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Cellular stress response in Eca-109 cells inhibits apoptosis during early exposure to isorhamnetin

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The flavonol aglycone isorhamnetin shows anti-proliferative activity in a variety of cancer cells. Previous work, from our laboratory showed that isorhamnetin inhibits the proliferation of human esophageal squamous carcinoma Eca-109 cells in vitro, but only after 72 h of exposure. This led us to propose that isorhamnetin exposure induces a cellular stress response that inhibits the antiproliferative and apoptotic effects of the compound during early exposure. To test this hypothesis, the present study examined the effects of isorhamnetin on Eca-109 cells during the first 72 h of exposure. Cell growth was assessed using the trypan blue exclusion assay, and expression of IκBα, NF-κB/p65, NF-κB/p50, phospho-Akt, Bcl-2, COX-2, Mcl-1, Bax, p53 and Id-1 were analyzed by Western blot. During the first 72 h of exposure, NF-κB/p65 and NF-κB/p50 accumulated in nuclei and expression of COX-2, Bcl-2 and Mcl-1 increased. In contrast, expression of IκBα and Bax fell initially but later increased. Expression of phospho-Akt and p53 showed no detectable change during the first 48 h. Pretreatment with the NF-κB inhibitor MG132 before exposure to isorhamnetin blocked the nuclear accumulation of p50 and p65, thereby inhibiting cell proliferation. These results show that during early exposure of Eca-109 cells to isorhamnetin, the NF-κB signaling pathway is activated and COX-2 expression increases, and this increase in expression partially inhibits isorhamnetin-induced apoptosis. Beyond 72 h of exposure, however, the apoptotic effect of isorhamnetin dominates, leading to inhibition of the NF-κB pathway and of cellular proliferation. These results will need to be taken into account when exploring the use of isorhamnetin against cancer in vivo.

Key words: isorhamnetin, Eca-109, NF-κB, COX-2, cellular stress response

Esophageal cancer belong to highly malignant tumors and it has the sixth highest mortality rate among all known cancers [1]. Despite the numerous risk factors associated with esophageal cancer, all cases can be classified as either esophageal squamous cell carcinoma (ESCC) or adenocarcinoma [2, 3]. Except traditional therapies, new treatment methods of ESCC are under investigation.

Isorhamnetin is one type of flavonol aglycone from the sea buckthorn (*Hippophae rhamnoides L*). Isorhamnetin has been found to have numerous effects on cancer cells, including antioxidant activity and growth inhibition [4.5.6]. Our previous work showed that isorhamnetin inhibits the growth of Eca-109 cells from human esophageal squamous carcinoma and induces apoptosis [7]. However, these effects were not observed until after 72 h of exposure, leading us to suggest that isorhamnetin initially induces a cellular stress response that inhibits its antiproliferative and apoptotic effects.

The NF-κB signaling pathway has been closely linked to the cellular stress response [8]. The NF-κB family contains five members: p50, p52, p65 (RelA), c-Rel, and RelB, which are encoded, respectively, by the genes *NFKB1, NFKB2, RELA, REL,* and *RELB*. Since the p50/p65 heterodimers are responsible for most NF-κB activity in cells, it is usually referred to as NF-κB [9]. Some reports have shown that activated NF-κB exerts anti-oxidative and anti-apoptotic effects by regulating the gene expression of bcl-2, cytoplasmic Mn-superoxide dismutase, TNF-α and related genes, as well as the release of cytochrome C [10, 11]. In addition, stimulation of the NF-κB pathway in LNCaP cells from human prostate adenocarcinoma activates the Inhibitor of differentiation/DNA binding protein-

1 (Id-1), which appears to promote cell survival in a number of human cancers by upregulating the expression of Bcl-xL and ICAM-1 [12]. A number of factors have been shown to activate the NF-κB pathway, including the serine/threonine kinase Akt, which is in turn activated by numerous growth factors and other factors. In the Jurkat T-cell line, Akt induces expression of transcription factors of the NF-κB family [13]. Whether activation of NF-κB also involves Akt phosphorylation in isorhamnetin-treated cells remains a question.

