Ha-Ras is modified by isoprenoid on Cys\textsuperscript{186} and by reversibly attached palmitates at Cys\textsuperscript{181} and Cys\textsuperscript{184}. Ha-Ras loses 90% of its transforming activity if Cys\textsuperscript{181} and Cys\textsuperscript{184} are changed to serines, implying that palmitates make important contributions to oncogenicity. However, study of dynamic acylation is hampered by an absence of methods for acutely manipulating Ha-Ras palmitoylation in living cells. S-nitrosocysteine (SNC) and, to a more modest extent, S-nitrosogluthathione were found to rapidly increase \[^{[3]}\text{H}\]palmitate incorporation into cellular or oncogenic Ha-Ras in NIH 3T3 cells. In contrast, SNC decreased \[^{[3]}\text{H}\]palmitate labeling of the transferrin receptor and caveolin. SNC accelerated loss of \[^{[3]}\text{H}\]palmitate from Ha-Ras, implying that SNC stimulated deacylation and permitted subsequent reacylation of Ha-Ras. SNC also decreased Ha-Ras GTP binding and inhibited phosphorylation of the kinases ERK1 and ERK2 in NIH 3T3 cells. Thus, SNC altered two important properties of Ha-Ras activation state and lipidation. These results identify SNC as a new tool for manipulating palmitate turnover on Ha-Ras and for studying requirements of repalmitoylation and the relationship between palmitate cycling, membrane localization, and signaling by Ha-Ras.

Ha-Ras is a monomeric GTPase that has two types of lipid modifications, both of which must occur in order for the protein to bind efficiently to the plasma membrane (1–3). A C15 farnesyl isoprenoid is attached through a permanent thioether linkage to cysteine 186 at the C terminus (4–6). Farnesylation has been shown to be a prerequisite to, but by itself not sufficiently strong for, full Ha-Ras membrane binding (7–9). Ha-Ras requires a second lipid to stabilize its membrane interaction. This second lipid is the fatty acid palmitate (10), attached through thioester bonds to cysteine 181 or 184 (11, 12). Acylation of Ha-Ras is dynamic, with the palmitates having a half-life considerably shorter than the polypeptide and undergoing repeated cycles of removal and replacement (13, 14). Very little is known of how this S-acyl modification occurs or of its possible regulation, in part because purification of enzymes that might attach palmitate has proven to be difficult (15–18). Further, no structural motifs or signal sequences for palmitoylation have been identified (7). Only recently has a cytosolic acyl-protein thioesterase (APT1) been isolated that can remove palmitate from heterotrimeric G protein \(\alpha\)-subunits and from Ha-Ras \textit{in vitro} (19). Despite the transience of a thioacyl group, the presence of cysteines that can be palmitoylated dramatically increases the extent of farnesylated Ha-Ras membrane binding from \(\sim 10\%\) to \(\sim 95\%\) (7, 20).

For Ha-Ras, palmitoylation and plasma membrane targeting are clearly necessary for biological activity, because Ha-Ras mutants that lack both palmitates are poorly transforming in NIH 3T3 cells (7, 8, 20). Furthermore, an Ha-Ras that has only one site for palmitoylation is partially misdirected to internal membranes and is only weakly active (7). Thus, permanent interference with palmitoylation decreases Ha-Ras function. These results suggest that regulation of palmitoylation could provide a novel approach for controlling Ha-Ras oncogenicity.

There is growing evidence (11, 21–25) that acylation of a variety of signaling proteins, including Ha-Ras, may be important for targeting and organizing a portion of these proteins in specialized subdomains of membranes (“rafts,” caveoli, or detergent-resistant membranes). However, the relationships between palmitoylation, submembrane location, and signaling by any of these proteins (26) are difficult to study, since there are few techniques through which the acylation state of such signaling proteins can be varied.

In a small number of palmitoylated proteins, acylation can be regulated by agonist stimulation (27). Isoproterenol interaction with the \(\beta\)-adrenergic receptor results in activation of the receptor and of the \(G_\alpha\) subunit, and this activation correlates with increased palmitate turnover on both the receptor and the \(G_\alpha\) protein (28–32). Serotonin treatment of membranes derived from rat brain cells is reported to increase palmitate labeling of a number of G protein \(\alpha\)-subunits (\(G_{\alpha q}, G_{\alpha i}, G_{\alpha o}\), and \(G_{\alpha}^\prime\)) (33), and agonist-promoted palmitate exchange has been reported for \(G_\alpha\) (34). For these \(G_\alpha\) proteins, deacylation appears to be an important regulated step in changes that occur in response to ligand. In endothelial cells, a bradykinin-triggered increase in depalmitoylation of the endothelial nitric-oxide synthase has been observed, coincident with activation of the protein (35, 36). Thus, palmitoylation of some proteins is, through as yet unknown mechanisms, responsive to external signals. However, for Ha-Ras proteins, no changes in palmitoylation following the rapid and transient activation of growth factor-dependent pathways have been reported. The compound 2-bromo-palmitate has recently been reported to decrease palmitate labeling of the Src family kinases Fyn and Lck but had very little effect on membrane binding of an Ha-Ras protein (37).

Another agent that may affect protein palmitoylation is ni-
tric oxide (NO\(^{-}\) or its metabolites, NO\(_x\)). The nitrosothiol compound S-nitrosocysteine (SNC)\(^1\) has been reported to cause a decrease in palmitate incorporation into many proteins in neuronal cells (38). Two of these proteins were identified as the synaptic vesicle protein, SNAP-25, and the growth cone-associated protein, GAP-43. This work suggested that S-acylation of neuronal growth cone proteins could be manipulated by exogenous exposure of neurons to nitric oxide or NO\(_x\)-producing compounds.

Recent studies have reported that activation of Ras proteins can also be affected by nitric oxide. In the Jurkat T cell line, and especially in Jurkat cells depleted of glutathione by buthionine sulfoximine treatment, more of the endogenous Ras proteins were reported to be GTP-bound after exposure to nitric oxide (39). In vitro studies also indicated that the cysteine at position 118 becomes nitrosylated during treatment of recombinant Ha-Ras with nitric oxide or SNC (40). Additionally, in rat and mouse cortical neuron cultures, the endogenous Ras proteins were reported to bind increased amounts of GTP after exposure to NO\(_x\) donors or stimulation of nitric-oxide synthase activity by N-acetyl-L-aspartate (41). These results indicate that Ras itself and/or proteins that regulate its GTP state were susceptible to nitric oxide or its metabolites (42). However, no information on the effects of nitric oxide or other oxidants on Ha-Ras palmitoylation has been reported.

