Regulation of IGF binding protein-1 in Hep G2 cells by cytokines and reactive oxygen species

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Lang, Charles H., Gerald J. Nystrom, and Robert A. Frost. Regulation of IGF binding protein-1 in Hep G2 cells by cytokines and reactive oxygen species. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G719–G727, 1999.—The liver is a major site of synthesis for insulin-like growth factor binding protein (IGFBP)-1. Because IGFBP-1 inhibits many anabolic actions of IGF-I, increases in IGFBP-1 may be partly responsible for the decrease in lean body mass observed in catabolic/inflammatory conditions. This study aimed to determine in Hep G2 cells 1) the sensitivity of IGFBP-1 synthesis to treatment with interleukin (IL)-1, tumor necrosis factor-α (TNF-α), and IL-6, 2) the ability of reactive oxygen species (ROS) to enhance IGFBP-1 production, and 3) the role of ROS in mediating cytokine-induced increases in IGFBP-1. Hep G2 cells responded to IL-1β, TNF-α, and IL-6 with maximal 8- to 10-fold increases in IGFBP-1 production. Although the maximal responsiveness of cells treated with TNF-α and IL-6 was 20–30% less than that with IL-1β, cells demonstrated a similar sensitivity to all cytokines (half-maximal responsive dose of ~10 ng/ml). A low concentration (3 ng/ml) of all three cytokines had an additive effect on IGFBP-1 production. Cytokines also increased IGFBP-1 mRNA. The half-life of IGFBP-1 mRNA was ~4 h and not altered by IL-1. Incubation with ROS, including H2O2 and nitric oxide (NO) donors, resulted in a relatively smaller increase in IGFBP-1. However, preincubating Hep G2 cells with various free radical scavengers and NO synthase and eicosanoid inhibitors failed to prevent or attenuate cytokine-induced increases in IGFBP-1. Finally, preincubating cells with pyrrolidinedithiocarbamate (PDTC) but not SN50 (inhibitors of nuclear factor-κB activation and nuclear translocation, respectively) attenuated increases in IGFBP-1 induced by IL-1. These results indicate that 1) proinflammatory cytokines directly enhance IGFBP-1 synthesis by stimulating transcription without altering mRNA stability, 2) addition of exogenous ROS also stimulates IGFBP-1 production but to a smaller extent than cytokines, and 3) the cytokine-induced increase in IGFBP-1 production is not mediated by endogenous production of ROS or eicosanoids but appears to at least partially involve a PTDC-sensitive pathway.

tumor necrosis factor-α; interleukins-1 and -6; free radicals; nitric oxide; nuclear factor-κB; liver; insulin-like growth factor

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The purpose of the present investigation was to determine 1) the sensitivity of IGFBP-1 synthesis in Hep G2 cells treated with TNF-\(\alpha\), IL-1, or IL-6, 2) the ability of ROS to enhance IGFBP-1 production, and 3) the role of endogenous ROS in mediating cytokine-induced increases in IGFBP-1.

MATERIALS AND METHODS

Cell culture. In general, Hep G2 cells have traditionally been used to elucidate mechanisms for hormone- and stress-induced changes in IGFBP-1 mRNA and protein secretion (23, 38) and continue to be used in this regard (6, 45). Specifically, Hep G2 cells were used in the present study because they are cytokine responsive (30), similar to that observed in isolated hepatocytes (14). Moreover, these cells and rat H4-II-E hepatoma cells also have a constitutive secretion of IGFBP-1, and the hormonal regulation of gene expression is essentially identical to that reported in hepatocytes (26, 48). Finally, because this cell line is derived from human cells, the IGFBP-1 secreted into the media can be easily quantitated by a commercially available immunoradiometric assay (IRMA).

