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Novel roles for palmitoylation of Ras in IL-1β-induced nitric oxide release and caspase 3 activation in insulin-secreting β cells☆

Hai-Qing Chen, Marie Tannous, Rajakrishnan Veluthakal, Rajesh Amin, Anjaneyulu Kowluru*

Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University and β Cell Biochemistry Research Laboratory, John D. Dingell VA Medical Center, Detroit, MI 48201, USA

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Abstract

We recently demonstrated that functional inactivation of H-Ras results in significant reduction in interleukin 1β (IL-1β)-mediated effects on isolated β cells. Since palmitoylation of Ras has been implicated in its membrane targeting, we examined the contributory roles of palmitoylation of Ras in IL-1β-induced nitric oxide (NO) release and subsequent activation of caspases. Preincubation of HIT-T15 or INS-1 cells with cerulenin (CER, 134 μM; 3 hr), an inhibitor of protein palmitoylation, significantly reduced (−95%) IL-1β-induced NO release from these cells. 2-Bromopalmitate, a structurally distinct inhibitor of protein palmitoylation, but not 2-hydroxymyristic acid, an inhibitor of protein myristoylation, also reduced (−67%) IL-1β-induced NO release from HIT cells. IL-induced inducible nitric oxide synthase gene expression was markedly attenuated by CER. Further, CER markedly reduced incorporation of [^{3}H]palmitate into H-Ras and caused significant accumulation of Ras in the cytosolic fraction. CER-treatment also prevented IL-1β-induced activation of caspase 3 in these cells. Moreover, *N*-monomethyl-L-arginine, a known inhibitor of inducible nitric oxide synthase, markedly inhibited IL-induced activation of caspase 3, thus establishing a link between IL-induced NO release and caspase 3 activation. Depletion of membrane-bound cholesterol using methyl-β-cyclodextrin, which also disrupts caveolar organization within the plasma membrane, abolished IL-1β-induced NO release suggesting that IL-1β-mediated Ras-dependent signaling in these cells involves the intermediacy of caveolae and their key constituents (e.g. caveolin-1) in isolated β cells. Confocal light microscopic evidence indicated significant colocalization of Ras with caveolin-1. Taken together, our data provide the first evidence to indicate that palmitoylation of Ras is essential for IL-1β-induced cytotoxic effects on the islet β cell.

Keywords: Ras; Palmitoylation; IL-1β; Nitric oxide; Cerulenin; Insulin-dependent diabetes mellitus; Caspase 3

1. Introduction

The majority of low molecular-mass guanine nucleotide-binding regulatory proteins (G-proteins) and the γ subunits of trimeric G-proteins undergo post-translational modifications at their C-terminal cysteine residue [1–4]. Such modifications include, incorporation of a 15-carbon (i.e. farnesyl group) or a 20-carbon (i.e. geranylgeranyl group)-derivative of mevalonic acid. Using lovastatin, an inhibitor of the biosynthesis of mevalonic acid from HMG-CoA, and hence isoprenoids, we and others have demonstrated that protein isoprenylation plays a critical regulatory role in physiologic insulin secretion [5,6]. Recent studies from our laboratory have confirmed this formulation through the use of novel inhibitors of protein prenylation [7]. For example, using specific inhibitors of protein isoprenylation (e.g. 3-allyl or 3-vinyl farnesols and geranylgeraniols), we have demonstrated a marked attenuation in glucose- and calcium-induced insulin secretion from insulin-secreting βTC3 cells [7]. Moreover, using the farnesyltransferase inhibitors (e.g. 3-allyl or 3-vinyl farnesols), we reported marked inhibition of IL-1β-induced NO release from clonal β cells [7]. Taken together, these data indicated positive as well as

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* Corresponding author. Tel.: +1-313-576-4478; fax: +1-313-576-1112.
E-mail address: akowluru@med.wayne.edu (A. Kowluru).

Abbreviations: IL-1β, interleukin 1β; NO, nitric oxide; CER, cerulenin; G-proteins, guanine nucleotide-binding regulatory proteins; SAM, *S*-adenosyl-L-[methyl-^{3}H]methionine; Cav-1, caveolin-1; mβcd, methyl-β-cyclodextrin; L-NMMA, *N*-monomethyl-L-arginine.

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negative modulatory roles for protein prenylation in β cell function.

Besides isoprenylation, certain small G-proteins (e.g. H-Ras) undergo additional modifications at cysteine residues, which are upstream to the C-terminal cysteine. Such modifications include, incorporation of fatty acyl CoA-derivatives (e.g. palmitoyl-CoA) *via* a thioester linkage [8–10]. Several previous studies have demonstrated [8–12] that protein palmitoylation is acutely regulated by the concerted actions of palmitoyltransferases and palmitoylesterases, which subserve the functions of incorporation and deletion of palmitic acid from the palmitoylated proteins, respectively. While the exact functional significance of protein palmitoylation remains to be defined, convincing recent data indicate that this post-translational modification step renders ''firm anchoring'' of substrate proteins into their target membrane sites for optimal interaction with their respective effector proteins [8–12].

Using specific inhibitors of protein palmitoylation (e.g. CER), several groups, including our own, have identified regulatory roles for these reactions in normal β cell function. For example, it has been shown that CER inhibits nutrient-induced insulin secretion from normal rat islets [5,13,14]. Using clonal β cell lines, Deeney *et al*. [15] have demonstrated inhibition by CER of palmitoyl CoA-mediated insulin secretion as well as fusion of secretory granules with plasma membrane in isolated β cells. Together, these data indicate key functional roles for protein palmitoylation in normal β cell function. In the context of protein palmitoylation, extant studies in multiple cell types, have demonstrated that H-Ras undergoes palmitoylation [16–18], which facilitates anchoring of the latter into caveolar microdomains of the plasma membrane [16]. Using immuno-histochemical and electron microscopic techniques, we have recently demonstrated localization of caveolin-1 (Cav-1), a key constituent of caveolar invaginations of the plasma membrane, in HIT cells.[1] We also observed that exposure of isolated β cells to IL-1β resulted in transient phosphorylation of Cav-1 at a tyrosine residue, and that specific inhibitors of protein tyrosine phosphorylation (e.g. genistein), markedly attenuated IL-1β-induced tyrosine phosphorylation of Cav-1 and subsequent NO release from HIT cells (see footnote 1). Our recent data, together with observations from several laboratories [19–21] prompted us to examine, the putative roles of these protein interactions in the sequence of events leading to IL-1β-induced NO release, in isolated β cells.

In the present study, we have examined contributory roles of protein palmitoylation, specifically that of H-Ras, in IL-1β-mediated release of NO and caspase activation in INS and HIT cells. Our data indicate a marked attenuation by CER or 2-bromopalmitate, two inhibitors of protein palmitoylation, of IL-1β-induced NO release from HIT and INS cells. We also demonstrate that CER-treatment of either of these cells normalizes IL-1β-induced activation of caspase 3. Lastly, we provide the first evidence to suggest that depletion of membrane-bound cholesterol, and hence the caveolar organization, prevents IL-1β-induced NO release suggesting critical regulatory roles for caveolar integrity, and possibly, Cav-1/Ras interactions in this signaling step. Based on these findings, we propose a model for the putative involvement of Ras in IL-1β-induced dysfunction and demise of the pancreatic β cells.

[1] M. Tannous, R. Veluthakal, R. Amin, A. Kowluru. Endocrinology (2003), revised manuscript in preparation.

2. Materials and methods

2.1. Materials

CER, Griess reagent, 2-hydroxymyristic acid, 2-bromopalmitic acid, *N*-monomethyl-L-arginine (L-NMMA) and methyl-β-cyclodextrin (mβcd) were purchased from Sigma. IL-1β was obtained from R&D Systems. Antisera directed against H-Ras and Cav-1 were purchased from Santa Cruz Biotechnology and Transduction Laboratories, respectively. [^{14}C]Palmitate, [^{14}C]arachidonate, [^{3}H]palmitate and *S*-adenosyl-L-[methyl-^{3}H]methionine (SAM) were purchased from NEN Life Sciences. *N*-Acetyl-*S*-*trans*,*trans*-farnesyl cysteine (AFC) was purchased from Biomol Research Labs Inc. Caspase assay kit was purchased from BioVision Research Products. All other reagents were of analytical grade and highest purity available.

