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Molecular mechanisms of Id2 down-regulation in rat liver after acetaminophen overdose. Protection by N-acetyl-L-cysteine

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Abstract

Id2 is a pleiotropic protein whose function depends on its expression levels. Id2-deficient cells show increased cell death. This study explored the molecular mechanisms for the modulation of *Id2* expression elicited by GSH and oxidative stress in the liver of acetaminophen (APAP)-intoxicated rats. APAP-overdose induced GSH depletion, *Id2* promoter hypoacetylation, RNApol-II released and, therefore, *Id2* down-regulation. *Id2* expression depends on c-Myc binding to its promoter. APAP-overdose decreased c-Myc content and binding to *Id2* promoter. Reduction of c-Myc was not accompanied by decreased *c-myc* mRNA, suggesting a mechanism dependent on protein stability. Administration of N-acetyl-cysteine prior to APAP-overload prevented GSH depletion and c-Myc degradation. Consistently, c-Myc was recruited to *Id2* promoter, histone-H3 was hyperacetylated, RNApol II was bound to *Id2* coding region and *Id2* repression prevented. The results suggest a novel transcriptional-dependent mechanism of *Id2* regulation by GSH and oxidative stress induced by APAP-overdose through the indirect modulation of the proteasome pathway.

Keywords: *GSH, c-Myc, Id2, N-acetyl-L-cysteine, histone acetylation, ChIP assay*

Introduction

The inhibitor of differentiation-2 (Id2) belongs to a family of helix-loop-helix (HLH) proteins known to play different functions in the same or different cell type depending on the surrounding microenvironment [1,2].

The molecular mechanism responsible for the biological activities of Id2 remain elusive. Data in the literature point out to the transcriptional control of *Id2* as a key event in the modulation of its function. In this sense, high levels of Id2 have been shown to promote cell survival in different cell types [3–7]. Moreover, *Id2* over-expression blocks the TGF-β-induced apoptosis and knockdown of *Id1* and *Id2* gene expression induce apoptosis in gut epithelial cells [8].

We and others have recently demonstrated that c-Myc binding to E-box motifs in the *Id2* promoter turns on its expression [9–12]. Moreover, we observed that *Id2* expression, upon c-Myc binding to its promoter, is induced in response to GSH depletion in the liver of rats treated with, l-buthionine-(S, R)-sulphoximine (BSO), the inhibitor of γ-glutamylcysteine synthetase [9]. On the other hand, in a model of liver regeneration after partial hepatectomy, known to have important fluctuations in GSH content, we have shown an increase of *Id2* gene expression modulated by c-Myc [10]. Conversely, TGF-β which induces oxidative stress and GSH depletion in a variety of cell types [13] has been shown to promote *Id2* down-regulation [11]. These apparently contradictory data suggest that, although GSH appears to be a key element mediating the transcriptional regulation of *Id2*, the molecular mechanisms of its effect are not yet well established.

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Acetaminophen (APAP)-induced intoxication produces a dramatic GSH depletion in the liver. APAP excess, metabolized by the cytochrome P450 system, gives rise to the formation of N-acetyl-p-benzoquinoneimine (NAPQI). This derivative will then react with GSH, inducing its rapid depletion within the liver and generating oxidative stress. The hepatotoxicity of APAP-overdose is the consequence of the additive effect of NAPQI formation, GSH depletion, oxidative stress and the generation of protein adducts [14]. The prominent role of Id2 in the survival pathways triggered in the context of pathologies or experimental models with important fluctuations of GSH content suggests that Id2 could be involved in the molecular events of APAP toxicity. Moreover, it would represent important evidence for the role of GSH and oxidative stress in the modulation of *Id2* expression in response to a cell death stimulus.

We report here for the first time that *Id2* is repressed in the liver of rats treated with APAP-overdose. We show that *Id2* repression is mediated by a decreased in c-Myc content induced by APAP intoxication. Moreover, prevention of APAP-induced GSH depletion by pre-treatment with N-acetyl-cysteine (NAC) increases c-Myc stability, leading to *Id2* expression.

Materials and methods

Reagents

Acetaminophen and N-acetyl-cysteine were purchased from Sigma Chemical Co (St Louis, MO). Antibodies against *α*-c-Myc were from Cell signaling (XPtm) (Beverly, MA) or SantaCruz Biotechnology (Santa Cruz, CA). Antibodies *α*-P(S62/T58)c-Myc and *α*-ubiquitin were from Cell Signaling. Other antibodies were obtained from Santa Cruz Biotechnology. Reagents for qPCR were from Applied Biosystems (Foster city, CA).

Animals

Male Wistar rats (200–250 g of weight) were from Harlan (Barcelona, Spain). Animals were kept in individual cages in a controlled environment (12 h dark/12 h light cycle) and received water and food *ad libitum*. Studies satisfied the guidelines for human treatment of animals and were approved by the Research Committee of the School of Medicine from the University of Valencia. Acetaminophen-induced toxicity has been shown to produce less APAP derivatives in rats than in mice [15]. Since we wanted to dissect the effects caused by GSH depletion from those caused by the products of APAP metabolization, we used rats along the whole study.

APAP was prepared as already described [16]. Briefly, APAP was resuspended in sterile physiological saline and warmed to 42°C to dissolve. Animals were administered APAP (ip.) in a single dose of 600 mg/kg (previously described to be toxic in rats [15]) and were sacrificed at 2, 4, 6 and 24 h after. Rats received high doses of NAC that, although clinically irrelevant for human requests according to Smilkstein et al. [17], assure the prevention of GSH depletion induced by acute APAP-overdose. NAC (500 mg/kg) was administered either as a single dose immediately prior to APAP administration (NAC1) or plus two additional doses (500 mg/kg/day) during 2 days before APAP administration (NAC2+2+1). The control groups received either vehicle or NAC. Liver-tissue samples were collected at the indicated times after APAP treatment. Tissue samples from each experimental condition were formaldehyde-cross-linked or snap frozen in liquid nitrogen and stored at −80°C for future analysis.

