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ORIGINAL PAPER

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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

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Introduction

N-acetylation is one of the major routes in the detoxification of carcinogenic substances, drugs (e.g. isoniazid, sulfamethazine and procainamide), and is also involved in the melanin 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 gultathione 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), phenylmethylsulfonylfluoride (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 cm^2 tissue culture flasks and incubated at 37°C in a humidified, 5% CO_2 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 1×10^6 cells/ml in individual wells of a 24-well-cell 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 and5% CO_2. 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 solvent 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 1×10^6 cells/ml in individual wells of a 24-well-cell 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% CO_2. 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 pyrocarbonate)-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 $MgCl_2$, 0.2 mM dNTP mix, 20 pmoles of each primer (B-MDIEA-NAT1 and VPKHGD-X-NAT1 for NAT1, FP1-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′-CACCCGGATCCGGGATCATGGACATTGAAGC-3′, nt 435–454, GenBank accession number: X17059; VPKHGD-X-NAT1, 5′-GGTCCTCGAGTCAATCACCATGTTTGGGCAC-3′, nt 1295–1278, GENBANK accession number:X17059; FP1-NAT2, 5′-CTAGTTCCTGGTTGCTGGCC-3′, nt 79–98, GenBank accession number: NM-000015; RP1-NAT2, 5′-TAACGTG AGGGTAGAGAGGA-3′, nt 1073–1054, GenBank accession number: NM-000015; Act b1, 5′-GCTCGTCGTCGACAACGGCTC-3′, nt 94–114, GenBank accession number: NM-001101; Act2 b2, 5′-CAAACATGATCTGGGTCATCTTCTC-3′, nt 446–422, GenBank accession number: NM-001101 [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 AFF 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 upto 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

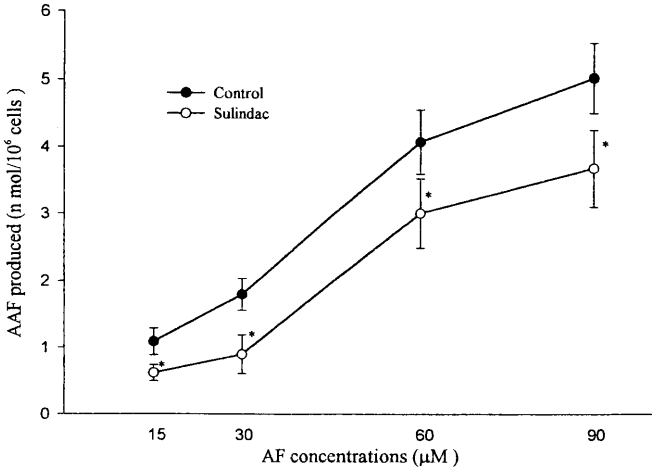

Fig. 1 Effects of 50 μM sulindac on N-acetylation of various concenations of AF by T24 cells. T24 cells were incubated as described for 24 h at various concentrations of AF co-treatment with 50 μM sulindac. The amounts of AAF were measured by HPLC assay. Each point represents the mean of triplicate assays of three incubations of cells. *mean differs between sulindac and control with $P < 0.05$

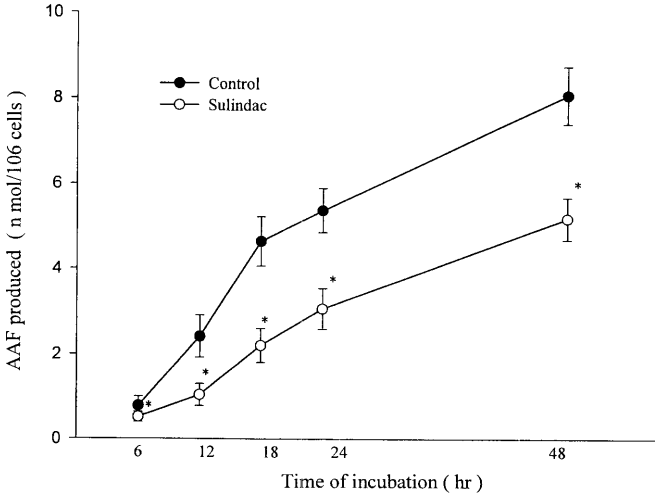

Fig. 3 Effects of incubation time on AAF production by T24 cells. T24 cells were incubated with AF at 60 μM with 50 μM sulindac co-treatment for the time shown. The amounts of AAF were measured by HPLC assay. Each point represents the mean of triplicate assays of three incubations of cells. *mean differs between sulindac and control with $P < 0.05$

