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INSIGHTS INTO THE MECHANISM OF ACTION OF BENZOYL PEROXIDE AS A TUMOR PROMOTER

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Abstract: A comparison of the mechanism of action of benzoyl peroxide, a tumor promoter was studied in three different cell lines i.e. NIH 3T3, HDCS and A431. Benzoyl peroxide was found to mediate its effect by inducing poly ADP-ribosylation in all the three cell types studied but to different extents, with histone H1 serving as a common acceptor for poly ADP-ribose. It also stimulated the activities of the antioxidant enzymes CuZn superoxide dismutase and catalase in NIH 3T3 and HDCS cells, but not in A431. Alterations in the experession of c-jun and c-fos were observed in NIH 3T3 and A431 cells. Benzoyl Peroxide appeared to mediate its effect via genetic and epigenetic mechanisms.

Key words: poly ADP-ribosylation, benzoyl peroxide, genes, protein kinase C, tumor promotion

INTRODUCTION

Chemical carcinogenesis is essentially a multistep process with tumor promotion as an integral mechanism. It involves alteration in gene expression, chromatin structure and function as well as distortion in signal transduction mechanism (1, 2). Benzoyl peroxide (BP) is among the bonafide oxidants which induce cellular prooxidant state acting as carcinogens, in particular as promoters and progressors (3). Tumor promoting activity of the peroxides can be inhibited *in vivo* suggesting that free radicals formed from peroxides are responsible for tumor promotion (4). BP is used as an initiator of free radical polymerization in plastics, as a bleaching agent in foods and as the active ingredient in non-prescription acne medications (5). BP has skin tumor promoting activity observed in several strains of mice following initiation with chemical carcinogens (6). The biological activity of BP in multistage carcinogenesis has been tested in the skin of SENCAR mice which suggests its ability to generate free radical derivatives, but the mechanism(s) by which BP facilitates tumor promotion remains to be elucidated (7). Hence, we have searched for the underlying mechanism of action of BP which might be useful for the pharmacological intervention.

METHODS

Cell culture : The isotopes (3H) NAD and [α^{32}-P] dCTP were from Amersham, USA, Benzoyl peroxide, phosphotidyl-L-serine, Cu-Zn superoxide dismutase, xanthine oxidase, nitroblue tetrazolium, xanthine, horse radish peroxidase, agarose, acrylamide were

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purchased from Sigma Chemical Co, St. Louis, USA, DMEM medium, fetal calf serum were from G1BCO-BRL, USA and phenyl boronate agarose matrix gel (PBA-30) from Amicon. Co, USA. The cell lines of human diploid kidney cells (HDCS), mouse embryo fibroblast cells (NIH 3T3) and human epidermoid carcinoma cells (A431) were obtained from NFATCC, Pune, India and grown in DMEM medium supplemented with 10% FCS and antibiotics in a 5% C02 atmosphere at 37°C in a humidified incubator. Cells were treated with BP (1mM) for varying time periods. At the end of treatment, cells were harvested, washed with phosphate buffered saline (PBS), pH7.4 and taken for the respective estimations.

Assay of poly ADPR polymerase activity: The cells were permeabilized and assayed for the enzyme activity as described by Jacobson et al (8).

Assay of superoxide dismutase activity : A xanthine/xanthine oxidase generating system was employed with nitroblue tetrazolium (NBT) as a detector (9). SOD activity was determined from the calibration curve of standard copper zinc superoxide dismutase (CuZnSOD). One unit of activity is defined as the amount of protein that led to 50% inhibition of the rate of NBT reduction.

Catalase assay : This was based on the disappearance of hydrogen peroxide in the presence of cellular extract at 25°C (10). The absorbance of chromogen was measured at 420nm. The enzyme activity was calculated using the rate constant and expressed as U/mg protein/min.

Assay of acceptor proteins for poly ADP-ribose : Untreated control cells ($3.5\text{-}4 \times 10^8$), as well as those treated with BP for 90 min were lysed, the nuclei isolated (11) and the histones extracted from the nuclear pellet (12). Separation of poly ADP-ribosylated proteins was performed by the method of Adamietz and Rudolph (11), as modified by Krupitza and Cerutti (12). SDS-PAGE gel was done as described by Laemmli (13).

