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Sulindac inhibited gene expression and activity of arylamine N-acetyltransferase and DNA-2-aminofluorene adduct formation in T24 human bladder tumor cells

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Abstract We demonstrated in vivo that non-steroidal anti-inflammatory drugs including sulindac can act as inhibitors of urinary bladder carcinogenesis in rats. The aim of the present study was to determine whether sulindac affects arylamine N-acetyltransferase (NAT) activity and gene expression and DNA-2-aminofluorene adduct formation in the T24 human bladder tumor cell line. The NAT activity (N-acetylation of 2-aminofluorene) was measured by high performance liquid chromatography assaying for the amount of acetylated 2-aminofluorene and the remaining 2-aminofluorene (AF). The results demonstrated that NAT activity in T24 cells were inhibited by the sulindac in a dose-dependent manner. The apparent values of Km and Vmax of NAT from T24 cells were also decreased by sulindac. This inhibition was not competitive. The amount of DNA-AF adduct formation in T24 cells was also inhibited by sulindac. The data also demonstrated that sulindac inhibited the NAT mRNA level in T24 cells.

Keywords Sulindac · N-acetyltransferase · 2-aminofluorene · DNA adduct · Bladder cancer · Gene expression

Introduction

N-acetylation is one of the major routes in the detoxification of carcinogenic substances, drugs (e.g. isoniazid, sulfamethazine and procaainamide), and is also involved in the melamin pathway, which is catalyzed by host cytosolic arylamine N-acetyltransferase (NAT) using acetyl coenzyme A as a acetyl group donor [31]. NAT is an important enzyme in the biotransformation of various xenobiotics which possess a primary aromatic or hydrazine structure, and which may play an important role in the etiology of colorectal, breast, and bladder cancer [17]. Two functional human NAT genes (NAT1 and NAT2) exist, located in chromosome 8. Both genes contain a 870-bp intronless protein-coding region that shows significant homology at the DNA level [25]. NAT activity has been reported in the colon [15], breast [22] and bladder [20] from humans. Thus, genetic variation in NAT activity may be indicative of different risks for arylamine-induced tumors in human populations.

Sulindac is a non-steroid, anti-inflammatory drug (NSAID) which has been used in the treatment of rheumatoid arthritis and osteoarthritis [3] and which has also been reported to lead to the regress of adenomatous polyps in patients with familial adenomatous polyposis [30]. Sulindac suppresses cell proliferation through the inhibition of prostaglandin synthesis [5] and enhances gastrointestinal glutathione S-transferase in rats [29]. Our previous studies have demonstrated that sulindac inhibits NAT activity in bacteria [8, 23] as well as in human colon tumor cells [7]. Other investigators have reported the potency of sulindac as an inhibitor of urinary bladder carcinogenesis [27]. Recently we reported that T24 human bladder tumor cells show NAT activity which is inhibited by berberine [11]. However, there is no available information on how sulindac affects NAT activity, gene expression or the DNA-2-aminofluorene adduct formation of the T24 cells. Thus, this study focuses on the effects of sulindac on NAT activity, gene expression and DNA adduct formation of the T24 human bladder tumor cell line.
Materials and methods

Chemicals and reagents

Sulindac, ethylenediaminetetraacetic acid (EDTA), 2-aminoﬂuorene (AF), N-acetyl-2-aminofluorene (AAF), p-aminobenzoic acid (PABA), N-acetyl-p-aminobenzoic acid (N-Ac-PABA), acetylcarnitine, dimethyl sulfoxide (DMSO), Tris, leupeptin, bovine serum albumin (BSA), phenylmethylsulfonyl ﬂuoride (PMSF), dithiothreitol (DTT), acetyl CoA, and carnitine acetyltransferase were obtained from Sigma (St. Louis, Mo.). All of the chemicals used were reagent grade.

T24 human bladder tumor cell line

The human bladder tumor (carcinoma) cell line T24 (human female; Caucasian; 81 year old) was obtained from the National Taiwan University Hospital (Taipei, Taiwan). The cells were placed into 75 cm² tissue culture ﬂasks and incubated at 37°C in a humididiﬁed, 5% CO₂ atmosphere in RPMI 1640 tissue culture medium (Sigma) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, N.Y.), 2% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin).

NAT activity determinations

The determination of acetyl CoA-dependent N-acetylation of PABA and AF was performed as described by Wu et al. [32]. Protein concentrations in the T24 cell cytosols were determined by the method of Bradford [4] with bovine serum albumin as the standard. All of the samples were assayed in triplicate.

