Suppression of Amphiregulin/Epidermal Growth Factor Receptor Signals Contributes to the Protective Effects of Quercetin in Cirrhotic Rats

María J. Cuevas, Juliana Tieppo, Norma Possa Marroni, María J. Tuñón, and Javier González-Gallego

Department of Clinical Nutrition, University of León, León, Spain; Laboratory of Experimental Hepatology and Physiology, Porto Alegre Clinical Hospital, Federal University of Rio Grande do Sul, Porto Alegre 90035903, Brazil; and Laboratory of Oxidative Stress, Lutheran University of Brazil, Canoas 92425900, Brazil

Abstract

The hepatic wound-healing response to chronic noxious stimuli may lead to liver fibrosis, a key feature of the preneoplastic cirrhotic liver. Fibrogenic cells activate in response to a variety of cytokines, growth factors, and inflammatory mediators. The involvement of members of the epidermal growth factor family in this process has been suggested. Amphiregulin is an epidermal growth factor receptor (EGFR) ligand specifically induced upon liver injury. We investigated the effects of quercetin on the amphiregulin/EGFR signal and on the activation of downstream pathways leading to cell growth. Rats were divided into 4 groups (8 rats/group): rats subjected to common bile duct ligation (CBDL), sham rats subjected to simulated CBDL, quercetin-treated sham, and quercetin-treated CBDL (CBDL-Q). Quercetin (50 mg/kg i.p. injection) was administered daily for 2 wk starting on d 14 after surgery. Overexpression of amphiregulin, EGFR, TNFα, IL-6, TGFβ, platelet-derived growth factor (PDGF), extracellular regulated kinase, protein kinase B (Akt), cycloxygenase (COX)-2, and glioma-associated oncogenes (GLI)-1 and-2 were observed in liver of CBDL rats after 4 wk of bile duct ligation. CBDL-Q rats had a significantly diminished expression of amphiregulin and EGFR compared with untreated CBDL rats. Furthermore, mRNA levels of TNFα, IL-6, TGFβ, and PDGF and the protein content of COX-2, GLI-1, and GLI-2 were significantly lower in CBDL-Q rats than in untreated CBDL rats. The findings indicate that quercetin ameliorated activation of survival pathways and downregulated the expression of genes related to inflammation and precancerous conditions. Suppression of amphiregulin/EGFR signals may contribute to this effect. J. Nutr. 141: 1299–1305, 2011.

Introduction

Hepatocellular injury induced by common bile duct ligation (CBDL) is a consequence of cholestasis, which causes hepatocellular injury and inflammation. Hepatocellular injury results in the release of cytotoxic and inflammatory cytokines, including tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), transforming growth factor-β (TGFβ), platelet-derived growth factor (PDGF), extracellular regulated kinase (ERK), and protein kinase B (Akt). These signaling cascades contribute to the activation of pro-inflammatory and anti-inflammatory responses, leading to the development of liver fibrosis.

This complex response aims at the preservation of hepatocyte survival; the restoration of functional liver mass involves profound changes in gene expression and is mediated by an intricate network of cytokines, mitogens, and growth factors. Within this context, signaling through the epidermal growth factor receptor (EGFR), a transmembrane protein endowed with tyrosine kinase activity, is deemed essential. In fact, mature hepatocytes express the highest levels of EGFR of any normal cell type, suggesting an important role for EGFR signaling in liver function. Furthermore, the expression and contribution of EGFR ligands such as TGFα, heparin-binding EGF-like growth factor (HB-EGF), and amphiregulin to EGFR activation and hepatoprotection during liver injury and inflammation has been demonstrated. Thus, it is known that the expression of amphiregulin, TGFα, and HB-EGF in liver tissue is markedly increased in liver injury toxicity and liver dysfunction due to the build-up of bile acids and other toxins in the liver. Under these conditions, when the liver defensive capacities are exceeded and the loss of parenchymal cells occurs, a potent regenerative response is triggered. This complex response aims at the preservation of hepatocyte survival; the restoration of functional liver mass involves profound changes in gene expression and is mediated by an intricate network of cytokines, mitogens, and growth factors. Within this context, signaling through the epidermal growth factor receptor (EGFR), a transmembrane protein endowed with tyrosine kinase activity, is deemed essential. In fact, mature hepatocytes express the highest levels of EGFR of any normal cell type, suggesting an important role for EGFR signaling in liver function. Furthermore, the expression and contribution of EGFR ligands such as TGFα, heparin-binding EGF-like growth factor (HB-EGF), and amphiregulin to EGFR activation and hepatoprotection during liver injury and inflammation has been demonstrated. Thus, it is known that the expression of amphiregulin, TGFα, and HB-EGF in liver tissue is markedly increased in liver injury.
induced by carbon tetrachloride (6) and that amphiregulin-deficient mice develop significantly less collagen accumulation, suggesting that this EGFR ligand plays a nonredundant role in hepatic fibrosis. The expression of amphiregulin is also upregulated in chronic experimental liver damage and human liver cirrhosis and is readily detected in rodent liver after partial hepatectomy. Moreover, amphiregulin seems necessary for adequate DNA synthesis during mouse liver regeneration (7).