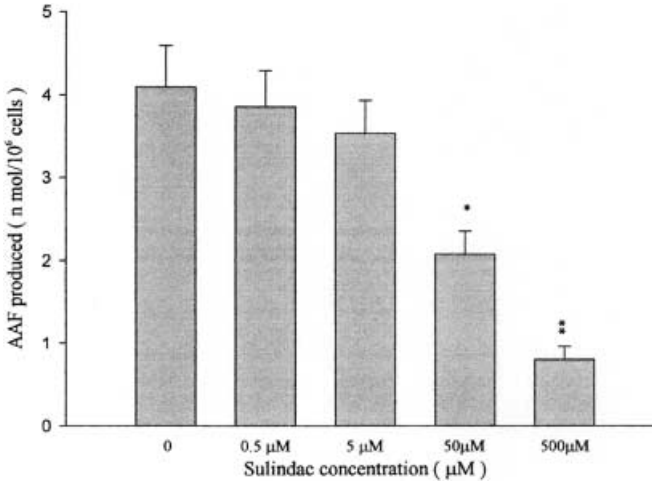

Fig. 2 Effects of various concentrations of sulindac on the production of AAF by T24 cells. T24 cells were incubated as described for 24 h at the 60 μM AF co-treatment with various concentrations of sulindac. The amounts of AAF were measured by HPLC assay. Each point represents the mean of triplicate assays of three incubations of cells. *mean differs between sulindac and control with $P < 0.05$

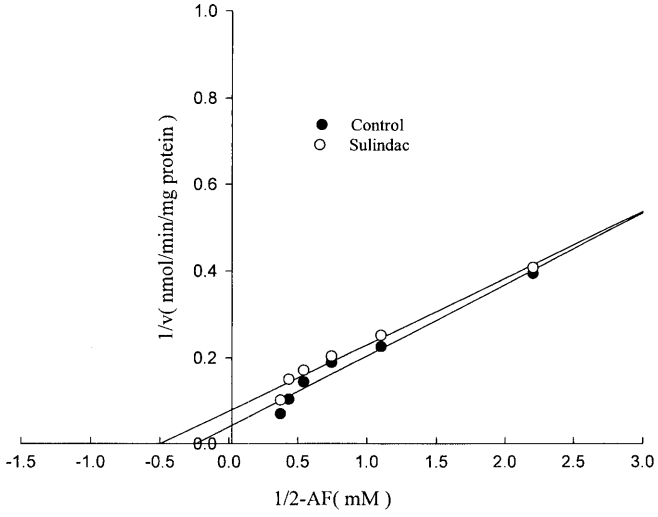

Fig. 4 Lineweaver-Burk double reciprocal plot of NAT in human T24 cells co-treatment with or without 50 μM sulindac. Cytosols of T24 cells containing 0.044, 0.088, 0.163, 0.303 and 0.709 mM AF with or without 50 μM sulindac were incubated for 10 min incubation. The amounts of AF and AAF, PABA and N-Ac-PABA were determined as. Lineweaver-Burk double reciprocal plots were calculated using a linear regression analysis of reciprocal substrate concentrations plotted against reciprocal initial velocities

mixture, the apparent values of Km and Vmax were decreased (Fig. 4 and Table 1).

Following 24 h incubation with AF in the presence or absence of sulindac, T24 cells were harvested and DNA was prepared, hydroylzed to nucleotides and the adducted nucleotides were extracted into butanol and analyzed by HPLC. The results indicated that T24 cells activated AF to a metabolite which was able to bind covalently with DNA. AF also induced dose-dependent AF-DNA adduct formation (Fig. 5). In the presence of 50 μM sulindac, DNA-AF adduct formation decreased at all AF concentrations ($P < 0.05$).