Cyclooxygenases play a key role in the biosynthesis of prostaglandins (PGs) from arachidonic acid, and some of these enzymes have been linked to cancer progression and metastasis. Three cyclooxygenase isozymes are known. COX-1 is a constitutively expressed housekeeping gene and appears to be responsible for the production of PGs. It is expressed in most tissues [14, 15]. In contrast, COX-2 shows basal expression, which in certain tissues rapidly increases in response to pro-inflammatory mediators [16, 17, 18, 19]. Meanwhile, a third isoform named COX-3, which is COX-1 splicing variant, was identified one decade ago; it plays a role in fever and pain processes [20]. COX-2 expression has been linked to carcinogenesis, tumor growth promotion, inhibition of apoptosis, angiogenesis and metastasis [21]. Its expression is induced by tumor promoters, growth factors and cytokines [22, 23, 24, 25], and this upregulation appears to occur via downregulation of p53 or activation of the Akt signalling pathway [26, 27, 28]. Isorhamnetin has also been shown to inhibit COX-2 expression, which may help to explain its anticancer effects [29].

In the present study, our aim was focused on the molecular events induced by isorhamnetin in Eca-109 cells within the first 72 h of exposure, in order to determine whether the cellular stress response causes the observed delay in the antiproliferative and apoptotic effects of the compound. To this end, we monitored the activation state of the NF-κB signaling pathway and COX-2 expression levels during early exposure to the compound.

Material and Methods

Reagents. Isorhamnetin was purchased from MEDCO Pharmaceutical (Chengdu, China), and cisplatin was purchased from West China Hospital of Sichuan University (Chengdu, China). MG132 was purchased from Calbiochem (Germany) and dissolved in dimethylsulfoxide (DMSO) to a final concentration of 2 mM. Antibodies used for Western blotting were purchased from the following manufacturers: anti-p53 (sc-99), anti-Bcl-2 (sc-492), anti-Id-1 (sc-488) and anti-NF-κB/p65 (sc-8008) antibodies were from Santa Cruz Biotechnology (USA); anti-COX-2 (ZA0243), anti-Mcl-1(ZA0569), anti-IκBα (ZA0509), anti-Akt(T308) (ZP0026) and anti-Akt(S473) (ZP0024) antibodies were from ABZoom (Chengdu, China); and anti-β-actin, anti-NF-κB/p50 and anti-Bax antibodies were from BOSTER (Chengdu, China). Horseradish peroxidase-conjugated secondary antibodies (sc-2004) were from Santa Cruz Biotechnology (USA).

Cell Culture and Treatments. The human ESCC line Eca-109 was obtained from the Institute of Biochemistry and Cell Biology (Beijing, China). Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 U/ml). And were maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

Isorhamnetin was dissolved in 0.1% NaOH, diluted with routine medium, and finally filtered through one 0.22μm microproefilter [7]. Since our previous work showed the 50% inhibitory concentration (IC50) of isorhamnetin to be 130μM(40μg/ml) for Eca-109 cells[7], this concentration was used in the present study and served as a treatment group. Eca-109 cells exposed to cisplatin (30 μM) served as a positive control for anticancer activity, and cells cultured in DMEM served as a negative control.

In a second experiment, cultures were treated for 90 min with the NF-κB inhibitor MG132 (2 μM) or 0.1% DMSO vehicle, these additives were washed out with several changes of fresh DMEM, and then the cultures were incubated with isorhamnetin for 48 h.

Trypan blue exclusion assay. This assay was carried out to determine the viability of Eca-109 cells treated with isorhamnetin. Briefly, equal numbers of Eca-109 cells (1.0×10^5) were seeded into 6-cm culture dishes and incubated overnight. Then these cells were treated with 130 μM isorhamnetin (experimental), 30 μM cisplatin (positive control), or left untreated (negative control), and incubated for 24, 48, 72, 96, 120 h. Viable cells from all three conditions were counted each day. Growth curves were generated in which each point was the average of three experiments.