2.2. Insulin-secreting cells and incubation conditions

INS and HIT cells were cultured in 24-well plates for 2 days prior to start of the study. They were incubated in the presence of different concentrations of inhibitors, or their respective diluents, as indicated in the text. The medium was collected after 24 hr of incubation and centrifuged at 1000 *g* for 5 min. Equal volumes of medium and the Griess reagent were mixed and the absorbance was measured at 540 nm [22]. To verify potential non-specific and cytotoxic effects of CER on β cells during our culture conditions, we quantitated total protein as well as nitrite release from HIT-T15 cells incubated in the presence of diluent alone or CER (67 or 134 μM). Data from these studies indicated no significant differences in the protein concentrations (taken as an index of no potential loss of β cells under the experimental conditions employed in the current study). The corresponding values (expressed as the amount of nitrite released per microgram of protein) are: 0.0162 ± 0.0014 ($N = 3$; diluent alone), 0.0161 ± 0.0021 ($N = 3$; 67 μM CER; $P = 0.99$ vs. control; not significant), 0.0178 ± 0.0033 ($N = 3$; 134 μM CER; $P = 0.6633$ vs. control; not significant). In addition, our estimates of metabolic cell viability using an MTT assay yielded no major differences between the control and CER-treated cells under conditions CER inhibited IL-induced NO

release (additional data not shown). Taken together, the assay conditions we used to determine the CER on IL-induced inducible nitric oxide synthase (iNOS) expression, NO release and caspase activation are free of any non-specific and cytotoxic effects of the compound. To further rule out such a possibility, we also examined the effects of 2-bromopalmitate, a structurally distinct inhibitor of protein palmitoylation on IL-induced NO release (see below).

2.3. Isolation of membrane and soluble fractions from β cells

Total membrane and soluble fractions were isolated from β cell homogenates by a single step centrifugation at 105,000 *g* for 60 min, using a Beckman TL-100 centrifuge as we described earlier [23–25].

2.4. Cellular labeling with [^{3}H]palmitate

Cells were cultured in 6-well plates and preincubated in the absence or presence of CER (134 μM; 3 hr) to facilitate its entry into the cells following which they were incubated with [^{3}H]palmitate (100 μCi/mL) for an additional 3 hr in normal culture medium consisting of cycloheximide (10 μg/mL). The cells were washed quickly (5×) in cold-PBS containing unlabeled palmitate and resuspended in radio-immunoprecipitation assay buffer. The lysates were pre-cleared with preimmune serum and protein G-agarose. The supernatants were incubated overnight at 4° with anti-H-Ras. Immune complexes were precipitated using protein G-agarose, and proteins in the immunoprecipitates were separated by SDS–PAGE. Gels were soaked in ENHANCE[2] (Amersham) as per the manufacturers' instructions, dried and exposed to an X-ray film for 3 weeks at −70° [23]. Protein concentrations in the samples were quantitated using a dye-binding assay [23–25].

2.5. Colocalization of Cav-1 and Ras by confocal light microscopy

HIT-T15 cells, plated on microscopic glass cover slips for 48 hr, were washed with PBS, fixed with ice-cold methanol for 20 min at −20°, followed by three washes with PBS. Cells were blocked by incubation for 20 min with 1% BSA plus 5% non-fat dry milk in PBS. Cells were then incubated with anti-H Ras antibody (1:200) for 1 hr at 37° followed by anti-mouse secondary antibody conjugated to FITC (1:200) for 1 hr at 37°. Cells were washed three times (5 min each) with 0.1% TBST and then probed with anti-Cav-1 antibody (1:200) for 1 hr at 37° and subsequently washed with 0.1% TBST. Final incubation was carried out in a medium consisting of anti-rabbit secondary antibody conjugated to rhodamine at a dilution of 1:200 for 1 hr at 37°. Cells were washed and visualized under a confocal light microscope (Zeiss LSM 510).

2.6. Quantitation of caspase 3 activity

Caspase 3 activity was assayed using a colorimetric assay kit according to the manufacturer's instructions. In brief, cells were incubated in the absence or presence of CER (134 μM for 3 hr). Following the incubation, the medium was replaced with fresh medium in the presence or absence of IL-1β (300 pM) and/or L-NMMA (500 μM) as indicated in the text. Incubation was continued for an additional 48 hr at which time point the cells were harvested. Using 300 μg of protein from cell lysate, caspase activity was quantitated by measuring proteolytic cleavage of chromogenic substrates for caspase 3 (Ac-DEVD-pNA) at 405 nm using a microtiter plate reader.

2.7. Immunoblot analysis of iNOS

HIT-T15 cells were preincubated in the absence or presence of CER (134 μM; 3 hr) prior to stimulation with IL-1β (600 pM) for an additional 24 hr. Proteins were separated by SDS–PAGE (12%) and the resolved proteins were transferred onto a nitrocellulose membrane. Blots were probed with anti-iNOS antibody (1:1000 dilution) then incubated with secondary antibody conjugated to HRP (1:1000 dilution). Immune-complexes were detected using ECL kit.

2.8. Quantitation of carboxyl methylation of Ras

To determine the degree of Ras carboxyl methylation, INS cell lysate proteins (60 μg) were incubated at 37° for 60 min in 50 mM sodium phosphate buffer, pH 6.8, containing 1 mM EGTA and SAM (100 μCi/mL; 100 μL total volume), in the presence or absence of AFC (100 μM). Proteins were extracted using a medium containing 20 mM HEPES, pH 7.4, 1% sodium cholate, 2 mM $MgCl_2$, and protease inhibitor cocktail. The contents of the tubes were sonicated (3 × 10 s) using a bath sonicator, and the tubes were rotated gently at 4° for 2 hr. Prior to the immunoprecipitation using anti H-Ras antibody, cholate extracts were precleared with serum and Pansorbin (Calbiochem). The immune complexes were captured by centrifugation, and the pellets were washed with Tris-buffer saline (3×) and the base labile [^{3}H]methanol release (an index of the degree of carboxyl methylation) was quantitated by vapor phase equilibration assay as we described in Refs. [26–28].

2.9. Effect of cholesterol depletion on IL-1β-induced NO release from HIT cells

This was carried out using mβcd according to published methods [29]. In brief, HIT cells were grown in 24-well

[2] R. Veluthakal, R. Amin, M. Tannous, A. Kowluru. Diabetes (2003), submitted.

plates and then pretreated with different concentrations (0–10 mM) of mβcd (2 hr) in serum-free media and then treated without or with IL-1β (600 pM) in fresh media for 24 hr. The media were collected after 24 hr and centrifuged at 1000 *g* for 5 min. Equal volumes of medium and the Griess reagent were mixed and the absorbance was measured at 540 nm.

2.10. Statistical analysis of the data

Where appropriate, data are expressed as mean ± SEM. Significance of difference between the control and experimental groups was determined using *t*-test and *P*-value <0.05 was considered significant.

3. Results

3.1. Inhibitors of protein acylation markedly attenuate IL-1β-induced NO release from insulin-secreting INS-1 and HIT-T15 cells

Data shown in Fig. 1 (panel A) indicate that preincubation of INS-1 cells with CER (0–134 μM) for as little as 60 min resulted in significant attenuation in IL-1β-induced NO release. Maximal inhibition (<95%) was demonstrable in the presence of 134 μM CER following 3 hr incubation. Data shown in Fig. 1 (panel B), which are pooled from multiple experiments utilizing INS-1 cells, demonstrate significant inhibition by CER (134 μM) of IL-1β-induced NO release. Likewise, CER-pretreatment caused substantial inhibition of IL-1β-induced NO release from HIT-T15 cells (Fig. 1; panel C). Taken together, these data indicate that similar CER-sensitive mechanisms are operable in HIT and INS cells for the generation of NO induced by IL-1β.