Protein kinase C (PKC) assay : 5×10^6 cells were treated with BP for 15 and 90 min and the subcellular fractions isolated, and the cellular protein was estimated in the cytosolic and the membrane fraction (14). PKC activity was determined by measuring Ca^{++} and phospholipid dependent phosphorylation of lysine rich histone by the incorporation of [γ^{32}P] into histone from [γ^{32}P] ATP. The radioactivity incorporated was quantitated by liquid scintillation spectroscopy (15) and PKC activity was expressed as pmoles/min/mg protein.

Northern blot analysis : Total RNA was isolated from the cells using RNAzol solution (Cinna/Biotech, Friendswood, Tx, USA) according to manufacturer's recommendation. 10 μg of total RNA was electrophoresed on 1.2% agarose/formaldehyde gel in MOPS buffer and transferred to nylon membrane. The membrane was dried in vacuum, exposed to UV for crosslinking of RNA to the filter. RNA of interest was located by prehybridization and hybridization with [α^{32}-P] labeled cDNA probe (16). The probes were purchased from Oncogene Science, USA. The membranes were washed post hybridization followed by autoradiography.

RESULTS

Stimulation of poly ADP-ribosylation by benzoyl peroxide : The effect of BP was studied on poly ADP-ribose polymerase activity. The maximum increase in the

enzyme activity of 1.9 fold was seen at 90 min in NIH 3T3 cells, of 1.6 fold at 3 h in HDCS and of 1.7 fold at 3h in A431 cells, post BP treatment (Fig. 1a, b, c).

Time specific alterations of intracellular antioxidant enzymes: As shown in Fig. 2, BP caused an initial drop in SOD activity at 90 min followed by a 2.5 fold increase at 3h in NIH 3T3 cells. In contrast the enzyme activity increased by 2.6 fold in HDCS cells at 90 min and declined thereafter but did not return to the basal levels even till 3 h. A431 cells showed a higher basal level of SOD activity in comparison to the other two cell types. BP stimulation increased this further by 1.6. fold at 90 min and then returned to the basal level at 3h.

The basal level of catalase (CAT) activity was more or less similar in all the three cell types. BP treatment stimulated the CAT activity by 1.6 fold in NIH 3T3 cells, by 1.5 fold in HDCS cells, both at 90 min, and dropped thereafter. In A431 cells BP showed a biphasic increase in enzyme activity (Fig. 3).

Histones served as acceptors for poly ADP-ribose: Histones H1, H3/H2A and H4 served as acceptors of the polymer in NIH 3T3 cells on BP treatment, with H1 and H3 being the major acceptors (Fig. 4a). In case of HDCS cells H3d, H1 H2B, H3, H2A and H4 served as acceptors, with H3d, H1 and H2B being the major acceptors (Fig. 4b).

Benzoyl peroxide did not affect PKC activity: Time dependent effects of BP on PKC activity in subcellular fractions were examined. In NIH 3T3 and HDCS cells the basal PKC activity was found essentially in the cytosol fraction and was about 5 fold higher as compared to the membrane fraction (Table I). But in A431 cells, the basal activity was 1.7

