Increased GADD Gene Expression in Human Colon Epithelial Cells Exposed to Deoxycholate*

By: DAVID W. SCOTT, SOPHIA MUTAMBA, ROBIN G. HOPKINS, and GEORGE LOO

Scott, D.W., Mutamba, S., Hopkins, R.G. and Loo, G. (2005) Increased GADD Gene Expression in Human Colon Epithelial Cells Exposed to Deoxycholate. J. Cell. Physiol. 202, 295-303. DOI: 10.1002/jcp.20135

Abstract:

The colonic epithelium is often exposed to high concentrations of secondary bile acids, which stresses the epithelial cells, leading potentially to activation of stress- response genes. To examine this possibility in vitro, the purpose of this study was to determine if expression of certain growth arrest and DNA damage- inducible genes (GADD) is upregulated in human colonic epithelial cells exposed to deoxycholate (DOC). DNA macroarray screening of a small cluster of stress/apoptosis-related genes in DOC-treated HCT-116 colonocytes revealed clearly higher expression of only GADD45, which was confirmed by gene-specific relative RT-PCR analysis. Subsequently, it was found that DOC also increased GADD34 mRNA expression. However, mRNA expression of GADD153 was increased most markedly in DOC-treated HCT-116 colonocytes, which express wild-type p53. However, the upregulation of GADD34, GADD45, and GADD153 mRNA expression apparently did not require p53, based on the finding that DOC increased expression of all three GADD genes in HCT-15 colonocytes, which express mutant p53. In further studying GADD153 in particular, the effect of DOC on GADD153 mRNA was prevented by actinomycin-D (Act-D), but not by antioxidants or MAPK inhibitors. DOC also caused GADD153 protein to be expressed in close parallel with increased GADD153 mRNA expression. Induction of GADD153 protein by DOC was prevented by either anisomycin or cycloheximide. These findings suggest that DOC-induced upregulation of GADD153 mRNA expression occurred at the level of transcription without involving reactive oxygen species and MAPK signaling, and that the expression of GADD153 protein was due also to translation of pre-existing, and not just newly synthesized, mRNA.

Article:

As emulsifying agents, bile acids facilitate the digestion and absorption of dietary fat. Primary bile acids, such as cholic acid and chenodeoxycholic acid, are synthesized as polar derivatives of cholesterol in the liver for subsequent storage in the gall bladder. When dietary fat enters the small intestine, bile containing the primary bile acids is secreted into the small intestine upon contraction of the gall bladder. Thus, high fat diets especially increase primary bile acid secretion. Although most of the primary bile acids are reabsorbed for reutilization, a small but significant amount escapes and reaches the large intestine for transformation by colonic bacteria into secondary bile acids or their salts, such as sodium deoxycholate (DOC). An early study (Stadler et al., 1988) determined that the concentrations of total bile acids in fecal water were as high as 1 mM, and DOC approached concentrations as high as 0.8 mM, in human subjects given additional fat in the diet. It is thought that the presence of excess secondary bile salts in the large intestine could have adverse effects on colonocytes. This possibility is supported by the results from in vitro studies. In particular, in our previous study (Powolny et al., 2001), evidence of DNA damage and apoptosis were found in HCT-116 and HCT-15 colonocytes exposed to DOC. It was concluded that DOC caused irreparable DNA damage that in turn was sufficient to trigger apoptosis. However, no experiments were conducted to ascertain if DOC increased the expression of specific stress-response and/or apoptotic genes as a potential consequence of the cellular DNA damage.

* Contract grant sponsor: North Carolina Agricultural Research Service; Contract grant sponsor: North Carolina Institute of Nutrition.

The expression of growth arrest and DNA damage- inducible genes (GADD), such as GADD34, GADD45, and GADD153, is often increased when cells are subjected to a stressful environment that may result in cell-cycle arrest and/or cell death. For example, GADD gene expression is upregulated when cells are deprived of essential nutrients, including glucose (Carlson et al., 1993), leucine (Bruhat et al., 1997), glutamine (Abcouwer et al., 1999), and zinc (Fanzo et al., 2001). Additionally, exposing cells to DNA-damaging agents such as peroxynitrite (Oh-Hashi et al., 2001), UV radiation (Tong et al., 2001), and anti-cancer drugs (Kim et al., 2002) increase GADD gene expression, as can situations that cause stress or malfunction in the endoplasmic reticulum (ER) (Nozaki et al., 2001). In terms of cellular function, GADD34 seems to have a direct role in initiating apoptosis, as supported by the observation that GADD34-transfected cells had nuclear fragmentation typically characteristic of apoptosis (Hollander et al., 2001). Although its main function is unclear (Sheikh et al., 2000), GADD45 may be involved in regulating cell growth, based on the finding that insertion of a GADD45 expression vector into cells arrested them in the G2-phase of the cell-cycle (Wang et al., 1999). Like GADD34, GADD153 also appears to have a direct role in initiating apoptosis, based on the occurrence of apoptosis in GADD153 expression vector-transfected cells (Maytin et al., 2001). Several subsequent studies (Conn et al., 2002; Kim et al., 2002; Lengwehasatit and Dickson, 2002; Xia et al., 2002) have suggested that GADD153 triggers the critical early events leading to the initiation of apoptosis.

To gain a better understanding of the molecular effects of bile acids on colonocytes, the aim of the present study was to determine whether the expression of certain members of the GADD gene family are upregulated as a result of exposing HCT-15 and HCT-116 colonocytes to DOC. Initial results are reported that show DOC increased mRNA expression of GADD34, GADD45, and GADD153 without an apparent requirement for the transcription factor, p53. Focusing on GADD153 because it was the most upregulated GADD gene in colonocytes exposed to DOC, evidence is presented to support the notion that DOC increased GADD153 gene expression at the level of transcription, which involved neither redox-sensitive steps nor the mitogen-activated protein kinase (MAPK) signaling pathway.

MATERIALS AND METHODS

Materials

HCT-116 and HCT-15 human colon adenocarcinoma cell lines were purchased from the American Type Culture Collection (Manassas, VA). PD098059 and SB203580 were from Calbiochem (San Diego, CA) and SP600125 from Tocris (Ellisville, MO). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Cell culture and treatment

HCT-116 and HCT-15 colonocytes were propagated in McCoy's 5A and RPMI-1640 media (Sigma Chemical Co.), respectively, that were supplemented with 100 ml/ L fetal bovine serum (BioWhittaker, Inc., Walkersville, MD), 2 mmol/L glutamine, 0.54 µmol/L fungizone, 100,000 U/L penicillin, and 100 mg/L streptomycin (last four items from Atlanta Biologicals, Atlanta, GA). Upon reaching 70–80% confluency, the cells were exposed to 0–300 µM DOC for 0–4 h. In some experiments, the cells were co-incubated with 5 µg/ml actinomycin-D (Act-D) and DOC. Cells were also co-incubated with PD098059 (50 µM), SB203580 (10 µM), SP600125 (0.1–10 µM), staurosporine (0.5–2 µM), or calphostin C (1–2 µM) and DOC. In other experiments, cells were pre-treated with 20 mM N-acetylcysteine (NAC), 0.1–0.2 mM α-tocopherol (a-TH), 100 µM epigallocatechin gallate (EGCG), 50 µM pyrrolidine dithiocarbamate (PDTC), or 5,000– 10,000 U/ml catalase for 0.5–2 h, prior to exposing the cells to DOC.