GSH determination in liver samples

Samples of 50 mg of liver tissue were homogenized in 4 vol. (v/w) of 6% $HClO_4$. The extracts were centrifuged at 1500 g for 10 min and the supernatant collected to measure GSH by the glutathione S-transferase method [18].

RNA isolation and real-time quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). Aliquots of 5 ng of RNA were reverse transcribed with the High capacity cDNA Reverse Transcription kit from Applied Biosystems. Next, 10% of the cDNA products were used for qPCR using the GeneAmp Fast PCR Master Mix (Applied Biosystems). All reactions were carried out in triplicate. Quantitative real-time PCR was run in a MicroAmp Optical 96-Well Reaction Plate using the 7900HT Fast Real-Time PCR System. Specific primers (pre-developed Taqman primers) for 18S, *c-myc* and *Id2* (with reference 4319413E, Rn00561507_m1, Rn01495280_m1, respectively) were purchased from Applied Biosystems. Results were normalized according to 18S quantification in the same sample reaction. Ct values were exported from the 7900HT Fast Real-Time SDS software into Microsoft Excel and normalized following the equation ΔCt = Ct target gene - Ct 18S. Gene expression values were achieved as *Relative amount* = $2^{-\Delta\Delta Ct}$ wherein ΔΔCt stands for the difference between ΔCt (treated rats) and ΔCt (untreated control).

Immunoblot analysis

Tissue samples were homogenized and protein extraction was performed as previously described [19].

Equal amounts of proteins were subjected to SDS-PAGE gel electrophoresis. Proteins were transferred to nitrocellulose membranes. The specific proteins were detected using the corresponding antibodies and secondary horseradish-conjugated antibodies. Blots were developed by enhanced chemoluminiscence (GE Healthcare). Equal loading of proteins was assessed with a specific antibody for β-actin. Protein levels were quantified by an image analyser and normalized by the values obtained for β-actin

Immunoprecipitation

Aliquots of 500 µg of proteins from whole liver tissue were pre-cleared with 30 µl of 50% (v/w) protein A-sepharose (GE Healthcare, Milwaukee, WI) at 4°C for 1 h. Supernatants were immunoprecipitated overnight with α-c-Myc (1:200) or 2 µg of normal serum IgG antibodies at 4°C. The protein-antibody complexes were pulled down, adding 50 µl of 50% (v/w) protein A-sepharose. Sample pellets were then subjected to several washes in lysis buffer (20 mM Tris-ClH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton x-100, protease and phosphatase inhibitors). The immunocomplexes were recovered from protein A-sepharose by boiling the samples in electrophoresis loading buffer. The immunocomplexes were analysed by Western blot with α-ubiquitin antibody as previously described.

ChIP assay

Formaldehyde-fixed tissue samples were immunoprecipitated according to Torres et al. [9]. Briefly, 2 g of fixed-liver tissue were homogenized and chromatin extracted as described. Cross-linked chromatin was subjected to sonication with a Vibra-Cell VCX-500 sonicator. The diluted chromatin was pre-cleared and supernatants incubated with 2 µg of the corresponding antibody at 4°C overnight. Immunocomplexes were incubated with blocked protein A/G-sepharose for an additional period of 4 h and recovered as previously described. An aliquot of cross-linked-chromatin was subjected to the same immunoprecipitation reaction in the presence of normal serum IgG and used as negative control. An aliquot of whole chromatin was collected and labelled as input.

PCR analysis of immunoprecipitated chromatin

DNA from the different collected fractions was purified with a PCR purification kit (Qiagen, Hilden, Germany) and analysed by PCR with specific primers for either *Id2* promoter (5'-acgggcattggctgc-gaacg-3' and 5'-gaggaaaggccgggagggag-3') or *Id2* coding region (5'-acaagaaggtgaccaagatggaa-3' and 5'-gcgatctgcaggtccaagat-3') and *c-myc* coding region (5'-acaaccgcaaatgctccagc-3'and 5'-ttccggtcagtttatg-cacc-3'). To determine the specificity of the assay, as negative control we used primers for skeletal α-actin promoter (5'-agggactctagtgcccaacacc-3' and 5'-ccca-cctccaccctacctgc-3') or coding region (5'-aggattc-ctacgtgggcgac-3' and 5'-tagagagacagcaccgcctg-3'). PCR fragments were size-fractionated in 2% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light.

Statistical analysis

Data are the means ± SE of at least three independent experiments. Representative blots and ChIPs are shown. Statistical significance was estimated with one-sample Student's *t*-test. A *p*-value of less than 0.05 was considered significant.

Results

Effect of acetaminophen toxicity on hepatic Id2 expression

To determine whether GSH was involved in the modulation of *Id2* expression in a pathological context, we treated animals with a single dose of acetaminophen known to induce liver-injury with GSH depletion [15].

As shown in Table I, GSH depletion occurred as early as 2 h after APAP intoxication and remained low for 6 h. As previously described [20], this phase of GSH depletion was followed by a recovery phase observed after 24 h of APAP overload. Therefore, we examined *Id2* mRNA levels during the early times after APAP-overdose concurrently with GSH declining. *Id2* mRNA was dramatically down-regulated in response to APAP-overdose at any of the tested time points (Figure 1A). On the other hand, *Id2* protein levels measured by western blot were in agreement with mRNA levels measured by real time PCR (Figure 1B).

