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Research Article

Apple polyphenols diminish the phosphorylation of the epidermal growth factor receptor in HT29 colon carcinoma cells

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Previously, we showed that an apple juice extract (AE) potently inhibits the protein tyrosine kinase (PTK) activity of the epidermal growth factor receptor (EGFR). In the present study, an apple pomace extract (APE) was found to exceed the EGFR inhibitory properties of AE in a cell-free system. The impact of the extracts on the phosphorylation status of the EGFR in intact cells (HT29) was sensitive to catalase, added to suppress the accumulation of hydrogen peroxide. In the absence of catalase, the formation of hydrogen peroxide was observed, achieving 1.1 ± 0.1 μM (AE) and 1.5 ± 0.1 μM (APE) after 45 min of incubation. In the presence of catalase, suppressing the hydrogen peroxide level to the solvent control, APE effectively suppressed EGFR phosphorylation, even exceeding the effects of AE. Both extracts inhibited the growth of HT29 cells, albeit the enhanced EGFR inhibitory properties of APE compared to AE were not reflected by a higher growth inhibitory potential. The results clearly show that the effect of apple extracts on the EGFR and cell growth are not simply artefacts of hydrogen peroxide formation. However, the formation of hydrogen peroxide has to be considered to modulate and/or mask cellular responses to apple extracts.

Keywords: Apple pomace extract / Autophosphorylation / Cell signalling / ErbB1 / Hydrogen peroxide

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1 Introduction

Colorectal cancer is one of the leading causes of cancer mortality in western countries. Lifestyle and diet are proposed to play an important role in colon carcinogenesis. Plant polyphenols appear to represent a rich source of compounds with potentially chemopreventive properties [1–4]. In a rat model, cloudy apple juice was found to suppress dimethylhydrazine induced formation of aberrant crypt foci [5]. Apple extracts have been repeatedly reported to inhibit the growth of human tumour cells *in vitro* [6–12]. Little is known so far about the underlying mechanism of action. Malignant transformation is associated with changes in cellular signalling cascades, conducting cell growth, differentiation and the induction of apoptosis [1, 13, 14]. The ERK/MAPK (extracellular-signal-regulated/mitogen-activated protein kinase) pathway is one of the key signalling cascades in the regulation of cell growth [2, 3, 6]. Inhibition of the upstream located epidermal growth factor receptor (EGFR) results in the suppression of the activity of the subsequent MAPK cascade, associated with the inhibition of cell growth and the induction of apoptosis [3, 15]. HT29 colon carcinoma cells express substantial amounts of EGFR, sensitive to the respective growth factor (EGF) [16]. A specific EGFR inhibitor (tyrphostin AG1478) was found to potently inhibit the growth of HT29 cells, comparable to the effect on A431 cells, known to overexpress the receptor (unpublished data). These findings indicate that the EGFR is involved in the regulation of proliferation in HT29 cells.

We reported previously that a polyphenol-rich extract of a consumer-relevant apple juice blend potently inhibits the protein tyrosine kinase (PTK) activity of isolated EGFR in a cell-free test system [6]. We, furthermore, identified quer-

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Abbreviations: AE, apple juice extract; **APE,** apple pomace extract; **EGFR,** epidermal growth factor receptor; **FCS,** fetal calf serum; **PTK,** protein tyrosine kinase; **QG,** quercetin glycoside mixture

Table 1. Constituents of an apple pomace extract (APE) in comparison to an apple juice extract (AE) and an APE mimetic mixture of quercetin glycosides (QG)

Known constituents	AE (mg/g)	APE (mg/g)	QG (mg/g)
Procyanidin B1	7.0	6.2	–
Procyanidin B2	15.1	18.4	–
(−)-Epicatechin	19.2	17.7	–
(+)-Catechin	n.d.	2.7	–
Phloretinxyloglucoside	66.2	31.7	–
Phloridzin	27.9	78.9	–
Chlorogenic acid	181.5	19.2	–
3-Coumaroylquinic acid	9.5	3.0	–
4-Coumaroylquinic acid	77.3	5.0	–
5-Coumaroylquinic acid	10.4	3.8	–
Caffeic acid	4.8	4.0	–
p-Coumaric acid	n.d.	4.2	–
Quercetin-3-xyloside	n.d.	18.1	–
Quercetin-3-arabinoside	n.d.	3.5	–
Quercetin-3-rhamnoside	4.1	25.1	25.1
Quercetin-3-galactoside	0.8	8.1	8.1
Quercetin-3-glucoside	1.4	12.3	12.3
Quercetin-3-rutinoside	2.6	49.1	49.1
Total	427.8	311.0	94.6

n.d., not detected.

