Ursodeoxycholic acid inhibits interleukin beta 1 and deoxycholic acid-induced activation of NF-κB and AP-1 in human colon cancer cells

Syed A. Shah*, Yuri Volkov, Qamrul Arfin, Mohamed M. Abdel-Latif and Dermot Kelleher

Department of Clinical Medicine and Dublin Molecular Medicine Centre, Trinity Centre for Health Sciences, St. James’s Hospital, Dublin, Ireland

Deoxycholic acid (DCA) has been implicated in colorectal carcinogenesis in humans with effects on proliferation and apoptosis, mediated at least in part by activation of transcription factors nuclear factor kappa B (NF-κB), activator protein 1 (AP-1) and protein kinase C (PKC) enzymes. Ursodeoxycholic acid (UDCA) is reported to reduce the frequency of colonic carcinogenesis in ulcerative colitis patients. Hence, we postulated that it might differ from DCA in its regulation of these transcription factors. The aim of the study was to determine effects of DCA and UDCA on NF-κB and AP-1 activation and explore its relationship to PKC.

Human colonic tumour cell lines HCT116 were treated with DCA, UDCA, alone or pretreated with UDCA followed by DCA or IL-1β. In other experiments, cells were pretreated with PKC inhibitors and then stimulated with DCA and IL-1β or PMA. Gel shift assays were performed on nuclear extracts of the cells for NF-κB and AP-1 analysis. Western blot analyses and immunofluorescence were performed for Rel A (p65) and IκB-α levels on the treated cells. DCA increased NF-κB and AP-1 DNA binding. UDCA did not increase DNA binding of NF-κB and AP-1 and UDCA pre-treatment inhibited DCA-induced NF-κB and AP-1 DNA binding. PKC inhibitors blocked DCA-induced NF-κB and AP-1 activation. These results were validated by Western blot analysis for RelA and IκB-α. In conclusion, UDCA did not induce NF-κB and AP-1 DNA binding but also blocked DCA-induced NF-κB and AP-1 activation. These findings suggest a possible mechanistic role for UDCA in blocking pathways thought to be involved in colon carcinogenesis.

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Key words: Colorectal cancer; deoxycholic acid; ursodeoxycholic acid; nuclear factor kappa B; activator protein-1

There is plethora of evidence from epidemiological studies that high fat diet predisposes to colon cancer. It has been suggested that high fat causes excessive secretion of bile acids in the intestine. Secondary bile acids, released as a result of bacterial metabolism are cytotoxic and cause crypt proliferation in colon carcinogenesis. Moreover, there is evidence from clinical studies in the pathogenesis of colorectal cancer. Many cancers including colon cancer are reported to exhibit aberrant and sustained activation of NF-κB. NF-κB activation is reported to induce resistance to apoptosis and hence may confer survival to transforming cells. AP-1 is another heterodimeric transcription factor consisting of various subunits including Fos and Jun family of proteins. AP-1 is a protooncogene and like NF-κB activates transcription of proinflammatory and other genes involved in cell proliferation and transformation. Bile acids activate both the transcription factors, NF-κB and AP-1 possibly through activation of upstream kinases including members of PKC family.

Protein kinase C (PKC) is a family of serine-threonine kinase isoformes, subclassified as classical PKC (cPKCs), novel PKC (nPKCs) or atypical PKCs (aPKCs) depending on cofactor requirements. Inactive PKC isoforms are located predominantly in the cytosol. Activated PKC isoformes translocate to a variety of intracellular sites including cell membrane, nucleus and membrane associated cytoskeleton. Bile acids are reported to activate PKC in colon cells and hence may impact indirectly on downstream transcription factors.

The aim of our study was first to explore the differential effects of DCA and UDCA on induction of transcription factors NF-κB and AP-1 and second to investigate the interactions between NF-κB, AP-1 and PKC signalling pathways.

