Terfenadine-induced apoptosis in human melanoma cells is mediated through Ca\textsuperscript{2+} homeostasis modulation and tyrosine kinase activity, independently of H1 histamine receptors

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In our previous works, we have demonstrated that terfenadine (TEF) induces DNA damage and apoptosis in human melanoma cell lines. In this present work, we have studied the effect of histamine on viability of A375 human melanoma cells and the cell-signalling pathways through which TEF may induce its apoptotic effect. We have found that exogenous histamine stimulates A375 melanoma cell proliferation in a dose- and time-dependent manner. Moreover, TEF-induced apoptosis seems to occur via other cell pathways independent of the histamine-signalling pathway since co-treatment of histamine with TEF did not protect melanoma cells from the cytotoxic effect of TEF, and alpha fluoromethylhistidine did not induce the same cytoxic effect of TEF. In addition, we have observed that knocking down the H1 histamine receptor (HRH1) by small interference RNA approach protects melanoma cells only slightly from TEF-induced apoptosis. To explore the molecular mechanisms responsible for histamine and TEF effect on the cell growth, we analysed intracellular cyclic nucleotides and Ca\textsuperscript{2+} levels. TEF did not modify intracellular levels of cyclic adenosine 3’,5’-monophosphate and cyclic guanine 3’,5’-monophosphate; however, TEF induced a very sharp and sustained increase in cytosolic Ca\textsuperscript{2+} levels in A375 melanoma cells. On the contrary, histamine did not modulate intracellular Ca\textsuperscript{2+}. TEF-induced Ca\textsuperscript{2+} rise and apoptosis appear to be phospholipase C (PLC) dependent since neomycin and U73122, two inhibitors of PLC, abolished cytosolic Ca\textsuperscript{2+} increase and protected the cells completely from cell death. Furthermore, inhibition of tyrosine kinase activity by genistein blocked cytosolic Ca\textsuperscript{2+} rise by TEF-induced apoptosis. These results suggest that TEF modulates Ca\textsuperscript{2+} homeostasis and induces apoptosis through other cellular pathways involving tyrosine kinase activity, independently of HRH1.

Introduction

Histamine is a well-known biogenic amine acting as a neurotransmitter in the central nervous system (1). It has been reported that histamine modulates cell proliferation in rapidly proliferating tissues such as the embryonic tissues, healed wound tissues and malignant tumours (2–4). Intracellular histamine levels are regulated by histidine decarboxylase, the only enzyme that catalyses the formation of histamine from L-histidine (5). Histamine acts on the cells through a group of receptors belonging to the G protein family called histamine receptors. They are classified into histamine H1, H2, H3 and H4 receptors (6,7). G protein-coupled receptors are the largest superfamily of cell-surface receptors. Melanoma cells are characterized by expressing H1, H2 and H3 histamine receptors; however, the expression of these receptors is cell line specific (8,9). Diverse clinical studies suggest that H2 antagonists have potential beneficial effects in the treatment of advanced melanoma (2,10). However, the role of histamine on melanoma is not fully understood.

H1 histamine receptors (HRH1) mediate the functional effects of histamine in multiple cell types through activation of the Gαq/11 heterotrimeric G protein and its downstream effector phospholipase C (PLC). PLC breaks down phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and 1,2-diacylglycerol. IP\textsubscript{3} acts on specific receptors in the endoplasmic reticulum (ER) membrane and mobilizes Ca\textsuperscript{2+} from ER stores, which activates certain proteins involved in the cell proliferation and survival process (11,12).

Terfenadine (TEF) is one of the second-generation HRH1 antagonists that penetrate poorly into the central nervous system (12). In addition to its anti-histaminic activity, TEF has been shown to block voltage-dependent ion channels and to reverse drug resistance in a variety of cell types via its interaction with P-glycoprotein (13). Apoptosis is a physiological type of cell death that plays an important role in normal development and tissue homeostasis, as well as in the pathogenesis and therapy of malignant diseases (14,15). Apoptosis is characterized by specific morphologic alterations such as cell shrinkage and rounding, membrane blebbing, chromatin condensation and margination and nuclear fragmentation (16) and biochemical changes as externalization of phosphatidylserine, internucleosomal breakdown of the chromosomal DNA and caspases cascade activation (17).

Ca\textsuperscript{2+} has strongly been implicated in regulation of the apoptotic signalling pathways and induction of apoptosis. Early and late increases in intracellular Ca\textsuperscript{2+} concentrations have been reported in apoptosis. The mode of cell death induced by Ca\textsuperscript{2+} depends on the level of intracellular Ca\textsuperscript{2+} rise, so that very high intracellular Ca\textsuperscript{2+} has been reported to promote cell death through necrosis, whereas lower intracellular calcium increases promote cell death by apoptosis (18). Two major ways whereby eukaryotic cells can increase their cytosolic Ca\textsuperscript{2+} concentration are the release of ions from intracellular stores (mainly the ER) and the entry of Ca\textsuperscript{2+} into the cell from the external medium (19).