DNA macroarray analysis of gene expression

Briefly, total RNA was isolated with a QIAGEN RNeasy kit. Following the instructions provided with the Human Stress and Apoptosis GEArray Kit (Superarray, Inc., Bethesda, MD), the isolated RNA was then used to generate biotin-labeled cDNA probes by reverse transcription. After hybridizing the biotinylated c-DNA

samples to the gene-specific oligomers pre-spotted on the nylon membrane, the chemiluminescence signal was generated with the streptavidin-alkaline phosphatase system and detected using Kodak X-ray film.

Determination of mRNA expression

Total RNA was isolated from the cells using a Qiagen RNeasy Mini kit. The expression levels of GADD34, GADD45, GADD153, and GRP78 mRNA were determined by multiplex relative RT-PCR analysis of total RNA using a Qiagen OneStep RT-PCR kit and gene-specific primers. The PCR primer base sequences for GADD34 and GADD45 (Oh-Hashi et al., 2001) were: GADD34 sense primer, 5'-ATG TAT GGT GAG CGA GAG GC-3'; GADD34 antisense primer, 5'-GCA GTG TCC TTA TCA GAA GGC-3'; GADD45 sense primer, 5'- AGA ACG ACA TCA ACA TCC TGC-3'; GADD45 antisense primer, 5'-AAT GTG GAT TCG TCA CCA GA-3'. The GADD153 PCR primer sequences (Mertani et al., 2001) were: sense, 5'-GCACCTCCCAGAGCCCTCACTCTCC-3' and antisense, 5'-GTCTACTCCAAGCCTTCCCCCTGCG-3'. The PCR primer base sequences for GRP78 were from the NCBI UniSTS data-base of NIH (UniSTS:48897) (forward primer: GCCTAAGCGGCTGTTTACTG, reverse primer: AACTTCCTACACCAGATGCACA). Either the β-actin or 18S rRNA QuantumRNA primer/competimer sets (Ambion, Inc., Austin, TX) were utilized to generate the internal standards. Thus, to observe the greatest electrophoretic separation between the targets and internal controls, GADD153 was multiplexed with B-actin, whereas GADD34, GADD45, and GRP78 were each multiplexed with 18S rRNA, during multiplex RT-PCR. The multiplex RT-PCR conditions were 30 min at 50°C followed by 15 min at 95°C (RT), then 0.5 min at 95°C, 0.5 min at 60°C, and 1 min at 72°C (PCR) for 24 cycles (GADD34), 25 cycles (GADD153), or 28 cycles (GADD45).

The multiplex RT-PCR conditions for GRP78 were 30 min at 48°C followed by 15 min at 95°C (RT), then 0.5 min at 94°C, 0.5 min at 48°C, and 1 min at 72°C for 32 cycles (PCR). The resulting cDNA products were separated by 2% agarose gel electrophoresis with ethidium bromide staining. For the target genes, the PCR product sizes were 110 bp (est),150 bp (est), 422 bp, and 175 bp for GADD34, GADD45, GADD153, and GRP78, respectively. For the internal controls, the PCR product sizes were 294 and 495 bp for β-actin or 18S rRNA, respectively.

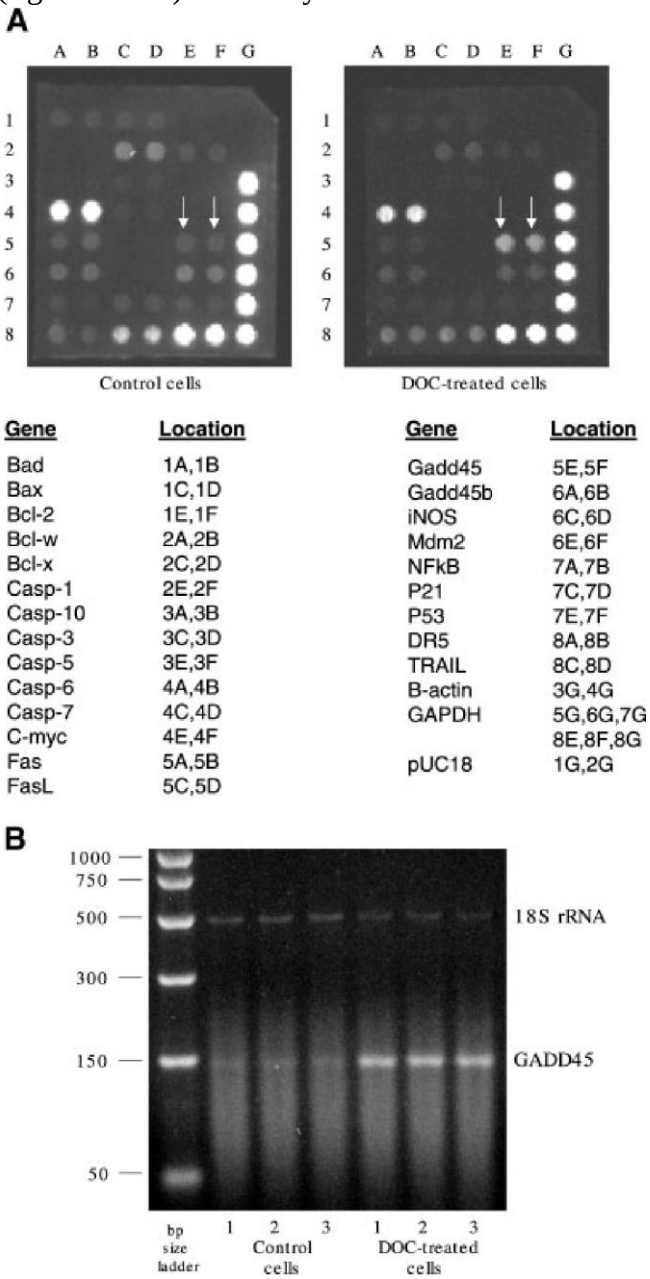
Western immunoblotting analysis of protein expression

After scraping, the cells were harvested and washed by centrifugation (500g for 5 min) in Hank's balanced salt solution (HBSS). The cell pellets were frozen at —80°C before thoroughly resuspending in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and Roche Complete Protease Inhibitor Cocktail, pH 7.4) and left on ice for 30 min. Next, the samples were centrifuged at 16,000g for 20 min at 4°C, and the supernatant was saved. The protein concentration of the supernatant was determined with a BCA protein assay kit (Pierce, Inc., Rockford, IL). Whole cell lysate supernatant (50 µg protein) was electrophoresed using Novex NuPAGE mini-gels (4– 12% Bis-Tris) before blotting the gel to nitrocellulose membrane. Blocking was performed for 1 h at 25°C in blocking buffer consisting of 5% skim milk powder in TBST (20 mM Tris-HCl,150 mM sodium chloride, 0.05% Tween-20, pH 7.4). The membrane was incubated overnight at 4°C with GADD153 rabbit polyclonal R-20 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in blocking buffer (1:5,000). After washing in TBST, the membrane was then incubated 2 h at 25°C with goat anti-rabbit IgG/HRP conjugate (Santa Cruz) in blocking buffer (1:100,000). Finally, after washing again in TBST, the membrane was placed in a plastic pouch for incubation with Pierce SuperSignal WestFemto Maximum Sensitivity Substrate for 5 min before analysis (Kodak digital science TM image station 440 CF). Afterwards, the membrane was kept in the plastic pouch overnight at 25°C, before reprobing for β-actin as a control for sample loading. The membrane was washed with TBST and incubated 2 h at 25°C with mouse monoclonal anti-β-actin antibody (Sigma Chemical Co.) in blocking buffer (1:50,000). After incubation for 1 h at 25°C with HRP-conjugated goat polyclonal anti-mouse IgG (Santa Cruz) in blocking buffer (1:100,000), the membrane was washed in TBST and processed for analysis as before.