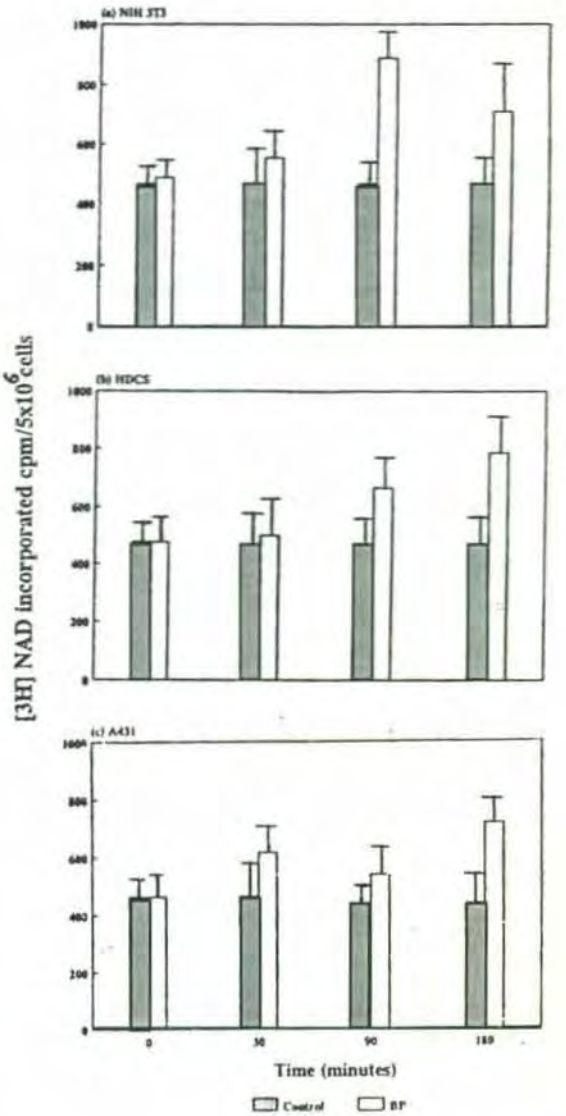

Fig. 1: Effect of benzoyl peroxide on poly ADPR polymerase activity:
NIH 3T3 (a), HDCS (b) and A431 (c) cells were treated with benzoyl peroxide for the indicated time periods and poly ADPR polymerase activity estimated. Each value is a mean ± SD of two seperate experiments, each performed in duplicate.

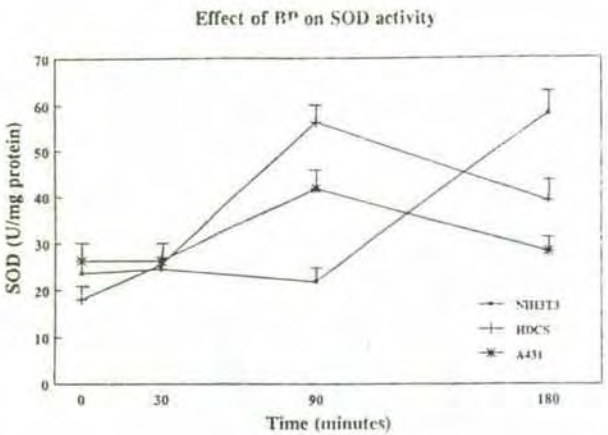

Fig. 2: Alterations in intracellular CuZn superoxide dismutase activity.
Cells were treated with benzoyl peroxide and sonicated. The cell sonicate was taken for CuZn SOD assay. Each value is a mean ± SD from two seperate experiments each performed in duplicate.

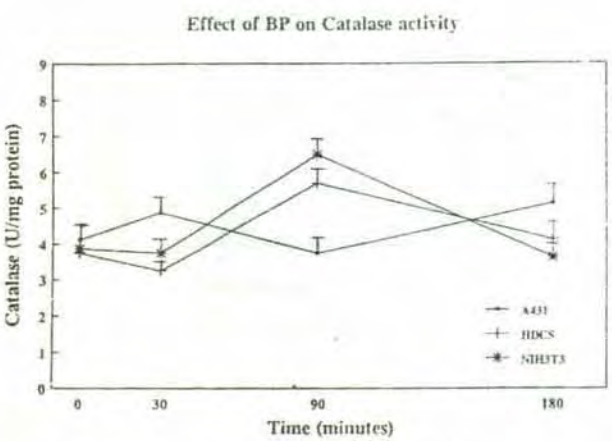

Fig. 3: Changes in catalase activity :
Cells were harvested post benzoyl peroxide treatment and sonicated. The sonicate was assayed for catalase activity. The values are expressed as mean ± SD of two seperate experiments, each performed in duplicate.

