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The differential NF-kB modulation by S-adenosyl-L-methionine, N-acetylcysteine and quercetin on the promotion stage of chemical hepatocarcinogenesis

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Abstract
S-adenosylmethionine (SAM), N-acetylcysteine (NAC) and quercetin exhibit a chemoprotective effect. Likely this effect is mediated by counteracting, oxidative stress and NF-kB activation. To test this hypothesis F344 rats were subjected to hepatocarcinogenesis with or without antioxidants. NAC decreased foci in number and area, SAM and quercetin decreased area. Lipid-peroxidation was decreased by antioxidants, but only SAM increased glutathione. SAM, in its regulation from IKK downwards, abolished the NF-kB activation. NAC decreased IKK and IkB-a phosphorylation, and Rel-A/p65 and NF-kB binding, though the last two were affected with less intensity compared to the NF-kB inhibitor. Quercetin decreased Rel-A/p65, without modifying upstream signalling. Although all antioxidants inhibited oxidative stress as shown by reduction of lipid peroxidation, not all exerted the same effect on NF-kB signalling pathway and only SAM increased GSH. The mechanisms exerted by SAM in the reduction of foci makes this compound a potential liver cancer therapeutic agent.

Keywords: *Oxidative stress, hepatocarcinogenesis, NF-κB, antioxidants*

Introduction

It is well accepted that oxidative stress is implicated in the development of conditions such as cancer, diabetes, ischemia/reperfusion and neuropathies, which are common diseases characterized by pro-oxidants shifting the thiol/disulphide redox state and inflammatory oxidative conditions [1]. A redox imbalance has been found in a wide variety of cancers including hepatocellular carcinoma (HCC), which is a common malignancy worldwide; thus, the development and application of antioxidant strategies to diminish oxidative stress in transformed cells is a major issue [2,3]. Increased oxidative DNA damage has been reported in patients with HCC [4]; genes related to the regulation of reduced glutathione (GSH) have been shown to be involved in the pre-neoplastic/neoplastic transition as an adaptive response of neoplastic cells [5]. High concentrations of reactive oxygen species (ROS) and reactive nitrogen species (RNS) can be toxic to cells in the initial stages of carcinogenesis, affecting DNA, proteins and lipids. Lipid peroxidation is a consequence of increased ROS production; malondialdehyde (MDA)

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and 4-hydroxynonenal are the principal lipid peroxidation products implicated in carcinogenesis [6]. ROS also participate in signal transduction pathways, playing a key role in carcinogenesis. Certain transcription factors such as NF-κB have been linked to carcinogenesis, because of their role in cell proliferation, apoptosis and inflammation. ROS has been postulated as a second messenger in NF-κB activation [7–10]; however, most studies of ROS signalling and transcription factors have been performed *in vitro* [11], while the mechanisms of redox signalling *in vivo* remain unknown. NF-κB belongs to the Rel family of proteins, which comprises five known proteins: Rel A/p65, p50, p52, cRel and relB. This inducible nuclear factor remains in the cytosol sequestered by inhibitory proteins (IkBs) until an extracellular stimulus induces IκB degradation by the 26S proteasome, thereby allowing NF-κB to enter the nucleus.

A diet that is low in antioxidants has been significantly associated with cancer risk; as a result, antioxidant strategies to reduce the oxidative stress in transformed cells have been performed [12,13]. Numerous antioxidants can modify transcription factors such as NF-κB through modulation of ROS production. Levels of S-adenosyl-methionine (SAM), a principal methyl donor and GSH precursor, are related to differentiation status; SAM depletion predisposes to liver injury and possibly to malignant degeneration in diethylnitrosamine-initiated rats subjected to the well established 'resistant hepatocyte' model [14]. Other reports have shown that decreased SAM in the liver is associated with DNA hypomethylation during the development of pre-neoplastic foci; however, if the levels of methyl donors are restored by administration of SAM after the development of persistent nodules in rat liver carcinogenesis, marked regression of the nodular lesions occurs [15,16]. The sulphydryl donor N-acetylcysteine (NAC) has been proved to exert a protective effect in MeIQx-induced rat hepatocarcinogenesis, due to its role as nucleophile antioxidant [17]. Similar studies have attempted to demonstrate the NAC protective mechanism in cultivated cells, in which NAC inhibited the upstream IκB kinase (IKK) activation induced by TNF-α [18] and reduced the increase of cell oxidants [19]. A further point of concern is the concentration of the antioxidant administered to obtain a protective effect. It was observed that, in antioxidant treatment with the flavonoid quercetin at concentrations ranging from 5–100 nmol/mL, the highest concentration showed the best effect on redox status and NF-κB activation [20] and reduced phosphorylation of IκB proteins was observed above 50 μm [21]. Although studies of a wide variety of antioxidant treatments have revealed some aspects of the mechanism of redox signalling, most have been described in *in vitro* systems [22]; the quimioprotective behaviour of antioxidants in the whole organism remains less clear. Elucidating the molecular mechanisms of antioxidant compounds linked to NF-κB activation of cancer in *in vivo* systems has emerged as an interesting approach to the development of new drugs for the treatment of cancer.

In this study, we determined the effect of SAM, NAC and quercetin on the induction of preneoplastic foci and modulation of the NF-κB activation pathway on the promotion stage of chemical hepatocarcinogenesis in our resistant hepatocyte modified model [23]. We demonstrated a close correlation between oxidative stress and carcinogenesis, observed by the expression reduction of two tumour markers. We found that NF-κB activation plays a critical role in the protective mechanism of antioxidants, focusing on its modulation by redox status. These results clarify the differences between the effects of the flavonoid and the GSH precursors on NF-κB activation and their protective mechanisms in the oxidative conditions that promote hepatocarcinogenesis. It is emphasized that SAM protective mechanism in early liver carcinogenesis could uncover a point of therapeutic approach.

Materials and methods

Animals and treatments

A total 35 male Fischer 344 rats weighing 180–200 g (UPEAL-Cinvestav, Mexico D.F., Mexico) were housed in a room at constant temperature. They had free access to water and laboratory diet food and all of them were subjected to a 10-day carcinogenic treatment (TC). The rats were initiated with a single dose of diethylnitrosamine (DEN, 200 mg/kg; Sigma-Aldrich, Toluca, Mexico) intraperitoneally and, 7 days later, as a promotion stimulus, received daily oral serial doses of 20 mg/kg of a suspension of 2-acetylaminofluorene (Sigma-Aldrich, Toluca, Mexico). On day 10, as a proliferation stimulus, the animals underwent two-thirds partial hepatectomy (HPx). The control group (CN) received no treatment. As shown in Figure 1, 15 rats received TC, in addition 18 rats received the antioxidants treatments, SAM (six rats) and NAC (eight rats) were started 24 h after the administration of DEN until 2 h before HPx. SAM was injected intramuscularly in the stable form of sulphate p-toluene sulphonate (Sigma-Aldrich, Toluca, Mexico) in freshly prepared solutions at a daily dose of 64 μmol/kg dissolved in injectable water. The selection of SAM dose was based on a previous reference [24]. NAC (Sigma-Aldrich, Toluca, Mexico) was dissolved in physiological Hank's solution (final pH 7.4) and administered intraperitoneally at a daily dose of 100 mg/kg. Quercetin (six rats) was administered in a single dose 24 h after the administration of DEN by gavage, dissolved in 0.5% of carboximethyl-cellulose at final dose of