This study was designed to determine if effects of nitric oxide on protein palmitoylation could also be observed in cells other than neurons and specifically whether palmitoylation of the Ha-Ras protein could be influenced by compounds that produce nitric oxide or its metabolites. The results identify S-nitrosocysteine and characterize parameters for its effective use as the first method for deliberate, external manipulation of palmitoylation of Ha-Ras.

**MATERIALS AND METHODS**

**Cell Culture—** COS-1 cells or NIH 3T3 cells expressing oncogenic v-Ha-Ras\(^{Arg12, Thr20}\), v-Ha-Ras\(^{Ser61}\) (B. Willumsen, Copenhagen), cellular (wild-type) Ha-RasWT, or a mutated form of Ha-RasWT, Ha-Ras(C118S) (L. Quillard, Indianapolis) were grown at 10% CO\(_2\) in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum (Hyclone, Logan, UT). RAW 264.7 cells were cultured at 10% CO\(_2\) in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum (Hyclone, Logan, UT). RAW 264.7 cells were cultured at 10% CO\(_2\) in RPMI medium supplemented with 5% fetal calf serum (Hyclone, Logan, UT). RAW 264.7 cells were cultured at 10% CO\(_2\) in RPMI medium supplemented with 5% fetal calf serum (Hyclone, Logan, UT). RAW 264.7 cells were cultured at 10% CO\(_2\) in RPMI medium supplemented with 5% fetal calf serum (Hyclone, Logan, UT). RAW 264.7 cells were cultured at 10% CO\(_2\) in RPMI medium supplemented with 5% fetal calf serum (Hyclone, Logan, UT). RAW 264.7 cells were cultured at 10% CO\(_2\) in RPMI medium supplemented with 5% fetal calf serum (Hyclone, Logan, UT). RAW 264.7 cells were cultured at 10% CO\(_2\) in RPMI medium supplemented with 5% fetal calf serum (Hyclone, Logan, UT). RAW 264.7 cells were cultured at 10% CO\(_2\) in RPMI medium supplemented with 5% fetal calf serum (Hyclone, Logan, UT).

**Preparation of NO Donors and Oxidants—** A 100 mM S-nitrosocysteine solution was prepared less than 30 min before each experiment by solubilizing equimolar amounts of sodium nitrate and L-cysteine in water and adding concentrated hydrochloric acid to initiate formation and release of nitric oxide gas (43). Control, nitric oxide-depleted SNC was prepared by allowing a solution of SNC to stand at least 48 h at room temperature. The acidic SNC was neutralized with NaOH before the addition to cells, and, to maintain pH during the various treatments, supplementary buffer (50 mM HEPES, pH 7.2) was included in all media. Additional 4 mM SNC was added every 30 min unless otherwise noted. S-Nitrosoglutathione solution was made and used similarly, substituting reduced glutathione (Sigma) for L-cysteine. Stock solutions of SNC were made by dilution in dimethyl sulfoxide.

**Radiolabeling, Immunoprecipitation, and Immunoblotting—** Cells were labeled with 0.5–1 mCi/ml \[^{3}H\]palmitate (NEN Life Science Prod-

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\(^1\) The abbreviations used are: SNC, S-nitrosocysteine; SNAP, S-nitroso-N-acetylsalicylic acid; GST, glutathione S-transferase; RBD, Ras binding domain; PAGE, polyacrylamide gel electrophoresis; ERK, extracellular signal-regulated kinase; SNG, S-nitrosoglutathione; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; GAP, GAP-activating protein; WT, wild type.
RESULTS

S-Nitrosocysteine Treatment Leads to both Increases and Decreases in Protein Palmitoylation—Previous results had shown that palmitate incorporation into SNAP-25 and GAP-43 decreased when the neuronal cell line, PC-12, or primary cultures of rat dorsal root ganglion cells were treated with [3H]palmitate in the presence of S-nitrosocysteine (49). However, preliminary experiments indicated that SNC caused little effect on palmitate labeling of Ha-Ras61L or Ha-RasWT expressed in PC-12 cells (data not shown). To determine if SNC could alter protein palmitoylation in another cell type (NIH 3T3 fibroblasts), three different classes of palmitoylated fibroblast proteins were examined: the transferrin receptor, which is localized in endocytotic vesicles and clathrin-coated pits in the plasma membrane (50); caveolin, a second transmembrane protein, localized in caveolae (51); and cellular (wild-type) Ha-Ras (Ha-RasWT), which is plasma membrane-associated through hydrophobic lipid modifications (4, 7).

When NIH 3T3 cells stably expressing Ha-RasWT were treated with SNC, a small decrease in palmitate incorporation into the transferrin receptor and a more pronounced decrease in labeling of caveolin were detected (Fig. 1A). This effect was similar to the broad decrease in palmitate labeling caused by SNC in neuronal cells. In contrast, [3H]palmitate incorporation into Ha-RasWT was increased by SNC treatment. Cells treated with NO-depleted SNC showed no changes in Ha-RasWT palmitoylation (data not shown), indicating that neither the cystine nor other components formed as nitric oxide is released were responsible for the observed effects on palmitoylation. No change in the amount of [3H]palmitate that entered cells or [3H]palmitoyl CoA that was produced had been observed previously in the SNC-treated neurons (49). The current results further confirmed that changes in protein palmitoylation did not arise from a general effect of NO on uptake of radioactive fatty acid or its activation to acyl-CoA, since two proteins responded to SNC with decreased labeling while a third, Ha-RasWT, showed increased labeling. These results demonstrated that SNC could alter protein palmitoylation in NIH 3T3 cells and indicated that specific proteins showed distinct responses to this compound.

