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Sodium Nitroprusside Promotes IRP2 Degradation via an Increase in Intracellular Iron and in the Absence of S Nitrosylation at C178

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In iron-replete cells the posttranscriptional regulator IRP2 undergoes ubiquitination and proteasomal degradation. A similar response occurs in cells exposed to sodium nitroprusside (SNP), an NO-releasing drug. It has been proposed that nitroprusside ($[Fe(CN)_5NO]^{2-}$) fails to donate iron into cells and that it promotes IRP2 degradation via S nitrosylation at C178. This residue is located within a stretch of 73 amino acids, earlier proposed to define an iron-dependent degradation domain. Surprisingly, we show that IRP2 bearing a C178S mutation or a Δ73 deletion is sensitive to degradation not only by ferric ammonium citrate (FAC) but also by SNP. Moreover, FAC and SNP attenuate the RNA-binding activities of IRP2 and its homologue IRP1 with similar kinetics. Actinomycin D, cycloheximide, succinylacetone, and dimethyl-oxalylglycine antagonize IRP2 degradation in response to both FAC and SNP, suggesting a common mechanistic basis. IRP2 is not only sensitive to fresh, but also to photodegraded SNP and remains unaffected by *S*-nitrosoglutathione (GSNO), an established nitrosation agent. Importantly, both fresh and photodegraded SNP, but not GSNO, promote a >4-fold increase in the calcein-accessible labile iron pool. Collectively, these results suggest that IRP2 degradation by SNP does not require S nitrosylation but rather represents a response to iron loading.

Iron regulatory proteins, IRP1 and IRP2, are posttranscriptional regulators of iron metabolism (13, 32). They coordinately control the expression of mRNAs containing iron-responsive elements (IREs), such as those encoding transferrin receptor 1 (TfR1) and ferritin. In iron-deficient cells, IRPs are activated for high-affinity IRE-binding, and IRE-IRP interactions stabilize TfR1 mRNA and inhibit ferritin mRNA translation. These homeostatic responses stimulate acquisition of extracellular iron via TfR1 and prevent its storage in ferritin.

IRPs control the expression of additional IRE-containing mRNAs encoding proteins with crucial functions in body iron homeostasis, such as the erythroid-specific enzyme of the heme biosynthetic pathway aminolevulinate synthase 2 and the iron transporters ferroportin 1 and DMT1 (13, 32). IRP1/IRP2 double-knockout mice exhibit early embryonic lethality (38). The targeted inactivation of IRP1 has yielded only minor phenotypic abnormalities in the kidney and in brown fat (29), whereas the disruption of IRP2 has been associated with a progressive adult onset neurodegenerative disorder (28) and/or microcytosis (5, 10).

Experiments with $IRP1^{-/-}$ and $IRP2^{-/-}$ cells showed that IRP1 and IRP2 have similar iron-sensing capacities under typical tissue culture conditions with 21% oxygen; however, when oxygen concentration was reduced to 3 to 6%, which is believed to mimic physiological conditions in tissues, only IRP2 responded to alterations in iron levels (30). It appears that under these conditions (30) and in animal tissues (29), IRP1 predominates in the cytosolic aconitase form and does not very efficiently respond to iron deficiency, which involves an iron-sulfur cluster switch (32). These data underscore the physiological significance of IRP2 as an iron sensor in vivo.

In iron-replete cells, IRP2 is subjected to proteasomal degradation by an incompletely characterized mechanism, which is sensitive to pharmacological inhibitors of 2-oxoglutarate dependent oxygenases (12, 39) and heme synthesis (11). Likewise, IRP2 undergoes proteasomal degradation in cells exposed to sodium nitroprusside (SNP) (22, 26), a nitric oxide (NO)-releasing drug with an iron moiety (nitrosylpentacyanoferrate(III) or nitroferricyanide; $[Fe(CN)_5NO]^{2-}$). SNP is commonly used in the laboratory for nitrosation reactions (6); in addition, it is clinically utilized for the treatment of hypertensive emergencies (9). It has been argued that SNP fails to donate iron into cultured cells and that the SNP-mediated degradation of IRP2 recapitulates a physiological response to NO (22, 25). Experimental data suggested that SNP promotes S nitrosylation of IRP2 at C178, which triggers its degradation by the proteasome (26).

Interestingly, C178, as well as C168 and C174, were previously thought to be critical for the iron-dependent proteasomal degradation of IRP2 by a mechanism involving their oxidative modification (16, 17). These residues lie within a cysteine and proline-rich stretch of 73 amino acids (aa) close to the N terminus of IRP2, which displays iron (21)- and heme (19)-sensing properties in vitro and has been reported to function as an "iron-dependent degradation domain" (15, 17, 41). However, the three cysteine residues C168, C174, and C178 (3, 39) and, moreover, the entire "73-aa domain" are not necessary for IRP2 degradation in iron-treated HEK293 (12) and H1299 (39) cells.

We demonstrate here that C178 and the "73-aa domain" are also dispensable for IRP2 degradation in cells exposed to SNP.

