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ORIGINAL CONTRIBUTION

Differential impact of cold and hot tea extracts on tyrosine phosphatases regulating insulin receptor activity: a focus on PTP1B and LMW-PTP

Massimo Genovese[1] · Simone Luti[1] · Elisa Pardella[1] · Mirella Vivoli-Vega[1] · Luigia Pazzagli[1] · Matteo Parri[1] · Anna Caselli[1] · Paolo Cirri[1] · Paolo Paoli[1]

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Abstract

Purpose The impact of tea constituents on the insulin-signaling pathway as well as their antidiabetic activity are still debated questions. Previous studies suggested that some tea components act as Protein Tyrosine Phosphatase 1B (PTP1B) inhibitors. However, their nature and mechanism of action remain to be clarified. This study aims to evaluate the effects of both tea extracts and some of their constituents on two main negative regulators of the insulin-signaling pathway, Low-Molecular-Weight Protein Tyrosine Phosphatase (LMW-PTP) and PTP1B.

Methods The effects of cold and hot tea extracts on the enzyme activity were evaluated through in vitro assays. Active components were identified using gas chromatography—mass spectrometry (GC–MS) analysis. Finally, the impact of both whole tea extracts and specific active tea components on the insulin-signaling pathway was evaluated in liver and muscle cells.

Results We found that both cold and hot tea extracts inhibit LMW-PTP and PTP1B, even if with a different mechanism of action. We identified galloyl moiety-bearing catechins as the tea components responsible for this inhibition. Specifically, kinetic and docking analyses revealed that epigallocatechin gallate (EGCG) is a mixed-type non-competitive inhibitor of PTP1B, showing an IC_{50} value in the nanomolar range. Finally, in vitro assays confirmed that EGCG acts as an insulin-sensitizing agent and that the chronic treatment of liver cells with tea extracts results in an enhancement of the insulin receptor levels and insulin sensitivity.

Conclusion Altogether, our data suggest that tea components are able to regulate both protein levels and activation status of the insulin receptor by modulating the activity of PTP1B.

Keywords Type 2 diabetes · Tea extracts · PTP1B inhibitors · Epigallocatechin gallate · Insulin resistance

Introduction

The rapid increase in diabetes prevalence recorded worldwide confirmed that this disease is approaching global pandemic proportions [1]. The World Health Organization (WHO) identified Type 2 Diabetes (T2D), also known as Non-Insulin Dependent Diabetes Mellitus (NIDDM), as the most prevalent form of this pathology, accounting for approximately 90% of all the diagnosed diabetes cases [2]. People affected by T2D usually produce lower insulin levels and suffer from insulin resistance. The combination of both these events results in increased blood glucose levels during fasting and/or after meal. Moreover, the unbalance of glucose homeostasis and the reduced activity of insulin lead over time to dysfunctions of the liver, muscle, adipose tissue, vascular endothelium and nervous system, thus contributing to the onset of diabetes-related pathologies [3, 4]. Although diabetic patients can rely on different types of oral antidiabetic drugs [5], most of them experience a progressive worsening of health conditions during their lifetime [6] and are exposed to an increased risk of incurring premature death [7]. For this reason, WHO strongly encourages the development of measures to prevent, rather than treat, diabetes, with the aim of reducing its spread and socio-economic impact.

✉ Elisa Pardella
elisa.pardella@unifi.it

[1] Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, Viale Morgagni 50, 50134 Florence, Italy

Considering that unbalanced diet is one of the major factors linked to the development of T2D, the observance of a healthy nutritional regimen is considered one of the best prevention strategies [3]. In this context, several studies demonstrated that the consumption of foods rich in bioactive molecules, such as whole grains, fruits and vegetables, can contribute to improve glycemic control and blood lipid profile in diabetic patients [8–10].

Tea is one of the most popular drinks worldwide and it is widely believed that its regular intake can help to prevent the onset of several chronic degenerative diseases, including diabetes [11]. Although several preclinical studies demonstrated that some tea components are able to improve insulin sensitivity, glucose uptake and glycogen synthesis in liver and muscle cells, many of these results were not confirmed by clinical trials on human volunteers [12]. The inconsistency of these results encouraged more in-depth studies to clarify whether tea extracts possess antidiabetic properties and to identify bioactive molecules responsible of these effects. Few years ago, Ma and colleagues outlined that tea extracts selectively inhibit Protein Tyrosine Phosphatase 1B (PTP1B), one of the most important negative regulators of the insulin receptor (IR). Unfortunately, the authors failed in identifying the molecules responsible for this inhibitory activity [13]. More recently, Kuban-Jankowska and co-authors reported that epigallocatechin inhibits PTP1B, with an IC_{50} value of 103 µM, and reduces cell viability of breast cancer cells [14]. However, this result is in conflict with the hypothesis formulated by Ma and colleagues that tea catechins behave as potent PTP1B inhibitors.

Given the great interest in identifying novel PTP1B inhibitors as lead compounds for the synthesis of antidiabetic drugs [15], we decided to perform more in-depth studies on tea extracts with the aim of identifying tea components responsible for the inhibition of PTP1B and Low-Molecular-Weight Protein Tyrosine Phosphatase (LMW-PTP), which are key tyrosine phosphatases involved in IR regulation [16]. We demonstrated that galloyl-catechins act as potent inhibitors of both PTP1B and LMW-PTP and we established their mechanism of action in vitro. Moreover, we pointed out that chronic treatment of liver cells with either tea extract or the tea component with the highest inhibitory potential against PTP1B strongly influences IR protein levels and improves glucose uptake.

In our opinion, data reported in this study provide relevant novel information about the impact of tea components on PTP1B, LMW-PTP and insulin signaling.

Materials and methods

Materials

All the reagents were obtained from Merck Life Science S.r.l. (Germany), unless otherwise specified. The following antibodies were used: pIR β subunit Y1162/1163 (sc-25103-R) and β-actin clone C-4 (sc-47778) were from Santa Cruz Biotechnology (Dallas, Texas, USA); IR β subunit clone CT-3 (MABS65) was from Merck-Millipore (Burlington, MA, USA); phospho-protein kinase B (pAkt) (9271S) and Akt (9272S) antibodies were from Cell Signalling Technology (Danvers, Massachusetts, USA). Secondary antibodies were from Santa Cruz Biotechnology.

Cell cultures

HepG2 liver cell line and C2C12 myoblasts were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HepG2 and C2C12 were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM)-high glucose (4500 mg/L) supplemented with 10% Fetal Bovine Serum (FBS, Euroclone), 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated at 37 °C in humidified atmosphere with 5% CO_2. To induce myoblast differentiation, 80% confluent myoblasts were incubated in differentiation medium (DMEM containing 2% horse serum) for 5 days.

Preparation of tea extracts

Cold tea extracts were prepared as follows. 2 g of dry tea leaves was added to 50 mL of deionized water and incubated at 25 °C for 1 h under agitation. Samples were then centrifuged, and supernatants filtered using a 0.22 µm filter to remove any debris. Obtained samples were stored at 4 °C. The protocol used to prepare hot tea extracts refers to the method described by Perva-Uzunalic and co-workers [17]. Specifically, to prepare hot tea extracts, 2 g of tea was diluted in 50 mL of boiling water. Before adding the tea, the heat source, that was used to boil water, was removed. The tea samples were incubated in hot water for 5 min and then transferred into a box containing water and ice to favor the rapid cooling of the extract. Once the samples reached the temperature of 25 °C, they were centrifuged, and supernatants filtered using a 0.22 µm filter to remove any debris. The samples were stored at 4 °C.