RESULTS

Effect of DOC on GADD45 mRNA expression in HCT-116 colonocytes as first screened with a DNA macroarray system

Differential gene expression was initially assessed in DOC-treated and untreated cells using a dedicated DNA macroarray system, which was limited to a relatively small number of relevant genes that included GADD45. Of the small panel of 23 stress/apoptosis-related genes examined (Fig. 1A), it appeared that the expression of GADD45 was increased to the greatest extent. More specifically, the most striking difference can be seen in comparing the duplicate 5E and 5F dot signal intensities (i.e., GADD45 gene) for control cells (left autorad) with that of DOC-treated cells (right autorad). To verify that



Gene	Location	Gene	Location
Bad	1A,1B	Gadd45	5E,5F
Bax	1C,1D	Gadd45b	6A,6B
Bcl-2	1E,1F	iNOS	6C,6D
Bcl-w	2A,2B	Mdm2	6E,6F
Bcl-x	2C,2D	NFkB	7A,7B
Casp-1	2E,2F	P21	7C,7D
Casp-10	3A,3B	P53	7E,7F
Casp-3	3C,3D	DR5	8A,8B
Casp-5	3E,3F	TRAIL	8C,8D
Casp-6	4A,4B	B-actin	3G,4G
Casp-7	4C,4D	GAPDH	5G,6G,7G
C-myc	4E,4F		8E,8F,8G
Fas	5A,5B	pUC18	1G,2G
FasL	5C,5D		

Fig. 1. Effect of deoxycholate (DOC) on GADD45 gene expression in HCT-116 colonocytes as screened by DNA macroarray analysis and confirmed by multiplex relative RT-PCR analysis. The cells were incubated with 300 μM DOC for 4 h. Total RNA was isolated for initial gene expression analysis utilizing a dedicated DNA macroarray system with chemiluminescence detection (**A**). The small arrows in each autorad of the macroarrays point to duplicate dots (5E and 5F) representing GADD45, which appeared to be the single gene most upregulated. For verification, multiplex relative RT-PCR was performed using gene specific primers for the target gene *GADD45* and the internal control gene 18S rRNA (**B**). The PCR products were separated on 2% agarose gel and stained with ethidium bromide. The results are for three different experiments.

DOC actually increased GADD45 gene expression, the total RNA samples from above were subjected to multiplex relative RT-PCR analysis, using gene-specific primers for GADD45 and also 18S rRNA as the internal control (Fig. 1B). The agarose gel shows separation of the two PCR products, namely, that corresponding to the mRNA of GADD45 and 18S rRNA. Control cells constitutively expressed barely detectable GADD45 mRNA levels. In contrast, DOC-treated cells expressed noticeably higher GADD45 mRNA levels.

Effect of DOC on GADD34 and GADD153 mRNA expression in HCT-116 colonocytes

To consider the possibility that DOC increased the expression of other GADD genes, GADD34 and GADD153 mRNA expression was assessed by multiplex relative RT-PCR analysis. Exposing HCT-116 colonocytes to 300 µM DOC for 4 h increased GADD34 mRNA expression slightly (Fig. 2A), whereas there was a marked increase in GADD153 mRNA expression (Fig. 2B).

Significance of p53 for the effects of DOC on GADD mRNA expression

Whereas HCT-116 colonocytes express wild-type p53, HCT-15 colonocytes express mutant p53 (Chinery et al., 1997). Because of this cellular difference in p53 gene status and the fact that at least one GADD gene is responsive to p53 (Kastan et al., 1992), the two distinct cell lines were utilized to evaluate the requirement for p53 in mediating DOC-induced GADD gene expression. As shown by the data, the conclusion is that mRNA expression of GADD34 (Fig. 3A), GADD45 (Fig. 3B), and GADD153 (Fig. 3C) was increased by DOC in HCT-15 colonocytes, suggesting that p53 was not required for activating any of the three GADD genes as caused by DOC.

Because GADD 153 was affected to the greatest extent by DOC of the three GADD genes investigated, further experiments were conducted to generally characterize the increase in GADD153 mRNA expression in DOC-treated HCT-116 colonocytes. Additionally, several substances were evaluated for their abilities to influence DOC-induced upregulation of GADD153 mRNA expression. The results of these experiments are detailed below.

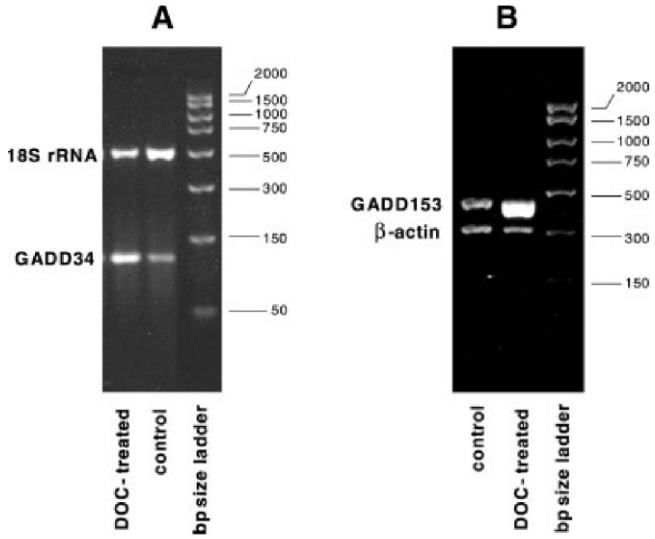


Fig. 2. Effect of DOC on mRNA expression of GADD34 (**A**) and GADD153 (**B**) in HCT-116 colonocytes. The cells were incubated with 300 µM DOC for 4 h. Total RNA was isolated for subsequent multiplex relative RT-PCR analysis using gene specific primers for the target genes GADD34 and GADD153, and also the internal control genes 18S rRNA and β-actin. The PCR products were separated on 2% agarose gel and stained with ethidium bromide. The results are representational of three different experiments.

DOC-induced upregulation of GADD153 expression in HCT-116 colonocytes in concentration-dependent and time-dependent manners

The effect of DOC on GADD 153 mRNA expression was concentration-dependent and time-dependent. With a 4-h incubation time, DOC was able to increase GADD153 mRNA expression at a concentration of 200 µM,

with additional upregulation at 300 µM (Fig. 4A). No effects were evident at 100 µM and 50 µM. Regarding time of incubation of HCT-116 colonocytes with DOC, a noticeable inductional effect of 300 µM DOC on GADD153 mRNA was seen after only 1 h of incubation, with further induction occurring with longer incubation (Fig. 4B).