Transcriptional regulation of *Id2* has been shown to be a key event in the control of *Id2* levels. The presence of RNApol II at the coding region of the gene has been shown to represent the transcription of the gene in

Table I. Rats received a single dose of APAP administered i.p. as 600 mg/kg. Liver tissue samples were collected at 0, 2, 4, 6 and 24 h after treatment. GSH levels were determined in liver tissue samples.

Time after APAP overdose	GSH (µmol/g of liver tissue)
0	7.00 ± 0.41
2 h	5.66 ± 0.25
4 h	3.06 ± 0.38
6 h	3.80 ± 0.20
24 h	5.71 ± 1.87

Data ($n = 4$) are mean ± SE, $^{*}p < 0.05$ vs untreated rats.

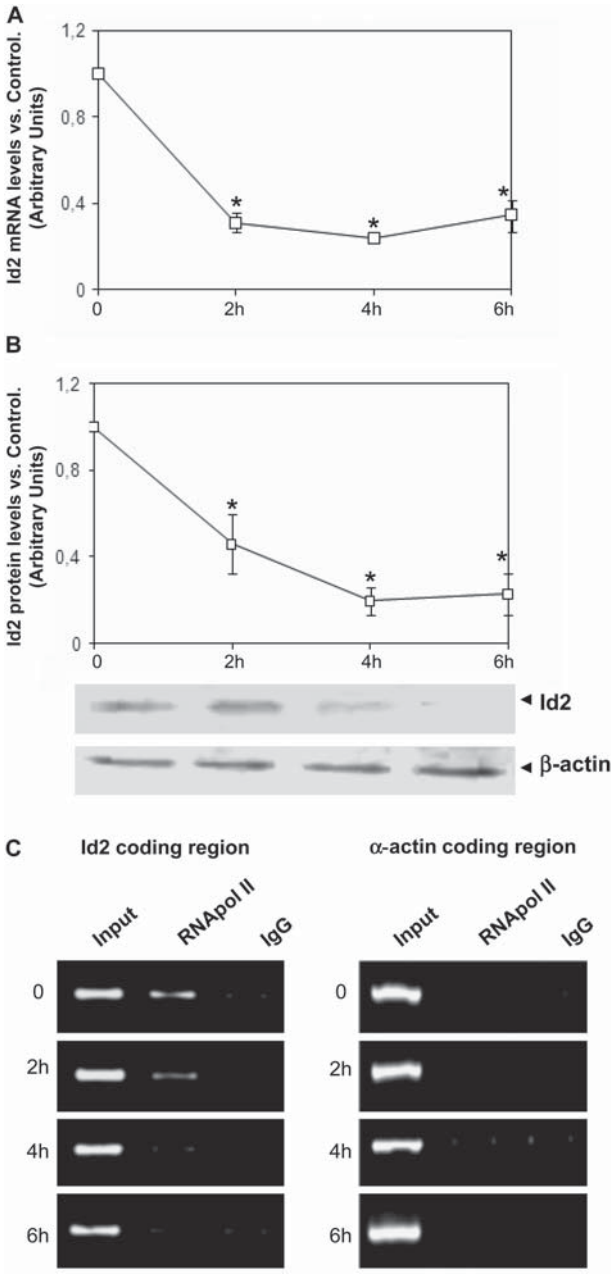

Figure 1. Time course of acetaminophen overdose. Analysis of *Id2* expression. Rats received a single dose of APAP (i.p. 600 mg/kg). Liver tissue samples were collected at 0, 2, 4 and 6 h after treatment and analysed. (A) Relative *Id2* expression during the time course of the experiment was analysed by quantitative real time PCR. Data were normalized according to 18S mRNA and quantified in the same sample reaction. Expression levels are displayed as fold-levels vs untreated controls. Data ($n = 4$) are means ± SE, $^{*}p < 0.05$ vs untreated controls. (B) *Id2* total protein levels were analysed by Western blot at the indicated times after APAP overdose. Data were normalized by the expression levels of β-actin and quantified. Expression levels were represented as fold vs untreated controls (means ± SE, $^{*}p < 0.05$). A representative analysis ($n = 4$) is shown. (C) RNApol II binding to *Id2* coding region at the indicated times was assessed by ChIP assay using antibodies for either RNApol II or normal serum IgG. PCR was performed using specific primers for *Id2* coding region (left). To verify, the specificity of the experiment samples were also analysed with primers for the skeletal α-actin coding region (right). A representative analysis ($n = 3$) is shown.

progress [21]. We tested the presence or absence of RNApol II at the coding region of *Id2* in response to APAP-overdose by ChIP assay. RNApol II was present at the coding region of *Id2* under basal conditions. Nevertheless, RNApol II was progressively released in response to APAP-overdose, suggesting a transcriptional down-regulation of *Id2* (Figure 1C, left panel). On the other hand, we could not detect RNApol II on the skeletal *α*-actin coding region, a gene which is not expressed in normal liver [22]. Therefore, the specificity of the results obtained in this experiment was confirmed (Figure 1C, right panel).

Role of GSH on Id2 down-regulation induced by acetaminophen overdose

Previous data have demonstrated that N-acetylcysteine (NAC) treatment before APAP-induced hepatotoxicity prevents liver injury [23]. It is generally thought that NAC, as a precursor of GSH, prevents its depletion after APAP-overdose supporting the detoxification of NAPQI. To determine the role of GSH on APAP-induced *Id2* gene repression, rats received NAC as a pre-treatment to APAP. It has been shown that, when administered after APAP-overdose, although protecting from hepatic necrosis, it takes several hours to restore GSH levels [23]. At that time *Id2* down-regulation would have already occurred. Therefore, in order to study the role of GSH on *Id2* expression, we administered NAC as a pre-treatment to APAP-overdose. Administration of a single dose of NAC immediately prior to APAP injection did not prevent GSH depletion induced by APAP dosing (data not shown). However, when NAC was administered during 2 days and immediately prior to APAP injection, GSH depletion in response to APAP-induced toxicity was prevented (Table II).