Materials and methods

Cell culture and materials

HCT116, a cell line derived from adenocarcinoma patient with Lynch’s syndrome, were obtained from American Type Culture Collection (ATTC, Rockville, MD), cultured in McCoy’s 5a medium with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin ( Gibco BRL, Grand Island, NY). The oesophageal epithelial cell line OE33 (derived from the adenocarcinoma of the Mwer oesophagus; Barrett’s metaplasia) was obtained from the European Collection of Animal Cell Cultures, ECACC (Porton Down, Salisbury, UK). OE33 cells were grown in RPMI 1640 medium supplemented with 10% filtered foetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. The human T cell lines

Abbreviations: AP-1, Activator Protein 1; DCA, deoxycholic acid; EGFP, Enhanced Green Fluorescent Protein; NF-κB, Nuclear factor-kappa B; PKC, Protein Kinase C; PMA, phorbol myristate acetate; UDCA, ursodeoxycholic acid.

*Correspondence to: Department of Clinical Medicine and Dublin Molecular Medicine Centre, Trinity Centre for Health Sciences, St. James’s Hospital, Dublin 8, Republic of Ireland. Fax: +353-1-4542043; E-mail: syedshah@doctors.org.uk Received 26 December 2004; Accepted after revision 24 May 2005 DOI 10.1002/ijc.21365 Published online 16 August 2005 in Wiley InterScience (www.interscience.wiley.com).
leukaemic HuT 78 cell line (ATCC, Manassas, VA) were maintained in RPMI-1640 (GIBCO/BRL, Grand Island, NY). HuT 78 cells were used as positive control for NF-κB in gel shift assays. The cells were used between passages 5–20 and maintained at 37°C in a humidified incubator containing 5% CO2. Media was renewed every third day and cells split every fifth day. Cells were grown to 70–90% confluence depending on the experiment.

NF-κB and AP-1-binding consensus oligonucleotide were obtained from Promega Corp. (Madison, WI). Polyclonal antibodies to IkB-α and RelA, anti-p50 (sc-114X), anti-RelA (sc-109X) and anti-RetA (sc-70X) and polyclonal antibodies against Jun family (sc-044X) and Fos family (sc-253X) (c-Jun, Jun D, Jun B, and anti-c-Rel (sc-70X) and polyclonal antibodies against Jun (c-Jun, Jun D, Jun B, c-Fos, Fos-B, Fra-1 and Fra-2) or NF-κB/Rel subunits (anti-p50, anti-RelA and anti-c-Rel) or 50-fold molar excess of unlabeled oligonucleotide was preincubated with nuclear extract for 30 min at room temperature prior to the addition of the labelled probe.

Preparation of total cell extracts

Cells were pelleted by centrifugation at 1,400 rpm for 5 min. The pellet was resuspended in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% (v/v) sodium dodecyl sulphate (SDS), 150 mM NaCl, 1 mM EDTA, 1 mM EDTA, 0.5 mM phenylmethylsulphonyl fluoride (PMSF) and leupeptin (10 μg/ml) and then the cells were solubilized by boiling for 5 min.