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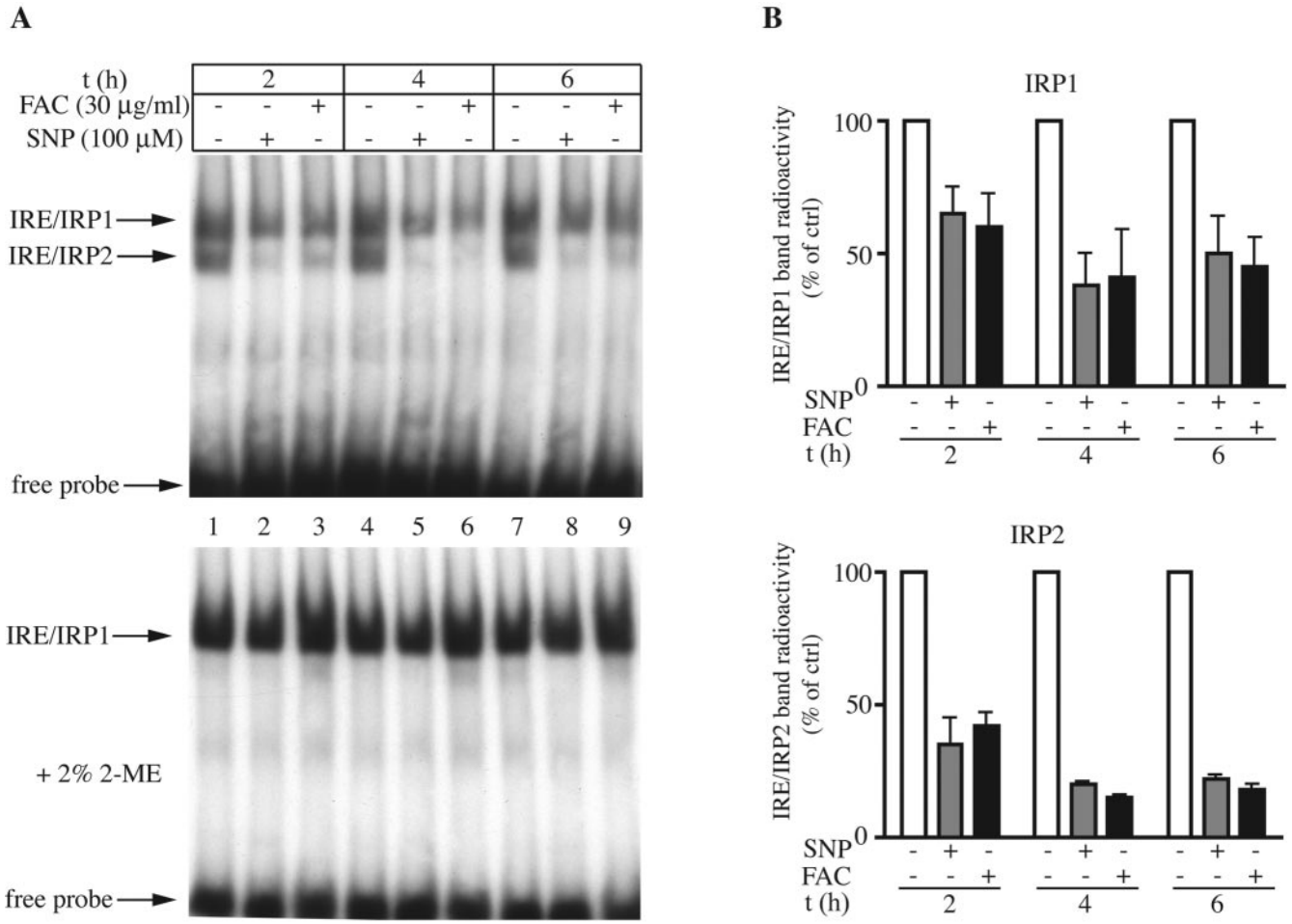

FIG. 1. Time-dependent inactivation of IRP1 and IRP2 by SNP and FAC. RAW 264.7 cells were either left untreated as control (lanes 1, 4, and 7) or exposed to 100 μM SNP (lanes 2, 5, and 8) or 30 μg of FAC/ml (lanes 3, 6, and 9) for the indicated time intervals. (A) Cytoplasmic extracts (25 μg) were analyzed for IRE-binding activity with a ^{32}P-labeled IRE probe in the absence (top) or presence of 2% 2-mercaptoethanol (2-ME; bottom). The positions of the IRE/IRP1 and IRE/IRP2 complexes and of excess free probe are indicated by arrows. (B) The band intensities of three representative experiments were quantified by phosphorimaging and plotted relative to control (mean ± the standard deviation [SD]).

Kinetic and pharmacological data reveal additional similarities in the response of IRP2 to iron and to SNP. Finally, experiments with fresh and photodegraded (nitric oxide-exhausted) SNP solutions further suggest that the degradation of IRP2 does not involve S nitrosylation but is rather mediated via the iron of nitroprusside.

MATERIALS AND METHODS

Materials. SNP ($Na_2[Fe(CN)_5NO]$), *S*-nitrosoglutathione (GSNO), potassium ferricyanide ($K_3[Fe(CN)_6]$), potassium cyanide (KCN), ferric ammonium citrate (FAC), hemin, cycloheximide, actinomycin D, succinyl acetone, and MG132 were obtained from Sigma (St. Louis, MI). Desferrioxamine (DFO) was from Novartis (Dorval, Canada). Dimethyl-oxalylglycine (DMOG) was synthesized and kindly provided by Andreas Schleifenbaum and Karsten Schulz (EMBL, Heidelberg, Germany).