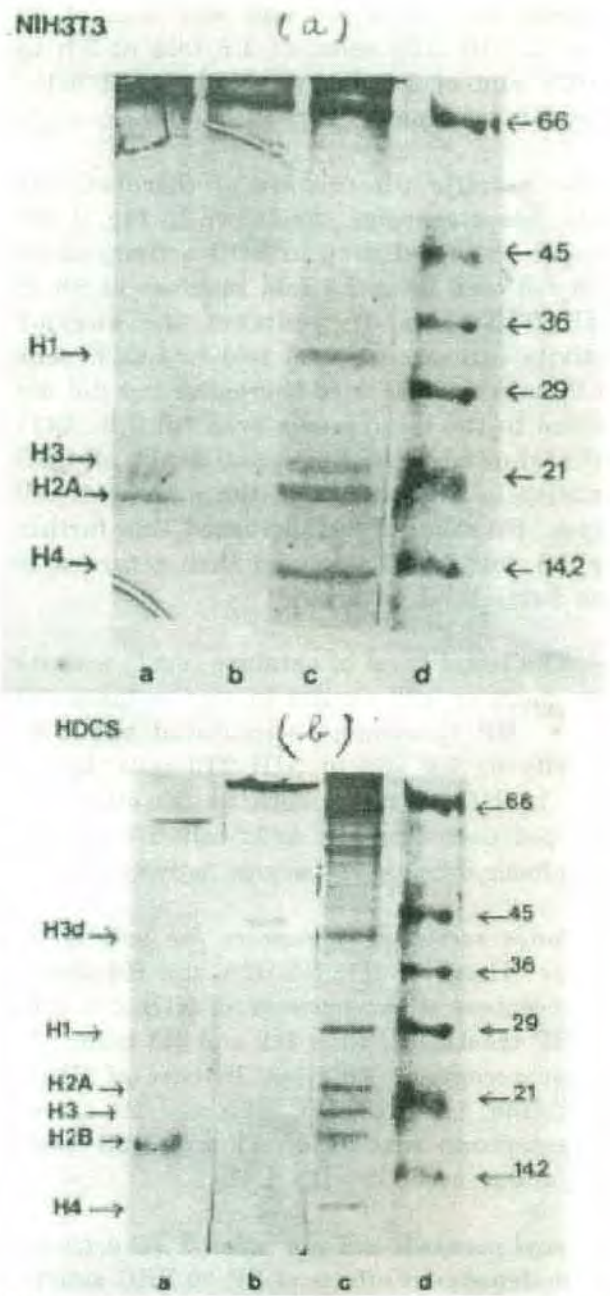

Fig. 4: Analysis of poly ADP-ribosylated histone proteins post BP treatment :
SDS-PAGE analysis of poly ADP-ribosylated histone proteins, post benzoyl peroxide treatment for 90 min in NIH 3T3 cells (a) and in HDCS cells (b). Lane (a) shows histone 2 A marker, lane (b) control cells, lane (c) benzoyl peroxide treated cells and lane (d) is molecular weight marker.

fold higher in the membrane fraction as compared to the cytosol fraction could be seen post BP treatment in membrane or cytosol fractions in the cells studied.

TABLE I : Subcellular distribution of protein kinase C activity:

Treatment	*Fraction*	*PKC activity (pmoles/min/mg protein)*	
		15 min	*90 min*
Control	Particulate	55 ± 9	51 ± 4
	Soluble	300 ± 27	286 ± 26
Benzoyl peroxide	Particulate	50 ± 5	50 ± 7
	Soluble	311 ± 35	291 ± 30
HDCS			
Control	Particulate	60 ± 7	56 ± 6
	Soluble	220 ± 21	195 ± 21
Benzoyl peroxide	Particulate	80 ± 6	52 ± 8
	Soluble	182 ± 31	198 ± 27
A431			
Control	Particulate	110 ± 12	120 ± 15
	Soluble	65 ± 7	60 ± 6
Benzoyl peroxide	Particulate	116 ± 15	118 ± 14
	Soluble	65 ± 6	58 ± 7

Differential expression of c-fos and c-jun by benzoyl peroxide: The levels of c-fos and c-jun mRNAs were compared in NIH 3T3 and A431 cells post BP treatment by Northern blot analysis of total RNA. 7s mRNA was used as an internal standard to monitor equal loading of the samples. Treatment with BP led to induction of c-jun mRNA in NIH 3T3 cells (Fig. 5) at 1h post treatment which persisted till 6h and then declined to the basal level. In A431 cells a decrease in c-jun mRNA was seen at 1h post BP treatment which persisted till 6h and declined at 24 h. However, a transient increase in c-fos mRNA level was seen in NIH 3T3 cells at 6h and in A431 cells at 1h post BP treatment. The results suggest the possible involvement of c-jun and c-fos in BP mediated action.

