

Modulation of Serum Growth Factor Signal Transduction in Hepa 1–6 Cells by Acetaminophen: An Inhibition of *c-myc* Expression, NF- κ B Activation, and Raf-1 Kinase Activity

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Acetaminophen (APAP) is a widely used analgesic and antipyretic that can lead to severe liver damage when taken at excessive doses. APAP toxicity results when cytochrome P450-generated APAP metabolites trigger an oxidative stress and covalently modify target proteins. APAP has also been reported to inhibit cells from completing S-phase through a cytochrome P450-independent mechanism, raising the possibility that APAP may directly suppress liver regeneration and repair. Here we show that APAP also inhibits entrance of Hepa 1–6 cells into the cell cycle by blocking a number of events associated with the G0-G1 transition. We have found that APAP inhibits serum growth factor activation of *c-myc* expression, NF- κ B DNA binding, and Raf kinase. Therefore, the ability of APAP to inhibit passage of cells through both G1 and S phases might interfere with organ regeneration and thus exacerbate acute liver damage caused by APAP.

Key Words: acetaminophen; *c-myc*; NF- κ B; Raf-1 kinase; I- κ B; Hepa 1–6 cells; liver; hepatotoxicity.

Acetaminophen (APAP) is a widely used over-the-counter analgesic and antipyretic drug. It is considered a good alternative to aspirin for individuals with blood coagulation disorders or for those that cannot tolerate aspirin. Although safe at therapeutic doses, APAP at higher doses can result in liver and kidney damage in humans and in laboratory animals (Boyd and Bereczky, 1966; Boyer and Rouff, 1971; Mitchell *et al.*, 1973a; Thomas, 1993). APAP is metabolized to glucuronide and sulfate conjugates, which are readily excreted in the urine (Jollow *et al.*, 1974). APAP can also be bioactivated by the cytochrome P450 mixed-function oxidase system to form a highly reactive intermediate, N-acetyl-p-benzoquinonimine (NAPQI) (Dahlin *et al.*, 1984). NAPQI is detoxified by covalently binding to intracellular glutathione (GSH), but as GSH becomes depleted,

NAPQI covalently binds to cellular proteins and initiates toxicity (Jollow *et al.*, 1973; Mitchell *et al.*, 1973b). The role of covalent binding of NAPQI to cellular proteins in cell killing is not fully understood. However, a close association has been established between the extent of covalent binding to target proteins and hepatotoxicity (Beierschmitt *et al.*, 1989; Hinson *et al.*, 1994; Nelson and Pearson, 1990; Vermeulen *et al.*, 1992). In fact, compounds which inhibit cytochrome P450-mediated biotransformation of APAP to NAPQI prevent its toxicity (Brady *et al.*, 1988; Mitchell *et al.*, 1973a; Roberts *et al.*, 1986). Furthermore, when cellular GSH levels are increased by N-acetylcysteine (NAC) administration, the level of covalent binding is decreased, as is toxicity (Corcoran *et al.*, 1978; Piperno and Berssenbruegge, 1976; Piperno *et al.*, 1978). Other events associated with APAP toxicity include chromosomal aberrations, apoptotic DNA fragmentation, disruption of calcium homeostasis, oxidative stress, and unscheduled DNA synthesis (Corcoran and Wong, 1986; Holme *et al.*, 1988; Hongslo *et al.*, 1994; Ray *et al.*, 1990; Rannug *et al.*, 1995; Shen *et al.*, 1991; Topinka *et al.*, 1989). An important challenge to understanding APAP toxicity is to determine which APAP effects are responsible for cell killing. From the existing literature it would appear that APAP toxicity likely involves several cellular perturbations, which collectively, contribute to the initiation and progression of liver damage (Cohen and Kahairallah, 1997).

In addition to the described cytochrome P450-dependent events, APAP also has several other effects on cells. APAP has been shown to alter cell proliferation in numerous cell types, including V79 cells (Holme *et al.*, 1988; Hongslo *et al.*, 1990; Hongslo *et al.*, 1989; Hongslo *et al.*, 1994), Hepa 1–6 cells (Navarro *et al.*, 1994) and HepG2 cells (Dai and Cederbaum, 1995). These cell lines do not express the cytochrome P450 responsible for APAP bioactivation, suggesting that the effects on cell proliferation are independent of cytochrome P450 activation (Dai and Cederbaum, 1995; Navarro *et al.*, 1994). APAP treatment leads to the oxidation of ribonucleotide reductase, the enzyme which converts ribonucleotides to deoxyribonucleotides (dNTPs) (Holme *et al.*, 1988; Hongslo *et al.*, 1990, 1994; Richard *et al.*, 1991; Rannug *et al.*, 1995). In

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association with this oxidation, APAP inhibits DNA synthesis by depleting dNTPs in the cell, thus preventing the completion of S phase (Hongslo *et al.*, 1989; Hongslo *et al.*, 1994; Rannug *et al.*, 1995). If this inhibition of cell proliferation by APAP occurs *in vivo* in conjunction with the cytochrome P450 mediated events, this could contribute to toxicity through the suppression of tissue regeneration during APAP poisoning.

The present study investigates whether APAP may also interfere with cell proliferation at steps other than the oxidation of ribonucleotide reductase. Growth factor signaling and the G0-G1 transition were studied for a number of reasons. Entrance of cells into the cell cycle is critical for liver maintenance and regeneration following injury (Fausto *et al.*, 1995). Growth factor signaling also appears to play an important role in counteracting the pro-apoptotic effects of transforming growth factor- β (TGF- β), a factor that plays an important role for regulating cell death in the liver (Michalopoulos and DeFrances, 1997). An interference with growth factor signaling might therefore contribute to apoptosis, which has been reported in the liver after exposure to toxic levels of APAP (Ray *et al.*, 1990, 1996), and/or interfere with liver regeneration triggered by NAPQI damage. Determining the influence of APAP on growth factor signaling in hepatocytes could therefore suggest strategies to stem APAP-induced liver damage through the enhancement of tissue regeneration. To evaluate the ability of APAP to inhibit entrance into cell cycle, three events associated with the G0-G1 transition were examined: (1) activation of *c-myc* expression, (2) activation of the transcription factor NF- κ B (an activator of *c-myc*), and (3) activation of the Raf-1 kinase, the first kinase of the growth factor-activated MAP kinase cascade.