In studies in which the IGFBP-1 concentration was quantitated in the medium, Hep G2 cells were grown in 24-well plates (Falcon, B-D, Lincoln Park, NJ). Each well contained 500 \(\mu\)l of MEM (Sigma, St. Louis, MO) supplemented with 5% calf serum, penicillin (100 U/ml), streptomycin (100 \(\mu\)g/ml), and amphotericin B (25 \(\mu\)g/ml). Cells were used 5–7 days after subculture, at which time they were near confluence. On the day of the experiment, the medium was replaced with serum-free MEM containing 0–300 ng/ml of either recombinant human (rh) IL-1\(\beta\) (rhIL-1\(\beta\); gift of Biological Response Modifiers Program, Division of Cancer Treatment/NCI), rhTNF-\(\alpha\) (gift of Amgen, Thousand Oaks, CA), or rhIL-6 (Calbiochem, La Jolla, CA). After an 18-h incubation period, culture medium was collected to determine IGFBP-1 protein concentration. In studies in which IGFBP-1 mRNA abundance was assessed, Hep G2 cells were cultured in 100-mm plates containing 8 ml of MEM. IGFBP-1 mRNA stability was examined by incubating control and cytokine-treated cells with the inhibitor of transcription 5,6-dichloro-\(\beta\)-d-ribofuranosyl-\(\beta\)-D-ribofuranosylbenzimidazole (DRB, 75 \(\mu\)M; Calbiochem) and then quantitating mRNA at various times thereafter (7). The IGFBP-1 concentration in the conditioned medium from these cells was also quantitated.

In the second series of studies, the ability of ROS to stimulate IGFBP-1 secretion was investigated. Cells were incubated for 18 h with either \(\mathrm{H}_2\mathrm{O}_2\) or tert-butyl-\(\mathrm{H}_2\mathrm{O}_2\) (100 and 300 \(\mu\)M) or a combination of xanthine (500 \(\mu\)M) and xanthine oxidase (10 \(\mu\)M/ml). Other cells were incubated with the NO donor sodium nitroprusside (SNP; 0.1, 0.5, and 1 mM) or S-nitroso-N-acetylpenicillamine (SNAP; 0.1, 0.5, and 1 mM). Comparable doses of these ROS donors have been previously demonstrated to stimulate acute phase protein synthesis and other processes in hepatocytes (20, 40). NO and superoxide anion can combine to produce peroxynitrite. However, because peroxynitrite rapidly decomposes, cells were incubated with 3-morpholinosydnonimine (SIN-1; 500 \(\mu\)M).

In the third series of studies, Hep G2 cells were preincubated for 30 min with one of several oxygen free radical scavengers or inhibitors of NO or eicosanoid synthesis before addition of cytokine (50 ng/ml). The oxygen free radical scavengers used included ascorbic acid (1 mM), \(\alpha\)-tocopherol (500 \(\mu\)M), N-acetyl-L-cysteine (NAC; 1 mM), and DMSO (1%). The doses of these scavengers used have been previously demonstrated to inhibit various cytokine- or ROS-induced changes in hepatic cells (42, 43). The NO synthesis inhibitors used in this study included N\(^\text{G}\)-monomethyl-L-arginine (L-NMMA; 5 mM), N\(^\text{G}\)-nitro-L-arginine methyl ester (L-NAME; 5 mM), and aminoguanidine (0.5 mM). The doses of these inhibitors used have been previously demonstrated to inhibit cytokine-induced changes in hepatic cells (16, 21). Inhibitors of eicosanoid biosynthesis were also used and included the cyclooxygenase inhibitor indomethacin (50 \(\mu\)M) and the lipoygenase inhibitor nordihydroguaiaretic acid (NDGA; 50 \(\mu\)M). On the basis of measurements of lactate dehydrogenase (LDH) released into the medium, none of the above treatments at the doses employed resulted in significant cytotoxicity or overt morphological alterations during the 18-h incubation period (data not shown).

Both IL-1 and ROS have been demonstrated to induce nuclear factor-\(\kappa\)-B (NF-\(\kappa\)-B) in Hep G2 cells (33, 39), and activation of this DNA-binding protein has been implicated in mediating a diverse array of biological responses (1). To determine whether the IL-induced increase in IGFBP-1 was regulated in part by NF-\(\kappa\)-B activation, cells were pretreated with pyrroldinedithiocarbamate (PDTC; 100 \(\mu\)M) 30 min before addition of IL-1\(\beta\). In addition, because PDTC does not inhibit NF-\(\kappa\)-B directly, Hep G2 cells were incubated with SN50 (18 \(\mu\)M), which is a more specific cell-permeable peptide inhibitor of NF-\(\kappa\)-B nuclear translocation, for 1 h before addition of IL-1\(\beta\). This peptide contains the nuclear localization sequence (NLS) for the p50 NF-\(\kappa\)-B subunit and the amino-terminal sequence of Kaposi fibroblast growth factor to promote cell permeability (28).