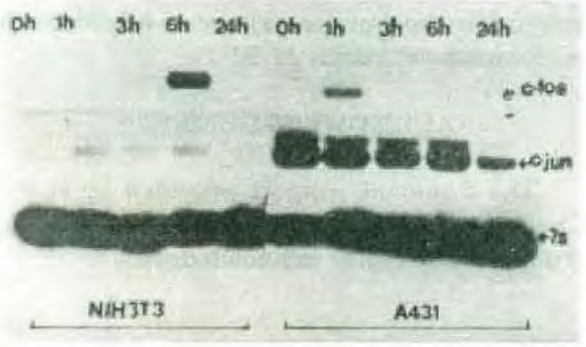

Fig. 5 : Northern blot analysis of c-fos and c-jun mRNA in cells exposed to benzoyl peroxide.
Cells were exposed to benzoyl peroxide for varying time periods. 10 µg to total RNA was loaded per lane, transferred to nylon membrane and hybridized with cDNA probes to fos and jun. 7s cDNA was used as a standard.

DISCUSSION

Poly ADP-ribosylation, a post-translational modification of chromatin proteins, is stimulated in response to DNA strand breaks (17). In the present study BP exposure led to an early stimulation of poly ADPR polymerase activity in NIH 3T3 but showed a delayed response in HDCS and A431 cells. The fall in enzyme activity could be due to partial inactivation of the enzyme or due to its degradation. It has been reported that this nuclear enzyme can also act as a self acceptor for the polymer, which results in cleavage of the enzyme thereby rendering it inactive (2). Our results are in agreement with earlier reports showing stimulation of this enzyme by BP (18, 19, 20). BP is known to induce DNA strand breaks (5) and the stimulation of poly ADP-ribosylation of chromatin proteins is a metabolic consequence of the DNA strand breakage (18). Thus BP induces poly ADP-ribosylation of chromatin proteins.

Cells have an elaborate defence system consisting of antioxidant enzyme like superoxide dismutase and catalase against reactive oxygen species (1, 3). It appears that BP generates active oxygen species which

could be the cause of the observed stimulation of CuZnSOD and CAT activities, to scavenge these active oxygen species. In the transformed A431 cells since there was higher basal level of catalase hence the observed initial decrease may be because the cells are overcoming BP induced oxidative stress. Phorbol ester, a tumor promoter also causes similar decrease in CAT activity (21). While investigating the acceptor proteins for the polymer we found that BP led to poly ADP-ribosylation of core and linker histones H3, H2A and H1, similarly, in both NIH3T3 and HDCS cells. Apart from these, two additional histones H3d and H2B served as acceptors of the polymer in HDCS cells. Histones H1 and H2B have been shown to serve as the polymer acceptors in rat liver and HeLa cells (22) Preferential poly ADP-ribosylation of H2B has been reported in dimethyl sulphate (DMS) treated rat hepatoma cells (11), and of H2B and H3 in isolated nuclei from DMS treated cells (23). Thus it appears that the linker histone H1 serves as a major acceptor of poly ADP-ribose post BP treatment, irrespective of the cell type in question.

The basal level of PKC activity was highest in NIH 3T3 followed by HDCS and A431 cells. In none of these cell types BP appeared to alter the PKC activity or its translocation, thereby suggesting the non-involvement of PKC in BP mediated response. The higher level of basal PKC in membrane fraction as well as the lower total PKC level in A431 cells suggests that perhaps these cells experience a chronic stimulation of PKC activity resulting in accompanying down regulation of the enzyme.

Nuclear proteins involved in gene regulation appear to function as transcription factors or transcription modulators and their interactions are crucial in determining whether the expression of a specific gene is activated or repressed (24). Genes belonging to fos and jun family encode nuclear proteins that are found to be associated in a number of transcriptional complexes. The observed fos induction in this study may be an early response to BP induced oxidant stress. Active oxygen causes both phosphorylation as well as poly ADP-ribosylation of fos protein (25). Induction of c-fos by BP in NIH 3T3 and A431 cells in a time dependent manner was similar to the results obtained by Muehlematter et al (3). BP showed a repression of c-jun mRNA in A431 cells but an induction in NIH 3T3 cells. This transitory increase of c-jun level could be due to an immediate cellular response to BP. Hence the induction of immedate early genes by BP may play a role in oxidant tumor promotion via poly-ADP-ribosylation. Our study indicates that both genetic and epigenetic mechanism (s) are involved in the mechanism of action of BP.

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