ELSEVIER

Biochemical Pharmacology 62 (2001) 1459–1468

Biochemical Pharmacology

Positive modulation by Ras of interleukin-1β-mediated nitric oxide generation in insulin-secreting clonal β (HIT-T15) cells

Marie Tannous[a,b], Rajesh Amin[a,b], Michel R. Popoff[c], Carla Fiorentini[d], Anjaneyulu Kowluru[a,b,*]

[a]*Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, 619 Shapero Hall, Wayne State University, Detroit, MI 48202, USA*

[b]*John D. Dingell VA Medical Center, Detroit, MI 48201, USA*

[c]*Unit of Microbial Toxins, Pasteur Institute, Paris, France*

[d]*Department of Ultrastructures, Istituto Superiore di Sanità, Rome, Italy*

Received 22 January 2001; accepted 10 May 2001

Abstract

In the present study, we have shown that exposure of insulin-secreting clonal β (HIT-T15) cells to interleukin-1β (IL-1β) results in a time- and concentration-dependent increase in nitric oxide (NO) release. These effects by IL-1β on NO release were mediated by induction of inducible nitric oxide synthase (iNOS) from the cells. Preincubation of HIT cells with *Clostridium sordellii* lethal toxin-82, which irreversibly glucosylates and inactivates small G-proteins, such as Ras, Rap, Ral, and Rac, but not Cdc42, completely abolished IL-1β-induced NO release. Pre-exposure of HIT cells to *C. sordellii* lethal toxin-9048, which monoglucosylates and inhibits Ras, Cdc42, Rac, and Rap, but not Ral, also attenuated IL-1β-mediated NO release. These data indicate that activation of Ras and/or Rac may be necessary for IL-1β-mediated NO release. Preincubation of HIT cells with *C. difficile* toxin-B, which monoglucosylates Rac, Cdc42, and Rho, had no demonstrable effects on IL-mediated NO release, ruling out the possibility that Rac may be involved in this signaling step. Further, two structurally dissimilar inhibitors of Ras function, namely manumycin A and damnacanthal, inhibited, in a concentration-dependent manner, the IL-1β-mediated NO release from these cells. Together, our data provide evidence, for the first time, that Ras activation is an obligatory step in IL-1β-mediated NO release and, presumably, the subsequent dysfunction of the pancreatic β cell. Our data also provide a basis for future investigations to understand the mechanism of cytokine-induced β cell death leading to the onset of insulin-dependent diabetes mellitus.

Keywords: Interleukin 1β; Pancreatic β cell; Nitric oxide; GTP-binding proteins; Apoptosis; Insulin-dependent diabetes mellitus; Ras

1. Introduction

IDDM develops as a consequence of the selective destruction of insulin-producing β cells due to autoimmune aggression [1]. It has been shown that such cytotoxic effects are mediated by cytokines (e.g. IL-1β) secreted by the infiltrating immune cells [2]. Cytokine-induced pancreatic β-cell death is attributed primarily to the induction of iNOS, leading to the generation of NO within the cell, which, in turn, culminates in cell death by apoptosis and necrosis [3]. Such cellular events have been shown to occur in normal rat islets, human islets, and in clonal β cells [3]. Together, these observations have led to the suggestion that IL-1β (alone or in combination with other cytokines) may be the key mediator of autoimmune destruction of β cells during the course of the onset of IDDM. The IL-1β effects are felt to be mediated largely due to the induction of iNOS. NO exerts multiple effects on β-cell function, including inhibition of glucose oxidation [4] and adenine and guanine nucleotide generation [5]. In intact β-cells, IL-1β has been shown to increase intracellular free sodium, prostaglandin E_2 (PGE_2), heat shock protein synthesis, c-*fos* protooncogene expression, and Mn^{2+}-dependent superoxide dismutase [6]. It has also been shown that NO initiates a cascade of events

* Corresponding author. Tel.: +1-313-576-4478; fax: +1-313-576-1112.

E-mail address: akowluru@wizard.pharm.wayne.edu (A. Kowluru).

Abbreviations: IL-1β interleukin-1β; NO, nitric oxide; iNOS, induced nitric oxide synthase; IDDM, insulin-dependent diabetes mellitus; LT, lethal toxin; CNF1, cytotoxic necrotizing factor 1; PTMs, post-translational modifications; and L-NMMA: *N*-monomethyl-l-arginine monoacetate.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00818-8

Table 1
Specificity of probes used in the present study

Probe	Concentration	Effects on function	Target G-proteins	Reference(s)
LT-82	200 ng/mL	Inhibition by glycosylation	Ras, Rap, Rac, Ral, but not Cdc42	29, 30
LT-9048	200 ng/mL	Inhibition by glycosylation	Ras, Cdc42, Rac, Rap, but not Ral	31
Toxin-B	200 ng/mL	Inhibition by glycosylation	Rac, Cdc42, Rho	9, 10
C3-Exoenzyme	12.5–25 μg/mL	Inhibition by ribosylation	Rho	9, 10, 15
CNF-1	200–400 ng/mL	Activation by deamidation	Rho, Rac, Cdc42	15
Manumycin A	10 μM	Inhibition of farnesylation	Ras	17
Damnacanthal	20 μg/mL	Inhibition	Ras	17

leading to mitochondrial dysfunction, including the release of cytochrome *c* and activation of caspases [7]. In turn, activation of caspases leads to proteolytic cleavage of several proteins, such as nuclear lamin-B, culminating in chromatin condensation and DNA fragmentation and terminating in cell death [8]. However, despite a growing body of biochemical evidence as to how IL-1β causes cellular dysfunction in multiple cell types, the exact nature and the mechanism(s) underlying the generation of intracellular signals in insulin-producing cells mediated by IL-1β have not been elucidated completely.

In this context, several groups, including our own, have shown that G-proteins play key roles in the physiologic regulation of insulin secretion (see Ref. 9 for a recent review). Using various biochemical and physiologic approaches, we identified some of these G-proteins (e.g. Cdc42, Rac, and Rap) that may be responsible for glucose- and calcium-mediated insulin secretion from isolated β cells [10,11]. During the course of these studies, we also observed that inactivation of specific G-proteins, either by long-term GTP depletion or by inhibition of their PTMs such as isoprenylation, resulted in apoptotic β-cell death [12,13]. Indeed, our observations are strengthened further by recent reports of involvement of specific G-proteins in fas- or ceramide-mediated apoptosis in other cell types as well [14].

Several recent studies have suggested key functional roles for the low molecular mass Rho subfamily of G-proteins in mitogenesis and survival. We recently reviewed these in Refs. 9 and 13. It has been possible to demonstrate such roles through the use of specific inhibitors of G-protein functions. These include bacterial toxins, such as *Clostridium difficile* and *sordellii* [12,15–17], which specifically monoglucosylate threonine residues on these G-proteins and inactivate them. A second class of inhibitors is comprised of inhibitors of PTMs (e.g. isoprenylation) of these G-proteins, which have been shown to play important roles in the translocation of these G-proteins to critical subcellular compartments for interaction with their effector proteins in the islet β cell [9,18–21]. We have shown earlier that exposure of isolated islet β cells to either class of these inhibitors markedly reduces both glucose- and calcium-mediated insulin secretion, suggesting the criticality of these signaling proteins in β-cell function [9,18–21]. In view of our recent observations demonstrating that selective inactivation of G-proteins by long-term exposure of islet β cells to these inhibitors leads to the apoptotic demise of the pancreatic β cell [12], we undertook the current study to examine the regulatory roles, if any, of the Rho subfamily of small G-proteins in the phenomenon of IL-induced NO release from isolated β cells.