Cell culture. Clones of human H1299 human lung cancer cells engineered for tetracycline-inducible expression of hemagglutinin (HA)-tagged wild-type $IRP2_{wt}$, or mutant $IRP2_{3CS}$ or $IRP2_{\Delta 73}$ (39), and murine RAW 264.7 macrophages were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U of penicillin/ml, and 0.1 ng of streptomycin/ml. The H1299 clones were maintained in the presence of 2 μg of tetracycline/ml, 2 μg of puromycin/ml, and 250 μg of G418/ml.

Western blotting. The expression of HA-IRP2, HIF-1α, and control β-actin was analyzed by Western blotting as described preciously (39).

Electrophoretic mobility shift assay. Cytoplasmic lysates were analyzed for IRE-binding activity with a radiolabeled ferritin IRE probe, as previously described (31, 39).

Photodegradation of SNP. For accelerated photodegradation of SNP, freshly prepared solutions were maintained for 2 days under a table lamp (100 W) at room temperature (36).

Nitrite assay. A total of 0.5 ml of culture medium was mixed with 0.5 ml of Griess reagent (Merck, Germany). After 10 min of incubation at room temperature, the absorbance was measured at 543 nm.

Measurement of LIP. Relative alterations in the levels of "labile iron pool" (LIP) were determined with the fluorescent metalosensor calcein (27). A total of 5×10^6 RAW 264.7 macrophages were washed three times with warm DMEM. The cells were resuspended in DMEM and then incubated with 0.125 μM calcein-AM (Fluka) for 25 min at 25°C. After three washes with cold DMEM to remove unbound calcein, the cells were resuspended in cold phosphate-buffered saline. The decrease in fluorescence was monitored under constant stirring on a Perkin-Elmer LS55 luminescence spectrometer (488-nm excitation and 517-nm emission) at 25°C. Traces of extracellular calcein were quenched with 100 μM high-molecular-weight DFO (Biomedical Frontiers, Minnesota). After stabilization of the signal, intracellular calcein-bound iron was released upon addition of 100 μM isonicotinoyl-hydrazone salicylaldehyde, a fast-permeating lipophilic iron chelator (kindly provided by Prem Ponka, Montreal, Quebec, Canada). The