S-Nitrosoglutathione Also Increases Ha-Ras Palmitoylation Incorporation—To determine if other nitric oxide producers or oxidants might also be useful agents for study of palmitoyl turnover on Ha-Ras, NIH 3T3 cells expressing Ha-RasWT were labeled for 1 h with [3H]palmitate and simultaneously treated with NO-depleted (C) or 4 mM freshly prepared SNC. The endogenous transferrin receptor, caveolin, or transfected Ha-RasWT proteins were isolated by immunoprecipitation, separated by SDS-PAGE, and transferred to poly(vinylidene d difluoride) membranes. Membranes were exposed to film for 69 days (transferrin receptor) or 28 days (caveolin and Ha-RasWT). Recovery of an equivalent amount of protein under both conditions was monitored after film exposure by detecting each protein on the membrane by immunoblotting with appropriate antibodies. B, NIH 3T3 cells expressing Ha-RasWT or v-Ha-Ras were labeled for 30 min with [3H]palmitate in medium containing NO-depleted SNC (C), 4 mM SNC, 4 mM SNAP, 1.1 mM SNAP, 4 mM SNAP, 2 mM SIN-1, 2 mM SNAP, 1.1 mM SNAP, and 200 mM H2O2. Ha-Ras was immunoprecipitated, resolved by SDS-PAGE, and detected by fluorographic exposure for 7 days (upper panel). Palmitate incorporation was corrected for variations in protein recovery as quantified by immunoblotting membranes after film exposure (lower panel). Numbers below each lane are averages of three (SNC), or two (all others) determinations of protein-corrected [3H]palmitate incorporation expressed relative to that of Ha-Ras from control (C) cells. C. NIH 3T3 cells expressing v-Ha-Ras were labeled for 1 h with [3H]palmitate in medium containing NO-depleted SNC (C), 4 mM freshly prepared SNC, or 4 mM SNG. v-Ha-Ras was immunoprecipitated, resolved by SDS-PAGE, and detected by fluorographic exposure for 21 days. Protein recovery was monitored by immunoblotting with antibody 3E4–146. D, NIH 3T3 cells expressing v-Ha-Ras were treated for 30 min with either NO-depleted SNC (C) or 4 mM freshly made SNC. Subcellular fractions were prepared; equal portions of the total (T), soluble (S), and particulate (P) fractions were resolved by SDS-PAGE, and v-Ha-Ras was detected by immunoblotting. Data are representative of three different experiments.
through a cGMP-dependent pathway, cells expressing Ha-RasWT were labeled with [3H]palmitate and simultaneously treated with 8-bromo-cyclic GMP. No change in palmitate incorporation was detected in cells treated with 8-bromo-cyclic GMP for 30 min (Fig. 1B) or in two additional experiments that tested the effect of 8-bromo-cyclic GMP on palmitate incorporation over a 2-h time course (data not shown). This implied that the observed increase in palmitate incorporation caused by SNC occurred independently of possible effects of SNC on guanylyl cyclase.

Characteristics of SNC-triggered Increases in Ha-Ras Palmitate Incorporation—To further characterize the effect of SNC on Ha-Ras palmitoylation, cells expressing Ha-RasWT were labeled with [3H]palmitate and simultaneously treated with a broad range of concentrations of SNC in the presence of 10% calf serum. Serum was included in the medium during SNC treatment because it contains growth factors whose removal would affect Ras-GTP binding and conformation and because this better replicates the in vivo conditions in which cells in tissues will encounter nitric oxide. However, serum proteins (e.g. serum albumin) can be nitrosylated and delayed entry or reduce the amounts of NO available to enter the cells (54, 55). Millimolar amounts of SNAP have been reported to be needed to produce micromolar amounts of free NO in intact cells (56). Therefore, SNC concentrations ranging from micromolar to millimolar were tested.

Using micromolar amounts of SNC, palmitate incorporation into Ha-RasWT was unaffected (neither increased nor decreased) after a 30-min exposure (Fig. 2). Between 1 and 2 mM SNC a stimulatory effect on palmitate labeling of Ha-RasWT became observable. At 4 mM SNC, palmitate incorporation was an average of 3.5-fold higher than in untreated cells. In three additional experiments, a 1-h treatment with 4 mM SNC induced increases in Ha-RasWT palmitate incorporation that ranged from 2.5- to 6.1-fold (data not shown). At a concentration of 8 mM, Ha-RasWT palmitate labeling was increased 3-fold at 15 min, but loss of cellular adhesion began to occur by 30 min, and additional samples were not collected. These data suggested that SNC could be used most effectively at 4 mM to induce increased Ha-Ras palmitate incorporation.

To determine how quickly SNC affected palmitoylation and if these changes were transient or sustained, cells expressing Ha-RasWT were again labeled in the presence of 4 mM SNC. In control, untreated cells, Ha-RasWT [3H]palmitate labeling was detectable within 15 min and reached maximal [3H]palmitate incorporation by 1–2 h. SNC treatment produced a rapid increase in Ha-RasWT [3H]palmitate labeling, doubling the amount of [3H]palmitate incorporated at the earliest time point, 15 min (Fig. 3A) and reaching maximal incorporation by 1 h. After 2 h of treatment, the amount of radioactive [3H]palmitate still present on Ha-RasWT in the SNC-treated cells diminished, so that control samples and SNC-treated samples showed nearly equal amounts of total [3H]palmitate attached. This decrease did not reflect death of the treated cells, because cells showed no loss of viability after a 2-h exposure if SNC-containing medium was replaced with regular growth medium (data not shown). In addition, the cellular signaling pathway involving the ERK proteins could regain activity after SNC exposure (see below).

To examine in more detail the ability of SNC to stimulate palmitoylation of an activated, oncogenic Ras protein, cells expressing v-Ha-Ras(Arg12, Thr59) were labeled in the presence of 4 mM SNC. As with Ha-RasWT, SNC noticeably increased the amount of [3H]palmitate incorporated into the v-Ha-Ras protein (Fig. 3B). The increase over controls was rapid, doubling the amount of [3H]palmitate incorporated within 15 min. Compared with Ha-RasWT, [3H]palmitate labeling into control v-Ha-Ras appeared to reach maximal incorporation more rapidly (within 30 min). For the SNC-treated cells, [3H]palmitate incorporation again declined after reaching a maximum between 30 and 60 min. Additional experiments showed SNC-induced increases in maximal v-Ha-Ras palmitate incorporation ranging from 1.7- to 4.5-fold. Thus, the effects of SNC on palmitoylation were not limited to cellular Ha-Ras. SNC could be used to increase the rate of [3H]palmitate labeling of both Ha-RasWT and oncogenic v-Ha-Ras within minutes, and this effect could be sustained for at least 1 h.