Effect of DOC on GRP78 mRNA expression

The GADD153 gene can be activated as a result of stress in the ER (Nozaki et al., 2001). To address the possibility that DOC induces ER stress in HCT-116 colonocytes, expression of the ER stress gene, GRP78, was determined (Fig. 4C). DOC did not affect the expression of GRP78 mRNA.

Sensitivity of DOC-induced upregulation of GADD153 mRNA expression to Act-D, cycloheximide, antioxidants, and MAPK inhibitors

To help determine if the effect of DOC on GADD153 mRNA expression could be due to transcription, the RNA synthesis inhibitor, Act-D, was used to block transcription in DOC-treated HCT-116 colonocytes to see if GADD153 mRNA still increased (Fig. 5A, upper part). DOC markedly increased GADD153 mRNA expression (lane 2), when compared to the control cells (lane 1). However, when colonocytes were co-treated with Act-D and DOC, the increased expression of GADD153 mRNA caused by DOC was prevented (lane 3). Therefore, these data suggest that the increased expression of GADD153 mRNA caused by DOC was most likely due to increased synthesis, rather than stability, of the transcript. To determine if the increased GADD 153 mRNA caused by DOC required protein synthesis, GADD153 mRNA levels were assessed in the presence of cycloheximide (CHX), as shown in Figure 5A (lower part). As can be seen, CHX noticeably attenuated DOC-induced upregulation of GADD153 mRNA expression. This finding would imply that DOC induced transcription of a specific transcription factor(s), whose subsequent translation would be needed for downstream activation of GADD153 gene transcription.

It is known that GADD genes become activated when cells are subjected to oxidative stress (Oh-Hashi et al., 2001), suggesting that reactive oxygen species could be involved in GADD gene activation. Moreover, there is

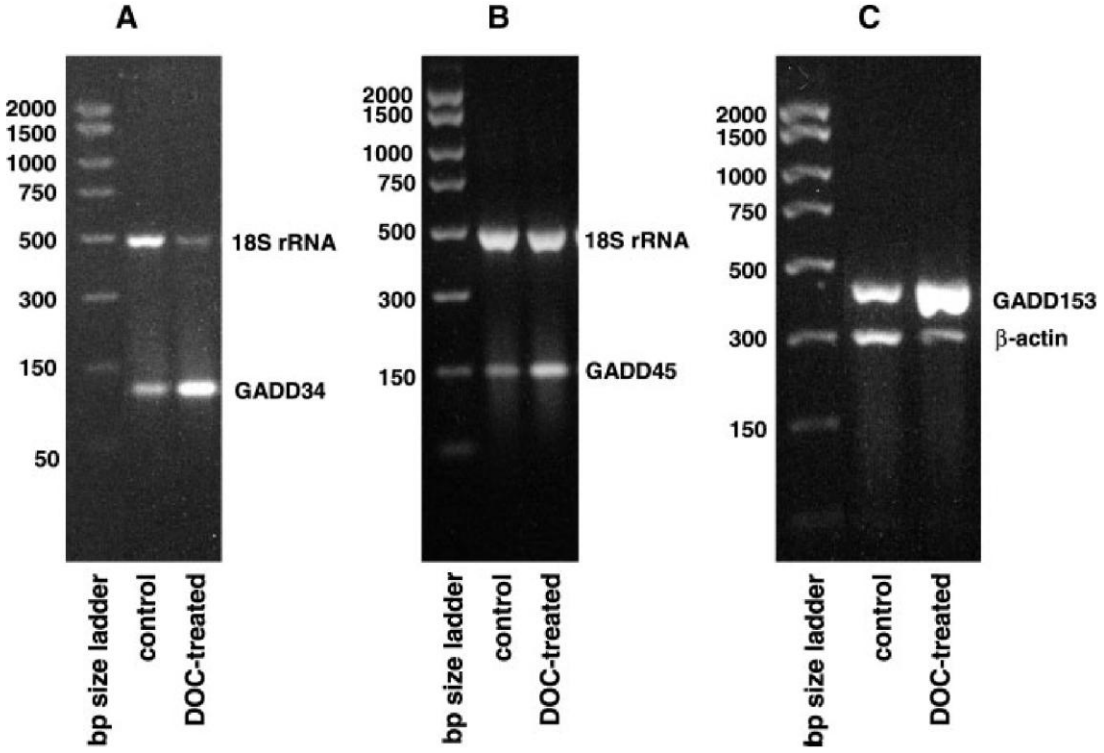


Fig. 3. Effect of DOC on mRNA expression of GADD34 (**A**), GADD45 (**B**), and GADD153 (**C**) in HCT-15 colonocytes. Cells were incubated with 300 µM DOC for 4 h. Total RNA was isolated for subsequent multiplex relative RT-PCR analysis using gene specific primers for the target genes (*GADD34*, *GADD45*, and *GADD153*) and the internal control genes (18S rRNA and β-actin). PCR products were separated on 2% agarose gel and stained with ethidium bromide. The results are representational of three different experiments.

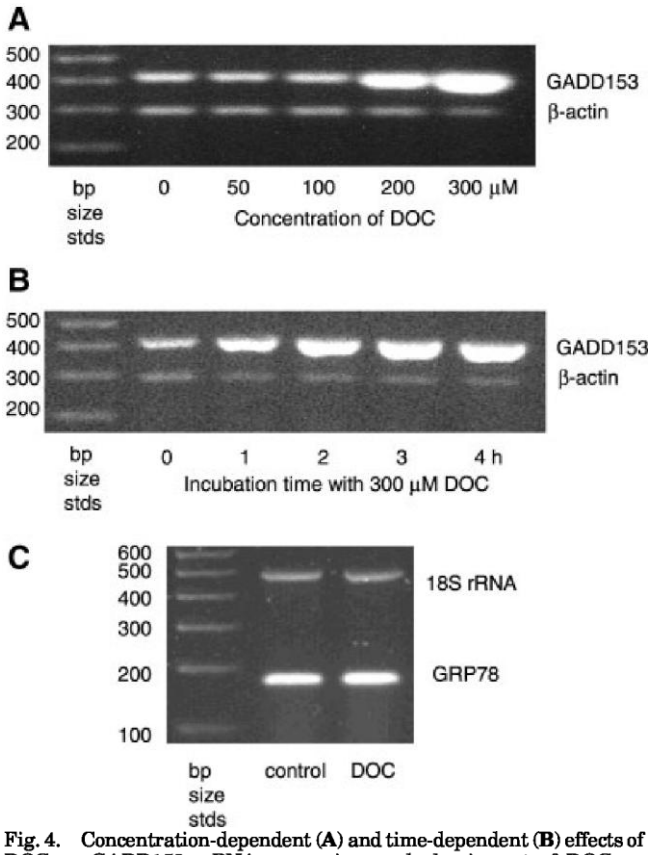


Fig. 4. Concentration-dependent (**A**) and time-dependent (**B**) effects of DOC on GADD153 mRNA expression, and also impact of DOC on GRP78 mRNA expression (**C**), in HCT-116 colonocytes. Cells were exposed to 0–300 μM DOC for 4 h (A). Additionally, cells were exposed to 300 μM DOC for 0–4 h (B), or in another experiment, to just 300 μM DOC for only 4 h (C). After exposure, multiplex relative RT-PCR analysis was performed, using gene specific primers for the target genes, and also the respective internal control genes. The PCR products were separated on 2% agarose gel and stained with ethidium bromide. The results are representational of three different experiments.

evidence to support the concept that exposing cells to DOC promotes the formation of reactive oxygen species (Sokol et al., 1993). Therefore, an experiment was simultaneously performed to determine if the antioxidant, NAC that raises intracellular reduced glutathione levels (Meister, 1991), could prevent DOC-induced upregulation of GADD153 mRNA expression (Fig. 5A, upper part). To the contrary, NAC was unable to prevent the increased GADD153 mRNA expression caused by DOC (lane 4). In another experiment, PDTC and EGCG (Fig. 5B), as well as catalase and α-tocopherol (Fig. 5C) also could not prevent DOC-induced GADD 153 mRNA upregulation.