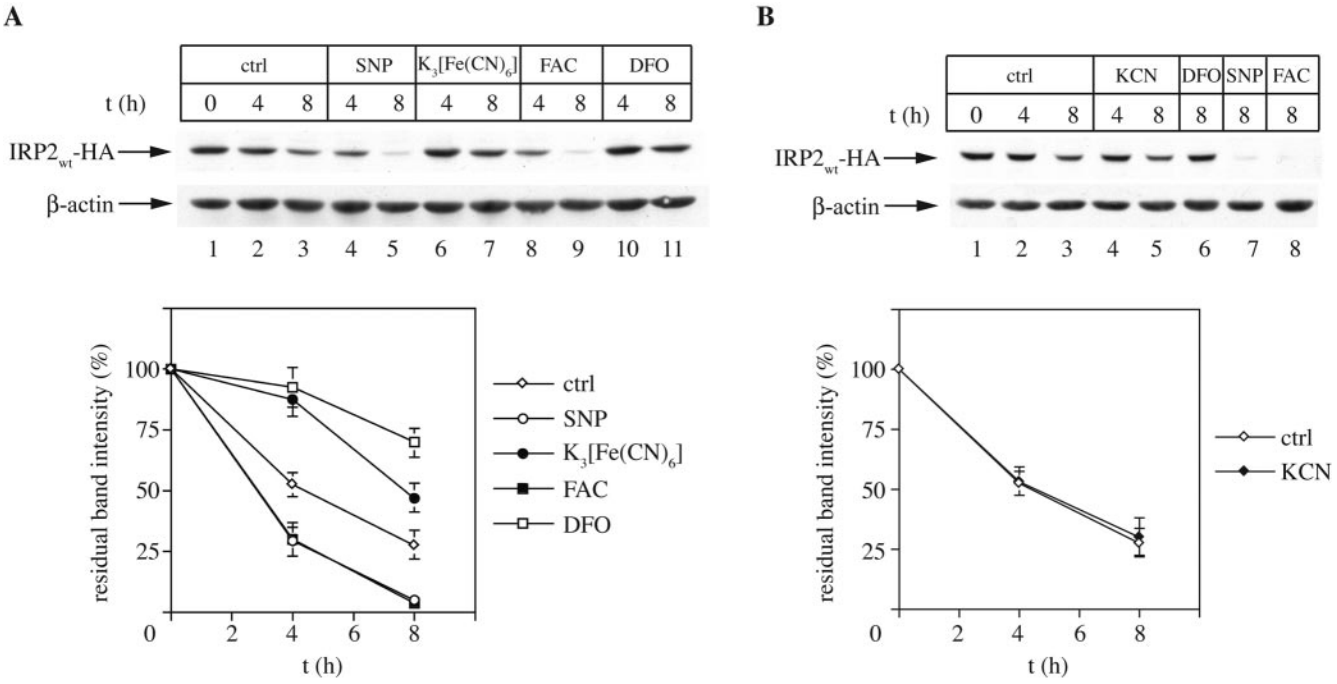

FIG. 2. Accelerated turnover of IRP2 by SNP and FAC. H1299 cells were plated for 24 h in tetracycline-free medium to express $IRP2_{wt}$-HA. Tetracycline (2 μg/ml) was then added back to shut off the transcription of the $IRP2_{wt}$-HA cDNA. (A) After 1 h, the cells were either left untreated (lanes 1 to 3) or exposed to 100 μM SNP (lanes 4 to 5), 100 μM $K_3[Fe(CN)_6]$ (lanes 6 to 7), 30 μg of FAC/ml (lanes 8 to 9), or 100 μM DFO (lanes 10 to 11) for the indicated time intervals. (B) Likewise, the cells were either left untreated (lanes 1 to 3) or exposed to 100 μM KCN (lanes 4 to 5), 100 μM DFO (lane 6), 100 μM SNP (lane 7), or 30 μg of FAC/ml (lane 8). Cell lysates were subjected to Western blotting with HA (top) and β-actin (bottom) antibodies. The immunoreactive bands were quantified by densitometric scanning. The $IRP2_{wt}$-HA/β-actin ratios are plotted (mean ± the SD) over time to illustrate the effects of the treatments on IRP2 decay.

resulting increase in fluorescence was used to calculate the LIP. Relative alterations were expressed as percentage of the control.

RESULTS

Unexpected similarities in the responses of IRP2 to FAC and SNP. Experiments were designed to evaluate a potential role of iron in nitroprusside (nitroferricyanide) on the regulation of IRP2. First, RAW 264.7 macrophages were exposed to SNP or FAC (an established iron source), and the IRE-binding capacities of IRP2 and IRP1 were analyzed at different time intervals by electrophoretic mobility shift assay (Fig. 1A, top panel). Both SNP and FAC elicited a partial (~50%) decrease in IRP2 activity within 2 h (lanes 1 to 3) and a further decline to <10% of control levels after 4 h (lanes 4 to 9). Likewise, both treatments promoted a time-dependent, albeit more modest decrease in IRP1 activity, which was already evident (~80% of control) after 2 h and reached a maximum (~50% of control) after 4 h. Quantifications of IRE/IRP2 and IRE/IRP1 band intensities are illustrated in Fig. 1B. The decrease in IRP1 activity by SNP and FAC was unrelated to alterations in its stability, since its full IRE-binding capacity could be recovered by a treatment of cell extracts with 2% mercaptoethanol (Fig. 1A, bottom panel). This agent activates latent IRP1 in vitro (31); under these conditions, IRE/IRP2 complexes are hardly visible (33). In conclusion, it is noteworthy that SNP and FAC triggered similar patterns of responses for IRP2 and IRP1. Furthermore, the kinetics of IRP2 and IRP1 inactivation by these treatments were similar.

To directly compare the effects of SNP and FAC on IRP2 stability, we utilized H1299 cells engineered to express HA-tagged wild-type IRP2 in a tetracycline-inducible fashion (tet-off system) (39). The cells were allowed to accumulate $IRP2_{wt}$-HA in tetracycline-free media. Subsequently, tetracycline was added back to block further production of the protein and the cells were either subjected to iron perturbations or treated with SNP for 4 or 8 h to analyze the stability of the $IRP2_{wt}$-HA pool. Both SNP (lanes 4 to 5) and FAC (lanes 8 to 9) accelerated the degradation of $IRP2_{wt}$-HA with similar kinetics, whereas levels of control β-actin remained unaffected (Fig. 2A). As expected, the iron chelator DFO elicited opposite responses (lanes 10 to 11). In agreement with previous observations (22), a treatment with potassium ferricyanide (the closest analogue of nitroferricyanide) did not destabilize $IRP2_{wt}$-HA but rather protected it from degradation (lanes 6 to 7). The half-life of $IRP2_{wt}$-HA was not affected by cyanide (Fig. 2B), a potential product of SNP decomposition (2, 37).

We further examined how pharmacological inhibitors interfere with IRP2 destabilization by SNP or FAC (Fig. 3). The iron chelator DFO, the transcriptional inhibitor actinomycin D, and the protein synthesis inhibitor cycloheximide efficiently blocked the degradation of $IRP2_{wt}$-HA in response to both SNP (Fig. 3A) and FAC (Fig. 3B). Likewise, dimethyl-oxalylglycine (DMOG) and succinyl acetone, which antagonize the pathway for iron-dependent degradation of IRP2 by inhibiting 2-oxoglutarate dependent oxygenases (11, 39) or heme biosynthesis (11, 39), respectively, partially protected $IRP2_{wt}$-HA