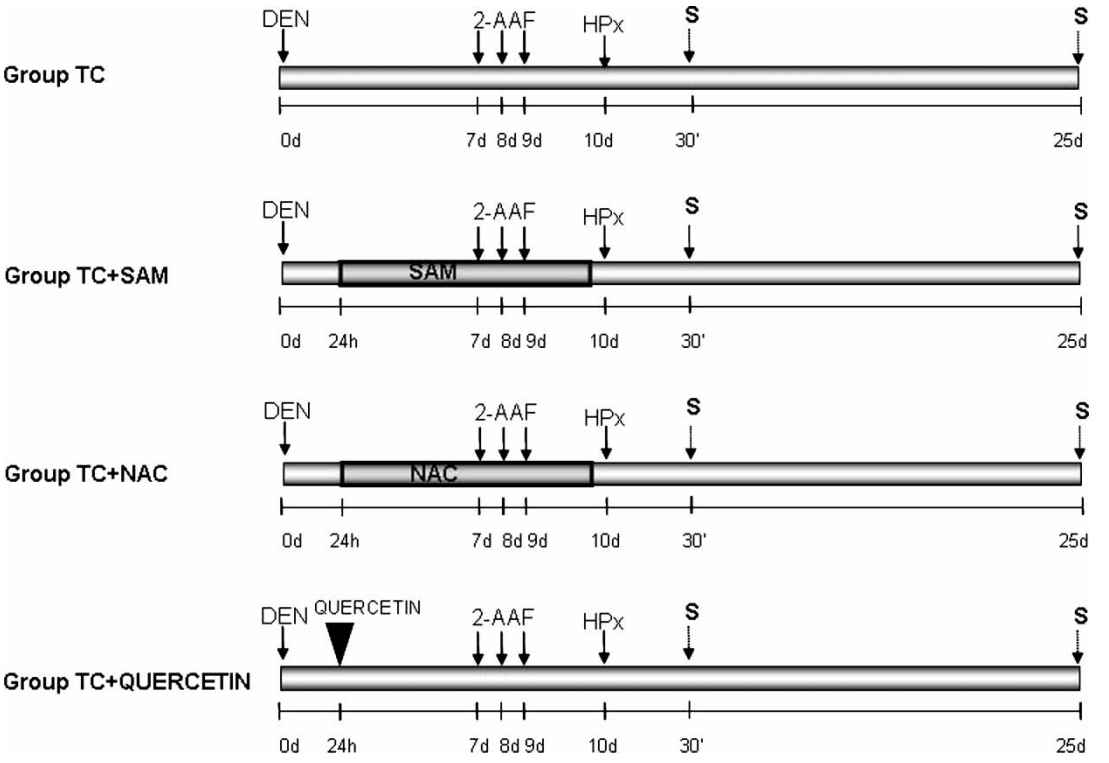

Figure 1. Experimental design. Four groups of Fisher 344 rats were subjected to modify Semple–Roberts hepatocarcinogenesis model and three of them received additionally antioxidant treatments. The animals were sacrificed (S) by cervical dislocation at 30 min after HPx for NF-kB analysis and 25 days after DEN administration for tumour marker expression. A normal liver group (CN) received no treatment.

5 mg/kg. Three rats per group were sacrificed by cervical dislocation 30 min after HPx, for NF-kB activation and redox status analysis. The remaining rats in each group were sacrificed 25 days after initiation for tumour markers analyses. Livers were excised and washed in physiological saline solution and frozen in liquid nitrogen; other sections were frozen in 2-methylbutane for cryoprotection and stored at -79°C until use.

Histological analysis of tumour markers

Thick sections (20 μm) from liver slices were stained for GGT activity according to Rutenburg et al. [25]. For immunostaining, formalin-fixed paraffin liver sections were blocked for 1 h in 0.1% H_2O_2 in phosphate-buffered saline, pH 7.4. They were then incubated with commercial monoclonal antibodies specific to GST-p (DakoCytomation, Glostrup, Denmark), diluted 1:100 (v/v) in blocking buffer overnight. After washing with phosphate-buffered saline, the primary antibody was detected using an avidin-biotin complex immunoperoxidase technique (Zymed Laboratories Inc., Carlsbad, CA). No staining was observed when the primary antibody was substituted with mouse isotype control.

Thiobarbituric acid reactive substances (TBARS) assay

Samples of frozen liver were homogenized in a buffer containing 10 mmol/L Tris, PMSF and NaCl. Protein concentration was determined using a Lowry assay kit (Biorad, Richmond, CA). Lipid peroxidation was measured in terms of TBARS, a widely used method [26]. In brief, 650 μg of protein liver homogenates plus 300 μL of 0.4% thiobarbituric acid in 20% acetic acid, pH 3.0, were mixed and heated at 100°C for 45 min. The samples were then cooled, 200 μL of 1.2% KCl and 0.5 mL of 1:15 pyridine/butanol (v/v) were added and the mixtures were centrifuged at 3 $500 \times g$ for 10 min. The absorbency of the supernatant was measured at 532 nm. Due to the instability of MDA, the data were interpolated in a standard curve of tetrametoxipropane (TMP), since hydrolysis of one mole of TMP produces 1 mol of MDA. Data were correlated using the extinction coefficient, $E = 1.56 \times 10^5$, and TBARS were expressed as nmol of MDA/mg/protein.

Determination of reduced glutathione (GSH)

One hundred and fifty milligrams of frozen liver samples were homogenized in a precipitant solution (EDTA 5mM in 5% sulphosalicilic acid). The resultant thiol extract was assayed by the method of Ellman with previously reported modifications [27]. The samples were then centrifuged at $8500 \times g$ for 20 min; 2.1 mL of phosphate solution (Na_2HPO_4 0.3 M) and 250 μL of Ellman's reagent were added to the supernatants (100 μL) and the samples were mixed and incubated for 10 min. The absorbance was

measured at 412 nm and total thiols levels (including GSH) were expressed as nmol/g of humid tissue.

Immunoblot analysis

For the analysis of Rel A/p65, IKKα/IKKβ-p, IκB-α-p and IκB-α, nuclear and cytosolic extracts were prepared from frozen liver samples as described previously [28–30]. Twenty or 45 μg of protein extracts were resolved using 8% SDS-PAGE gels for IKKα/IKKβ-p, 10% for Rel A/p65, and 12% for IκB-α-p and IκB-α, under denaturing and reducing conditions. The separated proteins were transferred to PVDF membranes. Non-specific binding was blocked by pre-incubation of the PVDF in 1% non-fat milk (5% w/v) and Tween 20 (0.1% v/v) in phosphate buffered saline before incubation overnight at 4°C with polyclonal anti-p65 (Upstate, NY), anti-IκB-α (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-IKKα (Ser^{180})/IKKβ (Ser^{181}) (Cell Signaling Technology Inc.), and anti-phospho-IκB-α ($Ser^{32/36}$) (Calbiochem, San Diego, CA) antibodies in 3% non-fat milk, 0.05% bovine serum albumin (Sigma-Aldrich) and Tween 20 in phosphate-buffered saline. Bound primary antibody was detected using horseradish peroxidase conjugated anti-rabbit, anti-goat or anti-mouse antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) by chemiluminiscence using a Luminol kit (Santa Cruz Biotechnology, Santa Cruz, CA) and developed in a photographic plaque (Konica, JP). The samples were normalized using β-actin and lamin B (Santa Cruz Biotechnology, Santa Cruz, CA). The density of the respective protein bands was quantified using imaging densitometer software (ImageJ).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described previously [28]. All buffers contained a protease inhibitor cocktail to prevent nuclear factor proteolysis. Protein concentration was measured using a Lowry assay protocol (Biorad, Richmond, CA). Twenty-five microgram samples were incubated on ice with 500 ng of poly[(dI-dC)] as a non-specific competitor (Amersham Biosciences) and 1 ng of [^{32}P]-end-labelled and non-labelled double-stranded oligonucleotides as follows: NF-κB, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; NF-κB mutant, 5′-AGT TGA GGC GAC TTT CCC AGG C-3′, and Sp-1, 5′-ATT CGA TCG GGG CGG GGC GAG C-3′.