Therefore, we analyzed the effect of NAC on *Id2* down-regulation induced by APAP. In correlation with GSH levels, we observed that NAC completely prevented *Id2* mRNA down-regulation induced by APAP toxicity (Figure 2A). Furthermore, *Id2* protein levels also remained comparable to the untreated controls when APAP was administered in combination with NAC (Figure 2B). NAC by itself had no

Table II. Rats were administered i.p saline, NAC, APAP (600 mg/kg) or both NAC/APAP, as described in Materials and methods section. GSH levels were determined in liver tissue samples collected 4 h after APAP administration.

Treatment	GSH (μmol/g of tissue)
Saline	7.00 ± 0.408
NAC (2+2+1)	8.65 ± 0.770
APAP	3.06 ± 0.377^{*a}
NAC (2+2+1)/APAP	6.08 ± 0.523^{*b}

Data ($n = 4$) are mean ± SE; $^{*}ap < 0.05$ vs saline injected rats; $^{*}bp < 0.05$ vs APAP treated rats.

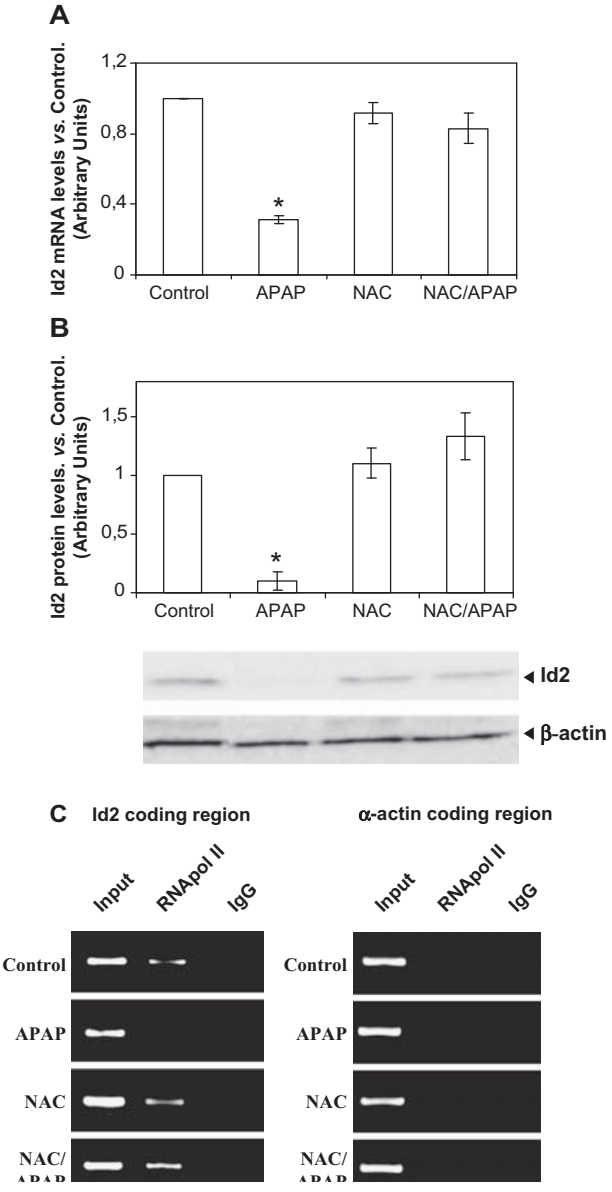

Figure 2. N-acetyl-cysteine protection from acetaminophen-induced toxicity. Effect on *Id2* expression. Rats were treated with saline, NAC, APAP (600 mg/kg) or both NAC and APAP. NAC (i.p. 500 mg/kg/day) was administered during 2 days before APAP administration and immediately prior to APAP treatment (NAC2+2+1). Liver samples were collected at 4 h after APAP administration and analysed. Expression levels were plotted as fold increase vs untreated controls. Representative blots are shown. (A) Total RNA was extracted and relative *Id2* expression analysed by quantitative real-time PCR. Data were normalized according to 18S mRNA and quantified in the same sample reaction. Data ($n = 3$) are mean ± SE $^{*}p < 0.05$ vs vehicle injected rats. (B) Analysis by Western blot of *Id2* in protein extracts from liver tissue samples under the indicated experimental conditions. Data were normalized by the expression levels of β-actin and quantified. Data ($n = 3$) are mean ± SE $^{*}p < 0.05$ vs vehicle injected rats. (C) The presence of RNApol II on Id2 coding region was assessed by ChIP assay using antibodies for RNApol II or normal serum IgG in samples from each experimental condition (left panel). PCR was performed using specific primers for Id2 coding region. Samples were also analysd with primers for skeletal α-actin coding region (right panel) to verify the specificity of experiments. A representative analysis ($n = 3$) is shown.

effect on *Id2* mRNA or protein basal levels. We analysed by chromatin immunoprecipitation the presence of RNApol II at the coding region of *Id2* in each experimental condition. As shown in Figure 2C, NAC prevented the APAP-induced release of RNApol II from *Id2* coding region. The specificity of the experiment was confirmed assessing the absence of RNApol II from the *α*-actin coding region (Figure 2C, right panel). These results suggest that NAC and most likely GSH prevents the effect of APAP on *Id2* expression through a molecular mechanism involving the transcriptional control of this gene.