SNC Increases Depalmitoylation of Ha-Ras—An increase in [3H]palmitate labeling can reflect attachment of [3H]palmitate...
to previously unmodified cysteines on mature Ha-Ras or accelerated removal of unlabeled palmitates with rapid replacement by \(^{[3]H}\)palmitates. The use of cycloheximide during the labeling periods prevented synthesis of Ha-Ras with newly created nonacylated cysteines. However, the stoichiometry of Ha-Ras palmitoylation is unknown, so it was unclear if mature Ha-Ras with one nonacylated cysteine might be present and be a possible source of the available sites for increased palmitoylation. A pulse-chase technique was therefore used to determine if turnover of previously attached palmitates remained constant or if SNC altered the rate of Ha-Ras depalmitoylation.

Using Ha-Ras that was prelabeled with \(^{[3]H}\)palmitate, a gradual loss of radioactivity from control samples was observed. However, in Ha-Ras from SNC-treated cells, less \(^{[3]H}\)palmitate remained at each time point. This was true for Ha-RasWT and both the phosphorylated and unphosphorylated forms of v-Ha-Ras (Fig. 4A). S-Nitrosglutathione also stimulated removal of \(^{[3]H}\)palmitate from Ha-RasWT (data not shown) and thus mimicked SNC in this effect. These results indicated that SNC hastened removal of \(^{[3]H}\)palmitate from already acylated Ha-Ras protein. In the previous labeling experiments, it appeared that during short exposure times SNC accelerated removal of unlabeled palmitates, which were rapidly replaced with \(^{[3]H}\)palmitate, leading to the observed increase in palmitate labeling. It was possible that the decline in radioactive \(^{[3]H}\)palmitate attached to SNC-treated Ha-Ras that was seen after longer SNC exposures (see Fig. 3) was the result of continued SNC-stimulated removal of palmitates (by then largely \(^{[3]H}\)palmitates) that was no longer matched by replacement with radioactive lipid. SNC thus appeared to be able to increase Ha-Ras depalmitoylation for 1–2 h and perhaps longer.

To examine in more detail the kinetics of both normal and SNC-stimulated palmitate removal, the data from multiple pulse-chase experiments were analyzed. In control, untreated cells, two phases of loss of \(^{[3]H}\)palmitate from Ha-RasWT could be detected (Fig. 4B). A more rapid rate predominated during the early stages (0–30 min) of the chase procedure. A second, slower phase of deacylation also appeared to be occurring during this time and became more evident at longer time points (1–24 h). It was necessary to follow deacylation for over 12 h in order to observe a decline of greater than 80% of the initial \(^{[3]H}\)palmitate levels. Because of the compound nature of the two overlapping deacylation curves, simple visual extrapolation of palmitate loss to the time when 50% of the initial amount was removed was difficult. In addition, a single phase exponential decay curve fit the data poorly (data not shown; note that the graphs have linear y axes, not logarithmic). Similar complex kinetics of palmitate removal have also been reported by others (13). The data were therefore analyzed using a double exponential decay model. The calculations for a pulse-chase experiment using control, untreated cells indicated that 50% of the \(^{[3]H}\)palmitate initially present in Ha-RasWT would be lost in 2.8 h if all loss occurred at the faster rate of palmitate removal.

Because Ha-Ras has two sites for palmitoylation, it was of interest to learn if the two phases of palmitate removal occurred because of different rates of deacylation of Cys\(^{181}\) and Cys\(^{184}\). To test this possibility, a pulse-chase experiment was performed with a v-Ha-Ras(C181S) protein with only Cys\(^{184}\) available for palmitoylation. Loss of ~75% of \(^{[3]H}\)palmitate from the single Cys\(^{184}\) site occurred very rapidly, in less than 30 min, but the remaining radioactivity continued to show the slow phase of decline (Fig. 4B). This result agrees well with the
much faster deacetylation of Ha-Ras(Ser\textsuperscript{181}) observed in COS-1 cells (13). This experiment indicated that Cys\textsuperscript{184} was easily deacetylated when it was the only site for palmitoylation. Despite this very rapid palmitate removal even in untreated, control cells, the Ser\textsuperscript{181}, Cys\textsuperscript{184} protein, which is farnesylated, retained 50–70% of the membrane binding of fully lipitated v-Ha-Ras (Ref. 7 and data not shown). Because the farnesyl group of completely nonacylated v-Ha-Ras(Ser\textsuperscript{181}, Ser\textsuperscript{184}) is, by itself, unable to support more than 10% membrane binding (7, 20), it therefore appeared that Cys\textsuperscript{184} of v-Ha-Ras(Ser\textsuperscript{181}, Cys\textsuperscript{184}) must be repalmitoylated rapidly and continuously in order to sustain this significant level of membrane attachment. Further advances in detection techniques for palmitates will be needed to distinguish if Cys\textsuperscript{181} and Cys\textsuperscript{184} might have different but swift rates of deacetylation.

For the current studies, however, it was important that deacetylation of Cys\textsuperscript{184} continued to show the prolonged phase of decline in \textsuperscript{[\textit{H}]palmitate. This suggested that the later phase of decrease was likely to reflect repeated removal of \textsuperscript{[\textit{H}]palmitates and replacement with lipids of lower specific activity. Similar to the Ha-RasWT from control cells, the Ha-RasWT and v-Ha-Ras proteins from SNC-treated cells showed both phases of decline in \textsuperscript{[\textit{H}]palmitate (data not shown). Therefore, further analysis on the effect of SNC on deacetylation was focused on the initial removal of palmitates during the faster, early phase within the first 2 h.