All reagents were purchased from Sigma Chemical, except the following: aminoguanidine hemisulfate, SNP, SNAP, and SIN-1 (Research Biochemicals International, Natick, MA); L-NMMA and SN50 (Calbiochem); L-NAME (Bachem, Torrance, CA); and sodium meta-arsenite (Aldrich, St. Louis, MO).

Analytical methods. Conditioned medium was stored at −20°C until IGFBP-1 could be quantitated using a two-site IRMA obtained from Diagnostic System Laboratories (Webster, TX).

Briefly, IGFBP-1 mRNA expression was determined as follows. Total RNA was isolated (TRI Reagent LS, Molecular Research Center, Cincinnati, OH) from Hep G2 cells, following the manufacturer’s protocol. Samples (10 \(\mu\)g) of total RNA were processed under denaturing conditions on 1% agarose gels containing 6% formaldehyde. Northern blotting was performed by capillary transfer to Zeta-Probe GT blotting membranes (Bio-Rad Laboratories, Hercules, CA). A human IGFBP-1 oligonucleotide probe (Oncogene Research Products, Cambridge, MA) was end-labeled with \(\gamma\)-\(^{32}\)P\(\text{ATP}\) (Amersham, Arlington Heights, IL) using T4 polynucleotide kinase (Pharmacia Biotech, Piscataway, NJ). For normalization of RNA loading, a human \(\beta\)-actin oligonucleotide (gift of J. Floros, Pennsylvania State College of Medicine) was radiolabeled using terminal deoxynucleotidyl transferase. Membranes were baked, hybridized, and washed according to the formamide protocol provided with the Zeta-Probe GT. Finally, membranes were exposed to a Phosphor Imager screen (Molecular Dynamics, Sunnyvale, CA), and the resultant data were quantitated using the accompanying ImageQuant software.

Statistics. Values are means ± SE (n ≥ 5 sets of duplicate wells for each dose or treatment) or SD (n = 3 sets of duplicate plates). Data were analyzed by ANOVA, followed by Student-Newman-Keuls test. Statistical significance was set at \(P < 0.05\).
RESULTS

Cytokine-induced changes in IGFBP-1. Figure 1 illustrates that IL-1β produced a dose-dependent increase in the IGFBP-1 concentration in the medium of Hep G2 cells incubated for ~18 h. A small (12%), but statistically significant, increase in IGFBP-1 was detected at a concentration of 1 ng/ml, and a maximal response was observed between 50 and 300 ng/ml. Incubation of cells with IL-1α yielded quantitatively comparable results (data not shown). Stimulation of Hep G2 cells with either TNF-α or IL-6 also produced a dose-dependent increase in IGFBP-1 (Fig. 1). The minimum dose of these cytokines necessary to detect an increase in IGFBP-1 was 3 ng/ml (P < 0.05), whereas their ability to maximally increase IGFBP-1 concentration was 70–80% of that seen in cells treated with IL-1β (P < 0.05). However, despite the difference in maximal responsiveness of Hep G2 cells to these cytokines, there was no significant difference in the calculated ED50 for IL-1β (10 ± 3 ng/ml), TNF-α (12 ± 2 ng/ml), and IL-6 (13 ± 3 ng/ml).

Northern blot analysis revealed that IL-1β increased IGFBP-1 mRNA expression in a dose-dependent manner in Hep G2 cells incubated for 18 h (Fig. 2, A and B). IGFBP-1 mRNA abundance was also increased in cells incubated with TNF-α and IL-6, but the increment was smaller than that observed with IL-1β (data not shown). Data from the time course study indicated that IL-1β significantly increased IGFBP-1 mRNA expression by 5 h and that the incremental response was maximal and stable between 10 and 25 h (Fig. 2C). IL-1β induced comparable temporal changes in the IGFBP-1 protein concentration in the medium (data not shown).