2. Materials and methods

2.1. Materials

The HIT-T15 pancreatic β cell line was purchased from the American Type Culture Collection; *C. sordellii* LT-82 and LT-9048 were purified as described previously [22]. C3-exoenzyme was from Biomol, and CNF1 was purified as described previously [23–25]. Human recombinant IL-1β was from R&D; manumycin A, damnancanthal, and Griess reagent were from the Sigma Chemical Co.; F-12 medium and calf bovine serum were purchased from Gibco; anti-iNOS and anti-mouse horseradish peroxidase-IgG were from Transduction Laboratories; ECL reagent and Hyperfilm were from Amersham Pharmacia. L-NMMA was from Calbiochem. Anti-K Ras and anti-H Ras were purchased from Santa Cruz Biotechnology. All other reagents employed in this study were of analytical grade, and of the highest reagent purity available.

2.2. Treatment of HIT-T15 cells

HIT cells were cultured in F-12 medium supplemented with 10% fetal bovine serum, 100 IU/mL of penicillin, 100 IU/mL of streptomycin, and 2 mM L-glutamine. Cells were cultured in 24-well plates for 2 days prior to various experimental manipulations (see text). The culture medium was then replaced with medium consisting of either diluent alone or in the presence of IL-1β. The specificity of the probes used in the current study is given in Table 1. In experiments involving the variants of LT or CNF1, cells were pretreated with 200 ng/mL of the toxins for 24 hr prior to incubation with IL-1β. In studies involving Ras function inhibitors such as manumycin A (0–10 μM) and damnacanthal (0–20 μg/mL), cells were incubated with inhibitors

for 1 hr prior to stimulation with IL-1β for an additional 24 hr. To study the effect of C3-exoenzyme, cells were loaded with C3-exoenzyme (10 μg/mL) by the scrape-loading method as described in Ref. 26 prior to stimulation with IL-1β for 24 hr (see text for additional details).

2.3. Quantitation of nitrite release

Nitrite production was measured as described in Ref. 27. In brief, medium from the treated cells was collected, and centrifuged at 1700 *g* for 5 min at 4°; then 100 μL of supernatant was incubated with 100 μL of Griess reagent for 15 min. The absorbance at 540 nm was measured, and the nitrite concentration was calculated from a sodium nitrite standard curve. We observed no clear indication to suggest that toxin- or inhibitor-treated cells dissociated from the culture plates throughout the incubation, indicating that the cells were viable.

2.4. Quantitation of expression of iNOS by western blotting

After appropriate treatments, cells were homogenized, by sonication, in a medium consisting of 1% SDS, 1 mM sodium orthovanadate, 10 mM Tris (pH 7.4), and the sonicates were centrifuged at 1200 *g* for 10 min at 4°. Proteins in the supernatants from control and treated samples (30 μg) were separated by SDS–PAGE (8%), and the resolved proteins were transferred onto a nitrocellulose membrane for 12 hr at 50 mA in Tris-glycine transfer buffer. The blots were blocked for 1 hr at room temperature and then were incubated for an additional 1.5 hr at room temperature with primary antibody (anti-iNOS, host mouse; 1:5000 dilution). Following extensive washing, the blots were incubated with the secondary antibody (conjugated anti-mouse IgG:horseradish peroxidase, 1:2000 dilution) for 1 hr at room temperature. Immune complexes were detected using an ECL kit, and the intensity of the iNOS bands was quantified by densitometry.

2.5. Other methods

Protein concentrations in the samples were determined by the dye-binding method of Bradford [28], using bovine serum albumin as the standard.

3. Results

3.1. Induction by IL-1β of NO release from HIT-T15 cells in a time- and concentration-dependent manner

Western blot data given in Fig. 1 (top panel) indicate that IL-1β (0–600 pM) induced iNOS protein expression in a concentration-dependent manner in HIT-T15 cells. Intensity of the bands was quantitated by densitometry (Fig. 1, bottom panel). Compatible with these observations, IL-1β (0–300 pM) also induced NO release in a time- (0–72 hr) and concentration-dependent manner (Fig. 2). It was also possible to demonstrate similar effects of IL-1β on NO release in INS-1 cells (additional data not shown). To further confirm that the IL-1β-induced increment in NO release is due to an increase in iNOS activity, we examined the effects of L-NMMA (500 μM), a competitive inhibitor of iNOS [5], on IL-induced NO release from HIT-T15 cells. These data (Fig. 3) indicate a >80% inhibition in IL-induced NO release in cells incubated with L-NMMA. As indicated in Fig. 3, optimal inhibitory effects were demonstrable within 24 hr of incubation. Together, the data in Figs. 1–3 verify our

