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Phenolic antioxidants trolox and caffeic acid modulate the oxidized LDL-induced EGF-receptor activation

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1 Oxidized low density lipoproteins (oxLDL) are thought to play a major role in atherosclerosis. OxLDL act in part through alteration of intracellular signalling pathways in cells of the vascular wall. We recently reported that the EGF receptor (EGFR) signalling pathway is activated by lipid peroxidation products (among them 4-hydroxynonenal, 4-HNE) contained in oxLDL.

2 The use of phenolic antioxidants, such as trolox, alpha-tocopherol, caffeic acid and tyrphostins A-25, A-46 or A-1478, showed that the oxLDL-induced EGFR activation is constituted by two separate components, the first (early) one being antioxidant-insensitive, the second (late) being antioxidant-sensitive.

3 4-HNE derivatization of EGFR and EGFR activation induced by exogenous 4-HNE, suggest that the early (0.5–3 h) component of oxLDL-induced EGFR activation is mediated (at least in part) by 4-HNE (and possibly by other oxidized lipids). This early component is antioxidant-insensitive.

4 The second component (4–5 h) of the oxLDL-induced EGFR activation is antioxidant-sensitive, since it is blocked by antioxidants such as trolox, caffeic acid or PDTC, which act by blocking the cellular oxidative stress (H_2O_2 generation) evoked by oxLDL. Conversely, exogenous H_2O_2 induced EGFR autophosphorylation (thus mimicking the second component) and was also inhibited by antioxidants. This effect is mediated in part through inhibition by oxidative stress of protein tyrosine phosphatases involved in EGFR dephosphorylation.

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Abbreviations: Caf, caffeic acid; EGFR, EGF receptor; FeMb, ferrylmyoglobin; 4-HNE, 4-hydroxynonenal; [^{3}H]NSP, N-succinimidyl[2,3-^{3}H] propionate; OxLDL, oxidized LDL; PDTC, pyrrolidine dithiocarbamate; PTPase, protein tyrosine phosphatase; SMC, smooth muscle cells; αT, α-tocopherol; Trol, trolox

Introduction

Low density lipoproteins (LDL) are involved in the pathogenesis of atherosclerosis (Goldstein & Brown, 1977; Ross, 1993), after undergoing oxidative modifications (Witztum & Steinberg, 1991). The oxidative hypothesis is supported by various evidences: (i) ability of cultured vascular cells to oxidize LDL (Berliner & Heinecke, 1996); (ii) presence of oxidized LDL (oxLDL) in atherosclerotic lesions (Witztum & Steinberg, 1991) and (iii) (partial) antiatherogenic effect of antioxidants (Olsson & Yuan, 1996). LDL oxidation is a progressive process resulting in the formation of mildly oxLDL (defined by low lipid peroxidation derivatives content and only minor apoB modifications), and leading finally to extensively oxLDL (characterized by high levels of lipid peroxidation derivatives and severe apoB alterations). LDL oxidation is promoted *in vitro* by a variety of oxidizing agents (Berliner & Heinecke, 1996; Chisolm *et al*., 1999) and is believed to be mediated *in vivo* by reactive oxygen species and free radicals generated by vascular cells (Witztum & Steinberg, 1991; Berliner & Heinecke, 1996).

Oxidation induces dramatic changes in the biological properties of LDL (Witztum & Steinberg, 1991). OxLDL induce a variety of biological effects potentially involved in atherogenesis, such as foam cells and fatty streak formation, alterations in gene expression, cell migration, motility and contractility, cell proliferation, cell viability, local immune response, vasomotor tone (Witztum & Steinberg, 1991; Hajjar & Haberland, 1997). These biological responses are triggered by oxidized lipids contained in oxLDL that alter the activity of various cellular signalling pathways (Hajjar & Haberland, 1997), for instance calcium (Escargueil-Blanc *et al*., 1994), phospholipase D (Natarajan *et al*., 1995), trimeric G proteins (Parhami *et al*., 1995), protein kinase C and MAPkinase cascade (Deigner & Claus, 1996), ceramide (Auge

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et al. 1996), EGF-receptor (EGFR) (Suc *et al*., 1998), PI3-kinase (Martens *et al*., 1998), PPARgamma (Nagy *et al*., 1998), rho-kinase (Essler *et al*. 1999).

Antioxidants have been shown to slow down the atherosclerotic process, but their mechanisms of action are only poorly understood (Olsson & Yuan, 1996; Diaz, *et al*., 1997; Faggiotto *et al*., 1998). Antioxidants are able to modulate LDL oxidation (Heinecke, 1998), and also act at the cellular level by modulating the activity of various intracellular signalling pathways and inhibiting the pathogenic effects (e.g. cytotoxic and proliferative effects) of oxLDL (Schmitt *et al*., 1995; Azzi *et al*., 1997; Vieira *et al*., 1998).

We recently reported that oxLDL induce derivatization of various cell proteins by 4-hydroxynonenal (4-HNE) (Vieira *et al*., 2000) and other oxidized lipids (Chang *et al*., 1999). Among the cell proteins derivatized by 4-HNE, we have shown that EGFR is a target of 4-HNE from oxLDL which induce 4-HNE-adducts formation and activation of the EGFR (Suc *et al*., 1998).

EGFR belongs to the large family of receptor tyrosine kinases (RTKs) which are involved in the regulation of complex cellular processes, such as cell growth, motility, differentiation and death. In mammals, the EGFR family consists of four members, EGFR (or ErbB1), HER2 (or ErbB2/neu), HER3 (or ErbB3) and ErbB4 which are able to bind multiple peptide ligands (EGF, TGFα, amphiregulin, NDF, neuregulins, heregulins, HB-EGF, betacellulin and epiregulin) and, subsequently, to form homo- and heterodimeric complexes (Riese & Stern, 1998; Moghal & Sternberg, 1999; Hackel *et al*., 1999). Beside ligand-induced activation, EGFR can also be activated through ligand-independent mechanisms, for instance upon exposure of cells to ultraviolet radiations, oxidants, alkylating agents (Hackel *et al*., 1999) and oxidized lipids (Suc *et al*., 1998).

Activation of the EGFR pathway is characterized by the following sequence of events: (i) stimulation of its intrinsic tyrosine kinase and phosphorylation of its own tyrosine residues (of its cytoplasmic domain) and of intracellular substrate proteins; (ii) binding on phosphotyrosine of SH2- and PTB-domain containing proteins, such as enzymes (*src*, phospholipase C-γ1, phosphatidylinositol 3-kinase, SHP2 phosphotyrosine phosphatase, *ras*-GAP) or adaptor molecules (Shc isoforms, Grb2, Grb7, Nck, Cbl) which mediate downstream signalling (reviewed in Riese & Stern, 1998; Moghal & Sternberg, 1999; Hackel *et al*., 1999).

We report here that the oxLDL-induced EGFR activation is triggered by at least two mechanisms: the first one is mediated by 4-HNE, whereas the second one is mediated by intracellular peroxides (H_2O_2) and is inhibited by various phenolic antioxidants (α-tocopherol, trolox, caffeic acid, PDTC) which act through multiple mechanisms of action.

Methods

Chemicals

[γ-^{33}P]-ATP and [^{3}H]-thymidine were from ICN Biomedicals (Orsay, FR), anti-EGFR antibodies were from Santa Cruz (Tebu, Le Perray-Yvelines, France), anti-phosphotyrosine protein antibody (anti-PTyr-4-G10) was from UBI (Euromedex, Souffelweyersheim, France), RPMI-1640 (containing Glutamax®), penicillin, streptomycin and foetal calf serum from Gibco (Cergy-Pontoise, France), acrylamide 4X/bisacrylamide-2X solution from Bioprobe (Montreuil, France), 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate di(acetoxymethyl-ester (carboxy-H_2DCFDA-AM) from Molecular Probes (Eugene, OR, U.S.A.), 4-hydroxynonenal (4-HNE) was from Tebu-Biomol (Le Perray en Yvelines, France) and other chemicals from Sigma (St Quentin Fallavier, France) or Merck (Darmstadt, Germany).