Methods

Nuclear extract preparation

Nuclear extracts were prepared from the cells as follows. The cells were washed twice with ice-cold PBS. The cells were centrifuged at 1,400 rpm for 5 min and washed once and resuspended in (1 ml) buffer A (10 mM Hepes (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM PMSF and 0.5 mM dithiothreitol (DTT)). The cells were then centrifuged again at 10,000 rpm for 10 min. The supernatant was diluted with 4 vol of buffer D [10 mM Hepes (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 25% (w/v) glycerol and 0.5 mM PMSF] for 15 min on ice. After incubation, the nuclei were centrifuged at 10,000 rpm for 10 min and the supernatant was diluted with 4 vol of buffer D [10 mM Hepes (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 25% (w/v) glycerol and 0.5 mM PMSF]. The nuclear extracts were used immediately or stored at −70°C until required. The protein concentration was determined on nuclear extracts by the method of Bradford.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (4 μg of protein) were incubated with 10,000 cpm of the 32P-labelled NF-κB oligonucleotide or AP-1 oligonucleotide (that had been previously labelled with (γ32P) ATP (10 μCi/mmole) at the 5’-ends with T4 polynucleotide kinase for binding assay. The assay was performed in 20 μl of binding buffer [10 mM Tris (pH 7.5), 4% (w/v) glycerol, 5 mM DTT, 1 mM EDTA, 100 mM NaCl and 0.1 mg/ml calf thymus DNA (freeze-free BSA)] in the presence of 2 μg poly(dI-dC) as nonspecific competitor. The reaction mixture was then incubated for 30 min at room temperature after the addition of the probe DNA. The binding reaction was terminated using a loading dye [0.25% bromophenol, 0.25% xylene cyanol, 30% (w/v) glycerol in deionized water] prior to electrophoretic separation of the DNA-protein complexes on 5% polyacrylamide gels that had been preelectrophoresed for 30 min at 80 V. Gels were run at 150 V for 1–2 hr at room temperature. After electrophoresis, the gels were dried and autoradiographed at −70°C for 24–36 hr with intensifying screens. In supershift assays, rabbit polyclonal antibodies (400 ng) against various Fos and Jun (c-Jun, Jun D, Jun B, c-Fos, Fos-B, Fra-1 and Fra-2) or NF-κB/Rel subunits (anti-p50, anti-RelA and anti-c-Rel) or 50-fold molar excess of unlabeled oligonucleotide was preincubated with nuclear extract for 30 min at room temperature prior to the addition of the labelled probe.

Western blot analyses

Whole cell extracts or nuclear extracts (50 μg of protein/lane) were resolved by electrophoresis through polyacrylamide gels using 10% separating gels according to described protocols. Proteins were electrotransferred onto PVDF membrane using a semidry blotting apparatus (Altto). Blots were blocked with 5% (w/v) dried skim milk in PBS for 1 hr at room temperature and then incubated for 1 hr at room temperature with the appropriate primary antibody (anti-IκB-α or anti-RelA) at a dilution of 1:1,000. Blots were then incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (Zymed Laboratories, San Francisco, CA) (1/1,000) for 1 hr at room temperature. Immunodetection was performed by enhanced chemiluminescence. A minimum of 3 experiments were performed.

Immunofluorescent for NF-κB

HCT116 cells were cultured on 8-well Permanox glass chamber slides (Nune, Naperville, IL) for 24 hr to 60–70% confluence. These cells were treated with 300 μM DCA, 20 ng/ml IL-1β or 300 μM UDCA alone for 2 hr or pretreated with UDCA for 2 hr and then treated with DCA or IL-1β. The slides were subsequently prepared for NF-κB immunofluorescence. The slides were gently washed with sterile phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 10 min. The slides were incubated with primary antibody (anti-RelA) for 1 hr or overnight at room temperature, and then washed 3 times in 0.1% Tween 20 in PBS, followed by 30 min incubation with fluorescein isothiocyanate (FITC-conjugated secondary antibody, Clontech, Inc., Palo Alto, CA). Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. Coverslips were mounted and images were acquired on Nikon TE 300 inverted microscope equipped with Leica DC-100 colour digital camera.

Results

UDCA does not induce NF-κB binding and inhibits DCA and IL-1β-induced NF-κB binding (Fig. 1e,f).

To investigate the effects of DCA and UDCA on NF-κB DNA binding, HCT116 cells were treated with 300 μM DCA, 300 μM UDCA or 20 ng/ml IL-1β for 2 hr alone or pretreated with 300 μM UDCA for 2 hr and then treated with DCA or IL-1β. Oesophageal cell line, OE33 cells were treated with 300 μM DCA, 300 μM UDCA for 2 hr or 300 μM UDCA for 2 hr prior to the addition of 300 μM DCA to further evaluate the effects of the two bile acids on gastrointestinal cells (Fig. 1e). These experi-
FIGURE 1.