Taken together, these pieces of evidence demonstrate that the EGFR signaling system is an important component of the regenerative and wound-healing response of the liver. However, its perpetuation is thought to participate in the multistep process of hepatocarcinogenesis through the establishment of autocrine mechanisms for self-sustaining cellular growth (8). EGFR is highly expressed in normal and transformed hepatocytes and is thought to convey essential mitogenic and survival signals in transformed cells, including hepatocellular carcinoma (HCC) cells (9). Moreover, there is consistent evidence that amphiregulin, having both mitogenic and cytoprotective effects during liver injury and regeneration, is upregulated in human cirrhotic liver (10) and in HCC (11), being potentially implicated in liver tumorigenesis and cancer progression (12).

Results of epidemiological and intervention studies indicate that vegetables and fruits contain components that have a wide spectrum of pharmacological properties (13). Quercetin is one of the most common plant flavonoids found in the diet. This ubiquitous bioactive flavonoid has been found to induce a strong antioxidant, antiinflammatory, antifibrotic, antiproliferative, antiapoptotic, and antitumoral response. Most of above-mentioned effects of quercetin might result at least in part from the modulation of the EGFR signaling pathway. Thus, it has been reported that blockade of the EGFR activity by quercetin leads to growth inhibition and apoptosis of pancreatic cells (14) and suppresses growth in A431 cells (15). Moreover, quercetin acts as an inhibitor of the EGFR phosphorylation and suppresses the activation of the subsequent MAPK cascade in human colon carcinoma and vulva carcinoma cell lines (16). Very recently, it was shown that the downregulation of EGFR contributes to the antitumor effects of quercetin in Hela cells (17).

In this study, we investigated the potential of quercetin to ameliorate liver damage in cirrhotic rats by assessing its effects on the amphiregulin/EGFR signal and the activation of downstream pathways leading to cell growth, such as Akt and extracellular regulated kinase (ERK). Results obtained indicate that treatment with 50 mg kg⁻¹ of quercetin administered for 2 wk to bile duct-ligated rats, starting on day 14 after surgery, markedly decreased the inflammation associated with cirrhosis, ameliorated the activation of the most important survival pathways, and downregulated the expression of genes related to precancerous conditions.

**Materials and Methods**

**Materials.** Quercetin was purchased from Sigma Chemical. TaqMan primers and probes for IL-6 (GenBank accession nos. M26744.1 and Rn99999011_m1), TNFa (GenBank accession nos. X66539.1 and Rn99999017_m1), TGFβ (GenBank accession nos. X52498.1 and Rn00572010_m1), platelet-derived growth factor-β (PDGF-B) (GenBank accession nos. Z14117.1 and Rn01302393_m1), amphiregulin (GenBank accession nos. X55183.1 and Rn00567471_m1) genes and rat GAPDH endogenous control were derived from TaqMan-Gene Expression Assays (Applied Biosystems). Antibody against ERK1/2, pERK1/2, Akt1, pAkt1, EGFR, Amphiregulin, and glioma-associated oncoproteins (GLI)-1 and -2 were from Santa Cruz Biotechnology, antibody against cyclooxygenase-2 (COX-2) was from Abcam, and anti-GAPDH antibody was from Sigma.