Previously, we had found that various HRH1 antagonists induce DNA damage and apoptosis in different human tumour cell lines (20,21). We extended our study on human melanoma cells by analysing the effect of histamine on cell viability and the primary molecular mechanism beyond the effect of TEF on human melanoma cells. We found that TEF induces tyrosine kinase and PLC-dependent apoptosis in these cells through modulation of Ca\textsuperscript{2+} homeostasis.

Materials and methods

Chemicals and reagents

Histamine, cimetidine, TEF, alpha fluoromethylhistidine (α-FMH), Dulbecco’s modified Eagle’s medium (DMEM), 1-glutamine, streptomycin–penicillin antibiotic solution, dimethylsulfoxide (DMSO), ethyleneglycol-bis(amoenoxy-lether)-tetraacetic acid (EGTA), thapsigargin, U73122, genistein and Trition 

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X-100 were all obtained from Sigma Chemical Co. (St Louis, MO). Culture flasks were from Falcon Plastics, Becton-Dickinson Laboratories, Orangeburg, NY, and fetal bovine serum was from Biochrom AG, Berlin, Germany. Sodium 3-[1-(phenylaminocarbonil)-3,4-tetrazolim] –bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) viability assay kit was purchased from Roche Molecular Biochemicals Indianapolis, IN and the Annexin V FITC assay kit was from Oncogene (San Diego, CA). Polyclonal antibody anti-histamine receptor 1 (1.8 mg/ml) was purchased from Acris Antibodies GmbH (Hiddenhausen, Germany), whereas the anti-actin antibody was from Chemicon International (Temecula, CA). Goat anti-rabbit IgGs were from Zymed Laboratories (South San Francisco, CA). Polyclonal rabbit anti-cleaved caspase-3 antibody was obtained from Cell Signalling Technology (Danvers, MA) and ALEXA Fluor goat anti-rabbit secondary antibody from Molecular Probes (Europe BV, Leiden, The Netherlands). Fura-2 acetoxymethyl ester was from Molecular Probes (Eugene, OR) and neomycin from Calbiochem (Darmstadt, Germany). N\(^\circ\)\(^\circ\)\(^\circ\)\(^\circ\)\(^\circ\)\(^\circ\)\(^\circ\)\(^\circ\) \(\text{H}\) assay system was from Amersham Biosciences (Buckinghamshire, UK) and cyclic guanine 3',5'-monophosphate (cGMP) assay was from R&D Systems (Lille Cedex, France). Two small interference RNAs (siRNAs) from human HRH1 (NM_000861) were provided by Ambion, Huntingdon, Cambridgeshire, UK, sense, 5'-GGACAAGUGUGAGACCT-3' and antisense, 5'-GUCUGUCUCACACUGCCC-3' and sense, 5'-GGGUAAUUUUCUAACUCUATT-3' and antisense, 5'-UAGAGUUGAAGAAAUUACCC-3'. Silencer siRNA transfection II kit, KDalert™ glyceraldehyde-3-phosphate dehydrogenase (GAPDH) assay kit and Cells-to-Signal™ were purchased from Ambion. OPTI-MEM I medium was from Invitrogen (Karlruhe, Germany). The sequences of the specific primers as well as the amplified fragment size for each gene were as follows: Fw 5'-GCTGGGCTACATCAACTCCAC-3' and Rv 5'-CCCTTAGGAGCAATATGCAGAAA-3' for human HRH1 gene (103 bp) and Fw 5'-CCTGTGACAGTCAGGGCGTGG- AAATATGCAGA-3' for human GAPDH (102 bp). All primers were designed using the Primer Express Software, Version 2.0 (Applied Biosystems, Foster City, CA) and were synthesized commercially (Bonsai Technologies Group, Madrid, Spain).

Fig. 1. Effect of histamine, TEF and \(\alpha\)-FMH on cell viability of A375 melanoma. Cell cultures were treated with various concentrations of histamine for 24–48 h (A) and TEF for 2–8 h (B) in DMEM culture medium. Results are expressed as a percentage of untreated controls. Data represent means ±SDs of six determinations of three separate experiments. \(P<0.05\) and \(P<0.01\) (Student's t-test). (C) Cells were incubated with (100 nM–5 mM) histamine for 15 min followed by addition of 10 \(\mu\)M TEF for 8 h. (D) Cell cultures were incubated with \(\alpha\)-FMH (1–200 \(\mu\)M) for 24–72 h. Cell viability was assessed by XTT as described in Materials and Methods. Results are expressed as the percentage of untreated controls. Data represent means ±SDs of six determinations of three separate experiments. \(P<0.05\) and \(P<0.01\) (Student's t-test).
viability was determined by XTT assay after 72 h of siRNA transfection. HRH1 mRNA attenuation by siRNA treatment prevents the cytotoxic effect induced by TEF on melanoma cells in a 20%. Data represent means ±SDs of three experiments. (21). After three rinses with phosphate-buffered saline (PBS), the cells were incubated with 4,6-diamidino-2-phenylindole for 10 min at room temperature to stain the nucleus. Last, the cells were viewed and analysed with a confocal microscope (Olympus FV 500). Images were obtained by sequential acquisition to avoid overlapping. The cells of 10 microscopic fields were counted and the percentage of caspase-3-positive cells was calculated.