Dose-dependent changes of NAT1 mRNA levels in response to various concentrations of sulindac were examined (Fig. 6A, B). Data presented in Fig. 6A and 6B show that the NAT1 mRNA level decreased as the sulindac levels increased. NAT1 mRNA was present in human bladder tumor T24 cells. The results indicated that sulindac could affect the expression of NAT mRNA these cells.

Table 1 Kinetic data for acetylation of AF in human T24 cells. Values are means ± SD, $n = 3$. The acetyl CoA and sulindac concentrations were 0.5 mM and 50 μM, and the kinetic constants were calculated from the modified HYPER program of Cleland [12]

	Km (mM)	Vmax(nmol/min/mg protein)
Control	5.04 ± 0.94	25.18 ± 4.86
Sulindac	[a]2.12 ± 0.39	[b]12.25 ± 3.02

[a] 50 μM sulindac and control differ with $P < 0.005$
[b] 50 μM sulindac and control differ with $P < 0.001$

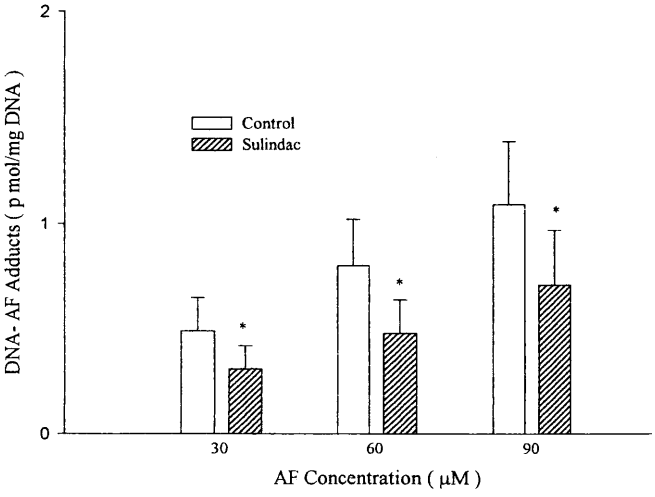

Fig. 5 Effects of 50 μM sulindac on DNA-AF adduct formation by intact human T24 cells. Following the 24 h incubation of human T24 cells with AF (30, 60 and 90 μM) in the presence of sulindac (50 μM), cells were harvested and DNA was prepared, hydrolyzed to nucleotides and the adducted nucleotides were extracted into butanol and analyzed by HPLC. Values are means ± SD of six separate preparations. *50 μM sulindac and control differ with $P < 0.05$

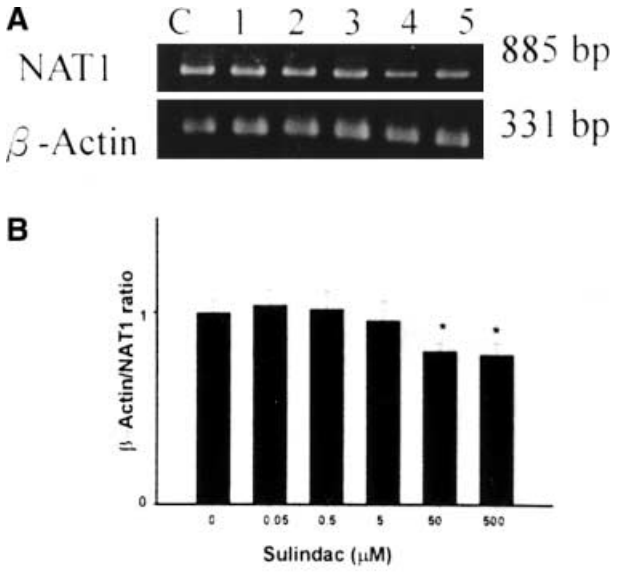

Fig. 6A, B Dose-dependent effect of sulindac on the expression of NAT mRNA in human T24 cells. The cells were incubated with various concentrations of sulindac for 24 h then collected to extract RNA. This was subjected to RT-PCR analysis using specific primers for NAT and β-actin, and then PCR-amplified cDNA derived from mRNA was applied to agarose gel-electrophoresis (**A**). The mRNA levels of NAT and β-actin on the gel-electrophoresis were quantified by densitometric analysis of a gel-photograph and expressed as NAT/β-actin ratio (**B**). * 50 μM sulindac and control differs with $P < 0.05$