**Rats and treatments.** The study protocol was approved by the institutional Animal Care Committee of the Hospital de Clínicas of Porto Alegre and conforms to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. A total of 32 male Wistar rats (Panlab) weighing ~200 g were used for the experiment. Rats were caged at 24°C with a 12-h-light:-dark cycle and free access to food (standard diet for rats Panlab A04). The composition of the standard diet was: 15.4% protein, 2.9% fat, 60.5% carbohydrate, 3.9% fiber, 5.3% minerals, and 12% water. Surgery for CBDL was carried out in 16 rats under sterile conditions. The common bile duct was gently exposed and doubly ligated with silk threads, after which a 2-mm segment was excised between the ligatures to prevent regeneration (18,19). A sham operation was performed in 16 rats in a similar manner to that for CBDL rats involving mobilization of the common bile duct but no ligation and excision. Four groups of rats (8 rats/group) were studied 4 wk after CBDL or a sham operation: an untreated sham-operated group (Sham), a quercetin-treated sham-operated group, an untreated CBDL group (CBDL), and a quercetin-treated CBDL group (CBDL-Q). Quercetin was suspended immediately before administration in a 0.2% Tween aqueous solution. Groups of sham and cirrhotic rats received daily a 50 mg kg⁻¹ i.p. injection of quercetin starting 14 d after surgery. Fourteen days after surgery, liver fibrosis is established, with an increase in collagen content, a characteristic pattern of perivenular and periportal deposition of α-smooth muscle actin, and the development of portal hypertension (20,21). The dose of quercetin was chosen based on our previous studies demonstrating that protective effects of this flavonoid in bile duct-ligated rats are maximal when administered at 50 mg kg⁻¹ d⁻¹ (18). After 28 d of obstruction, when there was a complete development of both cholestasis and cirrhosis and liver biochemical changes were clearly established (1,18), the rats were anesthetized using ketamine (100 mg kg⁻¹) and xylazine (10 mg kg⁻¹) and killed by exsanguination. Livers were collected and stored at −80°C.

**Real-time qRT-PCR.** Total RNA was extracted and reverse transcribed using a High-Capacity cDNA Archive kit (Applied Biosystems). cDNA was amplified using TaqMan Universal PCR Master Mix (Applied Biosystems) on a Step One Plus (Applied Biosystems). Each assay included a no-template control and a reverse transcriptase negative control. Relative changes in expression levels were determined using the 2⁻⁰ΔΔCT method (22). The cycle number at which the transcripts were detectable (CT) was normalized to the cycle number of GAPDH gene detection, referred to as ΔCT.

**Western-blot analysis.** Heparic lysates proteins (30 μg) were fractionated by SDS-PAGE and Western blotting was performed using the corresponding primary antibodies. Bound antibody was detected by enhanced chemiluminescence. Membrane rebinding with GAPDH antibody was performed for loading accuracy (23).

**Statistical analysis.** Results were expressed as means ± SEM. Data were analyzed using ANOVA with a 2 (quercetin) × 2 (cirrhosis) design. A Welch ANOVA was performed when Levene’s test indicated that variances were unequal. When a significant effect was found, post hoc comparisons were carried out by using the Tukey honestly significant difference test. Differences were considered significant at P < 0.05.

**Results**

Quercetin influences activation of amphiregulin/EGFR signaling system in cirrhotic rats. Liver EGFR protein level was significantly greater in untreated CBDL rats than in Sham rats. The effects were partly abrogated by quercetin treatment (Fig. 1A,B). After 4 wk of bile duct ligation, the amphiregulin protein level was significantly higher in CBDL rats and this effect was partly abolished in CBDL-Q rats (Fig. 1A,B). The transcriptional effect on the expression of amphiregulin gene was
also present at a post-translational level, as confirmed by Western blot. Analyses demonstrated that changes in protein concentration induced by bile duct ligation and quercetin treatment were parallel to those in mRNA levels (Fig. 1). Quercetin modulates activation of proliferation and survival signaling pathways in cirrhotic rats. To analyze whether inhibition of Akt phosphorylation is related to antiproliferative effects of quercetin, we measured the total and phosphorylated levels of this protein in the different experimental groups. Untreated CBDL rats had a marked expression of phosphorylated Akt. The protein level of phosphorylated Akt levels was less in the liver of CBDL-Q rats compared with the those of untreated CBDL rats (Fig. 2A,B). We also investigated whether quercetin modulated ERK. Similar to Akt regulation, phosphorylated ERK1/2 was overexpressed in untreated CBDL rats. Treatment of CBDL rats with quercetin inhibited ERK1/2 expression by decreasing the level of phosphorylated ERK1/2 (Fig. 2A,C).