In addition to CER, 2-bromopalmitate, a structurally distinct inhibitor of protein palmitoylation [30], markedly attenuated IL-1β-induced NO release from HIT cells. In these experiments, 2-bromopalmitate (100 μM; overnight) reduced IL-1β-induced (600 pM) NO release by 67% (8.8 ± 0.6 μM NO released in the presence of IL-1β vs. 3.0 ± 0.16 μM in the presence of IL-1β plus 2-bromopalmitate; N = 8 determinations in each case). In contrast, 2-hydroxymyristic acid (100 μM; overnight), an inhibitor of protein myristoylation [31], had no demonstrable effect on IL-1β-induced NO release. These values represented 8.8 ± 0.6 μM NO released in the presence of IL-1β alone vs. 11.24 ± 0.32 μM of NO released in the presence of IL-1β plus 2-hydroxymyristic acid (N = 8 determinations in each case). In the next series of experiments, we examined whether CER exerts a direct effect on the induction of the iNOS gene. Data shown in Fig. 2 demonstrate marked inhibition of IL-1β-induced iNOS gene expression by CER (Fig. 2). Taken together, these data (Figs. 1 and 2) suggest that palmitoylation (a post-translational modification), but not myristoylation (a co-translational modification) of proteins, may be required for IL-β1-induced NO release from these cells, and that CER-mediated inhibition of IL-β1-induced NO release is due to its inhibitory effects on IL-β1-induced iNOS gene expression.

3.2. Ras undergoes CER-sensitive palmitoylation in insulin-secreting cells

We have recently demonstrated that activation of Ras is essential for IL-1β-induced NO release from insulin-secreting cells [22], and since it was demonstrated that Ras undergoes palmitoylation in other cell types [32–34], we examined whether Ras is palmitoylated in the β cell, and if so, whether CER-mediated inhibition of NO release (Fig. 1; panels A–C) could, in part, be due to inhibition of palmitoylation of Ras. In this context, several earlier studies [9,10,32–34] have utilized [^{3}H] or [^{14}C]palmitate to study palmitoylation of proteins, although arachidonate and other fatty acids have also been shown to get incorporated into cellular proteins under similar conditions [35]. In this context, we observed that incubation of INS cells with either [^{14}C]arachidonate or [^{14}C]palmitate resulted in incorporation of these fatty acids into several β cell proteins, including those corresponding to the sizes of the α-subunits of trimeric G-proteins (37–42 kDa) as well as those corresponding to low molecular mass G-proteins (19–24 kDa). These data also indicated that palmitate is incorporated to a much larger degree as compared to arachidonate (additional data not shown). To determine whether Ras is palmitoylated in the β cell, HIT-T15 cells were labeled with [^{3}H]palmitate following preincubation in the presence of diluent or CER (134 μM; see Section 2). Labeled proteins were separated by SDS–PAGE and identified by autoradiography. These data (Fig. 3A) suggested that a protein in the molecular weight region of 19–22 kDa (corresponding to the size of Ras) significantly incorporated the label and CER (134 μM)-treatment markedly reduced the labeling of this protein. Further, CER markedly inhibited the incorporation of [^{3}H]palmitate into Ras, measured as the amount of radioactivity (as determined by autoflurography) in the anti-Ras immunoprecipitates (Fig. 3B). Additional immunoprecipitation experiments revealed that significant amount of radioactivity was incorporated into immunoprecipitates derived from lysates incubated with anti-Ras (Fig. 3C); findings compatible with those described in Fig. 3 (panels A and B). Taken together, our findings described in Fig. 3 suggest that H-Ras undergoes CER-sensitive palmitoylation in β cells.

3.3. Inhibition of palmitoylation results in altered cellular distribution of Ras

We next examined if inhibition of palmitoylation of Ras influences the subcellular localization (i.e. its relative

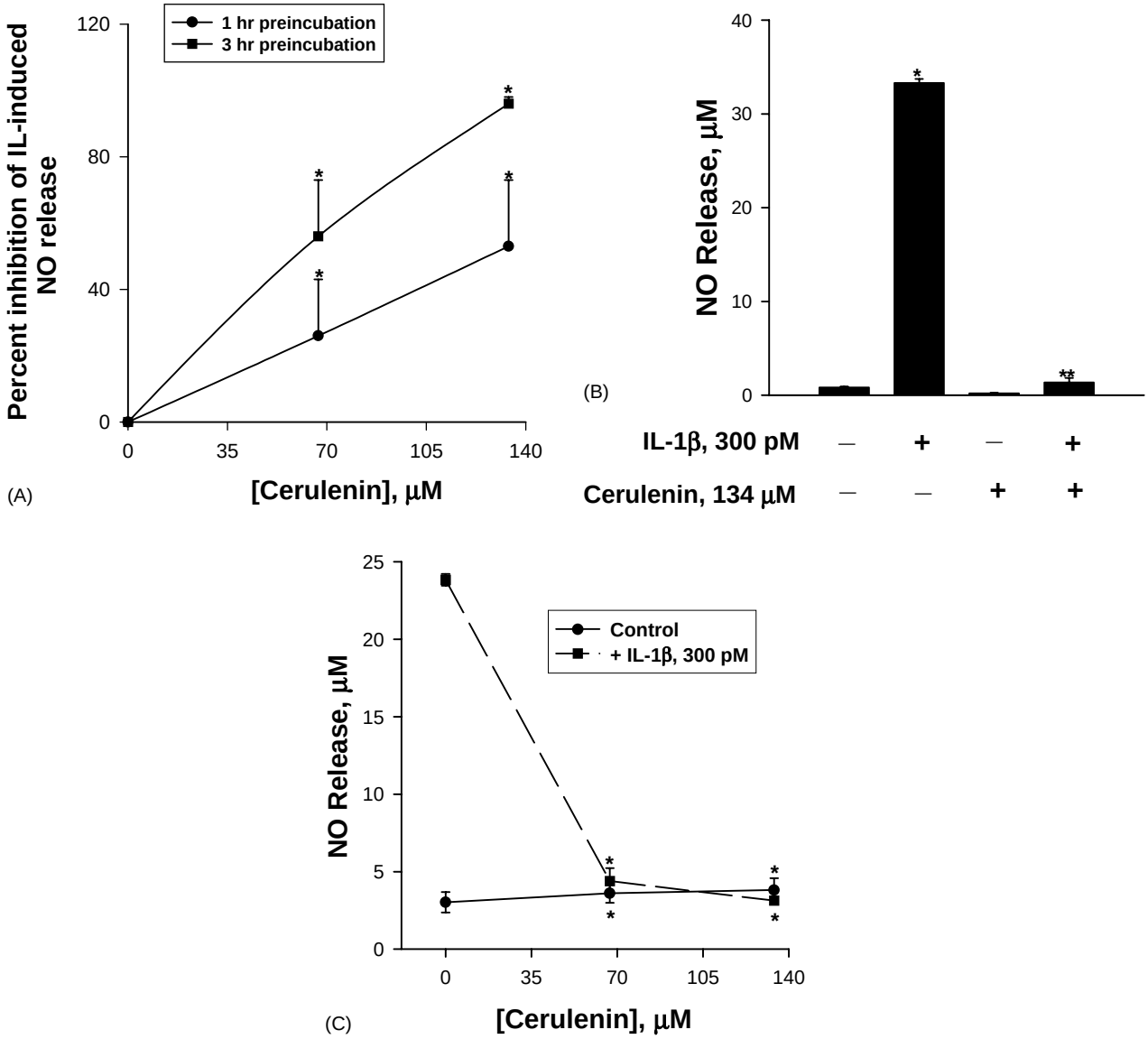

Fig. 1. Time- and concentration-dependent inhibition by CER of IL-1β-induced NO release from INS-1 and HIT-T15 cells. Panel A: INS-1 cells were preincubated in the presence of different concentrations of CER (0–134 μM) for either 1 or 3 hr as indicated in the figure. Following this, media were replaced by fresh media containing diluent alone or IL-1β (300 pM) for an additional 24 hr. NO released into the media was quantitated using Griess reagent. Data are expressed as percent inhibition of IL-1β-induced NO release by CER, and are mean ± SEM from three experiments. Asterisk (∗) represents $P < 0.05$ vs. control in the absence of CER. Panel B: INS-1 cells were preincubated in the absence or presence of CER (134 μM) for 3 hr as indicated in the figure. Following this, media were replaced by fresh media containing diluent alone or IL-1β (300 pM) for an additional 24 hr. NO released into the media was quantitated using Griess reagent. Data are expressed as NO released, and are mean ± SEM from at least three experiments. Asterisk (∗) represents $P < 0.05$ vs. control in the absence of IL-1β. Asterisks (∗∗) represents not different from control. Panel C: HIT-T15 cells were preincubated in the presence of either diluent or different concentrations of CER (0–134 μM) for 3 hr as indicated in the figure. Following this, media were replaced by fresh media containing diluent alone or IL-1β (300 pM) for an additional 24 hr. NO released into the media was quantitated using Griess reagent. Data are expressed as NO release and are mean ± SEM from three experiments. Asterisk (∗) represents no significant difference from NO release demonstrable in control cells in the absence of CER.

abundance in cytosolic vs. membrane fractions) of this protein, since several earlier studies have shown that inhibition of post-translational modifications of specific G-proteins leads to their selective accumulation in the soluble fraction in isolated β cells [5,6,36,37]. Data shown in Fig. 4 indicate a significant increase in the cytosolic to membrane ratios of the abundance of Ras in INS cells treated with CER, suggesting that inhibition of palmitoylation results in accumulation of unprocessed Ras in the cytosolic fraction. Taken together, data shown in Figs. 3 and 4 indicate that Ras is palmitoylated in isolated β cells and that inhibition of palmitoylation of Ras results in changes in its subcellular distribution; such changes are likely to impede its interaction with other effector proteins leading to inhibition of iNOS induction and NO release (see below).

