

International Journal for Pharmaceutical Research Scholars (IJPRS)



ISSN No: 2277 - 7873

RESEARCH ARTICLE

Induction of Apoptotic Cell Death by Naringenin in DEN Induced Hepatocarcinogenesis in Rats

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ABSTRACT

Naringenin (4,5,7-trihydroxyflavone) (NGEN) a flavanoid belonging to the subclass of flavanol is believed to play a pivotal role in apoptosis. In this study we found that co-treatment of Naringenin (50mg/kg body weight) for 16 weeks to *N*-Nitrosodiethylamine (DEN) induced rats thereby promoting apoptosis and blocking the down regulation of the PI3k/ Akt pathway. A better understanding of its biology could lead to improved treatment options. Generally, the goal of cancer treatment is to abolish cell proliferation and to induce necrotic or apoptotic cell death. Different apoptotic signals converge to induce caspase cascade activation. Caspase 3 and Caspase-9 is necessary for effective apoptotic cell death. Bcl-2 protein family regulates apoptosis. The Bcl-2 protein itself is a product of a proto-oncogene and has an antiapoptotic action. Analysis of morphological changes of hepatocytes and protein expressions of various apoptotic related markers, such as caspase-9, caspase-3, Bcl-2 Bax and Akt disclosed that the apoptotic activities of NGEN may be mediated through eliciting apoptotic mechanism in DEN-induced HCC condition.

KEYWORDS

Naringenin, N-Nitrosodiethylamine, Apoptosis, Caspase 9, Caspase 3, Akt

INTRODUCTION

Apoptosis, a programmed cell death initiated in neoplastic cells represent protective mechanisms against neoplastic transformation and development of tumours through elimination of genetically damaged cells or cells that may have been inappropriately induced to divide by mitogenic and/or proliferative stimuli.¹ Apoptosis is now studied as a cascade of a caspases and endonucleases responsible for the proteolytic cleavage of cellular proteins leading to the characteristic apoptotic features like plasma membrane blebbing, cell shrinkage, chromatin condensation, and fragmentation.²

*Address for Correspondence: Dr. Devaki Thiruvengadam Department of Biochemistry, University of Madras, Guindy Campus, Chennai 600 025, Tamilnadu, India. E-Mail Id: georget divya@yahoo.co.in However, most cancer cells block apoptosis, which allows malignant cells to survive despite genetic and morphologic transformations. Therefore, searching for agents that can trigger apoptosis in tumor cells has become an attractive strategy in anti-cancer drug discovery. Many agents that induce apoptosis often target the mitochondria and promote the activation of caspases, a family of cysteine acid proteases, which are important proteolytic enzymes responsible for the execution of apoptosis.³

Caspases, totally 14 family members to date, are synthesized as inactive zymogens, which must be proteolytically cleaved at two (or three in some cases) aspartate residues to generate the active mature enzyme. These now active initiator caspases^{2,8,9,10} in turn cleave and activate the downstream "executioner" caspases^{3,6,7}. These then cleave their target substrates to orchestrate the proteolytic dismantling of the cell. This sequence of events culminating in the activation of caspases has been broadly categorized into two pathways, the "extrinsic" pathway and the "intrinsic" pathway involving key mitochondrial events, such as anti-apoptotic protein Bcl-2, Bcl XL and pro-apoptotic protein Bax, Bak, Bik, Bad, and Bid.⁴

These proteins regulate the release of cytochrome C from mitochondria to cytosol; in both cases, an initiator caspase, via its interaction with a specialized adapter molecule, mediates its self-activation and ultimately, activation of the downstream effectors or executioner caspases. It is the activity of these caspases that ensure destruction of the cell. Akt, major downstream target of а phosphatidylinositol 3-kinase (PI3K), has been linked, through both indirect and direct mechanisms, to a wide variety of anti-apoptotic functions.^{5,6}

In particular, the activation of Akt signaling promotes cellular growth and survival.⁷ Several studies have shown that the PI3K/Akt signaling pathway's components are frequently altered in human cancers, and over-expression of Akt transformation malignant induces and chemoresistance.⁶ Furthermore, the PI3K/Akt pathway's inhibition combined with inhibitors of different signaling pathways, may provide an effective strategy to cancer cells by lowering the threshold for mitochondrial damage and apoptosis.

Thus, the Akt pathway presents an exciting new target for molecular therapeutics. Natural products with diverse bioactivities are becoming an important source of novel agents with pharmaceutical potential. Flavonoids are the most abundant antioxidants in humans diet and have attracted a great deal of attention in recent years for their role in the prevention of chronic diseases.⁸ Among them, naringenin (NGEN) is abundant in grapefruit and citrus fruits and juices.⁹ Recently NGEN, has been reported to induce apoptosis *in vivo*.

MATERIALS AND METHOD

Animals

Male Wistar albino rats (160–180 g) were procured from Kings Institute of Preventive Medicine, Guindy, Chennai, and fed with standard rat chow (Amrut Laboratory Animal Feed, Bangalore, India) and water *ad libitum*. Animal experiments were carried out in strict accordance with the guidelines set by the institutional ethical committee for the use of small animals in biomedical research at University of Madras, Chennai, India (IAEC No: 01/034/08).