Enzymatic assays and determination of IC_{50} values

Enzymatic assays were carried out as previously described [18]. The IC_{50} value of tea extracts and catechins against PTP1B and LMW-PTP was determined measuring the hydrolysis rate of the enzymes in the presence of 2.5 mM p-Nitrophenyl Phosphate (*p*-NPP) and increasing inhibitor concentrations (generally 14–16 different concentrations). Each test was carried out in triplicate. Experimental data were normalized respect to control samples and then fitted using a non-linear fitting software, as previously described [19]. A dilution test was carried out to evaluate whether tea extracts act as reversible or irreversible inhibitors of both PTP1B and LMW-PTP [19].

Determination of the mechanism of action of tea extracts and catechins on PTP1B and LMW-PTP

The inhibitory mechanism of tea extracts and catechins was determined studying the dependence between the main kinetics parameters (K_M and V_{max}) and the inhibitor concentrations. Data obtained were fitted using Michaelis–Menten equation and a non-linear fitting software. Then, data were analyzed using the double reciprocal plot (Lineweaver–Burk method). Secondary plots were used to calculate the Ki values.

HPLC analysis and sample fractioning

HPLC analyses were performed using a Thermo Scientific UltiMate 3000 UHPLC system equipped with a Variable Wavelength Detector and a Kinetex C18 column 5 µm, 100 Å, 250 × 4.6 mm (Phenomenex). In our study, we performed two different protocols for preparative or analytical run, using the following solvent system: 10 mM trifluoroacetic acid (TFA) in acetonitrile (solvent A); 10 mM TFA in water (solvent B). In both analyses, the gradients used were: 2–15% of A for 15 min, 15–30% of A for 10 min, 30–45% of A for 6 min, 45–100% of A for 4 min. The analytical run was carried out at a flow rate of 0.8 mL/min, using 10 µL injection volume. For all runs, detection was based on the absorbance at 214, 280 and 330 nm.

Sample fractionation was performed using a 100 µL injection volume loaded onto a Jupiter® C4, 5 µm, 300 Å, column 250 × 10 mm (Phenomenex) at 2.0 mL/min. Fractions were collected according to the retention time: fraction 1 8–12 min; fraction 2 12–16 min; fraction 3 16–20 min; fraction 4 20–24 min and fraction 5 24–28 min.

Gas chromatography–mass spectrometry (GC–MS) analysis

For SCAN mode, HPLC tea fractions were dried under vacuum and then dissolved in 60 µL of 2% methoxyamine hydrochloride in pyridine (Thermo Fisher Scientific, Waltham, MA, USA), and incubated at 30 °C for 2 h. After dissolution and reaction, 90 µL N-Trimethylsilyl-N-methyl trifluoroacetamide (MSTFA) + 1% trimethylchlorosilane (TMCS) were added and samples were further incubated at 37 °C for 60 min. Gas chromatographic runs were performed with helium as carrier gas at 0.6 mL/min. The split inlet temperature was set to 250 °C and the injection volume of 1 µL. A split ratio of 1:10 was used. The GC oven temperature ramp was from 60 to 325 °C at 10 °C/min. The data acquisition rate was 10 Hz. For the Quadrupole, an Electron Ionization (EI) source (70 eV) was used, and full-scan spectra (mass range from 50 to 600) were recorded in the positive ion mode. The ion source and transfer line temperatures were set, respectively, to 250 and 290 °C. The MassHunter data-processing tool (Agilent, Santa Clara, CA, USA) was used to obtain a global metabolic profiling. Fiehn Metabolomics RTL library (Agilent G1676AA) was used to identify metabolites.

Determination of total polyphenol content

The total polyphenol content of tea extracts was determined using Folin–Ciocalteu reagent, as previously described [18].

Molecular docking

Docking simulations were carried out with AutoDock (version 4.2.5.1) and AutoDock Tools (version 1.5.6rc3) [20] using the 1PTY structure of PTP1B downloaded from the Protein Data Bank. The grid map of the interaction energies for various atoms of the ligand with the macromolecule was calculated using AutoGrid software. Specifically, for 1PTY, the grid search size was 126 × 100 × 90 points with a spacing of 0.400 Å. The Lamarckian Genetic Algorithm (LGA) was used to identify potential binding pockets using the following parameters: the number of GA runs was 100, the number of individuals in the population was 150, the maximum number of energy evaluation was 250,000 and the maximum number of generations was 27,000. The LGA resulted in 100 ligand poses. These poses were clustered according to Root-Mean-Square-Deviation (RMSD), with a similarity of 10 rms. Among all, five poses were selected from the obtained clusters. Graphical representations of the ligand, the protein and the complexes were performed using UCSF Chimera [21].

Visualization of ligand–protein interactions

The potential intermolecular interactions between the ligand and the protein were calculated automatically by LigPlot$^+$ software (version 2.2), that generated 2D schematic representations of protein–ligand complexes displaying hydrogen

bonds and hydrophobic interactions within the binding pocket [22, 23].

Analysis of the insulin-signaling pathway

To evaluate the effect of tea extracts on the insulin-signaling pathway, HepG2 cells were grown in 35-mm culture dishes. Chronic stimulation was achieved incubating HepG2 cells for 90 min in the presence of starvation medium (DMEM supplemented with 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin) containing 5 µL/mL of tea extract. After this time, the medium was removed, cells were washed with PBS, and further incubated with fresh starvation medium for 24 h. The treatment was repeated four times before stimulation with 10 nM insulin (Humalog, lispro insulin) for 30 min. The impact of EGCG on C2C12 differentiated cells was evaluated by stimulating muscle cells for 30 min with EGCG concentrations in the range 0.1–10 µM or with cold Indian green tea extract before the analysis. Both treated and control HepG2 and C2C12 cells were lysed in Laemmli buffer 1X, containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue, 0.0625 M Tris–HCl pH 6.8, according to Lori et al. [18]. After that, SDS-PAGE was performed on whole cell extracts and then proteins were transferred on PVDF membranes. Membranes were incubated overnight at 4 °C with anti-IRβ, anti-pIRβ, anti-Akt and anti-pAkt primary antibodies diluted 1:1000 in PBS-Tween 0.1% containing 5% of bovine serum albumin (BSA). β-Actin was used as loading control for total protein lysates. After washing several times with PBS-Tween 0.1%, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies, diluted 1:5000 in PBS-Tween 0.1% containing 1% BSA. Images of the proteins were obtained by chemiluminescence using Clarity Western ECL substrate (BioRad) and Amersham Imager 600 luminometer (Amersham, Buckinghamshire, UK). Bands were quantified using the Amersham quantification software.

Glucose uptake

Glucose uptake assay was performed using C2C12 murine muscle cell line. Plates containing myotubes were starved for 20 h, and then treated for 30 min with 10 nM insulin or with increasing EGCG concentrations. Stimulated cells were then incubated in the presence of 0.5 mCi/mL of 2-deoxy-D-1,2-[^{3}H]-glucose (Perkin Elmer) for 15 min, washed with cold PBS and lysed using 0.2 M NaOH solution. The amount of radiolabeled glucose incorporated by cells was determined using αβ-scintillation-counter. Data were normalized on protein concentration and reported as percent respect to the control experiment.

Statistical analysis of data

Data are reported as means ± S.E.M. from at least three independent experiments. Data obtained were statistically analyzed using Unpaired Student's *T* test. p values ≤ 0.05 were considered statistically significant (* p value < 0.05, ** p value < 0.01).

Results

Tea extract preparation

Tea extracts were prepared using either cold or hot water. Seven different types of tea were used to obtain cold extracts: Indian earl grey and Indian English breakfast teas, Sri Lanka black tea, Indian, Chinese and Iranian green teas, and Chinese white tea. Hot extracts were prepared using Indian earl grey, and Indian and Iranian green teas. All extracts were filtered and stored at 4 °C in sterile tubes.