MAPK have been reported to be involved in oxidant (peroxynitrite)-induced upregulation of GADD153 mRNA expression (Oh-Hashi et al., 2001). To determine if MAPK are involved in increasing the expression of GADD 153 mRNA caused by DOC, HCT-116 colonocytes were pre-treated with selective chemical inhibitors of MAPK and then exposed to DOC. As shown by Figure 6, the three MAPK inhibitors (50 μM ERK inhibitor

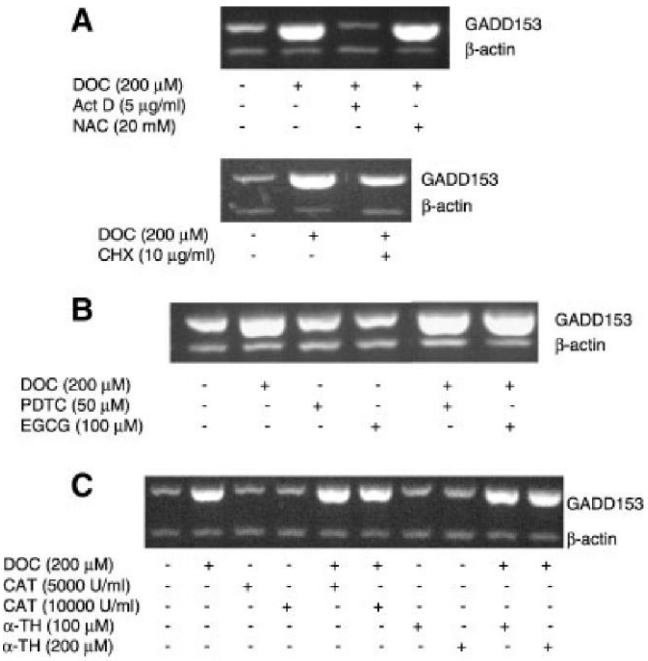


Fig. 5. Effects of actinomycin-D (Act-D), cycloheximide (CHX), and antioxidants on DOC-induced upregulation of GADD153 mRNA expression in HCT-116 colonocytes. Cells were co-treated for 4 h with DOC and either Act-D (**A**, upper part) or CHX (A, lower part). Additionally, cells were pre-treated with *N*-acetylcysteine (NAC) for 2 h, and then exposed to DOC for 4 h (A, upper part). In (**B**), cells were pre-treated with pyrrolidine dithiocarbamate (PDTC) or epigallocatechin gallate (EGCG) for 30 min, and then exposed to DOC for 4 h. In (**C**), cells were co-treated with catalase and DOC for 4 h. Other cells were pre-treated with α-tocopherol (α-TH) for 12 h and then exposed to DOC. Total RNA was subsequently isolated for multiplex relative RT-PCR analysis using gene specific primers for the target gene *GADD153* and the internal control gene β-actin. PCR products were separated on 2% agarose gel and stained with ethidium bromide. The results are representational of three different experiments.

PD098059, 10 μM p38 inhibitor SB203580, and 10 μM JNK inhibitor SP600125) each failed to prevent DOC-induced upregulation of GADD153 mRNA. That is, the MAPK inhibitors appeared to have only marginal effects at best, which is insufficient to definitively establish an involvement of MAPK in DOC-induced GADD153 gene activation. It is noteworthy that higher concentrations of SP600125 per se actually increased GADD 153 mRNA expression and caused apoptosis (data not shown). Even at the 10 μM concentration tested, SP600125 by itself appeared to increase GADD153 mRNA expression (Fig. 6A, lane 7). Therefore, lower concentrations of SP600125 were tested to consider if SP600125 could actually prevent or attenuate DOC-induced GADD153 mRNA upregulation. As can be seen (Fig. 6B), negative results were found.

In contrast to the selective MAPK inhibitors, the general protein kinase C (PKC) inhibitor, staurosporine, was able to prevent DOC-induced upregulation of GADD153 mRNA expression, as shown in Figure 6C. On the other hand, as further seen, a more specific PKC inhibitor (calphostin C) lacked this capability. Therefore, the potential role of PKC in DOC-induced GADD153 mRNA upregulation remains unclear.

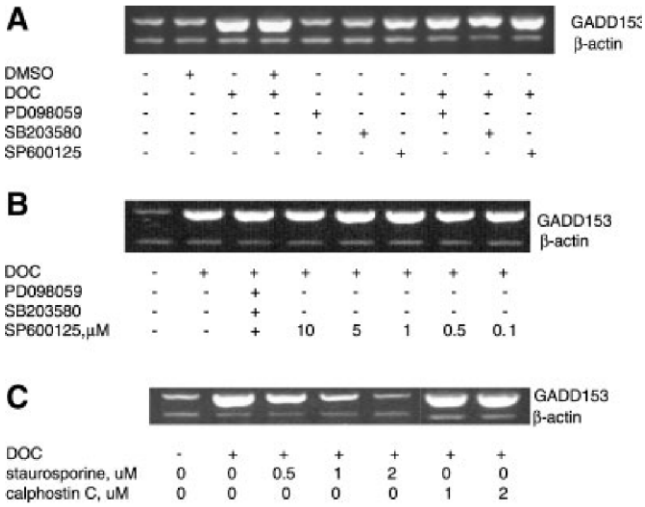


Fig. 6. Effect of MAPK (A, B) and PKC (C) inhibitors on GADD153 mRNA expression in HCT-116 colonocytes exposed to DOC. Cells were co-treated with PD098059 (50 μM), SB203580 (10 μM), SP600125 (0.1–10 μM), staurosporine (0–2 μM), or calphostin C (0–2 μM), and DOC (200 μM) for 4 h. Total RNA was subsequently isolated for multiplex relative RT-PCR analysis using gene specific primers for the target gene *GADD153* and the internal control gene β-actin. PCR products were separated on 2% agarose gel and stained with ethidium bromide. The results are representational of three different experiments.

