constituent proteins, such as Cav-1, in the IL-1β signaling pathway in the isolated β-cell. To address this, we first verified the localization of various forms of Cav-1 in islet β-cells. Western blot analyses of lysates derived from normal rat islets and clonal β-cells (HIT-T15 or INS-1) suggested that a protein with an apparent molecular weight of 22 kDa cross-reacted positively with an antibody directed against Cav-1 (Fig. 2*A*). In addition to Cav-1, lysates of rat islets and HIT-T15 cells also contained significant levels of Cav-2 (Fig. 2*B*). In one interesting finding, we noted that normal rat islets, unlike HIT-T15 cells (Fig. 2*B*), are devoid of Cav-3 (data not shown). Compatible with these observations (Fig. 2*A*) were our data in Fig. 3, which further demonstrated the localization of Cav-1 in HIT-T15 cells by electron microscopy. These data indicated localization of Cav-1 on the plasma membrane (Fig. 3, arrow). They suggest localization of Cav-1 in small vesicle-like structures within the β-cell. These data correlate well with data in multiple cell types where localization of Cav-1 has been shown to be associated with caveosomes, which represent intracellular Cav-1–containing, membrane-bound structures (29,30). Taken together, the findings depicted in Figs. 2 and 3 provide convincing evidence for the localization of Cav-1 in isolated β-cells.

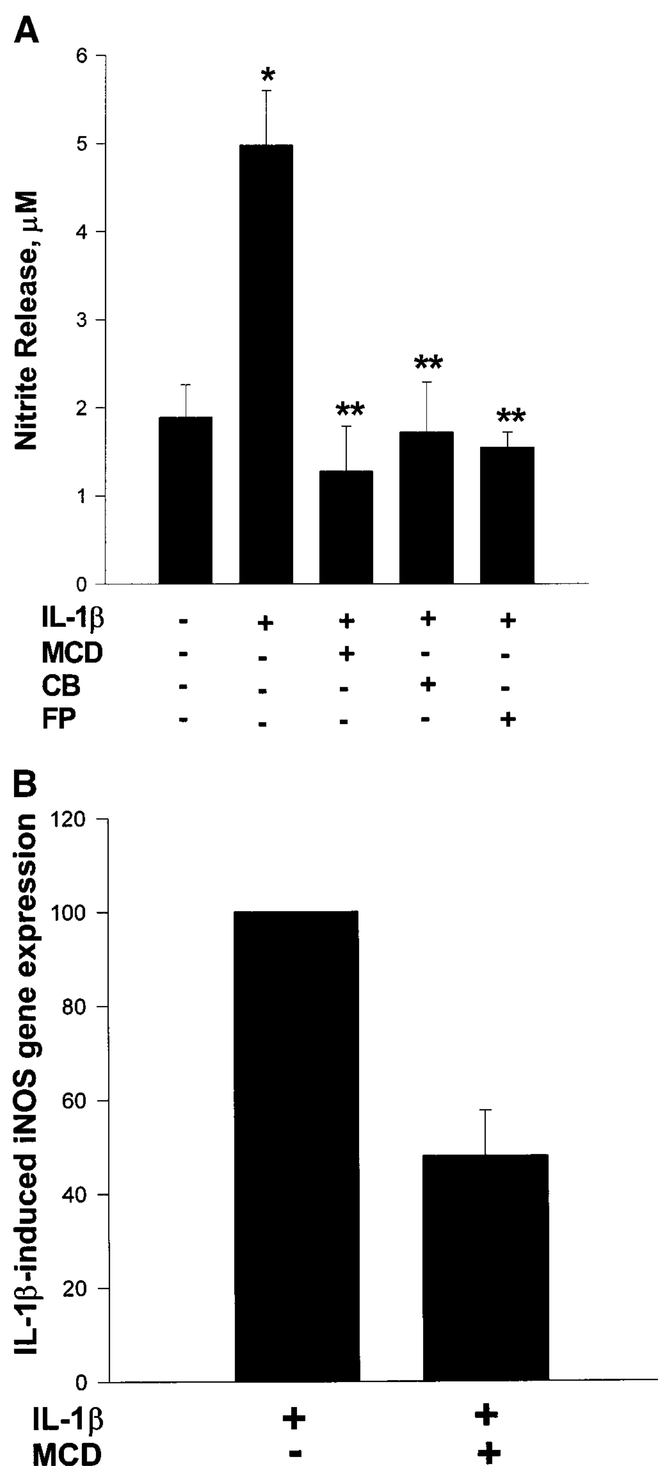

FIG. 1. *A*: Inhibition of IL-1β–induced NO release by cholesterol-depleting agents. INS-1 cells were treated with diluent alone, MCD (10 mmol/l), filipin (FP; 5 μg/ml), or cholera toxin B (CB; 20 μg/ml), as described in RESEARCH DESIGN AND METHODS. Afterwards, IL-1β–induced NO release was measured in the medium using Griess reagent. Data are means ± SE from three independent experiments. *$P < 0.05$ vs. control groups; **$P < 0.05$ vs. IL-1β–treated groups. Not shown are data indicating no demonstrable effects of MCD, CB, or FP on basal NO release. *B*: Inhibition of IL-1β–induced iNOS gene expression by MCD, a cholesterol-depleting agent. Lysate proteins from INS cells treated in the presence of diluents, MCD, or IL-1β (as in Fig. 1*A*) were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to immunoblot analysis with anti-iNOS antibody. Relative intensities of the iNOS bands were quantitated by densitometry and plotted. The intensity of iNOS bands in IL-1β–treated cells was taken as 100%. Data represent means ± variance from two independent experiments.