MATERIALS AND METHODS

Cell culture and treatments. Monolayers of Hepa 1-6 cells (obtained from American Type Culture Collection) were grown and maintained in Dulbecco modified minimal medium (DME) supplemented with 10% fetal bovine serum (FBS) and 3.5 g/liter glucose. For experiments involving serum starvation, cells were grown to an approximate density of 80% and then serum-starved for 24 to 36 h before treatment. Cells were then treated with 20% FBS for indicated times in the presence or absence of the indicated APAP concentrations. A concentration of 20% serum was chosen for these studies, based on previous reports (Arora *et al.*, 1996). To determine the effect of APAP on cell proliferation, cells were grown in the presence or absence of APAP and the number of cells in the culture determined by trypsinizing cells from the plate and counting them with a hemocytometer. In all cases, APAP was dissolved in medium prior to addition.

Cytoplasmic and nuclear extract preparations and electrophoretic mobility shift assay (EMSA). Cytoplasmic and nuclear extracts were prepared by the NP-40 lysis procedure, as described previously (Boulares *et al.*, 1996). Electrophoretic mobility shift assays (EMSAs) were performed using 32 P-end-labeled oligonucleotides that bind specifically to the transcription factor of interest as described (Boulares *et al.*, 1996). NF- κ B-binding reactions included 10 μ g of nuclear extract, while AP-1 binding assays contained 2 μ g. The sequence of the NF- κ B oligonucleotide was (5'-TCGACAGAGGGGACTT-TCGGAGAGGCTCGA-3') (synthesized by the Biotechnology Center at the University of Connecticut). The sequence for the AP-1 oligonucleotide was 5'-CTAGTGATGAGTCAGCCGGATC-3' (Promega).

Immunoblotting. Cytoplasmic extracts were prepared as described (Boulares *et al.*, 1996) with 50 μ g of protein run on a 10% SDS-PAGE and then transferred to Immobilon-P membranes (Millipore). I κ B α or Raf-1 proteins were detected with an affinity purified polyclonal anti-I- κ B or anti-Raf-1 antibodies (Santa Cruz Biotechnology), respectively, and visualized by enhanced chemiluminescent staining using the ECL reagents (Amersham).

RNA extraction and Northern blotting analysis. Cells were grown in 100-mm-diameter petri dishes (80% confluent), then treated as described in the figure legends. Media was removed and cells were lysed directly on the plates using TRIZOL LS reagent (GIBCO BRL, Life Technologies). Total RNA was extracted according to the manufacturer's recommendations. Twenty μ g of total RNA was separated in a formaldehyde-agarose gel and transferred to a nitrocellulose membrane (Schleicher and Schuell) (Sambrook *et al.*, 1989). Detection of *c-myc* mRNA was accomplished using a 32 P-labeled BamHI/HindIII fragment of 0.47 kb from pMMTV-Sma-myc plasmid coding for exon V of the murine *c-myc* gene (a kind gift from Dr. S. Bulera, University of Wisconsin). As a control, the mouse ribosomal protein L32 (rpl32) mRNA level was measured to normalize the loading of RNA samples using a 1.6 kb Sst fragment of the plasmid coding for L32 protein (a kind gift from Dr. C. Xu, University of Connecticut).

Immunoprecipitation and kinase activity. Immunoprecipitation and kinase assays were done according to Hu *et al.* (1995). Briefly, serum-starved cells were stimulated with 20% FBS in the absence or the presence of 10 mM APAP for 7.5 min. Control cells were left untreated. Cells were washed twice with ice-cold PBS, then lysed in 750 μ l of lysis buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1% NP-40, 10% glycerol, 1 mM sodium orthovanadate, 1 mM sodium fluoride) containing proteinase inhibitors (aprotinin 20 μ g/ml, leupeptin 20 μ g/ml, PMSF 0.5 mM). Eight hundred μ g of these protein extracts in a total volume of 500 μ l (adjusted with lysis buffer) were incubated with 3 μ l of anti-Raf-1 antibody for 1.5 h at 4°C. An equal amount of protein A-agarose conjugate was added to the mixture and incubated overnight at 4°C on a rocking platform. Immune complexes were then centrifuged at 12,000 g, washed 4 times with lysis buffer, one time with kinase buffer (20 mM Tris-HCl pH 7.5, 10 mM MnCl₂, 10 mM MgCl₂, 20 mM β -glycerophosphate) and resuspended in 15 μ l of kinase buffer.

Raf-1 autophosphorylation activity was assayed by incubating the immunoprecipitates with 3 μ l of 5X kinase buffer and 3 μ l of γ - 32 P-ATP in a final reaction volume of 30 μ l. The reaction was then incubated for 30 min at 25 °C and stopped by adding 30 μ l of 2X sample loading buffer. Proteins of the immunoprecipitate were separated by SDS-PAGE and Raf-1 autophosphorylation activity was visualized by autoradiography.