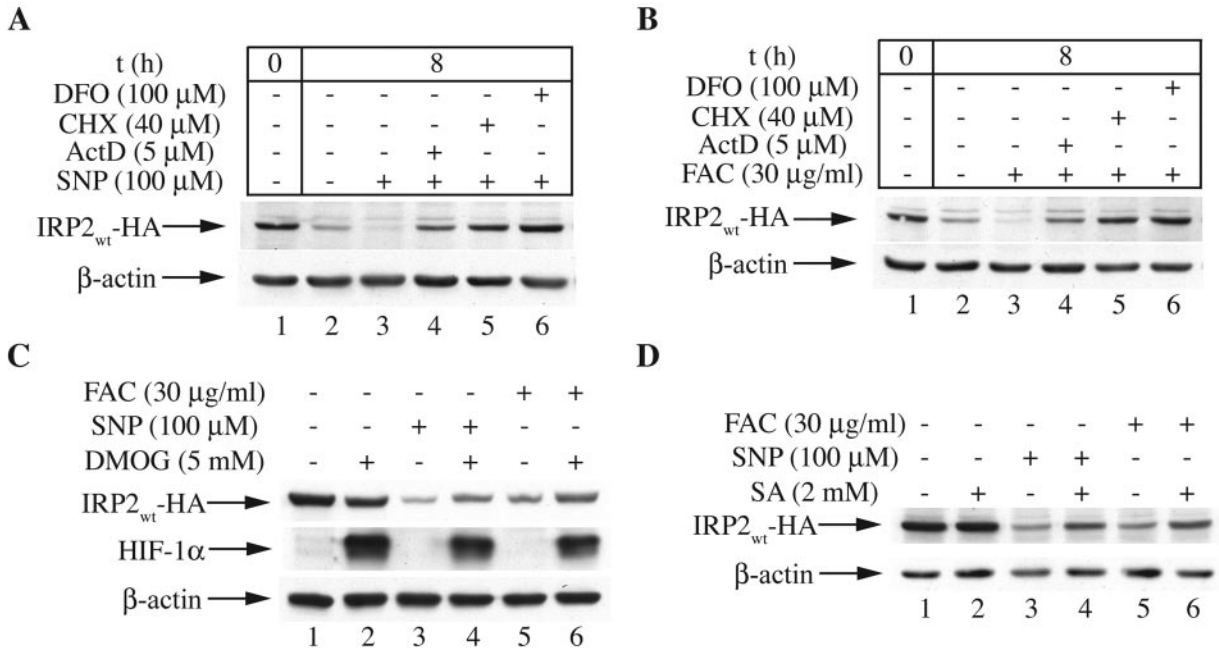

FIG. 3. Pharmacological inhibition of IRP2 degradation by SNP and FAC. H1299 cells were plated for 24 h in tetracycline-free medium to express $IRP2_{wt}$-HA. (A and B) Tetracycline (2 μg/ml) was added back to shut off the transcription of the $IRP2_{wt}$-HA cDNA. After 1 h, the cells were either harvested (lanes 1), left untreated (lanes 2), or treated with 100 μM SNP (A) or 30 μg of FAC/ml (B) in the absence (lanes 3) or presence of 5 μM actinomycin D (ActD; lanes 4), 40 μM cycloheximide (CHX; lanes 5), or 100 μM DFO (lanes 6) for 8 h. Lysates were analyzed by Western blotting with HA (top) and β-actin (bottom) antibodies. (C) After an overnight pretreatment with 100 μM DFO, the cells were either left untreated (lane 1) or treated for 6 h with 100 μM SNP (lanes 3 to 4) or 30 μg of FAC/ml (lanes 5 to 6) in the absence or presence of 5 mM DMOG (lanes 2, 4, and 6). Lysates were analyzed by Western blotting with HA (top), HIF-1α (middle), and β-actin (bottom) antibodies. (D) The cells were either left untreated (lane 1) or treated for 12 h with 100 μM SNP (lanes 3 to 4) or 30 μg of FAC/ml (lanes 5 to 6) in the absence or presence of 2 mM succinyl acetone (SA; lanes 2, 4, and 6). Lysates were analyzed by Western blotting with HA (top) and β-actin (bottom) antibodies.

against both SNP and FAC (Fig. 3C and D). The effectiveness of DMOG is illustrated in the activation of HIF-1α (18). Taken together, the experiments in Fig. 1 to 3 reveal unexpected similarities in the mechanisms for IRP2 degradation by SNP and FAC.

C178 and the "73-aa domain" are not required for IRP2 degradation by SNP. Previous data claiming that C168, C174, and C178 were crucial for iron-dependent degradation of IRP2 (17) could not be reproduced under rigorously controlled experimental conditions (3, 12, 39). Some of the discrepancies may be related to saturation in the IRP2 degradation machinery (39). Because the in vivo evidence for the requirement of C178 for SNP-mediated degradation of IRP2 was based on transient transfection experiments with a C178S mutant (26), which may have overlooked a possible saturating effect, we reevaluated the role of this residue. To this end, we utilized clones of H1299 cells expressing either $IRP2_{wt}$-HA, or $IRP2_{3CS}$-HA, which carries C168S, C174S, and C178S substitutions, or $IRP2_{\Delta 73}$-HA, containing a deletion of the "73-aa domain."