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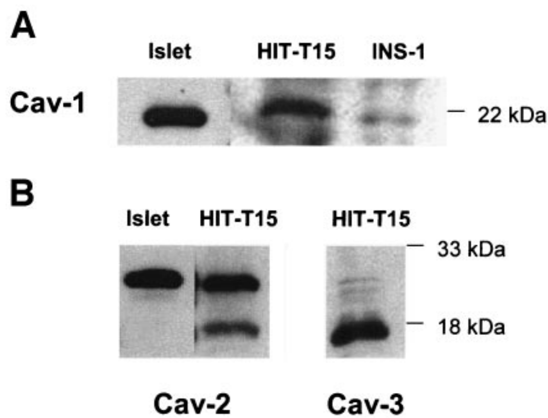

FIG. 2. Immunologic identification of caveolins in clonal β-cells and rat islets. Clonal β-cells (HIT-T15 or INS-1) or pancreatic islets were homogenized in a buffer containing 50 mmol/l Tris (pH 7.4), 150 mmol/l NaCl, 1% NP-40, 1 mmol/l EDTA, 0.25% Na-deoxycholate, 1 mmol/l Na_3V0_4, 1 mmol/l NaF, and protease inhibitor cocktail (Roche, Mannheim, Germany). The homogenates were centrifuged at 1,200*g* for 10 min to remove cell debris. Proteins from rat islets (12 μg protein), HIT-T15 cells (100 μg protein), or INS-1 cells (100 μg protein) (*A*) and protein from normal islets (12 μg protein) or HIT T-15 cells (60 μg protein) (*B*) were separated by SDS-PAGE and the resolved proteins were transferred onto a nitrocellulose membrane. The blots were probed with antibodies directed against Cav-1, -2, and -3 (1:1,000) and then incubated with the secondary antibody conjugated to horseradish peroxidase (1:1,000) for 1 h at room temperature. Immune complexes were detected using an enhanced chemiluminescence kit.

Protein tyrosine phosphorylation of Cav-1 may be required for IL-1β–induced NO release in HIT-T15 cells. Several lines of evidence suggest that Cav-1 undergoes phosphorylation at the Tyr-14 residue (15,16). Furthermore, earlier studies by Corbett and colleagues (18,19) have demonstrated marked attenuation by tyrosine kinase inhibitors of cytokine–induced NO release from isolated islets. Thus, in the next series of experiments, we verified

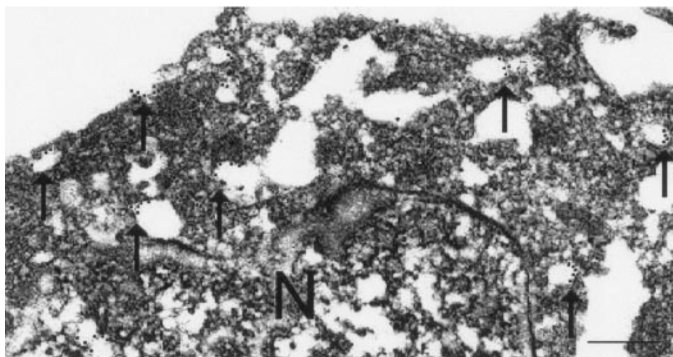

FIG. 3. Localization of Cav-1 in HIT-T15 cells by electron microscopy. Immunolocalization verified the presence of Cav-1 (*arrows*) in β-cells as seen by immunogold labeling. In brief, HIT-T15 cells were fixed in 2.5% paraformaldehyde and 0.15% glutaraldehyde in cacodylate buffer solution for 20 min at 4°C, then extensively washed, dehydrated, and permeabilized with increasing concentrations of LR white resin. Cells were then embedded in LR white resin and polymerized. Ultra-thin sections were sectioned and collected onto 200 mesh nickel grids using an RMC Ultramicrotome and a Diatome diamond knife. Tissue sections were etched using a saturated solution of sodium borohydride for 15 min, then washed extensively with distilled water and blocked using a 5% heat-inactivated goat serum for 1 h. Grids were floated on a rabbit Cav-1 antibody (1:200) solution overnight at 4°C, washed the next morning with PBS, then incubated in a goat anti-rabbit secondary antibody conjugated to 10 nm colloidal gold (1:100) for 2 h at room temperature. Grids were washed with PBS several times, and sections were counterstained using a saturated solution of uranyl acetate and lead citrate. Cav-1 localization was visualized using a Zeiss Transmission electron microscope. N, nucleus. Bar represents ~200 nm.