FIG 1a

DCA (μM)

Rest 100 200 300 400

NF-κB

HCT116 Cells

Fig 1c

DCA (μM)

Rest 100 200 300 400

NF-κB

OE33 Cells

Fig 1b

DCA (h)

Rest 1 2 3 4

NF-κB

HCT116 Cells

Fig 1d

DCA (h)

Rest 1 2 3 4

OE33 Cells

Fig 1e

DCA Rest IL-1β UDCA U+ U+ C

Free Probe

DCA IL-1β

HCT116 Cells

Fig 1f

Rest UDCA DCA U-DCA C

OE33 Cells

Fig 1g

Rest DCA UDCA IL-1β U+DCA U+IL-1β

IκB-α

Fig 1h

Rest DCA UDCA IL-1β U+DCA U+IL-1β

RelA

Fig 1i

DCA RelA P50 RelA/P50 c-Ral Unlab

Figure 1.
ments were performed with either UDCA left in or removed before activation with DCA with no effects on the results.

Figure 1e demonstrates that both DCA and IL-1β induced NF-κB DNA binding activity. By contrast, UDCA did not induce NF-κB DNA binding and UDCA treatment inhibited DCA and IL-1β-induced NF-κB DNA binding. UDCA pretreatment also inhibited DCA-induced NF-κB activation in OE33 cells (Fig. 1f). The Human T cell leukaemic, HuT 78 cell line constitutively expressing NF-κB, was used as positive control (C). Figure 1a,b shows dose and time response of NF-κB to DCA in HT116 and Fig 1c and 1d in OE33 cells as described in Materials and Methods.

**Effect of bile acids on IκB-α levels (Fig. 1g)**

Next, we investigated the effects of bile acids on IκB-α levels. NF-κB is found in the cytosol of the unstimulated cells bound to an inhibitory molecule 1κB-α. On activation, 1κB-α is phosphorylated and degraded, hence releasing NF-κB to translocate to the nucleus where it binds to DNA elements. HT116 cells were treated as described above. Western blot was performed on the total cell extracts of the treated cells to observe the effects on 1κB-α level. Figure 1g shows that DCA and IL-1β, but not UDCA, reduced 1κB-α levels. The reduction in 1κB-α levels by DCA and IL-1β was inhibited when cells were pretreated with UDCA. The reduction in 1κB-α levels by DCA and IL-1β, corresponds to their increase in NF-κB DNA binding, shown in Gel shift assay in Figure 1e.

**Effect of bile acids on Rel A levels (Fig. 1h)**

To further evaluate the effects of the bile acids on NF-κB, the cells were treated as above and incubated overnight with anti-NF-κB RelA antibody, followed by secondary antibody to detect RelA component of NF-κB in the nuclear extracts of the cells. Western blot analyses were performed on the nuclear extracts of the cells, for RelA levels. Figure 1h shows that both DCA and IL-1β induced RelA. UDCA, on the other hand, did not induce RelA. Additionally UDCA treatment inhibited DCA and IL-1β-induced increase in RelA levels. Taken together these data demonstrate that UDCA by contrast with DCA does not activate NF-κB but also inhibits DCA-induced NF-κB activation in colon cancer cells.

**Supershift assay (Fig. 1i)**

In order to identify the components of the NF-κB DNA-complex induced by DCA supershift assay was performed on the nuclear extracts of the treated cells. A panel of antibodies directed against various NF-κB subunits (p50, RelA and c-Rel) were preincubated with nuclear extracts from HCT116 cells treated with DCA (Fig 1i). Antibodies to RelA and p50 recognized this NF-κB-DNA complex. Moreover, competition assays with a 100-fold molar excess of unlabelled NF-κB binding nucleotide confirmed the specificity of labelled NF-κB DNA-complex induced by DCA and IL-1β.

**Immunofluorescence for NF-κB (Fig 2).**

To further validate the effects of bile acids on NF-κB activation, immunofluorescence was performed on the cells treated with DCA, IL-1β, UDCA alone or cells pretreated with UDCA first and then stimulated with DCA or IL-1β. After incubating the cells with RelA primary antibody overnight, FITC conjugated secondary antibody was used to visualize RelA. As shown in Figure 2, RelA is located in the cytosol in resting (untreated) cells. Both DCA and IL-1β caused translocation of RelA into the nucleus, but UDCA treatment alone did not alter localization of RelA. Pretreatment with UDCA prevented DCA and IL-1β-induced nuclear translocation. These data validate the observations made by gel shift assays in Figure 1a and Western blot analyses in Figure 1c.