RESULTS

APAP Inhibits Proliferation of Hepa 1-6 Cells

We initiated our analysis of Hepa 1-6 cells with the aim of establishing a transfectable cell line to study the role of individual proteins in APAP toxicity. It became apparent that APAP had a profound effect on Hepa 1-6 cell proliferation. As shown in Figure 1, APAP concentrations in the low millimolar range suppressed the number of cells present in the culture after 24 and 48 h. This result was surprising given the fact that these cells do not possess the cytochrome P450 system required for APAP metabolism to NAPQI. APAP also does not trigger protein arylation or glutathione depletion in these cells (Navarro *et al.*, 1994). Instead, the lower cell number observed in APAP treated cultures is the result of an inhibition of cell proliferation, rather than an increased rate of cell death (Navarro *et al.*, 1994). Since the APAP inhibition of cell proliferation may play a role in inhibiting liver regeneration following

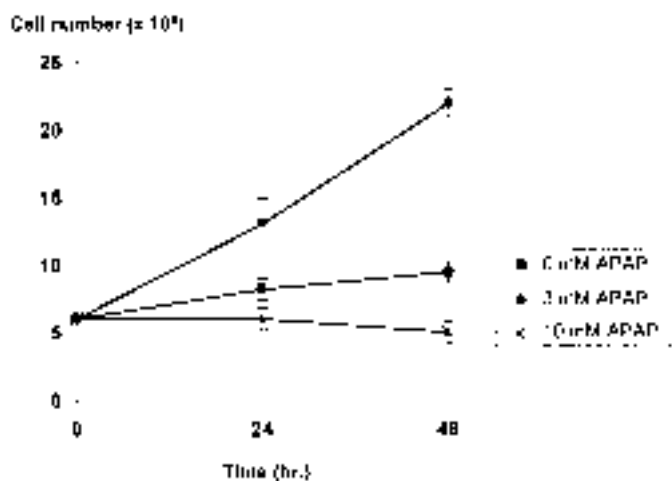


FIG. 1. APAP suppresses the proliferation of Hepa 1–6 cells, which were plated and retained under normal conditions for 24 h. The media was then adjusted to 0, 3 or 10 mM APAP, and cells were incubated for an additional 24 or 48 h. At these time points, the numbers of cells present in the culture were determined. The mean values of three replicate experiments are shown, with the error bars representing the standard deviations.

poisoning by APAP or other toxins, we decided to investigate this growth inhibition in more detail. Our studies focused on the influence of APAP on biochemical events that occur during commitment to the cell cycle in the G1 phase.

c-myc mRNA Synthesis Is Inhibited in APAP-Treated Cells

The *c-myc* protein is a transcription factor that activates a set of genes required for entrance into the cell cycle (Armelin *et al.*, 1984; Hanson *et al.*, 1994; Heikkila *et al.*, 1987). Since *c-myc* expression is up-regulated as the cell enters the cell cycle (LaRosa *et al.*, 1994); we examined the effect of APAP on the levels of *c-myc* transcripts in serum-stimulated cells to determine if the drug suppressed cells from entering G1 phase of the cell cycle.

To determine the ideal time point to test the effect of APAP on *c-myc* gene expression, we determined the kinetics of *c-myc* transcript synthesis in serum-treated cells. Total RNA was isolated from serum-treated cells at different time points and levels of *c-myc* transcripts were determined by Northern analysis using a ³²P-labeled BamHI/HindIII fragment from exon V of the coding sequence of the murine *c-myc* gene. Increases in *c-myc* transcripts were observed as early as 1 h after serum treatment (Fig. 2A, lane 2), reached a maximum at 3 h (lane 3), and decreased to the original levels after 6 h (lane 4). The peak of *c-myc* RNA synthesis was reproducibly observed at 3 h, which is consistent with published reports in other cell lines (Hanson *et al.*, 1994; LaRosa *et al.*, 1994). Based on these findings, the 3 h time point was selected for studying the effect of APAP on *c-myc* activation.

Serum-starved cells were stimulated by addition of fresh medium containing 20% FBS in the absence or the presence of

0.5, 3, or 10 mM APAP for 3 h. Total RNA was then isolated and levels of *c-myc* transcripts were determined by Northern analysis. Stimulation of serum-starved cells, as expected, caused an increase in *c-myc* mRNA (Fig. 2B, compare lanes 2 and 1). APAP caused a dose-dependent decrease in *c-myc* mRNA synthesis with inhibition detected at the lowest concentration tested (0.5 mM; compare Fig. 2B, lanes 2 and 3). When cells were treated with 10 mM APAP, the level of *c-myc* transcripts was reduced to the level observed in unstimulated cells (compare Fig. 2B, lane 5 with lanes 1 and 2). When the