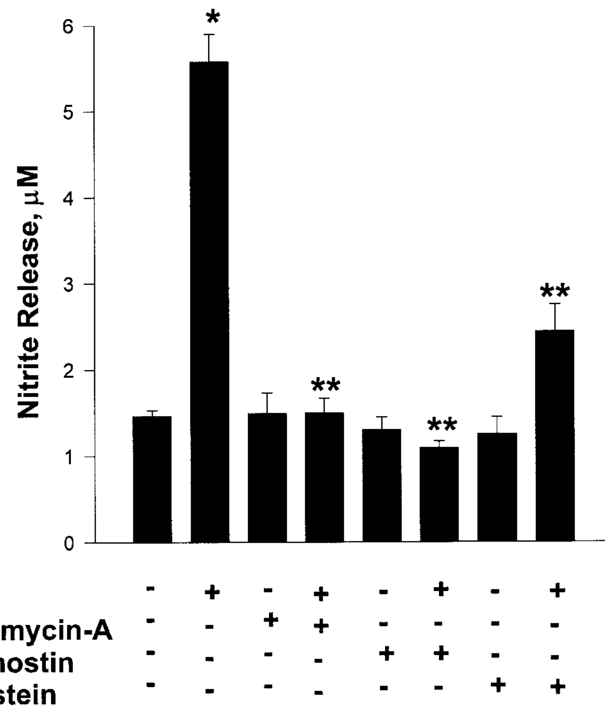

FIG. 4. Inhibition of IL-1β–induced nitrite release from HIT-T15 cells by tyrosine kinase inhibitors. HIT cells were cultured in the absence or presence of genistein (100 μmol/l), herbimycin-A (10 μmol/l), or tyrphostin (100 μmol/l) for 1 h before being stimulated with IL-1β for 24 h. Nitrite release was quantitated using Griess reagent. Data are means ± SE from three independent experiments. *P < 0.05 vs. control groups; **P < 0.05 vs. IL-1β−treated groups.

the putative regulatory roles, if any, of Cav-1 in IL-1β–induced NO release from HIT-T15 cells. We addressed this by examining the potential effects of IL-1β on tyrosine phosphorylation of Cav-1. First, we verified the effects of three structurally dissimilar inhibitors of protein tyrosine phosphorylation (genistein, herbimycin-A, and tyrphostin) on IL-1β–induced NO release. Figure 4 demonstrates that exposure of HIT-T15 cells to these inhibitors had no demonstrable effect on basal NO release from the control cells. However, IL-1β–induced NO release was markedly attenuated by all of these inhibitors (Fig. 4). These data further support our hypothesis that tyrosine phosphorylation of key signaling proteins, including Cav-1, may be necessary for IL-1β–induced effects on NO release.

IL-1β induces tyrosine phosphorylation of Cav-1 in HIT-T15 cells. To further verify the importance of tyrosine phosphorylation of Cav-1 in IL-1β–induced NO release, we assessed the level of phospho-Cav-1 in HIT-T15 cells exposed to IL-1β over time using an antiserum directed against Tyr-14−phosphorylated Cav-1. Maximal phosphorylation of Cav-1 by IL-1β was demonstrable within 15 min and appeared to decrease significantly (>90%) after a 3-h incubation with IL-1β (data not shown). Moreover, as indicated in Fig. 5*A*, we observed that pretreating HIT-T15 cells with genistein (100 μmol/l for 1 h) completely inhibited IL-1β–induced Cav-1 phosphorylation. Under these conditions, the total content of Cav-1 remained unchanged (Fig. 5*A*).

We further verified IL-1β–induced phosphorylation of Cav-1 by confocal microscopy using an antibody directed against phosphotyrosine Cav-1 (as in Fig. 5*A* studies).

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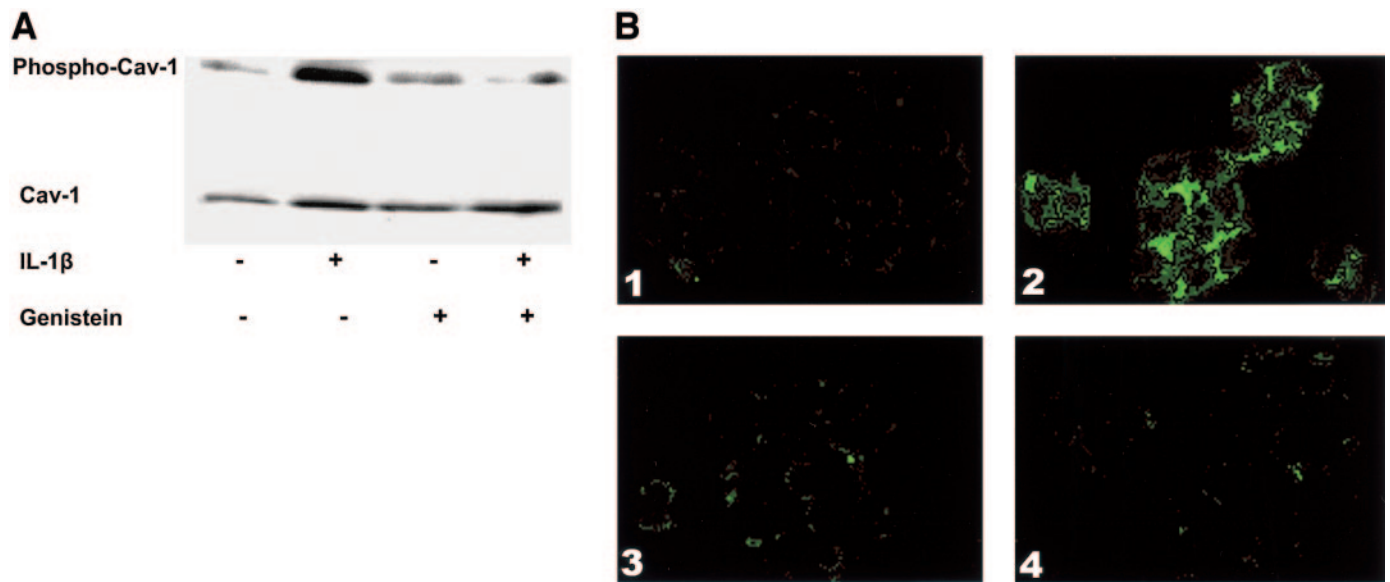