Western blotting. Each day, Eca-109 cells from the isorhamnetin-treated group were harvested using trypsin. Cells were lysed in SDS loading buffer [125 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1% (w/v) SDS, 1 mM phenylmethylsulfonyl fluoride], boiled for 5 min and centrifuged at 12,000 g for 10 min. Supernatants were collected and used as whole-cell extract. The nuclear protein fraction was obtained using NE-PER nuclear extraction reagents (Bioteke, Beijing, China). Subsequently, protein concentrations were estimated using the BCA Protein Assay Kit (Bioteke). Protein samples (30-50 μg) were fractionated by polyacrylamide gel electrophoresis (Bio-Rad) and transferred to a nitrocellulose membrane. After blocking with Blotto (5% non-fat milk) for 1 h at room temperature, membranes were incubated with one or more of the following primary antibodies overnight at 4 °C: Bax (1:400), Mcl-1 (1:400), Bcl-2 (1:400), NF-κB/p65 (1:400), NF-κB/p50 (1:400), IκBα (1:400), phospho-Akt (1:400), COX-2 (1:500), p53 (1:400), Id-1 (1:400), and β-actin (1:1000). Then membranes were incubated with horseradish peroxidase–conjugated secondary antibody (1:10000) for 1 h at room temperature. All antibody dilutions were prepared in Blotto. Immunoblots were visualized by enhanced chemiluminescence (ECL; Tiangen, Beijing, China) and bands were analyzed by densitometry using Quantity One 4.6.2 software (Bio-Rad, San Diego, USA).

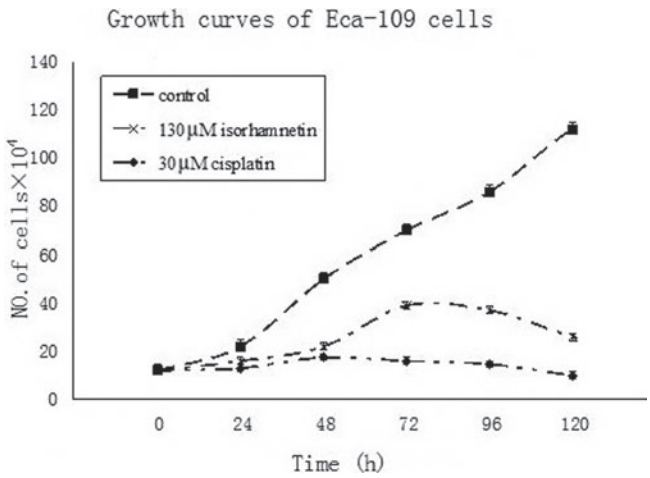

Figure 1. Growth curves of isorhamnetin-treated Eca-109 cells determined by the trypan blue exclusion assay. The number of viable cells treated with 130 μM isorhamnetin increased continuously during the first 72 h, in contrast to the cisplatin-treated cultures. However, the number of viable cells decreased significantly during the following 48 h.

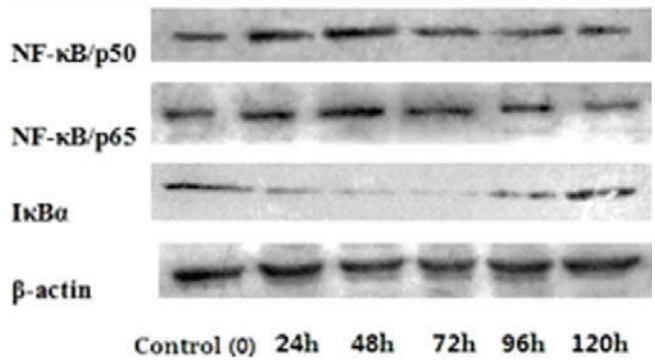

Figure 2. Effect of isorhamnetin on levels of NF-κB/p50, NF-κB/p65 and IκBα in Eca-109 cells. After treatment of cells with 130 μM isorhamnetin for 24, 48, 72, 96 and 120 h, cytoplasmic and nuclear proteins were isolated and analyzed by Western blot. During 0 to 72 h of treatment, nuclear levels of NF-κB/p50 and NF-κB/p65 increased dramatically, while the cytoplasmic level of IκBα decreased significantly. However, during 72 to 120 h of treatment, nuclear levels of NF-κB/p50 and NF-κB/p65 decreased and the cytoplasmic level of IκBα increased. Equal loading was confirmed by stripping the blot and reprobing for β-actin. Significant changes were determined based on three independent experiments.
***p<0.05 and ** p<0.01 compared with control (0 h of treatment).**

Statistical analysis. Data were obtained from at least three independent experiments. The one-way ANOVA test and Student's t test were used to analyze differences, and $p < 0.05$ was considered statistically significant. All statistical calculations were carried out using SPSS 16.0 (SPSS, Inc.; Chicago, USA). Eca-109 growth curves were plotted using Excel 2003.