**Quercetin reduces the upregulation of cytokines and growth factors.** Expression of IL-6 and TNFα mRNA levels were significantly higher in liver of CBDL rats than in Sham rats. Treatment with the flavonoid quercetin partly prevented IL-6 and TNFα overexpression in CBDL-untreated rats (Table 1). Dysregulation of the production of fibrogenic growth factors also plays an important role in hepatocarcinogenesis. In the present study, liver TGFβ and PDGF mRNA levels were significantly greater in CBDL rats than in the other experimental groups. Expression of TGFβ and PDGF was significantly lower in the CBDL-Q rats (Table 1).

**FIGURE 1** Quercetin influences activation of amphiregulin/EGFR signaling system in liver of cirrhotic rats. Representative Western-blot photographs for EGFR, amphiregulin, and GAPDH (A). Denisotmetric quantification of EGFR (B). Denisotmetric quantification of amphiregulin (C). mRNA levels of amphiregulin (D). Values are means ± SEM, n = 8 (means of duplicates). Significant effects of CBDL (C) and CBDL × Quercetin (C × Q) are shown. Means without a common letter differ, P < 0.05.

**FIGURE 2** Quercetin modulates activation of proliferation and survival signaling pathways in liver of cirrhotic rats. Representative Western-blot photographs for phospho-Akt, Akt, phospho-ERK1/2, ERK1/2, and GAPDH (A). Denisotmetric quantification of phospho-Akt (B). Denisotmetric quantification of phospho-ERK1/2 (C). Values are means ± SEM, n = 8 (means of duplicates). Significant effects of CBDL (C) and CBDL × Quercetin (C × Q) are shown. Means without a common letter differ, P < 0.05.
**TABLE 1** Effect of treatment with quercetin on liver mRNA levels of genes related to inflammation and fibrosis in rats with CLBD-induced cirrhosis.\(^1\)2

<table>
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<th></th>
<th>Sham</th>
<th>Sham-Q(^3)</th>
<th>CBDL</th>
<th>CBDL-Q</th>
<th>Q</th>
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<td>IL-6</td>
<td>1.0</td>
<td>1.4 ± 0.5(^a)</td>
<td>44 ± 3.6(^b)</td>
<td>32 ± 2.0(^b)</td>
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<td>1.1 ± 0.1(^a)</td>
<td>13 ± 0.7(^b)</td>
<td>8.1 ± 0.7(^b)</td>
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<td>0.0012</td>
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<tr>
<td>TGF(_\beta)</td>
<td>1.0</td>
<td>1.2 ± 0.3(^a)</td>
<td>4.1 ± 0.4(^b)</td>
<td>3.2 ± 0.2(^b)</td>
<td>0.08</td>
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<td>0.10</td>
<td>0.0014</td>
<td>0.0183</td>
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\(^1\)Values are means ± SEM, n = 8 (means of duplicates). Means without a common letter differ, P < 0.05.

\(^2\)Levels of mRNA were analyzed by real-time PCR assays and were normalized to GADPH.

\(^3\)Quercetin-treated sham-operated group.

**Quercetin reduces expression of genes associated to cancer progression in cirrhotic rats.** To study molecular changes in liver cirrhosis associated with HCC, it is necessary to identify molecules such as COX-2, GLI-1, and GLI-2, which have been associated with precancerous conditions. COX-2 protein levels were significantly higher in untreated CBDL rats compared with CBDL-Q rats (Fig. 3A,B). Moreover, CBDL rats showed a marked protein expression of GLI-1 and GLI-2 that was absent in CBDL-Q rats (Fig. 3A,C,D).

**Discussion**

Cirrhosis in a setting of chronic liver cell injury, with inflammation, hepatocyte necrosis, and regeneration, is a particular breeding ground for hepatocyte dedifferentiation and HCC (24). We previously reported that in the CBDL animal model of cirrhosis, treatment with quercetin attenuates the liver damage, as verified by macroscopic and histological findings; the improvement on markers of hepatic damage, including alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and bilirubin; and the downregulation of various profibrogenic molecules (19). In the present study, we further investigated the potential of quercetin to inhibit amphiregulin/EGFR signaling pathways and its contribution to liver damage in cirrhotic rats.