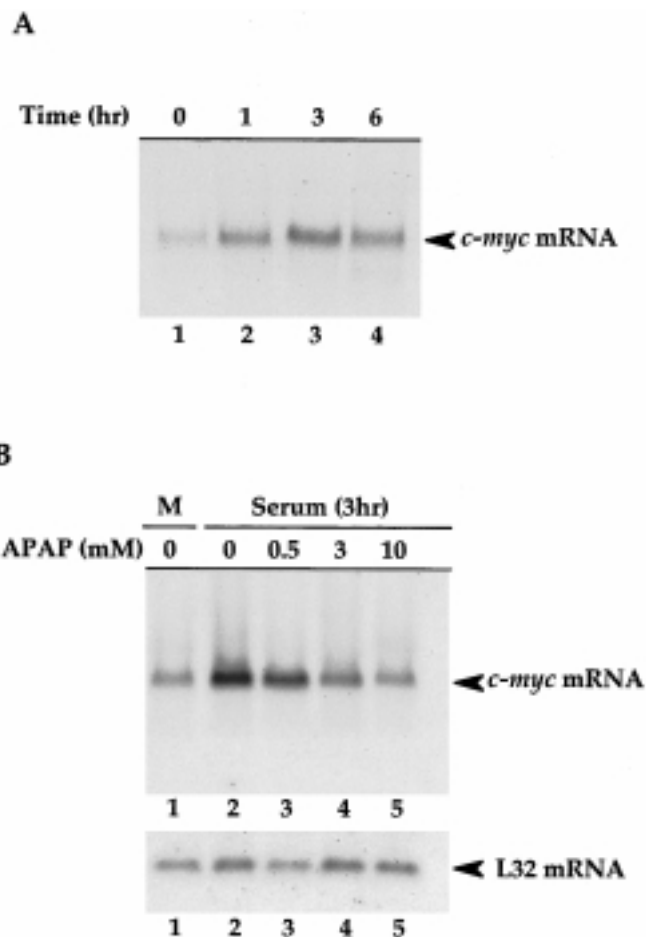


FIG. 2. APAP treatment inhibits serum induction of *c-myc* mRNA synthesis. (A) Serum-starved Hepa 1–6 cells were stimulated with 20% FBS for various time intervals. Total RNA (5 μ g) was extracted and subjected to Northern Blot analysis by hybridizing with the *c-myc* cDNA probe as described in Material and Methods. Levels of *c-myc* mRNA from: lane 1, unstimulated cells (0 h); lanes 2–4, cells stimulated with 20% FBS for 1, 3, or 6 h, respectively. (B) Serum-starved Hepa 1–6 cells were stimulated with 20% FBS in the presence of 0, 0.5, 3, or 10 mM APAP. After 3 h of treatment, total RNA (5 μ g) was isolated and subjected to Northern blot analysis as in Figure 1A. Levels of *c-myc* mRNA from: lane 1, unstimulated cells; lane 2, cells stimulated with 20% FBS; lanes 3–5, cells stimulated with 20% FBS in the presence of 0.5, 3, or 10 mM APAP, respectively. The same blot, stripped and reprobed for ribosomal protein L32, is shown in the bottom panel. This, and all other results presented in this manuscript, have been reproduced one or more times.

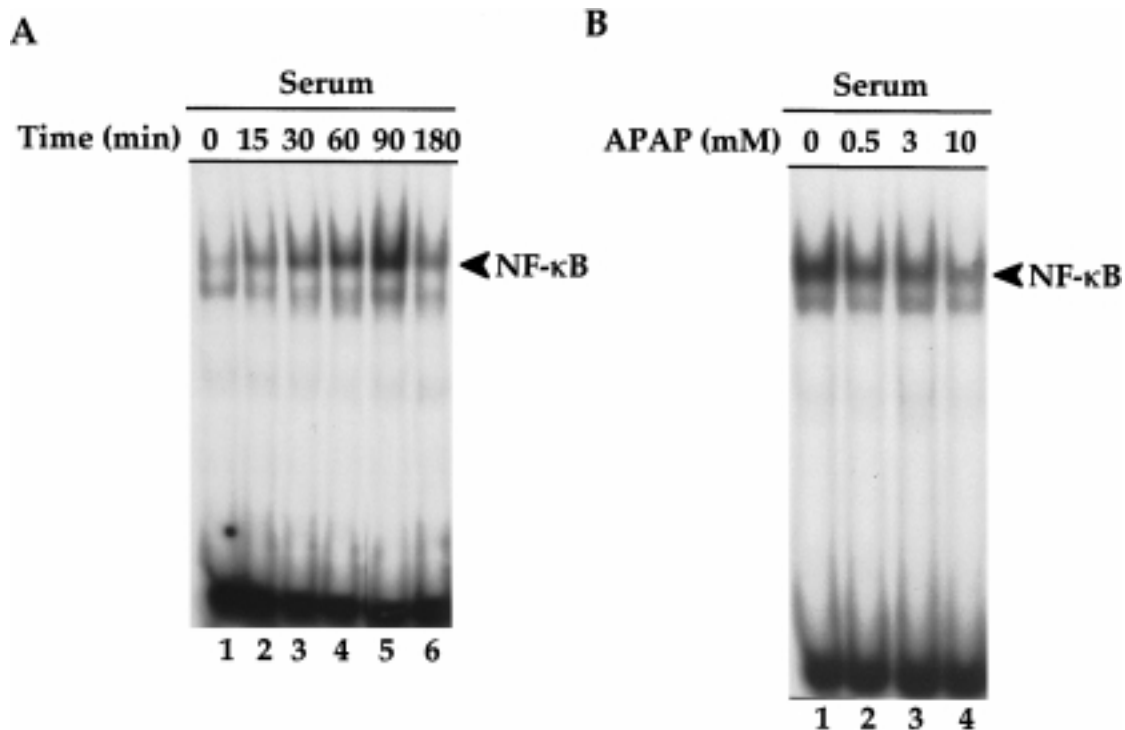


FIG. 3. APAP treatment inhibits serum growth factor activation of NF- κ B in Hepa 1-6 cells. (A) Time course of NF- κ B activation in serum stimulated cells. Serum-starved Hepa 1-6 cells were stimulated with 20% FBS for various time points. At each time point, nuclear extracts were prepared as described in Material and Methods and tested for NF- κ B DNA binding activity. Lanes 1-6, cells stimulated with FBS for 0, 15, 30, 60, 90, and 180 min, respectively. (B) Effect of APAP on NF- κ B activation by serum. Lanes 1 through 4: NF- κ B DNA binding activity in nuclear extracts from cells stimulated with FBS for 90 min in the presence of 0, 0.5, 3, and 10 mM APAP, respectively. Results representative of 3 experimental replicates.

same filter was hybridized with a probe to detect transcripts of the constitutively expressed ribosomal protein gene L32, used here as an internal control for loading, the level of transcripts remained approximately the same in the course of APAP treatment. This suggests that the APAP inhibitory effect on *c-myc* mRNA synthesis was not due to a general inhibition of cellular mRNA synthesis. These results suggest that APAP may be interfering with the progression of Hepa 1-6 into the cell cycle, in part by inhibiting *c-myc* gene expression.