Results

Isorhamnetin fails to inhibit proliferation of Eca-109 cells completely during early exposure. Eca-109 cells (1.0×10^5 cells/well) were plated into 24-well dishes. After exposure to 130 μM isorhamnetin and 30μM cisplatin for 24, 48, 72, 96, and 120 h respectively, cells viability was assessed using the trypan blue exclusion assay. Compared with counterpart of the control group, the number of viable cells from isorhamnetin-treated group increased gradually but significantly during the first 72 h, then fell dramatically over the following 48 h; on the other hand, viable cells in cisplatin group did not increase during 120 h exposure (Fig. 1).

Exposure to isorhamnetin activates NF-κB in Eca-109 cells. We then set out to determine whether the ability of Eca-109 cells to proliferate after brief exposure to isorhamnetin was associated with activation of cell stress response genes. Western blot analysis of nuclear extracts of isorhamnetin-treated cells showed nuclear accumulation of NF-κB/p50 and NF-κB/p65 proteins after 24 and 48 h of exposure. At the same time, the cytoplasmic level of IκBα decreased, confirming that NF-κB was activated (Fig. 2, Table 1). Between 48 and 120 h of exposure, however, when the number of viable cells was decreasing (Fig. 1), cytoplasmic levels of IκBα increased and nuclear levels of NF-κB/p50 and NF-κB/p65 decreased (Fig. 2, Table 1).

Exposure to isorhamnetin induces COX-2 expression in Eca-109 cells. The COX-2 expression pattern of Eca-109 cells during 120 h exposure to isorhamnetin corresponds to the

Table 1. Densitometric analysis of nuclear NF-κB/p50 and NF-κB/p65 and of cytoplasmic IκBα in Eca-109 cells following isorhamnetin

Time(hr)		Realative Optcal Density		
	N	IκBα	NF-κB/p50	NF-κB/p65
0	3	5078.33±227.48	5391.67±143.30	4886.00±210.69
24	3	2596.67±181.62**	8668.67±159.63**	6694.67±237.59**
48	3	2059.00±138.86**	11190.67±423.75**	9491.00±577.43**
72	3	1086.33±68.82**	5769.33±120.08	7961.67±339.16**
96	3	3175.33±186.80**	5474.00±193.03*	5274.67±198.56*
120	3	4881.00±279.61*	4681.00±60.90**	2789.33±265.48**

*P<0.05 *vs.* control group ** P<0.01 *vs.* control group

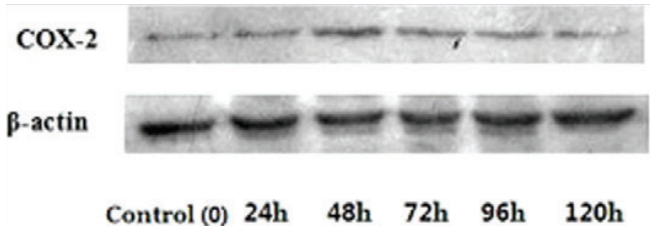

Figure 3. Effect of isorhamnetin on COX-2 expression in Eca-109 cells. Cells were treated with 130 μM isorhamnetin for 24, 48, 72, 96 and 120 h and whole-cell extracts were analyzed by Western blot. COX-2 expression peaked at 48 h, and it began to decrease sharply at 72 h. Equal loading was confirmed by stripping the blot and reprobing for β-actin. Significant changes were determined based on three independent experiments.
***p<0.05 and ** p<0.01 compared with control (0 h of treatment).**

proliferation trend of these treated cells. COX-2 expression increased during the first 48 h treatment (Fig. 3, Table 2), which is consistent with the gradually enhanced proliferation during the same period (Fig. 1). Between 72 and 120 h of exposure, however, COX-2 expression declined (Fig. 3, Table 2), which is in line with the decreased number of viable cells (Fig. 1).

Table 2. Densitometric analysis of COX-2 following isorhamnetin

Time (hr)	N	Relative Opitical Density
		COX-2
0	3	3232.00±100.26
24	3	3791.00±213.49*
48	3	5162.00±196.07**
72	3	3364.67±218.28*
96	3	2543.67±147.71**
120	3	2326.33±124.64**

*P<0.05 *vs.* control group ** P<0.01 *vs.* control group

Activation of NF-κB and upregulation of COX-2 expression during early exposure of Eca-109 cells to isorhamnetin does not involve Akt phosphorylation. Western blot analysis was used to determine whether phosphorylation of Akt was implicated in the activation of NF-κB. The results show no significant changes in the levels of phospho-Akt (S473) and phospho-Akt (T308) between 0 and 48 h of isorhamnetin exposure (Fig. 4A, Table 3). These results suggest that Akt was not responsible for NF-κB activation and peak of COX-2 expression at 48 h.