FIG. 5. *A*: Genistein, a tyrosine kinase inhibitor, markedly attenuates IL-1β–induced phosphorylated Cav-1 as determined by Western blot analysis. HIT-T15 cells were pretreated with diluent or genistein (100 μmol/l) for 1 h before being stimulated with IL-1β (600 pmol/l) for 15 min. Lysate proteins were separated by SDS-PAGE and the degrees of abundance of phosphorylated and total Cav-1 were determined by Western blot analysis. Data are representative of two experiments. *B*: Genistein markedly reduces the phosphorylation of Cav-1 as determined by confocal microscopy. HIT-T15 cells were pretreated with genistein (100 μmol/l) for 1 h before stimulation with IL-1β (600 pmol/l) for 15 min. Fixed HIT cells were labeled with mouse monoclonal phospho-Cav-1, then by anti-mouse IgG-FITC. Shown are cells treated with diluent alone (*slide 1*), IL-1β alone (*slide 2*), genistein alone (*slide 3*), or IL-1β plus genistein (*slide 4*). Data are representative of two experiments yielding similar results.

Figure 5*B* indicates that very little phospho-Cav-1 is present in β-cells under basal conditions (*slide 1*). However, a brief exposure (15 min) of HIT-cells to IL-1β resulted in a significant increase in the phosphorylation of Cav-1, as evidenced by the increase in the intensity of phospho-Cav-1 labeling (*slide 2*). Moreover, cotreatment with genistein markedly reduced the labeling of phospho-Cav-1 in these cells (*slide 4*), a result compatible with data in Fig. 5*A*. Genistein by itself had no demonstrable effect on the labeling of Cav-1 in control cells in the absence of IL-1β (*slide 3*). Together, the data in Fig. 5*A* and *B* conclusively demonstrate that Cav-1 undergoes a transient, IL-1β−inducible, genistein-sensitive tyrosine phosphorylation in HIT-T15 cells.

The expression of a Cav-1 mutant lacking Tyr-14 significantly attenuates IL-1β–induced NO release. To further determine the functional consequences of Tyr-14 phosphorylation of Cav-1 on IL-1β−induced NO release, INS-1 cells were transfected with a Cav-1 construct lacking the tyrosine phosphorylation site (Y14F). Figure 6*A* shows comparable degrees of expression of the recombinant Cav-1 constructs in these cells. Although the expression of wild-type Cav-1 had no effect on IL-1β–induced NO release, the overexpression of the Cav-1 mutant (Y14F) resulted in a significant reduction in IL-1β–induced NO release from these cells (Fig. 6*B*). We observed no significant difference in the degree of IL-1β–induced NO release from cells expressing vector alone and cells transfected with wild-type Cav-1 (data not shown). Based on activity determinations of β-Gal (cotransfected with Cav-1 mutants), we estimated that our transient transfection efficiency was 20–30% (data not shown). Therefore, the magnitude of inhibition in cells expressing the recombinant Cav-1 constructs may have been significantly greater than what is shown in Fig. 6*B*. To further substantiate our hypothesis that tyrosine phosphorylation of Cav-1 is necessary for IL-1β–induced iNOS gene expression and NO release, we performed confocal microscopy to determine the levels of IL-1β–induced expression of iNOS in INS cells expressing the wild-type or Y14 F mutant. Figure 6C (*slide A*) shows a significant degree of expression of iNOS (green) in cells treated with IL-1β alone (600 pmol/l for 24 h). These observations were further verified using DAPI (blue), a stain that specifically binds to chromatin material within the nucleus. Figure 6C (*slide B*) shows the expression of wild-type type (C-myc tagged) Cav-1 (red) in control cells. IL-1β–induced expression of iNOS (green) was demonstrable in cells expressing the wild-type Cav-1 mutant (red), as evidenced by the yellow color (*slide C*; cells indicated by arrows). In contrast, very little expression, if any, of iNOS (green) was demonstrable in cells overexpressing the C-myc−tagged Y14F mutant (red) after they were exposed to IL-1β (Fig. 6D, *slides B* and *C*), as evidenced by the lack of yellow color. These data further support our formulation that phosphorylation at Tyr-14 is necessary for IL-1β–induced iNOS gene expression and NO release.

siRNA-mediated knock down of Cav-1 results in inhibition of IL-1β–induced NO release. To further confirm our hypothesis that Cav-1 is important for IL-1β–induced NO release, INS-1 cells were transfected with a control siRNA that does not map to mammalian mRNA or with Cav-1 siRNA. Figure 7*A* depicts a significant reduction (~40%) in the expression of Cav-1 in cells transfected with the Cav-1 siRNA but not the control siRNA. Under these transfection conditions, we also observed a significant reduction in IL-1β–induced NO release (~32%), but only in cells in which Cav-1 was knocked down using Cav-1 siRNA (Fig. 7*B*). These data further implicate Cav-1 in IL-1β