The reaction mixtures were incubated for 20 min on ice and electrophoresed in 8% polyacrylamide gels using a low ionic strength 0.5X TBE buffer. The gels were dried and exposed to an autoradiographic film. For competition assays, the reaction mixtures were pre-incubated with the indicated non-labelled oligonucleotides (NF-κB, mutant NF-κB and Sp-1) probes for 15 min before addition of labelled kB site oligo.

Statistical analysis

Data were expressed as mean ± SD. All experiments were conducted on at least three animals per treatment group. Statistical significance between groups was determined by ANOVA test and Student's *t*-test; $p < 0.05$ was considered statistically significant.

Results

Antioxidants prevent GGT and GST-p lesions during in vivo *hepatocarcinogenesis*

The antioxidant effect of SAM, NAC and quercetin on the development of pre-neoplastic lesions was quantified by determination of the tumour marker GGT. The number of GGT-positive liver foci was significantly reduced by administration of SAM or NAC, by 64% and 69%, respectively, compared with the TC group. Three liver histological sections were randomly chosen and analysed (Figure 2A). All three antioxidant treatments (SAM, NAC and quercetin) reduced the GGT-positive area, by 75%, 72.3% and 72.7%, respectively (Figure 2B). We found non-focal hepatic GGT expression in some samples, which has been previously reported unrelated to carcinogenesis [31,32]; this increased expression of GGT could produce an error in the quantification of pre-neoplastic lesions and we therefore decided to use more than one tumour marker to help to distinguish hepatic foci from non-carcinogenic GGT staining. GST-p-positive pre-neoplastic lesions were analysed by immunohistochemistry. In contrast to the GGT results, only NAC and quercetin reduced GST-p liver foci, by 57% and 45%, respectively (Figure 3). All three antioxidant treatments caused a statistically significant decrease in the GST-p-positive area compared with TC group, by 67%, 73% and 86%. These results support the role of oxidative stress as a key factor in the induction of pre-neoplastic lesions and the protective effect of antioxidants is mainly on the size of foci.

Lipid peroxidation and GSH levels modulated by antioxidant treatment in hepatocarcinogenesis model

To evaluate the modulation of redox status by hepatocarcinogenesis and the effect of the antioxidants SAM, NAC and quercetin, we determined the levels of GSH and lipid peroxidation at 30 min post-HPx. The GSH precursors, SAM and NAC, and the flavonoid quercetin significantly decreased the lipid peroxidation induced by TC by 33%, 21% and 41%, respectively (Figure 4A). Only SAM significantly increased the intracellular GSH levels above of those found in normal liver (Figure 4B). Thus, all three antioxidant

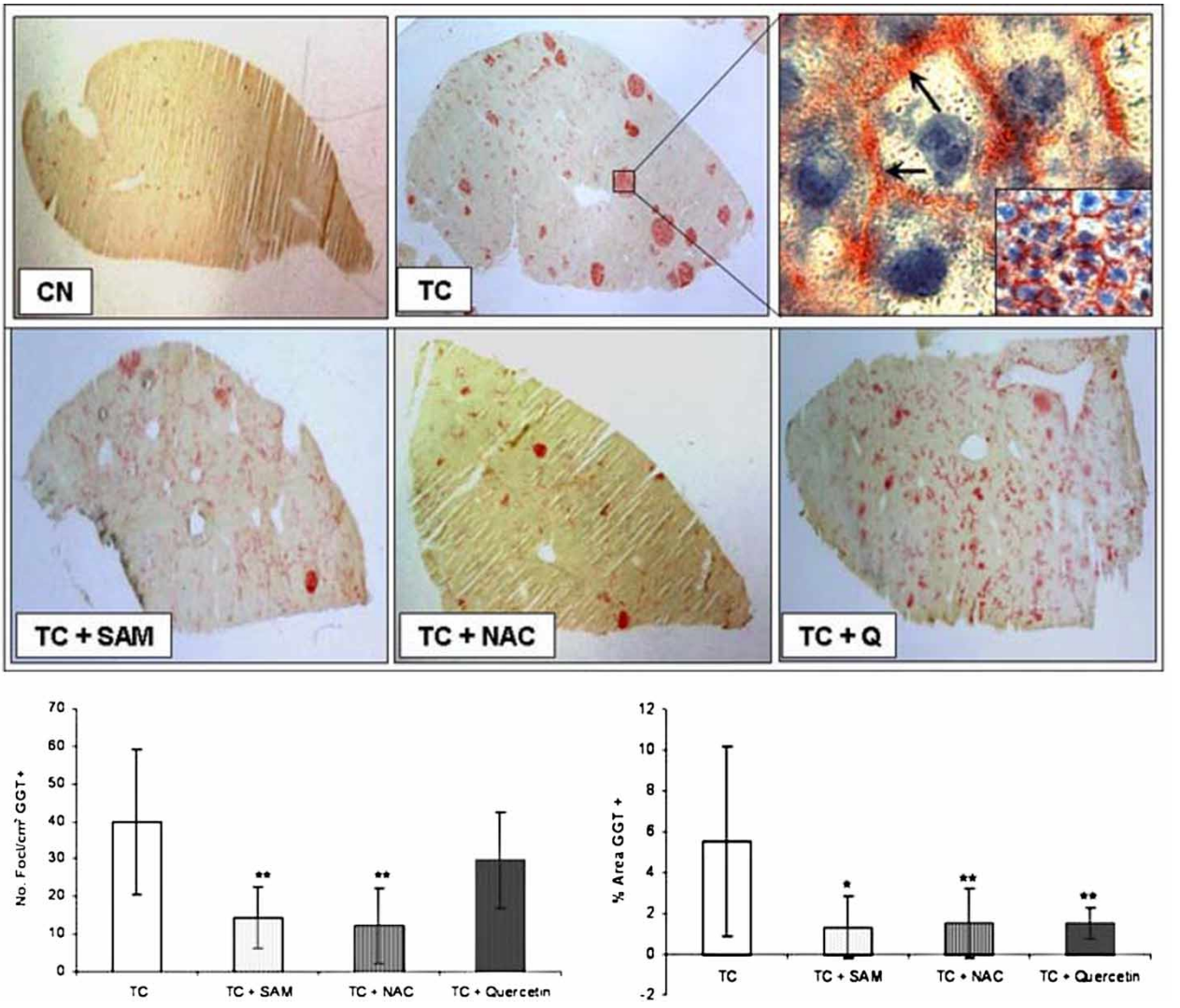

Figure 2. Expression of GGT tumour marker showing the effect of antioxidant treatments. (A) Images are from representative histochemistry of liver sections of each treatment. (B) Foci area and number were quantified and the data are presented in bar graphs. Results are expressed as the mean ± SD (SD foci number of all groups, TC group ±19.43, TC+SAM group ±8.23, TC+NAC group ±10.14 and TC+Q group ±12.74; SD foci area, TC group ±4.66, TC+SAM group ±1.49, TC+NAC group ±1.68 and TC+Q group ±0.78) (*$p \leq 0.05$, **$p \leq 0.001$ and ***$p \leq 0.0001$, compared with TC group).

treatments prevented the lipid peroxidation caused by TC; only SAM increased the cellular GSH pool.