Caspase-3 activation was detected in A375 cells using an immunofluorescence technique. Melanoma cells were pretreated with 25 μM genistein for 2 h, and then incubated with 10 μM TEF for 4 h. The cells were fixed, blocked and labelled with polyclonal rabbit anti-cleaved caspase-3 antibody as described in Jangi et al. (21). After three rinses with phosphate-buffered saline (PBS), the cells were incubated with 4,6-diamidino-2-phenylindole for 10 min at room temperature to stain the nucleus. Last, the cells were viewed and analysed with a confocal microscope (Olympus FV 500). Images were obtained by sequential acquisition to avoid overlapping. The cells of 10 microscopic fields were counted and the percentage of caspase-3-positive cells was calculated.

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Real-time reverse transcription–PCR

cDNAs were directly synthesized from 4 μl of cell lysates from the negative
siRNA control sample and both 1846 and 1935 HRH1 siRNA-transfected
samples, using the Cells-to-Signal™ Reverse Transcription Kit. Real-time
PCR assays were performed with an iCycler™ PCR platform (Bio-Rad, Her-
cules, CA). The reaction mixture contained 5 μl of cDNA from the reverse
transcription reaction together with forward and reverse specific primers (250
nM each) and iq™ SYBR Green Supermix (Bio-Rad) in a final reaction
volume of 20 μl. Thermal cycling conditions were as follows: an initial poly-
merase-activating step at 95°C for 3 min followed by 50 cycles with a 15 s at
95°C denaturation step, a 15 s at 60°C annealing step and a 15 s at 72°C
extension step during which data were collected. Each assay included no-
template control. Expression data were generated from four amplification re-
actions with samples and controls run in triplicate and performed on three
cDNA samples reverse transcribed from cell lysates prepared from three in-
dependent culture assays. Optical data obtained by real-time PCR were ana-
lysed using the MyiQ™ Single-Color Real-Time PCR Detection System
Software, Version 1.0 (Bio-Rad). The dynamic range of detection for each
gene was determined by preparing 2-fold serial dilutions from a 5-fold dilution
of the negative control cDNA sample. The reliability of real-time PCR was
defined by regression analysis of average cycles threshold versus the log2 of
the target copy number. Efficiency of PCRs was ~85% with all primer pairs.
Melt curve analysis of each PCR assay and agarose gel electrophoresis analysis
of randomly selected samples were performed to confirm the specificity of the
amplification products. Expression data obtained by quantitative PCR analysis
of the HRH1 gene were normalized to those of the housekeeping GAPDH gene,
using the Bio-Rad Gene Expression Macro™ Software Version 1.1 derived
from the algorithms outlined by Vandesompele et al. (22).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and
immunoblotting

A375 control cells and 1846 and 1935 HRH1 A375-transfected cells
were harvested by trypsinization. Cell pellets were resuspended in a lysis
buffer (sodium dodecyl sulfate 1%, sodium orthovanadate 1 mM and Tris-
HCl 10 mM, pH 7.4) and protease inhibitor cocktail (Roche, Molecular
Biochemicals) at 2 x 10⁶ cells/100 μl. Forty micrograms of each protein
supernatant was subjected to electrophoresis using 15% polyacrylamide gel,
transferred and developed as described by Asumendi et al. (23). An anti-
histamine receptor 1 polyclonal antibody was used at a dilution of 1/30 and an-antiantibody at a 1/200 dilution was used as a control.

