

Silibinin inhibits constitutive and TNF α -induced activation of NF- κ B and sensitizes human prostate carcinoma DU145 cells to TNF α -induced apoptosis

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Prostate cancer (PCA) is one of the most common invasive malignancies of men in the US, however, there have been limited successes so far in its therapy. Even most potent agents (e.g. TNF α) are ineffective in killing human PCA cells possibly due to constitutive activation of NF- κ B that subsequently activates a large number of anti-apoptotic genes. In such a scenario, strong apoptotic agent TNF α , further induces NF- κ B activation rather than inducing apoptosis. In several recent studies, we have demonstrated both cancer preventive and anti-cancer efficacy of silymarin and its constituent silibinin in a variety of experimental tumor models and cell culture systems. Here we examined whether silibinin is effective in inhibiting constitutive NF- κ B activation in human PCA cells, which would help in overcoming TNF α -insensitivity. Our studies reveal that silibinin effectively inhibits constitutive activation of NF- κ B in advanced human prostate carcinoma DU145 cells. Consistent with this, nuclear levels of p65 and p50 sub-units of NF- κ B were also reduced. In the studies assessing molecular mechanism of this effect, silibinin treatment resulted in a significant increase in the level of I κ B α with a concomitant decrease in phospho-I κ B α . Kinase assays revealed that silibinin dose-dependently decreases IKK α kinase activity. The effect of silibinin on IKK α seemed to be direct as evidenced by the *in vitro* kinase assay, where immunoprecipitated IKK α was incubated with silibinin. This shows that silibinin does not necessarily need an upstream event to bring about its inhibitory effect on IKK α and downstream effectors. Additional studies showed that silibinin also inhibits TNF α -induced activation of NF- κ B via I κ B α pathway and subsequently sensitizes DU145 cells to TNF α -induced apoptosis. These results indicate that silibinin could be used to enhance the effectiveness of TNF α -based chemotherapy in advanced PCA.

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Introduction

NF- κ B/Rel A family of transcription factors are known to participate in the regulation of numerous genes and are potential intracellular targets for compounds such as cytokines and growth factors, and also various environmental stimuli, infectious agents, oxidative stress and ultraviolet radiation (Baldwin, 1996; Baeuerle, 1998). NF- κ B is a heterodimer, composed of p50 and p65 subunits and is retained in the cytoplasm as an inert form through interaction of p65 subunit with a family of inhibitory proteins, such as I κ B α (Baeuerle and Baltimore, 1996). In response to various stimuli, I κ B α gets phosphorylated by an upstream IKK α at serine residues 32 and 36 (Zandi and Karin, 1999), which in turn triggers ubiquitination and degradation of I κ B α thereby facilitating the translocation of NF- κ B into the nucleus. NF- κ B is known to induce and control a broad spectrum of genes, that include inflammatory cytokines (such as TNF α), chemokines, cell adhesion molecules, growth factors, interferons, MHC proteins and viruses (Baeuerle and Henkel, 1994; Miyamoto and Verma, 1995). Recent studies indicate that NF- κ B is constitutively active in a number of hematological and solid tumors such as Hodgkin's lymphoma, melanoma, cutaneous T-cell lymphoma, squamous cell carcinoma as well as advanced prostate carcinoma (Bargou *et al.*, 1997; Dong *et al.*, 1999; Giri and Aggarwal, 1998; Reuther *et al.*, 1998; Shattuck-Brandt and Richmond, 1997; Palayoor *et al.*, 1999). NF- κ B is also known as cell survival factor and is known to activate a number of anti-apoptotic genes. Now there is growing evidence to support the role of NF- κ B in promoting cell survival and also in protection against programmed cell death (Waddick and Uckun, 1999). This constitutive activation of NF- κ B makes these cancer cells resistant to TNF α -based chemotherapy, which seems to be one of the possible reasons for the TNF α -insensitivity observed in advanced human prostate carcinoma DU145 cells.

Being the second largest leading cause of cancer-related deaths and accounting for more than 200 000 new cases/year, prostate cancer (PCA) has been one of

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the major concerns in the field of cancer therapy. PCA is a disease of aging and oxidative stress is shown to be a major factor in the promotion/progression of this malignancy (Sohal and Weindruch, 1996). Furthermore, activation of many kinases involved in NF- κ B pathway is shown to be dependent on oxidative stress (Baeuerle and Baichwal, 1997; Stancovski and Baltimore, 1997). Reactive oxygen species are shown to cause prolonged NF- κ B DNA binding activity and antioxidants have been shown to diminish this activity (Ripple *et al.*, 1999). Together, based on above studies, one approach to control PCA growth and progression could be inhibition of constitutive NF- κ B activation, however, limited efforts have been made in this direction.

Another important issue related to controlling the advanced stage of PCA is that conventional therapeutic measures such as surgery and chemotherapy have been proven to be only of limited success. Accordingly, use of diet/diet-related components either singly or in combination is gaining widespread attention for the management of this malignancy (Sporn and Suh, 2000). The association between consumption of certain diets of diet-related constituents and decreased cancer risk has already been established (Wattenberg, 1997). Number of flavonoids and other antioxidant compounds derived from dietary constituents have been proved to be valuable as chemopreventive and/or chemotherapeutic agents against a variety of cancers (Davis *et al.*, 1999). Silymarin (isolated from *Silybum marianum*) is one such agent that is a mixture of stereoisomers namely silibinin (the major active constituent) together with small amounts of isosilybin, dihydrosilybin, silydianin, and silychristin (Wagner *et al.*, 1974). Both silymarin and silibinin are used clinically for the treatment of liver diseases (Letterton *et al.*, 1990), and are largely free of toxic effects and well-tolerated even at large doses (Wellington and Jarvis, 2001).