Effect of SAM, NAC and quercetin on NF-κB activation

Once we had confirmed the antioxidant effect of SAM, NAC and quercetin, we investigated whether these antioxidants exerted an inhibitory action on NF-κB activation. We analysed NF-κB activation 30 min post-PH, which we have reported previously to be the time of maximal activation during the promotion stage of hepatocarcinogenesis model [33], and also incremented at 25 days after initiation [23]. Initially, we ensured that the carcinogenic process was under way at 30 min after PH; by means of tumour marker staining, we demonstrated the appearance of small groups of around 30 initiated cells at 30 min post-PH and scattered positive staining for the GGT or GST-p tumour markers (Figure 5). Then, we compared nuclear levels of Rel A/p65 and NF-κB binding activity between the three antioxidant treatments, with respect to caffeic acid phenethyl ester (CAPE), a selective NF-κB inhibitor [34] that we have reported reduces the pre-neoplastic lesions [23,35]. SAM and quercetin markedly decreased the nuclear levels of Rel A/p65 induced in the hepatocarcinogenesis model, by 86% and 96%, respectively ($p < 0.001$ vs TC) (Figure 6). NAC produced a decrease of 55% with respect to the TC group; this was contrasted with the effect of CAPE, which produced an inhibition of 93% (Figure 6). We analysed the DNA binding activity of NF-κB complexes by the EMSA assay. To verify the specificity of NF-κB binding activity, we used a competition assay with an unlabeled NF-κB oligonucleotide, a mutant sequence κB and a non-related Sp-1 sequence. These non-labelled oligonucleotides were added 15 min

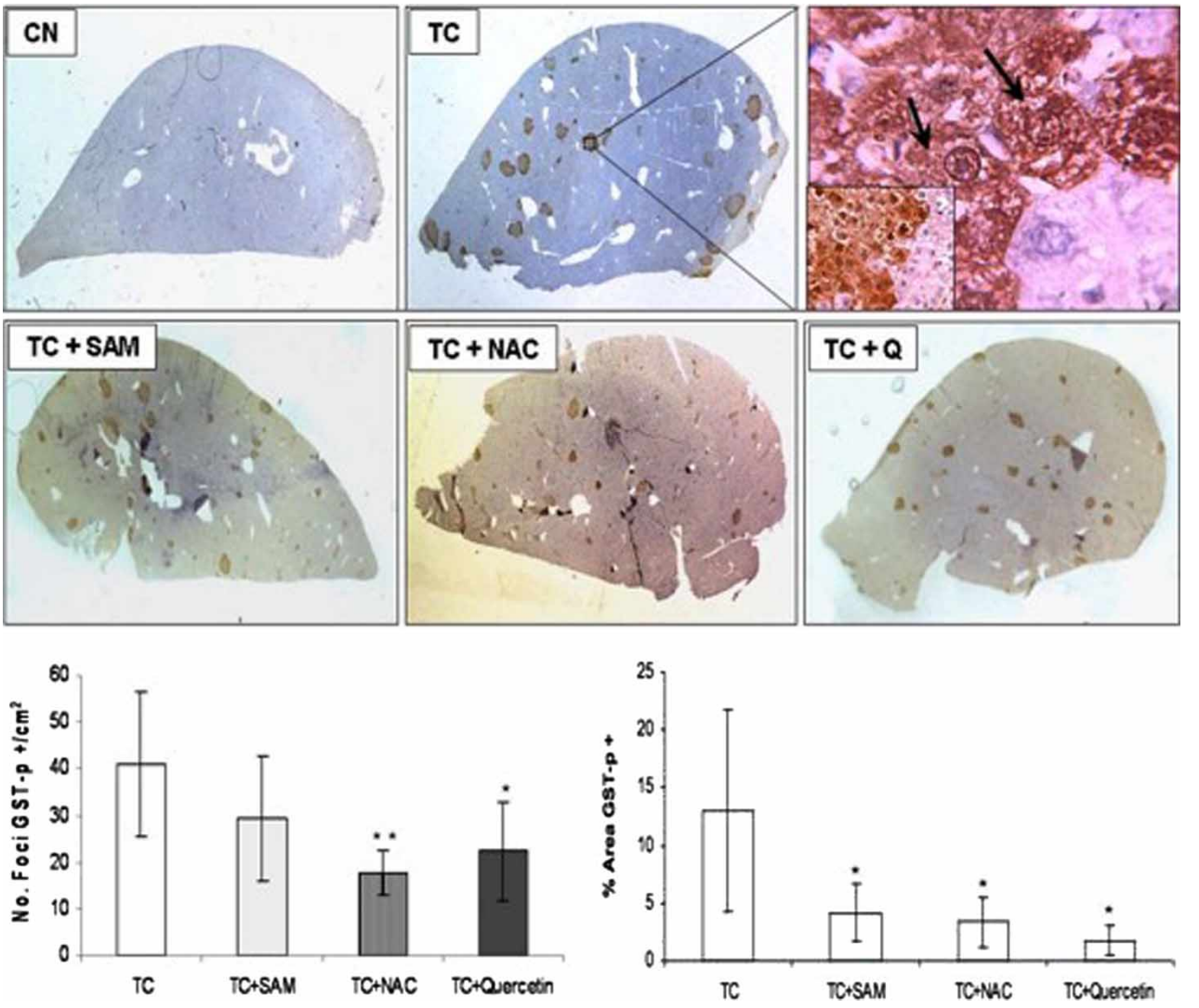

Figure 3. Effect of antioxidant treatments on GST-p-positive liver foci. Three liver histological sections were randomly chosen and analysed. Images are from representative histochemistry of liver sections of each treatment. Foci area and number were quantified and the data are presented in bar graphs. Results are expressed as the mean ± SD (*$p \leq 0.05$, **$p \leq 0.001$ and ***$p \leq 0.0001$, compared with TC group).

before the addition of labelled kB site. The NF-κB complex disappeared with the homologous competitor (Figure 7A), but the NF-κB oligonucleotide mutant version and the non-related Sp-1 sequence did not compete with the labelled NF-κB normal site. As shown in Figure 7B, SAM treatment significantly decreased NF-κB binding activity, by 85% with respect to that promoted by TC. All three antioxidants decreased the NF-κB binding activity in comparison to TC; nevertheless, SAM was able to decrease it beyond the CAPE inhibitor levels.