**UDCA inhibits DCA and PMA-induced AP-1 binding (Fig. 3a,b)**

In these experiments, we investigated the effects of bile acids on AP-1 activation. Gel shift assays were performed on the nuclear extracts of the cells treated with DCA, UDCA or 20 nM PMA alone for 4 hr or cells pretreated with UDCA for 2 hr and then treated with DCA or PMA. Figure 3a shows DCA and PMA induced AP-1 DNA binding, UDCA did not induce AP-1 DNA binding. Additionally UDCA treatment inhibited DCA and PMA-induced AP-1 DNA binding. To identify the components of the AP-1 complex stimulated by DCA, supershift assays were performed. HCT116 cells treated with 300 μM DCA for 4 hr and nuclear extracts were prepared. Antibodies against Fos and Jun proteins were added to detect AP-1 components. The results show that Jun D, Fra-1, and c-Fos created supershift bands. (Fig. 3b). This indicates that Jun D, c-Fos and Fra-1 are the components involved in the DCA-induced AP-1 DNA binding in HCT116 cells.

**Role of PKC Signalling pathway on NF-κB and AP-1 activation by the bile acids (Fig. 4a-c)**

The interactions between PKC and NF-κB were further investigated using PKC inhibitors, Calphostin C and Bisindolylmaleimide. The cells were pretreated with PKC inhibitors for half an hour at different doses and then activated with either DCA or IL-1β. Figure 4a shows that both PKC inhibitors Calphostin C and Bisindolylmaleimide prevented DCA-induced NF-κB DNA binding at standard doses but not that caused by IL-1β. Western blot analyses on the total cell extracts of similarly treated cells showed that PKC inhibitors blocked the reduction in 1κB-α level, induced by DCA but not by IL-1β (Fig 4b). This may indicate the activation...
Figure 2 – HCT116 cells treated with DCA, IL-1β, UDCA alone or cells pretreated with UDCA first and then stimulated with DCA or IL-1β. Cells were incubated with primary RelA antibody followed by FITC conjugated secondary antibody. Immunofluorescent microscopy shows that DCA and IL-1β induced nuclear translocation of RelA. Pretreatment of the cells with UDCA inhibited DCA and IL-1β-induced RelA nuclear translocation.
tion of NF-κB by DCA and IL-1β by separate pathways. Figure 4c shows that DCA and PMA-induced AP-1 DNA binding was blocked by pretreatment of the cells with PKC inhibitors, suggesting that activation of AP-1 by these two agents may occur through common signalling pathways.

Discussion

Transcription factors NF-κB and AP-1 are emerging as important targets in the pathogenesis of colon cancer. NF-κB is upregulated in colorectal cancer and overexpression of NF-κB may be associated with adenoma to carcinoma transition.26,27 Many genes involved in oncogenesis, cell proliferation and apoptosis are under the transcriptional regulation of NF-κB.30,31 Increased and sustained expression of NF-κB is associated with resistance to apoptosis and inhibition of NF-κB is reported to promote chemosensitivity of the colon tumour cells.32 This hypothesis is supported by the finding that CPT-11, used in colorectal cancer increases chemosensitivity of the tumour cells,33 IκB-α is an inhibitory protein that binds to and prevents NF-κB nuclear translocation. NF-κB also plays important role in the regulation of cyclo-oxygenase-2 (COX-2), which is overexpressed in many aggressive colon tumours.34 A number of agents with potential chemopreventive effects in cancer such as curcumin and sulindac are reported to suppress the activation of NF-κB.35,36

AP-1 is another transcription factor implicated in colorectal carcinogenesis. Recent data has demonstrated that AP-1 is critical modulator of colorectal cancer proliferation and that dominant negative c-Jun significantly blocked both cell proliferation and tumour growth.37 AP-1 regulates many genes involved in cell transformation, oncogenesis including COX-2 in intestinal epithelial cells.38,39 These two transcription factors are reported to play a synergistic role in many biological processes such as cell proliferation and cross-coupling of these transcription factors has been reported.17,40 Hence interventions targeting NF-κB and AP-1 may play significant role to suppress the colorectal tumour growth.