In several recent studies by us, both silymarin and silibinin showed strong growth inhibitory effect against different human carcinoma cells in culture by inhibition of a number of crucial growth stimulatory/mitogenic signaling pathways (Bhatia *et al.*, 1999; Zi *et al.*, 2000; Sharma *et al.*, 2001). For example, we showed that silibinin (or silymarin) protects against skin tumor promotion and progression, induces antioxidant enzymes, and inhibits cytokine expression and receptor tyrosine kinase signaling (Ahmad *et al.*, 1998; Zhao *et al.*, 1999; Lahiri-Chatterjee *et al.*, 1999). Silymarin has also been shown to inhibit the activation of NF- κ B in human lymphoma U-937 cells (Manna *et al.*, 1999). More recently, we also showed the inhibitory effects of silibinin on both IGF1 signaling (Zi *et al.*, 2000) as well as MAPK/ERK1/2 activation (Sharma *et al.*, 2001); the upstream kinase cascades involved in NF- κ B activation (Stancovski and Baltimore, 1997) and possibly contributory to its constitutive activation in human PCA cells (Palayoor *et al.*, 1999).

As mentioned earlier, NF- κ B is constitutively active in DU145 human PCA cells and that these cells are highly resistant to TNF α -induced apoptosis; in fact TNF α is known to further activate NF- κ B in these cells

(Palayoor *et al.*, 1999). Accordingly, our aims of the present study were whether silibinin inhibits constitutive activation of NF- κ B in DU145 cells, and whether it is effective in inhibiting TNF α -induced activation of NF- κ B that in-turn sensitizes DU145 cells to TNF α -induced apoptosis. Our results are clearly indicative of the inhibitory effect of silibinin on constitutive as well as TNF α -induced activation of NF- κ B, and also indicate that silibinin sensitizes DU145 cells to TNF α -induced apoptosis.

Results

Silibinin inhibits constitutive activation of NF- κ B, and nuclear translocation of p65 and p50 subunits in DU145 cells

To examine the effect of silibinin on constitutive activation of NF- κ B, DU145 cells were treated with various concentrations of (0, 10, 25 and 50 μ M) silibinin for indicated times. Ten μ g of protein from nuclear extract was used for assessing NF- κ B activity by electrophoretic mobility shift assay (EMSA). Silibinin inhibited constitutive activation of NF- κ B following both 12 and 24 h of treatment in a dose-dependent manner (Figure 1a,b). The level of inhibition at 10, 25 and 50 μ M doses of silibinin was found to be 20, 40 and 70% after 12 h of treatment and 20, 50 and 80% following 24 h of treatment, respectively (Figure 1a,b). In a time-response study, the inhibitory effect of silibinin on constitutive NF- κ B activation in DU145 cells was also evident after 3 and 6 h of its treatment (data not shown). In the studies analysing the specificity of NF- κ B band, addition of unlabeled NF- κ B probe resulted in a decrease or disappearance of the band whereas Sp1 probe showed no change (Figure 1c). In other assay systems to determine that the NF- κ B band in EMSA is indeed the case, nuclear extracts were first incubated with anti-p50 or anti-p65 antibody followed by EMSA. This showed a strong super-shift in case of anti-p50, but a weak shift for anti-p65 to a higher molecular weight band suggesting that the observed NF- κ B band consisted of these two subunits (Figure 1c).

Consistent with its inhibitory effect on constitutive activation of NF- κ B in DU145 cells, Western blot analysis of nuclear extracts from silibinin treated samples revealed that nuclear levels of p65 and p50 subunits were also decreased dose-dependently (Figure 2a,b). Compared to vehicle control, the decrease in nuclear p65 levels were 30–80% and 10–30% after 12 and 24 h of silibinin treatment, respectively (Figure 2a). Similar changes in nuclear p50 levels were also observed where silibinin treatments for 12 and 24 h resulted in 40–80% and 20–50% decrease, respectively (Figure 2b). Consistent with the strong decrease in the nuclear levels of p65, cytoplasmic levels of p65 were increased up to 1.6-, 2.6- and 2.5-fold after 12 h and up to 1.3-, 1.3- and 1.5-fold after 24 h of silibinin treatments at above-mentioned doses, respectively