Source of Chemicals and Antibodies

Anti-Bcl-2, anti-Bax, anti-Akt, anti-caspase-9 and anti-caspase-3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruze, CA). Naringenin and DEN were purchased from Sigma– Aldrich, USA and all other chemicals used were of analytical grade.

Experimental Protocol

The experimental animals were divided into four groups, each group comprising of six animals for a study period of 16 weeks as follows: group 1, normal control rats fed with standard diet and pure drinking water. In the preliminary studies performed on various dosages of Naringenin the optimal dosage for its hepatoprotective efficacy was found to be 50mg/kg body weight.

In group 2 rats, 50 mg/kg of Naringenin mixed diet was administered for 16 weeks. Group 3 rats were induced with DEN (200 mg/kg bodyweight) alone in drinking water for 16 Naringenin co-administered weeks. was 50mg/kg body weight to group 4 rats with DEN in drinking water (200 mg/kg bodyweight) for 16 weeks. After 16 weeks, experimental rats (n = 6 per group) were anaesthetized with sodiumpentothal after overnight fasting and euthanized. The liver was excised immediately and rinsed in ice-cold saline. A portion of the liver was homogenized in 0.1M Tris buffer, pH 7.4 and used for the assays.

Western Blot Analysis

The expression of caspase-3, caspase9, Bcl, Bax and AKT activity in liver tissues was detected by Western blot as described by Fido et al.¹⁰ Tissue protein was extracted in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 M Tris, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS in 500 ml water) and quantified by the Folin-Ciacalteu method of Lowry et al After the complete transfer of proteins, nitrocellulose membranes were blocked overnight in blocking buffer containing 10% non-fat dry milk and the membranes were incubated with rabbit polyclonal primary antibody for caspase-3,Caspase-9,Bax,Bcl and AKT (Santa Cruz Biotechnology, CA) and β -actin (H300-amino acids 76-375 of actin, human; Santa Cruz Biotechnology, CA) in 1:1000 dilutions for 4 h in 0.25% non-fat dry milk at room temperature. The membranes were probed for goat anti-rabbit IgG-peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, CA) used at a dilution of 1:15,000 in 0.25% non-fat dry milk, incubated for 1 h and washed twice with Tween-20 TBS and TBS for 10 min each. The protein bands were quantified using 'Quantity One' Software (Bio-Rad gel documentation system). The results are expressed in OD units reactive to β actin.

Statistical Analysis

Data were evaluated with SPSS/15 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference [LSD] test. p values of less than 0.05 were considered to indicate statistical significance. All these results were expressed as mean \pm S.D for six animals in each group.

RESULTS

Expression of Bcl-2 and Bax Proteins

Fig. 1 and 2 represents the expression of Bcl-2 and Bax respectively and Fig 3 and 4 represents the densitometry analysis of Bcl-2 and bax proteins respectively in experimental group of rats. The expression of Bcl-2 protein was found to be elevated in the HCC induced rats when compared to that of control (group I) while the administration of NGEN decreased the expression markedly in group IV when compared with group III. Decreased expression of Bax was observed in HCC induced group IV when compared to that of control (group I) and it was up-regulated in NGEN treated HCC induced rats (group III) when compared to that of HCC induced rats (group III).



Figure 1: Expression of Bcl-2, Lanes 1 - 4 corresponds to liver tissues of group I - IV respectively











Figure 4: Densitometric Analysis of Bax p<0.05 compared with ^agroup I, group III, ^cgroup IV

Expression of Caspase-9 and 3

Fig 5 represents the expression pattern of both caspase-9 and caspase-3 of liver tissues from the experimental groups and Fig 6 and Fig 7 represents the densitometry analysis of caspase-9 and Caspase-3 respectively. There was a decreased expression of caspase-9 and caspase-3 in HCC induced rat liver (group III) when compared with control (group I), while administration of NGEN significantly increased the caspase-9 and 3 when compared with HCC induced group



Figure 5: Expression of Caspase-9 and Caspase-3, Lanes 1 - 4 corresponds to liver tissues of





Figure 6: Densitometric Analysis of Caspase-9 p<0.05 compared with ^agroup I, ^bgroup III, ^cgroup IV





Effects of NGEN on protein expression of Akt

Western blotting analysis was performed to determine the expression Akt of protein in control and experimental groups. The proteins of Akt Fig 8 showed significantly high level of expression in HCC induced rats (group III) when compared to that of control rats, Fig 9 shows the densitometry analysis of the protein Akt. In NGEN treated group, there was a low levels of expression of both Akt when compared to that of HCC induced group.