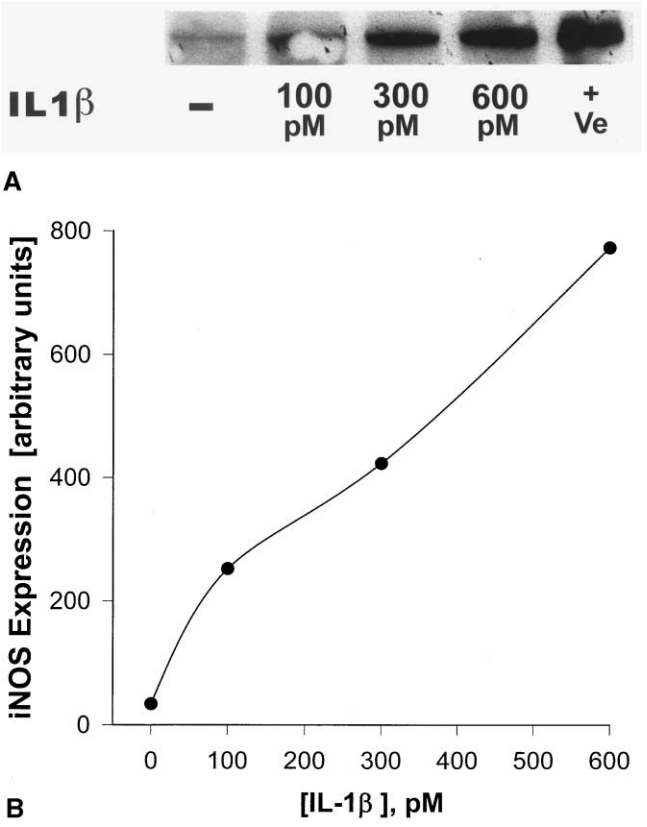

Fig. 1. Concentration-dependent stimulation by IL-1β of iNOS expression in HIT cells. Top panel: HIT-T15 cells were cultured for 24 hr in the presence of increasing concentrations (0–600 pM) of IL-1β. After appropriate treatments, cells were homogenized, by sonication, in a medium consisting of 1% SDS, 1 mM sodium orthovanadate, 10 mM Tris (pH 7.4), and the sonicates were centrifuged at 1200 *g* for 10 min at 4°. Proteins in the supernatants from control and treated samples (30 μg) were separated by SDS–PAGE (8%), the resolved proteins were transferred onto a nitrocellulose membrane, and the expression levels of iNOS were determined by western blotting (see "Materials and methods" for additional details). The last lane, marked as +Ve, represents the positive control of iNOS. Data are representative of three experiments carried out in triplicate. Since each lane contained the same amount of HIT cell lysate protein, the intensity of the iNOS bands (as an index of iNOS expression) was quantitated by densitometry (see bottom panel). Bottom panel: These data represent densitometric scanning of the intensity of iNOS protein bands from the representative experiment described in the top panel. The degree of intensity is expresssed as arbitrary densitometric units.

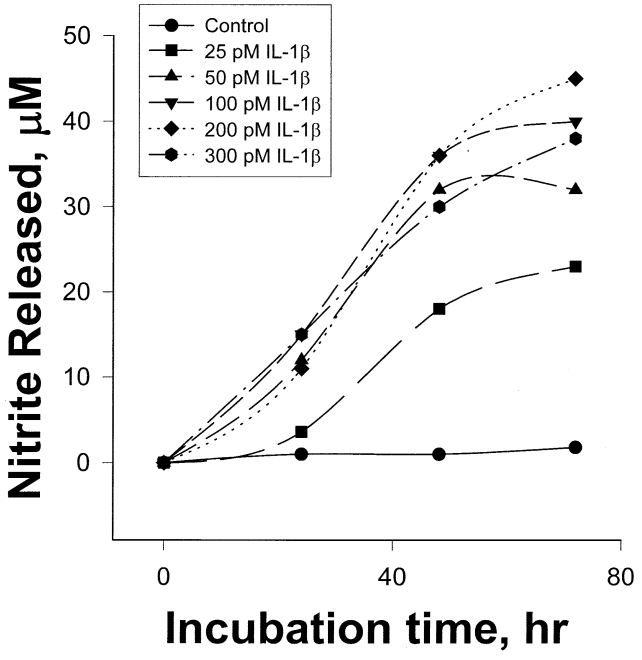

Fig. 2. Time- and concentration-dependent stimulation by IL-1β of NO release in HIT-T15 cells. HIT cells were incubated in the presence of different concentrations (0–300 pM) of IL-1β for different time intervals (0–72 hr) as indicated in the figure. Nitrite released in the medium was quantitated using Griess reagent. Data are representative of at least three experiments with identical results.

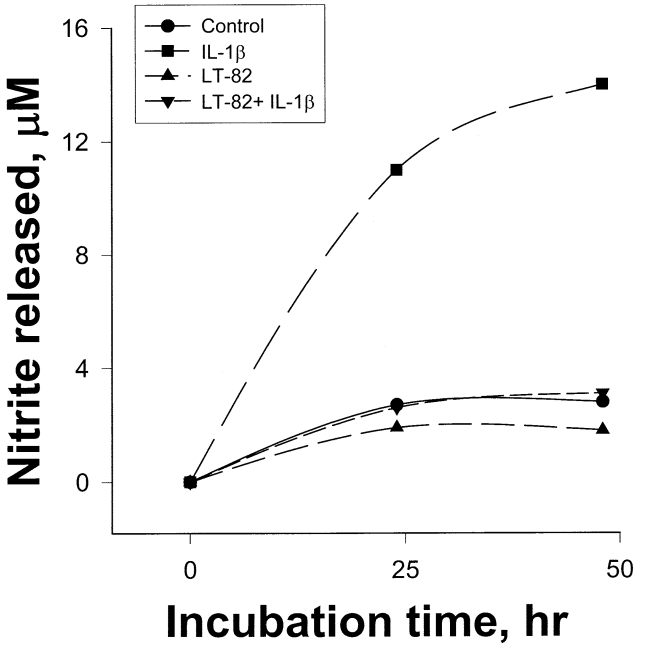

Fig. 4. Abolition of IL-1β-induced NO release from HIT-T15 cells pre-exposed to LT-82. HIT cells were cultured in the absence or presence of LT (200 ng/mL) for 24 hr as described under "Materials and methods." Following this, cells were incubated in the absence or presence of IL-1β (100 pM) for up to 48 hr. The nitrite released was quantitated using Griess reagent. Data are representative of at least three experiments with identical results.

experimental model system, which, as expected, demonstrates that the IL-1β-mediated increase in NO release is due to an increase in iNOS activity.

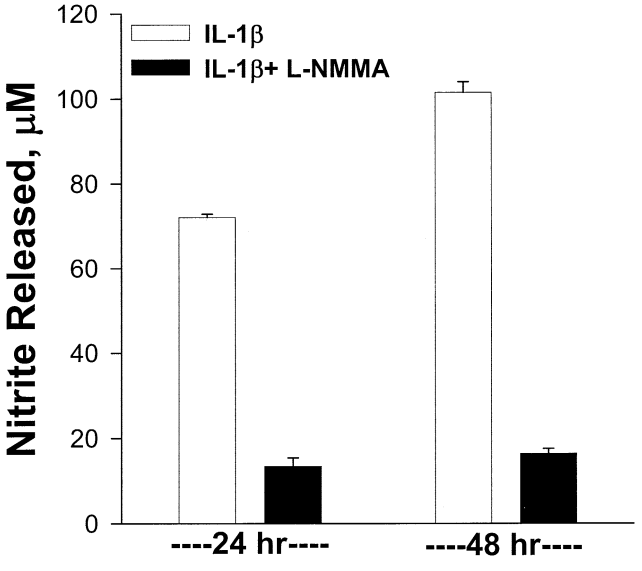

Fig. 3. Inhibition of IL-induced NO release by L-NMMA. HIT-T15 cells were cultured in the absence or presence of 500 μM L-NMMA, a competitive inhibitor of iNOS, for 24 or 48 hr as indicated in the figure. Nitrite was measured using Griess reagent. Data are the means ± SEM from six individual determinations.