Cell culture

The human cell line ECV-304 (CRL-1998) was from ATCC (Rockville MD, U.S.A) and the bovine aortic smooth muscle cells GM-08133A from the NIGMS (Camden, NJ, U.S.A.). Cells were routinely grown in RPMI 1640 medium (Life Technologies-Gibco) containing 10% foetal calf serum (Biowhittaker, Gagny, France) as described (Suc *et al*., 1998; Vieira *et al*., 2000). The B82LK+ cells (transduced with wild type EGFR) (Wright *et al*., 1995), were a generous gift from Dr M. Weber (Charlottesville, VA, U.S.A.), and were grown in DMEM containing 10% foetal calf serum. All passages were made at a splicing ratio 1/4. Twenty-four hours before addition of oxLDL, the standard medium was replaced by a serum-free medium.

LDL isolation and oxidation

LDL from human pooled sera were isolated by ultracentrifugation, dialyzed (against 150 mmol l^{-1} NaCl with 0.1 mmol l^{-1} EDTA), sterilized on 0.2 μm Millipore membrane and stored at 4°C under N_2 (up to 4 weeks). ApoB concentration was determined by immunonephelometry (Behring kit).

Mildly oxidized LDL were obtained by ferrylmyoglobin treatment. Ferrylmyoglobin mediated-LDL oxidation was carried out by incubation of LDL samples (2 mg apoB ml^{-1}, in PBS, pH 7.4) with 18 μM metmyoglobin/27 μM H_2O_2 during 2 h at 37°C, under the previously used conditions (Vieira *et al*., 2000).

LDL oxidation was evaluated by the content in thiobarbituric acid reactive substances (TBARS) (Yagi, 1987) and lipid hydroperoxides (FOX-2 method of Wolff, 1994), by the relative electrophoretic mobility (REM) on Hydragel (Sebia, Paris, France) and by the level of trinitrobenzene sulphonic acid (TNBS)-reactive amino groups (Steinbrecher *et al*., 1990). 4-HNE was evaluated according to Esterbauer *et al*. (1985).

In (FeMb)oxLDL, lipid hydroperoxides ranged between 21 and 45 nmol mg^{-1} apoB, TBARS between 3.9 and 9.2 nmol mg^{-1} apoB, 4-HNE between 8 and 18 nmol mg^{-1} apoB. REM between 1.1 and 1.2%, and TNBS-reactive amino groups between 89 and 95% (as per cent of the non-oxidized LDL). In non oxidized LDL, the ranges were 1.5–3.8 nmol hydroperoxide mg^{-1} apoB, 0.3–0.8 nmol TBARS mg^{-1} apoB.

Determination of EGFR-free amino groups

Free amino groups were evaluated on EGFR immunoprecipitates obtained from ECV-304 cells pre-incubated without

(control) or with EGF (10 nM for 15 min) or oxLDL (200 μg apoB ml^{-1} for 5 h) in the absence or presence of antioxidants. Free amino groups were labelled by an amine-reactive probe, N-succinimidyl[2,3-^{3}H]-propionate ([^{3}H]-NSP, Amersham, 99.0 Ci mmol^{-1}) (10 μCi in borate buffer 0.5 M, pH 8.5, 15 min. in an ice bath) (Staros *et al*., 1986), as previously described (Suc *et al*., 1998). The immunoprecipitates were washed three times in borate buffer, boiled in SDS-containing buffer and were resolved by SDS–PAGE. The 170 kDa bands were recovered and the radioactivity was determined by liquid scintillation counting.

Immunoprecipitation and Western blot analysis

Subconfluent cells grown and treated with oxLDL, 4-HNE, H2O2, EGF or other agonists, under conditions indicated in the text, were washed in cold phosphate buffered saline (PBS) containing (mM): NaF 20, sodium pyrophosphate 20, orthovanadate 1 and EDTA 5. Then, cells were lysed on ice for 30 min, in solubilization buffer (mM): Tris 50 pH 7.4, NaCl 150, EDTA 5, sodium vanadate 1, sodium pyrophosphate 10, sodium fluoride 160, phenylmethylsulphonyl fluoride 2.5, and leupeptin 10 μM, pepstatin A 2 μM, aprotinin 10 mg l^{-1}, triton X-100 10 g l^{-1} and deoxycholate 5 g l^{-1} derived from the method used by Wickramasinghe *et al*. (1996) and Suc *et al*. (1998). Fifty μg of protein cell extracts (determined using the bicinchoninic acid method) were resolved by electrophoresis in a 7.5% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane (Hybond-C, Amersham), and probed with anti-phosphotyrosine (4-G10, UBI), anti-EGFR (LA22, Santa-Cruz) or other antibodies, under the previously used conditions (Suc *et al*., 1998). Detection of 4-HNE adducts was performed by using polyclonal antibodies anti-4-HNE-protein (K5-4412) (Jürgens *et al*., 1993) on immunoblot of immunoprecipitated EGFR. The bound primary antibodies were visualized by using a peroxidase-coupled secondary antibody and the ECL detection system (Amersham).

When indicated, EGFR was immunoprecipitated before Western blot, according to method of Wickramasinghe *et al*. (1996), slightly modified by Suc *et al*. (1998). Briefly, after treatments with agonists or inhibitors, cells (from 7–8 large flasks 80 cm^2, i.e. around 1–1.5 mg cell protein) were washed in cold PBS, lysed in solubilization buffer (described above) and immunoprecipitated with anti-EGFR antibody (LA22, overnight at 4°C). Then, anti-EGFR immunoprecipitates were recovered on protein G-sepharose (1 h at 4°C), carefully washed in solubilization buffer, eluted by boiling in SDS-containing buffer and analysed by SDS–PAGE and the spots were revealed by immunoblotting (as used above). When required, semi-quantitative evaluation of Western blot spots was performed by densitometry.

Determination of in vitro *autophosphorylation and tyrosine kinase activity*

EGFR immunopurified from unstimulated cells was pre-incubated with antioxidants and then incubated with EGF for the indicated time. Autophosphorylation was evaluated by incubating the immunoprecipitates with 20 μM ATP in phosphorylation buffer (mM): HEPES 50, pH 7.5, NaCl 150, $MnCl_2$ 10, $MgCl_2$ 10, Na_3VO_4 10 μM, 0.2% Triton X-100, in a final volume of 50 μl. After incubation at 37°C for 15 min, the autophosphorylation of EGFR was analysed by Western-blots as above described.

The tyrosine kinase activity was evaluated under the same conditions, according to the previously used procedure (Suc *et al*., 1998), by determining the phosphorylation of polyGlu-Tyr (0.15 mg assay^{-1}) in the above used phosphorylation buffer containing 5 μCi of [γ-^{33}P]-ATP (3000 Ci mmol^{-1}, Isotopchim). After incubation at 37°C, the reaction was stopped by spotting an aliquot of the mixture on phosphocellulose membranes (Life Technologies), washed (10% TCA, 20 mM $Na_4P_2O_7$) and the radioactivity was counted.

Determination of protein tyrosine phosphatase (PTPase) activity and EGFR dephosphorylation rate

PTPases activity was determined according to Buscail *et al*. (1995). Briefly, cells incubated with agonists or/and inhibitors, under the conditions indicated below, were washed twice in PBS and homogeneized in 200 μl of Tris-HCl buffer 50 mM pH 7.0. Total PTPase activity of cell homogenate was determined by [^{33}P]-poly(GluTyr) dephosphorylation. [^{33}P]-poly(GluTyr) was prepared by incubating 7 mg poly(GluTyr) with 1 mg of A-431 cell membranes (which overexpress EGFR), in Tris-HCl buffer containing 0.1 μM EGF, 250 μM [^{33}P]-ATP (500 d.p.m. pmol^{-1}), 100 μM orthovanadate, 5 mM $MnCl_2$ and 5% Igepal. The assay mixture contained 30,000 c.p.m. [^{33}P]-poly(GluTyr) (final concentration 0.3 μM), and up to 20 μg cell homogenate in Tris-HCl buffer 50 mM pH 7.0 containing 0.1% albumin, and 5 mM DTT. After 10 min incubation at 30°C, the reaction was stopped by the addition of 100 μl trichloracetic acid 30% and the radioactivity of the liberated [^{33}P]-phosphate was extracted using the molybdate extraction and counted. The results are expressed as per cent of the unstimulated control.