Preliminary screening and determination of IC_{50} values of tea extracts

Preliminary enzymatic screening assays underlined that cold extracts obtained from all the seven different types of tea behave as potent inhibitors of both PTP1B and LMW-PTP (Fig. 1). To confirm this, we determined the IC_{50} values of each cold tea extract on both enzymes (Table 1 and Supplementary Fig. 1, 2). Data obtained show that the inhibitory potency of tea extracts is dependent on the type of tea analyzed. In particular, Indian earl grey, and Indian and Iranian green teas are the most efficient in inhibiting the activity of both phosphatases. Thus, we selected these three types of tea and prepared their hot extracts to further investigate their inhibitory potential. Kinetic analyses on Indian earl grey, Indian green and Iranian green tea extracts reveal that hot extracts are more effective than the corresponding cold ones in inhibiting both PTP1B and LMW-PTP activities (Table 1 and Supplementary Fig. 3). Remarkably, Indian green tea appears to be the most potent, with its cold extract displaying IC_{50} values of 0.04 µL/mL and 0.03 µL/mL on PTP1B and LMW-PTP, respectively, and its hot extract showing IC_{50} values of 0.007 µL/mL and 0.03 µL/mL on PTP1B and LMW-PTP, respectively (Table 1).

Kinetic mechanism of Indian green tea extract

We performed further analyses using Indian green tea to evaluate differences in the mechanism of action between cold and hot tea extracts. We found that the cold extract is a reversible inhibitor of both enzymes (Supplementary Fig. 4A), while the hot extract acts as a non-reversible

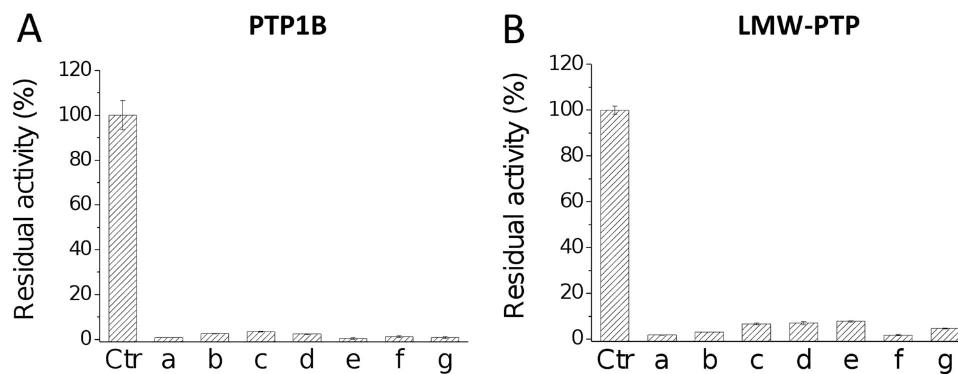

Fig. 1 Effect of cold tea extracts on the enzymatic activity of PTP1B (**A**) and LMW-PTP (**B**). To evaluate the inhibitory effects of cold tea extracts on PTP1B (**A**) and LMW-PTP (**B**) catalytic activity, tests were carried out diluting 5 μL of tea extracts in 1 mL of assay buffer. Each sample was marked with an alphabet letter: *Ctr* control, *a* Indian earl grey tea, *b* Indian green tea, *c* Chinese green tea, *d* Iranian green tea, *e* Chinese white tea, *f* Sri Lanka black tea, *g* Indian English breakfast tea. All tests were carried out in triplicate. All data obtained were normalized respect to control tests. Results reported in the figure represent the mean value ± S.E.M (n = 3)

Table 1 IC_{50} values calculated on PTP1B and LMW-PTP using both cold and hot tea extracts

Tea Extract	IC_{50} (μL/mL)			
	Cold extracts		Hot extracts	
	PTP1B	LMW-PTP	PTP1B	LMW-PTP
Indian earl grey	0.030 ± 0.002	0.450 ± 0.030	0.030 ± 0.002	0.044 ± 0.003
Indian green	0.040 ± 0.001	0.030 ± 0.002	0.007 ± 0.001	0.030 ± 0.004
Chinese green	0.040 ± 0.002	0.900 ± 0.150	–	–
Iranian green	0.040 ± 0.001	0.070 ± 0.010	0.020 ± 0.001	0.016 ± 0.002
Chinese white	0.050 ± 0.001	0.180 ± 0.020	–	–
Sri Lanka black	0.050 ± 0.001	0.080 ± 0.005	–	–
Indian English breakfast	0.090 ± 0.002	0.100 ± 0.005	–	–

inhibitor (Supplementary Fig. 4B). Furthermore, kinetic analyses revealed that the cold tea extract acts as non-competitive inhibitor of PTP1B, and as an uncompetitive inhibitor of LMW-PTP (Fig. 2 and Supplementary Fig. 5). Experimental data related to PTP1B in the secondary plot described a hyperbolic curve, indicating that more than one molecule binds to the enzyme surface (Fig. 2B). Conversely, in the case of LMW-PTP, experimental points reported in the secondary plot described a straight line, suggesting that the inhibition results from the binding of a single molecule (in this case the K_i value is 0.13 ± 0.1 μL/mL) (Fig. 2D).

HPLC analysis of the tea extracts

Qualitative analysis of both hot and cold tea extracts was carried out using an HPLC system. As expected, chromatograms obtained display several peaks with different elution times, thereby confirming that each sample contains different natural molecules (Supplementary Fig. 6). It is worth mentioning that the chromatograms generated by the hot and cold extracts are not perfectly superimposable, suggesting that the extracts are different both quantitatively and qualitatively (Supplementary Fig. 7). To confirm this hypothesis, the total polyphenol content of both cold and hot extracts was evaluated using the Folin–Ciocalteu reagent (Table 2). Data reported in Table 2 confirmed that the polyphenol content of hot extracts is higher than that of cold extracts. Finally, GC–MS analyses of both cold and hot extracts confirmed that these samples are qualitatively different (data not shown).

Identification of bioactive HPLC fractions

The components of the Indian green tea extract were separated using the HPLC apparatus and then each fraction was analyzed separately (Supplementary Fig. 8). We found that the most active fractions were eluted at high organic solvent concentrations, even though the inhibitory activity of each fraction changes according to the enzyme assayed and the extraction temperature (Fig. 3).