The cells were treated with SNP or FAC, and the expression of the IRP2 constructs was analyzed by Western blotting (Fig. 4). As expected on the basis of previous findings (26) and the results shown in Fig. 2 and 3, both SNP and FAC promoted the degradation of $IRP2_{wt}$-HA, and this was efficiently blocked by the proteasomal inhibitor MG132 (Fig. 4A). If IRP2 degradation depended on S nitrosylation at C178 (26), the iron-sensitive $IRP2_{3CS}$-HA and $IRP2_{\Delta 73}$-HA (39) should remain stable after SNP treatment. However, $IRP2_{3CS}$-HA (Fig. 4B) and $IRP2_{\Delta 73}$-HA (Fig. 4C) undergo degradation in response to both SNP and FAC. Thus, not only C178 but also the entire "73-aa domain" is dispensable for IRP2 degradation by SNP. These data cast doubt on whether SNP-mediated degradation of IRP2 necessitates S nitrosylation at C178 and further substantiate the hypothesis that SNP and FAC trigger IRP2 degradation by a common mechanism.

Evidence that SNP donates iron into cells. The results presented thus far would be consistent with a model where SNP may promote IRP2 degradation via its iron moiety rather than by NO release. To further explore this hypothesis, we used photodegraded SNP, which is exhausted in its capacity for NO release and retains redox-active iron (35, 36). H1299 cells were exposed to freshly prepared or photodegraded SNP for 8 h, and the expression of $IRP2_{wt}$-HA was analyzed by Western blotting (Fig. 5A). Both fresh and photodegraded SNP promoted the degradation of $IRP2_{wt}$-HA that could be rescued by MG132 (lanes 1 to 6). In contrast, GSNO that was previously reported to S nitrosylate IRP2 in vitro (26) and efficiently S nitrosylates other proteins in vivo (4) did not affect the expression of $IRP2_{wt}$-HA (lanes 7 to 8). We conclude that IRP2 degradation by SNP does not require the release of NO.

The efficacy of NO exhaustion in solutions of photodegraded

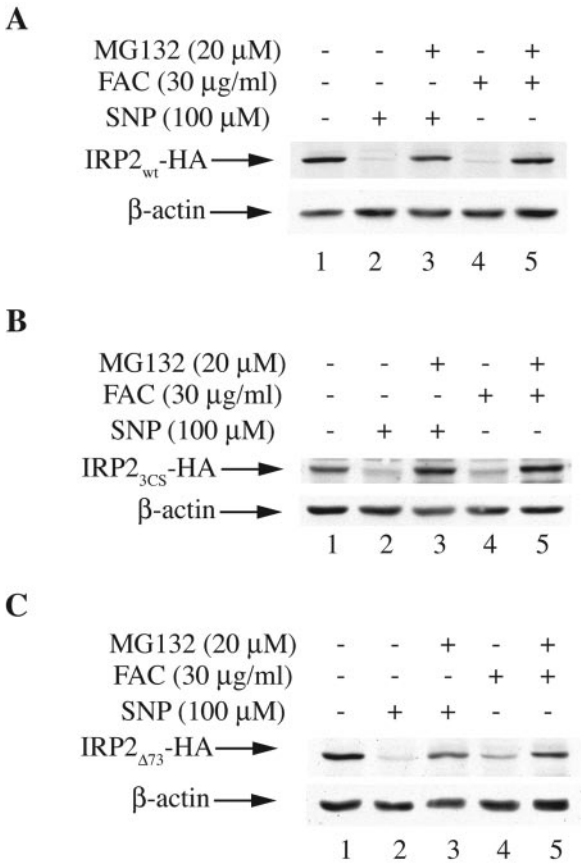

FIG. 4. IRP2 mutants bearing either C168S, C174S, and C178S substitutions or a deletion of the "73-aa domain" are sensitive to SNP-mediated degradation. H1299 cells were plated for 24 h in tetracycline-free media to express $IRP2_{wt}$-HA (A), $IRP2_{3CS}$-HA (B), or $IRP2_{\Delta 73}$-HA (C). The cells were either left untreated (lanes 1) or were treated for 12 h with 100 μM SNP (lanes 2 to 3) or 30 μg of FAC/ml (lanes 4 to 5) in the absence or presence of 20 μM MG132 (lanes 3 and 5). Lysates were analyzed by Western blotting with HA (top) and β-actin (bottom) antibodies.