For Ha-RasWT, SNC treatment decreased the calculated rate at which 50% of the palmitate would be removed at the faster rate from an average of 2.8 h to 0.4 h (Fig. 4C). SNC treatment thus caused an ~6-fold acceleration in this rate of deacetylation of Ha-RasWT. Depalmitoylation of v-Ha-Ras was also stimulated by SNC. The calculated rate at which 50% of the palmitate would be removed at the faster rate for v-Ha-Ras in control cells was 1.2 h. SNC accelerated this rate of deacetylation by approximately 4-fold, with a calculated loss of half of the original \textsuperscript{[\textit{H}]palmitate in 0.3 h (Fig. 4C). The ~2-fold faster basal rate of deacetylation of v-Ha-Ras (1.2 h) compared with Ha-RasWT (2.7 h) was reproducible and was most closely related to the activation state of Ha-Ras.\textsuperscript{2} Thus, SNC accelerated palmitate removal from both Ha-RasWT and v-Ha-Ras by 4–6-fold. During SNC treatment, deacetylation of both cellular and oncogenic forms occurred at similar rates. From the pulse-chase and labeling data it therefore appeared that SNC caused substantial and sustained acceleration of the deacetylation of both Ha-RasWT and v-Ha-Ras proteins, followed by rapid readdition of palmitates to the newly available sites. These results therefore indicated that SNC significantly increased overall palmitate cycling on Ha-Ras.

Examination of v-Ha-Ras membrane binding from SNC-treated cells showed that 30 min of exposure did not cause a perceptible decrease in membrane attachment (Fig. 1D). These data provided a further indication that SNC-accelerated deacetylation was likely to be balanced by readdition of palmitates and that membrane binding was maintained during modest lengths of exposure.

Ha-Ras GTP Binding Is Decreased during SNC Treatment—
SNC’s ability to stimulate Ha-Ras deacetylation might arise either from an effect on the proteins that regulate attachment or removal of palmitate or through more direct actions on the Ha-Ras protein itself. The proteins that regulate Ha-Ras palmitoylation in the cell are not yet identified and are thus not available for study. However, exposure of recombinant Ha-Ras to nitric oxide \textit{in vitro} had been reported to directly modify (nitrosylate) cysteine 118 in the GTP-binding pocket of Ha-Ras (57), and this nitrosothiol modification has been suggested to increase Ha-Ras guanine nucleotide binding. Thus, it was possible that SNC-triggered modification of Ha-Ras, potentially through a change in GTP binding, might produce a conformational change that enabled more rapid deacetylation of the C terminus. This model was evaluated in two stages: testing whether SNC would cause a change in Ha-Ras GTP status in the setting of an intact NIH 3T3 cell and testing if an Ha-Ras(Ser\textsuperscript{118}) protein (which should be insensitive to this direct nitrosylation) would also show SNC-triggered changes in GTP binding or palmitoylation.

A fragment of Raf kinase that binds preferentially to GTP-Ras was used as an affinity reagent to isolate active Ha-Ras proteins from stimulated cells. Lysates were prepared from Ha-Ras61L-expressing NIH 3T3 cells that had been treated for 15 min with NO-depleted or freshly prepared 4 mM SNC. Glutathione-agarose beads with a bound fusion protein of GST-RBD of Raf kinase were added to the lysates, and the GTP-Ha-Ras proteins that bound to the RBD domain were isolated, displayed by SDS-PAGE, and detected by immunoblotting with an anti-Ha-Ras antibody. In contrast to the increase in GTP binding to Ha-Ras that had been reported, SNC caused a decrease (Fig. 5A, middle panel), as indicated by the lower amount of Ha-Ras61L captured by the RBD in the SNC-treated sample. The assay was then scaled up so that the smaller amounts of GTP-bound cellular Ha-RasWT could be detected. The results with Ha-RasWT were similar to those with Ha-Ras61L. SNC treatment led to a decrease in the amount of Ha-RasWT that interacted with the Raf RBD (Fig. 5A, upper panel).

To determine more directly if this decrease in RBD association reflected a SNC-triggered decrease in Ha-Ras GTP binding, NIH 3T3 cells expressing Ha-RasWT or v-Ha-Ras were labeled with \textsuperscript{[\textit{H}]inorganic phosphate and treated for 30 min with either NO-depleted or 4 mM fresh SNC. Ha-Ras proteins were immunoprecipitated, the bound nucleotides were released, and GTP and GDP were separated by thin layer chromatography (Fig. 5B). Radioactive GTP and GDP were then quantified by scanning the films after autoradiography (Fig. 5C). With either 0.1 or 1 mM SNC, v-Ha-Ras GTP binding remained high (~60%), as expected for an activated v-Ha-Ras protein, and, importantly, no increase in GTP binding was detected (data not shown). With 2 mM SNC, a decrease in v-Ha-Ras GTP binding (to ~40%) was observed. At 4 mM SNC, the same concentration that accelerated deacetylation, an approximately 3-fold decrease in the amount of the GTP-bound form of v-Ha-Ras was observed, from an average of 59% to 19% of the total v-Ha-Ras protein. SNC also decreased the amount of GTP bound to the cellular form Ha-RasWT from 9% of total Ha-RasWT to 3%. Thus, low concentrations of SNC failed to produce an increase in v-Ha-Ras GTP-binding and higher doses of SNC, similar to those that affected palmitate replacement, caused a decrease in GTP binding to either the cellular or an activated form of Ha-Ras. Therefore, the effects of SNC on Ha-Ras expressed in NIH 3T3 cells appeared to differ from the reported effects of nitric oxide on the endogenous Ras proteins of neuronal or T lymphoma cells.

To address if the SNC-triggered decrease in GTP binding, like the suggested NO-triggered increase, might occur through a mechanism that utilized nitrosylation of Cys\textsuperscript{118}, the ability of SNC to alter GTP binding to an Ha-Ras(C118S) protein was determined. The percentage of GTP-bound C118S protein was found to decrease from an average of 9% to 3% after SNC exposure (Fig. 5C). This decrease was similar to the decrease caused by SNC in Ha-RasWT. It is essential to note that these results do not mean that Cys\textsuperscript{118} was not being nitrosylated in

\textsuperscript{2} T. L. Baker and J. E. Buss, manuscript in preparation.
the previous experiments but do indicate that neither Cys\textsuperscript{118} nor its nitrosylation were required for SNC to cause decreased GTP binding on Ha-RasWT. Therefore, the mechanism through which SNC decreased GTP binding on Ha-Ras was likely to be from an effect of SNC on some other region of Ha-Ras or from SNC effects on the guanine nucleotide exchange factors (GEFs) or GTPase-activating proteins (GAPs) that regulate the Ha-Ras GTP state in NIH 3T3 cells. More importantly, these results showed that, without manipulation of cellular glutathione levels and in serum-containing medium, exposure of intact cells to SNC could alter GTP binding on either cellular or activated forms of Ha-Ras.