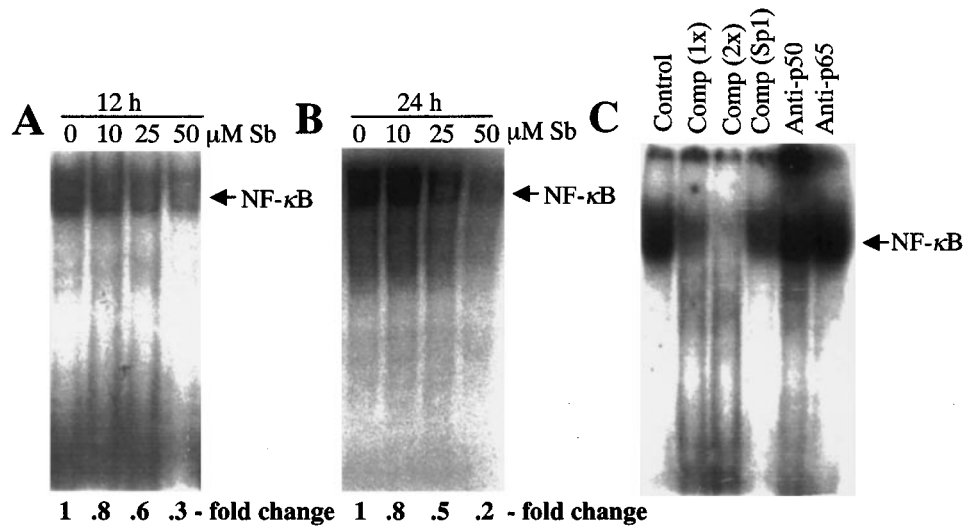


Figure 1 Inhibitory effect of silibinin on constitutive activation of NF- κ B in human prostate carcinoma DU145 cells. (a,b) DU145 cells at 70% confluency were switched to serum-free medium for 12 h, and then treated either with DMSO or 10, 25 and 50 μ M silibinin (final concentration in medium) in DMSO for indicated times. After these treatments, nuclear extracts were prepared and EMSA was performed for NF- κ B DNA binding activity as described in Materials and methods. Gels were then dried and subjected to autoradiography. Quantitation of bands was done by densitometric analysis, and is shown as fold change as compared to vehicle control at the bottom of the bands. (c) For competition study, reaction mixture was incubated with different concentrations of unlabeled NF- κ B cold probe, Sp1-specific cold probe, or antibody against p50 or p65, and EMSA was carried out as described in Materials and methods. Sb, silibinin; Comp, competition

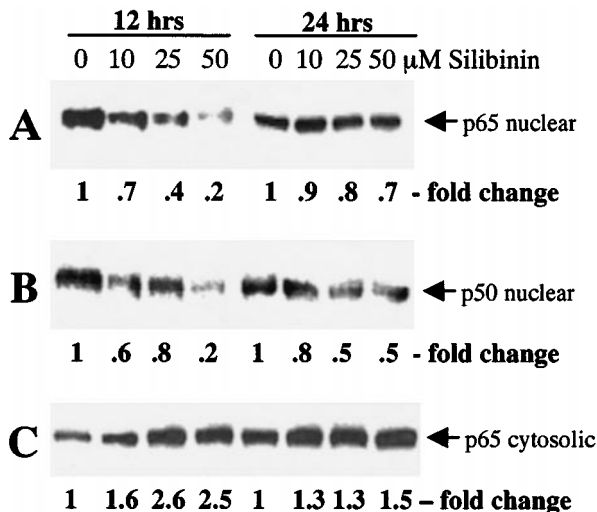


Figure 2 Effect of silibinin on nuclear levels of p65 and p50 and cytoplasmic level of p65 in human prostate carcinoma DU145 cells. Western blot analysis for p65 (a) and p50 (b) in nuclear extracts, and p65 (c) in cytoplasmic extract following 12 and 24 h of silibinin treatment. In all cases, DU145 cells were grown to 70% confluency and switched to serum free medium for 12 h followed by treatment with silibinin for indicated doses and time points. Equal amounts of protein from nuclear or cytoplasmic extracts was resolved on 12% SDS-PAGE, transferred on to a nitrocellulose membrane, probed with appropriate primary and secondary antibodies and visualized by ECL. Quantitation of bands was done by densitometric analysis, and is shown as fold change as compared to vehicle control at the bottom of the bands

(Figure 2c). The inhibitory effect of silibinin on the nuclear levels of p65 and p50 at 12 h treatment was more as compared to 24 h treatment, though its

inhibitory effect on NF- κ B DNA binding activity was comparable, at both the treatment duration. This is an intriguing observation, and needs further work in future studies.

Inhibitory effect of silibinin on NF- κ B is via I κ B α pathway

Phosphorylation and degradation of I κ B is essential for the nuclear translocation of NF- κ B (Baeuerle and Baltimore, 1988). To find out if silibinin acts by blocking the phosphorylation of I κ B, we examined the cytoplasmic levels of total and phosphorylated I κ B α . Treatment with 10, 25 and 50 μ M silibinin for 12 and 24 h resulted in a significant dose-dependent increase in the levels of total I κ B α (Figure 3a). Compared to vehicle control, the levels of induction observed, were 0.9-, 1.5- and 1.9-fold and 2.3-, 2.8- and 3.7-fold following 12 and 24 h silibinin treatments at these doses, respectively (Figure 3a). Concomitantly, the level of serine 32-phosphorylated I κ B α (pI κ B α) showed a dose-dependent decrease (Figure 3b). In this case, 12 h of silibinin treatment at 10 μ M dose was not effective, but 25 and 50 μ M doses showed 20 and 40% decrease in pI κ B α , respectively (Figure 3b). However, 24 h silibinin treatments at all three doses showed 20, 40 and 60% decrease in pI κ B α levels, respectively (Figure 3b). These results are indicative of the inhibitory effect of silibinin on the phosphorylation and degradation of I κ B α .