Effect of acetaminophen and N-acetylcysteine on c-myc expression

We and others have previously demonstrated that c-Myc modulates *Id2* transcription upon binding to its promoter [9–12]. On the other hand, we have recently shown that GSH depletion in rat liver induces *c-myc* expression [9]. Therefore, we explored the role of c-Myc as part of the molecular mechanisms involved in the transcriptional control of *Id2* after APAP intoxication. Surprisingly, as shown in Figure 3A, *c-myc* mRNA was induced at 2 h after APAP overload and remained high at 4 h and 6 h after treatment. Interestingly, NAC pre-treatment not only did not prevent *c-myc* up-regulation but it further increased mRNA steady state levels of *c-myc* (Figure 3B).

In order to understand the molecular mechanisms involved in the synergistic effect of NAC on the APAP-induced *c-myc* up-regulation, we studied the transcriptional control of *c-myc* by RNApol II ChIP. As shown in Figure 3C, RNApol II was present at *c-myc* coding region in response to APAP-overdose, indicating that *c-myc* mRNA up-regulation was caused by an increase in gene transcription. Nevertheless, we could not observe any difference in RNApol II bound to *c-myc* coding region in samples from either APAP or NAC/APAP-treated rats. We confirmed the specificity of our experiments assessing the absence of RNApol II from the skeletal *α*-actin coding region in any experimental condition. These results suggest that the synergistic effect of NAC on APAP-induced *c-myc* is not caused by increased transcription of the gene, but most probably by an increase of *c-myc* mRNA stability.

Acetaminophen-induced liver injury and protection by N-acetyl-cysteine: Role of c-Myc on Id2 down-regulation

Although c-Myc has been shown to up-regulate *Id2* expression, Myc and Mad compete for complex formation with Max in TGF-*β* mediated response. Heterodimers of Max/Mad repress transcription of *Id2* [11]. Moreover, mechanisms of Myc-driven transcriptional repression of *Id2* have been described

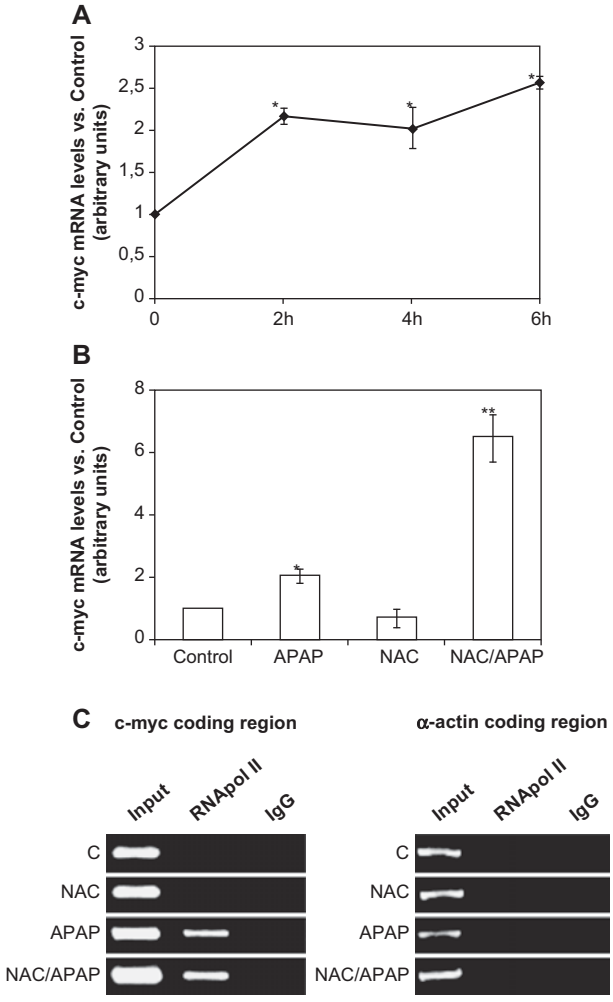

Figure 3. Effect of acetaminophen and N-acetyl-cysteine on c-myc expression. (A) Analysis of relative c-myc expression levels during the time course of APAP overdose. Data were normalized according to 18S mRNA and quantified in the same sample reaction. Expression levels are displayed as fold levels vs untreated controls. Data ($n = 4$) are means ± SE $^{*}p < 0.05$ vs untreated controls. (B) Rats were treated with saline, NAC, APAP or both NAC/APAP, as described in the Materials and methods section. Total RNA was analysed by quantitative real-time PCR for c-myc expression. Data were normalized according to 18S mRNA and quantified in the same sample reaction. Expression levels are displayed as fold increase vs saline injected controls. Data ($n = 3$) are means ± SE. $^{*}p < 0.05$ vs controls. (C) Rats were treated with saline, NAC, APAP or both NAC/APAP, as described. The presence of RNApol II on c-myc coding region was assessed by ChIP assay using antibodies for RNApol II and normal serum IgG. PCR was performed using specific primers for c-myc coding region (left panel). Samples were also analysed with primers for skeletal α-actin coding region (right panel) to verify the specificity of experiments. A representative analysis ($n = 3$) is shown.

which involve the recruitment of other transcription factors and histone deacetylases to the *Id2* promoter [24]. To unveil the role played by c-Myc in the transcriptional repression of *Id2* we explored the binding of these complexes to *Id2* promoter in our experimental model.

Surprisingly, as seen in Figure 4A, upper panel, c-Myc was not bound to *Id2* promoter despite the fact that *c-myc* mRNA was dramatically induced after APAP overdose (Figures 3A and B). On the other hand, we could not detect dimers of Max/Mad bound to *Id2* promoter in any of the experimental conditions (data not shown). More interestingly, NAC pre-treatment of APAP intoxicated animals strongly induced c-Myc binding to *Id2* promoter (Figure 4A, lower panel).