Alternatively, EGFR-PTPase activity was determined by evaluating the rate of EGFR dephosphorylation in the presence of EGFR tyrosine kinase inhibitor (AG1478 100 μM). When indicated, cells were pre-incubated with or without oxLDL or H_2O_2 for 1 or 5 h, then incubated with EGF for 5 min and finally incubated with AG1478 (but without agonist) for 30 min (or the indicated time). Then cells were harvested and EGFR phosphorylation was evaluated by Western blot, under the above described conditions.

Binding experiments

The ability of antioxidants to compete with the binding and uptake of [^{125}I]-EGF was determined according to Marikovsky *et al*. (1993) under the previously used conditions (Suc *et al*., 1998). Briefly, after 3 h preincubation in the absence or presence of antioxidants, tracer amounts of [^{125}I]-EGF (70,000 c.p.m. ml^{-1}) (NEN) (and unlabelled EGF when indicated) were added for the indicated time (at 25°C). Then, after washing the cells twice in PBS containing 0.5% BSA and once in PBS alone, the cell-associated radioactivity was counted (Minaxi gamma Packard).

Determination of intracellular hydroperoxides and of extracellular H_2O_2

Intracellular hydroperoxides were determined by using the permeant probe carboxy-H_2DCFDA-AM (6-carboxy-2′,7′-

dichlorodihydrofluorescein diacetate, di(acetoxy-methyl-ester) (Molecular Probes C2938) which is retained into the cell after hydrolysis by cellular esterases and becomes fluorescent upon oxidation by peroxides (Hockenbery *et al*., 1993).

Cells, grown in 6-multiwells plates, were starved overnight in serum free medium and incubated with oxLDL (200 μg ml^{-1}) for variable times in the absence or presence of antioxidants. 30 min before stopping the experiments, 5 μM of H_2DCFDA-AM were added to each well. At the end, the cells were carefully washed in PBS, scraped off and centrifuged ($1500 \times g$ for 10 min). The cell pellet was sonicated in 0.8 ml of sterile water and the fluorescence of the homogenate was measured (Jobin-Yvon JY3C spectrofluorometer, excitation/emission wavelengths 495/527 for H_2DCFDA-AM).

H_2O_2 released in the culture medium was determined fluorometrically by the quenching (oxidation) of scopoletin in the presence of horseradish peroxidase (Loschen *et al*., 1973).

Protein determination

Proteins were determined using the bicinchoninic acid method of Smith *et al*. (1985).

Presentation of data and statistical analysis

Data are given as means ± s.e.mean. Estimates of statistical significance was performed by Anova (Student-Newman-Keuls Multiple comparison test).

Results

OxLDL-induced EGFR activation results from two separate components, the early one antioxidant-insensitive and the late one antioxidant-sensitive

OxLDL induced mild EGFR tyrosine phosphorylation, sustained for (at least) 5 h (Figure 1), whereas native LDL induced no (or only minor) EGFR activation (Suc *et al*., 1998 and Figure 2). EGF was used as positive control (maximal but transient EGFR tyrosine phosphorylation). EGFR was identified in preliminary experiments by immunoprecipitation with anti-EGFR antibody followed by immunoblot (Suc *et al*., 1998; see also Figure 2).

When ECV-304 cells were co-incubated with oxLDL and antioxidants (100 μM caffeic acid or 100 μM trolox), the time course of the oxLDL-induced EGFR autophosphorylation was partly altered, leading to discriminate between an early antioxidant-insensitive phase (0.5 – 3 h), and a late antioxidant-sensitive one (5 h) (Figure 1B,C) which was inhibited in a dose-dependent manner by antioxidants caffeic acid and trolox (Figure 1D,E). It may be noted that the early antioxidant-resistant EGFR autophosphorylation is not due to a delay in cell penetration of antioxidants, because similar results were obtained when cells were pre-incubated for 6 h with antioxidants (data not shown).

These data were not restricted to ECV-304 cells since similar oxLDL-induced EGFR tyrosine phosphorylation was also observed in smooth muscle cells (Figure 1F) and in B82LK+ (cells overexpressing human EGFR wild type) (Figure 1G). In these cells, the late (5 h) component of oxLDL-induced tyrosine phosphorylation of EGFR was also inhibited by caffeic acid, trolox, α-tocopherol, or by the receptor tyrosine kinase inhibitors tyrphostin A25 or A46.

As assessed by co-immunoprecipitation experiments, the late phase of oxLDL-induced EGFR autophosphorylation was associated with the recruitment of SH2-containing substrates/adaptor protein (such as PLC-γ1, SHP-2, SHC) which was inhibited by antioxidants, caffeic acid or trolox (Figure 2).

These data strongly suggest that the sustained oxLDL-induced EGFR activation is constituted by two phases, an early (0.5 – 3 h) antioxidant-insensitive component followed by a late (4 – 5 h) antioxidant-sensitive component.

Both oxLDL-induced 4-HNE-EGFR adduct formation and 4-HNE-induced EGFR autophosphorylation are antioxidant-insensitive

We recently reported that oxLDL lipid extracts induced *in vitro* derivatization by 4-HNE and activation of immunopurified EGFR (thus suggesting that EGFR is a target of 4-HNE and, perhaps, of other oxidized lipids); moreover, 4-HNE mimicked these effects of oxLDL both *in vitro* and in intact living cell (Suc *et al*., 1998). As antioxidants caffeic acid or trolox did not inhibit the early component of the oxLDL-induced EGFR activation (Figure 1), it was hypothesized that caffeic acid or trolox were unable to inhibit the EGFR activation induced by 4-HNE. As expected, antioxidants caffeic acid or trolox did not inhibit the 4-HNE-EGFR adduct formation induced by oxLDL or 4-HNE and subsequent activation of EGFR were not (or only slightly) inhibited by caffeic acid or trolox (Figure 3).

These data strongly suggest that the mild activation of EGFR triggered by low concentrations of 4-HNE (either derived from oxLDL or directly added to the culture medium) is not sensitive to antioxidants. This is consistent with the hypothesis that the early phase of oxLDL-induced EGFR activation (which is antioxidant-insensitive) may be mediated by EGFR derivatization induced by 4-HNE (or other oxidized lipids).

Mechanisms of action of antioxidants in the late antioxidant-sensitive phase of the oxLDL-induced EGFR activation

Several hypothetical mechanisms (potentially regulated by antioxidants) may be involved in the late phase of the oxLDL-induced EGFR activation: (i) cultured cells may promote a further oxidation of mildly oxLDL, thus increasing the level and the activity of oxidized lipids in the culture medium during incubation (in this case antioxidants act extracellularly); (ii) oxLDL may induce a cellular oxidative stress which may in turn activate EGFR (in this case antioxidants act intracellularly) and (iii) some antioxidants may regulate (inhibit) directly the EGFR.