To further confirm that the observed inhibitory effect of silibinin on NF- κ B is by blocking the I κ B α phosphorylation, we used ALLN, a proteasomal inhibition, which prevents the degradation of I κ B α without having any effect on its phosphorylation (Whiteside *et al.*, 1995).

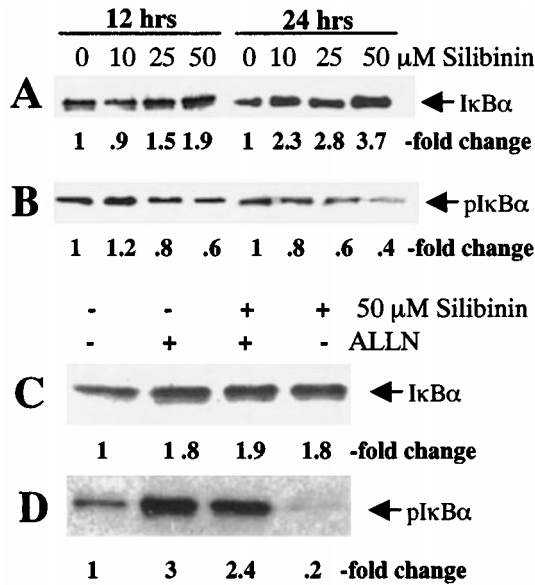


Figure 3 Effect of silibinin on cytoplasmic levels of I κ B α and pI κ B α , and degradation of I κ B α by blocking its phosphorylation in human prostate carcinoma DU145 cells. DU145 cells were grown at 70% confluency and switched to serum free medium for 12 h followed by treatment with silibinin for indicated doses and time points. For Western blot analysis of I κ B α (a) and pI κ B α (b), equal amounts of protein from cytoplasmic extracts was resolved on 12% SDS-PAGE, transferred on to a nitrocellulose membrane, probed with appropriate primary and secondary antibodies, and visualized by ECL. To assess whether silibinin inhibits degradation of I κ B α by blocking its phosphorylation, cells were treated either with DMSO or 50 μ M silibinin for 12 h followed by ALLN (100 μ g/ml) for 1 h. Cytoplasmic extracts were prepared, and subjected to Western blotting for I κ B α (c) and pI κ B α (d) protein levels as described above and in Materials and methods. In each case, quantitation of bands was done by densitometric analysis, and is shown as fold change as compared to vehicle control at the bottom of the bands

DU145 cells were treated with silibinin (50 μ M) for 12 h followed by ALLN (100 μ g/ml) for 1 h, and cytoplasmic extracts were analysed for total and phosphorylated I κ B α levels by Western blotting. The level of total I κ B α showed an increase up to 1.8–1.9-fold by treatment with either ALLN or silibinin alone or by both (Figure 3c). In case of pI κ B α levels in this experiment, treatment with ALLN resulted in a 3.0-fold increase in its level, however, pre-treatment with silibinin resulted in the hyper-phosphorylation level of I κ B α to 2.4-fold (Figure 3d). These results confirm that the inhibitory effect of silibinin on constitutive NF- κ B activation in DU145 cells is mediated, specifically, by blocking the phosphorylation of I κ B α . Treatment with silibinin (50 μ M) for shorter duration (6 h) resulted in almost similar changes in the levels of I κ B and pI κ B under identical experimental conditions (data not shown).

Silibinin inhibits phosphorylation of I κ B α via its inhibitory effect on IKK α kinase activity

Since the phosphorylation of I κ B α is mediated by IKK α (DiDonato et al., 1997), we next examined whether the inhibitory effect of silibinin on I κ B α phosphorylation is

mediated via IKK α kinase activity. As shown in Figure 4a, indeed, treatment of DU145 cells with 10, 25 and 50 μ M doses of silibinin for 12 and 24 h resulted in a strong decrease in IKK α associated kinase activity in a dose-dependent manner. The level of inhibition was 20–40% and 30–60%, respectively, following these treatments (Figure 4a). It is important to emphasize here that the degree of decrease observed in the kinase activity by silibinin was comparable to the decrease observed in the phosphorylation of I κ B α . The level of total IKK α was measured in the cytoplasmic extract as a control, which did not show any appreciable changes (Figure 4b). In an attempt to assess whether the inhibitory effect of silibinin on IKK α activity is a direct response or is mediated via an upstream event, we next performed an *in vitro* IKK α kinase activity assay in presence of silibinin. For this assay, equal amount of cytosolic protein from the control sample was immunoprecipitated with agarose-conjugated IKK α antibody and the immunocomplex was then incubated with 10, 25 and 50 μ M silibinin along with the substrate, for 30 min. Kinase assay was then performed as detailed in Materials and methods. Interestingly, the *in vitro* addition of above-mentioned doses of silibinin to assay incubations also resulted in 40–80% inhibition of IKK α kinase activity (Figure 4c). This observation suggests that the inhibitory effect of silibinin on IKK α kinase activity is rather a direct response and does not necessarily need an upstream event.