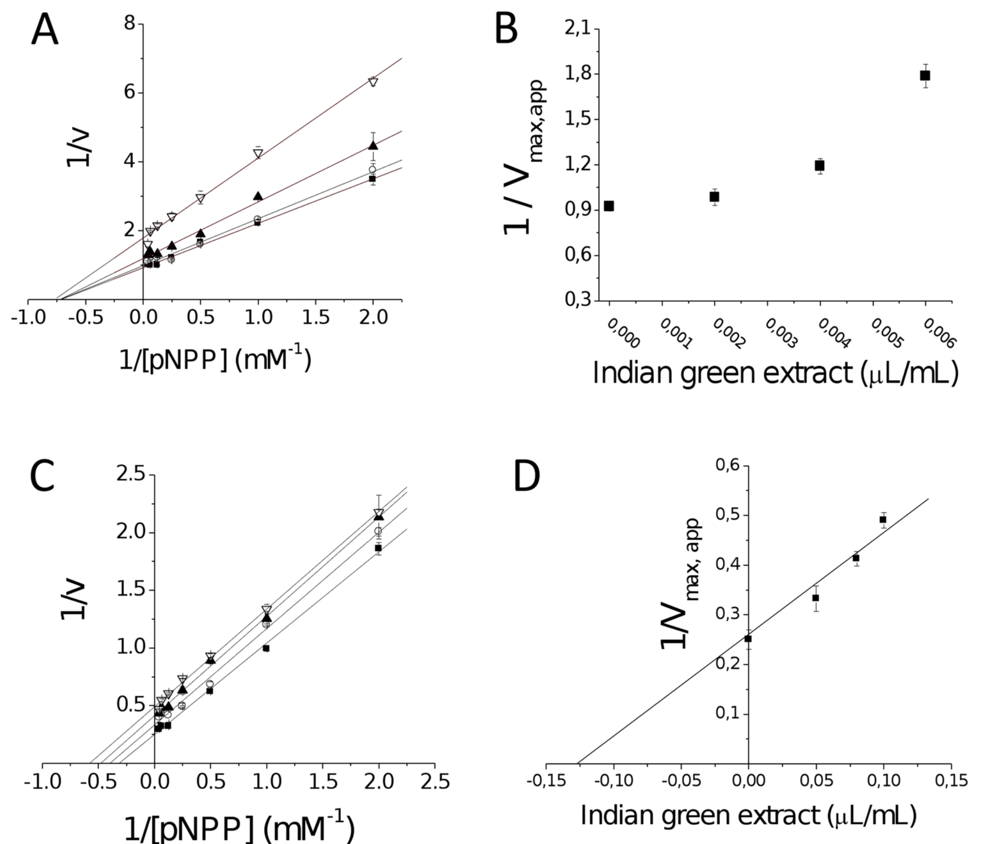

Fig. 2 Kinetic analysis of cold Indian green tea extract on PTP1B and LMW-PTP. To evaluate the mechanism of action of cold Indian green tea extract, we analyzed the dependence of K_M and V_{max} on the extract concentration. K_M and V_{max} were calculated measuring the catalytic rate of the enzyme in the presence of increasing p-NPP concentrations and plotting data using Michaelis–Menten equation. (A-B) For PTP1B: (**A**) double reciprocal plot. The final amounts of extract used were: (filled square), control; (circle), 0.002 µL/mL; (filled triangle), 0.004 µL/mL; (inverted triangle), 0.006 µL/mL; **B** secondary plot. **C**–**D** For LMW-PTP: **C** double reciprocal plot. The final amounts of extract used were: (filled square), control; (circle), 0.05 µL/mL; (filled triangle), 0.08 µL/mL; (inverted triangle), 0.1 µL/mL; **D** secondary plot. All tests were carried out in triplicate. Results reported in the figure represent the mean value ± S.E.M ($n = 3$)

Table 2 Determination of the total polyphenol content in tea extracts

Tea	Hot	Cold
	Total polyphenol content (µg/mL)	
Indian earl grey	2.4 ± 0.2*	1.4 ± 0.1
Indian green	3.7 ± 0.2*	3.0 ± 0.2
Chinese green	4.6 ± 0.5*	2.5 ± 0.7
Iranian green	1.9 ± 0.1*	1.7 ± 0.2
Chinese white	2.6 ± 0.1*	1.7 ± 0.1
Sri Lanka black	3.9 ± 0.3*	2.1 ± 0.1
Indian English breakfast	4.7 ± 0.5*	1.4 ± 0.1

*$p < 0.05$ hot versus cold extracts

§ The results were expressed as µg catechin equivalents (CEQ) reported as gallic acid

GC–MS analysis of the active fractions

The most active fractions of hot (fractions 3 and 5) and cold (fractions 3, 4 and 5) extracts from Indian green tea were analyzed using a GC–MS apparatus. Each fraction contains several molecules, including different types of catechins (Supplementary Table 1). Interestingly, some of the identified molecules have already been described as PTP1B inhibitors (Table 3) [24, 25].

Dissecting the inhibitory potency of catechins in vitro

We calculated the IC_{50} values of epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC), epicatechin (EC) and gallic acid on both PTP1B and LMW-PTP (Supplementary Fig. 9). Interestingly, we found that EGCG and ECG are more effective towards PTP1B compared to LMW-PTP, while EGC, EC and gallic acid are inactive against both phosphatases (Supplementary Table 2). Finally, kinetic analyses revealed that both EGCG and ECG act as reversible inhibitors of PTP1B (Supplementary Fig. 10) and EGCG behaves as a potent mixed-type noncompetitive inhibitor of PTP1B, with a K_i of 26 nM (Fig. 4).

Molecular docking calculations

The interaction mode of EGCG and PTP1B was evaluated using AutoDock 4.2 and 1PTY crystal structure of PTP1B [26]. Five ligand–protein conformations were then selected according to the docking energy. Specifically, the pose 1 belongs to the cluster with the most favorable binding energy. In this pose, the ligand forms four hydrogen bonds with several residues of the PTP1B active site (Ser216, Gly220, Arg221, Lys116 and Tyr46), and engages hydrophobic interactions with amino acids belonging to