Inhibition of IKKα/IKKβ activation by SAM and NAC

We next determined the effects of SAM, NAC and quercetin on upstream NF-κB signalling; that is, IκB kinase (IKK) phosphorylation. IKK is a multiprotein complex comprising several sub-units, including two highly homologous catalytic kinases, IKK/α and IKK/β [36]. The IKK complex is required for the phosphorylation of NF-κB inhibitors (IkBs), to promote its degradation by the 26S proteasome; and to allow the released NF-κB to then enter the nucleus. Hence, NF-κB activation requires IKK phosphorylation. We observed that TC significantly increased IKKα/IKKβ phosphorylation, by 88% compared with normal liver ($p < 0.05$ vs CN) (Figure 8). Treatment with SAM or NAC inhibited IKKα/IKKβ activation; IKKα/IKKβ phosphorylation was significantly decreased by 47% and 54% with respect to the TC group, beyond that produced by CAPE. Although quercetin decreased the IKKα/IKKβ phosphorylation, it was not comparable with CAPE inhibition. No statistically significant changes were observed in the quercetin group with respect to TC levels. These results indicate that NF-κB activation in hepatocarcinogenesis is governed by upstream IKK

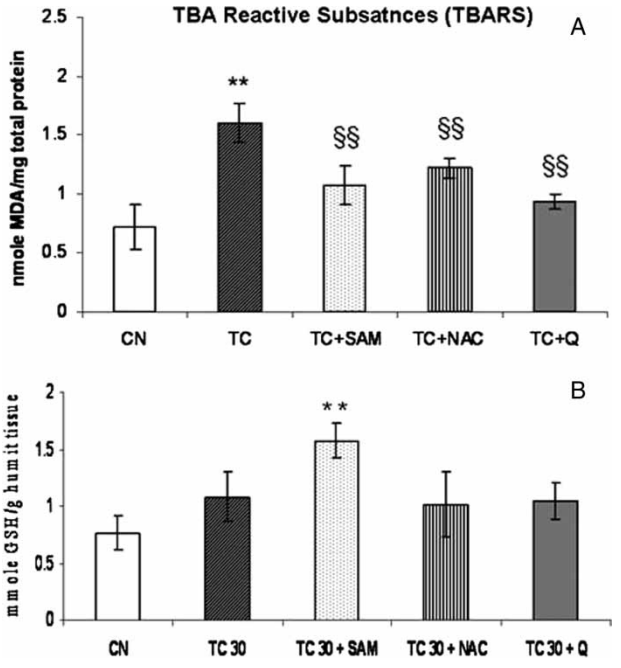

Figure 4. Inhibition of oxidative stress markers by different antioxidant treatments. (A) Lipid peroxidation was determined by detection of MDA concentration and expressed in terms of TBARS. The three antioxidant treatments produced a significant reduction of 33%, 21% and 41%. (B) GSH content was analysed by Ellman's reagent in liver homogenates. SAM increased the GSH content by 104%. Data represent the mean of lipid peroxidation and GSH levels $\pm$SD (**$p \leq 0.001$, compared with CN group; §§$p \leq 0.001$, compared with TC group).

activation and the antioxidant effects of SAM and NAC prevent this activation.

SAM and NAC prevented IκB-α phosphorylation in hepatocarcinogenesis

Another upstream event in the NF-κB activation cascade was characterized by measuring the phosphorylation of the inhibitory NF-κB peptide IκB-α by Western blot assay. In agreement with the IKK activation results, the ratio of phosphorylated IκB-α-p/IκB-α was significantly two-fold higher in hepatocarcinogenesis than in the normal liver group (Figure 9). SAM or NAC treatment prevented the increase in cytosolic IκB-α phosphorylation and decreased the IκB-α-p levels by 54% and 65% ($p <$ 0.05 vs TC) (Figure 9B). IκB-α-p levels were not statistically significant modified by quercetin or CAPE treatment in the hepatocarcinogenesis model. CAPE results are in agreement with previous reports that its administration to the human histiocytic cell line U937 had no significant effect on the TNF-induced rate of degradation of IκB-α, but delayed its resynthesis [34]. In summary, SAM and NAC inhibited the IκB-α phosphorylation induced by hepatocarcinogenesis; quercetin had no effect.

Discussion

Although the participation of ROS in chronic inflammation and degenerative diseases such as HCC is well established, the molecular mechanisms by which they induce cell proliferation and malignant transformation are not entirely understood. The principal aim of this study was to evaluate the antioxidant effects of the GSH precursors SAM and NAC and the flavonoid quercetin on oxidative status and NF-κB activation in the promotion stage of chemical hepatocarcinogenesis. These effects were compared with those produced by NF-κB specific inhibitor CAPE. Only NAC decreased both the number and the area of GGT and GST-p pre-neoplastic foci induced by hepatocarcinogenesis; SAM and quercetin reduced the area of foci as shown by both tumour markers. This decrease in liver focus area is a consequence of poor growth capacity of initiated cells, the dose and duration of SAM treatment being proportional to the decrease in number and area of GGT- and GST-p-positive foci [24,37]. In our experiments, the molar concentration of the NAC treatment was almost nine-fold higher than that of the SAM treatment and the quercetin group received a single dose of the antioxidant 24 h after the administration of DEN. SAM and quercetin had no effect on the number of liver foci and this may be because their principal action is on focus size, reflecting proliferative behaviour, and not on the emergence of initiated cells. These results strongly suggest that the dose, start time and duration of antioxidant treatment are critical parameters for the protective effect against the development of pre-neoplastic lesions. Oxidative stress is a key factor in hepatocarcinogenesis, but other events must be involved; the tumour markers were not completely abolished when antioxidants were administered after initiation, suggesting that ROS contributes to but is not the only factor required for hepatocarcinogenesis.

A principal factor in cell membrane degradation is the interaction of ROS with double bonds of polyunsaturated fatty acids to yield lipid hydroperoxydes. MDA is the principal lipid peroxidation product and is considered as a mutagenic and carcinogenic compound [38]. In accordance with pervious reports, we found an increase in lipid peroxidation, which has been related to a malignant transformation tendency [39]. This increase was abolished by all three antioxidant treatments (SAM, NAC and quercetin), perhaps due to their antioxidant ability to block the oxidant burst promoted by the metabolism of carcinogens [40]. One mechanism proposed for the non-enzymatic inhibition of lipid peroxidation by SAM is Fe^{2+} chelation and increased GSH synthesis [41]. GSH is the main non-enzymatic antioxidant defense within the cell, reducing peroxides, hydroperoxydes and radicals (alkyl, peroxyl, etc.) [42]. The particular