Effect of DOC on GADD153 protein expression in HCT-116 colonocytes

To determine whether the changes in GADD153 mRNA levels produced corresponding increases in GADD 153 protein levels, Western-immunoblotting analysis was lastly performed on DOC-treated cells. As shown in Figure 7A, GADD153 protein was undetectable in HCT-116 colonocytes not exposed to DOC. Faint expression of GADD153 protein (apparent MW: 27 kDa) was detectable in cells exposed to 100 μM DOC, but with much stronger expression in cells exposed to 200 and 300 μM DOC. GADD153 protein expression started to become apparent after 2 h of incubation (Fig. 7B). Thus, it seems that newly synthesized GADD153 mRNA available as early as 1 h after exposure of the colonocytes to DOC (Fig. 4B) is rapidly translated into GADD153 protein. On the other hand, because some GADD153 mRNA is detectable in HCT-116 colonocytes even without any stimulation as shown above, these findings suggest that the pre-existing GADD153 mRNA could have been translated into protein in contributing to increased GADD 153 protein expression in DOC-treated colonocytes. Furthermore, it is likely that the increase in GADD153 protein was more a result of translation, rather than increased protein half-life, because the protein synthesis inhibitors, ASM and CHX, each prevented the effect of DOC on GADD153 protein expression (Fig. 7C).

DISCUSSION

Bile acids carry out an important physiological function in the small intestine as they facilitate the processing and eventual absorption of various lipids present in foods that are ingested. However, after

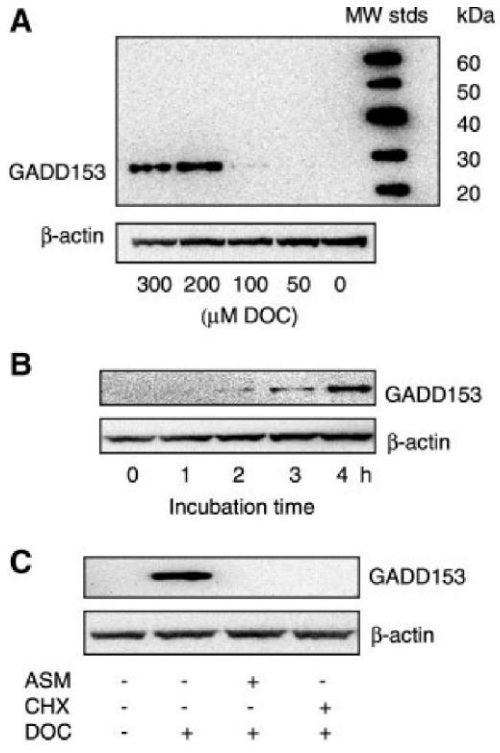


Fig. 7. GADD153 protein expression in HCT-116 colonocytes as affected by DOC concentration, incubation time, and protein synthesis inhibitors. Cells were exposed to different concentrations of DOC (0–300 μM) for 4 h (**A**) or to 300 μM DOC for different time periods ranging from 0–4 h (**B**). In (**C**), cells were exposed for 4 h–200 μM DOC in the presence of 10 μg/ml cycloheximide (CHX) or anisomycin (ASM). Whole cell lysates were prepared for subsequent Western-immunoblotting analysis for GADD153 protein and also β-actin, as detailed under "Materials and Methods."

performing this task, bile acid metabolites such as DOC eventually become present in the large intestine, especially when high amounts of fat are consumed.

It is thought that chronic exposure of the colonic epithelium to DOC has undesirable effects on the epithelial cells that can result in disease. This notion is supported by epidemiological, animal and cellular studies. For example, there is a link between the incidence of colon cancer and diets high in fat (Reddy, 1995), which may be explained by the cancer-promoting abilities of DOC (Owen, 1997). Elevated levels of secondary bile acids are present in the colon residual fluid or fecal material of human subjects with colon cancer (Imray et al., 1992). When administered to rats, DOC increased the proliferation of colonic crypt cells (Hori et al., 1998) and potentiated the stomach tumorigenic effect of the chemical carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (Kobori et al., 1984). In cultured colonocytes, DOC caused activation of protein kinase C (Huang et al., 1992), which is a common event triggered by many tumor promoters. Additionally, activation of the transcription factors, AP-1 (Hirano et al., 1996) and NF-κB (Payne et al., 1998), as well as the NF-κB-responsive cyclooxygenase-2 (COX-2) gene (Zhang et al., 1998), has been reported in DOC-treated cells. Transcriptional activation of COX-2 may promote colon tumor cell growth and proliferation, since expression of COX-2 has been found in colon tumor tissue but not normal colon tissue (Sano et al., 1995) although such distinct localization of COX-2 apparently has been refuted more recently (Garewal et al., 2003).

Genotoxic and cytotoxic effects of DOC have also been previously reported. For example, in our earlier study (Powolny et al., 2001), DOC induced single-strand breaks in DNA of HCT-116 and HCT-15 colonocytes. Soon after the initial occurrence of DNA damage, recognizable features characteristic of apoptosis became evident. Hence, these previous findings suggested to us that the expression of certain stress/apoptosis-related genes, but particularly those genes induced by DNA damage and involved in initiating apoptosis such as the GADD genes, might be changed in DOC-treated colonocytes. As the present study shows, mRNA expression of GADD34, GADD45, and GADD153 was increased as a result of exposing either HCT-116 or HCT-15 colonocytes to

DOC. However, GADD153 mRNA expression was most upregulated. The upregulation of GADD153 by DOC in HCT-116 colonocytes corroborates recent findings by Qiao et al. (2002).

Consideration was given to p53 as a possible regulator of the effect of DOC on GADD gene expression. This redox-sensitive transcription factor is activated when cells are subjected to agents that induce oxidative stress and DNA damage (Renzing et al., 1996). When incubated with isolated rat hepatocytes (Sokol et al., 1993) or HCT-116 colonocytes (Crowley-Weber et al., 2003), DOC induced the formation of reactive oxygen species. Moreover, DNA damage was detected in DOC-treated HCT116 colonocytes (Powolny et al., 2001). Thus, p53 activation would be expected, although this was not assessed in the present study. Nevertheless, in comparing GADD gene expression in HCT-116 colonocytes (wild-type p53) and HCT-15 colonocytes (mutant p53) colonocytes, it was found that DOC increased mRNA expression of GADD34, GADD45, and GADD153 in both cell lines. Therefore, these findings imply that upregulation of GADD gene expression occurred independently of p53 and suggest the involvement of other transcription factors. Although it is well established that p53 can bind to a regulatory element of the GADD45 gene (Kastan et al., 1992) in upregulating GADD45 mRNA levels, it is now known that a regulatory element for AP-1 also exists within intron 3 of the GADD45 gene (Graunke et al., 1999). More recently, it has been shown that the transcription factors, Oct-1 and NF-YA, can bind to distinct GADD45 regulatory elements to result in p53- independent induction of GADD45 gene expression (Jin et al., 2001). Regarding the GADD153 gene, it has been reported that there is a regulatory element for AP-1 besides regulatory elements for C/EBP, interleukin 6, and Sp-1 (Guyton et al., 1996). The GADD34 gene promoter contains regulatory elements for cyclic AMP (Hollander et al., 1997) and also ATF4 (Ma and Hendershot, 2003).