Trolox inhibits the cell-mediated progression of oxLDL oxidation

Incubation of mildly oxLDL with subconfluent ECV-304 cells (in RPMI-1640 serum-free medium), led to enhance the level of oxLDL TBARS (Table 1). This cell-mediated additional

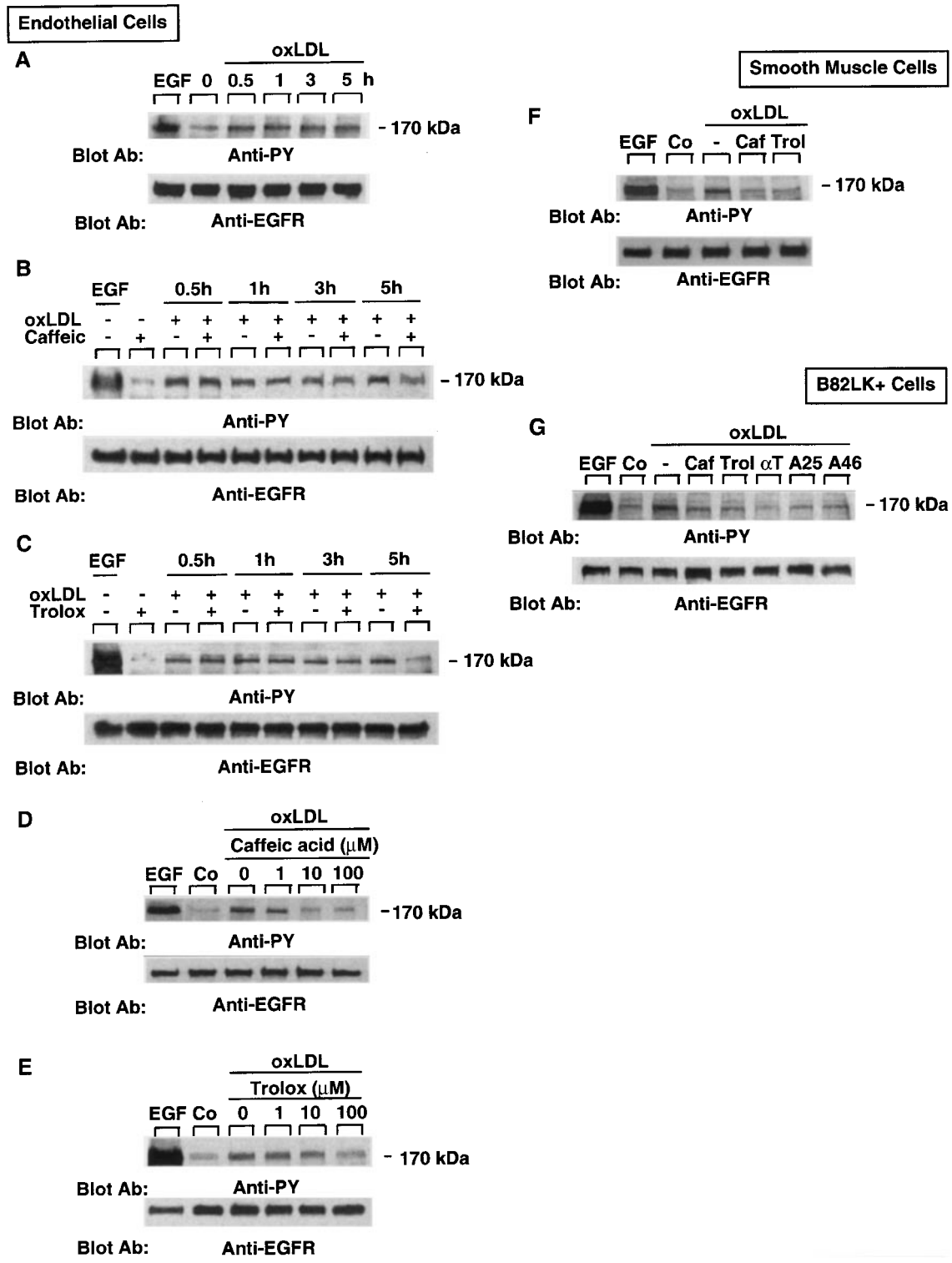

Figure 1 OxLDL-induced EGFR tyrosine phosphorylation and inhibition by antioxidants and tyrosine kinase inhibitors. Cells ((A–E) ECV-304 endothelial cells; (F) GM-08133A smooth muscle cells; (G) B82LK+ cells transduced with wild type EGFR) were incubated with oxLDL (200 μg apoB ml^{-1}) for the indicated time, then harvested and used for Western blots, labelled with anti-phosphotyrosine and anti-EGFR antibodies. EGF (10 nM for 15 min) was used as maximal positive control. (A–C) Time course of EGFR autophosphorylation induced by oxLDL in the absence (A), or presence (B,C) of antioxidants, caffeic acid (100 μM) or trolox (100 μM). (D,E) Inhibition of the oxLDL-induced EGFR autophosphorylation by increasing concentrations of caffeic acid (D) or trolox (E) in ECV-304 EC. Cells were co-incubated for 5 h with or without antioxidants and oxLDL. (F,G) Inhibition of the oxLDL-induced EGFR autophosphorylation at 5 h, by caffeic acid (100 μM), trolox (100 μM), α-tocopherol (100 μM) or tyrphostins A25 (5 μM) or A46 (20 μM), in GM-08133A smooth muscle cells (F) or in B82LK+ cells (transduced with and overexpressing wild type EGFR) (G). Representative data of 3–5 experiments.

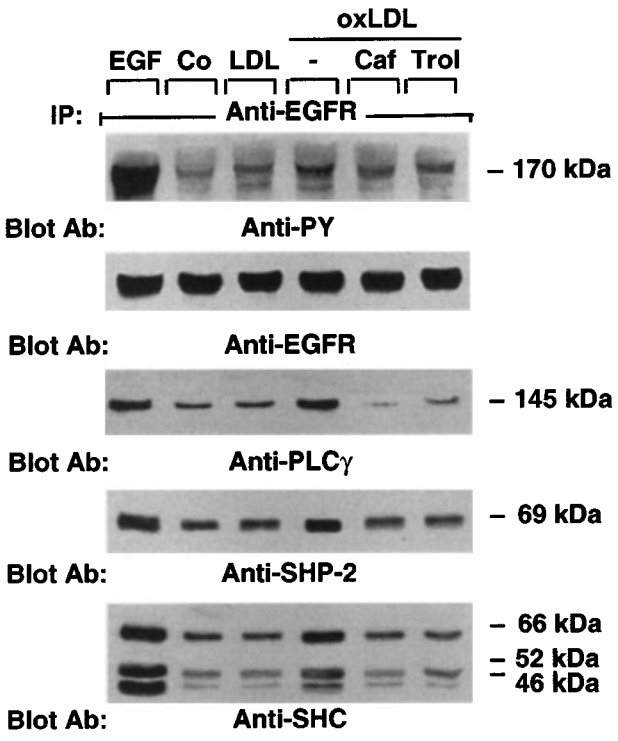

Figure 2 OxLDL-induced recruitment of EGFR substrates (i.e. activation of the EGFR signalling pathway) and inhibition by antioxidants. ECV-304 cells were incubated with oxLDL (200 μg apoB ml^{-1}) for 5 h (or 10 nM EGF for 15 min, used as maximal control) in the presence or absence of caffeic acid (100 μM) or trolox (100 μM). Cells were lysed in solubilization buffer (the used solubilization buffer allows co-immunoprecipitation of SH_2-proteins bound to EGFR) and (1.5 mg cell protein) were immunoprecipitated with anti-EGFR antibody (LA22, overnight at 4°C). Then, anti-EGFR immunoprecipitates were recovered on protein G-sepharose (1 h at 4°C), washed, eluted and analysed by SDS–PAGE. The spots were revealed by immunoblotting with anti-phosphotyrosine, anti-EGFR, anti-PLCγ, anti-SHP2 or anti-SHC antibodies. Representative data of three experiments.

oxidation of oxLDL was partly reduced by 100 μM caffeic acid and completely inhibited by 100 μM trolox (Table 2). Under the used conditions, trolox was more potent than caffeic acid to inhibit LDL oxidation progression, but, as expected, these antioxidants did not reduce the level of preformed TBARS (aldehydes). Therefore, as an appreciable level of bioactive aldehydes remains in the oxLDL (even when the progression of oxidation is inhibited by trolox), it is suggested that this extracellular effect of antioxidants (inhibition of the extracellular progression of oxLDL by trolox or caffeic acid) plays only a minor role in the inhibition of the oxLDL-induced EGFR activation.