To examine IGFBP-1 mRNA stability, cells were incubated overnight with or without IL-1β. The next morning, all cells received fresh medium (without IL-1) containing the transcription inhibitor DRB. RNA was extracted, and then mRNA was quantitated at various time points thereafter (Fig. 3, A and B). DRB effectively reduced IGFBP-1 mRNA in both control and IL-1β-treated cells at each time point examined, compared with time 0 values. The calculated apparent half-life for IGFBP-1 mRNA was essentially identical for control cells and those incubated with IL-1β (3.8 or 4.1 h, respectively). Cells pretreated with IL-1β continued to synthesize and secrete measurable amounts of IGFBP-1 protein for the first 10 h after treatment with DRB (Fig. 3C). A negligible amount of IGFBP-1 protein...
accumulated in the medium between 10 and 15 h, and this was consistent with the 90% reduction in IGFBP-1 mRNA at the 15-h time point (Fig. 3A). In the absence of DRB, cells that had been pretreated with IL-1β continued to secrete IGFBP-1 even though the cytokine had been removed from the cultures (Fig. 3D). Consistent with its effect on IGFBP-1 mRNA, DRB inhibited IGFBP-1 protein synthesis and secretion by 85% over a 24-h time period (Fig. 3D).

Insulin has been previously demonstrated to be a dominant negative modulator of IGFBP-1 secretion (26, 48). In the present study, incubation of cells with a maximally effective insulin concentration (100 nM) resulted in a 55% reduction in IGFBP-1 mRNA expression, compared with cells treated with IL-1β alone (Fig. 4). Again, the insulin- and IL-induced changes in IGFBP-1 protein secretion were comparable to those observed for mRNA expression (Fig. 4, inset).

To determine whether the stimulating effect of IL-1, TNF-α, and IL-6 on IGFBP-1 production was additive or synergistic, Hep G2 cells were incubated with a relatively low concentration (3 ng/ml) of each cytokine separately or in combination (Fig. 5). Similar to data presented earlier, both IGFBP-1 protein and mRNA expression were increased by each cytokine individually and to a greater extent in those cells treated with IL-1β. Addition of all three cytokines together resulted in an additive, or possibly synergistic, effect on the production of IGFBP-1 (Fig. 5, top). The cytokine combination also enhanced IGFBP-1 mRNA expression, and this effect appeared to be additive (Fig. 5, bottom).

Oxidative stress-induced changes in IGFBP-1. t-Butyl-H2O2 increased the medium IGFBP-1 concentration at both 100 and 300 µM (137 and 180%, respectively; Fig. 6, top). A qualitatively similar, albeit quantitatively smaller, increase in IGFBP-1 was observed at the highest dose of H2O2 (73%). The in vitro generation of H2O2 by addition of xanthine and xanthine oxidase also resulted in a small (68%) but statistically significant increase in the IGFBP-1 concentration (Fig. 6, top). IGFBP-1 secretion was also stimulated by the NO donors SNAP and SNP (Fig. 5, bottom). Higher doses of exogenous ROS were not used in this study because...
preliminary data indicated that they caused cell death, as evidenced by the increased release of LDH into the medium. Endogenous generation of peroxynitrite produced by the decomposition of SIN-1 increased IGFBP-1 levels (45.66 vs. 94.612 ng/ml), and the increment was comparable to that seen in response to other ROS described above. Although incubation of cells with t-buty1-H2O2 (300 µM) or SNAP (0.5 µM) stimulated IGFBP-1 production, no significant increase in IGFBP-1 mRNA was detected at the 18-h time point (data not shown).

Simultaneous treatment of Hep G2 cells with H2O2 and IL-1b increased the IGFBP-1 concentration (Fig. 7). However, the increment in IGFBP-1 induced by this combination was not different from that seen in cells treated with cytokine alone. Hence, the ability of H2O2 and IL-1b to increase IGFBP-1 appears to be neither additive nor synergistic.

The increase in IGFBP-1 does not appear to be a generalized stress response of Hep G2 cells, however, because the IGFBP-1 secretion was not increased by arsenite (100 µM) or endotoxin (1 µM) (data not shown).

Role of ROS, eicosanoids, and NF-κB on cytokine-induced changes in IGFBP-1. Table 1 illustrates that preincubation of hepatoma cells with either ascorbic acid, α-tocopherol, or NAC did not significantly alter basal IGFBP-1 secretion. All cells were incubated for 18 h. Values are means ± SE; n = 5–6 sets of duplicate wells. *P < 0.05 compared with control value.

t-buty1-H2O2 (300 µM) or SNAP (0.5 µM) stimulated IGFBP-1 production, no significant increase in IGFBP-1 mRNA was detected at the 18-h time point (data not shown).