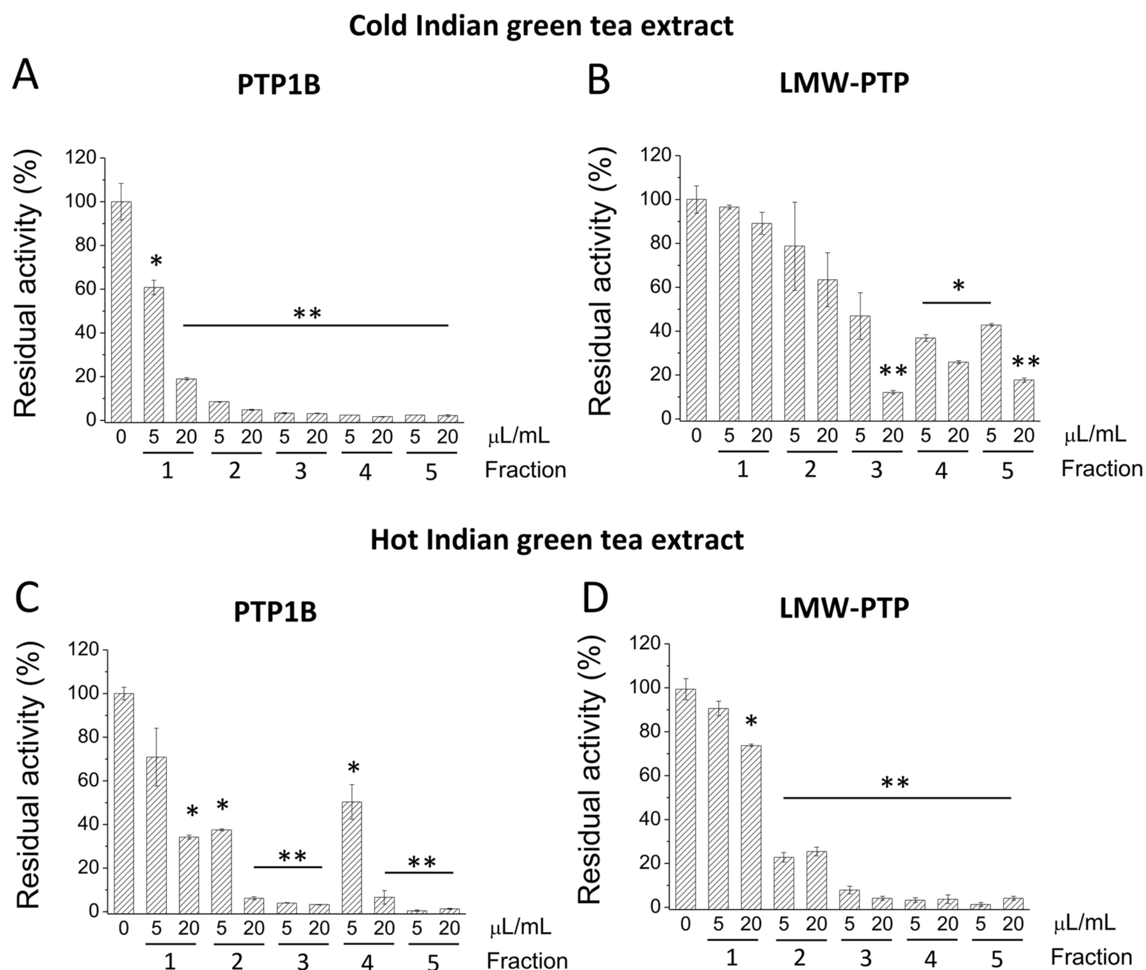

Fig. 3 Effect of cold and hot Indian green tea extract fractions, collected by HPLC, on the enzymatic activity of PTP1B and LMW-PTP. By equipping HPLC apparatus with a semi-preparative column and increasing the content of hydrophobic solvent, **A**–**B** 100 µL injection volume of cold Indian green tea extract was analyzed and five fractions were collected (for more details, see "Materials and methods"). Their inhibitory activity on **A** PTP1B and **B** LMW-PTP was tested by diluting, respectively, 5 and 20 µl/ml of each fraction in 1 ml of assay buffer. **C**–**D** 100 µL injection volume of hot Indian green tea extract was analyzed using HPLC apparatus equipped with semi-preparative column and five fractions were collected (for more details, see "Materials and methods"). Their inhibitory activity on **C** PTP1B and **D** LMW-PTP was tested by diluting, respectively, 5 and 20 µl/ml of each fraction in 1 ml of assay buffer. All tests were carried out in triplicate. All data obtained were normalized respect to control tests. Results reported in the figure represent the mean value ± S.E.M ($n = 3$). * indicates a statistically significant difference according to Unpaired Student's *T* test data analysis (*$p < 0.05$, **$p < 0.01$)

WPD loop (Asp181, Phe182) and the Q-loop (Gln262) (Fig. 5B). Similarly, in the pose 2, the EGCG establishes three hydrogen bonds with Gln262 and maintains hydrophobic interactions with Phe182 and hydrogen bonds with Ala264, belonging to WPD loop and the α-6 helix, respectively (Fig. 5C). In the pose 3, the ligand establishes both hydrogen bonds and hydrophobic interactions with residues in the α-6 helix (Asp265, Phe269), in the WPD loop (Thr178, Pro180, Phe182, Gly183, Val184, Pro185), and in the α-3 helix (Glu186) (Fig. 5D). Furthermore, in the pose 4, the ligand interacts with the amino acids belonging to helices α-6 (Glu276, Lys279, Ile275, Leu272), α-7 (Leu294) and α-3 (Ser187, Pro188, Ala189) (Fig. 5E). Finally, in the pose 5, the EGCG forms hydrogen bonds with Ile275 in the α-6 helix, and Asp284 in the loop connecting α-6 and α-7 helices. Moreover, it establishes hydrophobic interactions with residues in the α-6 helix (Lys279, Ala278), in the α-7 helix (Val287) and in the loop connecting these two helices (Gly283, Met282) (Fig. 5F).

Modulation of the insulin-signaling pathway activity by tea extracts and catechins

With the aim of evaluating the effects of EGCG on the insulin-signaling pathway in myoblasts, further tests were performed on C2C12 cells. We found that 30 min treatment with EGCG increased IR phosphorylation at all used concentrations (Fig. 6A, B), reaching phosphorylation

Table 3 Known PTP1B inhibitors found in cold and hot Indian green tea extracts

Metabolites	Cold Indian green tea extract			Hot Indian green tea extract		References
	Fraction 3	Fraction 4	Fraction 5	Fraction 3	Fraction 5	
Epicatechin	–	✓	–	–	–	[13]
Palmitic acid	✓	✓	✓	✓	✓	[24, 25, 46]
Behenic acid	–	–	✓	–	–	[24, 25]
Catechin	✓	✓	✓	✓	✓	[13]
Epicatechin gallate	–	–	✓	–	✓	[13]
Chlorogenic acid	✓	–	–	✓	–	[47, 48]
Stearic acid	✓	✓	✓	✓	✓	[25]
Linoleic acid	✓	✓	✓	✓	✓	[25]
Oleic acid	–	–	–	✓	–	[24]
Epigallocatechin gallate	✓	✓	–	–	–	[13]
Epigallocatechin	✓	✓	✓	✓	✓	[13]
Acetophenone	✓	✓	✓	–	–	[49]
Myristic acid	–	✓	✓	–	–	[25]
Salicylic acid	–	✓	–	–	–	[50, 51]
Gallic acid	✓	✓	✓	✓	✓	[52]

levels close to those obtained treating C2C12 cells with 10 nM insulin. Furthermore, glucose uptake in C2C12 cells is increased only after treatment with 10 µM EGCG (Supplementary Fig. 11). Further tests were carried out to assess the effects of chronic treatment of liver cells with cold Indian green tea extract, that resulted to be the most potent extract in inhibiting the in vitro activity of the tyrosine phosphatases. We found that in non-insulin-stimulated cells the chronic treatment resulted in a significant increase of IR levels (Fig. 6C, D), and a decrease of both pIRβ and pIRβ/IRβ ratio (Fig. 6C, E, F). However, by evaluating the relative increase of pIRβ/IRβ ratio after insulin stimulation, we found that the strongest increase was observed in extract-treated cells, suggesting that IR becomes more responsive to insulin stimulation after tea extract administration. Moreover, in C2C12 cells, treatment with either cold Indian green tea extract alone or in combination with 10 nM insulin strongly increases Akt phosphorylation levels (Supplementary Fig. 11B). Finally, we also analyzed the effect of hot Indian green tea extract on HepG2 cells (Supplementary Fig. 12A). Surprisingly, we observed a small decrease of both IR and phosphorylation levels when cells were chronically treated with hot tea extract (Supplementary Fig. 12A-C). Besides, the pIRβ/IRβ ratio of cells treated or untreated with tea extract remains substantially unchanged (Supplementary Fig. 12D). Nonetheless, no significant differences in the pIRβ/IRβ ratio were observed between untreated and tea extract-treated cells stimulated with insulin, suggesting that treatment with hot tea extract does not significantly improve insulin sensitivity in liver cells (Supplementary Fig. 12D).

Discussion

T2D is a pathology difficult to diagnose especially in the early stages. The onset of T2D is generally preceded by a series of pathological manifestations (prediabetes phase) that can persist for years and denote a transient imbalance of the mechanisms responsible for glycemic control. Prediabetes is considered a reversible pathological condition and it can be treated by a strict control of caloric uptake and regular physical activity that usually restore normoglycemia [27]. In this context, consumption of functional foods containing bioactive molecules can result in significant benefits, contrasting systemic inflammation and improving insulin sensitivity [28].

Tea, one of the most popular drinks in the world, is considered a functional food able to counteract the onset of chronic degenerative diseases, such as diabetes [29]. Although the evidence that tea contains many bioactive constituents [30], its antidiabetic activity remains to be confirmed [12].