Intracellular cAMP and cGMP levels

The intracellular levels of cAMP were determined by using the cAMP assay
kit—Amersham Biosciences (UK). The assay is based on the competition be-
tween unlabelled cAMP and a fixed quantity of the tritium-labelled compound for
binding to the cAMP-specific antibody. Cells were seeded into a Petri dish at a den-
sity of 10⁶ cells/ml and incubated in serum-free DMEM in the absence (control)
or presence of TEF (10 μM) for 15, 30 and 60 min. The samples were duplicated.
At the indicated times, the medium was eliminated and the cells washed twice
with PBS. Cells were removed with a rubber policeman from the plates, resus-
pended in PBS into a Petri dish and centrifuged. Five hundred microlitres of
acidic ethanol (1 ml 1 N HCl/100 ml ethanol) was added to the pellet, mixed
and left to stand for 5 min at room temperature. The samples were centrifuged
at 13 000 r.p.m. for 10 min, and the supernatant was collected. The precipitate
was washed with 1 ml ethanol:water (2:1) and centrifuged. Supernatants were com-
bined and evaporated to dryness at 50°C under vacuum. The residue was resus-
pended in 75 μl of Tris/ethylenediaminetraacetic acid buffer. Fifty microlitres
were assayed for cAMP quantification according to the manufacturer’s protocol.
The intracellular levels of cGMP were determined using the cGMP assay kit
from R&D Systems. This assay is based on the competitive binding technique
by which cGMP present in a sample competes with a fixed amount of horse-
radish peroxidase-labelled cGMP for sites on a rabbit polyclonal antibody.
During the incubation, the polyclonal antibody bound to the goat anti-rabbit
antibody coats onto the microplate. Following a wash to remove excess of
conjugate and unbound sample, a substrate solution is added to the wells to
determine the bound enzyme activity. Colour development is stopped and the
absorbance is read at 450 μM. The intensity of the colour is inversely pro-
portional to the concentration of cGMP in the samples. Untreated and TEF-
treated cells (5 x 10⁶ cells) for 15, 30 and 60 min were removed and lysed
before assaying according to the manufacturer’s protocol. Two hundred micro-
litres of cell lysates was required to perform the assay in duplicate. The sen-
sitivity of the assay was 1.14 pmol/ml.

Cytosolic Ca²⁺ measurement

Trypsinized cells (3 x 10⁶ cells/ml) were resuspended in DMEM containing
5% fetal bovine serum. Fura-2 loading was achieved by incubating cells with

2 μM Fura-2 acetoxyethyl ester dissolved in DMSO (0.2% final concentra-
tion) for 40 min at room temperature. After dye loading, the cells were washed,
resuspended in HEPES-buffered saline (145 mM NaCl, 5 mM KCl, 1 mM
MgCl₂ and 10 mM HEPES, pH 7.4), supplemented with 10 mM glucose to
a cell density of 1 x 10⁶ cells/ml and kept at room temperature under constant
rolling agitation until use. Control cells (used for autofluorescence measure-
ments) received the same volume of DMSO and were incubated under similar
conditions.

Fura-2 fluorescence of A375 cells was measured in a Perkin-Elmer LS-50
fluorometer. Samples (1.5 ml) of cell suspensions were added to the cuvettes,
equilibrated at 30°C and gently mixed during measurement. CaCl₂ (1 mM) or
1 mM EGTA (Ca²⁺-free medium) was added to the cuvettes 1 min before any
further addition. Fluorescence was registered continuously at excitation wave-
lengths of 340 and 380 nm and an emission wavelength of 505 nm. [Ca²⁺]
was calculated as described previously (24) from the ratio of 340:380 fluorescence
from the following formula: [Ca²⁺] = k x S380/S380 (R – Rmin)/Rmax – R; where Kd is 200 nM, S380/S380 is the ratio of the intensities of the free
and bound dye forms at 380 nm, R is the fluorescence ratio (340:380 nm) of the
intracellular fura-2, Rmax and Rmin are the maximal (Ca²⁺-saturated condition)
and minimal (Ca²⁺-free condition) fluorescence ratios obtained after additions
of 0.2% Triton X-100 and 4 mM EGTA, respectively. All traces are typical of at
least three experiments.

Comet assay

Treated and untreated A375 melanoma cells were harvested by trypsinization,
centrifuged and diluted into PBS 1 x. The comet assay was basically performed
as described previously (21). In brief, the cells seeded on the standard
slides coated with low and normal melting agarose gel and electrophoresis
was done at 4°C for 20 min at 1.6 V/cm and 300 mA under yellow light
or in the dark to prevent additional DNA damage. After staining with

Fig. 3. TEF-induced apoptosis is independent of cyclic nucleotides. (A) cAMP intracellular levels in A375 melanoma cells. Cells (10⁶ per plate) were
cultured in serum-free DMEM in the absence (control) or presence of TEF (10 μM) for 15–60 min. After incubation, intracellular cAMP levels were
determined by radioimmunoassay as described in Materials and Methods.
Data represent the mean ±SD of three experiments. (B) Effect of adenylate
cyclase and phosphodiesterase inhibitors on cell viability of A375 melanoma
cells. Cell cultures were treated with SQ-22536 (adenylate cyclase inhibitor),
IMBX (phosphodiesterase inhibitor) for 2 h and then TEF 10 μM was added for
4 h in DMEM culture medium. Also, dibutyl cAMP (300 μM) was added
to the culture cells to study the effect of cAMP on viability of the melanoma
cells. Results are expressed as a percentage of untreated controls.
Data represent means ±SDs of three determinations of three separate
experiments. *P<0.05 (Student’s t-test).

Tyrosine kinase and Ca²⁺-dependent apoptosis in melanoma cells by terfenadine
4,6-diamidino-2-phenylindole (1 μg/ml), digital images were taken using a Leica digital camera under a Leitz DMRB fluorescence microscope.