3.2. Inhibition by two LT variants of IL-1β-induced NO release from HIT-T15 cells

We then examined the contributory roles of the Rho subfamily of G-proteins in IL-1β-induced NO release from HIT-T15 cells. For this purpose, we examined the effects of two variants of *C. sordellii* toxin, i.e. LT-82 and LT-9048, on IL-1β-mediated NO release from HIT cells. Data from several laboratories, including our own, have shown that these toxins irreversibly monoglucosylate and inactivate specific G-proteins. For example, LT-82 monoglucosylates and inhibits Ras, Rac, Ral, and Rap, but not Cdc42 ([29, 30]; Table 1), whereas LT-9048 glucosylates and inactivates Ras, Cdc42, Rac, and Rap, but not Ral ([31]; Table 1). We have demonstrated previously the specificity of *Clostridium* toxins in normal rat islets and HIT cells [10]. Data in Fig. 4 demonstrate that exposure of HIT cells to LT-82 (200 ng/mL) had no effect on basal NO release from control cells even up to 48 hr of incubation. However, LT-82 treatment completely attenuated the IL-1β-induced NO release from these cells. Such inhibitory effects of LT-82 on IL-1β-mediated NO release were demonstrable with a 24-hr exposure to the toxin. These data suggest that activation of its substrate G-proteins (i.e. Ras, Rap, and Rac, but not Cdc42) may be necessary for IL-1β-induced NO release. These studies were repeated using LT-9048, which monoglucosylates and inactivates Cdc42, Rac, Rap, and Ras, but

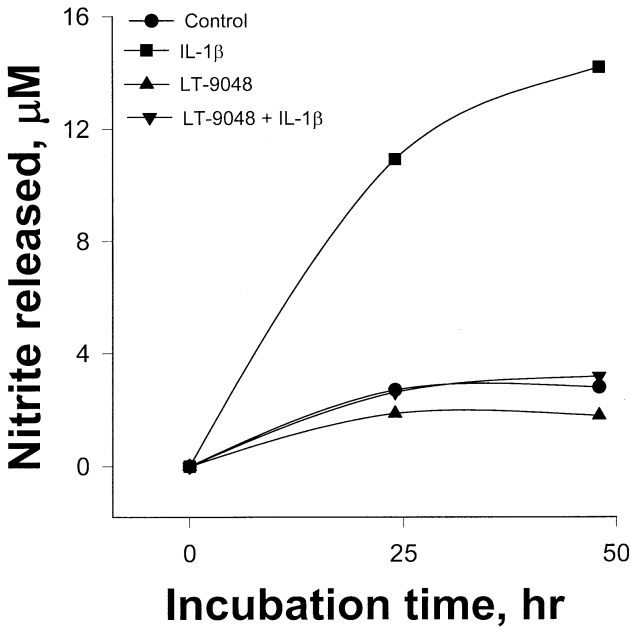

Fig. 5. Abolition of IL-1β-induced NO release from HIT-T15 cells exposed to LT-9048. HIT cells were cultured in the absence or presence of LT 9048 (200 ng/mL) for 24 hr as described under "Materials and methods." Following this, cells were incubated in the absence or presence of IL-1β (100 pM) for up to 48 hr. The nitrite released was quantitated using Griess reagent. Data are representative of at least three experiments with identical results.

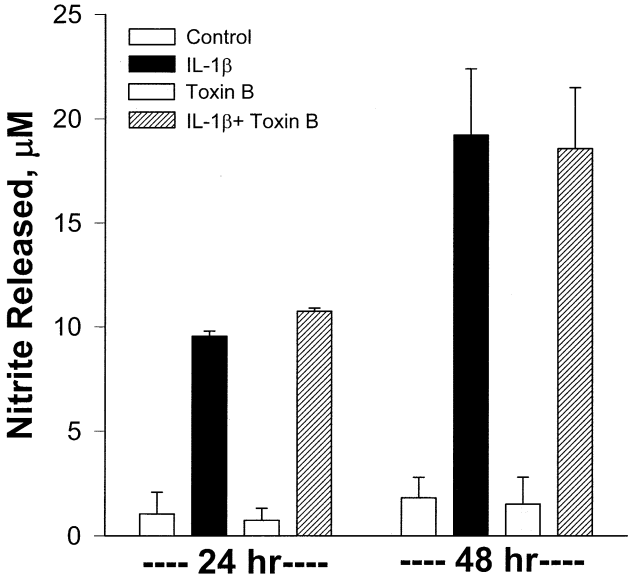

Fig. 6. Lack of effect of toxin-B on IL-mediated nitrite release from HIT-T15 cells. HIT cells were cultured in the absence or presence of toxin-B (200 ng/mL) for 24 hr as described under "Materials and methods." Following this, cells were incubated in the absence or presence of IL-1β (100 pM) for 24 or 48 hr as indicated in the figure. The nitrite released was quantitated using Griess reagent. Data are means ± SEM from two experiments carried out in triplicate.

not Ral (Table 1). Data from these studies (Fig. 5) yielded virtually identical inhibitory effects of LT-9048 on IL-mediated NO release from HIT cells. Together, our data in Figs. 4 and 5 suggest that activation of Ras and Rac, but not Cdc42 and Rap, is required for IL-1β-induced NO release from isolated β cells.

3.3. Further evidence that activation of Rac and Rho is not necessary for IL-induced NO release from HIT-T15 cells

In the next series of experiments, we examined the effects of *C. difficile* toxin-B, which monoglucosylates and inactivates Cdc42, Rac and Rho, on IL-induced NO release from HIT-T15 cells. In these studies, the effects of toxin-B were studied following 24 and 48 hr of exposure of the HIT cells to this toxin. Data in Fig. 6 indicate no demonstrable effects of toxin-B on IL-induced insulin release. Thus, these data rule out the possibility that Rac and Rho are involved in IL-mediated NO release.

To further rule out the possibility that Rho is not involved in this signaling cascade, we examined the effects of C3-exoenzyme (which inactivates Rho by ADP-ribosylation; [9,10,15]; Table 1) and of CNF-1 (which activates Rho, and to a lesser degree Rac and Cdc42, by deamidation; [15]; Table 1) on IL-1β-mediated NO release from HIT cells. C3-exoenzyme selectively modified (and inhibited) Rho with much less, or no, modification of other Rho family GTPases, such as Cdc42 or Rac. In our earlier studies [10], we demonstrated no subsequent ribosylation of Rho in cells during an acute exposure to C3-exoenzyme, indicating that most, if not all, Rho endogenous to HIT cells had been modified. Furthermore, C3-exoenzyme-treated cells also rounded up similar to LT- or toxin-B-treated cells, indicating disturbances in cytoskeletal organization [10]. Similar morphological changes were demonstrable in HIT cells scrape-loaded with C3-exoenzyme (current studies; additional data not shown).

Data in Fig. 7 indicate no clear effects of C3-exoenzyme on IL-induced NO release. Note that the degree of NO release in C3-treated cells was lower (nearly 2-fold) compared with that seen normally (see above). This may be due, in part, to the fact that the C3-exoenzyme was scrape-loaded for optimal incorporation, in contrast to the normal incubation of cells with the other toxins, such as LT-82 and LT-9048 and toxin-B (see "Materials and methods"). Nonetheless, data in Fig. 7 indicate no demonstrable effects of C3-exoenzyme on IL-induced NO release from HIT-T15 cells. Likewise, CNF-1 treatment (and, hence, Rho activation) of HIT-T15 cells elicited no changes in IL-1β-induced NO release from these cells (Fig. 8). These data thus provide evidence to indicate that neither activation nor inhibition of Rho is necessary for IL-induced NO release. Together, the above data (Figs. 4–8) rule out the involvement of Cdc42, Rap, Rac, Rho, but not Ras, in IL-induced NO release from HIT-T15 cells, which was further confirmed in the following experiments.