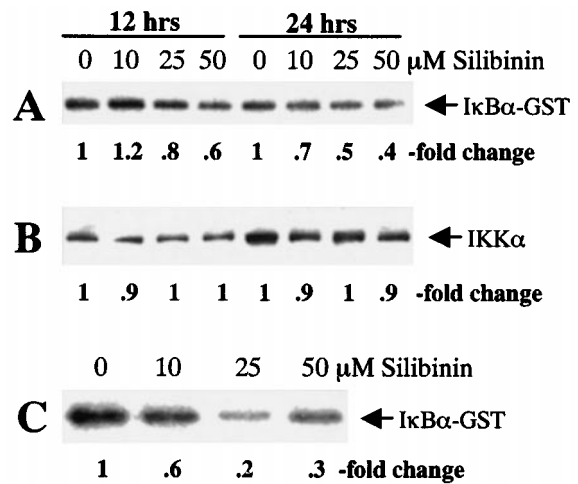


Figure 4 Effect of silibinin on IKK α kinase activity. (a) 200 μ g of protein per cytoplasmic extract from control or silibinin treated cells was immunoprecipitated with agarose-conjugated IKK α antibody overnight and kinase assay was carried out as described in Materials and methods using I κ B α -GST as substrate. Total IKK α protein level (b) was measured in the cytoplasmic extracts by Western blotting and probing with the appropriate primary and secondary antibody. In *in vitro* kinase activity assay (c), employing equal amount of protein from control cytoplasmic extract, IKK α was immunoprecipitated and then incubated with indicated doses of silibinin followed by kinase activity assay. The gels were then fixed, dried and visualized by autoradiography. In each case, quantitation of bands was done by densitometric analysis, and is shown as fold change as compared to vehicle control at the bottom of the bands

Silibinin also inhibits TNF α -induced activation of NF- κ B via I κ B α pathway, and significantly sensitizes DU145 cells to TNF α -induced apoptosis

To assess the effect of silibinin on TNF α -induced activation of NF- κ B, serum starved cells were treated with 50 μ M silibinin for 12 h either prior to or after TNF α (10 ng/ml) treatment for 30 min. Vehicle treated control and only TNF α treated cultures were used for comparisons. Cytoplasmic and nuclear extracts were prepared from these cultures, and nuclear extracts were analysed by EMSA for NF- κ B DNA binding activity. Compared to vehicle control, treatment with TNF α resulted in a 2.8-fold increase in NF- κ B DNA binding activity while pre- and post-treatment with silibinin decreased this activity to 1.7- and 1.2-fold, respectively, suggesting that silibinin inhibits TNF α -induced NF- κ B activation, and that pre- or post-silibinin treatments have comparable inhibitory response (Figure 5a). In other studies, analysis of cytoplasmic extracts from this experiment, for total I κ B α and pI κ B α , revealed that treatment with TNF α resulted in the decrease (40%) in total I κ B α , which was partially restored only in case of post-treatment with silibinin to 80% (Figure 5b). However, TNF α -induced pI κ B α levels (5.1-fold compared to control) were strongly inhibited by both pre- and post-treatment with silibinin to 3.0- and 2.8-fold, respectively (Figure 5c). Almost similar results were observed by post- and pre-

treatment with 50 μ M silibinin for 6 h in both TNF α -induced NF- κ B DNA-binding activity, and levels of I κ B α and pI κ B α (data not shown).

Since TNF α -induced NF- κ B activity is the major factor in DU145 cells that make them resistant to apoptosis, we checked if silibinin is effective in sensitizing these cells to TNF α -induced apoptosis, possibly by virtue of its ability in inhibiting NF- κ B activity. As observed by Annexin V-PI staining, TNF α treatment of DU145 showed comparable apoptotic cell death to that of vehicle treated controls; silibinin alone did show an increase in apoptotic death over controls but was not significant (Figure 5d). However, pre-treatment of cells with silibinin followed by TNF α resulted in a significant increase ($P < 0.001$) in the percentage of apoptotic cells (Figure 5d). This observation clearly suggests that silibinin sensitizes DU145 cells to TNF α -induced apoptosis and that possibly this effect of silibinin is mediated via inhibition of TNF α -induced NF- κ B activation in DU145 cells.

Discussion

Although PCA is treated by androgen-ablation at initial stages of development, it becomes androgen-insensitive as the disease progresses to an advanced stage, resulting in tumor progression and poor