Table 3. Densitometric analysis of IκBα, COX-2, p-Akt (T308)/(S473) following isorhamnetin

Time (hr)	N	Relative Optical Density			
		IκBα	Cox-2	p-Akt(T308)	p-Akt(S473)
0	3	1192.50±123.62	1227.09±116.42	1198.84±116.80	3144.03±223.89
24	3	927.03±120.89*	2771.55±128.62*	1137.49±121.65	3699.09±237.52
48	3	383.93±118.91**	5439.43±161.60**	1085.58±121.60	3828.75±210.72

*P<0.05 *vs.* control group ** P<0.01 *vs.* control group

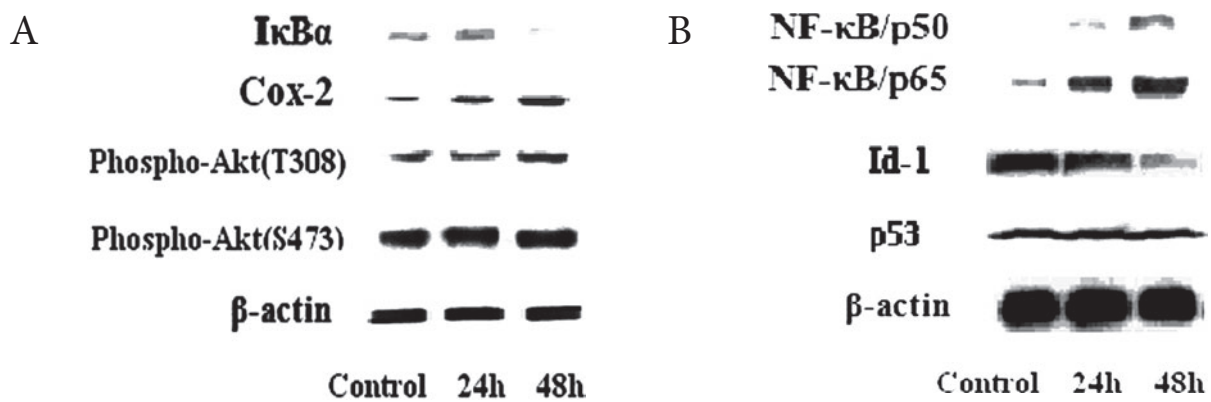

Figure 4. Relationship among NF-κB, p-Akt, Id-1, p53and COX-2 expression in Eca-109 cells during the first 48 h of exposure to isorhamnetin. Cells were treated with 130 μM isorhamnetin for 24 or 48 h and whole-cell extracts were analyzed by Western blot. (A) Phospho-Akt(T308)/(S473) was not detected under these conditions, whereas the level of NF-κB peaked at 48 h and the level of COX-2 increased sharply from 0 to 48 h. (B) The level of Id-1 decreased significantly from 0 to 48 h, while the nuclear levels of NF-κB/p50 and NF-κB/p65 increased. No significant change in the levels of p53 was observed. Equal loading was confirmed by stripping the blot and reprobing for β-actin. Significant changes were determined based on three independent experiments.
***p<0.05 and ** p<0.01 compared with control (0 h of treatment).**

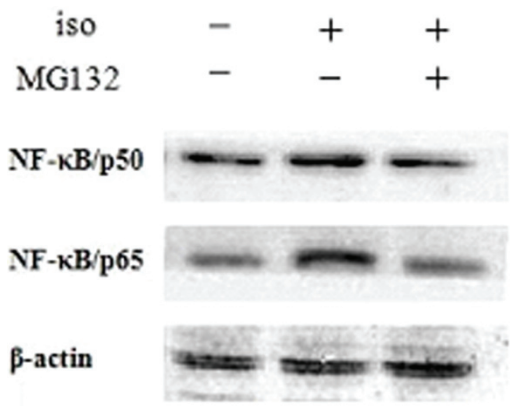

Figure 5. Growth curves of Eca-109 cells pretreated with the NF-κB inhibitor MG132 and then exposed to isorhamnetin. Cells were treated for 90 min with MG132 (2 μM) or 0.1% DMSO vehicle, these additives were washed out with several changes of medium, and then the cells were treated for 48 h with 130 μM isorhamnetin. Pretreatment with MG132 led to significantly less proliferation than treatment with isorhamnetin alone.