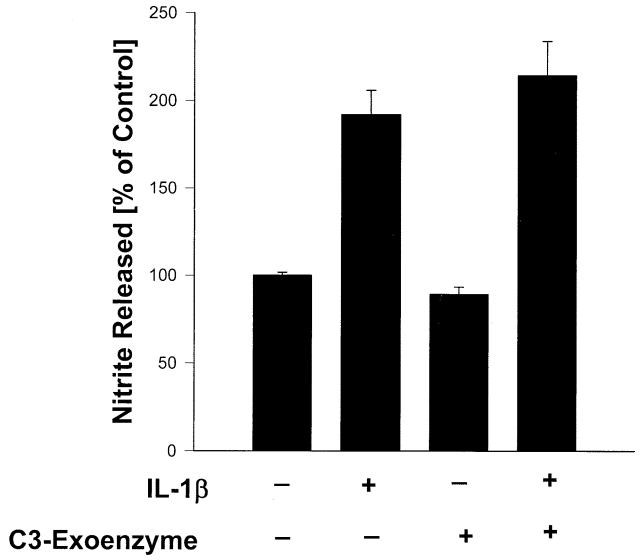

Fig. 7. Lack of effect of C3-exoenzyme on IL-mediated nitrite release from HIT-T15 cells. HIT cells were scrape-loaded with diluent or C3-exoenzyme (see "Materials and methods"), and NO release from these cells was monitored following incubation with IL-1β (300 pM; 24 hr) or diluent. Data are the means ± SEM from three experiments carried out in triplicate. The amount of NO released from control cells (expressed as 100%) corresponded to 5.79 μM.

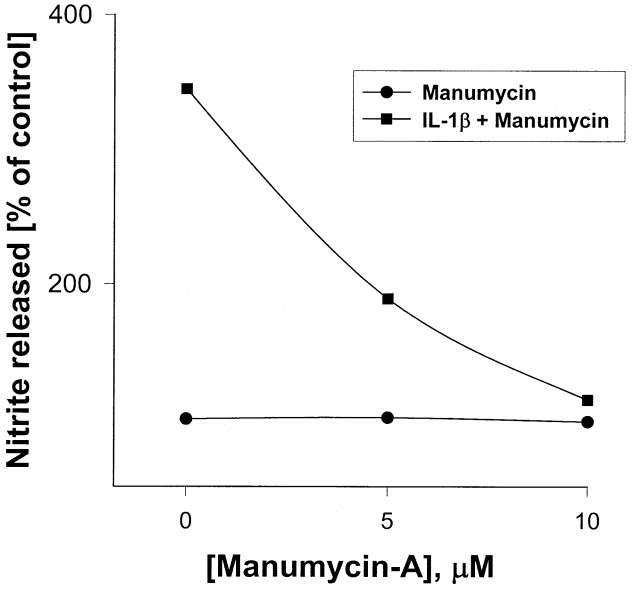

Fig. 9. Effect of manumycin A, a Ras inhibitor, on IL-1β-induced NO release from HIT-T15 cells. HIT cells were incubated in the absence or presence of increasing concentrations (0–10 μM) of manumycin-A or manumycin-A + IL-1β (300 pM). The NO released was quantitated as described under "Materials and methods." The amount of NO released from the control cells (expressed as 100%) corresponded to 3.2 μM. Data are representative of three experiments with a comparable degree of inhibition.

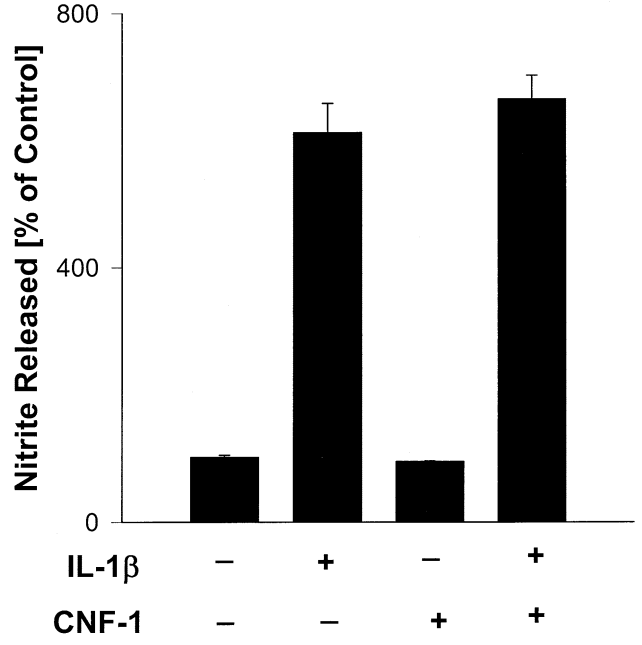

Fig. 8. Lack of effect of CNF-1, a Rho activating protein, on IL-mediated NO release from HIT-T15 cells. HIT cells were cultured with CNF-1 (200 ng/mL; 24 hr) and then were incubated with IL-1β (300 pM; 24 hr) or diluent; the NO released was measured in the control and IL-1β-treated cells. Data are the means ± SEM from two experiments carried out in triplicate. The amount of NO released from the control cells (expressed as 100%) corresponded to 7.4 μM.

3.4. Inhibition of IL-induced NO release from HIT-T15 cells by two structurally dissimilar inhibitors of Ras function

To further examine the role of Ras in IL-1β-mediated NO release, we studied the effects of two structurally dissimilar, but specific inhibitors of Ras function (Table 1). Data in Fig. 9 indicate that manumycin A inhibited IL-1β-mediated NO release from these cells in a concentration-dependent manner. Half-maximal inhibition was demonstrable at 5–10 μM manumycin A, compatible with published values of its inhibitory potency [17]. A similar degree of inhibition of IL-1β-mediated NO release from HIT cells was also demonstrable (Fig. 10) in the presence of damnacanthal, another inhibitor of Ras function [17]. Together, the toxin and inhibitor data indicate that Ras activation is necessary for IL-1β-mediated NO release from isolated β cells.