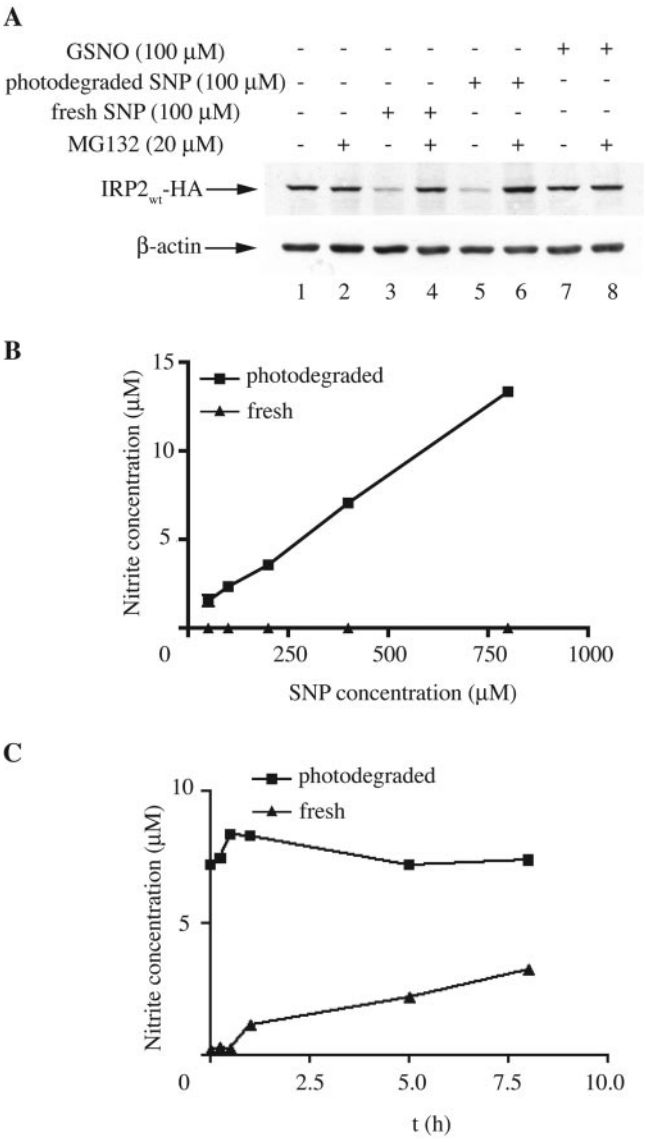

FIG. 5. IRP2 undergoes proteasomal degradation in response to fresh and photodegraded SNP, but remains unaffected by GSNO. (A) H1299 cells were plated for 24 h in tetracycline-free medium to express $IRP2_{wt}$-HA. The cells were either left untreated (lane 1) or were treated for 12 h with 100 μM fresh (lanes 3 to 4) or photodegraded (lanes 5 to 6) SNP or 100 μM GSNO (lanes 7 to 8) in the absence or presence of 20 μM MG132 (lanes 2, 4, 6, and 8). Lysates were analyzed by Western blotting with HA (top) and β-actin (bottom) antibodies. (B) Solutions of freshly prepared and photodegraded SNP were analyzed for the presence of nitrite by using Griess reagent. (C) H1299 cells were exposed to 1 mM fresh or photodegraded SNP for the indicated time intervals, and the nitrite levels in the culture supernatant were determined by using the Griess reagent.

SNP is illustrated by the presence of nitrite, which accumulates in a linear and dose-dependent fashion (Fig. 5B). Since NO is not spontaneously liberated in the absence of light (2), no nitrite was detected in solutions of freshly prepared SNP, where photodegradation was negligible. Upon administration of SNP to cells, NO release is mediated by reducing agents, such as thiols, NADH, or NADPH (2, 34). Thus, nitrite gradually accumulated in the supernatant of H1299 cells exposed to a 1 mM concentration of freshly prepared SNP in a time-dependent manner (Fig. 5C). In contrast, the concentration of nitrite in the supernatant of cells treated with photodegraded SNP did not change significantly over time, suggesting that no additional NO was liberated in the course of the treatment and that the drug was indeed NO exhausted.

If IRP2 degradation were triggered by the iron of nitroprusside, it is expected that exposure of cells to the drug would result in an increase of the LIP that reflects cellular iron status (20). To address this issue, RAW 264.7 macrophages were treated with FAC, hemin, fresh SNP, photodegraded SNP, ferricyanide, or GSNO for 30 min, and relative alterations in the LIP were monitored by the calcein assay (Fig. 6). The treatments with fresh, as well as with photodegraded, SNP resulted in profound expansions of the LIP by 9- and 7-fold, respectively. Interestingly, treatments with FAC and hemin resulted in a more modest increase of the LIP by 2- and 5-fold, respectively. Consistent with their inability to destabilize IRP2 (Fig. 2 and 5), ferricyanide and GSNO largely did not affect the

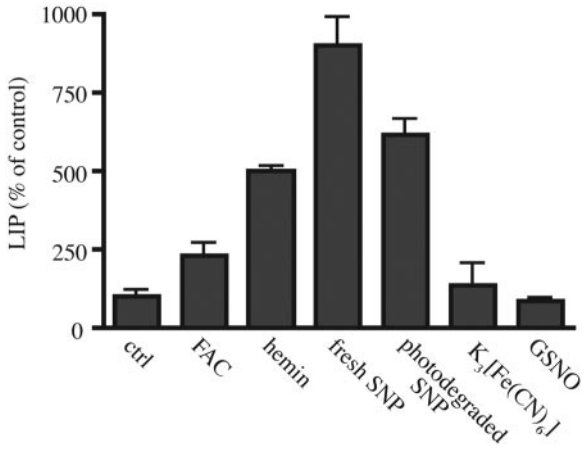

FIG. 6. Fresh and photodegraded SNP, but not GSNO, promote an increase in the LIP. RAW 264.7 cells were either left untreated (ctrl) or were treated for 30 min with 30 μg of FAC/ml or with 100 μM hemin, fresh SNP, photodegraded SNP, $K_3[Fe(CN)_6]$, or GSNO. Relative alterations of the LIP were registered with the calcein assay upon addition of isonicotinoyl-hydrazone salicyladehyde. Values correspond to triplicate samples (mean ± the SD).

levels of the LIP. These data provide strong evidence that SNP donates iron into cells and promotes IRP2 degradation via an increase of the LIP.