OxLDL induce a cellular oxidative stress which participates in the EGFR activation and is inhibited by caffeic acid and trolox

As (i) oxLDL are able to induce a cellular oxidative stress (Schmitt *et al*., 1995; Thomas *et al*., 1993); (ii) oxidative stress may trigger EGFR autophosphorylation (Gamou *et al*., 1995) and (iii) α-tocopherol and trolox are able to prevent peroxidation of cellular lipids induced by oxLDL (Thomas

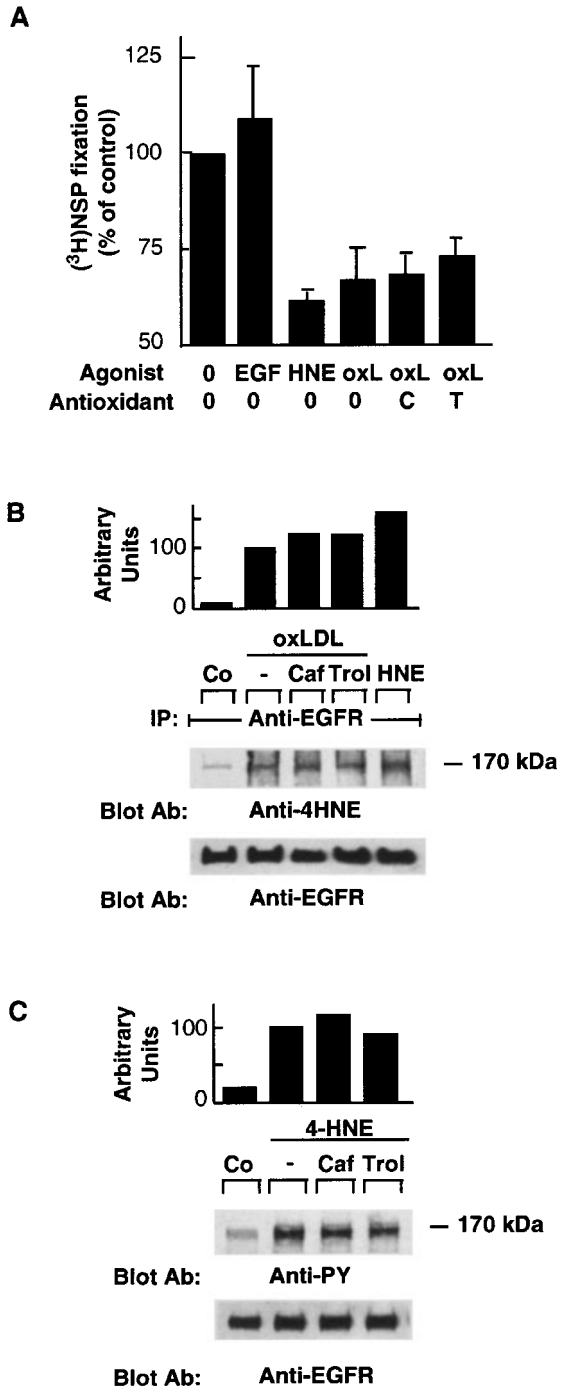

Figure 3 OxLDL- and 4-HNE-induced EGFR derivatization and activation. Lack of effect of antioxidants. (A,B) ECV-304 cells were incubated (for 3 h) with or without oxLDL (200 μg apoB ml^{-1}) and with or without caffeic acid (100 μM) or trolox (100 μM) or with 4-HNE (1 μM). Then cells were harvested and lysed and EGFR was immunoprecipitated. In (A), free amino groups were labelled by [^{3}H]-NSP, EGFR was resolved by SDS–PAGE and the radioactivity of the 170 kDa bands was counted. Mean ± s.e.mean of three experiments. In (B) EGFR was immunoprecipitated, resolved by SDS–PAGE and 4-HNE adducts was detected by polyclonal antibodies anti-4-HNE-protein (K5-4412) or anti-EGFR antibodies. (C) ECV-304 cells were incubated for 1 h with or without 4-HNE (1 μM) and with or without caffeic acid (100 μM) or trolox (100 μM) or α-tocopherol (100 μM), then harvested and lysed. Immunoprecipitated EGFR was resolved by SDS–PAGE and labelled with anti-phosphotyrosine and anti-EGFR antibodies. Representative data of four experiments. In B and C, semi-quantitative evaluation of Western blot spots was performed by densitometry.

Table 1 Progression of oxidation of oxLDL (pre-oxidized by FeMb under standard conditions) during cell culture with ECV-304 cells. Values are expressed as mean ± s.e.mean

Incubation time with cells (h)	*0*	*1*	*2*	*3*	*5*
LDL oxidation (nmol TBARS mg^{-1} apoB)	5.7 ± 0.5*	6.8 ± 0.9	7.3 ± 0.7	7.9 ± 1.2	9.9 ± 1.1

Table 2 Inhibition of the progression of oxidation of oxLDL (pre-oxidized by FeMb under standard conditions) during cell culture with ECV-304 cells (5 h incubation) in the presence or absence of antioxidants (100 μM caffeic acid or trolox)

	a	*b*	*c*	*d*	*e*
Experimental conditions					
Pre-incubation time with FeMb (h)	0	2	2	2	2
Incubation time with cells (h)	0	0	5	5	5
Antioxidant[†]	0	0	0	Caf	Trol
LDL oxidation: (nmol TBARS mg^{-1} apoB)					
Experiment 1	0.6 ± 0.1*	5.7 ± 0.5*	9.7 ± 0.6	7.5 ± 0.5*	5.5 ± 0.7*
Experiment 2	0.8 ± 0.1*	9.2 ± 0.1*	16.2 ± 0.9	11.3 ± 0.6*	8.7 ± 0.3*

[†]Antioxidants (caffeic acid or trolox) were added during the 5 h incubation of oxLDL with cells. *Statistical significance (Anova one way, Student-Newman-Keuls test) $P < 0.05$ (comparison of a, b, d, e with c).

et al., 1993), we hypothesized that the cellular oxidative stress triggered by oxLDL may also take part in the oxLDL-induced EGFR activation.

The oxidative stress induced by oxLDL was monitored by using the fluorogenic carboxy-H_2DCFDA probe sensing the intracellular peroxides. In ECV-304 cells, oxLDL induced a rapid rise of the fluorescence (resulting from oxidation of the carboxy-H_2DCFDA probe), indicating a cellular oxidative stress occurring rapidly in the first hour of the pulse (Figure 4A). In contrast, native LDL induced no oxidative stress (data not shown). This intracellular oxidative stress was followed by a release of H_2O_2 in the culture medium (beginning at 2 h), as assessed by scopoletin quenching (Figure 4A). This rise of both intracellular peroxides and H_2O_2 generation triggered by oxLDL was inhibited by 100 μM caffeic acid or 100 μM trolox (Figure 4B,C).

This led us to examine the role of H_2O_2 in the oxLDL-induced EGFR activation and the possible effect of caffeic acid and trolox, in comparison with pyrrolidine dithiocarbamate (PDTC), a classical H_2O_2 scavenger. Incubation of ECV-304 cells with 200 μM H_2O_2 induced a progressive rise of intracellular H_2CFDA fluorescence (Figure 5A) and a subsequent EGFR tyrosine phosphorylation which was reduced in part by caffeic acid (100 μM) and completely inhibited by trolox (100 μM) or PDTC (100 μM) (Figure 5B,C). Moreover, it may be noted that the late phase of oxLDL-induced EGFR activation was also blocked by PDTC (Figure 5C).

These data support the hypothesis that oxLDL-induced H_2O_2 generation may participate in the EGFR activation and that this mechanism is antioxidant-sensitive (i.e. inhibited by trolox or caffeic acid).