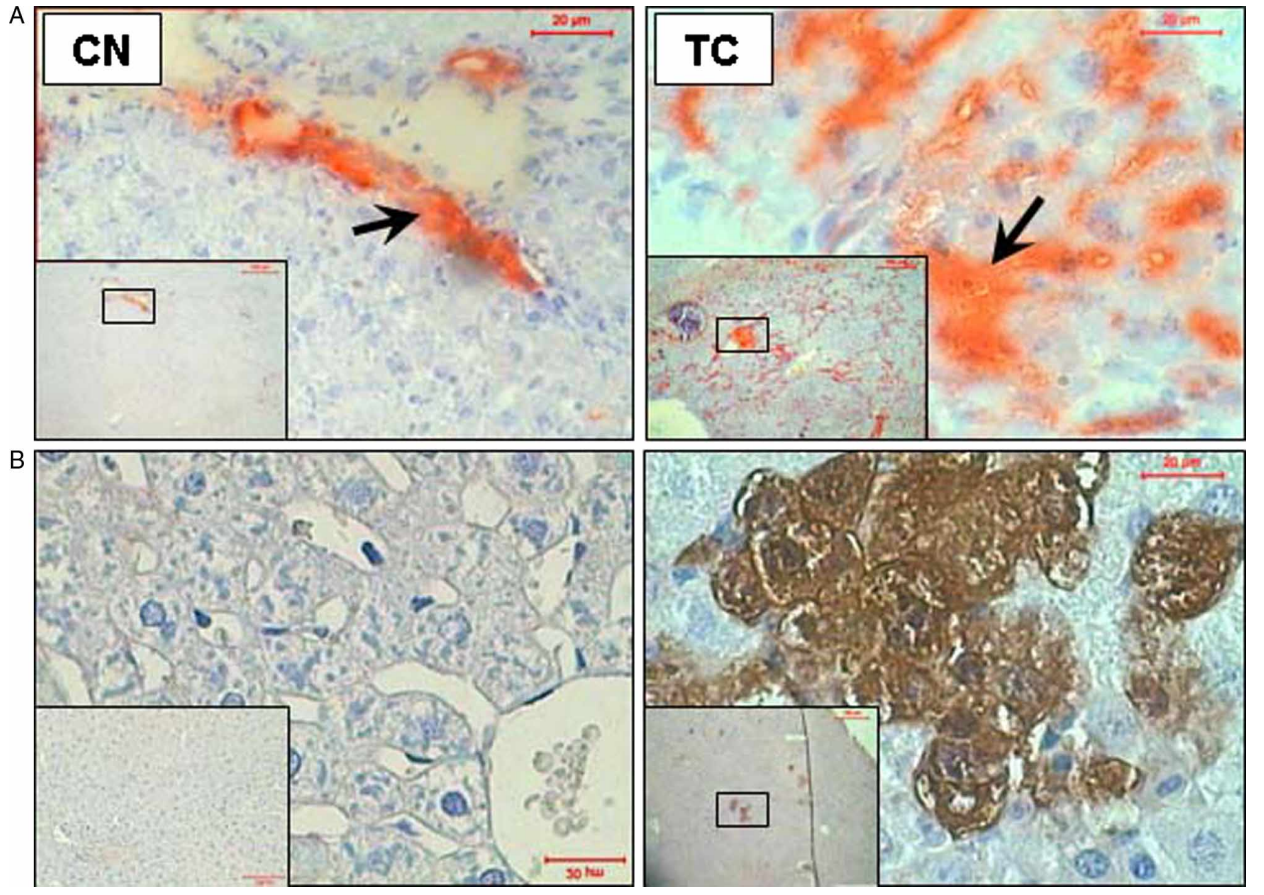

Figure 5. Tumour marker expression after partial hepatectomy in the promotion stage of hepatocarcinogenesis. (A) Expression of GGT in liver at 30 min after PHx. The arrows indicate the GGT expression of biliar duct cells in normal liver (CN) and carcinogenesis-related expression of parenquima cells in the TC group. (B) Immunohistochemical detection of GST-p expression at 30 min after PHx. Small groups of around 30 GST-p-positive cells were observed. Non-staining was observed in normal liver.

effect of SAM in increasing GSH levels could be attributed to the trans-sulphuration pathway, which converts SAM to homocysteine, which is subsequently converted to cysteine, one of the three principal amino acids of GSH. Although NAC reduced lipid peroxidation levels, it was unable to restore the GSH pool; hence, it could not be acting as a GSH precursor like SAM, but as a nucleophile and a sulphydryl donor, a role that has been attributed to its protective mechanism against chemical carcinogenesis [43]. The antioxidant activity of several flavonoids is attributed to OH groups at positions 5 and 7 of the A ring and at position 4 of the B ring [44]. Therefore, the fact that GSH synthesis was not increased by quercetin treatment is in agreement with the idea that its antioxidant role is as an ROS scavenger and antioxidant effectors inductor [45,46].

It has previously been shown that NF-κB is a redox-sensitive transcription factor that is crucial to a series of cellular processes such as cell proliferation, inflammation, immunity and apoptosis [9,47–49]. The activation of various redox-sensitive transcription factors in rat liver depends on the ROS level [50]. Low oxidative stress induces Nrf2 activation, but an intermediate amount of ROS triggers an inflammatory

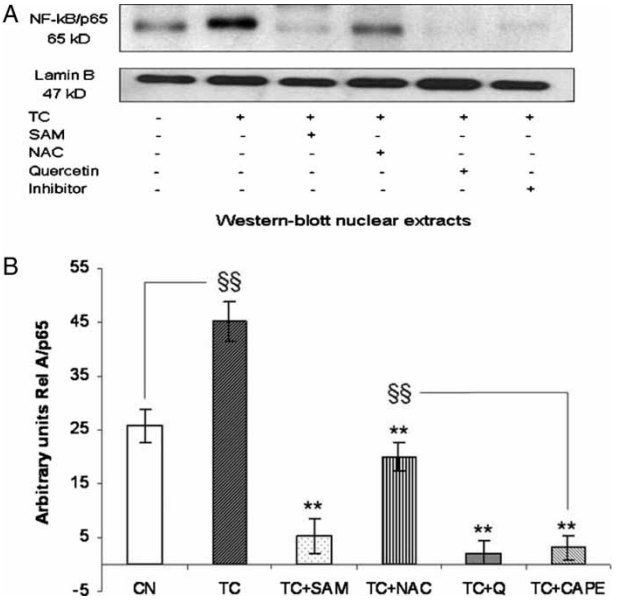

Figure 6. Effect of SAM, NAC and quercetin on nuclear levels of Rel A/p65. (A) Representative Western blot of Rel A/p65 from nuclear extracts. (B) The bar graphs indicate the relative amounts of Rel A/p65 after normalization with lamin B. SAM and quercetin significantly decreased Rel A/p65, by 86% and 96%, respectively. Mean value $\pm$SD, from three rats in triplicate (**$p \leq 0.001$, compared with TC group; §§ $p \leq 0.001$).