A Cysteine at Position 118 Is Not Required for SNC to Affect Ha-Ras Palmitoylation—Although Cys\textsuperscript{118} was not needed in order for SNC to cause decreased GTP binding, it might still be needed in order for SNC to affect palmitoylation. To test this possibility, palmitoylation of the C118S mutant was examined in SNC-treated cells. \( ^{3} \text{H} \) Palmitate labeling of C118S was increased 2–3-fold when cells were exposed to SNC (Fig. 6A). This stimulation of C118S palmitate labeling by SNC was almost identical to the increase caused by SNC on Ha-RasWT. Additionally, the decylation rate of C118S was also accelerated by SNC treatment (Fig. 6B). SNC therefore continued to produce effects on palmitate turnover in a mutant Ha-RasWT protein that lacked Cys\textsuperscript{118}. These data implied that the accelerated palmitate turnover caused by SNC was not a secondary effect of nitrosylation of Cys\textsuperscript{118}. Thus, neither the ability of SNC to decrease Ha-Ras GTP binding nor its acceleration of Ha-Ras depalmitoylation required Cys\textsuperscript{118} to be present.

SNC Inhibits Phosphorylation of ERK1 and ERK2 in NIH 3T3 Cells—From the above experiments, SNC could alter two important properties of Ha-Ras: its activation state and its lipidation. To examine if SNC also altered Ras signaling, the phosphorylation of the mitogen-activated protein kinases ERK1 and ERK2, a property that parallels activation of these kinases, was examined in SNC-treated NIH 3T3 cells. Relative changes in phosphorylation were detected using an antibody specific for the phosphorylated form, and in addition, the portion of total ERK1 and ERK2 that was phosphorylated was measured using antibody that detected both the unphosphorylated and phosphorylated forms that had been separated by SDS-PAGE. In the first experiments, untransfected NIH 3T3 cells, containing only endogenous cellular Ras proteins, were incubated in the absence of serum for 2 h to deactivate the ERK1/ERK2 proteins. The serum-free medium was then replaced with medium containing 10% bovine calf serum (the same amount present in the palmitoylation experiments). The control plates received medium that contained nitric oxide-depleted SNC solution. A second set of plates received a single treatment with medium containing freshly made SNC. A third set of plates received medium containing SNC and had additional SNC added every 30 min over the course of 2 h.

In the control cells, phosphorylation of both ERK1 and ERK2 was stimulated by the serum addition. This serum-stimulated

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**Fig. 5.** SNC decreases Ha-Ras GTP-binding. A, NIH 3T3 cells expressing either Ha-RasWT (top panel) or Ha-Ras61L (middle panel) or PC12 cells expressing Ha-Ras61L (bottom panel) were treated for 15 min with either NO-depleted SNC (C) or 4 mM freshly made SNC. As a positive control, one culture of Ha-RasWT-expressing cells was treated for 5 min with 50 ng/ml PDGF. Cells were lysed, and samples (500 μg of total protein) were incubated with a GST fusion protein of the RBD of Raf-kinase bound to glutathione agarose. Beads were washed, protein was resolved by SDS-PAGE, and bound Ras-GTP was detected with anti-Ras antibody. The data are representative of three separate experiments. B, NIH 3T3 cells expressing Ha-RasWT, v-Ha-Ras, or Ha-Ras(C118S) were labeled with \(^{32}\text{P}\) inorganic phosphate for 4 h and then treated with either NO-depleted SNC or 4 mM freshly made SNC for 15 min (for C118S) or 30 min. Ha-Ras proteins were immunoprecipitated, and the bound nucleotides were released, separated by thin layer chromatography, and detected by autoradiography. C, radioactive GTP and GDP were quantified by scanning films of TLC-separated nucleotides and calculating the percentage of total nucleotides that were GTP. The bars show average and range of two experiments. Black columns represent control samples, and gray columns represent SNC-treated samples.

**Fig. 6.** Cysteine at position 118 is not required for SNC to affect Ha-Ras palmitoylation in NIH 3T3 cells. A, NIH 3T3 cells expressing Ha-RasWT(C118S) were labeled for the times indicated with \(^{3} \text{H} \) palmitate in medium with no additions (—), with nitric oxide-depleted 4 mM SNC (C), or with fresh 4 mM SNC re-added every 30 min. Ha-Ras was detected by fluorographic exposure for 19 days and quantified as in Fig. 2. In graphs the hatched black line and diamonds indicate untreated samples; control samples treated with NO-depleted SNC are shown by the solid black line and squares; SNC-treated samples are designated by the gray line and triangles. B, NIH 3T3 cells stably expressing Ha-Ras(C118S) were labeled with \(^{3} \text{H} \) palmitate for 3 h and then incubated with medium containing 200 μM palmitate and either NO-depleted SNC (Control) or 2 mM freshly made SNC. SNC was added every 30 min. Ha-Ras was detected by fluorographic exposure for 7 days and quantified as in Fig. 2. The solid black line and squares represent the control samples, and the gray line and triangles depict the SNC-treated samples.
phosphorylation was maintained for the entire 2 h (Fig. 7A). However, in the SNC-treated cells, serum-stimulated ERK phosphorylation was inhibited. In cells that received a single addition of SNC, decreased phosphorylation of the ERK proteins could be detected within 5 min (data not shown). Phosphorylation of ERK1 and ERK2 was decreased by ~75% within 30 min and remained depressed for longer than 1 h. After 2 h, the effectiveness of SNC began to wane, and small amounts of phosphorylated ERK1 and ERK2 became detectable. In plates that received multiple additions of SNC (as in the palmitoylation experiments), a complete lack of ERK phosphorylation was maintained. Variation of the amount of SNC applied showed that inhibition of ERK1 and ERK2 required the same concentrations of SNC (2–4 mM) that were needed to increase Ha-RasWT palmitate turnover and decrease GTP binding (data not shown). Lower concentrations of SNC (0.1 and 1 mM) neither inhibited nor stimulated ERK1 or ERK2 phosphorylation in NIH 3T3 cells. SNC also inhibited ERK phosphorylation in growing NIH 3T3 cells that were not serum-starved, as well as in cells in which ERK phosphorylation was restimulated with the specific growth factor epidermal growth factor or platelet-derived growth factor rather than serum (data not shown). Thus, SNC interfered with both serum- and growth factor-induced activation of the ERK pathway in NIH 3T3 cells.