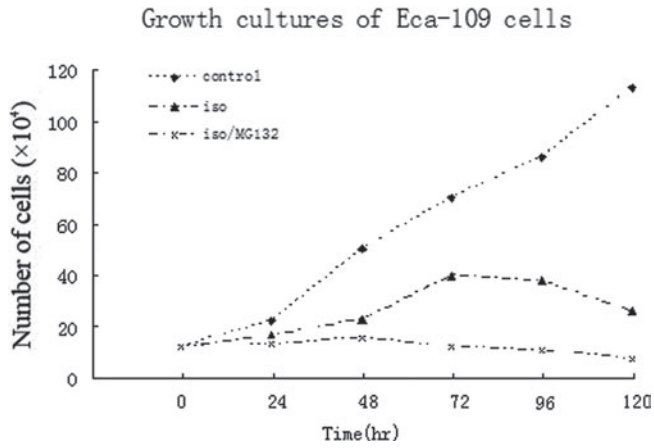

Figure 6. Inhibition by MG132 of isorhamnetin-induced activation of NF-κB. Eca-109 cells were pretreated with MG132 or DMSO vehicle and then treated with isorhamnetin as described in Fig. 5. Nuclear levels of NF-κB/p50 and NF-κB/p65 were significantly lower in cells pretreated with MG132 than in those treated with isorhamnetin alone. Results shown are from three independent experiments.
**** p<0.01 compared with control cells (no pretreatment)**

Isorhamnetin treatment downregulates the expression of Id-1 but fails to alter the levels of p53 protein. Isorhamnetin decreased Id-1 expression in isorhamnetin treated Eca-109 cells. Forty-eight-hour exposure resulted in two thirds less protein level of Id-1, compared with that of Eca-109 cells from the time point 0 h (Fig. 4B , Table 4). Nevertheless, this drug did not cause any significant change in levels of p53 (Fig. 4B, Table 4).

Activation of NF-κB is essential for proliferation of Eca-109 cells during early exposure to isorhamnetin. To confirm the role of NF-κB in Eca-109 cell proliferation, cells were exposed to isorhamnetin after pretreatment with the NF-κB inhibitor MG132. The inhibitory effect of MG132 was confirmed by Western blot (Fig. 5, Table 5). Under these conditions, no significant proliferation of cells was observed at any time during exposure to isorhamnetin (Fig. 6). In fact, the number of viable cells was significantly lower in the cells pretreated with MG132 than that in the cells exposed only to isorhamnetin at all time points examined (Fig. 5). These results, together with the increased in COX-2 expression, indicate that the cellular stress response partially inhibited the antiproliferative effects of isorhamnetin during early exposure.

Exposure of Eca-109 cells to isorhamnetin induces changes in the expressions of the apoptotic proteins Bcl-2, Bax, and Mcl-1. Expressions of Bcl-2 and Mcl-1 shared the same pattern. Their expressions increased significantly

Table 4. Densitometric analysis of NF-κB/p50, NF-κB/p65, Id-1 and p53 following isorhamnetin

Time (hr)	N	Relative Optical Density			
		NF-κB/p50	NF-κB/p65	Id-1	P53
0	3	0	996.11±145.48	7490.65±151.99	4174.37±119.79
24	3	1155.41±170.33**	3186.72±141.90**	6658.93±110.53**	3911.78±132.48
48	3	2272.86±104.36**	5633.82±191.77**	2503.72±119.79**	3868.16±130.24

*P<0.05 *vs.* control group ** P<0.01 *vs.* control group

Table 5. Densitometric analysis of nuclear NF-κB/p50 and NF-κB/p65 following MG132/isorhamnetin, isorhamnetin

Time (hr)	N	Relative Optical Density	
		NF-κB/p50	NF-κB/p65
Control	3	6324.00±200.50	4086.00±184.50
Iso	3	11239.00±526.00**	9660.00±369.70**
Iso/MG132	3	5464.00±109.60	4539.00±234.20