APAP Inhibition of c-myc Gene Expression Occurs at the Level of NF- κ B Activation

Evidence has been obtained that NF- κ B may be a crucial transcription factor for *c-myc* gene activation (Duyao *et al.*, 1990; Schauer *et al.*, 1996): it constitutes one of the early factors to be activated after serum stimulation (Baldwin *et al.*, 1991; Olashaw *et al.*, 1992). Therefore, we determined whether APAP suppressed the activation of this transcription factor.

Initially, the kinetics of NF- κ B activation in Hepa 1-6 cells was analyzed. Serum-starved cells were stimulated with 20% FBS and incubated for various times as indicated in Figure 3A. The negative control cells were incubated in fresh media without serum. Nuclear extracts were then prepared, and the DNA binding activity of NF- κ B was assayed by EMSA using a 32 P-labeled dsDNA NF- κ B binding sequence as probe. As

expected, NF- κ B was rapidly activated in serum treated cells (Fig. 3A). DNA binding activity was detected as early as 15 min after serum treatment (lane 2), peaked at 90 min after stimulation (lane 5) and decreased by 3 h (lane 6), the last time point tested. Similar kinetics of DNA binding activity were detected when PMA was used to stimulate cells (data not shown). This rapid activation is consistent with published reports in which other stimuli such as PDGF (Olashaw *et al.*, 1992), TNF- α and IL-2 (Beg *et al.*, 1993), and virus infection (Boulares *et al.*, 1996) were used. The association between *c-myc* mRNA expression and NF- κ B activation is consistent with NF- κ B's role as a positive activator of this gene (Baldwin *et al.*, 1991).

To determine the effect of APAP on NF- κ B activation, serum-starved cells were stimulated with 20% FBS in the presence of APAP (0, 0.5, 3, or 10 mM) for 90 min. Cells were then collected and nuclear extracts were prepared. NF- κ B DNA binding activity was partially decreased in extracts from cells that were treated with 0.5 and 3 mM APAP (Fig. 3B, compare lanes 2 and 3 with lane 1) and was almost abolished by 10 mM APAP (Fig. 3B, compare lane 4 with lane 1). Approximately 80% inhibition is obtained at 10 mM APAP, as quantified by a Packard-Instant Imager (Packard Instruments). These results are consistent with the inhibition of *c-myc* mRNA synthesis (Fig. 2B), suggesting that *c-myc* gene expression is

inhibited by APAP at the level of NF- κ B activation. It should be noted, however, that we cannot rule out the possibility that APAP is affecting *c-myc* mRNA stability. The fact that L32 mRNA is unaltered by APAP indicates that there is not a general decrease in mRNA stability.

APAP Treatment Does Not Directly Interfere with AP-1 Activation in Serum-Treated Cells

To determine whether APAP similarly affects other transcription factors involved in cell proliferation, we tested its effect on serum activation of the AP-1 transcription factor. The AP-1 complex is composed of Jun/Jun homodimers or Jun/Fos heterodimers (Yoshioka *et al.*, 1995). AP-1 has the ability to control transcription of genes whose products are also important for cell proliferation (Kovary and Bravo, 1991). Serum-starved cells were stimulated with serum in the presence or absence of 10 mM APAP and incubated for 1 h. Nuclear extracts were prepared and their DNA binding activity to a 32 P-oligonucleotide containing the AP-1 binding site was tested. We reasoned that if APAP was to have an effect on AP-1, it would be apparent at this relatively high concentration. Figure 4 shows that 10 mM APAP treatment had no detectable effect on AP-1 DNA binding activity. These results suggest that APAP is acting with some specificity when it inhibits NF- κ B activation in response to serum stimulation.

APAP Does Not Interfere with NF- κ B Binding to DNA

To determine whether APAP directly blocked DNA binding by NF- κ B, nuclear extract from serum-stimulated cells (90 min) was incubated in the presence of increasing concentrations of APAP *in vitro* at 37°C for 15 min. Labeled κ B probe was then added and DNA binding activity of NF- κ B was analyzed. No effect on NF- κ B-DNA binding was observed (Fig. 5, compare lanes 2–4 with lane 1), indicating that the APAP inhibition of NF- κ B activation is not direct and that APAP is, instead, likely interfering with cellular signal transduction.

The Inhibition of NF- κ B Activation in APAP-Treated Cells Occurs at the Level of I κ B α Degradation

A crucial step in NF- κ B activation is the phosphorylation and subsequent degradation of the inhibitor protein I κ B α (Miyamoto *et al.*, 1994; Thanos and Maniatis, 1995; Verma *et al.*, 1996). To examine the fate of I κ B α in APAP-treated cells, we first determined the kinetics of I κ B α degradation in serum-stimulated cells. Cells were stimulated with serum, and cytoplasmic extracts were prepared at selected time points. I κ B α was detected by immunoblotting, using an anti-I- κ B α /MAD3 antibody. I κ B α was rapidly degraded in serum-treated cells, and maximal degradation was observed between 30 and 60 min (Fig. 6A, compare lanes 3 and 4 with lane 1). I κ B α levels increased at 90 min and reached nearly control levels by 3 h (compare lanes 5 and 6 with 1). Similar kinetics of I κ B α degradation was observed in PMA-treated cells (data not