Inhibitory effects of a single addition of SNC were sustained for at least 1 h and could be maintained for longer periods by repeated additions.

In NIH 3T3 cells stably expressing larger amounts of Ha-RasWT, SNC also decreased serum-stimulated phosphorylation of ERK1 and ERK2 (Fig. 7A). However, in NIH 3T3 cells transformed by the oncogenic v-Ha-Ras protein, SNC failed to decrease ERK1 or ERK2 phosphorylation (Fig. 7A). This lack of effect of SNC on ERK phosphorylation in transformed cells indicated that SNC was not a direct chemical depressor of ERK phosphorylation. In addition, the distinct ERK responses of cells expressing Ha-RasWT and v-Ha-Ras, despite the similar SNC-triggered effects on Ha-Ras palmitoylation, suggested that SNC affected ERK phosphorylation and Ha-Ras palmitoylation through separate mechanisms.

The mechanism of SNC-triggered ERK inactivation was further examined to determine if the decrease in ERK1 and ERK2 phosphorylation reflected only decreased activation of the Ha-Ras/Raf/MEK/ERK pathway or also a SNC-triggered increase in activity of an ERK phosphatase. ERK phosphorylation was evaluated using NIH 3T3 cells expressing Ha-RasWT and the phosphatase inhibitor, sodium orthovanadate, to prevent removal of tyrosine phosphates from the ERK proteins. Successful inhibition of phosphatase activity by vanadate was demonstrated by elevation of ERK1 and ERK2 phosphorylation in the vanadate-treated cells (Fig. 7B). The addition of serum to the vanadate-treated cells produced the expected increased increase in ERK phosphorylation. In the vanadate-treated cells, SNC still diminished serum-stimulated ERK phosphorylation but importantly was not able to fully inhibit ERK phosphorylation, as it could in cells in which phosphatases remained active (Fig. 7B). Thus, when the activity of phosphatases was limited by vanadate, the inhibition of ERK phosphorylation caused by SNC was lessened. This suggested that vanadate-sensitive phosphatase(s) contributed to the decreased phosphorylation of ERK1 and ERK2 in SNC-treated cells.

To learn if SNC was distinct from nitric oxide in inhibiting phosphorylation seen in the vanadate-treated cells.

FIG. 7. SNC effects on ERK1/ERK2 are transient and vary in different cell types. A, untransfected NIH 3T3 cells were incubated in serum-free medium for 2 h and then incubated in fresh, serum-containing medium with either NO-depleted SNC (C) or 4 mM freshly prepared SNC. Every 30 min, additional fresh SNC was added to one set of plates (2×, 4×). No additional SNC was added to the single addition (1×) samples. NIH 3T3 cells expressing Ha-RasWT were also incubated in medium lacking serum for 2 h and then were treated for 30 min with fresh, serum-containing medium with either no addition (−), NO-depleted SNC (C), or fresh 4× mM SNC. Cells expressing v-Ha-Ras were treated similarly except without incubation in serum-free medium. Cells were lysed at the times indicated, proteins were separated by SDS-PAGE, and ERK proteins were detected by immunoblotting with an antibody (phospho-ERK antibody) that specifically recognizes the phosphorylated forms of ERK1 and ERK2 (upper panel) or an ERK antibody that recognizes both phosphorylated and unphosphorylated ERK1 and ERK2 proteins (total ERK antibody). The phosphorylated forms of ERK1 and ERK2 are indicated with arrowheads. B, NIH 3T3 cells expressing Ha-RasWT were incubated in medium lacking serum for 1 h, and then sodium vanadate (1 mM) was added to the indicated plates and incubation continued for an additional 1 h. Medium was replaced with medium containing 10% serum and, as indicated, vanadate, 4 mM NO-depleted SNC (−), or 4 mM fresh SNC, and samples were collected after 30 min. ERK phosphorylation was detected by immunoblotting as in A. COS1 cells were incubated in serum-free medium for 2 h and then treated for 30 min with fresh, serum-containing medium with either no addition (−), NO-depleted SNC (C), or fresh 4× mM SNC. Raw 264.7 and PC-12 cells were placed directly in fresh, serum-containing medium with the indicated additions. ERK1/ERK2 phosphorylation was detected as in A. Phosphorylated ERK2 in the COS1 monkey cells has a mobility equivalent to unphosphorylated ERK1, so only three bands are detected on the blot. Similar results were observed in multiple experiments (n > 3).
SIN-1 treatment, whereas palmitate labeling of the Gα protein did not appear to be a substrate for the APT1 thioesterase that can deacylate Ha-Ras (35). The dissimilarity in the effects of SNC on palmitate labeling of Ha-Ras (increase) and the transferrin receptor and caveolin (decreases) also indicates that not all palmitoylated proteins or the putative enzymes that regulate their acylation will respond to SNC in the same way.