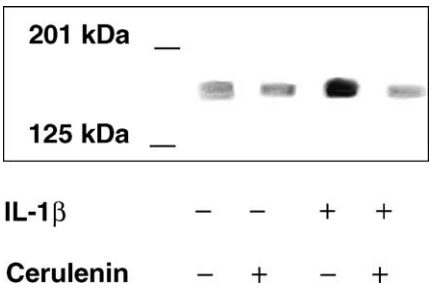

Fig. 2. Inhibition by CER of IL-induced iNOS expression: HIT-T15 cells were preincubated in the absence or presence of CER (134 μM; 3 hr) prior to stimulation with IL-1β (600 pM) for an additional 24 hr. Proteins were separated by SDS–PAGE (12%) and the resolved proteins were transferred onto a nitrocellulose membrane. Blots were probed with anti-iNOS antibody (1:1000) then incubated with the secondary antibody conjugated to HRP (1:1000). Immune-complexes were detected using ECL kit.

3.4. In addition to farnesylation and palmitoylation, post-translational carboxyl methylation of Ras may also be required for IL-1β-induced NO release

Earlier studies have shown that farnesylation of Ras is necessary for IL-1β-induced NO release from clonal β cells [22]. Our current findings suggest that palmitoylation of Ras is also critical for IL-1β-induced effects on isolated β cells. As demonstrated earlier [10,17,18] and in the current studies, farnesylation and palmitoylation of Ras occurs at different cysteine residues, and that such modifications appear to dictate the degree of association of Ras with the plasma membrane. We and others have demonstrated earlier [26–28,38] that in addition to farnesylation, the C-terminal cysteine is also subjected to additional post-translational modifications (e.g. carboxyl methylation); such a modification has been shown to increase the hydrophobicity and the subsequent association of the modified proteins (e.g. Cdc42) with the plasma membrane. Therefore, we investigated whether the carboxyl methylation of Ras is also critical for IL-1β-induced effects. Data in Fig. 5 (panel A) demonstrate that incubation of INS cell lysates with [^{3}H]*S*-adenosyl methionine results in incorporation of the label into Ras, as determined by immunoprecipitation of Ras. Furthermore, AFC (100 μM), an inhibitor of carboxyl methyl transferase [39], markedly reduced (nearly by 50%) the incorporation of the label into Ras, indicating that Ras is carboxyl methylated in the pancreatic β cell (Fig. 5; panel A). We also examined the effects of AFC on IL-1β-induced NO release from INS-1 cells to determine putative regulatory roles of the carboxyl methylation of Ras in this signaling cascade. Increasing concentrations of AFC (up to 400 μM) had modest, but significant inhibitory effects on IL-induced NO release (Fig. 5; panel B). Together, these data demonstrate that Ras undergoes AFC-sensitive carboxyl methylation in isolated β cells and akin to farnesylation and palmitoylation steps, the carboxyl methylation of Ras appears to play regulatory roles in IL-1β-induced NO release as well.

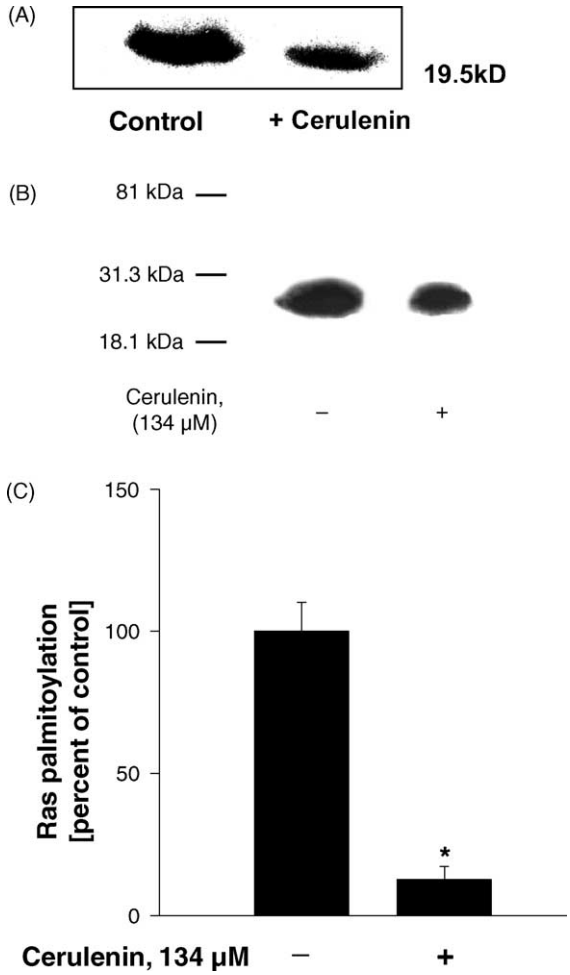

Fig. 3. CER inhibits incorporation of [^{3}H]palmitic acid into a 19–21 kDa β cell protein (panel A); identification of that protein as Ras by immunoprecipitation (panel B); and quantitation of degree of inhibition of by CER of Ras palmitoylation (panel C). Panel A: HIT-T15 cells were preincubated in the absence or presence of CER to facilitate its entry following which they were labeled in the presence of [^{3}H]palmitate (see Section 2 for additional details). Labeled proteins were separated by SDS–PAGE and identified by autofluorography. These data indicate CER-sensitive incorporation of the label into a protein with an apparent molecular weight in the range of 19–21 kDa. Panel B: HIT cells were incubated in the absence (diluent alone) or presence of CER (134 μM) as indicated in the figure, followed by additional 3 hr of incubation in the presence of [^{3}H]palmitate (see Section 2 for additional details). Ras was immunoprecipitated from the lysates of labeled cells and radioactivity associated with pellets was detected by SDS–PAGE. Immune complexes were precipitated using protein G-agarose, and proteins in the immunoprecipitates were separated by SDS–PAGE. Gels were soaked in ENHANCE (see footnote 2) as per the manufacturers' instructions, dried and exposed to an X-ray film for 3 weeks at −70°. Labeled proteins were detected by autoflurography. Panel C: HIT-T15 cells were incubated in the presence of CER (134 μM) as indicated in the figure, followed by an additional 3 hr of incubation in the presence of [^{3}H]palmitate (see Section 2 for additional details). Ras was immunoprecipitated from the lysates of labeled cells and radioactivity associated with pellets was quantitated by scintillation spectrometry. Data are expressed as a percent control of Ras palmitoylation, and are mean ± SEM of three individual immunoprecipitations in each case. Asterisk (∗) represents $P < 0.05$ vs. control.