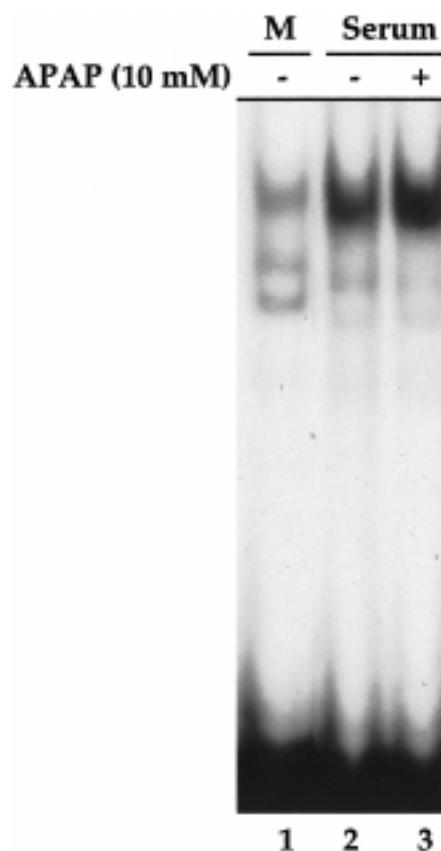


FIG. 4. Effect of APAP on serum activation of AP-1 DNA binding activity. AP-1 binding activity from: lane 1, unstimulated cells; lane 2, serum-stimulated cells; lane 3, serum-stimulated cells in the presence of 10 mM APAP.

shown). This fast degradation of I κ B α is also seen when cells are subjected to other stimuli such as TNF- α and IL-2 (Beg *et al.*, 1993; Gilmore and Morin, 1993; Israel, 1995; Verma *et al.*, 1996). The degradation of I κ B α correlated, as expected, with the activation of NF- κ B DNA binding activity shown in Figure 3A.

To determine the fate of I κ B α in APAP-treated cells, cytoplasmic extracts were prepared from serum-stimulated cells that were left untreated or treated with 0.5, 3, or 10 mM APAP for 45 min. The degradation of I κ B α was nearly complete in cytoplasmic extracts from serum-treated cells when compared to untreated cells (Fig. 6B, compare lanes 1 and 2). This degradation was greatly inhibited in APAP-treated cells, even at concentrations as low as 0.5 mM (Fig. 6B, lane 3). These results suggest that APAP inhibits NF- κ B activation by interfering with serum-stimulated I κ B α degradation.

APAP Interferes with Serum Signal by Inhibiting Raf-1 Kinase Activity

Raf-1 kinase has been shown to be necessary for growth in many cells (Kolch *et al.*, 1991; Li *et al.*, 1991, 1995; Li and Sedivy, 1993; Morrison, 1995). It has been well established that upon serum stimulation, Raf-1 is recruited to the mem-

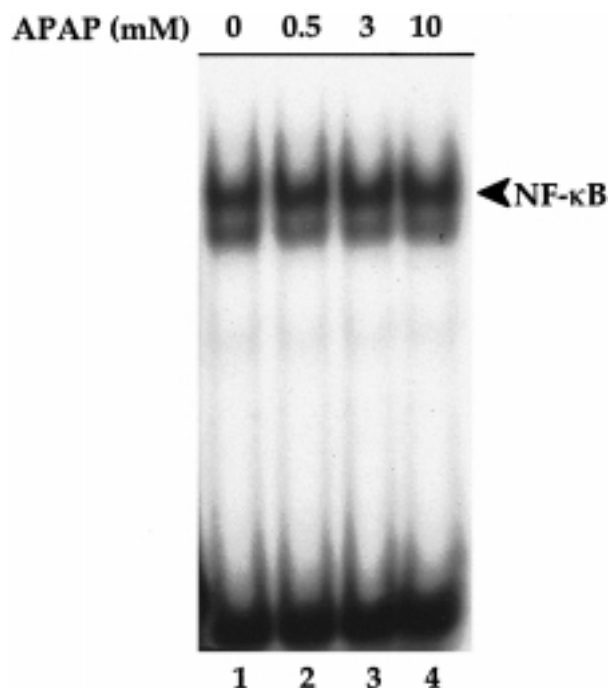


FIG. 5. Effect of APAP on *in vitro* NF- κ B DNA binding. Nuclear extracts from serum-stimulated cells were incubated with APAP and their NF- κ B binding activity was tested. NF- κ B binding activity in the presence of 0 (lane 1), or 0.5, 3, and 10 mM APAP (lanes 2–4, respectively) is shown.

brane by the GTP-binding protein, Ras (Dent *et al.*, 1995; Stokoe *et al.*, 1994), and activated by a mechanism that is still unclear. Therefore, we wished to determine whether APAP suppressed Raf-1 kinase activation.

Raf-1 kinase activation was determined by the autophosphorylation assay described by Hu *et al.* (1995). This assay is based on the observation that activated Raf-1 in immunoprecipitated complexes catalyzes an autophosphorylation reaction. Cells were stimulated with serum in the absence or presence of 10 mM APAP for 7.5 min. Cells were then lysed and Raf-1 protein was immunoprecipitated with anti-Raf-1 antibody. Immunoprecipitated Raf-1 complexes were then tested for their autophosphorylation activity. Serum stimulation induced Raf-1 autophosphorylation when compared to Raf-1 immunocomplex from unstimulated cells (Fig. 7A top panel, compare lanes 1 and 2). APAP treatment impaired the ability of Raf-1 to induce autophosphorylation (Fig. 7A top panel, lane 3). As revealed by silver staining of the same gel viewed by autoradiography in the top panel of Figure 7A, the apparent decrease in Raf-1 autophosphorylation was not a protein-loading artifact (Fig. 7A, bottom panel). These results indicate that APAP interferes with growth factor activation of Raf-1 kinase, an event required for growth factor-activated cell proliferation.