Statistical analysis
The level of statistical significance between sample means was determined by using the Student’s t-test; P < 0.05 was considered statistically significant. Non-linear regression analysis was performed using GraphPad Prism Version 3 for Windows (GraphPad Software, San Diego, CA) to calculate the half maximal inhibitory concentration for tested products.

Results
**TEF-induced cytotoxicity on A375 melanoma cells is independent of HRH1**
First, we assessed the effect of histamine, cimetidine and TEF on cell proliferation and viability in A375 melanoma cells by XTT assay kit. We found that 10 nM histamine did not modify cell proliferation after 24 h, whereas after 48 h of treatment the same dose exerted a significant stimulating effect (Figure 1A). The effect was also dose dependent and reached the peak at 10 μM after 48 h with an half maximal effective concentration of 650 nM. In contrast, TEF induced a dose- and time-dependent cytotoxicity on A375 human melanoma cells. The effect was apparent after 2 h of treatment and reached the maximum after 8 h of 10 μM TEF with an half maximal inhibitory concentration of 6.7 μM (Figure 1B). Cimetidine did not show any significant effect on A375 melanoma cells at the tested doses (up to 1 mM) and time points (up to 48 h) (data not shown).

In order to verify whether TEF-induced cytotoxicity is mediated through HRH1 on the human melanoma cells, we studied the effect of co-treatment of histamine with TEF on A375 cell viability and proliferation. Figure 1C shows that histamine can protect the cells only...
slightly from cytotoxicity induced by TEF. The protecting action was significant at a dose of 100 μM and reached the peak at a dose of 1 mM when the cell viability increased by 20%. To further establish the role of endogenous histamine on A375 melanoma cell viability, cells were treated with different concentrations of α-FMH, a histidine decarboxylase enzyme inhibitor, for 24–72 h. We found that the cell viability decreased in a dose- and time-dependent manner. The effect was significant at 100 μM after 72 h when the cell viability was reduced by 20% (P < 0.05) (Figure 1D). These data suggest that histamine blockage does not seem to be the only mechanism by which TEF induces apoptosis on A375 melanoma cells.

To further explore the role of HRH1 in TEF-induced apoptosis, siRNA specific for two sequences of HRH1 mRNA was used to knock down its expression in melanoma cells. Transfection optimization was performed using GAPDH mRNA as target and siPORT amine as transfection agent. The remaining GAPDH activity in GAPDH siRNA-transfected cultures relative to negative control was 30% and the viability was 85%. Using the same culture conditions, two different siRNAs specific for HRH1 were transfected on melanoma cells. After 72 h of transfection, a quantitative SYBR Green PCR was done from cell lysates. As shown in Figure 2A, the relative levels of HRH1 mRNA were significantly decreased by 80 and 40% using 1846 and 1935 siRNAs, respectively. HRH1 protein on A375 melanoma cells also decreased significantly when analysed by western blot (Figure 2B). The viability of melanoma cells measured by XTT assay after siRNA transfection experiments was not affected significantly, except when HRH1 was down-regulated by HRH1 1846 siRNA. In this case, viability decreased by 20%. When these transfected cells were treated with TEF 10 μM for 4 h, viability decreased significantly at 50% (Figure 2C). In the negative control siRNA-transfected cells and in the rest of samples, viability decreased 70%. These results suggest that the cytotoxic effect of TEF on melanoma cells seems to be independent of HRH1.

**TEF-induced apoptosis is independent of cyclic nucleotides**

To evaluate the involvement of cyclic nucleotides in the apoptosis induced by TEF, the intracellular cAMP and cGMP levels were measured. Also, the effect of adenylate cyclase and phosphodiesterase inhibitors on cell viability was analysed. We found that the intracellular cAMP levels in TEF-treated melanoma cells did not change significantly at any time studied (Figure 3A). Moreover, the addition of dibutyryl cAMP on the culture cells did not modify cell viability (Figure 3B). Intracellular cGMP levels were measured in the same cells and culture conditions, but cGMP intracellular levels were almost undetectable. cGMP values were always included between 0 and 2.1 pmol of the standard curve and TEF treatment did not change these values (data not shown). Furthermore, adenylate cyclase and phosphodiesterase inhibitors did not modify the viability of the melanoma cells (Figure 3B). In the combined treatments of adenylate cyclase and phosphodiesterase inhibitors with TEF, the decrease of cell viability seems to be induced only by TEF.
TEF increases cytosolic Ca\(^{2+}\) in A375 melanoma cells by mobilizing intracellular Ca\(^{2+}\) stores and activating Ca\(^{2+}\) influx.