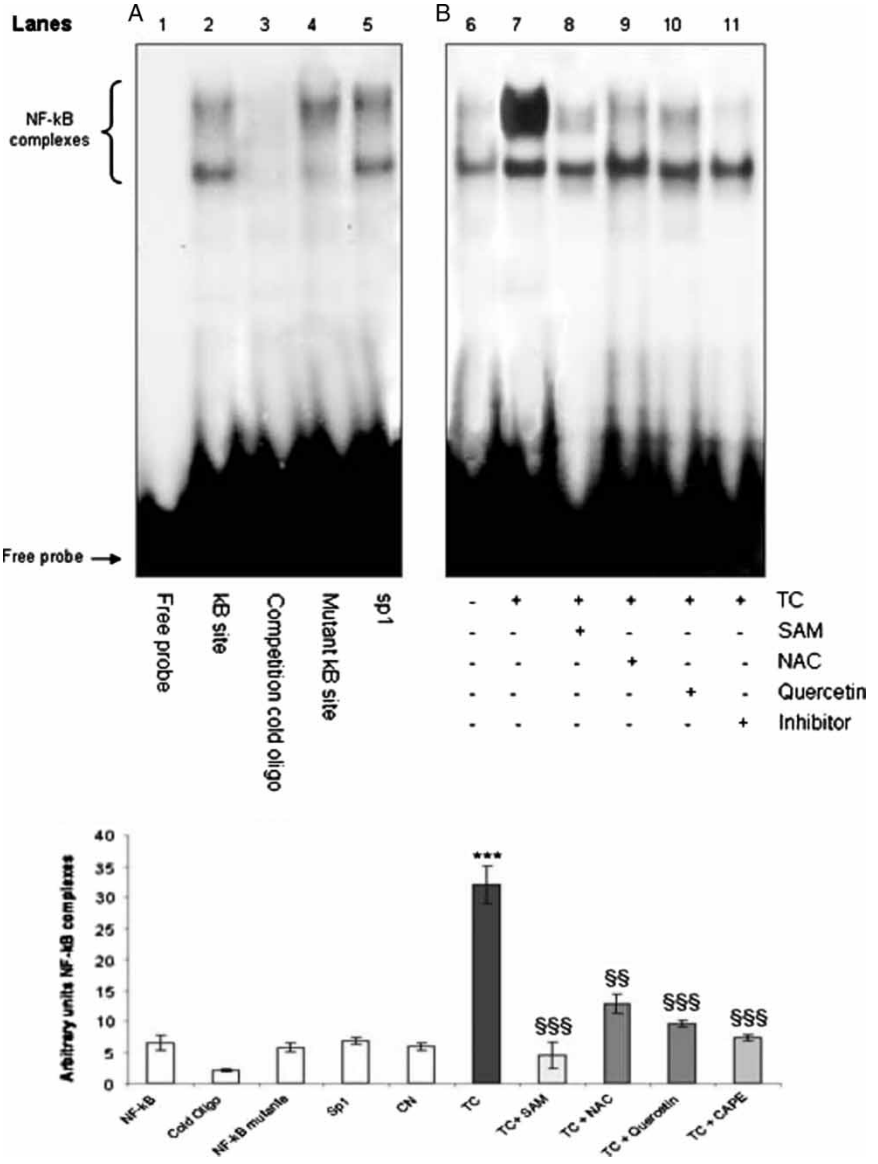

Figure 7. Inhibition of NF-κB binding activity by SAM in early liver carcinogenesis. (A) Competition assay. The specificity in NF-κB complex formation was tested using nuclear extracts with the indicated double-stranded non-labelled NF-κB sequence (3), NF-κB mutant (4) and non-related Sp1 (5) oligonucleotides as competitors. The non-labelled oligonucleotides were added 15 min before to the addition of labelled kB site. (B) EMSA analysis of three antioxidant treatments in the hepatocarcinogenesis model. SAM significantly decreased NF-κB binding activity by 85%, comparable with NF-kB inhibitor levels. Representative images from three rats (***$p \leq 0.0001$, compared with CN group; §§$p \leq 0.001$, §§§$p \leq 0.0001$, compared with TC group, $n = 3$ in all groups).

response through the activation of AP-1 and NF-κB [51]. H_2O_2 is the principal ROS demonstrated to act as a second messenger in the NF-κB signal transduction pathway due to its capacity to inhibit tyrosine phosphatase through oxidation of cysteine residues, which in turn activates tyrosine kinases and downstream signalling [52]. Many NF-κB inducers trigger the formation of ROS [51], which is abolished by various antioxidant treatments [53]. Most studies on redox signalling have focused on antioxidant compounds and these studies suggest that NF-κB activation by oxidative stress is highly cell-type specific [54], that antioxidants have broad effects on cellular physiology and that various different mechanisms are involved. In our model, NAC decreased the nuclear levels of Rel A/p65 and the NF-κB binding activity induced by carcinogenesis, but it was not comparable to the intensity of the inhibition induced by CAPE; however, it significantly reduced IKKα/IKKβ and IκB-α phosphorylation. Although several studies have indicated inhibition of NF-κB by NAC [19,55], others have controversially established that NAC is unable to suppress NF-κB activation at sub-toxic and physiologically relevant concentrations. NAC inhibited the phosphorylation of IKKα/IKKβ and IκB-α induced by TNF-α, but had no effect on the phosphorylation of IKKα/IKKβ and IκB-α induced by IL-1 [18,56]. According to our results, these reports

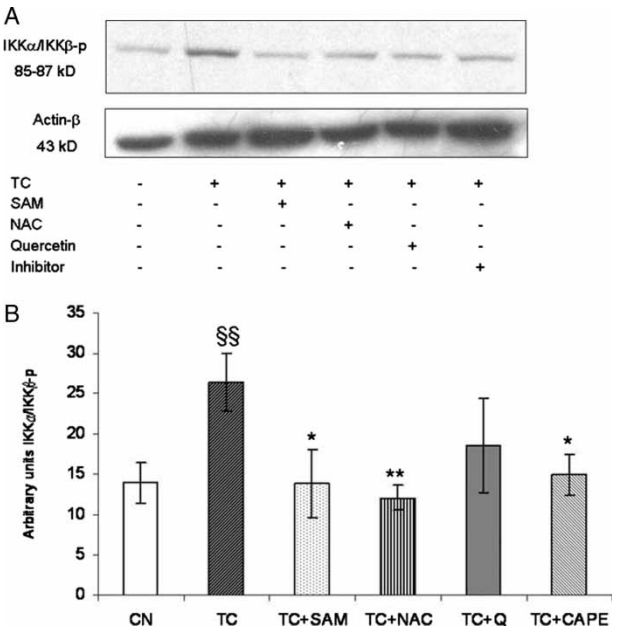

Figure 8. SAM and NAC inhibited IKK activation induced by hepatocarcinogenesis. (A) Representative Western blot of IKKα/IKKβ phosphorylation in cytosolic extracts. (B) Densitometric analysis of the 85–87 kD bands. The bar graphs indicate the relative amounts of IKK after normalization with actin β. SAM and NAC significantly decreased IKK phosphorylation, by 47% and 54%, respectively. Mean $\pm$ SD of three rats in triplicate ($\S\S p \leq 0.001$, compared with CN group; $^{*}p \leq 0.05$, $^{**}p \leq 0.001$, compared with TC group).

suggest that NAC acts specifically on early events in the NF-κB signalling induced by TNF-α, causing structural changes in TNF receptors by reducing disulphide bridges. Quercetin is known to modulate NF-κB activation with various cell types, diseases and inductors [21,57,58], possibly suppressing certain tyrosine kinases as has been previously reported [59]. The treatment with quercetin in our hepatocarcinogenesis model decreased nuclear Rel A/p65 levels and NF-κB binding activity; but did not have an effect on total phosphorylation of IKKα/IKKβ and IkB-α. NF-κB inhibition by quercetin has been shown to be dose dependent, as mentioned in a previous study [20] in which the administration of 5 μmol/L of quercetin produced no significant decrease of NF-κB activation in IL-1β-activated rat hepatocytes. The fact that quercetin had no effect on upstream NF-κB signalling suggests that another mechanism must be involved in the decreased NF-κB activation independent of IKK activation. Further studies must be conducted to clarify the role of quercetin in NF-κB activation in hepatocarcinogenesis.

Despite increasing evidence of the antioxidant effect of SAM in carcinogenesis [60], its protective mechanism has not been completely elucidated. It has been reported that SAM had no effect on JNK activity, which is known to be responsive to redox changes, in ethanol-induced apoptosis in primary hepatocyte cultures [61]. Contrary to this report, in our experiments we observed that SAM decreased nuclear Rel A/p65 levels, NF-κB binding activity and the upstream signalling phosphorylation induced by hepatocarcinogenesis surpassed exceedingly to CAPE inhibitor. These results are important in elucidating the NF-κB inhibition mechanism of SAM in early chemical hepatocarcinogenesis. We suggest that SAM inhibits NF-κB activation through the classical activation pathway in early liver carcinogenesis (Figure 10). The oxidative stress that participates in the induction of pre-neoplastic lesions is decreased by treatment with SAM. The inhibition of kinase activity of IKK by SAM prevents the phosphorylation of IκB-α and its degradation by the proteasome system to release Rel A/p65. As a result, NF-κB complexes are unable to enter the nucleus and promote the gene transcription related to cell proliferation and oncogenesis.