Although our results do not support an involvement of a modification of Cys118 in the effects of SNC on depalmitoylation, the three C-terminal lipid-modified cysteines are possible candidates for direct modification. The other cysteine residues in Ha-Ras are buried within the core of the folded Ha-Ras protein and do not react with nitric oxide or several other oxidants (61, 62). At least three of these four cysteines in Ha-RasWT can be shown to be S-nitrosylated after exposure of the protein to S-nitrosoglutathione (61). Of the C-terminal cysteines, Cys186 is stably modified with isoprenoid in the mature Ha-Ras protein and is available as a target only in the unmodified precursor form. If exposure to nitric oxide, pharmacological inhibitors of farnesyl synthesis (e.g., compactin), or farnesyltransferase inhibitors were to cause accumulation of this usually minor population, then S-nitroso modification of Cys186 could assume larger importance, since this would at least transiently prevent membrane attachment of Ha-Ras. It is possible that Cys181 or Cys184 are nitroso-modified, but the increase in [3H]palmitate labeling of Ha-RasWT occurs so rapidly that such modification, if it occurs, must be brief, since these sites are quickly available for repalmitoylation. It will be necessary to develop direct chemical methods to analyze C-terminal cysteines of Ha-Ras from SNC-treated intact cells to clarify if direct nitrosative or oxidative modifications take place.

Indirect SNC-induced conformational changes of the C terminus could also contribute to an increased susceptibility of Ha-Ras to decylation. APT1, a thioesterase that can deacylate Ha-Ras, has recently been reported to deacylate the endothelial nitric-oxide synthase and to do so more efficiently when endothelial nitric-oxide synthase is activated by Ca2+/calmodulin (35). However, little structural information is available for the Ha-Ras C terminus (and currently none for lipidated forms of Ha-Ras), so whether the observed decreases in Ha-Ras GTP binding triggered by SNC might also cause conformational changes in the C-terminal domain remains to be clarified. More detailed studies using SNC and selected C-terminal mutants in intact cells can now examine for how long a singly palmitoylated or nonpalmitoylated Ha-Ras may persist and begin to examine requirements for dynamic decylation and repalmitoylation.

Regardless of the mechanisms involved, SNC is the first compound to be identified that can be used to manipulate Ha-Ras palmitoylation in living cells. These results raise the question of whether SNC is mimicking a natural regulatory event. The present studies suggest that Ras palmitate turnover should be explored in situations where abundant nitric oxide is produced, such as during activation of macrophages or neutrophils (63). Palmitate turnover on Ras proteins may be among the several important properties of the Ras-dependent signaling pathways that are altered during nitrosative or oxidative stress.

**Effectiveness of SNC and SNG**—It is not yet clear why SNC is such a potent inducer of Ha-Ras palmitate turnover in NIH cells, because the mechanism by which increased rate of palmitoylation might occur can be envisioned: by stimulation of the activity of an Ha-Ras S-palmitoyl thioesterase or through modifications or conformational changes that increase accessibility of the Ha-Ras C terminus to a thioesterase. The possibility that SNC may change palmitoyl thioesterase activity is now amenable to study, since a candidate thioesterase that can depalmitoylate Ha-Ras and trimeric G proteins in vitro has recently been cloned (19). Of note is the report that caveolin does not appear to be a substrate for the APT1 thioesterase that can deacylate Ha-Ras (35). The dissimilarity in the effects of SNC on palmitate labeling of Ha-Ras (increase) and the transferrin receptor and caveolin (decreases) also indicates that not all palmitoylated proteins or the putative enzymes that regulate their acylation will respond to SNC in the same way.

**DISCUSSION**

**Manipulation of Ha-Ras Palmitoylation with SNC**—These studies show that SNC can alter two important properties of Ha-Ras: its activation state and its lipidation. These effects were observed in NIH 3T3 cells expressing the normal Ha-RasWT as well as in cells transformed by an activated v-Ha-Ras mutant.

The effects of SNC on palmitoylation were protein-specific and caused increased palmitate labeling and turnover on Ha-Ras but decreased incorporation of palmitate into the transferrin receptor and caveolin in the same cells. The nitric oxide donor compound SIN-1 has also been reported to alter protein palmitoylation (60). As with SNC, the effects of SIN-1 on palmitoylation were protein-selective; palmitate incorporation into the transmembrane β-adrenergic receptor was reduced by SIN-1 treatment, whereas palmitate labeling of the Gα subunit coupled to the receptor was unaffected (60). It thus appears that at least certain palmitoylated proteins, including Ha-Ras, are susceptible to nitrosylating agents but that each will display an individualized response, presumably resulting from regulatory mechanisms distinct from each protein. The search for the still elusive enzymes that regulate protein palmitoylation should benefit from the discovery of these compounds’ abilities.

Our results indicate that SNC can stimulate palmitate turnover on Ha-Ras and that the mechanism for this increase may be primarily through acceleration of palmitate removal. Additional possible effects through increases in palmitoylation of unoccupied sites in the mature protein have not been excluded rigorously. Although palmitate removal is enhanced, the overall state of Ha-Ras palmitoylation does not appear to be decreased during short term SNC exposure, since the cellular machinery for repalmitoylation rapidly compensates and replaces palmitates that are removed. Perhaps as a result of this reacylation, no general release of Ha-RasWT or v-Ha-Ras from membranes of the treated cells was detected. Lu and Hofmann (13) have suggested previously that access of Ha-Ras palmitates to decylation can occur without release of the protein from membranes. The possibility of a subtle redistribution of the Ha-Ras protein, either into or out of subdomains of the plasma membrane remains to be explored.

At least two mechanisms by which this increased rate of depalmitoylation might occur can be envisioned: by stimulation of the activity of an Ha-Ras S-palmitoyl thioesterase or through

3. T. L. Baker, M. A. Booden, and J. E. Buss, unpublished data.
that can be easily nitrosylated. The demonstration that SNC of free nitric oxide available from the added SNC is also likely effective in causing changes within NIH 3T3 cells. The amount for studies of the role of nitric oxide or oxidants (66, 71) in the this concerted effect of SNC in NIH 3T3 cells should be useful been reported to regulate cell growth and survival (56, 69, 70),

It is likely that the decreases in cellular Ha-RasWT GTP bind-
tyrosine-specific phosphatases instead of activating them (68).

formed cell lines may explain why ERK proteins in cells trans-
activation of a vanadate-sensitive ERK phosphatase. The ad-
that will enable study of how the two dynamic properties of

Our observation that the slightly larger nitrosothiol, S-nitroso-
and other RGS proteins that stimulate hydrolysis of Gz

A possible model already exists, based on studies with hetero-
and Pate Skene for supplying various cells and DNAs and Sharon

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