We studied the effect of TEF and histamine, either separately or in combination, on the intracellular Ca\(^{2+}\) homeostasis in A375 melanoma cells. In a medium containing physiological Ca\(^{2+}\) concentrations, TEF dose dependently increased cytosolic Ca\(^{2+}\), showing a maximum effect at 40 \(\mu\)M with an half maximal effective concentration value of 9 \(\mu\)M (Figure 4A). At a concentration of 25 \(\mu\)M, TEF immediately (20 s) increased cytosolic Ca\(^{2+}\) 4-fold over baseline. The cytosolic Ca\(^{2+}\) declined to a sustained phase of 200 nM at the time point of 400 s. When we studied the effect of histamine, interestingly we found that histamine had no effect on Ca\(^{2+}\) homeostasis at the tested range of doses (data not shown). At a concentration up to 5 mM, histamine did not modulate the Ca\(^{2+}\) response to the subsequent addition of 25 \(\mu\)M TEF (Figure 4D). In order to analyse whether Ca\(^{2+}\) influx from extracellular medium may contribute to the TEF-induced Ca\(^{2+}\) rise, cells were stimulated with TEF in the absence of extracellular Ca\(^{2+}\) (cells incubated in a medium containing 1 mM EGTA). EGTA was added to the cell cuvette 1 min before the addition of 25 \(\mu\)M TEF. Under these conditions, the initial rapid Ca\(^{2+}\) response induced by TEF was attenuated to a maximal Ca\(^{2+}\) concentration of 300 nM, accounting for a 25% reduction of the cytosolic Ca\(^{2+}\) levels detected in the presence of physiological Ca\(^{2+}\) concentrations in the medium. Furthermore, Ca\(^{2+}\) returned rapidly to the normal baseline level and it was not able to maintain the same sustained level induced by TEF in the presence of Ca\(^{2+}\) in the medium (Figure 4C). These results suggest that TEF stimulates both the mobilization of Ca\(^{2+}\) from intracellular stores and also Ca\(^{2+}\) influx from the extracellular medium.

We then examined whether ER Ca\(^{2+}\) stores were involved in the cytosolic Ca\(^{2+}\) rise induced by TEF. For this purpose, A375 melanoma cells were pretreated with 2 \(\mu\)M thapsigargin, an inhibitor of ER Ca\(^{2+}\)-ATPase. Thapsigargin increased cytosolic Ca\(^{2+}\) in a rapid monophasic manner to a similar level as that caused by TEF. The subsequent addition of 25 \(\mu\)M TEF failed to increase cytosolic Ca\(^{2+}\) (Figure 4D). Furthermore, pretreatment with 25 \(\mu\)M TEF for several minutes also totally blocked the effect of thapsigargin on Ca\(^{2+}\) homeostasis (Figure 4E). Our data indicate that TEF induces Ca\(^{2+}\) release from thapsigargin-sensitive ER stores and that this effect is necessary for the entrance of Ca\(^{2+}\) from outside the cell.

**TEF-increased cytosolic Ca\(^{2+}\) involves IP\(_3\)-PLC pathway**

The possibility that IP\(_3\)-PLC pathway may be involved in TEF-induced Ca\(^{2+}\) release and cytotoxicity was revealed by pretreating A375 melanoma cells with 0–5 mM neomycin and U73122 (2 \(\mu\)M), inhibitors of IP\(_3\)-PLC, for 30 min before challenging with 25 \(\mu\)M TEF. We found that both neomycin (Figure 5A) and U73122 (data not shown) prevented the effect of TEF on Ca\(^{2+}\) release in a dose-dependent manner. The maximum effect was observed at 5 \(\mu\)M neomycin; this concentration blocking completely TEF-induced cytosolic Ca\(^{2+}\) rise (Figure 5A). Furthermore, neomycin also dose dependently protected the cells from TEF-induced toxicity, with a maximum effect at 5 mM. Moreover, neomycin did not induce any cytotoxic effect, as determined by XTT viability assay (Figure 5B) and morphological changes by phase-contrast microscopy (Figure 5C).

**BAPTA-AM protects A375 cells from TEF-induced DNA damage and apoptosis**

To verify whether the cytosolic Ca\(^{2+}\) release was responsible for TEF-induced cytotoxicity on A375 melanoma cells, we studied the effect of TEF on the viability of cells previously depleted from intracellular Ca\(^{2+}\) by 1 h of pretreatment with the Ca\(^{2+}\)-chelator BAPTA in its acetoxymethyl hydrophobic form BAPTA-AM (1,2-bis-(o-Aminophenoxy)-ethane -N,N',N'-tetraacetic acid tetracetoxy-Methyl ester [BAPTA-AM] (1–10 \(\mu\)M). The treatment prevented markedly TEF-induced cytotoxicity. At a dose of 10 \(\mu\)M, BAPTA-AM reversed cell viability by ~40% in the TEF-treated cells (Figure 6A). We further studied the effect of TEF on DNA damage in the presence of 10 \(\mu\)M BAPTA-AM and we found that BAPTA-AM almost completely reversed the DNA damage induced by TEF alone (Figure 6B). This fact indicates that increase in cytosolic Ca\(^{2+}\) plays an important role in the mechanism of DNA damage and cell death induced by TEF in A375 melanoma cells.