cetin glycosides in the apple extract as effective EGFR inhibitors [6]. However, considering the low amounts of these constituents in the apple juice extract (AE) (Table 1), the impact on the overall EGFR inhibitory effect is expected to be limited. To further explore the EGFR inhibitory properties of this class of compounds, we included in the testing an apple pomace extract (APE), characterised by a substantially higher content of quercetin glycosides (Table 1) [17, 18]. In order to address the question, whether these constituents play a crucial role for the biological effects of APE, an APE mimetic mixture of quercetin glycosides, available so far (Table 1), was included in the testing.

Recent reports indicate that the use of apple extracts in cell culture experiments might lead to the formation of hydrogen peroxide, generated by yet unknown reactions of apple polyphenols with cell culture media constituents. Thus, it has been speculated whether cellular effects of apple polyphenols might represent artefacts arising from hydrogen peroxide formation. Several strategies to suppress the hydrogen peroxide accumulation under cell culture conditions have been suggested, including the use of catalase [8, 10, 18, 19].

In the present study, the question was addressed whether the EGFR activity in intact human colon tumour cells (HT29) is affected by apple extracts and whether quercetin glycosides contribute to the EGFR inhibitory properties of APE. We, furthermore, investigated whether the extracts differ with respect to the formation of hydrogen peroxide under cell culture conditions and whether the presence of catalase is sufficient for its suppression.

2 Materials and methods

2.1 Chemicals

The polyphenol-rich AE was produced as reported previously [6]. An APE was generated from a mixture of different table apple varieties (Melrose, Granny Smith, Golden Delicious, Jonagold) according to [17, 20]. The different quercetin glycosides were purchased from Extrasynthèse (Genay, France) (Table1). For all the assays, the compound or extract solutions were freshly prepared in DMSO directly before starting the experiment. Catalase (100 U/mL, Sigma, Taufkirchen, Germany) was added to the tested compounds before cell incubation [10].

2.2 Cell culture

The human vulva carcinoma cell line A431 [21] was cultured in minimum essential medium (MEM; Sigma, Taufkirchen, Germany) containing L-glutamine (4.5 g/L). The human colon carcinoma cell line HT29 was cultivated in DMEM with 4500 mg/L glucose, without sodium-pyruvate (Invitrogen^{M} Life Technologies, Karlsruhe, Germany) in humidified incubators (37°C, 5% CO_2). Both cell culture media were supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin.

2.3 Hydrogen peroxide assay

HT29 cells were seeded in 24-well plates (50 000 cells *per* well), in FCS (10%) containing medium and allowed to grow for 48 h. Thereafter, cells were incubated with the respective extract or quercetin glycoside mixture (QG) for 45 min in serum-free medium to mimic the conditions of the studies on phosphorylated EGFR. Alternatively, starting with 5000 cells *per* well, the incubation was performed for 72 h in 10% FCS containing medium, to mimic the conditions of the sulforhodamine B (SRB) assay. At the respective time points (45 min, 72 h), hydrogen peroxide was measured in the medium using the Amplex Red Hydrogen Peroxide Assay Kit (Molecular Probes, Invitrogen Life Technologies, Karlsruhe, Germany).

2.4 SRB assay

The SRB assay was performed according to a modified method of Skehan *et al.* [22] as reported previously [2, 3, 6].

2.5 Tyrosine kinase assay

The EGFR was isolated from A431 cells and purified by affinity chromatography using wheat germ lectin agarose (Pharmacia Biotech, Uppsala, Sweden) according to Kern *et al.* [6]. The ELISA was carried out as described previously [2, 3, 6].