There are a number of mechanisms by which APAP could suppress Raf-1 kinase activation. We have examined two possible mechanisms for this inhibition: (1) increased Raf-1 degradation, and (2) decreased association with the Raf-1 accessory protein, Hsp90 (Schulte *et al.*, 1995). To determine if

APAP triggered Raf-1 degradation, Raf-1 was immunoprecipitated from cell extracts, the immunoprecipitated proteins were run on an SDS-PAGE and immunoblotted with anti-Raf-1 antibody. The level of Raf-1 protein remained constant in immunoprecipitates from both APAP treated or untreated cells (Fig. 7B) suggesting that APAP treatment did not cause degradation of the Raf-1 protein in Hepa 1–6 cells.

Raf-1 protein binds to Hsp90 in the cytosol and when bound to Ras at the plasma membrane (Schulte *et al.*, 1995; Wartmann and Davis, 1994). The purpose of the association of Raf-1 to Hsp90 is not entirely clear, but it may facilitate the recruitment of Raf-1 to the plasma membrane (Schulte *et al.*, 1995). Since recruitment to the membrane is necessary for Raf-1 activation, we determined whether APAP treatment disrupted the association of Raf-1 protein with Hsp90. The filter shown in Figure 7B was stripped and reprobed with an anti-Hsp90 antibody (Fig. 7C). The level of Hsp90 binding to Raf-1

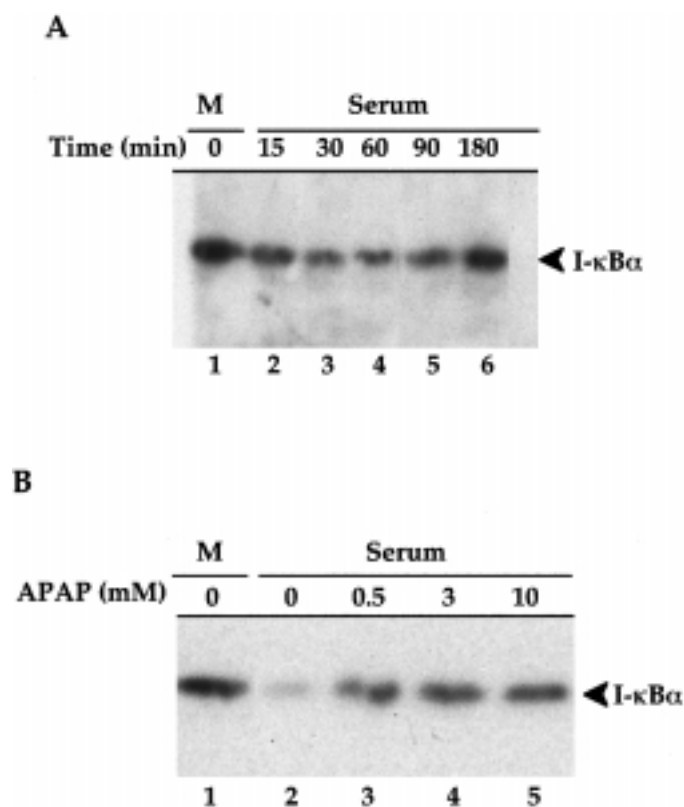


FIG. 6. APAP treatment inhibits I κ B α degradation in serum-stimulated cells. (A) Time course of I κ B α degradation in serum-stimulated cells. Cells were stimulated with FBS as described for Figure 3. Cytoplasmic extracts were then prepared and analyzed by Western blot using an I κ B α /MAD-3 antibody as indicated in Material and Methods. I κ B α levels in cytoplasmic extracts from: lane 1, unstimulated cells; lanes 2–6, cells serum-stimulated for 15, 30, 60, 90, and 180 min, respectively. (B) APAP inhibits I κ B α degradation. Cells were serum stimulated in the absence or presence of 0.5, 3, or 10 mM APAP. Cytoplasmic extracts were then prepared and analyzed by Western blot as described above. I κ B α levels in cytoplasmic extracts from: lane 1, unstimulated cells; lane 2, serum-stimulated cells, lanes 3–5, serum-stimulated cells in the presence of 0.5, 3, and 10 mM APAP, respectively.

effects of APAP on cell proliferation include an interference with growth factor signaling and the G0–G1 transition. These effects on cell physiology may have implications for APAP toxicity.

The liver has an impressive ability to recover from chemical and physical injury (Fausto *et al.*, 1995). Such repair entails the increased proliferation of hepatocytes. The ability of APAP to inhibit both the G0–G1 transition and S-phase could therefore suppress tissue repair processes initiated in response to damage incurred from reactive APAP metabolites. Determining the extent to which the inhibition of proliferation might impact liver recovery during APAP poisoning will require additional experimentation. Alterations in cell cycle progression and cell proliferation may also impact liver regeneration after damage incurred from surgical resection, viral infection, alcohol poisoning, or other injuries (Fausto *et al.*, 1995). The finding that multiple steps in the cell cycle are affected by APAP highlights the need for additional study on the effects of APAP on liver recovery from injury.

We have begun to unravel some of the molecular events associated with the inhibition of growth-factor signaling in Hepa 1–6 cells exposed to APAP. The earliest event assayed was activation of the Raf-1 kinase, the first kinase in the growth factor-activated MAP kinase cascade (Dent *et al.*, 1995; Avruch *et al.*, 1994). The inhibition of Raf-1 could result from an inhibition of any one of a number of steps from growth factor-receptor binding, through Ras activation, and Raf-1 recruitment to the Ras complex at the membrane. Each of these steps requires a battery of proteins, so each is a potential target for APAP's inhibitory activities. We did not, however, observe any changes in Raf-1 stability or its ability to associate with Hsp90. Additional studies are needed to determine the precise site at which APAP influences these very early stages of growth factor activation.