DISCUSSION

Previous work established that IRP2 undergoes proteasomal degradation in SNP-treated cells (22). We studied here the underlying mechanism. Since SNP is an iron complex, we first compared how SNP and FAC affect the IRE-binding activities of IRP1 and IRP2. Treatments of cells with either SNP or FAC inactivated both IRPs with similar kinetics (Fig. 1). Furthermore, no differences were observed in the kinetics of IRP2 degradation in response to SNP and FAC (Fig. 2). These time course experiments represent the first direct comparison of the effects of SNP and FAC on IRE-binding activities and on IRP2 stability. Indirect kinetic assessments (22, 23) led to the conclusion that SNP does not inactivate IRP1 at early time points and under conditions where it already destabilized IRP2; however, the design of separate experiments may not be optimal for comparative studies.

The data in Fig. 1 and 2 strengthen the idea that SNP and FAC may promote IRP2 degradation by a common pathway. This is further supported by pharmacological data showing that IRP2 degradation in response to both SNP and FAC is antagonized by DFO, actinomycin D, cycloheximide, DMOG, and succinyl acetone (Fig. 3). Thus, IRP2 degradation by both SNP and FAC is iron dependent and requires ongoing transcription and protein synthesis. In addition, it is, at least partially, dependent on the activity of 2-oxoglutarate-dependent oxygenases and on heme synthesis. It has been proposed that succinyl acetone fails to inhibit IRP2 degradation by SNP (26); however, this conclusion was entirely based on a transient-transfection assay that was not controlled for transfection efficacy. Moreover, such assays may be inappropriate for the analysis of IRP2 stability due to potential saturating effects on IRP2 degradation (39).

Notably, results from a transient-transfection assay with IRP2 point mutants were interpreted as evidence that C178 is crucial for SNP-mediated degradation of IRP2 (26). By using previously characterized clones of H1299 cells expressing iron-sensitive IRP2 variants (39), we found that not only $IRP2_{wt}$-HA but also $IRP2_{3CS}$-HA and $IRP2_{\Delta 73}$-HA undergo proteasomal degradation in response to SNP, as with FAC (Fig. 4). Even though C178 may indeed constitute a target for S nitrosylation in vitro (26), the data in Fig. 4 demonstrate that neither this residue, nor the "73-aa domain" are required for SNP-mediated degradation of IRP2.

IRP2 undergoes proteasomal degradation not only in response to fresh SNP but also in response to photodegraded SNP (Fig. 5A). Considering that only the former is an active NO donor (2) (Fig. 5B and C), this suggests that SNP-mediated degradation of IRP2 is independent of NO release. Further support is provided by the failure of GSNO, a drug that S nitrosylates the E3 ubiquitin ligase parkin (4), to destabilize IRP2 (Fig. 5A). Along these lines, the generation of NO by other pharmacological donors, such as *S*-nitroso-*N*-acetyl-penicillamine (SNAP) or the NONOate 1-hydroxy-2-oxo-3,3-bis-(3-aminoethyl)-1-triazene (NOC-18) has been shown to activate rather than inhibit IRP2 expression by antagonizing its degradation (33, 40).

Fresh and photodegraded SNP share a redox-active iron moiety (35, 36) that could possibly alter the cellular iron status. In fact, exposure of cells to either fresh or photodegraded SNP, but not to GSNO, resulted in a marked increase of the intracellular calcein-accessible LIP within 30 min (Fig. 6). Interestingly, the effects of SNP were more pronounced compared to those of FAC and hemin. These data strongly suggest that SNP functions as a direct, and possibly fast-permeating, iron donor. Alternatively, SNP could trigger a rapid mobilization of iron from intracellular iron stores. In any case, our data show that SNP-mediated degradation of IRP2 is associated with an increase in the LIP.

Importantly, evidence that SNP is an active iron donor can also be found in previous literature. Thus, the addition of SNP or other iron compounds efficiently protected various tumor cells from NO-mediated growth inhibition and apoptosis (7, 8). Furthermore, SNP rescued hypoxia-induced death of C6 glioma cells via the iron-dependent activation of the Na^+-Ca^{2+} exchanger (1).

It should be noted that a treatment with ferricyanide, the closest analogue of nitroferricyanide, did not promote an increase in the levels of the LIP. This result is consistent with the failure of ferricyanide to destabilize IRP2 (22) (Fig. 2). The differential response of IRP2 to SNP and ferricyanide has been used as an argument that SNP does not donate iron into cells (22, 26). Nonetheless, nitroferricyanide and ferricyanide remain distinct chemical entities. The former is decomposed by complex chemistry (37), while the latter is fairly stable; thus, it would not be surprising to exhibit contrasting biological properties.

Even though protein S nitrosylation is implicated in an increasing number of redox-based regulatory mechanisms (14), we conclude that IRP2 degradation by SNP is independent of this modification and rather represents a response to SNP-

mediated iron loading of the cells. Our results further suggest that the use of SNP does not faithfully recapitulate (patho-) physiological regulation of IRP2 by NO, as earlier postulated (22–24), and are consistent with the fact that biologically synthesized NO stabilizes IRP2 against proteasomal degradation (40). The potential of SNP to increase intracellular iron should be considered for biochemical and, possibly, also for clinical applications of this drug.

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