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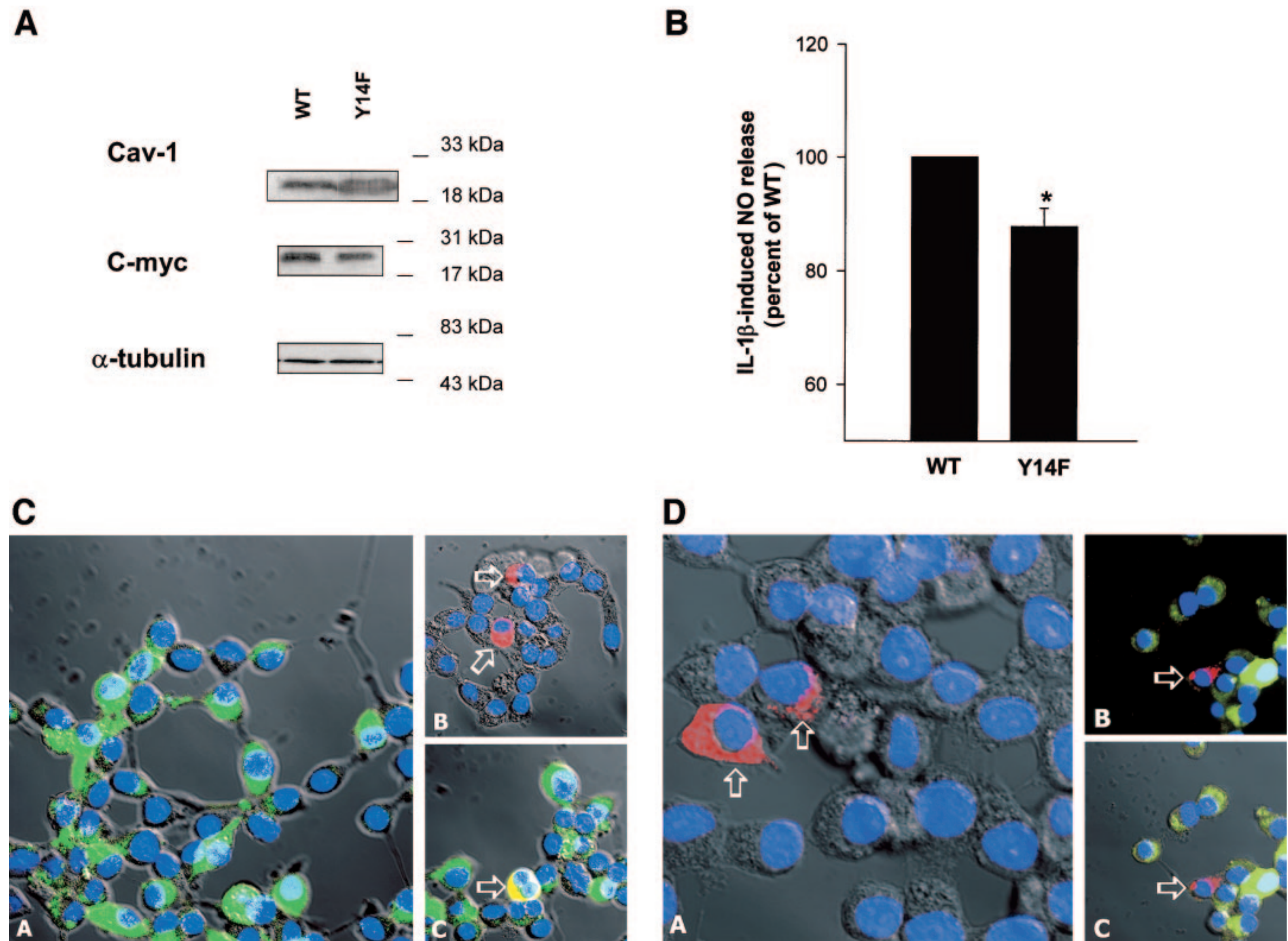

FIG. 6. *A*: Expression profiles of wild-type and inactive mutant of Cav-1 in transfected INS-1 cells. Lysates (30 μg protein) from cells transfected with wild-type Cav-1 construct (WT) or Cav-1 constructs lacking the phosphorylation site (Y14) were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with Cav-1, C-myc, or α-tubulin antibodies. Data are representative of four experiments with comparable results. *B*: Overexpression of Cav-1 mutants lacking the phosphorylation site (Y14) markedly attenuates IL-1β−mediated NO release from INS-1 cells. IL-1β–induced NO release was quantitated in INS-1 cells transfected with the WT Cav-1 construct and Cav-1 constructs lacking the phosphorylation site (Y14), as indicated. The media were collected and NO release was quantitated using Griess reagent. Data are expressed as the percent of total NO released in cells transfected with WT plasmid. Data are means ± SE from six independent experiments. $*P < 0.0022$ vs. cells transfected with wild-type Cav-1 and treated with IL-1β. *C*: Overexpression of WT Cav-1 mutant had no effect on IL-1β–induced iNOS gene expression. INS-1 cells treated with IL-1β (600 pmol/l for 24 h) showed a significant expression of iNOS (green; *slide A*). The expression of WT Cav-1 (red) in control cells (as indicated by arrows) is shown in *slide C*. IL-1β–induced iNOS gene expression is demonstrable in cells (indicated by arrows) expressing WT Cav-1, as evidenced by the yellow color (*slide C*). *D*: Overexpression of Cav-1 mutant lacking the phosphorylation site (Y14F) markedly attenuates IL-1β−mediated iNOS gene expression. Cells expressing Y14F (red) mutant (indicated by arrows) are shown in *slide A*. In *slides B* and *C*, a significant induction of iNOS gene expression in a large population of cells (green), except those expressing Y14F mutant (red alone), is shown.

signaling steps in the β-cell, specifically at the level of NO release.