This study aimed to clarify the effects of tea and/or some of its components on the activity of tyrosine phosphatases regulating the IR-mediated insulin-signaling pathway. Despite previous studies have already addressed this topic, the results obtained are largely incomplete and conflicting [13, 14]. Remarkably, we pointed out that both hot and cold tea extracts act as potent inhibitors of PTP1B and LMW-PTP, with evident differences in terms of mechanism of action. We hypothesize that such differences can be ascribed either to the higher number of biomolecules present in the hot extracts, and to the chemical modifications occurring with higher temperatures that are responsible of conversion

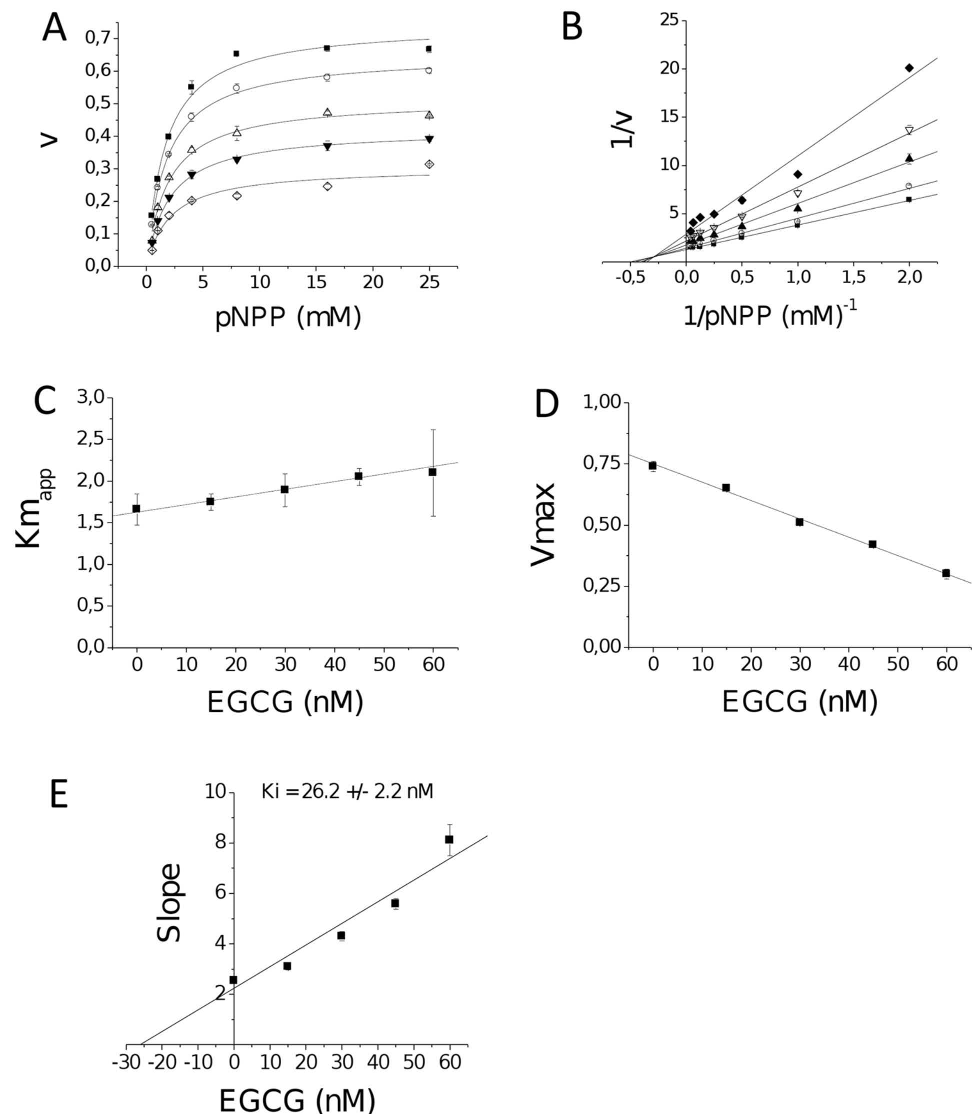

Fig. 4 Kinetic analysis of EGCG on PTP1B. To evaluate the mechanism of action of EGCG on PTP1B catalytic activity, we analyzed the dependence of K_M and V_{max} on EGCG concentration. **A** K_M and V_{max} were calculated measuring the catalytic rate of the enzyme in the presence of increasing p-NPP concentrations and plotting data using Michaelis–Menten equation. The final amounts of extract used were: (filled square), control; (circle), 15 nM; (triangle), 30 nM; (filled inverted triangle), 45 nM; (diamond), 60 nM. **B** Double reciprocal plot. **C** Dependence of K_M on EGCG concentration. **D** Dependence of V_{max} on EGCG concentration. **E** Secondary plot and K_i obtained (26.2 ± 2.2 nM). All tests were carried out in triplicate. All data obtained were normalized respect to control tests. Results reported in the figure represent the mean value ± S.E.M (n = 3)

of tea catechins in less active biomolecules [31, 32]. Ma and co-workers reported similar results, underlining that pre-incubation of PTP1B with hot tea extract improves its inhibitory effects. This finding indicated that tea extracts act as slow binding or non-reversible PTP1B inhibitors [13]. Our data confirmed that the hot extract obtained from Indian green tea is a non-reversible inhibitor of both enzymes, thereby reinforcing the hypothesis that high temperature of extraction could modify the biological properties of tea. Therefore, to eliminate such confounding factors, we focused our attention on the activity of the cold extract obtained from Indian green tea. Kinetic assays confirmed that this extract acts as a potent and reversible inhibitor of both PTP1B and LMW-PTP and that catechins present in this extract are crucial tea components responsible for such activity. Interestingly, we noted that only EGCG and ECG were active on both enzymes, while EGC, EC and gallic acid were not. Reasonably, we think that these differences can be attributed to the greater structural flexibility of EGCG and ECG respect to other catechins lacking the galloyl group [33]. In this context, our results disagree with data reported by Kuban–Jankowska and collaborators, showing that catechins possess a limited inhibitory activity against PTP1B [14]. Based on our data, we propose that structural flexibility of EGCG and ECG can explain the ability of such molecules to inhibit both PTP1B and LMW-PTP, two enzymes characterized by strong structural differences. Moreover, it is noteworthy that both EGCG and ECG showed higher affinity for PTP1B over LMW-PTP, suggesting that these catechins possess a marked specificity for the first enzyme. Accordingly, Ma and co-workers reported that both black and green tea extracts display a marked selectivity for PTP1B over TC-PTP, another member of the PTP family sharing a strong structural similarity with PTP1B [13]. Altogether, these data reinforce the evidence that EGCG and ECG display a significant selectivity for PTP1B over other PTPs.