*P<0.05 *vs.* control group ** P<0.01 *vs.* control group

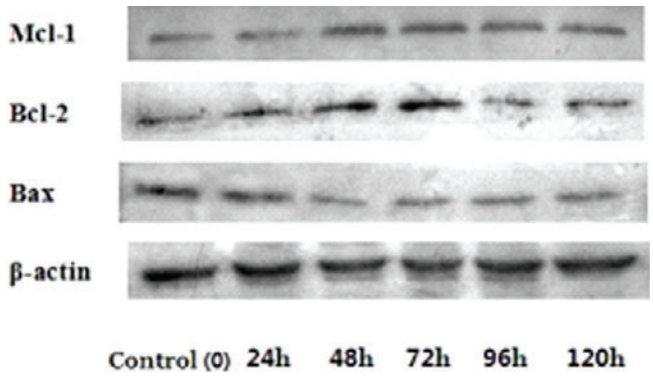

Figure 7. Effect of isorhamnetin on the expression of Mcl-1, Bcl-2 and Bax in Eca-109 cells. Cells were treated with 130 μM isorhamnetin for 24, 48, 72, 96 or 120 h and whole-cell extracts were analyzed by Western blot. The level of Mcl-1 gradually increased from 0 to 48 h of exposure to isorhamnetin. The level of Bcl-2 increased sharply, peaking at 72 h of exposure. On the contrary, the level of Bax decreased from the beginning of exposure, such that nearly no Bax was detected by 48 h. Results are from three independent experiments.
***p<0.05 and ** p<0.01 compared with control (0 h of treatment).**

immediately after exposure to isorhamnetin, peaked at the point of 72 h, and then gradually decreased during the last 48 h (Fig. 7, Table 6). In contrast, the expression of Bax decreased for 48 h and then increased for another 72 h (Fig. 7, Table 6).

Discussion

Isorhamnetin has been shown to have antioxidant and antiproliferative activities in a variety of cancer cell lines. In previous work, we found that this compound could inhibit the growth of ESCC Eca-109 cells and induced apoptosis, but only after longer exposure. In the present report, we provide evidence that these effects are delayed because the compound initially induces a cell stress response involving NF-κB activation and COX-2 upregulation, which allows the cells to grow slowly for at least 72 h. These results indicate that the cellular stress response will need to be taken into account when developing isorhamnetin as an anticancer drug.

Aberrant expression of COX-2 has been reported in several types of cancer [30,31,32,33,34], and constitutive COX-2 activity may stimulate cellular proliferation of ESCC cells [34]. Jones *et al* reported that isorhamnetin was a potent inhibitor of COX-2 activity in the human colorectal cancer cell line HCA-7. While the present study showed that isorhamnetin did downregulate COX-2 expression during longer exposure, expression was upregulated during the first 48 h. During this same early period of exposure, NF-κB/p50 and NF-κB/p65 accumulated in the nucleus. These results suggest that short exposure to isorhamnetin induces a cell stress response involving COX-2 upregulation and NF-κB activation in Eca-109 cells. These findings led us to examine the isorhamnetin-induced stress response in greater detail.

The transcription factor NF-κB plays a key role in the transcriptional regulation of adhesion molecules, enzymes and cytokines involved in chronic inflammatory diseases [35], and in transcriptional regulation in various cancer cell models, including esophageal carcinoma[36]. Activation of NF-κB is associated with numerous signalling events, including upregulation of COX-2 expression[37]. NF-κB normally exists in the cytoplasm as inactive complexes with inhibitory proteins of the IκB family. In the present study, we found that isorhamnetin induced significant degradation of IκBα and nuclear accumulation of NF-κB/p50 and NF-κB/p65 within 48 h. Consistent with a previous report that the NF-κB inhibitor MG132 blocks the disappearance of IκBα, we found that it blocked the isorhamnetin-induced degradation of IκBα under our conditions. We also found that it downregulated the expression of NF-κB/p50 and NF-κB/p65 in isorhamnetin-treated cells. Together with our previous work [7], the present study shows that isorhamnetin activates the NF-κB pathway.

This NF-κB activation may be part of a pro-survival response during isorhamnetin-induced endosplasmic reticular stress. This pathway may contribute to a premalignant phenotype not only by promoting cell proliferation and survival, but also by upregulating genes that control cell adhesion and angiogenesis[38] and that express proteins involved in antiapoptosis (Bcl-2, Bcl-xL) and proliferation (cyclin D1, c-Myc) [39].