**TEF-induced apoptosis is tyrosine kinase dependent**

To analyse whether tyrosine kinase activity is involved in the cytotoxic effect induced by TEF on A375 melanoma cells, we studied the effect of TEF on Ca\(^{2+}\) release and viability of cells previously treated with genistein, a tyrosine kinase inhibitor. We found that genistein significantly blocked TEF-induced cytotoxicity in a dose-dependent manner (Figure 7A). This result was confirmed by measuring the caspase-3 activity: we observed ~70% caspase-3-positive cells in TEF-treated cells compared with 8% caspase-3-positive cells in genistein pre-incubated cells before TEF treatment (Figure 7B). Furthermore, we found that genistein prevented the effect of TEF on Ca\(^{2+}\) release in a dose-dependent manner. TEF-induced cytosolic Ca\(^{2+}\) rise was completely blocked with 25 \(\mu\)M of genistein (Figure 6C, line c). These results suggest that TEF induces apoptosis through modulation of calcium level in human melanoma cells.

**Discussion**

Histamine plays an important role in various physiological and pathological processes. In melanoma cells, histamine has been reported to be
available at high levels (5,8,25). Although, histamine has been shown to stimulate melanoma cell growth (8,25), it has been used as an adjuvant to cytokine therapy in patients with metastatic melanoma (26).

In the present study, we have found that, in accordance with previous reports (8,27), histamine is a growth-stimulating factor in A375 melanoma cells and that TEF decreases cell viability and induces cell death by apoptosis. On the contrary, cimetidine, an H2 histamine receptor antagonist, does not modulate A375 melanoma cell growth. Other authors have also found similar pro-apoptotic effects of TEF in several types of human cancer cell lines (28,29) and normal thymocytes (30). However, apoptosis induced by TEF does not seem to be mediated by HRH1 since we have observed that pretreatment of melanoma cells with histamine protects the cells only partially from the cytotoxic effect induced by TEF. Moreover, inhibition of endogenous histamine synthesis by α-FMH decreased melanoma cell viability only by ~20%. To confirm the real role of HRH1 in the TEF-induced apoptosis, we tested the effect of TEF down-regulating HRH1 expression by siRNA on melanoma cells. We found that attenuation of HRH1 expression changed slightly the ratio of cytotoxicity induced by TEF. These results suggest that apoptosis induced by TEF on A375 melanoma cells is independent of HRH1 and that other cellular pathways are involved.

Fig. 7. TEF-induced tyrosine kinase-dependent apoptosis. (A) Cell viability assay. Cell culture were incubated with 0–50 µM genistein for 2 h; afterwards 10 µM TEF was added and the incubation continued for 4 h. Cell viability was assessed by XTT. Results are expressed as the percentage of untreated controls. Data represent mean ±SD of six determinations of three separate experiments. *P<0.05 (Student’s t-test). (B) Genistein abolished caspase-3 activation induced by TEF treatment. Untreated and treated cells with 25 µM genistein for 2 h were then incubated with 10 µM TEF for 4 h. The cells were collected, and after fixation and permeabilization, they were incubated with polyclonal rabbit anti-cleaved caspase-3 antibody. After three rinses, the cells were incubated with 4,6-diamidino-2-phenylindole for 10 min to stain the nucleus. Last, the cells were viewed and analysed with a confocal microscope. Bar = 20 µM. (C) Genistein blocks TEF-induced cytosolic Ca2+ rise in A375 melanoma cells. Fura-2-loaded cells were pretreated without (a) or with 6.25 µM (b) or 25 µM (c) genistein for 30 min, and then transferred to the fluorimetric cuvette and the fluorescence was registered. At the indicated time, 25 µM TEF was added to the cell suspension. The figure shows a representative datum set in one cellular suspension.
In order to study the signalling loops in TEF-induced apoptosis on melanoma cells, intracellular cAMP, cGMP and Ca²⁺ levels were determined. We found that cyclic nucleotide levels were unmodified after TEF treatment; however, a significant increase of cytosolic Ca²⁺ was detected. Intracellular Ca²⁺ has been shown to be commonly involved in cell death signal transduction. An unregulated elevation in cytosolic Ca²⁺ levels is often cytotoxic and induces cell death in most of the cells (18). As histamine is linked to the intracellular Ca²⁺ homeostasis through the activation of HRH1 (8,31), we focused our study on the intracellular Ca²⁺ signal as a molecular mechanism that may be involved in the response of melanoma cells to histamine and TEF treatment. Surprisingly, we found that the presence of TEF induced a rapid and sustained increase in cytosolic Ca²⁺ levels in a dose-dependent manner. However, histamine did not modulate Ca²⁺ homeostasis in A375 melanoma cells at doses even higher than those that induce cell growth. Moreover, when histamine was added to the culture medium before TEF, it was unable to block the effect of TEF on cytosolic Ca²⁺ modulation. These results, in accordance with those reported previously by other authors (13,32), support our previous conclusion that the cellular mechanism involved in apoptosis induced by TEF on melanoma cells may involve other cellular pathways independent of the histamine-signalling system.