Other cellular responses to growth-factor stimulation that were inhibited by APAP were I κ B α degradation, subsequent NF- κ B activation, and *c-myc* expression. Raf-1 has been implicated in I- κ B phosphorylation leading to its degradation by the proteasome (Li and Sedivy, 1993), so Raf-1 inhibition may relate directly to the observed inhibition of NF- κ B activation. However, a ubiquitin-inducible kinase has also been implicated in I- κ B phosphorylation and degradation (Thanos and Maniatis, 1995). This I- κ B kinase appears to be activated by the MEKK1 kinase of the JNK MAP kinase cascade (Lee *et al.*, 1997), raising the possibility that multiple MAP kinase pathways may be affected by APAP. Linking the APAP inhibition of Raf-1 to NF- κ B inhibition will require additional analysis. Finally, the observed inhibition of *c-myc* expression by APAP likely results (at least in part) from the inhibition of its positive activator, NF- κ B.

APAP was found to be a potent inhibitor of NF- κ B DNA binding in Hepa 1–6 cells, with some suppression observed at APAP concentrations that are not toxic to primary hepatocyte cultures (Bruno *et al.* 1985). The APAP inhibition of transcrip-

tion factor NF- κ B displayed a degree of specificity, since the serum stimulation of AP-1 was not affected. It is intriguing that APAP would inhibit one serum-responsive transcription factor, and not another. A similar result has, however, been obtained using other compounds. For example, the antioxidant pyrrolidinedithiocarbamate (PDTC) has been shown to inhibit NF- κ B activation, but not AP-1 (Meyer *et al.*, 1993; Pinkus *et al.*, 1996). Apparently the signal transduction pathways responsible for activating these transcription factors are sufficiently different to allow inhibition of one and not the other. A similar transcription-factor response has also been observed *in vivo*: APAP intoxication triggers a transient inhibition in NF- κ B DNA binding activity (4 to 24 h after exposure), without a corresponding decrease in AP-1 activity (Blazka *et al.*, 1995). The finding that APAP inhibits NF- κ B activity *in vivo* suggests that our findings in Hepa 1–6 cells may have implications for APAP toxicity *in vivo*. Our studies also indicate that this response to APAP occurs in the absence of extensive biotransformation to NAPQI (Navarro *et al.*, 1994), and can be explained by a direct response of hepatocytes to APAP, independent of Kupffer cell responses (Laskin, 1991). Hepa 1–6 cells are therefore a good model system for studying the APAP inhibition of NF- κ B. Finally, the ability of APAP to inhibit NF- κ B activation may be relevant to the possible induction of apoptosis, which has been observed during APAP poisoning (Ray *et al.*, 1996): NF- κ B is a positive regulator of a number of genes that suppress apoptosis (Grimm *et al.*, 1996; Wu *et al.*, 1996)

The exact mechanism of the hepatotoxicity of APAP is yet to be fully elucidated. Covalent binding to cellular proteins and oxidative damage to cellular macromolecules undoubtedly play a major role in APAP toxicity. However, the recent findings of apoptotic cell death, cell cycle perturbations, and NF- κ B inhibition by APAP suggests that a number of cellular signaling changes may also play important roles in the toxicity. Shown in Figure 8 is a working model that incorporates a role for cell cycle perturbations in APAP toxicity. In this model, reactive APAP metabolites such as NAPQI are envisioned to initiate cell injury and cell death. Liver damage inflicted by these reactive metabolites then provokes liver repair processes, which include increased cell proliferation. It is at this repair stage that APAP's influence on the cell cycle may come into play. The effects on growth-factor signaling are illustrated in Figure 8, but the effects at S-phase may also be critical. This inhibition of cell proliferation could theoretically occur at therapeutic doses. However, given APAP's half-life *in vivo* (Prescott *et al.*, 1968), such inhibition would likely be of little importance in the absence of other events associated with APAP overdose. It remains to be determined if therapeutic doses of APAP interfere with liver recovery from other types of injury. In the presence of hepatocyte injury, this inhibition of proliferation may tip the balance toward liver failure. Additional work is, however, required to determine if cell cycle perturbations play a significant role in APAP toxicity *in vivo*.

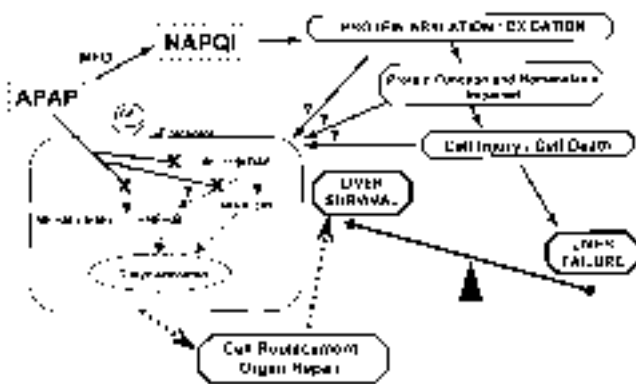


FIG. 8. APAP-induced hepatotoxicity: potential contribution of altered signal transduction. Activation of APAP by mixed function oxidases (MFO) results in the formation of the electrophilic metabolite, NAPQI. Excess NAPQI arylates or oxidizes critical cellular protein targets, disrupting protein function and cellular homeostasis. Cellular injury may progress to cell death and, ultimately, liver failure. The initial insult, consequent injury and cell death likely serve as signals to activate signal transduction pathways to repair, remove and/or replace injured cells. The present study documents that, in the absence of MFO activation, APAP also blocks signal transduction pathways (shown by large X). In the presence of MFO-mediated NAPQI injury, cell-cycle blockade by APAP may tip the balance from organ survival to organ failure. (Dotted lines indicate pathways diminished by APAP; GF, growth factor.)

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