Because earlier studies have demonstrated (31) localization of specific tyrosine kinases such as the *src* kinase in the caveolar fraction, we examined whether specific inhibitors of this kinase attenuate IL-1β–induced NO release. Data from these studies were inconclusive in that both PP1, a specific inhibitor of *src* kinase, and PP3 (its inactive analog) markedly attenuated IL-1β–induced NO release (data not shown). Together, these findings suggest that *src*-related tyrosine kinases may not mediate tyrosine phosphorylation of Cav-1 and subsequent signaling events leading to NO release.

IL-1β treatment of INS-1 cells increases translocation of H-Ras in the caveolar fraction. We recently reported (7–9) positive modulatory roles for H-Ras in IL-1β–induced iNOS gene expression and NO release from HIT cells. Our data plus data from other studies in multiple cell types (32,33) on the possible interaction of Cav-1 with members of the Ras signaling pathway prompted us to investigate if such interactions also take place in β-cells. Initial pull-down assays suggested a significant association between H-Ras and Cav-1 in HIT-T15 cells (data not shown). In subsequent studies, we examined potential localization of Ras-signaling proteins in the caveolar/lipid raft fractions to determine whether IL-1β treatment results in specific targeting of Cav-1 and Ras signaling proteins into this fraction.

To achieve this, we isolated purified Cav-1−enriched fractions by sucrose-density gradient centrifugation of lysates from insulin-secreting cells (24; see RESEARCH DESIGN AND METHODS for additional details). The relative abundance

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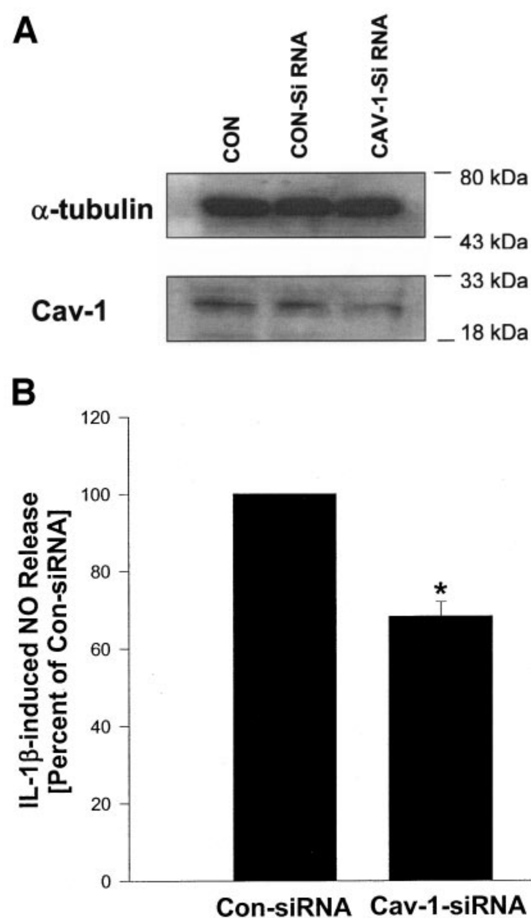

FIG. 7. ***A*: Expression profile of Cav-1 by siRNA-mediated knock down by Cav-1 siRNA. Lysates from untransfected cells (CON) or cells transfected with control (CON-Si RNA) or Cav-1 siRNA were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and probed for Cav-1. Data are representative of two separate experiments with comparable results. *B*: siRNA-mediated knock down of Cav-1 attenuates IL-1β–induced NO release. IL-1β–induced NO release was quantitated in INS-1 cells transfected with control or Cav-1 siRNA, as indicated. The media were collected and NO release was quantitated using Griess reagent. Data are expressed as the percent of total NO released in con-siRNA transfected cells. Data are means ± SE from two independent experiments carried out in replicate. *$P < 0.05$ vs. cells transfected with con-siRNA and treated with IL-1β.**

of Cav-1 was determined in these fractions by Western blotting (Fig. 8*A*). The relative de-enrichment of a nonlipid raft marker (Na^+/K^+ ATPase) in Cav-1−rich fractions was also determined by Western blot analysis (Fig. 8*A*). The distribution profile of Cav-1 and nonlipid markers (e.g., Na^{+}/K^+ ATPase) in these fractions is consistent with published reports (11,34). Figure 8*A* shows the localization of H-Ras in Cav-1−rich fractions.

Our next series of studies was aimed at determining the potential effects of IL-1β treatment on the distribution of Cav-1, H-Ras, and Na^+/K^+ ATPase within the lipid and nonlipid raft fractions. Pooled data from multiple experiments (Fig. 8*B*) indicated a modest but significant translocation of H-Ras (*upper panel*) and Cav-1 (*middle panel*) into lipid raft fractions after INS-1 cells were exposed to IL-1β. In contrast, we observed no significant effects of IL-1β treatment on the distribution of Na^+/K^+ ATPase (*lower panel*) in these fractions. These findings are compatible with the observations of Zhu et al. (35), who reported significant lipoprotein-mediated translocation of Cav-1 and Ras into caveolar fractions derived from human endothelial cells. Taken together, our findings appear to suggest potential IL-1β−mediated targeting of H-Ras and Cav-1 into the lipid rafts, which may be critical for iNOS gene expression and subsequent NO release (7–10).

DISCUSSION

Caveolae are flask-shaped structures that serve as platforms for the interaction between a host of signaling proteins in various cell types (36). Cav-1, which is a key component of caveolae-enriched lipid rafts of the plasma membrane, has been shown to play an important regulatory role in growth factor–induced signal transduction, primarily by interacting with several signaling proteins, including trimeric and monomeric G-proteins, as well as protein and lipid kinases (37–40). Additional cellular functions for caveolae have also been identified, including cholesterol transport (41,42), transcytosis of macromolecules (43,44), and signal transduction (45,46).