Acetylation of histone H3K9 is critical for the recruitment of the basal transcriptional machinery at the initiation of transcription and is enriched in transcriptionally active genes [25,26]. Histone H3K9 was hypoacetylated at *Id2* promoter after APAP intoxication (Figure 4A, upper panel), suggesting an APAP-induced repression of *Id2* transcription These results are in agreement with those data obtained from the *Id2* expression analysis and RNApol II ChIP (Figure 1C) Nevertheless, NAC injected prior to APAP-overdose strongly re-established histone H3K9 acetylation in accordance with an active transcription of *Id2* under these conditions (Figure 4A, lower panel). As expected, the acetyl histone H3K9 and c-Myc binding were not detected at the *α*-actin promoter (Figure 4A, right panels)

Study of c-Myc stability as part of the molecular mechanisms of Id2 down-regulation

The modulation of protein stability is one of the key events in the control of c-Myc abundance [27]. The increased *c-myc* mRNA induced by APAP-overdose was not translated into an increased binding of c-Myc to *Id2* promoter (Figure 4A). Therefore, we analyzed c-Myc protein levels in response to APAP toxicity by Western blot. As can be seen in Figure 4B, c-Myc protein levels diminished in response to APAP intoxication in a time-dependent manner, suggesting an increased c-Myc turnover after this treatment. Interestingly, NAC, as a pre-treatment to APAP, seemed to increased c-Myc stability, since levels of this protein remained as those from untreated controls (Figure 4C).

Although there are several phosphorylation sites on c-Myc, S62 and T58 are primarily responsible for the stabilization and degradation of c-Myc [27]. The analysis of phospho(S64/T58)-c-Myc by western blot revealed an increased phosphorylation of c-Myc in samples from both NAC and NAC/APAP-treated rats (Figure 4C, lower panel).

An increased phosphorylation of c-Myc that is not translated into a higher degradation of the protein suggests that the protein degradative pathway could be involved in the modulation of c-Myc. Ubiquitin-dependent degradation of other proteins has already been described in mice and cultured cells in response to APAP-overdose [28,29]. We analysed the ubiquitination of c-Myc in response to APAP exposure in the presence or absence of NAC. As can be seen in Figure 4D, ubiquitinated-c-Myc was accumulated in samples