2.6 Western blot analysis

An aliquot of 1.2×10^6 HT29 cells was seeded *per* Petri dish and allowed to grow for 48 h. Thereafter, cells were serum-reduced (1% FCS) for 24 h and incubated with catalase (100 U/mL) and the respective compound for 45 min in serum-free medium. The EGFR of HT29 cells was stimulated with EGF (100 ng/mL) within the last 15 min of incubation. Cells were abraded at 4°C in 0.2 mL RIPA buffer (50 mM Tris/HCl, pH 7.4, 250 mM NaCl, 1 mM EDTA, 1 mM NaF, 1% v/v Igepal; 1 mM PMSF, 1 mM sodium *ortho*-vanadate and 2% protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) were freshly added to RIPA buffer). Thereafter, the lysate was homogenised thoroughly and subsequently centrifuged for 10 min (20 000 × *g*, 4°C). The supernatant was separated by SDS-PAGE (7% polyacrylamide gel) and the proteins were transferred onto a nitrocellulose membrane. Western blot was performed using mouse monoclonal antibodies against human EGFR and phospho-EGFR Tyr1173 (175 kDa; Cell Signaling Technology, Beverly, MA, USA) and an antimouse IgG peroxidase conjugate (Santa Cruz, Heidelberg, Germany) as a secondary antibody. Alpha-tubulin was used as a loading control. The respective chemoluminescent signals (LumiGLO, Cell Signaling Technology, Beverly, MA, USA) were analysed using the LAS 3000 with the AIDA Image Analyzer 3.52 software for the quantification (raytest, Straubenhardt, Germany). Arbitrary Light Units were plotted as test over control [%].

3 Results

3.1 Hydrogen peroxide formation

The formation of hydrogen peroxide was determined in the cell culture medium of HT29 cells using the Amplex Red Hydrogen Peroxide assay kit. After 45 min incubation, in accordance with the sample preparation for Western blot analysis, hydrogen peroxide formation was observed with both extracts at concentrations ≥ 100 µg/mL (Figs. 1A, B). At higher extract concentrations, the hydrogen peroxide levels increased up to 1.1 ± 0.1 µM (AE) and 1.5 ± 0.1 µM (APE) (Figs. 1A, B). At 250 and 500 µg/mL, APE was found to exceed significantly the hydrogen peroxide generating properties of AE. In the presence of catalase (100 U/mL) the formation of hydrogen peroxide was effectively suppressed to the level of the solvent control (Figs. 1A, B). After incubation of HT29 cells with both extracts for 72 h, in accordance with the incubation conditions of the SRB assay, hydrogen peroxide was not detectable, independent of the presence of catalase (data not shown).

The incubation of HT29 cells with a mixture of quercetin glycosides in a concentration corresponding to 500 µg/mL of APE did not lead to the formation of hydrogen peroxide in the cell culture medium after 45 min or 72 h (data not shown).

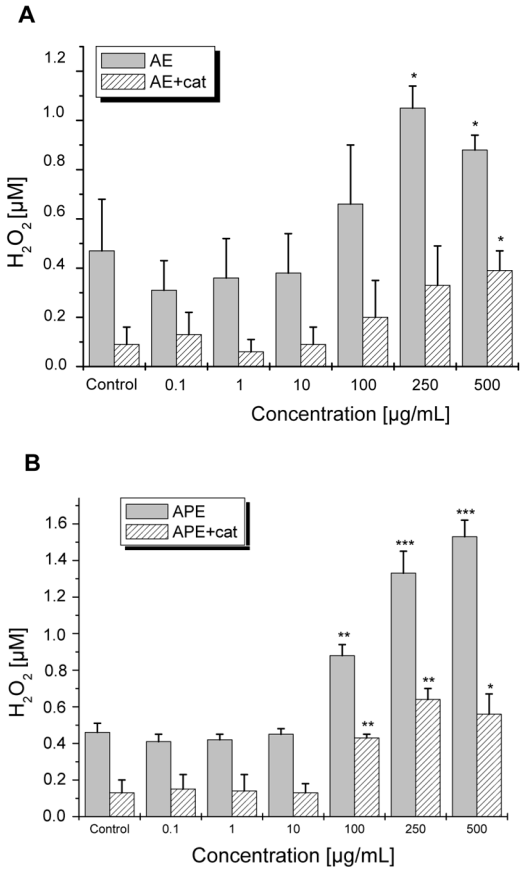

Figure 1. Hydrogen peroxide formation in cell culture medium (DMEM high glucose) by (A) AE and (B) APE in the presence and absence of catalase (cat, 100 U/mL) after 45 min of incubation. The data presented are the mean ± SD of at least two independent experiments, each performed in duplicate. * = $p <$ 0.05, ** = $p <$ 0.001, *** = $p <$ 0.005; The indicated significances refer to the respective control.