The EGFR signaling system is generally upregulated in acute and chronic liver injury (12) and is also involved in the regenerative and wound-healing response of the liver (25). There is solid evidence linking EGFR signaling to the progression of liver disease from chronic injury and inflammation to tumor development (12). Previous research has shown a sequential increase in the intra-hepatic expression of EGF at progressive time points (postoperative d 2, 7, 14, and 21) in bile duct-ligated rats (26). In agreement with other studies (16,17), we also found that quercetin is a potent inhibitor of EGFR, which markedly reduced the EGFR overexpression present in cirrhotic rats. Amphiregulin, a heparin-binding EGFR ligand, is upregulated in the premalignant cirrhotic human liver and experimental liver cirrhosis in rats (27). The expression of amphiregulin during liver injury serves protective and pro-regenerative functions that cannot be fully compensated for by other EGFR ligands, such as TGF\(_\alpha\) and HB-EGF, also induced during liver injury (9). It is worth noticing that whereas the expression of most EGFR ligands is detected in the liver under basal conditions, amphiregulin is induced only upon injury and inflammation and may represent an additional link between hepatic inflammation and fibrogenesis. In the present study, we provide by the first time, to our knowledge, evidence that the expression of amphiregulin is lower in cirrhotic rats treated with quercetin compared with untreated cirrhotic rats in parallel to the substantial attenuation of EGFR expression. It is known that reactive oxygen species induce tyrosine phosphorylation of EGFR and the expression of EGFR-related ligands, such as amphiregulin and HB-EGF, in cultured rat gastric epithelial cells (28). Another report demonstrated an upregulation of amphiregulin in bronchopulmonary dysplasia induced by oxidative stress (29). Therefore, although other mechanisms cannot be ruled out, the effects of quercetin on amphiregulin/EGFR signals...
could be mediated by the antioxidant properties of the flavonoid.

EGFR can activate potent prosurvival and mitogenic signaling cascades, which are of interest from a therapeutic perspective, because targeting them may help to induce hepatic stellate cell (HSC) apoptosis and attenuate liver fibrosis. Among then, Akt and ERK1/2 phosphorylation have been found to be activated in the livers of rats with hepatic fibrosis and a large proportion of human HCC (30). Amphiregulin treatment enhances the activation of Akt and ERK in lung epithelial cells (30) and stimulation of human and rodent liver cells with amphiregulin rapidly induces ERK1/2 and Akt phosphorylation (31,32). In addition, the suppression of amphiregulin blocks Akt signals involved in the progression of pancreatic cancer (33) and has also been reported to interfere with the regulation of cell growth through inhibition of the MAPK cascade in colon cancer cell lines (34). Quercetin has been reported to modulate the enzymes involved in proliferation and signal transduction pathways, including members of the MAPK family such as ERK and Akt (31,33). Our data indicate that quercetin decreased the levels of phosphorylated Akt and ERK1/2 in parallel to the impairment of amphiregulin/EGFR signals. This inhibition could contribute to a higher occurrence of cell death and therefore delay the progression to cancer.

The inflammatory reaction characteristic of chronic liver injury actively participates in the development of hepatic fibrosis as well as in the activation of the potent regenerative response of liver parenchyma (4,12). The production of cytokines such as TNFα and IL-6 is essential to trigger hepatocyte proliferation, liver regeneration, and animal survival after partial hepatectomy, as has been demonstrated in the corresponding genetically modified mice (TNF receptor type 1 and IL-6 knockout animals) (12). Moreover, other studies performed in experimental models of cancer and cultured cancer cells have demonstrated that these inflammatory mediators are good candidates to stimulate tumor growth and progression (36). It is known that activation of EGFR may be mediated by TGFβ release in NCI-H292 cells (37). TGFβ may be directly induced by stimulation with TNFα and indirectly by autocrine amphiregulin stimulation, acting as a secondary activator EGFR that prolongs the effect of TGFβ (38). It has also recently been shown that EGFR regulates the expression of IL-6 in human smooth muscle cells (39) and that amphiregulin gene silencing has a potent inhibitory effect on IL-6 secretion by salivary gland epithelial cells (40). The higher mRNA levels of TNFα and IL-6 due found in cirrhotic animals were markedly reduced with quercetin treatment. Therefore, quercetin could ameliorate liver injury, and therefore tumorigenesis, through an antiinflammatory effect, which in turn could be partly related to effects on amphiregulin/EGFR signals.