Discussion

The results from this study indicate that T24 cells contain NAT activity and this NAT belongs to NAT1 not NAT2 based on two observations: 1. the PCR product did not show NAT2 mRNA after using a NAT2 primer for reaction; 2. N-acetyl-SMZ was not detected after SMZ was used as substrate for T24 cell culture. PABA belongs to NAT1 but SMZ belongs to NAT2 due to the substrate specificity of NATs. However, T24 cells did acetylate PABA and AF. NAT activity of T24 cells were decreased by sulindac. NAT activity in several laboratory animal species and humans is genetically determined and the rapid NAT activity (rapid acetylator phenotype) has been shown to predispose humans to colorectal and breast cancer [19, 21], whereas the slow NAT activity (slow acetylator phenotype) is related to arylamine-induced bladder cancer [6, 31]. The substrate specificity for NAT1 and NAT2 is different in humans. AF is the common substrate for both NAT1 and NAT2 and this is why it was chosen for the present study [31]. It is impossible to conclude whether the NAT activity in T24 cells belong to rapid or slow acetylation, therefore further investigation is needed in the future. More important is the fact that the N-acetylation is a primary determinant in the elimination of several therapeutic compounds and arylamines.

The results from the present study demonstrated that sulindac can markedly decrease the Km and Vmax values in T24 cells and inhibit the NAT activity. The inhibition has a dose-dependent effect. Both kinetic constants (Km and Vmax) were shown to be decreased, thus in this reaction sulindac acts as a uncompetitive inhibitor. The results also show that sulindac decreased DNA-AF adduct formation in T24 cells. The initiation of carcinogenesis is related to the levels of DNA-AF adduct formation [10, 28]. Other investigators have already pointed out that the studies from bacteria and animals both indicated that N-(dGus-8-yl)-AF-adducts formation in the DNA is essential for the initiation of hepatocarcinogenesis and in the induction of mutations [24]. More importantly, N-hydroxyarylamines are ultimately bladder carcinogens and C8-deoxyguanosine substitution may represent an initiating lesion in bladder tumor formation [1]. The results from the present studies also demonstrated that sulindac decreases NAT mRNA levels in T24 cells. It is difficult at this point to extrapolate the quantity of sulindac that would be needed to be consumed by humans to potentially reduce NAT activity and gene expression and DNA-AF adduct formation in target cells in vivo.

Humans show considerable genetic variability in their ability to N-acetylate arylamine [16], and this variation has been implicated in colon cancer [18, 21] and the etiology of arylamine-induced bladder tumors [24]. Currently, our data (Fig. 5) also demonstrated that, in T24 cells, sulindac decreased DNA-AF adduct formation. At present it is not known whether this decrease in NAT activity and DNA-AF adduct in the cell culture would therefore result in decreased of tumor production in the animal in vivo or whether sulindac could prevent the development of bladder cancer in vivo. Nevertheless, DNA-carcinogen is the first step for carcinogen induced carcinogenesis in target organs or cells. DNA-carcinogen also plays an important role in the chemical carcinogenesis. More interesting is the fact that other investigators have already pointed out that the increased sensitivity to the mutagenic effects of many arylamines is associated with promoted NAT activity in bacteria [14]. Two disease processes, such as breast and bladder cancer, are associated with the attenuation of liver NAT activity [31]. Apparently, the changes of NAT activity are related to several diseases. Further investigation is needed to examine whether or not the decrease of NAT activity leads to the decrease of target organ malignancy after being exposed to arylamine carcinogens.

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