3.2 Growth inhibition

Growth inhibitory properties of the apple extracts were determined using the SRB assay [22]. As reported previously, the AE potently inhibited the growth of HT29 cells [6]. In the presence of catalase the growth inhibitory effect of AE was significantly diminished at concentrations ≥150 µg/mL (Fig. 2A). Also the APE exhibited growth inhibitory properties, albeit less effective than AE (compare Fig. 2A, B, Table 2). The presence of catalase significantly diminished the growth inhibitory effect of APE only at the highest concentration (500 µg/mL). Also in the presence of catalase both extracts mediated substantial growth inhibitory effects *in vitro*. In the presence and absence of catalase, AE showed a significantly higher growth inhibitory potency

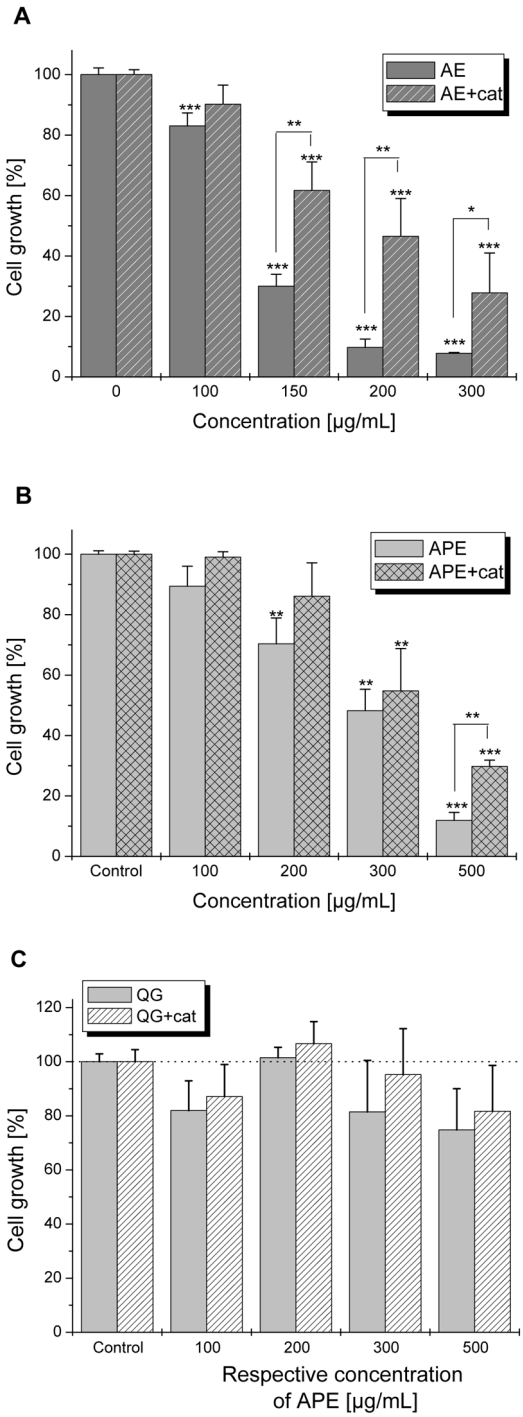

Figure 2. Inhibition of tumour cell growth *in vitro* determined in the sulforhodamine B (SRB) assay by (A) AE, (B) APE, (C) QG in the presence or absence of catalase (cat, 100 U/mL). HT29 cells were incubated for 72 h with the respective extract or QG. Growth inhibition was calculated as the percent survival of treated cells over control cells (treated with the solvent 1% DMSO) × 100(T/C%). The values given are the mean ± SD of at least three independent experiments, each performed in quadruplicate. * = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.005$; the indicated significances refer to the respective control.

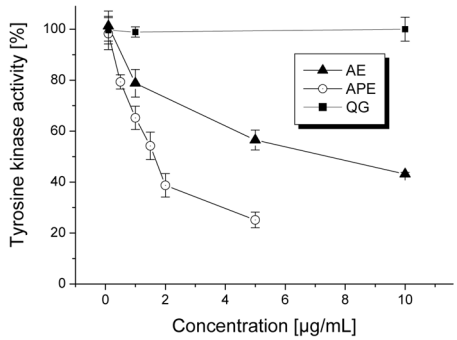

Figure 3. Inhibition of the tyrosine kinase activity of the EGF-receptor by AE, APE and an APE mimetic mixture of quercetin glycosides (QG). QG were applied according to their content in the respective APE concentrations. The phosphorylation of tyrosine residues of a peptide poly(Glu/Tyr) was determined by ELISA using an antiphosphotyrosine antibody linked to peroxidase. The data presented are the mean ± SD of at least three independent experiments, each performed in triplicate.

than APE (compare Figs. 2A, B; Table 2). A mixture of quercetin glycosides corresponding to growth-inhibitory concentrations of APE did not affect the growth of HT29 cells (Fig. 2C).