Approaches that inhibit HSC activation, proliferation, and matrix production at multiple locations may be more effective than those that target a single step of the fibrogenic cascade. Because TGFβ and PDGF are probably the most important profibrogenic cytokines (41), inhibition of their secretion, receptor activation, or downstream signal transduction should attenuate HSC activation and subsequent events (42). In this context, the interruption of TGFβ/PDGF downstream signal transduction through antioxidants appears to be an effective approach. Moreover, it is thought that the inflammatory environment contributes to shift the tumor-suppressive signaling of TGFβ on hepatocytes toward a migratory and mesenchymal phenotype, which is associated with tumorigenic progression (43). Antioxidants that are able to modulate the activity of these molecular mediators therefore represent potential therapeutic agents for the treatment of hepatic fibrosis and its complications. Results from the present research showed that quercetin administration downregulated the expression of TGFβ and PDGF in cirrhotic rats, suggesting that the flavonoid plays a role in inhibiting pathways connecting inflammation, fibrogenesis, and HCC growth, in which amphiregulin could be involved.

Published evidence indicates that the EGFR system undergoes extensive cross-talk with other inflammatory signaling pathways. Increased COX-2 expression and elevated prostaglandin 2 levels are found in chronic liver inflammation and cirrhosis, as well as human and experimental HCCs, and are thought to contribute to neoplastic transformation at early stages (12). COX-2-derived prostaglandin 2 is able to transactivate the EGFR receptor. In fact, combined treatment with TGFβ and components of EGFR pathway markedly increases COX-2 expression, and secretion of amphiregulin results in autocrine signaling through EGFR, providing a permissive stage for TGFβ to influence COX-2 expression (36). Our data demonstrate that upregulation of TGFβ and amphiregulin/EGFR in CBDL rats was associated with increased expression of COX-2, supporting that induction of COX-2 by TGFβ and amphiregulin may contribute to early stages of liver carcinogenesis. Furthermore, these effects were partly abrogated in cirrhotic rats receiving quercetin. The inhibitory effect of quercetin on COX-2 expression also has been observed in rat liver epithelial cells treated with arsenite, a metalloid carcinogen strongly associated with increased risk of liver cancer (34). The present research goes a step further by demonstrating the potential relevance of amphiregulin in the protective effect of quercetin against cirrhosis-induced COX-2 expression.

An additional finding was the reversal by quercetin of changes in the expression of Hedgehog (HH)/GLI signaling components induced by experimental cirrhosis. Persistent activation of the HH/GLI signaling pathway has been implicated in regulating growth, proliferation, and apoptosis of several cancer types, including HCC. The failure to terminate HH/GLI signaling leads to a persistent increase in GLI-1 and GLI-2 activity, which has been shown to account for the initiation and growth of HH-associated tumors (44). GLI activity also can be positively regulated by Akt, ERK, or TGFβ (45,46). Several recent studies have implicated the EGFR pathway in the modulation of GLI activity, and EGFR signaling seems to synergize with GLI-1 and GLI-2 to selectivity activate transcription of a subset of direct GLI target genes (47). Our data indicate that the higher GLI-1 and GLI-2 protein content in cirrhotic rats was coupled with higher levels of EGFR. Moreover, GLI expression was inhibited by quercetin treatment, supporting previous research that shows that several polyphenols, including quercetin, are able to decrease GLI1 mRNA concentrations in human and mouse prostate cancer cells (48). So, mechanistically, the HH/GLI signaling pathway could be a direct or indirect target of quercetin.

In summary, the results of our experiments demonstrate that quercetin treatment could prevent the progression of liver fibrosis and reduce the risk of liver carcinogenesis because of the beneficial effect on the inflammatory response, the regulation of several relevant growth factors, including amphiregulin and its receptor, and a sustained inhibition of the major survival signals, Akt and ERK. EGFR-targeted therapies have been shown to cause many side effects and it has been reported that EGFR agents can suppress ERK signal proliferation but not Akt survival signals (49). Therefore, the potential clinical usefulness of quercetin is worthy to be explored as an interesting alternative anti-EGFR therapeutic strategy in cirrhosis regression and in HCC chemoprevention and further efforts to investigate these possibilities are needed.
Acknowledgments

M.J.T., N.M., and J.G.G. designed research; M.J.T. and J.G.G. conducted research; M.J.C. and J.T. analyzed data; M.J.C., M.J.T., and J.G.G. wrote the paper; and J.G.G. had primary responsibility for final content. All authors read and approved the final manuscript.

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