Caffeic acid (but not trolox) inhibits EGF-induced EGFR autophosphorylation

Because of the structural analogy of caffeic acid with tyrphostins A25 and A46, it was hypothesized that, in addition to its antioxidant activity, it may inhibit the EGFR tyrosine kinase. This led us to investigate the effect of caffeic acid and trolox on the EGF-induced EGFR activation both in cultured living cells and in an *in vitro* biochemical system (using immunopurified EGFR).

EGFR tyrosine phosphorylation induced by EGF in cultured B82LK+ cells was inhibited by caffeic acid (100 μM) and by the EGFR kinase inhibitor tyrphostin A46 (20 μM), but not by trolox (Figure 6A). As the inhibitory effect of caffeic acid did not result from an inhibition of the binding of EGF to EGFR (Figure 6B), we examined the possibility that caffeic acid may inhibit more directly the EGFR tyrosine kinase, using an *in vitro* biochemical system, i.e. immunopurified EGFR stimulated *in vitro* by EGF. *In vitro* activation of the EGFR kinase by EGF, monitored by EGFR autophosphorylation and polyGluTyr phosphorylation was strongly inhibited by caffeic acid, but not by trolox (Figure 6C,D). This suggests that caffeic acid may inhibit the EGFR kinase activity, probably independently of its antioxidant activity.

Inhibition of protein tyrosine phosphatases (PTPases) by oxLDL (Figure 7)

A hypothetical mechanism involved in the late phase of oxLDL-induced EGFR activation may result from peroxide-mediated inhibition of protein tyrosine phosphatases (PTPases) implicated in EGFR dephosphorylation (Knebel *et al*., 1996). The validity of this hypothesis was examined by evaluating, under conditions of the late phase of oxLDL-induced EGFR activation: (i) the activity of PTPases and (ii) the rate of dephosphorylation of EGFR.

The whole cellular PTPase activity was not affected by 1 h incubation with oxLDL, whereas it was reduced by 45% in cells treated for 5 h with oxLDL (Figure 7A,B). This late oxLDL-induced inhibition of PTPases was reversed by antioxidants (Figure 7B).

The activity of EGFR-PTPase(s) was evaluated by determining the rate of EGFR dephosphorylation in cells

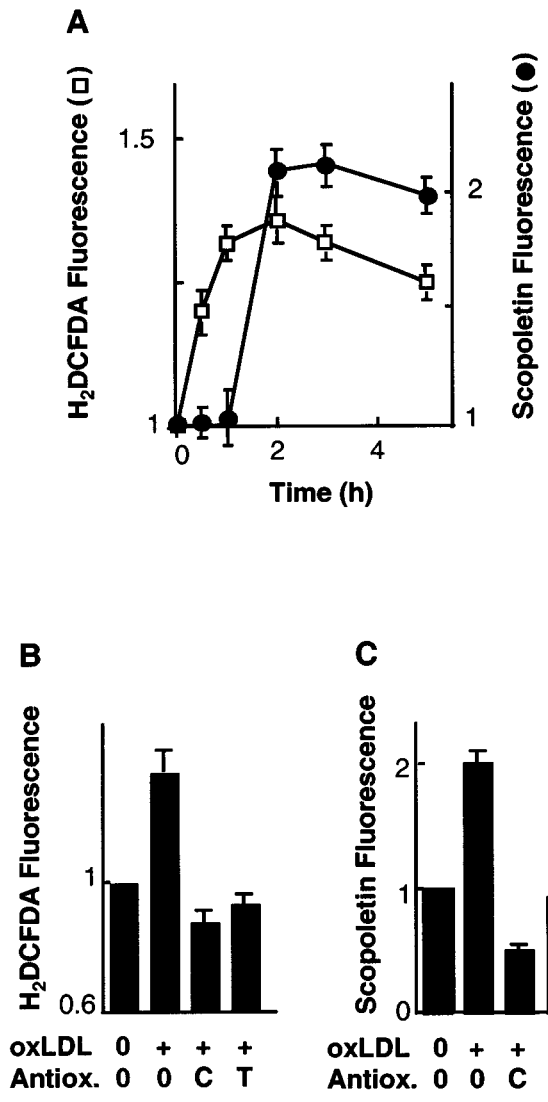

Figure 4 OxLDL induce oxidative stress in ECV-304 EC. Inhibition by caffeic acid or trolox. (A) Time course of the intracellular oxidative stress (squares) and extracellular liberation of H_2O_2 (circles) induced by oxLDL. Cells were incubated with oxLDL (200 μg apoB ml^{-1}) and, at the indicated time, cellular H_2DCFDA fluorescence was monitored, and H_2O_2 released in the cultured medium was determined by scopoletin fluorescence quenching method, as indicated in the Methods section. (B,C) Inhibition by caffeic acid or trolox of the intracellular oxidative stress (B) and extracellular liberation of H_2O_2 (C). Cells, were incubated for 3 h with or without oxLDL (200 μg apoB ml^{-1}) and with or without caffeic acid (100 μM) or trolox (100 μM). Then, the intracellular cellular oxidative stress (H_2DCFDA) and extracellular liberation of H_2O_2 (scopoletin) were measured as in Figure 5A. Fluorescence is expressed as arbitrary units. Mean$\pm$s.e.mean of 3–4 experiments.

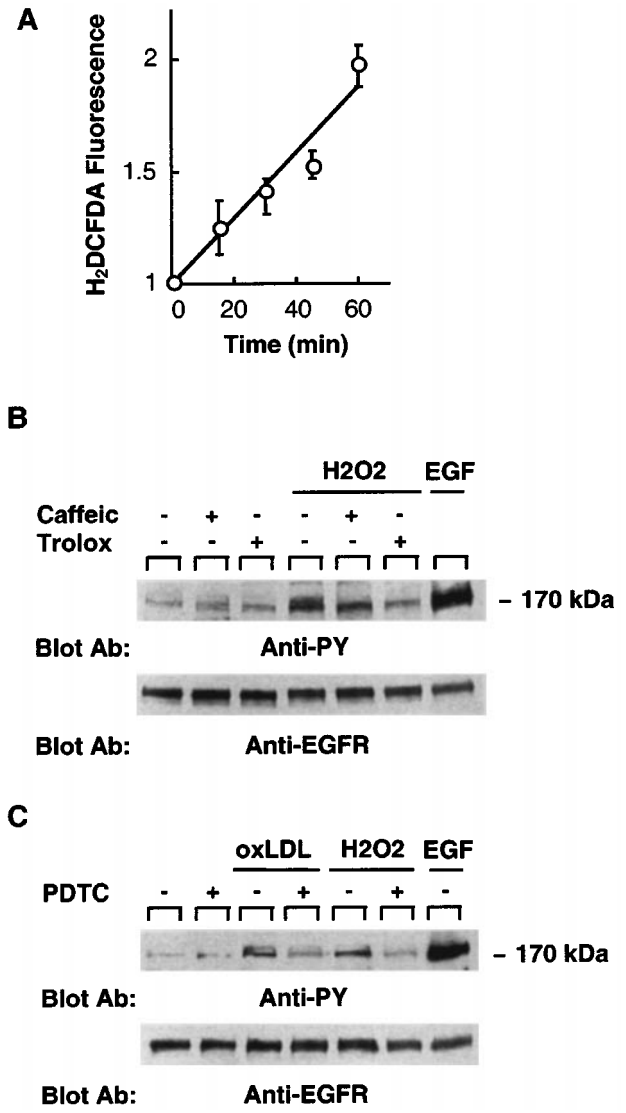

Figure 5 Cellular oxidative stress triggered by H_2O_2 induces EGFR activation in ECV-304 EC. (A) Time course of intracellular peroxides, monitored by using the fluorescence of the oxidation-sensitive H_2DCFDA fluorogenic probe, in cells incubated with H_2O_2 (200 μM). Mean$\pm$s.e.mean of three experiments. (B,C) Cells were incubated with 200 μM H_2O_2 for 3 h (B and C) or oxLDL (200 μg apoB ml^{-1}) for 5 h (C) and without (−) or with (+) antioxidants, caffeic acid (100 μM) or trolox (100 μM) (B) or PDTC (100 μM) (C). Western blot were revealed by anti-phosphotyrosine and anti-EGFR antibodies. In (B) and (C), representative data of three experiments.