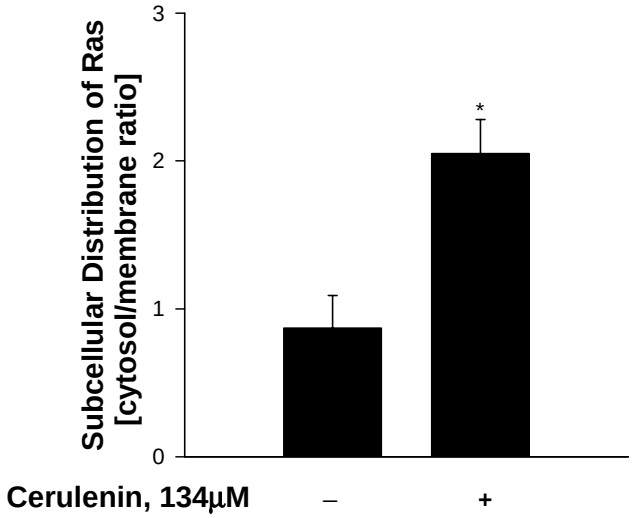

Fig. 4. Inhibition of palmitoylation by CER results in altered subcellular distribution of Ras in INS-1 cells. INS-1 cells were incubated in the presence of diluent alone or CER (134 μM) for 3 hr and total membrane and soluble fractions from the control and CER-treated cells were isolated by a single-step centrifugation method as described in Section 2. Proteins from each fraction were separated by SDS–PAGE and Ras was identified by Western blotting. Intensity of Ras bands was quantitated by densitometry. Data are expressed as densitometric units and are mean ± SEM from three individual preparations of each fraction. Asterisk (∗) represents $P < 0.05$ vs. control.

3.5. CER inhibits IL-1β-mediated activation of caspase 3

It is well established that one of the downstream signaling steps subsequent to IL-1β-induced NO release is the release of cytochrome *c* from the mitochondrial fraction, which, in turn, activates various caspases [40–43]. The first of such effector proteins is caspase 3, the activation of which leads to the proteolytic cleavage of procaspase 6 to yield caspase 6 [40–43]. In isolated β cells, activation of caspases leads to the degradation of several intracellular proteins, including nuclear lamins and protein kinases (see footnote 2). Herein, we examined if CER treatment, and hence inhibition of Ras function, prevents IL-1β-induced activation of caspase 3 in isolated β cells. To verify this, INS and HIT cells were pretreated with CER then treated with either diluent or IL-1β, followed by quantitation of caspase 3 activity. Data shown in Fig. 6 demonstrate that IL-1β treatment results in an increase in caspase 3 activity in INS (panel A) and HIT (panel B) cells. Pretreatment with CER completely prevented IL-1β-induced activation of caspase 3 in either of these cells. CER, by itself, had no demonstrable effects on this activity in control cells (Fig. 6; panels A and B).

In the next series of experiments, we examined whether IL-induced CER-sensitive activation is mediated *via* IL-induced iNOS gene expression and NO release as we described above (Figs. 1 and 2). To verify this, we quantitated IL-induced activation of caspase 3 in HIT cells in the absence or presence of L-NMMA, a known inhibitor of

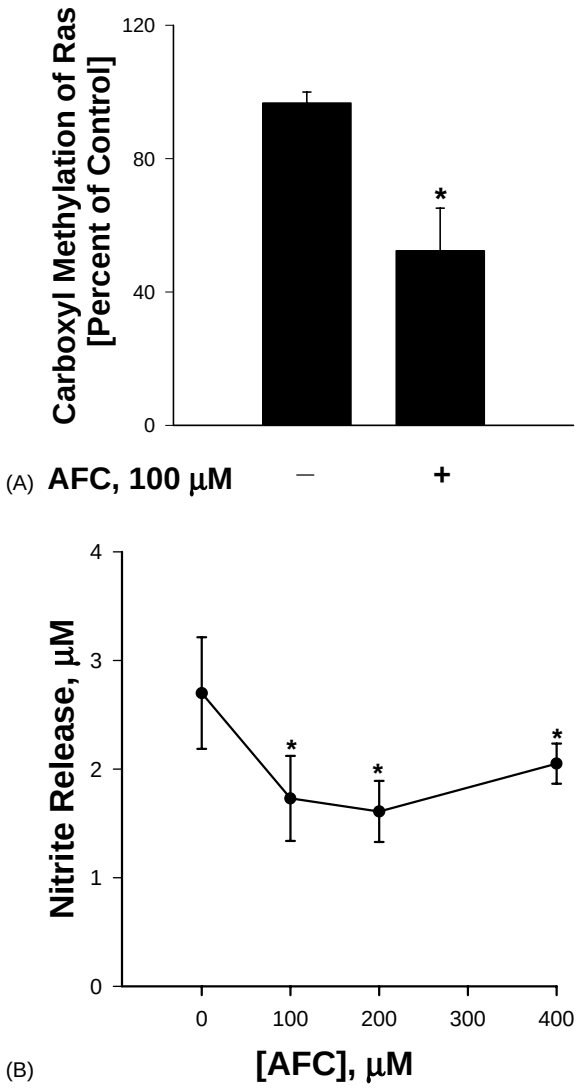

Fig. 5. AFC-sensitive carboxyl methylation of Ras (panel A) and a modest, but significant inhibition by AFC of IL-1β-induced NO release. Panel A: INS cell lysate proteins were methylated using [^{3}H]*S*-adenosyl methionine as the methyl donor in the absence or presence of AFC (100 μM) as indicated in the figure. Ras was immunoprecipitated from the lysates using a specific antiserum directed against Ras (see Section 2 for additional details), and the degree of carboxyl methylation of Ras was determined by vapor phase equilibration assay. Comparable results were obtained in HIT cells. Data are mean ± SEM from three individual immunoprecipitations. Asterisk (∗) represents $P < 0.05$ vs. control (i.e. in the absence of AFC). Panel B: IL-1β-induced NO release was quantitated in HIT-T15 cells cultured in the absence or presence of increasing concentrations of AFC (0–400 μM) as indicated in the figure. Data are mean ± SEM from two experiments carried out in triplicates. Asterisk (∗) represents $P > 0.05$ vs. control (i.e. no added AFC).

iNOS, and subsequent NO release. Data shown in Fig. 6; panel C indicate marked reduction in IL-induced activation of caspase 3 by L-NMMA. Taken together data described in Fig. 6 suggest that, in isolated β cells, IL-induced activation of caspase 3 is mediated *via* activation of iNOS gene expression, and that inhibition of Ras palmitoylation, by CER, results in significant inhibition of IL-1β-sensitive signaling pathways, including those involving NO release and caspase activation.