Simultaneous treatment of Hep G2 cells with H2O2 and IL-1β increased the IGFBP-1 concentration (Fig. 7). However, the increment in IGFBP-1 induced by this combination was not different from that seen in cells treated with cytokine alone. Hence, the ability of H2O2 and IL-1β to increase IGFBP-1 appears to be neither additive nor synergistic.

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IGFBP-1 induced by either TNF-α or IL-6 (data not shown). Likewise, incubation of cells with various inhibitors of NO synthesis also failed to alter IL-1β- or TNF-α-induced increases in IGFBP-1 (Table 1 and data not shown). Finally, incubation with either a cyclooxygenase (indomethacin) or lipoxygenase (NDGA) inhibitor failed to significantly reduce the increment in IGFBP-1 produced by IL-1β (Table 1).

Cytokine-induced activation of NF-κB is known to increase the expression of a wide range of genes. When cells were incubated with PDTC, an inhibitor of NF-κB activation, the IL-1β-induced increase in IGFBP-1 protein secretion and mRNA expression was significantly reduced by 53 and 30%, respectively (Fig. 8). However, preincubation of Hep G2 cells with a cell-permeable inhibitor of NF-κB nuclear translocation (SN50) failed to significantly alter either the basal synthesis of IGFBP-1 or the ability of IL-1β to stimulate IGFBP-1 synthesis (data not shown).

**DISCUSSION**

There is now considerable evidence implicating cytokines in the control of various components of the growth hormone-IGF axis, particularly on the regulation of IGFBP-1. It has been previously demonstrated that in vivo administration of IL-1β, IL-6, or TNF-α markedly increases circulating levels of IGFBP-1 (9, 13, 41). Moreover, pretreating animals with a specific IL-1 receptor antagonist has been shown to completely prevent the increase in IGFBP-1 in blood and liver produced by bacterial infection (22). Neutralizing anti-

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**Table 1. Effect of reactive oxygen species scavengers and nitric oxide synthase and eicosanoid inhibitors on IL-1β-induced increases in IGFBP-1 concentration**

<table>
<thead>
<tr>
<th>Treatment (no additions)</th>
<th>IGFBP-1 Concentration, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no additions)</td>
<td>55 ± 9</td>
</tr>
<tr>
<td>+ Ascorbic acid (1 mM)</td>
<td>48 ± 7</td>
</tr>
<tr>
<td>+ α-Tocopherol (500 µM)</td>
<td>47 ± 6</td>
</tr>
<tr>
<td>+ NAC (1 mM)</td>
<td>59 ± 9</td>
</tr>
<tr>
<td>IL-1β (50 ng/ml)</td>
<td>587 ± 89*</td>
</tr>
<tr>
<td>+ Ascorbic acid (1 mM)</td>
<td>551 ± 78*</td>
</tr>
<tr>
<td>+ α-Tocopherol (500 µM)</td>
<td>647 ± 106*</td>
</tr>
<tr>
<td>+ NAC (1 mM)</td>
<td>591 ± 68*</td>
</tr>
<tr>
<td>Control (no additions)</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>+ L-NMMA (5 mM)</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>+ L-NAMMA (5 mM)</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>+ Aminoguanidine (0.5 mM)</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>IL-1β (50 ng/ml)</td>
<td>321 ± 41*</td>
</tr>
<tr>
<td>+ L-NMMA (5 mM)</td>
<td>345 ± 38*</td>
</tr>
<tr>
<td>+ L-NAME (5 mM)</td>
<td>291 ± 34*</td>
</tr>
<tr>
<td>+ Aminoguanidine (0.5 mM)</td>
<td>334 ± 46*</td>
</tr>
<tr>
<td>Control (no additions)</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>+ Indomethacin (50 µM)</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>+ NDGA (50 µM)</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>IL-1β (50 ng/ml)</td>
<td>378 ± 42*</td>
</tr>
<tr>
<td>+ Indomethacin (50 µM)</td>
<td>355 ± 45*</td>
</tr>
<tr>
<td>+ NDGA (50 µM)</td>
<td>396 ± 39*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4–5 sets of duplicate wells. Cells were preincubated with scavengers or inhibitors for 30 min before addition of interleukin-1β (IL-1β). IGFBP-1, insulin-like growth factor-1 binding protein; NAC, N-acetyl-L-cysteine; L-NMMA, N⁵-mononodemethyl-L-arginine; L-NAMMA, N⁵-nitro-L-arginine methyl ester; NDGA, nordihydroguaiaretic acid. *P < 0.05 compared with control value for each agent.
bodies to TNF-α have also been shown to partially attenuate the increased production of IGFBP-1 induced by either endotoxin or peritonitis (9, 11). Hence, not only are cytokines capable of stimulating IGFBP-1 secretion but the above-mentioned inhibitor studies suggest that they are critical components in mediating the increase observed during various inflammatory conditions. The significance of changes in IGFBP-1 during inflammation as well as the alterations observed during more physiological adaptations such as fasting and development remain to be elucidated. However, numerous in vitro studies have demonstrated the ability of IGFBP-1 to inhibit IGF-I-mediated responses (24).