DOC increased GADD gene expression probably as an early transcriptional response to the cellular DNA damage caused by DOC, based on the results of the experiments focusing on GADD 153. The ability of Act-D to prevent DOC-induced upregulation of GADD153 mRNA expression supports the view that the effect of DOC on GADD153 mRNA was due to increased transcription rather than greater mRNA stability. However, other inducers of GADD153 gene expression increase GADD153 mRNA levels in distinct ways. For example, depriving cells of leucine (Bruhat et al., 1997) increased GADD153 mRNA expression that was attributed to both increased transcription and mRNA stability. Glutamine deprivation also induced GADD153 mRNA expression (Abcouwer et al., 1999), but the primary way was by mRNA stabilization. On the other hand, it is conceivable that the upregulation of GADD gene expression caused by DOC could have resulted from potential DOC-induced stress in the ER. It is known that stress or malfunction in the ER can lead to activation of GADD153 (Nozaki et al., 2001). A reporter gene construct for GRP78, which is recognized as a marker for ER stress, was activated in HepG2 hepatoma cells when exposed to DOC (Crowley-Weber et al., 2003), but at a higher concentration of DOC than used in the present study. As the current data show, GRP78 mRNA expression was not increased in DOC-treated HCT116 colonocytes, suggesting that the concentration of DOC used did not promote ER stress and that DOC-induced upregulation of GADD153 mRNA expression was not due to ER stress.

The molecular cascade leading to GADD gene activation may involve reactive oxygen species and MAPK, as suggested by certain previous studies. For example, when p38/SAPK2 was activated by anisomycin in Jurkat T-lymphocytes, the levels of GADD153 transcript increased, but this effect was nullified with a selective chemical inhibitor of p38/SAPK2 (Rolli-Derkinderen and Gaestel, 2000). Exposing HCT-116 human colon adenocarcinoma cells to UV radiation, which promoted the formation of reactive oxygen species, induced transcriptional activation of GADD45 that involved ERK and JNK signaling based on the findings that a selective chemical inhibitor of ERK and a dominant negative mutant JNK1 expression vector blunted reporter construct activation (Tong et al., 2001). In some cases of apoptosis, MAPK are required to increase gene expression of GADD genes, including GADD153, and to subsequently mediate apoptosis (Sarkar et al., 2002). Collectively, such studies suggest that reactive oxygen species and MAPK would be involved in DOC-induced upregulation of GADD153 mRNA expression, especially in view of the capability of DOC to induce oxidative stress (Sokol et al., 1993; Crowley-Weber et al., 2003) and MAPK signaling (Rao et al., 2002) in association with GADD gene activation.

To the contrary, unexpected results were obtained in assessing the potential of either antioxidants or selective MAPK inhibitors to counteract the effect of DOC on GADD 153 mRNA expression. That is, several antioxidants (NAC, (x-TH, PDTC, EGCG, and catalase) and MAPK inhibitors (PD098059, SB203580, and SP600125) failed to prevent the increased GADD 153 mRNA expression caused by DOC. Therefore, these findings suggest that DOC-induced upregulation of GADD153 mRNA expression does not depend to a significant extent on reactive oxygen species nor MAPK. In considering other possibilities, it was found that the general PKC inhibitor, staurosporine, prevented GADD 153 mRNA upregulation caused by DOC. In contrast, the more specific PKC inhibitor, calphostin C, had no such effect. Thus, the role of PKC in DOC-induced GADD153 gene activation remains unclear. The involvement of specific PKC isoforms needs to be examined.

Although the full significance of upregulation of GADD gene expression in DOC-treated cells is unclear, the effect of DOC on GADD153 in particular might contribute to the capacity of DOC to induce apoptosis. There is substantial evidence that GADD153 has a role in triggering apoptosis (Maytin et al., 2001; Conn et al., 2002; Kim et al., 2002; Lengwehasatit and Dickson, 2002; Xia et al., 2002). Therefore, it is conceivable that the expression of GADD153 protein, which clearly preceded the appearance of recognizable features of apoptosis, in DOC-treated HCT-116 colonocytes could be involved in triggering apoptosis in these cells as well. This is not to say, however, that other apoptosis-related genes or their protein products are unimportant in the apoptosis induced by DOC. Further studies will need to be conducted to determine whether GADD153 protein has a role in initiating apoptosis in colonocytes exposed to DOC.

LITERATURE CITED

- Abcouwer SF, Schwarz C, Meguid RA. 1999. Glutamine deprivation induces the expression of GADD45 and GADD153 primarily by mRNA stabilization. J Biol Chem 274:28645–28651.
- Bruhat A, Jousse C, Wang XZ, Ron D, Ferrara M, Fafournoux P. 1997. Amino acid limitation induces expression of CHOP, a CCAAT/ enhancer binding protein-related gene, at both transcriptional and post-transcriptional levels. J Biol Chem 272:17588–17593.
- Carlson SG, Fawcett TW, Bartlett JD, Bernier M, Holbrook NJ. 1993. Regulation of the C/EBP-related gene gadd153 by glucose deprivation. Mol Cell Biol 13:4736–4744.
- Chinery R, Brockman JA, Peeler MO, Shyr Y, Beauchamp RD, Coffey RJ. 1997. Antioxidants enhance the cytotoxicity of chemotherapeutic agents in colorectal cancer: A p53-independent induction of p21WAF1/CIP1 via C/EBPbeta. Nat Med 3:1233–1241.
- Conn KJ, Gao WW, Ullman MD, McKeon-O'Malley C, Eisenhauer PB, Fine RE, Wells JM. 2002. Specific up-regulation of GADD153/CHOP in 1-methyl-4-phenyl-pyridinium-treated SH-SY5Y cells. J Neurosci Res 68:755–760.
- Crowley-Weber CL, Dvorakova K, Crowley C, Bernstein H, Bernstein C, Garewal H, Payne CM. 2003. Nicotine increases oxidative stress, activates NF-kappaB and GRP78, induces apoptosis and sensitizes cells to genotoxic/xenobiotic stresses by a multiple stress inducer, deoxycholate: Relevance to colon carcinogenesis. Chem Biol Interact 145:53–66.
- Fanzo JC, Reaves SK, Cui L, Zhu L, Wu JY, Wang YR, Lei KY. 2001. Zinc status affects p53, gadd45, and c-fos expression and caspase-3 activity in human bronchial epithelial cells. Am J Physiol Cell Physiol 281:C751–C757.
- Garewal H, Ramsey L, Fass R, Hart NK, Payne CM, Bernstein H, Bernstein C. 2003. Perils of immunohistochemistry: Variability in staining specificity of commercially available COX-2 antibodies on human colon tissue. Dig Dis Sci 48:197–202.
- Graunke DM, Fornace AJ, Jr., Pieper RO. 1999. Presetting of chromatin structure and transcription factor binding poise the human GADD45 gene for rapid transcriptional up-regulation. Nucleic Acids Res 27:3881–3890.
- Guyton KZ, Xu Q, Holbrook NJ. 1996. Induction of the mammalian stress response gene GADD153 by oxidative stress: Role of AP-1 element. Biochem J 314:547–554.