3.3 Inhibition of the PTK activity of the isolated EGFR

Effects on the PTK activity of isolated EGFR were determined using an ELISA. Both extracts were found to effectively inhibit the PTK activity of the isolated EGFR (Fig. 3, Table 2). In contrast to the results in the SRB assay, APE clearly exceeded the EGFR inhibitory properties of AE. However, a mixture of quercetin glycosides, corresponding to the EGFR inhibitory concentrations of APE, showed no effect on the activity of the isolated EGFR (Fig. 3). Even up to quercetin glycoside concentrations present in 500 µg/mL APE, no significant inhibition of the EGFR activity was observed (data not shown).

3.4 Modulation of EGFR autophosphorylation

We further addressed the question whether the inhibitory effects of the extracts are limited to the isolated EGFR preparation in the ELISA or are also of relevance within intact colon tumour cells. The activation of the EGFR by respective ligands is mirrored by its phosphorylation status. We investigated the effect of both extracts on the level of phosphorylated EGFR in HT29 cells by Western blot analysis.

In the absence of catalase, a significant decrease in phosphorylated EGFR was already seen after incubation with 1 µg/mL AE for 45 min (Figs. 4A, B). A concentration-dependent decrease in EGFR phosphorylation was determined upto 100 µg/mL AE. However, at higher concentrations the recurrence of phosphorylated EGFR upto the level

Table 2. Growth inhibitory properties of an apple juice extract (AE) and an APE in the sulforhodamine B (SRB) assay (HT29 cells, 72 h incubation) in the presence or absence of catalase (100 U/mL) and inhibition of the EGFR activity in the ELISA in comparison to effects on the phosphorylation status of the receptor in HT29 cells

Compound	Growth inhibition IC_{50} (μg/mL)		EGFR ELISA IC_{50} (μg/mL)	EGFR autophosphorylation IC_{50} (μg/mL)	
	Without catalase	Catalase (100 U/mL)		Without catalase	Catalase (100 U/mL)
AE	134 ± 18[a)]	188 ± 35	10 ± 5[a)]	5 ± 2	90 ± 12
APE	301 ± 28	365 ± 17	1.6 ± 0.2	–[b)]	48 ± 4

a) See [6].
b) No significant inhibition up to 500 μg/mL (Fig. 5A).