We have previously demonstrated, using Western blot analysis, that proinflammatory cytokines can increase the relative concentration of IGFBP-1 in conditioned medium from Hep G2 cells (41). This observation has been confirmed and extended by the results of the present study, in which the cytokine-induced increases in IGFBP-1 have been quantitated by a specific IRMA. On the basis of IGFBP-1 protein secretion, Hep G2 cells have approximately the same sensitivity to the stimulatory effects of IL-1, IL-6, and TNF-α (ED50 ~10 ng/ml). However, the ability of IL-1 (either α- or β-isofrom) to maximally stimulate IGFBP-1 production was greater than for either IL-6 or TNF-α. Cytokine-induced increases were seen at a nominal concentration of ~1–3 ng/ml, levels that may be present either in the blood or the local hepatic environment during inflammation (8, 15). Moreover, a low dose of all three cytokines together appeared to have an additive or synergistic effect on IGFBP-1 production.

This study clearly indicates that these cytokines also produce a corresponding increase in IGFBP-1 mRNA abundance. The magnitude of the cytokine-induced increase in IGFBP-1 mRNA was comparable to that seen in hepatocytes and rat hepatoma cells incubated with dexamethasone (26, 37). The IL-1-induced increase in IGFBP-1 expression appears to be regulated at the transcriptional level because cytokine treatment did not significantly alter IGFBP-1 mRNA stability. Our estimate of IGFBP-1 mRNA half-life in Hep G2 cells under basal conditions is in agreement with that reported for rat hepatoma cells (36). The present findings are the first to indicate that IGFBP-1 mRNA is transcriptionally regulated by IL-1 in hepatic cells. This is the same mechanism by which insulin and glucocorticoids control IGFBP-1 synthesis (36, 48).

Similarly, the time course for the IL-1β-induced increase in IGFBP-1 mRNA was comparable to that previously observed in cells incubated with dexamethasone (36), with the half-maximal increase occurring at ~5 h. Moreover, insulin appears to partially suppress both the IL-1- and dexamethasone-induced increase in IGFBP-1 synthesis (26).

The half-life of IGFBP-1 mRNA deduced by DRB treatment and Northern blotting is consistent with the finding that IGFBP-1 protein increases in the medium of Hep G2 cells during the first 10 h of DRB treatment but that additional accumulation is negligible after 15 h when more than 93% of the IGFBP-1 mRNA has been lost from the cells. DRB significantly inhibited IGFBP-1 mRNA and protein accumulation in both control cells and those treated with IL-1β. DRB was chosen as the transcription inhibitor for these experiments after a preliminary study demonstrated that another transcription inhibitor, actinomycin D, paradoxically stimulated the synthesis and secretion of IGFBP-1 and increased IGFBP-1 mRNA at time points after 12 h. This response to actinomycin is similar to the superinduction of NF-κB that has been previously observed in epithelial cells treated with actinomycin D (34) or the superinduction of IGFBP-1 mRNA in Hep G2 cells treated with cyclohexamide (35).