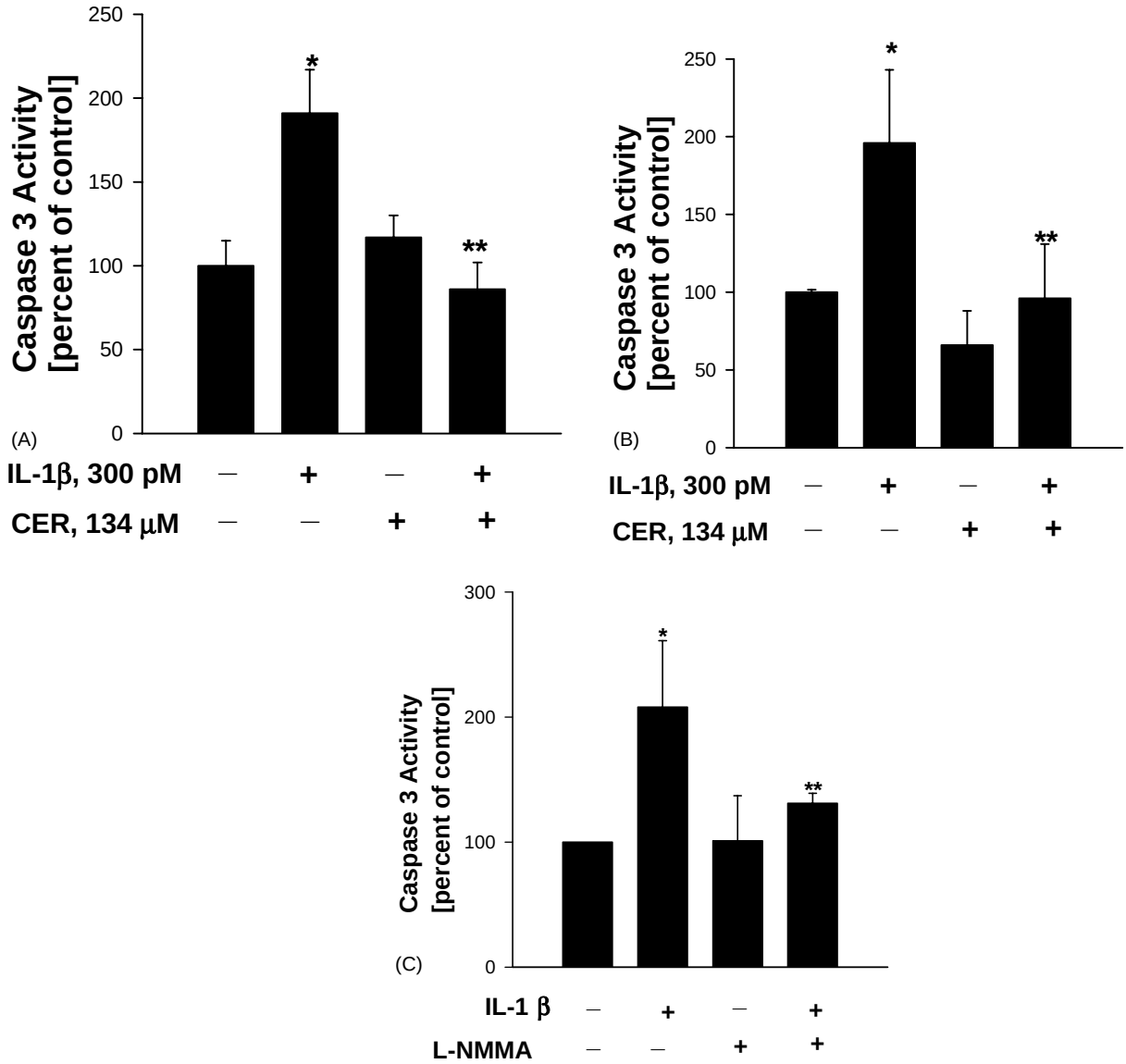

Fig. 6. CER prevents IL-1β-induced activation of caspase 3 in INS and HIT cells INS cells (panel A) and HIT cells (panel B) were incubated with CER (134 μM for 3 hr). Following the incubation, the media was replaced with fresh medium in the presence or absence of IL-1β (300 pM). Incubation was continued for 48 hr at which time point cells were harvested and caspase 3 activity was measured using a colorimetric assay. Data are expressed as percent of control and are mean ± SEM from three experiments. Asterisk (∗) represents $P < 0.05$ vs. control in the absence of IL-1β. Asterisks (∗∗) represents no significant difference from control. Panel C: L-NMMA, a known inhibitor of iNOS and NO release, inhibits IL-1β-induced activation of caspase 3 in HIT cells HIT cells were incubated in the presence or absence of IL-1β (300 pM) and/or L-NMMA (500 μM) as indicated in the figure. Incubation was continued for 48 hr at which time point cells were harvested and caspase 3 activity was measured using a colorimetric assay (see Section 2 for additional details). Data are expressed as percent of control and are mean ± SD from two experiments. Asterisks (∗∗) represents no significant difference from control (i.e. in the absence of IL-1β and L-NMMA).

3.6. Depletion of membrane-associated cholesterol prevents IL-1β-induced NO release from HIT cells

Earlier studies [19–21] have demonstrated that Cav-1, a key constituent of the caveolar microdomains of the plasma membrane, interacts with Ras, and such an interaction is critical for Ras to attain its active (GTP-bound) conformation. Recent studies have utilized mβcd to verify the roles of membrane-bound cholesterol and the caveolar organization in signal transduction processes [29,44], since this agent has been shown to selectively deplete cholesterol pools from the plasma membrane and disrupt caveolae while preserving other structural features of the membrane [44]. Pretreatment of HIT cells with mβcd markedly attenuated IL-1β-induced NO release from HIT cells; maximal inhibition was demonstrable at 10 mM of mβcd following 2 hr of incubation (Fig. 7). These conditions (concentration of mβcd and duration of incubation) for optimal effects of cholesterol depletion are compatible with observations from other laboratories [29,44].

In the next series of experiments, we examined whether Ras and Cav-1, a key constituent of caveolae colocalize in

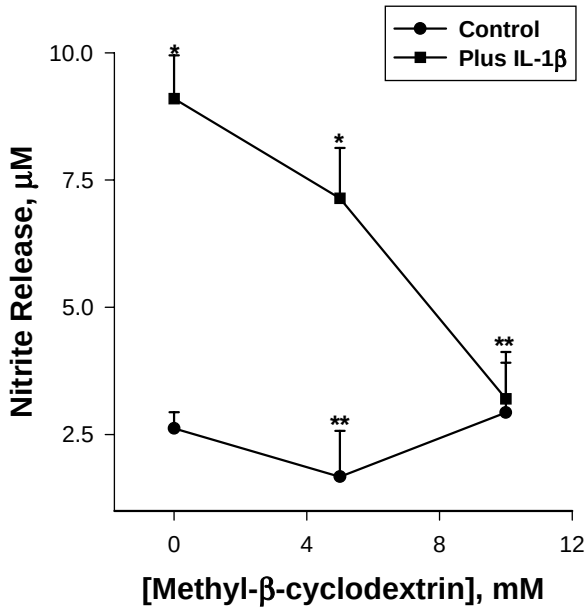

Fig. 7. Depletion of membrane-bound cholesterol using mβcd results in inhibition of IL-1β-induced NO release from HIT-T15 cells. HIT cells were grown in 24-well plates and were pretreated with different concentrations (0–10 mM) of mβcd for 2 hr in serum-free media and then treated with or without IL-1β (600 pM) in fresh media for 24 hr. Data are mean ± variance from two experiments carried out in triplicates. Asterisk (∗) represents $P < 0.05$ vs. control and asterisks (∗∗) represents no significant difference from control.

the isolated β cells. We verified this by confocal light microscopy (see Section 2 for additional details). Data shown in Fig. 8 demonstrate immunolocalization of Cav-1 (right panel; in red) and Ras (middle panel; in green) in HIT-T15 cells. An overlay of the two images (right panel) demonstrates colocalization of Ras with Cav-1 (indicated by arrows in the right panel; Fig. 8) as evidenced by the intense yellow color within the caveolar compartment of the β cell. Taken together, data shown in Figs. 7 and 8 suggest that Ras and Cav-1 exist as a complex in the isolated β cells and that membrane-bound cholesterol and caveolar microdomain organization within the plasma membrane are critical for IL-1β-mediated signaling mechanisms within the β cell. These data further support a critical role for Cav-1 in IL-1β-induced signaling pathway in isolated β cells (see below).

4. Discussion

IL-1β-induced β cell death is widely used as a model for IDDM since the latter results from an autoimmune aggression of the pancreatic β cell [45]. However, the exact cellular mechanisms underlying cytokine-mediated dysfunction and demise of the islet β cell remain partially understood [46]. One of the main objectives of the present study was to examine the contributory roles of protein palmitoylation, specifically that of H-Ras, in IL-1β-mediated NO release from isolated β cells. Salient features of our current studies are the following: (a) two structurally-dissimilar inhibitors of protein palmitoylation (e.g. CER and 2-bromopalmitate) markedly reduced IL-1β-induced NO release from isolated β cells; (b) H-Ras is one of the signaling proteins in the IL-1β-signaling pathway that is palmitoylated in a CER-sensitive fashion in insulin-secreting β cells; (c) CER elicits significant alterations in the subcellular distribution of Ras (i.e. increase in cytosolic to membrane ratios); (d) IL-induces caspase 3 activation *via* iNOS gene expression and NO generation; (e) CER prevents IL-1β-induced activation of caspase 3; (f) post-translational modifications (e.g. palmitoylation of Ras appear to be critical for IL-induced NO release); and (g) depletion of membrane-bound cholesterol and disruption of caveolar microdomains of the plasma membrane using mβcd, results in marked inhibition of IL-1β-induced NO release. To the best of our knowledge, this is the first report, which demonstrates a regulatory role for protein palmitoylation, specifically of H-Ras, in IL-1β-induced effects on the islet β cell.