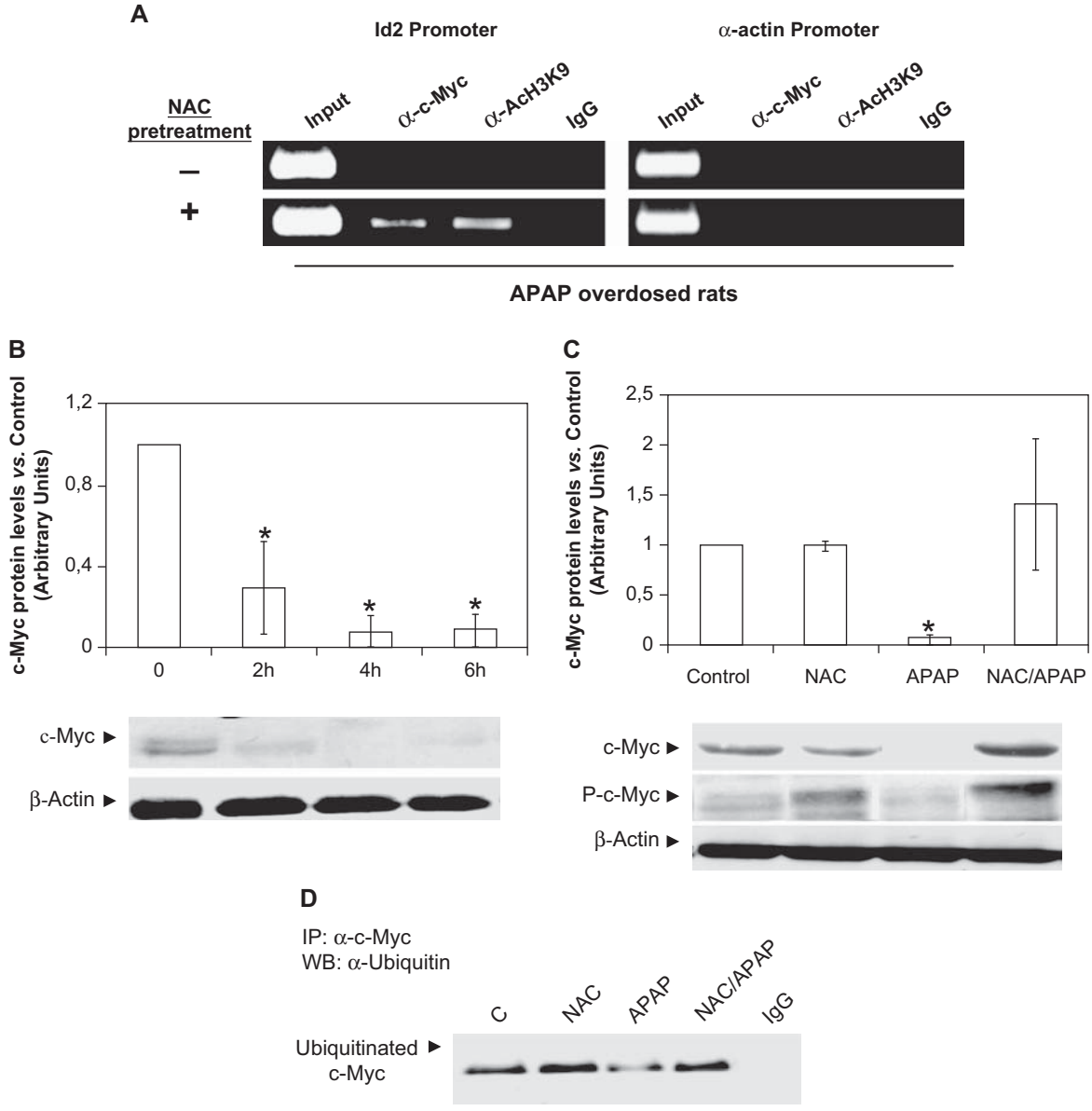

Figure 4. Modulation of *Id2* expression by c-Myc in response to acetaminophen overdose. Role of N-acetyl-cysteine. (A) Histone H3 hyperacetylation and c-Myc binding to *Id2* promoter (left panel) were analysed by ChIP assay in liver samples from APAP treated rats in the absence or presence of NAC (2+2+1) pre-treatment using antibodies either for c-Myc, Ac-H3K9 or normal serum IgG. PCR was performed using primers specific for *Id2* promoter. Samples were also analysed with specific primers for skeletal α-actin promoter to verify the specificity of experiments (right panel). A representative analysis ($n = 3$) is shown. (B) c-Myc total protein levels were analysed by Western blot at the indicated times after APAP overdose. Data were normalized by the expression levels of β-actin and quantified. Expression levels were represented as fold vs untreated controls ($^*p < 0.05$). A representative analysis ($n = 4$) is shown. (C) Rats were treated with saline, NAC, APAP or both NAC and APAP, as described in the Materials and methods section, and c-Myc and p-c-Myc protein levels analysed by western blot. Data for c-Myc protein levels were normalized by the expression of β-actin, quantified and represented as fold vs controls. A representative analysis ($n = 3$) is shown. Data are means ± SE, $^*p < 0.05$ vs controls. (D) Ubiquitinated c-Myc was analysed in protein samples from rat liver under the indicated experimental conditions. Protein extracts were immunoprecipitated with either normal serum IgG or α-c-Myc antibodies. The immunoprecipitated c-Myc proteins were analysed by Western blot with α-Ubiquitin antibody.

from both NAC and NAC/APAP-treated animals. This result might suggest an inhibition of the protein degradative pathway by NAC

Discussion

Id2 is a pleiotropic protein involved in the modulation of multiple biological processes by different mechanisms. We have previously shown that GSH content is involved in the modulation of hepatic *Id2* expression [9]. This prompted us to investigate, in a well-established pathological model of oxidative stress, the role of GSH on *Id2* expression. We used a single dose of APAP (600 mg/Kg) that, although far above the dose known to be toxic in humans, is the minimal dose described to be toxic in rats [15].

As previously reported [20], the intoxication by APAP-overdose induced an acute GSH depletion, taking 4–6 h to initiate the recovery phase in which GSH was re-synthesized (Table I). It has been shown that the toxic and reversible events in APAP toxicity occur during the first phase of GSH depletion [30]. Therefore, we analysed *Id2* levels during the early times after APAP-overdose. Concomitantly with the GSH depletion we observed a dramatic transcriptional-dependent decrease of *Id2* expression in response to APAP toxicity (Figure 1). Conversely, we recently published that *Id2* is induced in the liver of rats treated with BSO, an experimental model of GSH depletion in the absence of a concomitant pro-oxidant challenge [9]. Therefore, our present results might suggest that the combination of GSH depletion, oxidative stress and APAP derivatives that are produced after APAP intoxication (depicted in Figure 5) may be responsible for the observed *Id2* repression.

In agreement with previously published data in rats treated with BSO [9], we observed that *c-myc* mRNA was clearly induced after GSH depletion stimulated by APAP-overdose (Figure 3A and depicted in Figure 5B). Moreover, the presence of RNApol II at the coding region of this gene revealed that APAP overload stimulated the transcriptional activity of *c-myc* (Figure 3C). However, the mRNA steady state levels did not correlate with the protein levels analysed by Western blot (Figure 4B) and consistently c-Myc was not bound to *Id2* promoter in response to APAP-overdose (Figure 4A, upper panel). APAP-overdose has been shown to induce the degradation of specific proteins [28,29] and c-Myc down-regulation has been shown to be involved in the repression of *Id2* [12]. Accordingly, it would be expected that a decrease in c-Myc protein levels induced by APAP overload would lead to *Id2* repression. It is noteworthy to mention that the time-dependent degradation of c-Myc in response to APAP overload could be of high relevance not only for the modulation of Id2, but also for the regulation of other genes, since *c-myc*-centred interactomes have been identified as the most significant networks of proteins associated with liver injury [31].

Our data show that NAC prevents APAP-induced *Id2* down-regulation through a transcriptional-dependent mechanism (Figures 2 and 4) most likely by two different ways (depicted in Fig.5g and 5f).