A portion of the hepatic response to cytokines is mediated via the production of ROS, including reactive intermediates of oxygen (i.e., H2O2) and nitrogen (i.e., NO). Hence, the ability of cytokines to increase IGFBP-1 synthesis may be regulated by the secondary production of one or more ROS. To determine whether IGFBP-1 production by Hep G2 cells could be induced by reactive oxygen intermediates (ROI), cells were incubated with either H2O2, the more stable t-butyl-H2O2, or the combination of xanthine plus xanthine oxidase, which generates ROI endogenously. This appears to be the first report that ROI are capable of stimulating IGFBP-1 protein release in vitro. In addition, reactive NO donors and peroxynitrite were also able to enhance IGFBP-1 production to a comparable extent. It is noteworthy, however, that the increase in IGFBP-1 production induced by ROS was considerably smaller than that observed in response to cytokine stimulation (2-fold vs. 8-10-fold). Finally, no synergistic or additive effect was detected in cells incubated with both ROS and IL-1β, suggesting that ROS and IL-1β enhanced IGFBP-1 production via a similar mechanism. In contrast to the response observed with cytokines, we did not detect an increase in IGFBP-1 mRNA in cells incubated with ROS. Although this lack of response has not been further examined, it is possible that ROS did increase IGFBP-1 mRNA but that the increase was too small to reliably detect or occurred relatively early after the addition of ROS and was transient in nature.

Although ROS were able to stimulate IGFBP-1 release, their role in mediating cytokine-induced changes in Hep G2 cells appears minimal. Our data indicate that various antioxidants, free radical scavengers, and inhibitors of inducible and/or constitutive NO synthase failed to prevent or attenuate the release of IGFBP-1 in response to cytokines. Similar doses of these inhibitors have been previously reported to prevent other cytokine-induced responses (as referenced in MATERIALS AND METHODS), and, therefore, the failure of these agents to effectively inhibit increases in IGFBP-1 does not appear related to the use of suboptimal doses. Although endogenously produced ROS did not appear to mediate cytokine-induced increases in IGFBP-1 under this particular experimental paradigm, this does not exclude the possibility that ROS are important regulators of
IGFBP-1 synthesis in other cell types or under different conditions.

Cytokines are known to stimulate arachidonic acid metabolism, and the eicosanoids generated appear capable of mediating gene expression (19). Enzymatic oxidation of arachidonic acid via the cyclooxygenase and lipooxygenase pathways leads to the synthesis of various prostaglandins, thromboxanes, and leukotrienes. However, pretreatment of Hep G2 cells with indomethacin, which selectively inhibits the cyclooxygenase pathway, or NDGA, which preferentially inhibits the lipooxygenase pathway at the dose used, had no detectable effect on the cytokine-induced increase in IGFBP-1.

Transcription factors, such as NF-κB and activator protein-1, are important in upregulating the expression of specific genes in response to extracellular signals, including cytokines and ROS (1). The inducible expression of many of these genes represents a central cellular response to stress, injury, and inflammation. In the present study, PDTC significantly attenuated the IL-induced increase in IGFBP-1 mRNA and protein. Because the dithiocarbamates are both inhibitors of NF-κB activity and potent antioxidants, the exact mechanism for the PDTC inhibition of the cytokine-induced increase in IGFBP-1 remains unclear. However, nuclear translocation of NF-κB does not appear necessary for upregulation of IGFBP-1 synthesis. Pretreatment of cells with SN50, a cell-permeable peptide containing the p50 NF-κB subunit NLS, failed to block the increase in IGFBP-1 induced by IL-1β. This peptide has previously been shown to maximally inhibit NF-κB translocation at the concentration and in the time frame used in the present study, whereas peptides with a mutated NLS had no effect (28). These findings are consistent with the ability of PDTC to inhibit activation of an IL-1β receptor-associated protein kinase and thus one of the earliest steps in IL-1 signaling after ligand binding to its receptor (46). Therefore, our data suggest that IL-1β increases IGFBP-1 synthesis through a PDTC-sensitive, but NF-κB-independent, pathway, which has been previously hypothesized to mediate other effects of IL-1β (5).

In summary, our data indicate that in human hepatoma cells IL-1, IL-6, and TNF-α produce dose-dependent increases in IGFBP-1 protein release and IGFBP-1 mRNA expression and that the increased rate of IGFBP-1 synthesis is due primarily to stimulation of transcription. Moreover, although ROS are capable of enhancing IGFBP-1 release, cytokine-induced increases in IGFBP-1 release are not mediated by endogenously produced ROS and also appear to be independent of eicosanoids. In contrast, part of the IL-induced increase is mediated via a PDTC-sensitive pathway.

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REFERENCES


Cytokines, ROS, and IGFBP-1

G727