preincubated (or not) with oxLDL (for 5 h), then incubated with EGF (for 5 min) and finally chased for 30 min in the presence (or absence) of tyrphostin AG1478 (a specific inhibitor of the EGFR kinase). If the hypothesis (PTPase inhibition by oxLDL) is right, preincubation of cells with oxLDL should inhibit EGFR-PTPase(s) and, consequently, the EGF-induced EGFR phosphorylation should be more sustained. Preliminary experiments have shown that, after stimulation by EGF (without preincubation with oxLDL) EGFR was almost completely dephosphorylated after 30 min chase in the presence of tyrphostin AG1478 (a specific EGFR-kinase inhibitor added after the 5 min-stimulation by EGF in order to stop further autophosphorylation) (Figure 7C, lanes 5, 6). In contrast, when cells were preincubated for 5 h with oxLDL before incubation with EGF and 30 min chase with tyrphostin AG1478, EGFR was only partly dephosphorylated (Figure 7C, lanes 4, 5). This suggests that the EGFR-PTPase(s) is (partly) inhibited by preincubation of cells with oxLDL. Antioxidants (added during the preincubation with oxLDL) reversed in part the oxLDL-induced inhibition of EGFR dephosphorylation (Figure 7D).

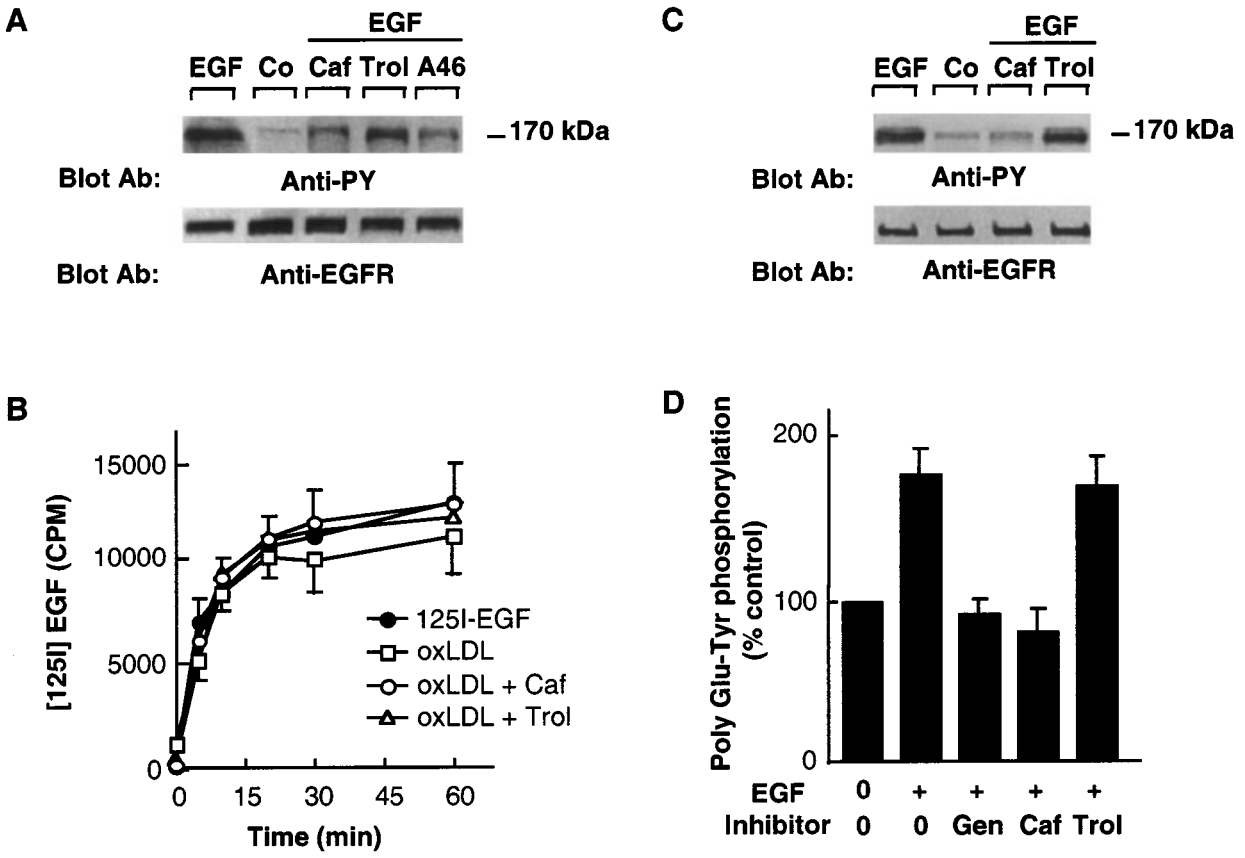

Figure 6 Caffeic acid and tyrphostin A46 (but not trolox) inhibit EGF-induced EGFR activation (inhibition of the EGFR tyrosine kinase). (A) B82LK+ cells, pre-incubated for 1 h with or without caffeic acid (100 μM), trolox (100 μM) or tyrphostin A46 (20 μM) before treatment with EGF (2 nM for 20 min) (Co, untreated control). EGFR tyrosine phosphorylation was visualized on Western blots labelled with anti-phosphotyrosine antibody. (B) Lack of influence of caffeic acid or trolox on the association (binding and uptake) of ^{125}I-EGF to ECV-304 EC. Cells were pre-incubated for 3 h with or without oxLDL (200 μg apoB ml^{-1}) and caffeic acid (100 μM) or trolox (100 μM); then a tracer amount of ^{125}I-EGF (70,000 c.p.m. ml^{-1}) was added for variable periods of time (up to 60 min). Pre-incubation conditions: black circles, no addition; empty squares, oxLDL (200 μg apoB ml^{-1}); empty circles, oxLDL and caffeic acid (100 μM); empty triangles, oxLDL and trolox (100 μM). (C,D) Caffeic acid (but not trolox) inhibits the *in vitro* EGF-induced tyrosine phosphorylation (C) and tyrosine kinase activation (D) of immunopurified EGFR. EGFR purified by immunoprecipitation from unstimulated B82LK+ cells, was incubated without (control, Co) or with EGF (5 nM) and without or with caffeic acid (100 μM) or trolox (100 μM) or genistein (25 μM) in the phosphorylation buffer for 15 min. In (C), EGFR tyrosine phosphorylation was visualized on Western blot labelled with anti-phosphotyrosine and anti-EGFR antibodies. In (D), EGFR tyrosine kinase activity was evaluated under the same conditions as in (C) but in the presence of [γ-^{33}P]-ATP and poly Glu-Tyr (as indicated in Methods). In (A) and (C), representative data of three experiments. In (B) and (D), Mean$\pm$s.e.mean of three experiments.

Altogether these data suggest that PTPases inhibition may be (in part) involved in the late phase of the oxLDL-induced EGFR activation and that antioxidants are able to prevent (partly) PTPases inhibition.

Discussion

Various signalling pathways activated by growth factors are potentially involved in atherogenesis (Ross, 1993). Growth factors of the EGF family (through activation of EGFR or related receptors), acting either alone or in combination with other mediators may participate in the regulation of vascular cell biology and may thereby play a role in atherogenesis (Ross, 1993). EGFR is a target of lipid peroxidation products from oxLDL which induce EGFR activation, independently of any autocrine secretion of growth factors of the EGF family (Suc *et al*., 1998). We report here that the oxLDL-induced EGFR activation results from two separate components, an early (0.5–3 h) antioxidant-insensitive component and a late (5 h) antioxidant-sensitive component which is inhibited by antioxidants, such as caffeic acid trolox, α-tocopherol and PDTC.