Using four different classes of selective inhibitors of Ras function, we have been able to demonstrate the involve-

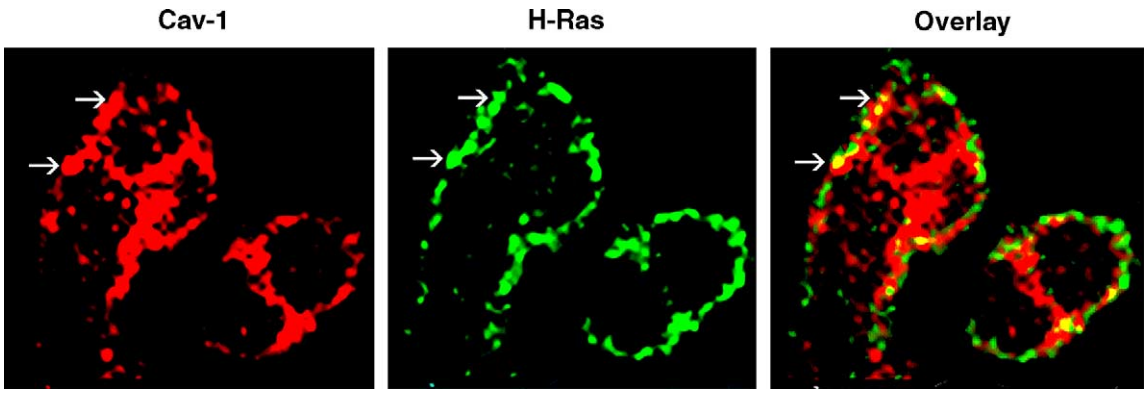

Fig. 8. Confocal light microscopic evidence for the association of Cav-1 with H-Ras in HIT cells. HIT cells were grown on glass coverslips, fixed, and immunocytochemical detection of H-Ras and Cav-1 was accomplished by confocal light microscopy using specific antisera directed against H-Ras and Cav-1 (see Section 2 for additional details). Cav-1 (in red, left panel) and H-Ras (in green, middle panel) were overlaid to demonstrate colocalization in the far right panel as indicated by the yellow color and arrows. Data are representative of one out of three experiments yielding comparable results.

ment of Ras in IL-1β-induced NO release [22,47]. These inhibitors include, bacterial toxins (e.g. *Clostridium sordellii* lethal toxins; LT-9048 and LT-82), which specifically glucosylate and inactivate the Rho subfamily of G-proteins; natural inhibitors of Ras farnesylation and function (e.g. manumycin and damnacanthal); peptidomimetic inhibitors of Ras function (e.g. FTI-277); and pro-drug inhibitors of Ras farnesyl transferase (e.g. 3-allyl and 3-vinyl farnesols). While bacterial toxins have been shown to inhibit Ras function by modification of a threonine residue [22,28,47], which resides in the GTP-binding domain, the farnesylation inhibitors block farnesylation of Ras, thus impeding its translocation to the plasma membrane for interaction with its effector proteins. In this context, several recent studies [16,48,49], including our own [50], have provided evidence to suggest that the membrane-associated, GTP-bound form of Ras recruits its effector protein Raf-1 into the plasma membrane for initiation of various signaling events leading to the release of NO. Compatible with this postulation are our current data to indicate that inhibition of palmitoylation by CER results in significant accumulation of H-Ras in the soluble fraction of the β cell. The current study further confirms our original hypothesis that Ras activation is necessary for IL-1β-induced NO release.

A growing body of recent experimental evidence has implicated palmitoylation as a critical step for the biological activity of Ras-related proteins [32–34]. For example, it was reported that the C-terminal Cys-186 of H-Ras undergoes farnesylation and the two-cysteine residues upstream to the farnesylated cysteine (i.e. Cys-181 and Cys-184) are palmitoylated. Mutational studies have demonstrated that substitution of Cys-181 and Cys-184 by serines results in a nearly 90% loss in the transforming activity; suggesting the requisite nature of palmitoylation in normal Ras function [51]. Studies of Dudler and Gelb [18] have demonstrated that palmitoylation is essential for ''firm'' anchoring of H-Ras into the membrane, since using mutational analysis these investigators provided evidence to suggest that a nonpalmitoylated, but farnesylated and carboxyl methylated Ras mutant, miscolocalizes into the cytosolic compartment. More recently, studies by Prior *et al*. [16] have shown that palmitoylation and farnesylation steps enable H-Ras targeting to lipid rafts and caveolae. They also provided evidence to indicate that GTP-loading of Ras facilitates in redistribution of Ras into the plasma membrane [52].

Extant studies have utilized CER to probe for the roles of protein palmitoylation in normal β cell function. In 1993, we first reported [5] that protein palmitoylation plays a positive modulatory role in glucose-induced insulin secretion; these studies were confirmed later by Yajima *et al*. [14] and Straub *et al*. [13]. Further, studies of Deeney *et al*. [15] have implicated protein palmitoylation step in insulin secretion from isolated β cells elicited by long-chain fatty acids. These investigators have suggested regulatory roles of protein palmitoylation in fusion of insulin-laden secretory granules with the plasma membrane [15]. Other researchers have used CER to examine the role of protein palmitoylation steps in internalization of insulin in adipocytes as well as insulin-mediated glucose uptake in these cells [53]. Together, these observations support a modulatory role for protein palmitoylation in normal functioning of cells, including the pancreatic β cell. It may be mentioned that there have been reports with regard to other biological effects of CER, including inhibition of fatty acid synthetase [54]. It is unlikely that such is the case in our studies, since we have observed CER effects on IL-1β-induced NO release following very short periods of incubation (i.e. as little as 60 min). Further, we have demonstrated that under similar experimental conditions, CER markedly inhibited the palmitoylation of Ras followed by significant alterations in its subcellular distribution, suggesting that CER-mediated effects, may, in part, be due to its effects on Ras palmitoylation. However, we cannot completely rule out such a possibility at the present time. Moreover, our data using 2-bromopalmitate, a structurally dissimilar inhibitor of protein palmitoylation, further afford strength to our model that palmitoylation of proteins (e.g. Ras) is essential for IL-1β-mediated effects. De Vos *et al*. have recently described [55] synthesis of novel CER analogs (e.g. *cis*-2,3-epoxy-4-oxonodecanamide and *cis*-2,3-epoxy-4-oxododecanamide), which inhibited protein palmitoyltransferase activity significantly, presumably by covalent alkylation of the enzyme. It will be interesting to investigate the effects of these new generation inhibitors as probes to study not only the role(s) of protein palmitoylation reactions in normal β cell function, but also in cytokine-mediated NO release culminating in the dysfunction and demise of the effete β cell. We also observed no clear effects of 2-hydroxymyristic acid, on IL-1β-induced NO release (current studies). These data suggest no clear evidence for a regulatory role for this post-translational modification step in IL-1β-induced signaling at least at the level of iNOS expression and NO release. Cadwallader *et al*. have shown previously, by mutation of cysteine 181, 184, and 186 to serine at the C-terminal end of H-Ras failed to associate with the membrane showing myristoylation alone is insufficient to direct the H-Ras to the membrane compartment [56]. Hawash *et al*. [57] have demonstrated that the oxygen-substituted palmitic acid analogue, 13-oxypalmitic acid, inhibits *Lck* localization to lipid rafts and T cell signaling. However, we failed to observe any demonstrable effects of 13-oxypalmitic acid on IL-1β-induced NO release from HIT cells (additional data not shown).

It may also be argued that CER also inhibits palmitoylation of other key proteins that are involved in the Ras signaling pathway. For example, recent studies [32] have demonstrated that H-Ras undergoes trafficking to the plasma membrane from the endoplasmic reticulum *via* the classical *trans*-Golgi secretory pathway to gain firm anchoring within the caveolae and the lipid rafts of the