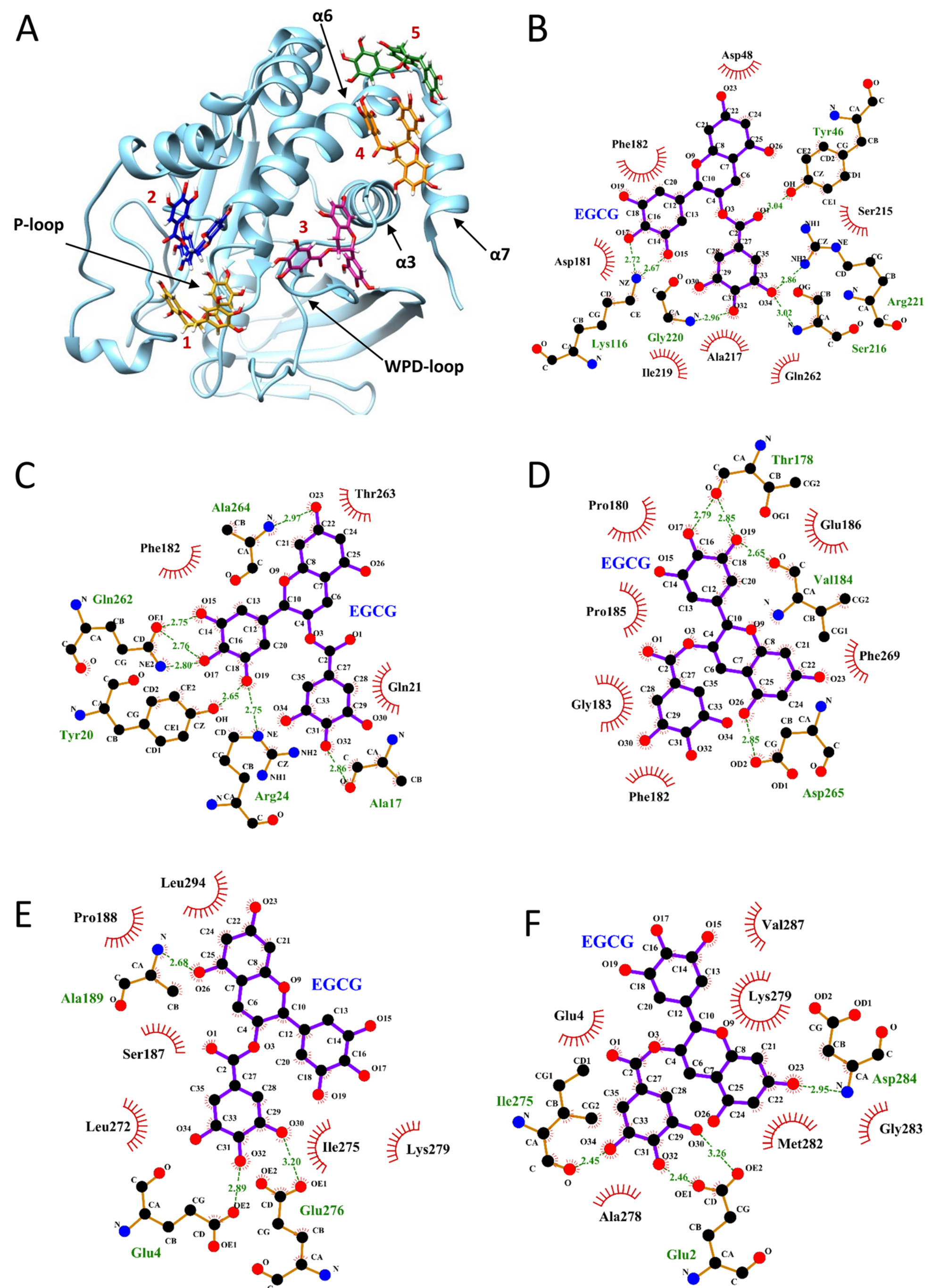

Fig. 5 Docking analyses of EGCG on PTP1B structure. Docking simulations of EGCG on PTP1B structure (PDB code: 1PTY) were carried out using AutoDock and Ligplot+ software as described in detail in "Materials and methods". **A** Several interaction sites of the ligand were found on PTP1B structure and indicated with numbers from 1 to 5. **B**–**E** 2D schematic representations of the intermolecular interactions between the enzyme and the ligand EGCG. Ligand bonds are reported as purple lines; EGCG interactions with PTP1B are reported as red lines for hydrophobic interactions, and as green lines for H-bonds. Specifically, **B** interactions between PTP1B and EGCG in position '1', related to the active site of the enzyme; **C** interactions between the enzyme and EGCG in position '2', close to the PTP1B active site; **D** interactions between PTP1B and EGCG in position '3' close to the WPD loop, which contains essential residues for the catalysis; **E**, **F** interactions between the phosphatase and EGCG in position '4' and '5', close to PTP1B allosteric sites

We wondered whether the selectivity of EGCG for PTP1B could depend on its ability to interact with allosteric sites previously described for this enzyme. Accordingly, we demonstrated that EGCG acts as a mixed-type non-competitive inhibitor of PTP1B, indicating that this molecule is able to bind to multiple sites on the enzyme surface. Furthermore, docking studies confirmed that EGCG interacts with amino acids located in the active site, the WPD loop [34], as well as with amino acids located between α3, α6 and α7 helices, where the ligand occupies pockets close to those already described for other allosteric PTP1B inhibitors [34]. Although further analyses will be necessary to identify the binding site(s) of EGCG on PTP1B structure, we believe that these molecules represent interesting lead compounds for the development of novel potent and selective inhibitors of PTP1B.

Our data showed that in vitro EGCG acts as a potent inhibitor of PTP1B. However, the physiological relevance of our results remains to be clarified. For instance, it is not