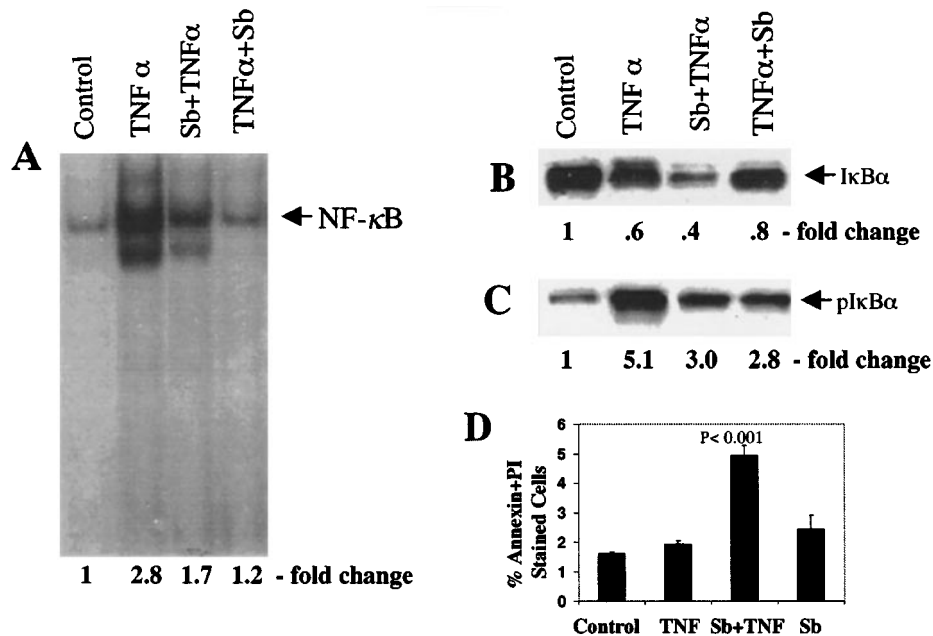


Figure 5 Silibinin inhibits TNF α -induced NF- κ B activation via I κ B pathway in human prostate carcinoma DU145 cells, and sensitizes them to TNF α -induced apoptosis. DU145 cells, at 70% confluency, were switched to serum free medium for 12 h, and then treated either with DMSO for 12 h, or with TNF α (10 ng/ml) for 30 min without or with pre- or post-silibinin treatment (50 μ M) for 12 h. At the end of these treatments, nuclear and cytoplasmic extracts were prepared. EMSA for NF- κ B (a) in nuclear extract, and Western blotting for I κ B α (b) and pI κ B α (c) in cytoplasmic extract were carried out as described in Materials and methods. In each case, quantitation of bands was done by densitometric analysis, and is shown as fold change as compared to vehicle control at the bottom of the bands. Sb, silibinin. For quantitative apoptosis (d), DU145 cells grown 30–40% confluency were either treated with vehicle, 50 μ M silibinin for 24 h, TNF α (10 ng/ml) for 12 h, or both silibinin and TNF α for 24 h (TNF α was added after 12 h). Cells were harvested and processed for FACS analysis of Annexin V-PI stained cells detailed in Materials and methods. The data are representative of three independent experiments with similar results. Sb, silibinin

prognosis. Conventional therapeutic approaches such as surgery and chemotherapy have been proved to have only limited success. Several chemotherapeutic drugs such as cisplatin, etoposide and physiological death inducers (FasL), though are capable of inducing apoptotic death of PCA cells, their use has been limited due to high systemic toxicity (Sinha *et al.*, 1995; Rokhlin *et al.*, 1997; Nakajima *et al.*, 1995). Furthermore, commonly used chemotherapeutic agent TNF α has been shown to be ineffective in killing the advance prostate carcinoma PC3 and DU145 cells (Yu *et al.*, 2000). Together, these limitations pose a major set back in the treatment of advanced PCA. Accordingly, several efforts are being made to identify or develop an effective chemotherapeutic agent that selectively kills PCA cells or sensitizes them to apoptosis, with minimal toxicity to the normal tissues. Consistent with these efforts, the central finding of the present study is that silibinin, an active constituent of a widely consumed dietary supplement and known as anti-hepatotoxic agent used clinically, strongly inhibits both constitutive and TNF α -induced NF- κ B activation in human PCA DU145 cells, and significantly sensitizes them to TNF α -induced apoptotic death.

Several reports in recent years show that NF- κ B plays an anti-apoptotic role in the survival of human carcinoma cells (Giri and Aggarwal, 1998; Sumimoto *et al.*, 1999). Furthermore, ineffectiveness of TNF α in inducing apoptotic death of cancer cells is mainly attributed by the activation of NF- κ B by TNF α (Van Antwerp *et al.*, 1996; Liu *et al.*, 1996; Sumimoto *et al.*, 1999), which further activates a number of anti-apoptotic genes (Wang *et al.*, 1996). Activation of NF- κ B by TNF α has been shown to block the activation of caspase 8 and thereby the downstream caspases such as caspase 3 that are crucial for apoptotic signaling (Wang *et al.*, 1998). Moreover, apoptosis inhibitor proteins cIAP 1 and cIAP 2 have been shown to be one of the target genes for NF- κ B (Chu *et al.*, 1997; Wang *et al.*, 1998), and therefore, inhibition of NF- κ B activity enhance apoptosis and diminishes anti-apoptotic signaling (Wang *et al.*, 1996). Further, it has been shown that overexpression of NF- κ B is correlated with the transformation and neoplastic phenotype *in vitro* and conversely, anti-sense inhibition of p65 reduces the ability of thyroid carcinoma cells to form soft agar colonies (Visconti *et al.*, 1997). Moreover, pro-metastatic genes such as interleukin-6, urokinase plasminogen activator, matrix metalloproteinase 9, and pro-angiogenic genes such as interleukin 8 and vascular endothelial growth factor are induced by NF- κ B (Bauerle and Henkel, 1994; Grumont *et al.*, 1999; Jones *et al.*, 1997; Lee *et al.*, 1999; Wang *et al.*, 1998; Zong *et al.*, 1999). Also, inhibiting NF- κ B pathway seems to be one of the major mechanisms of action through which proven anti-carcinogenic phytochemicals such as EGCG and curcumin are known to act (Nomura *et al.*, 2000; Plummer *et al.*, 1999).