3.5. Lack of evidence for the induction of Ras expression by IL-1β in HIT cells

In these studies, we examined the possibility as to whether the IL-mediated effects involve induction of Ras in HIT-T15 cells. For this purpose, HIT cells were incubated with IL-1β (300 pM for up to 48 hr), and the amount of Ras in cell lysates was determined by western blotting. Data in Fig. 11 indicate no clear effects of IL-1β on H-Ras expres-

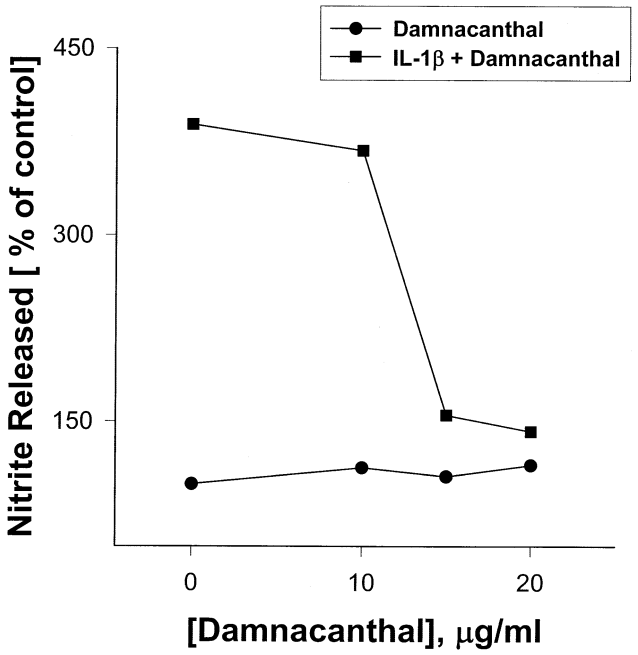

Fig. 10. Effect of damnacanthal, an inhibitor of Ras function, on IL-1β-induced NO release from HIT-T15 cells. HIT cells were incubated in the absence or presence of increasing concentrations (0–20 μg/mL) of damnacanthal or damnacanthal + IL-1β (300 pM). The NO released was quantitated as described under "Materials and methods." The amount of NO released from the control cells (expressed as 100%) corresponded to 4.1 μM. Data are representative of three experiments with a comparable degree of inhibition.

sion in these cells. Other isoforms of Ras (e.g. K-Ras) were not identifiable in these cells under the current set of experimental conditions. These data suggest that the IL-mediated effects may not involve an increase in the expression of H-Ras. However, we cannot rule out the possibility of Ras activation by IL-1β via other mechanisms. For example, it is likely that IL treatment of HIT cells might result in Ras activation, presumably involving post-translational mechanisms, including nitrosylation [32] and/or activation of other Ras regulatory proteins (e.g. guanine nucleotide releasing factor) that have been identified in β cells recently ([33]; see below). In conclusion, based on data using specific chemical inhibitors of Ras function and select bacterial toxins, we provide evidence that Ras, but not Rap, Rac, Rho, or Cdc42, is involved in this signaling cascade involved in IL-1β-mediated NO release from HIT-T15 cells.

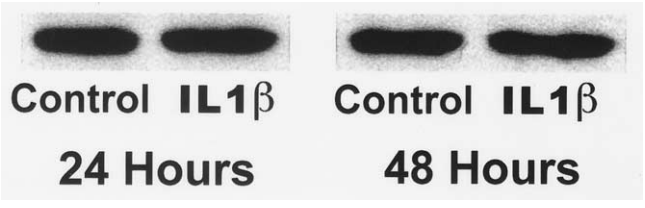

Fig. 11. Lack of stimulatory effects of IL-1β on Ras expression. Control and IL (300 pM)-treated (24 or 48 hr) HIT cell lysates were separated by SDS–PAGE and probed for the localization of H-Ras using a polyclonal antiserum directed against H-Ras (see "Materials and methods"). Data are representative of at least three experiments, with identical results.

4. Discussion

The primary objective of the current study was to examine the putative roles of the Rho subfamily of G-proteins in IL-1β-induced NO release from isolated β cells. To address this issue, using two distinct experimental approaches, i.e. bacterial toxins and specific chemical inhibitors of G-protein function, we studied the effects of IL-1β in cells where specific G-protein functions have been compromised. We chose NO release as the endpoint of our study, since it would offer a "window of opportunity" for defining mechanisms for reversing the noxious effects of IL-1β on pancreatic β cells. Our findings provide the first evidence to suggest that inactivation of Ras prior to IL-1β binding to its receptor results in inhibition of NO release from HIT-T15 cells.

Our current observations indicate that Ras plays key functional roles in IL-1β-mediated release of NO since inhibition of its function by two independent methods (i.e. bacterial toxins and chemical inhibitors) completely prevented IL-1β-induced NO release from isolated β cells. Based on our current data, we propose that Ras plays a positive modulatory role in IL-1β-mediated NO release from HIT-T15 cells. Putative contributory roles of this G-protein in IL-mediated dysfunction and, subsequently, the demise of the pancreatic β cell remain to be studied. Data derived from experiments involving LT, C3-exoenzyme, and CNF-1 indicated that Ras and Rac, but not Rap, Cdc42, or Rho, are involved in IL-1β-mediated NO release. To further determine whether Ras and Rac are involved in this signaling cascade, we studied the effects of toxin-B which specifically modifies Rac, Cdc42, and Rho on IL-mediated NO release. Data from those experiments revealed no measurable effects of toxin-B, suggesting that Rac activation may not be necessary for IL-mediated NO release. Further studies using two structurally different inhibitors of Ras function (e.g. manumycin A and damnacanthal) identified Ras as one of the key G-proteins involved in IL-mediated release of NO from HIT-T15 cells.

In this context, it may be germane to mention that several recent studies, including our own, have demonstrated that long-term inactivation of G-proteins by selectively impeding their isoprenylation with lovastatin, an HMG-CoA-reductase inhibitor, results in apoptotic cell death [12,34,35]. It may be pointed out that besides Rho G-proteins, certain nuclear proteins (e.g. lamin-B) have also been shown to undergo PTMs, including isoprenylation and carboxyl methylation. In the case of lamins, such modifications promote their association with nuclear components to facilitate nuclear envelope assembly [36]. Therefore, inhibition of these modification steps of lamins (i.e. inhibitors of isoprenylation, such as lovastatin or inhibitors of methylation,

such as methyl thioadenosine) results in disassembly of nuclear envelope [36]. This, in turn, facilitates the proteolysis of "death substrates" such as poly(ADP-ribose) polymerase (PARP) and lamins to "initiate" the process of chromatin condensation and DNA degradation, culminating in apoptotic cell death [37]. These data clearly indicate that PTMs of some of these relevant proteins are crucial for normal cellular function.