Effect of various concentrations of sulindac on NAT activity: N-acetylation of AF

T24 cells (in 1 ml RPMI 1640 media with glutamine and 10% fetal bovine serum) were incubated with various concentrations of AF (15, 30, 60 and 90 µM) at 1 x 10⁶ cells/ml in individual wells of a 24-well culture plate with or without sulindac (ﬁnal concentrations are 0.5, 5, 50 and 500 µM) co-treatment for 18 h at 37°C in 95% air and 5% CO₂. At the end of incubation, the cells and media were harvested by centrifugation. The supernatant was immediately extracted with ethyl acetate/methanol (95:5), the solvents evaporated, and the residue redissolved in methanol and assayed for AAF as described above [9, 32].

Effects of incubation time of sulindac on NAT activity: N-acetylation of AF

T24 cells (in 1 ml RPMI 1640 media with glutamine and 10% fetal bovine serum) were incubated with 60 µM AF at 1 x 10⁶ cells/ml in individual wells of a 24-well culture plate with or without sulindac (ﬁnal concentration is 50 µM) co-treatment for various incubation time (6, 12, 18, 24 and 48 h) at 37°C in 95% air and 5% CO₂. At the end of incubation, the cells and media were harvested by centrifugation. We then determined the amounts of acetylated and non-acetylated AF as described above.

Effects of sulindac on the kinetic constants of NAT

The cytosols of T24 cells co-treated with or without 50 µM sulindac and selected concentrations of AF were examined for NAT activity as described above [32]. All reactions were run in triplicate.

Detection and measurement of DNA adducts

Detection and measurement of DNA adducts were performed as described previously [32].

Effects of various concentrations of sulindac on NAT gene expression

After co-treatment with or without different concentrations of sulindac, the total amount of RNA was extracted from T24 cells by using Qiagen RNeasy Mini Kit 24 hr. Then total RNA (1.5 µg), 0.5 µg of oligo-dT primer and DEPC (diethyl pyrocarboximate)-treated water were combined into a microcentrifuge tube to a ﬁnal volume of 12.5 µl. The entire mixture was ﬁrst heated at 70°C for 10 min and then chilled on ice for at least 1 min. The subsequent procedures for conducting reverse transcription conformed with instruction manual (First-strand cDNA synthesis kit, Novagen). The reverse transcription products from total RNA served as a template for PCR. The components in 50 µl of solution were as follows: when amplifying target cDNA: 1.5 mM MgCl₂, 0.2 mM dNTP mix, 20 pmoles of each primer (B-MDIEA-NAT1 and VPKHGDX-NAT1 for NAT1, FPI-NAT2 & RP1-NAT2 for NAT2, Act b1 and Act b2 for beta-actin), cDNA template corresponding to the amount synthesized from 50 ng of total RNA and 2 U of DyNAzyme DNA polymerase. The sequence of primers was: B-MDIEA-NAT1, 5′-CAC-CGGATCCGGATCATGGACATTGAAGC-3′, nt 435–454, GenBank accession number: X17059; VPKHGDX-NAT1, 5′-GGTTCCTCAGTCAAACATGGTTGGCCAC-3′, nt 1295–1278, GENBANK accession number:X17059; FPI-NAT2, 5′-CTAGTTCCTGGTTTCGAGC-3′, nt 79–98, GenBank accession number: NM-000015; RP1-NAT2, 5′-TAACTGTTAGGG- TAGAGGGA-3′, nt 1073–1054, GenBank accession number: NM-000015; Act b1, 5′-GCCTGTGCACACGCT-3′, nt 94–114, GenBank accession number: NM-000110; Act b2, 5′-CAAACATGATGGGGTATCTTCTC-3′, nt 446–422, GenBank accession number: NM-000110 [2, 13, 16, 26].

Statistical analysis

Statistical analysis of the data was performed with an unpaired Student’s t-test. The kinetic constants were calculated with the Cleland HYPER program [12] that performs linear regression using a least-squares method.

Results

The possible effects of sulindac on NAT activity in T24 cells were determined by HPLC assessing the percentage of acetylated and non-acetylated AF. The means ± SD of AF N-acetylation co-treated with or without sulindac with AF as the substrate are given in Figs. 1 and 2. The data indicated that there was a decrease in AAF associated with increasing concentrations of sulindac in intact T24 cells.

To determine the time course effect of 50 µM sulindac on the N-acetylation of AF in T24 cells, the cells were incubated at 37°C with or without sulindac and harvested at 6, 12, 18, 24, and 48 h, respectively. An increased time of incubation led to increased AAF production for up to 48 h (Fig. 3) and the presence of 50 µM of sulindac decreased the amounts of AAF by about 6–36%.

In the presence or absence of 50 µM sulindac, specific concentrations of AF (0.373, 0.435, 0.543, 0.745, 1.102, and 2.205 mM, respectively) were added to the recycling mixture for determining T24 cell NAT kinetic constants. When 50 µM sulindac was added to the cytosol reaction...