Mortality associated with PCA is generally attributed to the metastasis and development of chemoresistance, and NF- κ B activation, which is constitutively active in

advanced human PCA cells, has a major contribution in both these events. Accordingly, it is logical to target the inhibition of NF- κ B activation in advanced human prostate carcinoma cells to make them stop growing and also to sensitize them to death by apoptosis inducing agents such as TNF α . Previous approaches in this direction have focused either in using protein synthesis inhibitors such as cyclophosphamide or proteosomal inhibitors such as ALLN, both of which are highly cytotoxic to normal cells due to non-specific nature of these compounds (Yu *et al.*, 2000). Silibinin, on the other hand is a well known non-toxic agent widely consumed by human population as a dietary supplement and used clinically as an anti-hepatotoxic agent (Letterton *et al.*, 1990; Wellington and Jarvis, 2001). Although silibinin has been proved to have exceptional growth inhibitory properties against both early and advanced stages of PCA, as shown in this study and reported earlier also, it is less effective in inducing apoptotic death in these cells (Zi and Agarwal, 1999).

In the present study we explored whether PCA cells growth inhibitory effects of silibinin are attributed, at least in part, via its effect of NF- κ B activation. As evidenced by gel retardation assay, silibinin showed a dose-dependent inhibitory effect on the constitutive activation of NF- κ B in DU145 cells. NF- κ B activation is primarily regulated by its inhibitory protein I κ B α that is phosphorylated at serine residues 32 and 36 by an upstream kinase IKK α , releasing p65/p50 that translocates to the nucleus and exert transcriptional activities (Zandi and Karin, 1999). Therefore, I κ B α phosphorylation is the key event in NF- κ B activation pathway, and its blocking has been shown to be effective in inhibiting NF- κ B activation (Muenchen *et al.*, 2000). In this study, we report that silibinin effectively inhibits the phosphorylation of I κ B α in a dose-dependent manner. Since phosphorylation of I κ B α is mediated by a family of kinases known as IKKs, we also assessed the effect of silibinin on the kinase activity of IKK α , which plays a major role in I κ B α phosphorylation (Stancovski and Baltimore, 1997). As observed by immuno-complex kinase assay measuring GST-I κ B α phosphorylation, silibinin treatment also resulted in a dose-dependent inhibition if IKK α kinase activity. The decrease in I κ B α phosphorylation seemed to directly correlate with the decrease in IKK α kinase activity when compared with their levels of inhibitions. We next assesses whether silibinin directly inhibits IKK α kinase activity or it is effected via an upstream signaling event. Using IKK α immuno-complex in an *in vitro* assay in presence of similar silibinin concentrations to those in cell culture studies, we found even higher inhibition if IKK α kinase activity suggesting a direct effect of silibinin on IKK α kinase activity. IKKs are generally regulated by several members of MEKK kinase family, including NIK and MEKK1 (Lee *et al.*, 1998). Although we have already shown the inhibitory effect of silibinin on some of these upstream kinase cascades (Zi *et al.*, 1998b, 2000), present study provides additional evidence that at least part of the inhibitory effects of silibinin on IKK α are mediated possibly via a direct effect.

As mentioned earlier that human PCA cells are resistant to TNF α -induced apoptosis possibly because it activates NF- κ B (Yu *et al.*, 2000; Sumimoto *et al.*, 1999). Whereas TNF α is a very effective chemotherapeutic agent against various cancers (Rath and Aggarwal, 1999), its cytotoxic effects in many cells are evident only if RNA or protein synthesis is inhibited (Beg and Baltimore, 1996; Yu *et al.*, 2000). It appears that TNF α has a dual role, one that is involved in the induction of apoptosis and the other in the induction of anti-apoptotic genes via the activation of NF- κ B. Some of the factors that determine the modes of action of TNF α include type of the cell, NF- κ B status and TNF α threshold of the cell (Beg and Baltimore, 1996). For example, in those cells that have a constitutive activation of NF- κ B, as in case of DU145 cells, TNF α induces NF- κ B activity rather than apoptosis. Moreover, since TNF α is one of the target genes of NF- κ B, there is constitutive activation of TNF α and practically doses that are multifold higher than the determined LD₁₀ (10 ng/ml) also do not induce apoptosis (Muenchen *et al.*, 2000). This seems to be the reason for the TNF α -insensitivity exhibited in DU145 cells.

Based on above studies and our data showing the inhibitory effect of silibinin on constitutive activation of NF- κ B, we next assessed whether silibinin could inhibit TNF α -induced NF- κ B activation in DU145 cells that would overcome TNF α -insensitivity towards apoptosis in these cells. Our results provided convincing evidence that pre- and post-treatment of cells with silibinin results in significant inhibition of NF- κ B activation, and that this effect was mediated via I κ B α pathway. This observation raises the possibility that treatment of DU145 cells with silibinin and TNF α combination could make them more sensitive to apoptosis since the anti-apoptotic signaling elicited by TNF α -induced NF- κ B activation is effectively blocked by silibinin. Indeed, this possibility is in accordance with our results where pre- and post-treatment with silibinin effectively sensitized DU145 cells to TNF α -induced apoptosis. Although there is strong evidence for the anti-apoptotic role of NF- κ B, there are also contradictory reports, for example, in the study where NF- κ B activity was blocked specifically by transfecting with mutated I κ B α suggest that inhibition of NF- κ B is not sufficient to allow apoptosis or to increase cytotoxic response to TNF α (Bentires-Alj *et al.*, 1999). However, in advanced human carcinoma cells, like DU145, where a number of anti-apoptotic and mitogenic signaling pathways are constitutively 'on', turning one of the signals 'off' may not be sufficient in inducing apoptosis. Silibinin on the other hand has proved to be very potent in inhibiting a number of mitogenic as well as cell survival signaling pathways, in prostate, breast and skin cancer cells (Ahmad *et al.*, 1998; Zi *et al.*, 1998a,b, 2000; Sharma *et al.*, 2001).