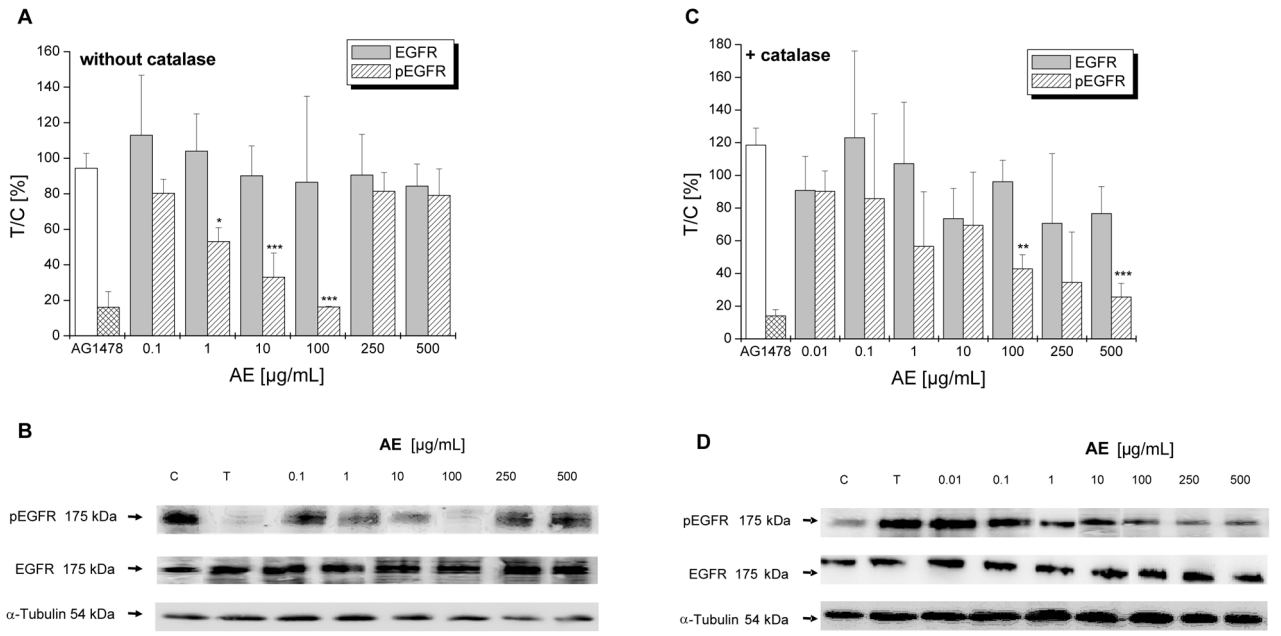

Figure 4. Western blot analysis of total EGFR protein and the phosphorylated EGFR in HT29 cells after 45 min treatment with AE. (A) Without catalase. (B) Representative Western blot of an experiment without catalase. (C) In the presence of catalase (100 U/mL). (D) Representative Western blot of an experiment in the presence of catalase (100 U/mL). The plotted data are the mean ± SD of $n = 4$ independent experiments with similar outcome. The data are presented as % of the solvent control stimulated by EGF (100 ng/mL). * = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.005$; the indicated significances refer to the respective control. C, solvent control (1% DMSO as final concentration); T = tyrphostin AG1478, EGFR specific inhibitor (5 μM).

of the solvent control was observed. In the presence of catalase, a statistically significant decrease in phosphorylated EGFR was found at concentrations ≥ 100 μg/mL AE, but without recurrence of the phosphorylated receptor upto 500 μg/mL (Figs. 4C, D).

In the absence of catalase, APE showed only marginal effects on the level of phosphorylated EGFR in HT29 cells. Only at a concentration of 100 μg/mL APE, a slight but significant decrease was observed, followed by a recurrence of the phosphorylated receptor upto the level of the solvent control (Figs. 5A, B). In contrast, in the presence of catalase APE effectively diminished the level of phosphorylated EGFR in a concentration-dependent manner without recurrence of the phosphorylated receptor upto 500 μg/mL (Figs. 5C, D). Thus, the presence of catalase substantially enhanced the inhibitory properties of APE.

An APE mimetic mixture of quercetin glycosides did not affect the activity of the phosphorylated EGFR in HT29 cells, irrespective of the presence or absence of catalase (data not shown).

4 Discussion

In the present study we showed that an AE and an APE inhibit the growth of the human colon carcinoma cell line HT29 *in vitro*. These results are inline with earlier reports on growth inhibitory properties of apple extracts, an effect which is