Our current studies examined the roles of Rho G-proteins in IL-1β-mediated NO release. Admittedly, these are very early steps in the cascade of events leading to IL-1β-mediated dysfunction and demise of the pancreatic β cell. Our findings identify at least one of presumably several loci at which these signaling proteins could exert potential regulatory roles in cytokine-mediated β cell dysfunction. Rho G-proteins (e.g. Cdc42 and Rac) have been implicated in both mitogenic and apoptotic pathways [38–40]. These proteins are known to trigger activation of the mitogen-activated protein kinase pathways, namely of the C-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK). Recent evidence also indicates that this event is mediated by specific Rac/Cdc42 effectors, such as the p21Rac/Cdc42-activated kinases [41,42]. In the case of apoptotic regulation of low molecular mass G-proteins, a recent study by Gulbins and coworkers [14] showed that fas- and ceramide-mediated apoptosis in human leukemic Jurkat cells is effected by Rho subfamily G-proteins, especially Rac and Ras. Their data suggested simultaneous activation of these G-proteins and Jun N-terminal kinase/p38 kinase cascade. Activation of these G-proteins appears to be a key component of this cascade since fas- and ceramide-mediated apoptosis was prevented by inactivation of these G-proteins [14,43]. Interestingly, activation of these G-proteins was followed by their translocation to the cytoskeletal fraction. These data strongly suggest that activation of specific G-proteins of the Rho subfamily is necessary for causing cell death and that such activation may be downstream to ceramide generation. It must be emphasized that while there is a general consensus that Rho subfamily G-proteins are involved in cytokine/fas/ceramide-mediated apoptosis, the exact loci where these G-proteins might exert their modulatory effects still remain poorly understood. For example, Rho G-proteins have been shown to stimulate arachidonic acid generation, presumably via activation of cytosolic phospholipase A_2 [44], and its subsequent activation of sphingomyelinase, which generates ceramides from sphingomyelin. Therefore, it seems likely that these G-proteins may play regulatory roles at multiple loci in bringing about changes within the cell to cause β cell dysfunction. Furthermore, these data appear to contrast with observations of Gómez *et al.* [45], who described that activation of Rho prevents apoptosis through Bcl-2 expression in murine T cells. Therefore, these G-proteins may subserve the functions of apoptosis suppressors as well as inducers. Furthermore, the identity, the nature, and the exact loci of action of these G-proteins seem to be different among cells and the type of apoptotic stimulus studied. We have demonstrated earlier that long-term inactivation of specific G-proteins either by depletion of GTP or by inhibition of PTMs (using lovastatin) results in the apoptotic demise of isolated β cells. These studies do not identify a locus at which Ras might exert its action leading to inhibition of NO release. Recent studies by Palsson *et al.* [17] have shown that selective inhibition of Ras function by the use of bacterial toxins or by chemical inhibitors markedly reduces the ability of Ras to activate the mitogen-activated protein (MAP) kinase pathway. It is likely that the MAP kinase pathway is involved in the IL-1β-mediated generation of NO since recent data from the laboratory of Newgard have indicated a MAP kinase activation step in IL-1β-mediated β cell dysfunction [46].

Finally, our western blot data indicate localization of H-Ras, but not K-Ras in HIT-T15 cells. Furthermore, we have obtained no supporting evidence in favor of induction of Ras protein by IL-1β. However, it still is likely that IL-1β could contribute to Ras activation by other mechanisms, including its nitrosylation [32] and increase in its post-translational C-terminal cysteine modification (e.g. farnesylation) and redistribution within the cellular compartments of the β cell [9–12,18–20,36]. Other activation mechanisms include functional regulation of Ras regulatory proteins, such as guanine nucleotide release factors that have been localized within the β cell recently [33]. These studies are currently in progress in our laboratory.

In conclusion, our data provide evidence, for the first time, that Ras activation is an obligatory step in IL-1β-mediated NO release and, presumably, the subsequent dysfunction of the pancreatic β cell. It is likely that activation of other G-proteins is downstream to the site of regulation by Ras. Clearly, our current data provide a basis for our ongoing investigations to understand the mechanism of cytokine-induced β-cell death, leading to the onset of insulin-dependent diabetes mellitus.

Acknowledgments

This research was supported by grants (to A.K.) from the Department of Veterans Affairs (Merit Review and Research Enhancement Award Program), the National Institutes of Health (DK-56005–01), the American Diabetes Association, and the Burroughs Wellcome Foundation. We thank Naimei Tang and Hai-Qing Chen for skillful technical assistance. A portion of these studies was presented at the 60th Annual Meeting of the American Diabetes Association in San Antonio, TX, in June 2000.

References

[1] Bach JF. Insulin-dependent diabetes mellitus as an autoimmune disease. Endocr Rev 1994;15:516–42.