Taken together, the results of the present studies along with those published recently, raise an exciting possibility that combining silibinin with TNF α could be highly effective in treating patients with advance PCA. Additional studies employing *in vivo* pre-clinical PCA models are needed to substantiate this possibility.

Materials and methods

Cell lines and reagents

Human prostate carcinoma DU145 cells were obtained from ATCC (Manassas, VA, USA). RPMI 1640 medium, all other cell culture materials and TNF α were obtained from Life Technologies Inc., (Gaithersburg, MD, USA). Silibinin and ALLN (N-acetyl leucyl leucyl norleucinal) were from Sigma Chemical Co. (St Louis MO, USA). Anti-p65, anti-p50 antibodies, I κ B α -GST substrate and agarose-conjugated IKK antibody were procured from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA). Anti-I κ B α and pI κ B α antibodies were from Cell Signaling (Fremont, CA, USA). Anti-IKK α antibody was from Oncogene Research Products (Boston, MA, USA). Annexin V-Vybrant apoptosis kit was from Molecular Probes (Eugene, OR, USA).

Cell culture

DU145 cells were cultured in RPMI 1640 medium containing 10% FBS and 1% Penicillin-Streptomycin under standard culture conditions. At 70% confluency, cultures were switched to serum-free medium for 12 h, and then treated with specified doses of silibinin in DMSO or DMSO alone (maximum final concentration 0.1%, v/v), or with/without TNF α , or with/without ALLN for indicated times. After desired treatments, medium was aspirated and cells were harvested by the addition of Trypsin-EDTA, and cytosolic and nuclear extracts were prepared as described previously (Schreiber *et al.*, 1989).

Electrophoretic mobility shift assay (EMSA)

For EMSA, NF- κ B-specific oligonucleotide (3.5 pmol) was end-labeled with γ -³²P-ATP (3000 Ci/mmol at 10 mCi/ml) using T₄ polynucleotide kinase in 10 \times kinase buffer as per Manufacturer's protocol (Promega, Madison, WI, USA). The labeled double-stranded oligo probe was separated from free γ -³²P-ATP using G-25 Sephadex column. The consensus sequences of the oligonucleotide used were 5'-AGT TGA GGG GAC TTT CCC AGG C-3' and 3'-TCA ACT CCC CTG AAA GGG TCC G-5'. EMSAs were conducted as described below. 8 μ g protein from nuclear extracts was first incubated with 5 \times gel shift binding buffer (20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl and 0.25 mg/ml poly dI-dC.poly dI-dC) and then with ³²P end-labeled NF- κ B consensus oligo nucleotide for 20 min at 37°C. In super shift assays either unlabeled oligo or oligo specific for SP1 was co-incubated with labeled oligo or incubated with anti-p65/p50 antibody before the addition of ³²P end-labeled NF- κ B oligo. DNA-protein complex thus formed was resolved on 6% DNA retardation gels (Invitrogen, Gaithersburg, MD, USA). The gels were dried and bands were visualized by autoradiography.

Western blot analysis

To assay the nuclear levels of p65 and p50, and cytoplasmic levels of p65, total I κ B α , pI κ B α and total IKK α level, 30–50 μ g protein was resolved on 12% Tris-glycine gels and proteins were electrotransferred on to nitrocellulose membrane. The membranes were then probed with appropriate primary and secondary antibodies. Protein was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway).

IKK α kinase activity assay

For this assay, 200 μ g of cytoplasmic protein was pre-cleared with A-G beads and immunoprecipitated with agarose-conjugated IKK antibody at 4°C overnight. The immunocomplex thus obtained was washed 2–3 times each with buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and kinase buffer, and then incubated with GST-I κ B substrate (1 μ l), 50 μ M cold ATP and 0.5 μ Ci γ ³²P-ATP for 15 min at 37°C. The reaction was stopped by the addition of 20 μ l 2 \times SDS sample buffer, boiled for 6 min and subjected to SDS-PAGE. Gels were then dried and analysed by autoradiography. In *in vitro* kinase assay, cytoplasmic extract from controls was used and immunocomplexes were obtained as described above, and were incubated with specified doses of silibinin along with the substrate. Kinase assay was performed as described above.

Detection of apoptosis

DU145 cells were plated in 60 mm dishes. At 30–50% confluency, cells were switched to serum-free media for 12 h and then treated either with TNF α (10 ng/ml) only, or with

silibinin (50 μ M) only for 24 h or co-treated with TNF α for 12 h (TNF α was added after 12 h from the start of silibinin treatment). After these treatments, cells were harvested and processed for Annexin V-PI staining using Vybrant Apoptosis Detection kit as per Manufacturer's protocol. The samples were then analysed for apoptotic cells by FACS analysis.

Densitometry and statistical analysis

Quantitation of the bands was done by ScionImage Software (NIH, Bethesda, MD, USA). In each case, the gel data and the corresponding densitometric values, shown are representative of at least 2–3 independent studies with reproducible results. Statistical significance of difference in the percentage of apoptotic cells was calculated by Student's *t*-test (SigmaStat 2.0, Jandel Scientific).

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