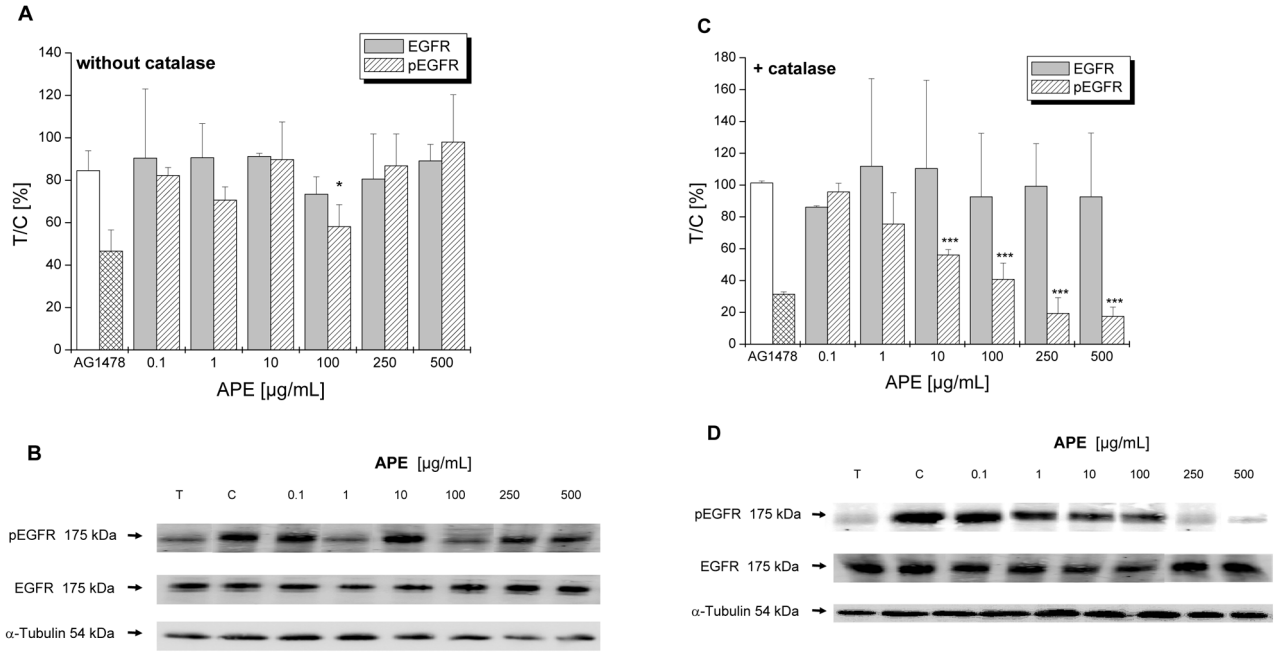

Figure 5. Western blot analysis of total EGFR protein and the phosphorylated EGFR in HT29 cells after 45 min treatment with APE. (A) Without catalase. (B) Representative Western blot of an experiment without catalase. (C) In the presence of catalase (100 U/mL). (D) Representative Western blot of an experiment in the presence of catalase (100 U/mL). The plotted data are the mean ± SD of $n = 4$ independent experiments with similar outcome. The data are presented as% of the solvent control stimulated by EGF (100 ng/mL). * = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.005$; the indicated significances refer to the respective control. C, solvent control (1% DMSO as final concentration); T = tyrphostin AG1478, EGFR specific inhibitor (5 µM).

mainly attributed to the comprised polyphenols and flavonoids [6–12]. The preparation process to generate the pomace extract led to substantial differences in the polyphenol composition compared to a respective juice extract (Table 1). Notably, a decrease in the content of coumaroylquinic acids and chlorogenic acid was observed, whereas quercetin glycosides were clearly enriched from a total of 8.9 mg/g (AE) to 116.2 mg/g (APE) (Table 1). Quercetin-3-galactoside and quercetin-3-glucoside were already reported to represent inhibitors of the PTK activity of the EGFR in a cell-free system [6], raising the question whether quercetin glycosides might substantially contribute to the cellular effects of APE. Thus, an APE-mimetic mixture of the comprised quercetin glycosides was included in the testing.

Recently, several studies indicated that the use of apple extracts in cell culture experiments might lead to the formation of hydrogen peroxide, probably generating experimental artefacts [8, 10, 18, 19]. Therefore, we addressed the question whether the observed cellular effects in our cell culture experiments were modulated by the presence of catalase, suppressing hydrogen peroxide accumulation. After 45 min treatment of HT29 cells with AE or APE, indeed, the formation of hydrogen peroxide was observed at concentrations ≥ 100 µg/mL (Figs. 1A, B). APE was found to exceed the hydrogen peroxide generating properties of AE significantly. In the presence of 100 U/mL catalase, the accumulation of hydrogen peroxide was effectively suppressed to the level of the solvent control (Figs. 1A, B). After 72 h of incubation with either extract, hydrogen peroxide was not detectable any more independent of the presence of catalase (data not shown). Respective results with an APE mimetic mixture of quercetin glycosides show that these constituents did not contribute to the hydrogen peroxide generating properties of APE.

The growth inhibitory effect of AE was found to be sensitive to the presence of catalase already at concentrations ≥ 150 µg/mL AE (Fig. 2A). However, the resulting IC_{50}-values with and without catalase were not significantly different (Table 2). The growth inhibitory effect of APE was found to be less sensitive to the presence of catalase (Fig. 2B). In the presence and absence of catalase, AE showed a significantly higher growth inhibitory potency than APE (Table 2). An APE mimetic mixture of quercetin glycosides did not affect the cell growth upto a concentration corresponding to 500 µg/mL APE (quercetin-3-glucoside 13 µM, quercetin-3-galactoside 9 µM, quercetin-3-rhamnoside 28 µM, quercetin-3-rutinoside 40 µM), indicating that these constituents do not play a crucial role for the growth inhibitory properties of APE (Fig. 2C). These results are inline with earlier studies with the single compounds, showing that these glycosides are not mediating substantial growth inhibitory effects in the SRB assay [6].