To the best of our knowledge, ours was the first study to examine the contributory role of Cav-1 in the IL-1β signaling pathway in the pancreatic β-cell. The salient features of this study are as follows: *1*) the disruption of membrane-associated cholesterol significantly attenuates IL-1β–induced iNOS gene expression and NO release; *2*) IL-1β specifically stimulates tyrosine phosphorylation of Cav-1, which is inhibited by three structurally dissimilar inhibitors of protein tyrosine phosphorylation, inhibitors that concomitantly attenuate IL-1β–induced NO release in cognate β-cell preparations; *3*) the overexpression of Cav-1 mutants lacking the primary tyrosine phosphorylation site results in the inhibition of IL-1β–induced iNOS gene expression and NO release; *4*) siRNA-mediated Cav-1 knock down attenuates IL-1β–induced NO release from these cells; and *5*) IL-1β acutely targets Cav-1 and H-Ras into membrane lipid rafts, an observation we previously showed to be involved in IL-1β–induced NO release. Taken together, these data substantiate a novel regulatory role(s) for Cav-1 in the IL-1β signaling pathway leading to NO release from islet β-cells.

Our findings provide further support to our original hypothesis that IL-1β–induced NO release involves tyrosine phosphorylation of Cav-1, which in turn could initiate its interaction with the Ras/Raf-1 signaling cascade, leading to the activation of signaling machinery required for iNOS expression and NO release (9,47,48). Our data are compatible with the original observations of Song et al. (17), who reported copurification and direct interaction of Ras with Cav-1 in MDCK cells. Using mutational analysis, those authors found that the Ras binding domain of Cav-1 is localized within the 41–amino acid membrane proximal region of the cytosolic NH_2-terminal domain of Cav-1. They further demonstrated that the interaction between these two proteins was highly specific. Those findings provide additional support to our current observations demonstrating potential colocalization of Cav-1 with H-Ras in isolated β-cells, specifically in the caveolar fraction.

Our data indicate that IL-1β–induced tyrosine phosphorylation of Cav-1 and NO release from these cells were attenuated to a significant degree by treating these cells with genistein. These data are compatible with the earlier

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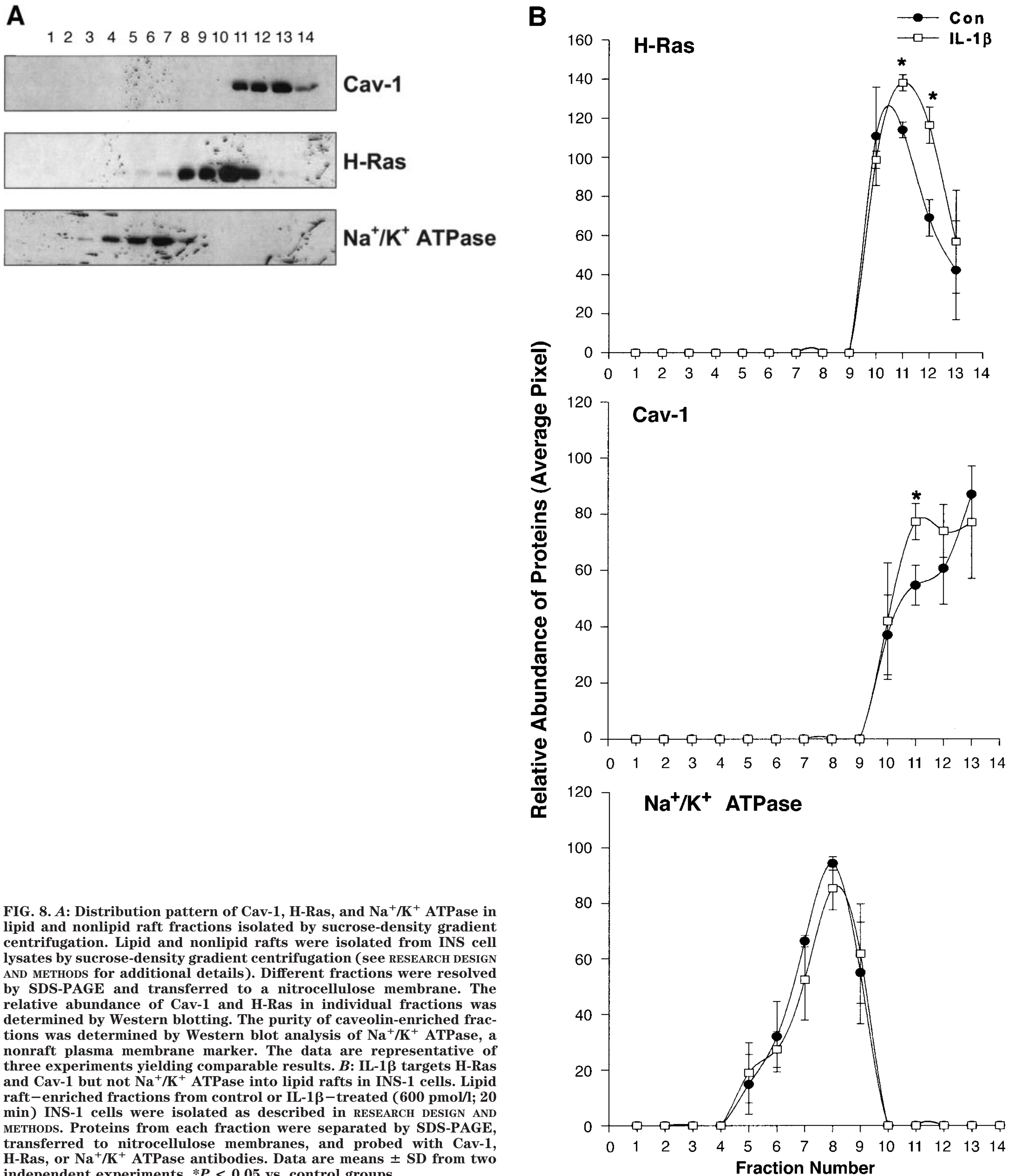