In our model, TEF appears to act mainly by releasing intracellular Ca²⁺ from ER stores since thapsigargin, which inhibits the ER Ca²⁺-ATPase and prevents refilling of ER Ca²⁺ stores, totally abolished the Ca²⁺ response to TEF. Furthermore, TEF-induced cytosolic Ca²⁺ rise appears to be PLC dependent because both PLC inhibitors, neomycin and U73122, were able to block completely the TEF-induced Ca²⁺ rise. However, Ca²⁺ influx from the extracellular medium into the cells may also contribute to the cytosolic Ca²⁺ rise induced by TEF since in a Ca²⁺-free medium TEF induced a lower cytosolic Ca²⁺ rise that returned more rapidly and completely to its normal baseline level. In non-excitatory cells, Ca²⁺ influx may occur through a group of channels on the cell membrane known as store-operated Ca²⁺ channels (SOC) (32). Upon depletion of Ca²⁺ stores in the ER, calcium influx factor is released from the ER and stimulates plasma membrane-bound phospholipase A2, which in turn activates SOC (33). Melanoma cells have been demonstrated to express SOC that can be stimulated by various agents such as thapsigargin (34,35). We propose a similar mechanism of action for TEF in A375 melanoma cells. Furthermore, it is known that PLC controls Ca²⁺ homeostasis through IP³, which acts exclusively on IP₃ receptors on the ER without acting directly on voltage-dependent Ca²⁺ channels (36,37). Thus, the complete blocking effect of the IP₃-PLC inhibitors on TEF-induced Ca²⁺ rise indicates that TEF has no direct effect on these voltage-dependent channels in the studied melanoma cells. It is well known that PLC inhibitors, acting by inhibiting the hydrolysis of phosphatidylinositol 4,5-bisphosphate to IP₃, prevent the increase of cytosolic-free calcium. Furthermore, it has been reported that IP₃ may induce IP₃,R-SOC conformational coupling that ends by activation and opening of SOC without depending on the depletion of Ca²⁺ stores in the ER (38). Moreover, TEF has been shown to have the ability to block voltage-dependent ion channels in the cells (13). All these facts support our hypothesis that the only way through which Ca²⁺ influx may occur in our model is by SOC.

Although depletion of ER Ca²⁺ stores has been reported to play an important role in the apoptotic process (38), unregulated cytosolic Ca²⁺ rise is often cytotoxic and induces cell death in most of the cells (18). In this context, we have demonstrated that cytosolic Ca²⁺ rise is involved in TEF-induced cytotoxicity of A375 melanoma cells because the presence of an intracellular Ca²⁺-chelating agent in the culture medium of TEF-treated melanoma cells reverted markedly the cytotoxic effect and DNA damage induced by TEF. These results, in accordance with other reports (39), indicate that DNA damage and apoptosis induced by TEF are a consequence of the intracellular calcium level modulation.

Since PLCy isoforms can be phosphorylated and activated by tyrosine kinase receptors and non-receptor protein tyrosine kinases (40,41), we examined the possible role of tyrosine kinase activity in the apoptosis and Ca²⁺ mobilization induced by TEF on A375 melanoma cells. Surprisingly, when tyrosine kinase activity was inhibited by genistein before TEF treatment, apoptosis was almost completely suppressed and no increase in cytosolic Ca²⁺ concentration was observed. These results indicate that tyrosine kinase activity is required for Ca²⁺ mobilization and apoptosis.

It has been reported that genistein, a potent in vitro inhibitor of the epidermal growth factor receptor, does not inhibit the activation of growth factor receptors in tumour cells, but a target further down-stream in the signal transduction (42). In the HRH1-signalling pathway activated by TEF in melanoma cells, we think that protein tyrosine kinases might phosphorylate a number of intracellular substrates and activate signalling cascades that include activation of PLCy. Moreover, protein tyrosine kinases could be involved in the tyrosine phosphorylation of the Gₓ subunit proteins, in the transactivation of tyrosine kinase receptors from stimulated G protein-coupled receptors or in the receptor tyrosine kinase/G protein-coupled receptor signal integration (43,44).

In summary, all these findings suggest that histamine is an important growth factor in A375 melanoma cells and that TEF induces DNA damage and apoptosis through the modulation of Ca²⁺ homeostasis and the cellular pathway involving tyrosine kinase activity. This fact might have an important therapeutic application in the treatment of melanoma.

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References

Tyrosine kinase and Ca\textsuperscript{2+}-dependent apoptosis in melanoma cells by terfenadine


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