With respect to the potential cellular mechanisms, the impact of the extracts on the PTK activity of isolated EGFR was determined in a cell-free test system. In contrast to the results in the SRB assay, APE showed a higher potency for the inhibition of the EGFR activity than AE (Fig. 3, Table 2). The QG did not affect the isolated EGFR activity when applied according to EGFR-inhibitory concentrations of the APE (Fig. 3). Even upto concentrations corresponding to 500 μg/mL of APE, the QG showed no significant effect on the EGFR activity (data not shown). Whereas quercetin-3-glucoside and quercetin-3-galactoside effectively inhibit the EGFR activity when applied as single compounds [6], the inhibitory effects appear to be compensated in an APE mimetic mixture of the four glycosides.

We, furthermore, addressed the question whether the potent inhibition of isolated EGFR by AE and APE is also of relevance within intact tumour cells. The activity of the EGFR is mirrored by its phosphorylation status, which is crucial for the interaction of the PTK domain with downstream elements of the MAPK cascade [14, 23, 24]. Inhibition of the autophosphorylation of the EGFR has already been shown for several flavonoids such as the green tee catechin (–)-epigallocatechin-3-gallate, the flavonol quercetin or the flavone luteolin [3, 4, 6, 25 – 27].

Treatment of HT29 cells with the polyphenol-rich AE in the absence of catalase was found to effectively suppress EGFR autophosphorylation in a concentration range of 1 – 100 μg/mL (Fig. 4A, Table 2). These results correspond to the PTK-inhibitory concentration in the EGFR ELISA (Table 2). However, AE concentrations ≥ 250 μg/mL, found to generate substantial amounts of hydrogen peroxide (Fig. 1A), led to the recurrence of the phosphorylated EGFR to the level of the solvent control (Figs. 4A, B). In the presence of catalase, the suppression of EGFR phosphorylation by AE was observed, albeit less distinct in the concentration range from 1 – 100 μg/mL. Noteworthy, a recurrence of phosphorylated EGFR at higher concentrations was completely prevented when the accumulation of hydrogen peroxide was suppressed by the addition of catalase (Figs. 1A, 4C, D). In contrast to AE, APE showed only marginal effects on the phosphorylation status of the EGFR in the absence of catalase (Figs. 5A, B). An initial inhibitory effect at 100 μg/mL APE was followed by a complete recurrence of the phosphorylated receptor at higher concentrations. However, APE was found to effectively inhibit the phosphorylation of the EGFR when hydrogen peroxide was degraded by catalase (Figs. 1B, 5C, D, Table 2). Thus, for both extracts, the presence of catalase prevented the recurrence of phosphorylated EGFR at concentrations ≥ 250 μg/mL, indicating that hydrogen peroxide formation counteracts the inhibitory effect of the extracts in that concentration range.

The effects of catalase in the concentration range 1 – 100 μg/mL were contradictory for the two extracts. The presence of catalase diminished the inhibitory effect of AE to some extent but enhanced the effectiveness of APE. However, the results clearly showed that also in the presence of catalase AE suppressed the phosphorylation of the EGFR in HT29 cells. At least in the presence of catalase, APE was found to diminish EGFR phosphorylation, even exceeding the potency of AE, inline with the results in the cell-free system. However, the enhanced effectiveness of APE was not mirrored by an increase in growth inhibitory properties (Table 2). The EGFR and the subsequent signalling cascade are known to play an important role in the regulation of cell growth [2 – 4, 6, 23, 24]. The apparent discrepancy between the effectiveness against the EGFR and cell growth indicates that additional cellular targets might be affected involved in the regulation of cell proliferation. It is tempting to speculate that the complex mixture of polyphenols in the apple extracts affects several potential cellular targets which might at least partially compensate or enhance each other.

In summary, in growth-inhibitory concentrations apple extracts were found to potently inhibit the activity of the EGFR, an effect which was not limited to the cell-free system. Also in intact cells a decrease in phosphorylated EGFR was observed, at least in the presence of catalase. The results clearly show that the growth inhibitory effects and the impact on the EGFR by apple extracts are not simply artefacts of hydrogen peroxide formation. However, the formation of hydrogen peroxide has to be considered to modulate cellular responses to apple extracts and/or to mask cellular effects as seen for the recurrence of phosphorylated EGFR in the absence of catalase.

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