FIG. 8. *A*: Distribution pattern of Cav-1, H-Ras, and Na^+/K^+ ATPase in lipid and nonlipid raft fractions isolated by sucrose-density gradient centrifugation. Lipid and nonlipid rafts were isolated from INS cell lysates by sucrose-density gradient centrifugation (see RESEARCH DESIGN AND METHODS for additional details). Different fractions were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The relative abundance of Cav-1 and H-Ras in individual fractions was determined by Western blotting. The purity of caveolin-enriched fractions was determined by Western blot analysis of Na^+/K^+ ATPase, a nonraft plasma membrane marker. The data are representative of three experiments yielding comparable results. *B*: IL-1β targets H-Ras and Cav-1 but not Na^+/K^+ ATPase into lipid rafts in INS-1 cells. Lipid raft−enriched fractions from control or IL-1β−treated (600 pmol/l; 20 min) INS-1 cells were isolated as described in RESEARCH DESIGN AND METHODS. Proteins from each fraction were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with Cav-1, H-Ras, or Na^+/K^+ ATPase antibodies. Data are means ± SD from two independent experiments. *$P < 0.05$ vs. control groups.

observations of Corbett et al. (19), who demonstrated significant inhibition of iNOS gene expression and subsequent NO release by tyrosine kinase inhibitors in human islets. In addition to genistein, marked inhibition of IL-1β–induced NO release was also demonstrable in the presence of other inhibitors of tyrosine kinases, such as tyrphostin and herbimycin. These pharmacological data were further confirmed via molecular biological analyses. Our data from the expression of the Cav-1 mutant devoid of the tyrosine phosphorylation site also demonstrated an inhibition of IL-1β–induced iNOS gene expression and NO release, thus further suggesting a role for Cav-1 in this

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signaling cascade. Our data from siRNA-mediated knock down of Cav-1 also demonstrated an inhibition of IL-1β–induced NO release. These observations therefore implicate Cav-1 in the IL-1β-signaling pathway, specifically at the level of NO release from the islet β-cell.

The Cav-1 phosphorylation step has been implicated in cellular function and regulation in NIH 3T3 cells (15) exposed to a variety of stress conditions and in 3T3L1 adipocytes exposed to insulin (16). It is interesting that insulin-sensitive tyrosine phosphorylation of Cav-1 was not blocked by inhibitors of either mitogen-activated protein kinase or phosphatidylinositol 3-kinase. Furthermore, insulin-mediated phosphorylation of Cav-1 was resistant to inhibitors of Fyn, a member of the *src* family of kinases. Based on these data, these investigators (16) concluded that the insulin receptor directly catalyzes the phosphorylation of Cav-1. Along these lines, preliminary data from our laboratory (see RESULTS) appear to rule out the possibility that *src*-related kinases may not be involved in IL-1β−mediated tyrosine phosphorylation of Cav-1. A recent report by Sanguinetti and Mastick (49) identified c-Abl kinase as the putative kinase mediating oxidative stress−induced phosphorylation of Cav-1 at the Tyr-14 residue. Additional studies are needed to identify and characterize the putative IL-1β−sensitive tyrosine kinase that mediates the phosphorylation of Cav-1 in the β-cell.

A number of studies have demonstrated the localization and potential cross-talk among various signaling proteins in the lipid rafts (37–40), including Raf-1 (50). In this context, studies by Mineo et al. (32) have demonstrated the localization of H-Ras/Raf-1 in the caveolar fraction after stimulation with epidermal growth factor. Others (51) have also demonstrated IGF-mediated tyrosine phosphorylation and targeting of Cav-1 into lipid rafts. Studies by Zhu et al. (35) have demonstrated that lipoproteins promote the translocation of Cav-1 and H-Ras into the caveolae. Compatible with these studies are our findings, which indicate a significant translocation of H-Ras and Cav-1 into Cav-1−rich compartments from isolated β-cells after brief exposure to IL-1β. These data indicate potential cross-talk between H-Ras and Cav-1 in the IL-1β−mediated signaling cascade, leading to iNOS gene expression and NO release. Further studies will need to verify whether posttranslational phosphorylation and/or fatty acylation of Cav-1 are required for optimal interaction of Ras/Raf-1 signaling proteins with Cav-1.

Thus, based on extant data and the current findings from our laboratory, we propose that IL-1β–induced iNOS gene expression and subsequent NO release involves tyrosine phosphorylation of Cav-1 and subsequent interaction among members of the Ras signaling pathway within the membrane lipid microdomains. We also propose that such an interaction between Ras and its signaling proteins leads to activation of nuclear transcription factors, such as nuclear factor-κB, which might be required for the induction of the iNOS gene and the accompanying release of NO.

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