In vitro studies support the idea that COX-2 over-expression inhibits apoptosis and promotes tumor angiogenesis

Table 6. Densitometric analysis of Mcl-1, Bcl-2 and Bax following isorhamnetin

Time (hr)	N	Relative Optical Density		
		Mcl-1	Bcl-2	Bax
0	3	2669.67±174.95	2962.33±91.52	7259.22±139.67
24	3	2598.00±126.89	3772.33±191.82**	6048.00±303.48**
48	3	6548.00±145.49**	5276.00±252.48**	2303.33±272.09**
72	3	6413.67±124.54**	6247.33±178.30**	2752.33±209.00**
96	3	4503.00±80.47**	2017.67±111.43**	3481.33±145.84**
120	3	4110.00±50.86**	2340.67±68.13*	4018.00±189.41**

*P<0.05 *vs.* control group ** P<0.01 *vs.* control group

[40,41,42]. Indeed, several studies support a role of COX-2 overexpression in tumor growth promotion [41,43]. Previous work showed isorhamnetin to be a potent inhibitor of COX-2 activity in the human colorectal cancer cell line HCA-7[29]. However, we observed in the present study that the level of COX-2 was initially upregulated and peaked at 48 h. These data suggest that COX-2 may participate in the isorhamnetin-induced stress response by preventing apoptosis.

Akt kinase is activated when it is phosphorylated on threonine 308 or serine 473 by phosphoinositide 3-kinase (PI-3K). This phospho-Akt has been proposed to activate NF-κB by phosphorylating IKK [13,44]. Some reports have also suggested that Akt directly regulates the expression of COX-2 [45]. However, our results show that isorhamnetin-induced NF-κB activation and upregulation of COX-2 expression in Eca-109 cells did not involve Akt phosphorylation. These findings suggest that NF-κB activation does not necessarily involve Akt phosphorylation, especially during exposure to isorhamnetin.

Recent reports have associated upregulation of Id-1 protein with cell proliferation in several human cell lines [46,47]. In prostate cancer cells, NF-κB activation may regulate the growth-promoting effect of Id-1 in order to protect against apoptosis [12]. In contrast to these reports, we found that under our experimental conditions, the level of Id-1 protein decreased during the first 48 h of exposure to isorhamnetin, while the expression of NF-κB increased. One possible explanation for these discrepant results is that isorhamnetin targets the gene encoding Id-1 and represses its transcription. Another possible explanation is that in Eca-109 cells, isorhamnetin-induced NF-κB activation does not involve Id-1.

COXs catalyze the oxidative metabolism of arachidonic acid into prostaglandin H2, which is the precursor of other prostaglandins including prostaglandin G2 (PEG2) [48,49]. PEG2 has been reported to inhibit Fas-induced apoptosis and markedly increase expression of Mcl-1 in KMBC cholangiocarcinoma cells [50]. Mcl-1 is a member of the Bcl-2 family and a potent inhibitor of the mitochondrial pathway of apoptosis. Thus, one way in which COX-2 inhibits apoptosis is by upregulating Mcl-1. Consistent with these previous studies, we found that the level of Mcl-1 increased during early exposure to isorhamnetin. In addition, the ratio of Bax to Bcl-2 proteins noticeably decreased from 0 to 48 h, and a decrease in this ratio is considered an indicator of apoptosis inhibition in mammalian cells.

The present study does not explain how isorhamnetin upregulates COX-2 expression. While previous work indicates that such upregulation occurs via downregulation of p53 or activation of the Akt signalling pathway [26,27,28], we did not observe changes in the levels of either p53 or phosphorylated Akt under our experimental conditions. Future studies should examine whether the COX-2 upregulation in this cell line occurs by a novel or known signaling pathway.

In conclusion, we provide here evidence that isorhamnetin causes a positive stress response in Eca-109 cells. NF-κB activation and upregulation of COX-2 are involved in this stress response during the first 72 h of exposure. The activation of the IκBα/NF-κB/COX-2 signaling pathway and the reduction in Bax/Bcl-2 ratio promote cell survival and proliferation during this period. Further studies are needed to determine whether additional molecules help to modulate the antiproliferative and apoptotic effects of isorhamnetin. In particular, although previous studies have shown that downregulation of p53 induces COX-2 overexpression, we did not see evidence that p53 is involved in the cell stress response under our experimental conditions. Further studies are needed to clarify the molecular events behind this stress response, which may help us understand the cellular processes that weaken or negate the anticancer properties of isorhamnetin.

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