We describe the mechanism by which SAM decreased the liver foci area and its role in NF-κB activation. Due its protective effect on pre-neoplastic lesions and also because it is commercially available as a nutritional supplement, it could be considered a promising chemoprotective compound.

In summary, our results indicate that oxidative stress is related to hepatocarcinogenesis and that the mechanism involved must be comprehensively explored. We found that the effectiveness of the

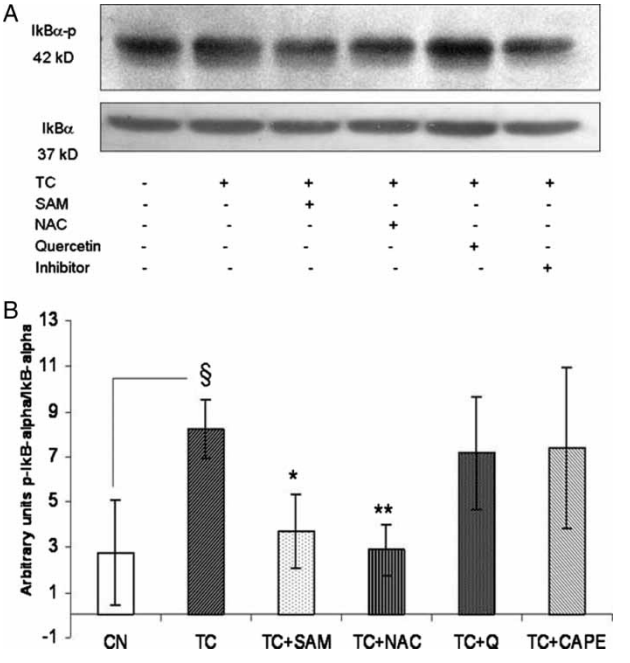

Figure 9. Preventive effect of SAM and NAC on IκB-α phosphorylation. (A) Representative Western blot of IκB-α phosphorylation in cytosolic extracts. (B) Densitometric analysis of the 42 kD bands. The bar graphs indicate the p-IκB-α/IκB-α ratio. SAM and NAC significantly decreased IκB-α phosphorylation, by 54% and 65%, respectively. Mean $\pm$ SD of three rats in triplicate ($\S p \leq 0.05$, compared with CN group; $^{*}p \leq 0.05$, $^{**}p \leq 0.001$, compared with TC group).

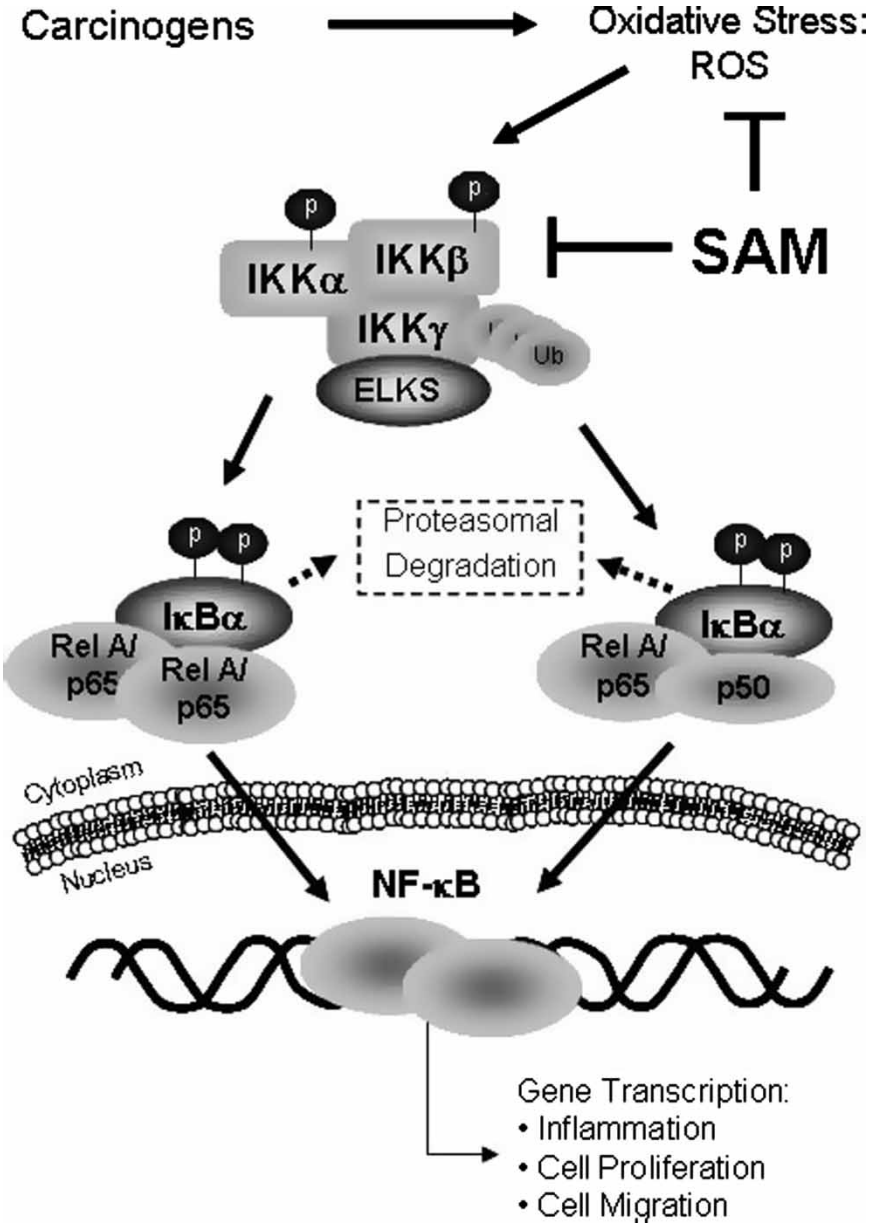

Figure 10. Proposed mechanism for NF-kB inhibition by SAM in hepatocarcinogenesis. The carcinogens metabolism induces oxidative stress, which is able to stimulate IKK activation and subsequent NF-κB translocation into the nucleus. Activation of NF-κB occurs predominantly through IKK activation, where all inducers converge. This kinase phosphorylates to IκB proteins and allows its degradation by proteasomal system, then NF-κB released translocates into the nucleus. This process can be blocked by the antioxidant S-adenosyl-methionine, which reduces the oxidative burst and inhibits IKK activation, IκB-α phosphorylation and NF-κB binding activity.

protection of different antioxidant treatments is linked to the dose and timing of the start of administration. All three treatments tested decreased oxidative stress; though not all exerted the same inhibitory effect on the NF-κB activation pathway cascade. SAM showed to block the NF-κB activation upstream signalling by the classic activation pathway. Since we achieved to describe the mechanism through which SAM decreased the liver foci area in early liver carcinogenesis, protective antioxidant strategies focused on SAM could be a promising therapeutic approach in liver cancer.

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