- Hirano F, Tanada H, Makino Y, Okamoto K, Hiramoto M, Handa H, Makino I. 1996. Induction of the transcription factor AP-1 in cultured human colon adenocarcinoma cells following exposure to bile acids. Carcinogenesis 17:427–433.
- Hollander MC, Zhan Q, Bae I, Fornace AJ, Jr. 1997. Mammalian GADD34, an apoptosis- and DNA damage-inducible gene. J Biol Chem 272:13731–13737.
- Hollander MC, Sheikh MS, Yu K, Zhan Q, Iglesias M, Woodworth C, Fornace AJ. 2001. Activation of Gadd34 by diverse apoptotic signals and suppression of its growth inhibitory effects by apoptotic inhibitors. Int J Cancer 96:22–31.
- Hori T, Matsumoto K, Sakaitani Y, Sato M, Morotomi M. 1998. Effect of dietary deoxycholic acid and cholesterol on fecal steroid concentration and its impact on the colonic crypt cell proliferation in azoxymethane-treated rats. Cancer Lett 124:79–84.
- Huang XP, Fan XT, Desjeux JF, Castagna M. 1992. Bile acids, nonphorbol-ester-type tumor promoters, stimulate the phosphorylation of protein kinase C substrates in human platelets and colon cell line HT29. Int J Cancer 52:444–450.
- Imray CH, Radley S, Davis A, Barker G, Hendrickse CW, Donovan IA, Lawson AM, Baker PR, Neoptolemos JP. 1992. Faecal unconjugated bile acids in patients with colorectal cancer or polyps. Gut 33:1239– 1245.
- Jin S, Fan F, Fan W, Zhao H, Tong T, Blanck P, Alomo I, Rajasekaran B, Zhan Q. 2001. Transcription factors Oct-1 and NF-YA regulate the p53-independent induction of the GADD45 following DNA damage. Oncogene 20:2683–2690.
- Kastan MB, Zhan Q, El-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B, Fornace AJ, Jr. 1992. A mammalian cell- cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell 71:587–597.
- Kim D-G, You K-R, Liu M-J, Choi Y-K, Won Y-S. 2002. GADD153- mediated anticancer effects of N-(4-hydroxyphenyl)retinamide on human hepatoma cells. J Biol Chem 277:38930–38938.
- Kobori O, Shimizu T, Maeda M, Atomi Y, Watanabe J, Shoji M, Morioka Y. 1984. Enhancing effect of bile and bile acid on stomach tumorigenesis induced by N-methyl-N'-nitro-N-nitrosoguanidine in Wistar rats. J Natl Cancer Inst 73:853–861.
- Lengwehasatit I, Dickson AJ. 2002. Analysis of the role of GADD153 in the control of apoptosis in NS0 myeloma cells. Biotechnol Bioeng 80:719–730.
- Ma Y, Hendershot LM. 2003. Delineation of a negative feedback regulatory loop that controls protein translation during endoplasmic reticulum stress. J Biol Chem 278:34864–34873.
- Maytin EV, Ubeda M, Lin JC, Habener JF. 2001. Stress-inducible transcription factor CHOP/gadd153 induces apoptosis in mammalian cells via p38 kinase-dependent and -independent mechanisms. Exp Cell Res 267:193–204.
- Meister A. 1991. Glutathione deficiency produced by inhibition of its synthesis, and its reversal: Applications in research and therapy. Pharmacol Ther 51:155–194.
- Mertani HC, Zhu T, Goh EL, Lee KO, Morel G, Lobie PE. 2001. Autocrine human growth hormone (hGH) regulation of human mammary carcinoma cell gene expression. Identification of CHOP as a mediator of hGH-stimulated human mammary carcinoma cell survival. J Biol Chem 276:21464–21475.
- Nozaki S, Sledge GW, Jr., Nakshatri H. 2001. Repression of GADD 153/CHOP by NF-kappaB: A possible cellular defense against endoplasmic reticulum stress-induced cell death. Oncogene 20: 2178–2185.
- Oh-Hashi K, Maruyama W, Isobe K. 2001. Peroxynitrite induces GADD34, 45,153 via p38 MAPK in human neuroblastoma SH-SY5Y cells. Free Radic Biol Med 30:213–221.
- Owen RW. 1997. Faecal steroids and colorectal carcinogenesis. Scand J Gastroenterol222(Suppl):76–82.
- Payne CM, Crowley C, Washo-Stultz D, Briehl M, Bernstein H, Bernstein C, Beard S, Holubec H, Warneke J. 1998. The stress- response proteins poly(ADP-ribose)polymerase and NF-κB protect against bile salt-induced apoptosis. Cell Death Different 5:623– 636.

- Powolny A, Xu J, Loo G. 2001. Deoxycholate induces DNA damage and apoptosis in human colon epithelial cells expressing either mutant or wild-type p53. Int J Biochem Cell Biol 33:193–203.
- Qiao D, Im E, Qi W, Martinez JD. 2002. Activator protein-1 and CCAAT/enhancer-binding protein mediated GADD153 expression is involved in deoxycholic acid-induced apoptosis. Biochim Biophys Acta 1583:108–116.
- Rao YP, Studer EJ, Stravitz RT, Gupta S, Qiao L, Dent P, Hylemon PB. 2002. Activation of the Raf-1/MEK/ERK cascade by bile acids occurs via the epidermal growth factor receptor in primary rat hepatocytes. Hepatology 35:307–314.
- Reddy BS. 1995. Nutritional factors and colon cancer. Crit Rev Food Sci Nutr 35:175–190.
- Renzing J, Hansen S, Lane DP. 1996. Oxidative stress is involved in the UV activation of p53. J Cell Sci 109:1105–1112, Rolli-Derkinderen M, Gaestel M. 2000. p38/SAPK2-dependent gene expression in Jurkat T cells. Biol Chem 381:193–198.
- Sano H, Kawahito Y, Wilder RL, Hashiramoto A, Mukai S, Asai K, Kimura S, Kato H, Kondo M, Hla T. 1995. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res 55:3785–3789.
- Sarkar D, Su ZZ, Lebedeva IV, Sauane M, Gopalkrishnan RV, Valerie K, Dent P, Fisher PB. 2002. mda-7 (IL-24) mediates selective apoptosis in human melanoma cells by inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK. Proc Natl Acad Sci USA 99:10054–10059.
- Sheikh MS, Hollander MC, Fornace AJ, Jr. 2000. Role of Gadd45 in apoptosis. Biochem Pharm 59:43–45.
- Sokol RJ, Devereaux M, Khandwala R, O'Brien K. 1993. Evidence for involvement of oxygen free radicals in bile acid toxicity to isolated rat hepatocytes. Hepatology 17:869–881.
- Stadler J, Stern HS, Sing-Yeung KA, McGuire V, Furrer R, Marcon N, Bruce WR. 1988. Effect of high fat consumption on cell proliferation activity of colorectal mucosa and on soluble faecal bile acids. Gut 29:1326–1331.
- Tong T, Fan W, Zhao H, Jin S, Fan F, Blanck P, Alomo I, Rajasekaran B, Liu Y, Holbrook NJ, Zhan Q. 2001. Involvement of the MAP kinase pathways in induction of GADD45 following UV radiation. Exp Cell Res 269:64–72.
- Wang XW, Zhan Q, Coursen JD, Khan MA, Kontny HU, Yu L, Hollander MC, O'Connor PM, Fornace AJ, Jr., Harris CC. 1999. GADD45 induction of a G2/M cell-cycle checkpoint. Proc Natl Acad Sci USA 96:3706–3711.
- Xia Y, Wong NS, Fong WF, Tideman H. 2002. Upregulation of GADD153 expression in the apoptotic signaling of N-(4-hydroxyphenyl)retinamide (4HPR). Int J Cancer 102:7–14.
- Zhang F, Subbaramaiah K, Altorki N, Dannenberg AJ. 1998. Dihydroxy bile acids activate the transcription of cyclooxygenase2. J Biol Chem 273:2424–2428.