[2] Rabinovitch A, Suarez-Pinzon WL. Cytokines and their roles in pancreatic islet β-cell destruction and insulin-dependent diabetes mellitus. Biochem Pharmacol 1998;55:1139–49.
[3] Saldeen J. Cytokines induce both necrosis and apoptosis via a common Bcl-2-inhibitable pathway in rat insulin-producing cells. Endocrinology 2000;141:2003–10.
[4] Beggs M, Beresford G, Clarke J, Mertz R, Espinal J, Hammonds P. Interleukin-1β inhibits glucokinase activity in clonal HIT-T15 β-cells. FEBS Lett 1990;267:217–20.
[5] Metz S, Meredith M, Rabaglia M, Corbett J. Dual functional effects of interleukin-1β on purine nucleotides and insulin secretion in rat islets and INS-1 cells. Diabetes 1996;45:1783–91.
[6] Borg LA, Calgliero E, Sandler S, Welsh N, Eizirik DL. Interleukin-1β increases the activity of superoxide dismutase in rat pancreatic islets. Endocrinology 1992;130:2851–7.
[7] Subauste MC, Herrath M, Bernard V, Chamberlain CE, Chuang TH, Chu K, Bokoch GM, Hahn K. Rho family proteins modulate rapid apoptosis induced by cytotoxic T lymphocytes and Fas. J Biol Chem 2000;275:9725–33.
[8] Wong GHW, Goeddel DV. Induction of manganous superoxide dismutase by tumor necrosis factor: possible protective mechanism. Science 1988;242:941–4.
[9] Kowluru A, Robertson RP, Metz SA. GTP-binding proteins in the regulation of pancreatic β-cell function. In: Leroith D, Taylor SI, Olefsky JM, editors. Diabetes mellitus, a fundamental and clinical text. Philadelphia: Lippincott Williams & Wilkins, 2000. p. 78–94.
[10] Kowluru A, Li G, Rabaglia M, Segu V, Hofmann F, Aktories K, Metz S. Evidence for differential roles of the Rho subfamily of GTP-binding proteins in glucose- and calcium-induced insulin secretion from pancreatic β cells. Biochem Pharmacol 1997;54:1097–108.
[11] Kowluru A, Seavey S, Li G, Sorenson RL, Weinhaus AJ, Nesher R, Rabaglia ME, Vadakekalam J, Metz SA. Glucose-, and GTP-dependent stimulation of the carboxyl methylation of CDC42 in rodent, and human pancreatic islets, and pure β cells, evidence for an essential role of GTP-binding proteins in nutrient-induced insulin secretion. J Clin Invest 1996;98:540–55.
[12] Li G, Segu VB, Rabaglia ME, Luo RH, Kowluru A, Metz SA. Prolonged depletion of guanosine triphosphate induces death of insulin-secreting cells by apoptosis. Endocrinology 1998;139:3752–62.
[13] Metz SA, Kowluru A. Inosine monophosphate dehydrogenase: a molecular switch integrating pleiotropic GTP-dependent β-cell functions. Proc Assoc Am Physicians 1999;111:335–46.
[14] Gulbins E, Brenner B, Koppenhoefer U, Linderkamp O, Lang F. Fas or ceramide induce apoptosis by Ras-regulated phosphoinositide-3-kinase activation. J Leukoc Biol 1998;63:253–63.
[15] Lerm M, Selzer J, Hoffmeyer A, Rapp UR, Aktories K, Schmidt G. Deamidation of Cdc42 and Rac by *Escherichia coli* cytotoxic necrotizing factor 1: activation of c-Jun N-terminal kinase in HeLa cells. Infect Immun 1999;67:496–503.
[16] Von Eichel-Streiber C, Boquet P, Thelestam M. Large clostridial cytotoxins—a family of glycosyltransferases modifying small GTP-binding proteins. Trends Microbiol 1996;4:375–82.
[17] Palsson E, Popoff M, Thelestam M, O'Neil L. Divergent roles for Ras and Rap in the activation of p38 mitogen-activated protein kinase by interleukin-1. J Biol Chem 2000;275:7818–25.
[18] Kowluru A, Metz SA. GTP and its binding proteins in the regulation of insulin exocytosis. In: Draznin B, LeRoith D, editors. Molecular biology of diabetes. Tottawa, NJ: Humana Press, 1994. p. 249–83.
[19] Kowluru A, Li G, Metz SA. Glucose activates the carboxyl methylation of γ subunits of trimeric GTP-binding proteins in pancreatic β cells. Modulation in vivo by calcium, GTP, and pertussis toxin. J Clin Invest 1997;100:1596–610.
[20] Kowluru A, Metz SA. Subcellular distribution and posttranslational modifications of GTP-binding proteins in insulin-secreting cells. Methods Neurosci 1996;29:298–318.
[21] Metz SA, Rabaglia ME, Stock JB, Kowluru A. Modulation of insulin secretion from normal rat islets by inhibitors of the post-translational modifications of GTP-binding proteins. Biochem J 1993;295:31–40.
[22] Popoff MR. Purification and characterization of *Clostridium sordellii* lethal toxin and cross-reactivity with *Clostridium difficile* cytotoxin. Infect Immun 1987;55:35–43.
[23] Falzano L, Fiorentini C, Donelli G, Michel E, Kocks C, Cossart P, Cabanie L, Oswald E, Boquet P. Induction of phagocytic behavior in human epithelial cells by *Escherichia coli* cytotoxic necrotizing factor type 1. Mol Microbiol 1993;9:1247–54.
[24] Flatau G, Lemichez E, Gauthier M, Chardin P, Paris S, Fiorentini C, Boquet P. Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine. Nature 1997;387:729–33.
[25] Fiorentini C, Fabbri A, Flatau G, Donelli G, Matarrese P, Lemichez E, Falzano L, Boquet P. *Escherichia coli* cytotoxic necrotizing factor 1 (CNF1), a toxin that activates the Rho GTPase. J Biol Chem 1997;272:19532–7.
[26] Malcolm KC, Elliott CM, Exton JH. Evidence for Rho-mediated agonist stimulation of phospholipase D in rat1 fibroblasts. Effects of *Clostridium botulinum* C3 exoenzyme. J Biol Chem 1996;271:13135–9.
[27] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [^{15}N]-nitrate in biological fluids. Anal Biochem 1982;126:131–8.
[28] Bradford MM. A rapid, and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[29] Just I, Selzer J, Hofmann F, Green GA, Aktories K. Inactivation of Ras by *Clostridium sordellii* lethal toxin-catalyzed glucosylation. J Biol Chem 1996;271:10149–53.
[30] Popoff MR, Chaves-Olarte E, Lemichez E, von Eichel-Streiber C, Thelestam M, Chardin P, Cussac D, Antonny B, Chavrier P, Flatau G, Giry M, de Gunzburg J, Boquet P. Ras, Rap, and Rac small GTP-binding proteins are targets for *Clostridium sordellii* lethal toxin glucosylation. J Biol Chem 1996;271:10217–24.
[31] Ben El Hadj N, Popoff MR, Marvaud JC, Payrastre B, Boquet P, Geny B. G-protein-stimulated phospholipase D activity is inhibited by lethal toxin from *Clostridium sordellii* in HL-60 cells. J Biol Chem 1999;274:14021–31.
[32] Mallis RJ, Buss JE, Thomas JA. Oxidative modification of H-Ras: *S*-thiolation and *S*-nitrosylation of reactive cysteines. Biochem J 2001;355:145–53.
[33] Arava Y, Seger R, Walker MD. GRFβ, a novel regulator of calcium signaling, is expressed in pancreatic β cells, and brain. J Biol Chem 1999;274:24449–52.
[34] Wang IK, Lin-Shiau SY, Lin JK. Induction of apoptosis by lovastatin through activation of caspase-3 and DNase II in leukaemia HL-60 cells. Pharmacol Toxicol 2000;86:83–91.
[35] Tan A, Levrey H, Dahm C, Polunovsky VA, Rubins J, Bitterman PB. Lovastatin induces fibroblast apoptosis *in vitro* and *in vivo*. A possible therapy for fibroproliferative disorders. Am J Respir Crit Care Med 1999;159:220–7.
[36] Kowluru A. Evidence for the carboxyl methylation of nuclear lamin-B in the pancreatic β cell. Biochem Biophys Res Commun 2000;268:249–54.
[37] Lindahl T, Satoh MS, Poirier GG, Klungland A. Post-translational modification of poly(ADP-ribose) polymeRase induced by DNA strand breaks. Trends Biochem Sci 1995;20:405–11.
[38] Majumdar M, Seasholtz TM, Goldstein D, de Lanerolle P, Brown JH. Requirement for Rho-mediated myosin light chain phosphorylation in thrombin-stimulated cell rounding and its dissociation from mitogenesis. J Biol Chem 1998;273:10099–106.
[39] Kim BC, Yi JY, Yi SJ, Shin IC, Ha KS, Jhun BH, Hwang SB, Kim JH. Rac GTPase activity is essential for EGF-induced mitogenesis, Mol Cells 1998;28:90–5.

[40] Brown JH, Sah V, Moskowitz S, Ramirez T, Collins L, Post G, Goldstein D. Pathways and roadblocks in muscarinic receptor-mediated growth regulation. Life Sci 1997;60:1077–84.
[41] Coso A, Chiariello M, Yu J, Teramoto H, Crespo P, Xu N, Miki T, Gutkind J. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell 1995;81:1137–46.
[42] Minden A, Lin A, Claret F-X, Abo A, Karin M. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell 1995;81:1147–57.
[43] Subauste MC, von Herrath M, Bernard V, Chamberlain E, Chuang TH, Chu K, Bokoch GM, Hahn KM. Rho family proteins modulate rapid apoptosis induced by cytotoxic T lymphocytes and Fas. J Biol Chem 2000;13:9725–33.
[44] Kim B-C, Kim J-H. Exogenous C2-ceramide activates c-*fos* serum response element via Rac-dependent signalling pathway. Biochem J 1998;330:1009–14.
[45] Gómez J, Martinez-A C, González A, Garciá A, Rebollo A. The Bcl-2 gene is differentially regulated by IL-2, and IL-4: role of the transcription factor NF-AT. Oncogene 1998;17:1235–43.
[46] Chen G, Hohmeier HE, Gasa R, Vien Tran V, Newgard CB. Selection of insulinoma cell lines with resistance to interleukin-1β- and rquote 08γ-interferon-induced cytotoxicity. Diabetes 2000;49:562–70.