Figure 9: Densitometric Analysis of Akt, p<0.05 compared with ^agroup I, ^bgroup III, ^cgroup IV

DISCUSSION

Effect of Naringenin on the Expression of Bcl-2, Bax

As lack of apoptosis is an abetting mechanism of cancer cells, this study also deals with the study of expression of apoptosis-inhibiting and apoptosis-promoting proteins in the liver of HCC implanted rats and in NGEN treated rats. Proteins of Bcl-2 family are known to regulate both promote and inhibit apoptosis.³ The antiapoptotic function of Bcl-2 may be explained by its ability to control several key steps of death signalling. Bcl-2 can form ion channels in biological membranes and its ion channel activity may control apoptosis by influencing intracellular membrane's permeability and ion's release into the cytoplasm.^{11,12} Members of the Bcl-2 family are over-expressed in many cancers including liver cancer. Recent studies have demonstrated that the Bcl-2 family significantly regulates apoptosis, either as an activator (Bax) or as an inhibitor (Bcl-2).¹³ The Bax protein is known to be an apoptosis inducing factor because of the formation of homo and hetero-dimers with the apoptosis suppression factors Bcl-2 and Bcl-XL.¹⁴ It is said that in oesophageal carcinoma, Bcl-2 and COX-2 play in conjunction culminating in antiapoptotic effect in the cell.¹⁵ The current study demonstrated that NGEN treatment significantly decreased the expression of Bcl-2 protein and increased the expression of Bax protein in NGEN treated HCC induced rats when compared to HCC induced rats. These results demonstrate that NEGN-induced apoptosis in HCC induced rats is related to augmentation levels of the Bax protein and down regulation of that of Bcl-2 in accordance with Park *et al.*¹⁶ It was also suggested that the NGEN seemed to increase Bax/Bcl-2 ratio which is reported as a key factor in regulating apoptosis^{17,18} and stimulate the intrinsic pathway of apoptosis.

Moreover, this apoptotic response of NGEN with an up-regulation of Bax, down-regulation of Bcl-2 is associated with caspase activation.¹⁹ Caspase signalling is initiated, propagated by proteolytic autocatalysis; also by the cleavage of downstream caspases and substrates such as poly (ADP-ribose) polymerase (PARP) and lamin A.¹¹ Kanno *et al.*²⁰ reported that the triggering of apoptosis in leukemia cells by NGEN *via* the promotion of caspases activation, similarly in the current investigation, there was an increase in the expression of caspase-9 and caspase-3 in tumors induced NGEN treated rats was observed. This triggering of apoptosis is also evidenced from the ultra-structure studies of liver tissues of NGEN treated HCC induced rats showing the characteristic features of apoptosis.

It could be argued based on the above results, that NGEN plays an important role in induction of apoptosis in liver cancer by its potential of up-regulating the Bax expression, downregulating that of Bcl-2 in addition to the activation of caspases (3 and 9).

Effect of Naringenin on PI3K/PKB Pathway

The possibility of selective targeting of tumour cells by specific pharmacologic inhibitors of members of the PI3K/PKB pathway opens up new perspectives for a targeted molecular therapy of malignant tumors.²¹ Activation of this pathway likely contributes to the development of HCC and to their exquisite resistance to standard therapies. Although in *vitro* biochemical studies have identified putative components of the PI3K pathway, it remains unclear which of these elements plays a salient role in HCC development in vivo. Identification of these key elements is important because they likely represent the most promising molecular targets for signal transduction inhibitors.²²

In the current study, the activation of Akt pathway which has a key role in regulating proliferation,^{23,24} anti-apoptotic functions (Nicholson *et al.*, 2002) has been encountered in HCC induced rat liver tissues. It has been reported that increased PI3K activity due to loss of PTEN, gene amplification and mutation of catalytic and regulatory subunits of PI3K is common in high grade cancers.²⁵ Obviously, HCC induced rat liver registered the activation of AKT leading to a positive influence on the cell survival mechanism. When the expression in NGEN treated HCC rats was observed, it is prompting to interpret the results on the basis of reduction in the number of proliferating of cells.

Naringenin is a flavanone has been shown to an important negative regulator of Akt in hepatocellular experimental carcinogenesis model and targets their important apoptotic downstream like caspase-9, caspase 3, Bcl-2, Bax, thus initiating apoptosis. NGEN, a flavanone, has been reported to have inhibitory activity at a number of protein kinases including PI3K.²⁶ The mechanism underlying the counteracting action of the flavanone, NGEN on PI3K/Akt pathway, observed in the current study remains unclear. However, by virtue of its inhibitory effect on the pathway were putatively seem to be involved in mediating the apoptotic and anti-proliferative. Park et al.¹⁶ report also supported the current finding that by blocking of PI3K/Akt culminate in apoptosis.

From the observations encountered in the current investigation, NGEN may be considered as an excellent candidate drug in a battle against HCC.

CONCLUSION

The current lack of effective cure for HCC prompted the present investigation to screen NGEN a potent flavanone, for its antiapoptotic activity. The investigations were carried out *in vivo* which exhibited protein expressions of various apoptotic related markers, such as caspase-9, caspase-3, Bcl-2 and Bax disclosed that the chemopreventive activities of NGEN may be mediated through eliciting apoptotic mechanism in DEN-induced HCC condition. NGEN seemed to target PI3K/Akt pathway by decreasing the expression of these protein.

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