Several data support the hypothesis that the early phase of the oxLDL-induced EGFR activation is mediated (at least in part) by 4-HNE (Suc *et al*., 1998) and is an antioxidant-insensitive event: (i) free (chemically active) 4-HNE is present in oxLDL (beside the 4-HNE-adducts to apoB or lipids) (Esterbauer *et al*., 1987) or may be continuously formed by decomposition of polyunsaturated fatty acid hydroperoxides (Esterbauer *et al*., 1992); (ii) both the oxLDL-induced EGFR derivatization and activation are mimicked by incubation of cultured cells with exogenous 4-HNE; (iii) *in vitro*, 4-HNE

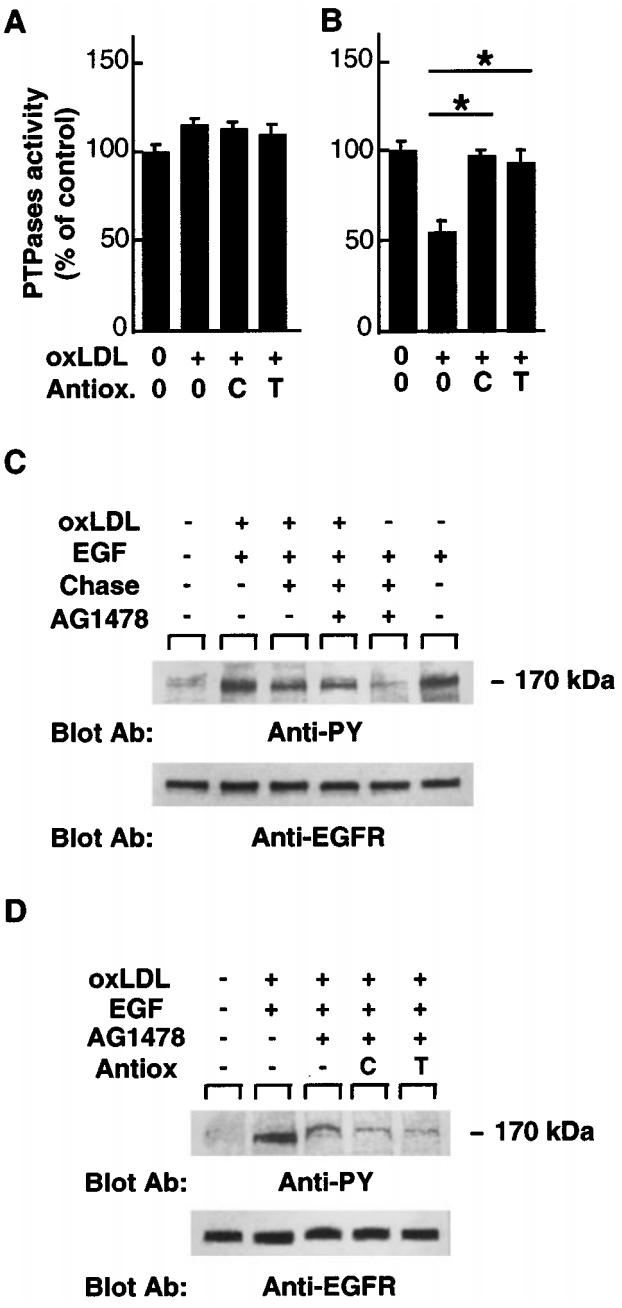

Figure 7 Evaluation of PTPase activity and EGFR dephosphorylation in cells incubated with oxLDL and antioxidants. (A,B) PTPase activity was evaluated in ECV-304 EC preincubated (or not) with oxLDL (200 μg apoB ml^{-1} of oxLDL) for 1 h (A) or 5 h (B) with (+) or without (0) antioxidants caffeic acid, C or trolox, T. (C,D) ECV-304 EC were preincubated with (+) or without (−) oxLDL (5 h preincubation with 200 μg apoB ml^{-1} of oxLDL), then were incubated with EGF (2 nM for 5 min) and finally were chased (without EGF) for 30 min with (+) or without (−) tyrphostin AG1478 (100 nM) (C), and, when indicated, with antioxidants caffeic acid, C or trolox, T (added during preincubation of cells with oxLDL) (D). Western blot were revealed by anti-phosphotyrosine and anti-EGFR antibodies. In (C) and (D), representative data of three experiments.

induces concomitantly both 4-HNE-adduct formation and tyrosine phosphorylation of immunopurified EGFR, thus supporting the idea of a direct link between 4-HNE-derivatization and activation of EGFR and (iv) both events (derivatization and activation of EGFR) induced by oxLDL or by exogenous 4-HNE are antioxidant-insensitive. Of course, it cannot be excluded that other (yet unknown) mechanisms may take part in this oxLDL-induced EGFR activation.

Several lines of evidence suggest that the second phase of the oxLDL-induced EGFR activation is mediated by oxidative stress triggered by oxLDL: (i) the time course of H_2O_2 (or other ROS or peroxides) generated during incubation with oxLDL is compatible with the hypothesis of a role of oxidants in the second phase of EGFR activation; (ii) PDTC, trolox and caffeic acid inhibit effectively both the H_2O_2 generation (and the cellular oxidative stress) and the late phase of oxLDL-induced EGFR activation and (iii) exogenous H_2O_2 triggers both a cellular oxidative stress and EGFR activation which are concomitantly inhibited by antioxidants. These data and conclusion are in agreement with previous reports demonstrating that H_2O_2 is able to activate EGFR tyrosine phosphorylation *in vitro* and in the intact living cell (Gamou *et al*., 1995) and that H_2O_2 is also generated during EGF-induced EGFR activation and is involved in the activation of the EGFR signaling pathway (Bae *et al*., 1997). This is also consistent with the concept that oxidative stress (possibly H_2O_2) acts as intracellular messenger (Rhee, 1999). Hypothetically, this sustained EGFR activation may result from the inhibition of phosphotyrosine phosphatases (PTPases) which are known to be inhibited by oxidants such as H_2O_2 (Knebel *et al*., 1996) or sulphydryl reagents (Monteiro & Stern, 1996). Direct determination of PTPases activity and evaluation of the rate of EGFR dephosphorylation in cells preincubated with oxLDL, suggest that, after 5 h-incubation with oxLDL, PTPase activity and EGFR dephosphorylation are inhibited by around 50%. This led us to conclude that the late phase of the oxLDL-induced EGFR activation may be mediated (at least in part) through EGFR-PTPase inhibition (resulting from the oxLDL-induced cellular oxidative stress). However, as PTPase inhibition is only partial, it cannot be excluded that other yet unknown mechanism(s) may play a role in the late phase of the oxLDL-induced EGFR activation.

From a (patho)physiological point of view, as oxLDL are present in atherosclerotic lesions (Witztum & Steinberg, 1991), it may be speculated that oxLDL-induced EGFR activation may occur in atherosclerotic areas. Antioxidants, from dietary (such as caffeic acid or tocopherol) or synthetic (such as trolox or PDTC) origin, may prevent at least in part this effect of oxLDL, either by preventing LDL oxidation (Esterbauer *et al*., 1992) or by acting at the cellular level by reducing the oxidative stress triggered by oxLDL (this study). But it is to note that antioxidants were unable to prevent the part of oxLDL-induced EGFR activation mediated by 4-HNE.

Finally our data suggest that the oxLDL-induced activation of the EGFR pathway is probably mediated by at least two mechanisms, an early antioxidant-insensitive EGFR derivatization mediated by 4-HNE and a late antioxidant-sensitive component resulting from cellular oxidative stress (H_2O_2 generation triggered by oxLDL). Antioxidants may act on multiple molecular targets (LDL oxidation, EGFR, oxidative stress) and through multiple

mechanisms of action (antioxidant effect and inhibition of the EGFR kinase) and may thereby counteract the deleterious effect of oxLDL.

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