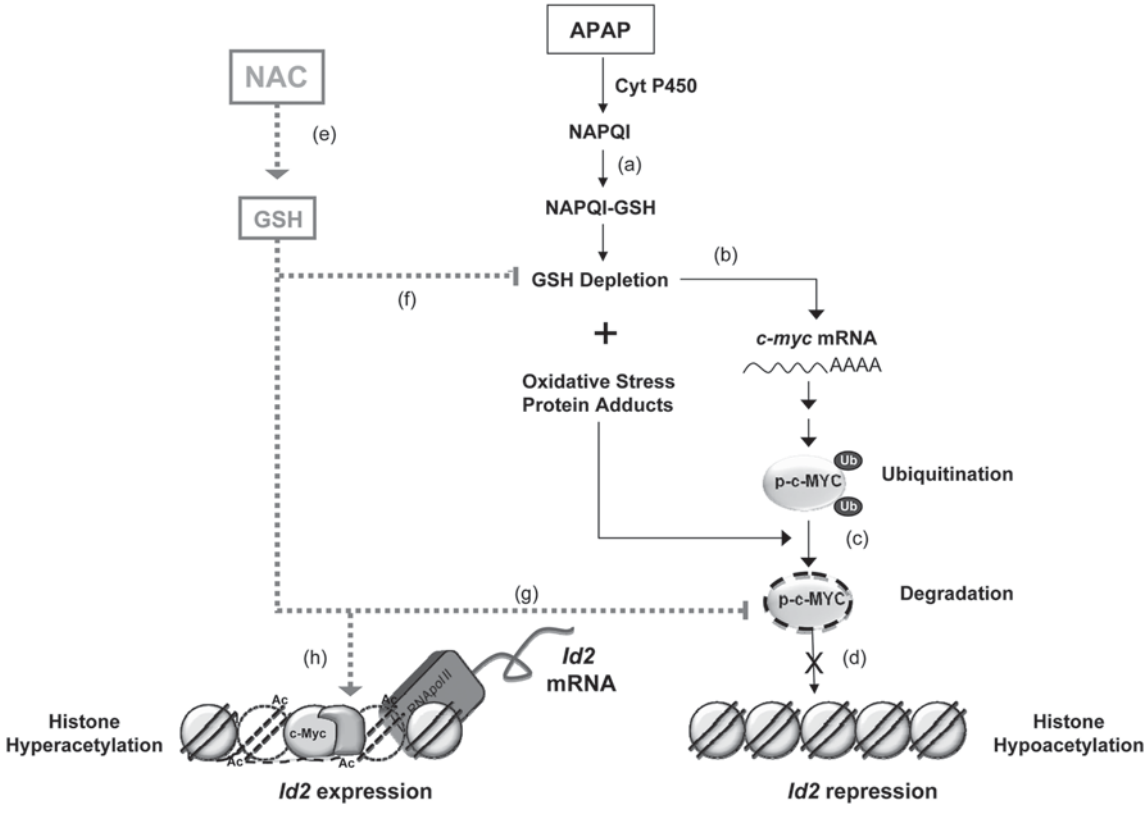

Figure 5. Model for the regulation of *Id2* expression in rat liver after Acetaminophen overdose and protection by N-acetylcysteine. APAP overdose is metabolized by the cytochrom P450 system giving rise to the highly reactive derivative NAPQI. This derivative will be detoxified by covalent binding to GSH (a), which will be soon depleted inducing c-myc expression (b). As NAPQI exceeds the hepatic GSH stores, oxidative stress and protein adducts formation will induce c-Myc degradation (c). The histones hypoacetylation and absence of c-Myc from *Id2* promoter will block *Id2* basal expression (d). NAC, which is rapidly converted to cysteine within the liver, replenishes GSH stores (dotted line) (e), increases the efficiency of the detoxification system, prevents GSH depletion and protein adduct formation (f) and consequently the proteasome pathway activation. In the presence of NAC, c-Myc degradation is prevented (g). *Id2* promoter becomes hyperacetylated and c-Myc will bind to *Id2* promoter supporting its basal expression (h).

Indeed, NAC rapidly converted to cysteine replenishes GSH stores leading to increased c-myc mRNA stability (Figures 3B and C). We have already described a similar effect of GSH on c-myc stability [9]. In those experiments, we observed that once *c-myc* transcription was triggered, the restoration of GSH levels seemed to increase *c-myc* mRNA stability. Furthermore, in liver regeneration after PH, described to have important fluctuations in GSH content, an early peak of c-myc transcription has been shown, followed by a second peak of increased mRNA stability [32].

On the other hand, NAC, either preventing oxidative stress and protein adducts formation or directly by itself, seems to block c-Myc degradation (Figures 4 and 5g), amplifying the extent of c-Myc abundance. Consequently, c-Myc will bind to *Id2* promoter (Figures 4A and 5h), leading to the expression of the gene (Figure 2).

Indeed, NAC pre-treatment to APAP-overdose induced accumulation of phosphorylated and ubiquitinated c-Myc, suggesting that while the ubiquitination pathway was intact, most probably the proteasome pathway was somehow inhibited (Figure 4D). These results are in accordance with reports showing that NAC inhibits the 26S proteasome activity, increasing the stability and accumulation of ubiquitinated IκBα and p53 [33]. On the other hand, c-Myc down-regulation has been shown to induce GSH depletion [34], thus NAC, by preventing the degradation of c-Myc induced by APAP, might be indirectly replenishing GSH content.

Covalent binding of NAPQI to mitochondrial proteins has been shown to induce mitochondrial dysfunction and enhances the formation of reactive oxygen species [35]. The effective protection of high doses of NAC on APAP-induced liver damage has been attributed to the recovery of hepatic and mitochondrial GSH levels. Nevertheless, NAC, apart from being a GSH pre-cursor, has reducing and antioxidant properties acting as a direct scavenger of free radicals. In our model, we cannot exclude the contribution of NAC as a direct scavenger of free radicals, although treatment of cultured hepatocytes with NAC before APAP exposure has been shown to ameliorate but not block oxidative stress [30]. Altogether our data suggest that NAC by itself and/or GSH seem to improve the efficiency of the detoxifying system, preventing, among other events, Id2 down-regulation.

In conclusion, we propose a novel mechanism of *Id2* regulation by GSH and oxidative stress in the context of a wide spread pathology such as the intoxication by APAP. Moreover, since both *Id2* and c-Myc take part of the transcriptional regulatory pathway of multiple genes, the significance of this mechanism might be extended to the modulation of other genes in response to APAP-overdose or, even more interestingly, in the context of other biological and pathological processes.

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