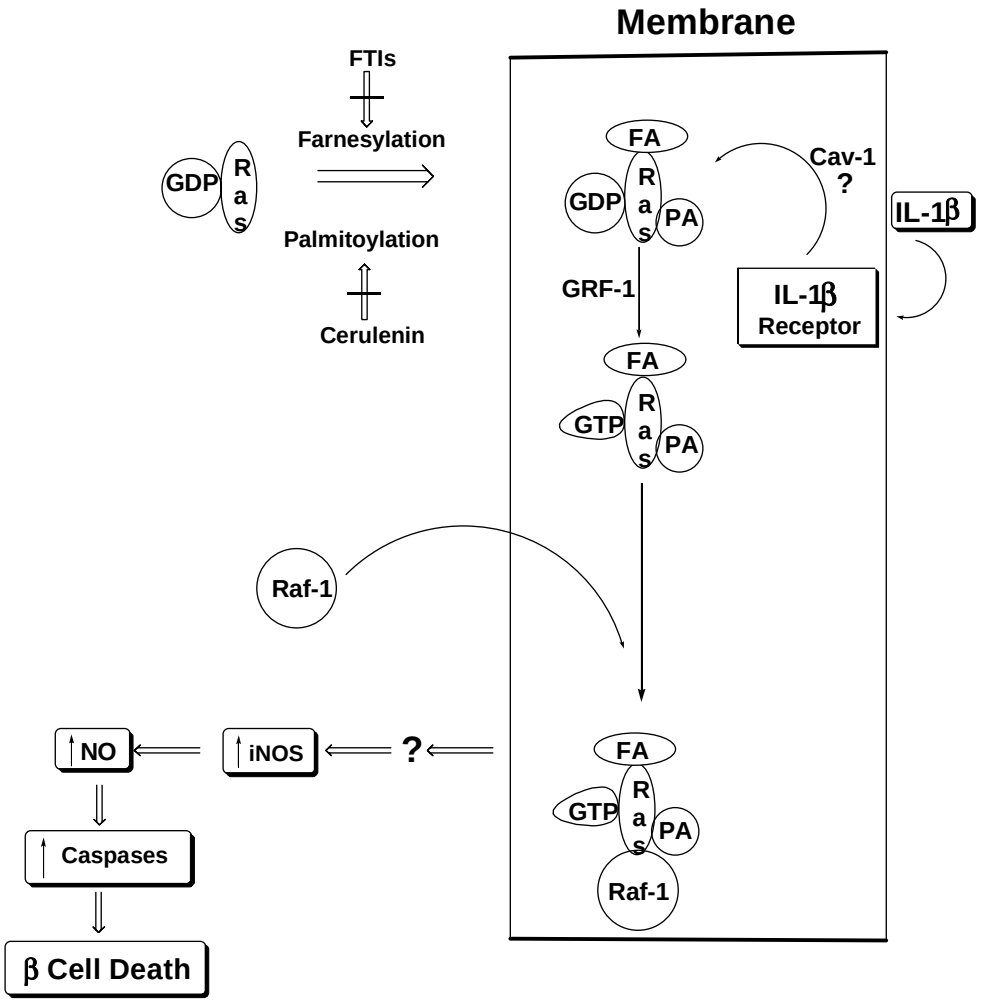

Fig. 9. Proposed model for a role of palmitoylation of Ras in IL-1β-induced dysfunction of the β cell. Based on our data, we propose the following model for the involvement of Ras in IL-1β-induced NO release from the pancreatic β cell. We propose that GDP-bound inactive form of Ras, undergoes farnesylation and/or palmitoylation; such modifications enable Ras to translocate to the plasma membrane for its activation (i.e. to attain its GTP-bound conformation). The conversion of GDP-bound inactive Ras to its GTP-bound active conformation is brought about by the putative guanine nucleotide releasing factors (e.g. GRF-1), which we have recently identified in the β cell [61]. Inhibition of farnesylation (by farnesyl transferase inhibitors; FTIs) or palmitoylation (by CER or bromopalmitate) results in its accumulation within the cytosolic compartment. Following its activation in the membrane fraction, it recruits cytosolic Raf-1 to the plasma membrane (which we have also identified in the β cell; [61]), resulting in the formation of GTP-Ras/Raf-1 complex. This in turn, initiates a cascade of signaling events, including activation of other signaling proteins leading to increased expression of iNOS and generation of NO (see text for additional details). Not shown here is the possibility of carboxyl methylation-dependent translocation of cytosolic Ras to the membrane fraction. Our current data also indicate colocalization of Ras with Cav-1 and that disruption of caveolar ultrastructure *via* methyl-β-cyclodextrin facilitated depletion of membrane-associated cholesterol leads to significant reduction in IL-1β-induced NO release. These data raise an interesting possibility for potential regulatory roles for Cav-1 in IL-signaling pathway. FA: farnesylated Ras, and PA: palmitoylated Ras.

plasma membrane before attaining its GTP-bound active conformation [52,58,59]. It has been shown that Cav-1, which constitutes a key protein in the caveolar compartment, has also been shown to undergo palmitoylation [60]. Therefore, it is likely that CER or bromopalmitate could have inhibitory effects on the palmitoylation of other proteins (e.g. Cav-1) involved in the Ras signaling pathway. Recent evidence from our laboratory also indicates measurable degree of incorporation of [^{3}H]palmitate into Cav-1.[3] Therefore, it is possible that CER or bromopalmitate-treatment results in inhibition of palmitoylation of Cav-1, in addition to Ras, as we have demonstrated in the current studies. Under such conditions, inhibition of palmitoylation of these two proteins could result in alterations in their subcellular distribution and targeting (e.g. caveolae) for optimal response to IL-1β challenge. We are currently investigating putative regulatory roles of post-translational modifications of Cav-1 (e.g. phosphorylation and palmitoylation) in IL-1β-mediated signaling within the islet β cell, specifically at the level of its interaction with Ras and Raf-1. Indeed, along these lines, we have obtained convincing evidence to indicate that Cav-1 forms complexes with Ras (current study) and Raf-1 in isolated β cells (see footnote 1).

In support of this, our current data also demonstrate that depletion of membrane-associated cholesterol using mβcd

[3] A. Kowluru and H.-Q. Chen, unpublished.

markedly attenuated IL-1β-induced NO release from HIT cells. Such a treatment is expected to disrupt caveolar organization *via* selective depletion of cholesterol from the plasma membrane [44]. Recent studies have demonstrated that disruption of caveolar structure results in dissociation of Cav-1-mediated signaling steps [29,44]. As indicated above we have recently demonstrated (see footnote 1) that IL-1β-mediates the tyrosine phosphorylation of Cav-1 in isolated β cells and that tyrosine kinase inhibitors selectively block IL-1β-induced phosphorylation of Cav-1 and NO release from these cells. These data suggest a critical contributory role for Cav-1 in IL-1β-signaling in the β cell. We have also shown (*via* immunoprecipitation and Western blot approaches), key members of Ras signaling pathway (e.g. Ras and Raf-1) are localized in the caveolar compartment (see footnote 1). These findings along with data derived from cholesterol depletion studies tend to support a formulation that Cav-1/Ras/Raf-1 interactions represent the key proximal events leading to the transcriptional regulation of iNOS expression, which leads to NO release, culminating in the dysfunction and demise of the β cell.

Data from the current studies clearly indicate that post-translational modifications, such as farnesylation, palmitoylation and the carboxyl methylation of Ras appear to be essential for IL-1β-induced signaling events leading to NO release. Therefore, it seems likely that such modification steps are critical for membrane association of Ras to gain access to the plasma membrane and to attain the GTP-bound, active-conformation necessary for its interaction with its effector proteins, such as Raf-1. Based on our recently published data [22,47,61] and the current findings, we propose a model for the putative involvement of Ras in IL-1β-induced NO release from the β cell (Fig. 9). Unmodified, GDP-bound Ras (and, possibly, Cav-1) undergoes palmitoylation by palmitoyltransferase (or farnesylation by farnesyl transferase), which results in an increase in the hydrophobicity of Ras thus enabling it to translocate to the plasma membrane for its activation (i.e. for attaining its GTP-bound conformation) by the putative guanine nucleotide releasing factors (e.g. GRF-1), which we have identified in the β cell [61]. There is growing experimental evidence to indicate that Ras is activated in the caveolar compartment following its binding to Cav-1 and other proteins, such as GRF-1. Following activation, it recruits cytosolic Raf-1 to the plasma membrane (which we have also identified in the β cell; [61]), resulting in the formation of GTP-Ras/Raf-1 complex. This, in turn, initiates a cascade of signaling events, leading to increased expression of iNOS and generation of NO. At least based on the preliminary data available to date,[4] our model predicts that IL-1β has very little effect, if any, on the translocation and membrane-association of Ras (i.e. *via* its effects on farnesylation and/or palmitoylation). Instead, we propose that binding of IL-1β to its receptor initiates signaling events including, phosphorylation of specific signaling proteins (Cav-1) (see footnote 1) and activation of GRF-1 to facilitate conversion of GDP-bound Ras (inactive) to its GTP-bound form (active conformation). These aspects of IL-1β-Ras pathway are being investigated in our laboratory currently.

[4] A. Kowluru, R. Amin and H.-Q. Chen, unpublished.

Acknowledgments

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