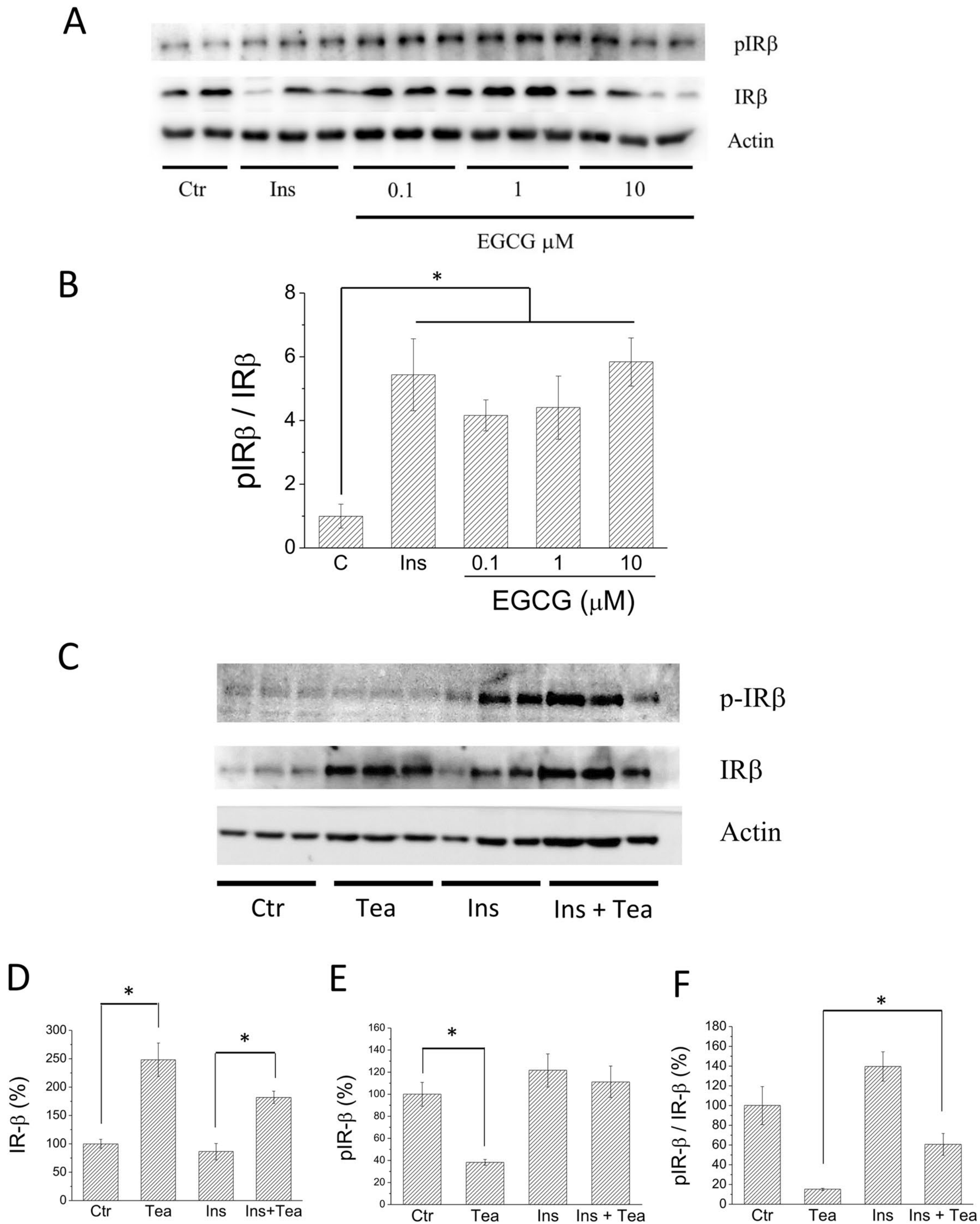

Fig. 6 Western blot analyses of C2C12 and HepG2 cells treated with EGCG and cold Indian green tea extract, respectively. **A** Representative images of western blot analysis of C2C12 cells treated for 30 min with 10 nM insulin or increasing amounts of EGCG (0.1, 1, 10 μM). Images show the expression levels of pIRβ and IRβ. Anti-β actin antibody was used to ensure equal loading. **B** Densitometric analysis of pIRβ/IRβ ratio. The EGCG treatment induces an increase in IR phosphorylation levels. No significant differences between samples treated with insulin alone or with increasing EGCG doses were detected. **C** Representative images of western blot analysis of HepG2 cells treated chronically for 4 days with cold Indian green tea extract and stimulated with insulin 10 nM for 30 min before cell lysis. Liver cells treated with cold extract resulted in a significant increase of IR expression levels **D**, and a decrease of both pIRβ **E** and pIRβ/IRβ ratio **F**. Results reported in the figure represent the mean value ± S.E.M ($n = 3$). *indicates a statistically significant difference according to Unpaired Student's *T* test data analysis (*$p < 0.05$)

clear whether in vivo EGCG can reach critical concentrations to inhibit PTP1B. Studies performed on human volunteers reported that, after taking a cup of tea, blood concentration of free catechins rapidly increases reaching a C_{max} of 25–55 nM, and then slowly decreases over several hours [35]. This evidence suggests that after the dietary intake of a cup of tea, blood contains a consistent reservoir of EGCG that may favor its accumulation in liver cells, allowing liver cells to accumulate quantities of EGCG sufficient to inhibit this enzyme. Accordingly, several preclinical studies confirmed that the administration of EGCG causes an increase in insulin sensitivity in liver cells [36, 37]. Validation of the ability of EGCG to cross cell membrane and affect insulin-signaling pathway comes from tests carried out using both HepG2 and C2C12 cells. HepG2 is a human hepatoma cell line that has been largely used for investigating insulin-signaling pathway. Although Molinaro and collaborators recently suggested that hepatoma cell lines do not accurately model primary hepatocytes in terms of insulin action, they demonstrated that in HepG2 cells the response of IR to hormone stimulation substantially recapitulates IR activity in primary hepatocytes [38]. In agreement with this finding, we used HepG2 cells to evaluate the impact of tea extracts and tea catechins only on IR phosphorylation status. Conversely, we selected C2C12 murine muscle cell line as experimental model to evaluate the impact of tea extracts on IR and Akt phosphorylation levels and on glucose uptake. This cell line is recognized as a suitable model to study the molecular mechanisms of insulin resistance and the potential of natural and drug/synthetic compounds in maintaining glucose homeostasis [39]. We observed that the acute treatment of myotubes with 0.1 μM EGCG results in increased phosphorylation of IR, confirming that EGCG can easily cross cell membrane and inhibit PTP1B. However, we also pointed out that only treatment with EGCG concentrations higher than 10 μM leads to a significant increase of glucose uptake in C2C12 cells, suggesting that the insulin-mimetic activity of EGCG is strictly dose-dependent. Remarkably, these data agree with results from a previous study showing that the treatment of primary hepatocytes with EGCG concentrations lower than 1 μM failed to activate insulin receptor substrate 1 (IRS1) or Akt, two downstream effectors of IR [40]. Therefore, our data confirmed that the acute effects of EGCG are strictly dose-dependent, and for this reason, it is difficult to reproduce them in a physiological context in which the amount of absorbed EGCG can be influenced by several factors. Interestingly, further tests showed that acute treatment of C2C12 cells with cold Indian green tea extract enhances Akt phosphorylation either when administered alone or in combination with insulin, confirming that in muscle cells the tea extract is able to potentiate insulin sensitivity.

Previous studies underlined that chronic treatment of diabetic rodents with EGCG reduces glucose-stimulated insulin secretion [41], suggesting that the chronic treatment with tea catechins could be more effective than acute one. Surprisingly, we observed that liver cells chronically treated with small amount (0.5%) of tea extract display higher IR levels and a strongly decreased ratio of phosphorylated IR over total IR (pIRβ/IRβ), compared to control samples, indicating that treated cells are enriched of the non-phosphorylated form of IR. However, by analyzing the relative increase of pIRβ/IRβ ratio after stimulation with insulin, we observed that tea-treated cells are more sensitive to insulin than untreated ones. To date, we are far from identifying the specific tea components responsible for this effect on cell lines, and from assessing whether the enhancement of IR levels is due to the increased rate of IR synthesis/recycling or to a slower rate of degradation. Nevertheless, a similar increase was not observed in muscle cells acutely treated with EGCG, thereby indicating that the upregulation of IR levels requires a long-time treatment. In our opinion, this observation could have important clinical implications. Indeed, changes in both the number and the affinity of liver IR have been already described following chronic insulin stimulation [42], treatment of liver cells with saturated fatty acids [43] or in mice models fed with high fat-diet [44], or that assumed alcohol [45]. In all these cases, the downregulation of IR is accompanied by the onset of liver insulin resistance. Noteworthy, our data suggest that IR downregulation is a transitory condition that could be reverted by the regular intake of tea. Intriguingly, none of these effects were detected in liver cells treated with the same dose of hot tea extracts, despite their higher total polyphenol content. These contradictory results could be due to chemical modifications or aggregation of tea components resulting from higher temperature of extraction, that leads to the extraction of molecules with decreased effects on the insulin-signaling pathway [32]. This hypothesis is in part confirmed by superimposing the HPLC fingerprints of hot and cold tea extracts, revealing that the composition of the two extracts is rather different both quantitatively and qualitatively. Therefore, the extraction temperature is overall a key variable influencing the beneficial effects of tea extracts on glucose homeostasis. To date, further studies are ongoing to identify tea components able to influence IR levels in liver cells, hoping that in future such compounds could be used as lead molecules to develop a new generation of drugs able to counteract the onset of liver insulin-resistance.

Conclusions

In this study, the bioactivity of tea extracts was characterized, focusing on their ability to modulate the activity of the main tyrosine phosphatases that regulate IR phosphorylation. Kinetic analyses revealed that all extracts act as potent inhibitors of both PTP1B and LMW-PTP, with a mechanism of action strongly dependent on the extraction temperature. Further investigations led to the identification of EGCG and ECG as critical tea components responsible for the inhibition of both the phosphatases. Specifically, from a mechanistic point of view, we demonstrated that EGCG acts as a mixed-type inhibitor, showing a Ki value of 26 nM. In addition, assays performed on muscle cells demonstrated that acute treatment with EGCG increases IR phosphorylation, suggesting that EGCG acts as an insulin-sensitizing agent. Besides, we showed that chronic treatment of liver cells with tea extracts produces the increase of IR levels and insulin sensitivity. In conclusion, data reported in this study provide useful information to understand the biological effects of tea and support the hypothesis that this beverage has a potential antidiabetic activity.

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Author contributions Conceptualization: LP, PP; methodology: SL, MVV; data curation: MG, MP; formal analysis and investigation: SL, MP, MVV, EP; visualization. EP, AC; writing—original draft preparation: PP; writing—review and editing: all the authors; funding acquisition: PP; resources: AC, LP, PP; supervision: PC, PP.

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Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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