Our study shows that bile acids DCA and UDCA have distinct and differential effects on the DNA binding of NF-κB and AP-1 in colon cancer cell lines. Secondary bile acid, DCA induced both NF-κB and AP-1 DNA binding. UDCA on the other hand did not induce DNA binding of either of the transcription factors. By contrast it inhibited DCA and PMA-induced AP-1 DNA binding. The effects of these two bile acids on IκB-α level were also distinct. DCA reduced IκB-α level in cells, suggesting possible degradation of IκB-α induced by DCA, while UDCA pretreatment blocked IκB-α degradation induced by DCA. Inhibition of IκB-α by UDCA has also been reported in microglial cells.41 As stated earlier, PKC and other members of kinase family are involved in the regulation of NF-κB. In our study pretreatment of the cells with PKC inhibitors prevented DCA-induced NF-κB DNA binding as well as IκB-α degradation, suggesting a possible role for PKC in DCA-induced NF-κB activation. An interesting observation was that IL-1β-induced NF-κB DNA binding was not blocked by the PKC inhibi-
tors. These findings are consistent with previous reports that NF-
κB DNA binding may be induced by multiple and diverse signal-
ling pathways. Phosphatidylinositol 3-kinase (PI 3-kinase) is
involved in IL-1β-induced NF-κB DNA binding while bile acids
like phorbol esters may induce NF-κB DNA binding through acti-
vation of PKC.42,43 Hence, these data suggest that the bile acid-
induced NF-κB DNA binding in colorectal cancer cells may be
modulated through the PKC signalling pathway.

DCA has previously been shown to induce AP-1 DNA binding
in colon cancer cells.16-21 The protein components of AP-1
induced by DCA are the products of oncogenes c-Fos and c-
Jun.16,21 Our data shows that UDCA pre-treatment of the cells
suppresses not only DCA-induced AP-1 DNA binding but also
that caused by PMA. The concentration of bile acid in the colon
varies with the fat content of the diet and concentrations as high as
800 μM DCA have been reported in subjects on high fat diet.7 In a
recent study, 500 μM DCA was used for EGFR/Raf-1/ERK signal-
ling in HCT116 cells.44

UDCA has been used for more than a decade for cholestatic
liver disease. More recently, UDCA is shown to have protec-
tive effect against colorectal cancer in animals as well as
humans.45,46 It has recently been reported that ursodeoxycholic
acid caused a dose-dependent decrease in the number of intestinal
tumours with equal efficacy throughout the entire intestine in ani-
mal model.47 Despite abundant evidence for the cytoprotective and
chemoprotective effects of UDCA in colorectal cancer, both the
target molecules and pathways for UDCA effects remain elusive.

Our data demonstrates UDCA suppresses DCA-induced activation
of both transcription factors, AP-1 and NF-κB at least partly
through PKC signalling pathways in colorectal cancer cells. This
may be one of the mechanisms behind the chemoprotective effects
of UDCA against colorectal tumours, reported in many studies.

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FIGURE 4 – (a–c) Effects of PKC inhibitors on bile acid-
induced NF-κB and AP-1 DNA binding. HCT 116 cells were
pretreated with PKC inhibitors Calphostin C and Bisindolylma-
leimide at different doses for 30 min prior to stimulation with
either DCA or IL-1β. Pretreat-
ment of the cells with PKC inhibi-
tors prevented DCA-induced
NF-κB DNA binding but had no
effect on IL-1β-induced NF-κB
DNA binding (a). Western blot
analyses on the total cell extracts
of similarly treated cells showed
that DCA-induced reduction in
NF-κB-α level was inhibited by
PKC inhibitors (b). Figure 4c de-
monstrates that DCA and PMA-
induced AP-1 DNA binding was
blocked by pretreatment of the
cells with PKC inhibitors.


