Activation of Epithelial Growth Factor Receptor Pathway by Unsaturated Fatty Acids

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Abstract—Nonesterified fatty acids (NEFAs) are acutely liberated during lipolysis and are chronically elevated in pathological conditions, such as insulin resistance, hypertension, and obesity, which are known risk factors for atherosclerosis. The purpose of this study was to investigate the effect and mechanism of action of NEFAs on the epithelial growth factor (EGF) receptor (EGFR). In the ECV-304 endothelial cell line, unsaturated fatty acids triggered a time- and dose-dependent tyrosine phosphorylation of EGFR (polyunsaturated fatty acids [PUFAs] were the most active), whereas saturated FAs were inactive. Although less potent than PUFAs, oleic acid (OA) was used because it is prominent in the South European diet and is only slightly oxidizable (thus excluding oxidation derivatives). EGFR is activated by OA independent of any autocrine secretion of EGF or other related mediators. OA-induced EGFR autophosphorylation triggered EGFR signaling pathway activation (as assessed through coimmunoprecipitation of SH2 proteins such as SHC, GRB2, and SHP-2) and subsequent p42/p44 mitogen-activated protein kinase (as shown by the use of EGFR-deficient B82L and EGFR-transduced B82LK cell lines). OA induced in vitro both autophosphorylation and activation of intrinsic tyrosine kinase of immunopurified EGFR, thus suggesting that EGFR is a primary target of OA. EGFR was also activated by mild surfactants, Tween-20 and Triton X-100, both in vitro (on immunopurified EGFR) and in intact living cells, thus indicating that EGFR is sensitive to amphiphilic molecules. These data suggest that EGFR is activated by OA and PUFAs, acts as a sensor for unsaturated fatty acids (and amphiphilic molecules), and is a potential transducer by which diet composition may influence vascular wall biology. (Circ Res. 1999;85:892-899.)

Key Words: fatty acid ★ growth factor ★ mitogen-activated protein kinase ★ EGF receptor

Nonesterified fatty acids (NEFAs) are physiologically liberated during postprandial lipolysis and are chronically elevated in plasma in various diseases (eg, obesity, mellitus diabetes, ketoacidosis, hypertension), known risk factors for vascular diseases and atherosclerosis.1-3 Saturated fatty acids (FAs) are thought to be atherogenic, whereas unsaturated FAs (UFAs), such as oleic acid (OA) and n-3 polyunsaturated FA (PUFA), are considered to be antiatherogenic,3 despite recent contradictory reports.4 The antiatherogenic effect of UFAs may result in part from their lowering effect on LDL cholesterol,5,6 from their “anti-inflammatory” effect on vascular cells (eg, UFAs inhibit the expression of endothelial proinflammatory proteins7), and from their anti-thrombotic effects (for n-3 PUFA).8 The mechanisms of action of NEFAs are only in part understood and are complex because NEFAs are involved at various stages of cell biology—namely, membrane structure, cell metabolism, energy production, and cell signaling.9,10 UFAs are able to modulate the activity of various intracellular signaling pathways mediated by calcium, protein kinase C (PKC), mitogen-activated protein kinases (MAPKs), and epithelial growth factor receptor (EGFR).9-17

EGFR, now considered to be a critical crossroad of multiple receptor pathways,19 is potentially implicated in the regulation of cell migration, proliferation, or differentiation and may be involved in atherogenesis.19 EGFR is a 170- kDa transmembrane receptor tyrosine kinase that is shared by several growth factors, such as EGF, heparin-binding EGF, tumor necrosis factor-α, amphiregulin, and betacellulin.20,21 Moreover, EGFR activation is modulated by various non specific factors, such as UV irradiation,22 H2O2,23 oxidized lipoproteins,24 UFAs, and their oxidation derivatives.16,25,26

Ligand binding induces EGFR dimerization, stimulation of its intrinsic tyrosine kinase, and autophosphorylation of its own tyrosine residues.20,21 Phosphotyrosines of the C-terminal domain of EGFR are binding sites for SH2 domains of adaptors or enzymatic proteins, including phospholipase Cγ1, GTPase-activating protein of p21ras (ras-GAP), SHP2, p85 subunit of phosphatidylinositol 3-kinase,
SHC, Nck, c-cbl, and GRB2-Sos. The activation of GRB2-Sos complex may in turn activate p21ras and the kinase cascade leading to MAPK activation. MAPK or extracellular-regulated kinase (ERK) (p44/ERK1 and p42/ERK2) can also be activated through receptors for growth factors, hormones or cytokines, or G-protein-coupled receptors, or in response to stress.

The aim of this study was to investigate whether FAs are able to activate EGFRs and whether they are active per se or only after metabolic activation (eg, after the generation of oxidized or other bioactive derivatives).

Our data show that (1) in intact living cells, UFAs induce EGFR autophosphorylation and activation, and subsequent MAPK activation; (2) UFA activity is related to the degree of unsaturation but is, at least in part, independent of FA metabolism; (3) in vitro, UFAs elicit autophosphorylation of the immunopurified EGFR, thus suggesting that EGFR may be considered a primary target of oxidized lipids; and (4) EGFR acts a sensor for amphiphiles (or for membrane fluidity changes).

Materials and Methods

Chemicals and culture reagents were obtained from Gibco. [125I]EGF (150 μCi/μg) was from New England Nuclear Research Products. Enhanced chemiluminescence reagent (ECL Kit), nitrocellulose, and autoradiography films were from Amersham Corp. FAs, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC), and general reagents were obtained from Sigma Chemical Co. Recombinant EGFR and antibodies against EGFR (monoclonal), EGFR antibodies against EGFR (monoclonal), and rabbit polyclonal), GRB2, SHP2, ERK1/ERK2 (MK12), SHC were from Santa Cruz/Peprotech/Tebu. Activated EGFR was isolated from UBI/Euromedex.

Cell Culture and Cell Extracts

Human endothelial ECV-304 cells (American Type Culture Collection) were grown in RPMI-1640 containing 10% FCS. The murine B22L, parental fibroblasts (EGFR deficient) and B22L* cells (transduced with wild-type EGFR), a generous gift from Dr M. Weber (Charlottesville, Va), and SrcK (transduced with wild-type EGFR), a generous gift from Dr M. S. Parsons (Charlottesville, Va), were grown in Laemmli’s buffer. Immunoprecipitation and immunoblotting. The OA-induced EGFR phosphorylation (Figure 1) that was identified as EGFR through immunoprecipitation and immunoblotting. The OA-induced EGFR phosphorylation began rapidly (2 minutes) and was sustained for ≥45 minutes (Figure 1A). EGFR phosphorylation increased progressively with OA concentration, apparently without saturation up to 100 μmol/L (Figure 1B). In the presence of BSA (50 μmol/L), the OA-induced EGFR phosphorylation was clearly visible with a molar ratio (OA/BSA) of 1:1 and was more intense when the ratio was ≥2 (Figure 1C). This suggests that the activity may result from unbound OA.

EGFR phosphorolyzing activity of FAs was dependent on chain length and unsaturation. Short chain FAs were inactive, and PUFAs were more effective (C20:5, or eicosapentaenoic acid, and C22:6, or docosahexaenoic acid, were the most effective) (Figure 1). In the present study, we used preferentially OA (although less potent than PUFA) because (1) OA is the major circulating UFA and (2) we sought to understand whether UFAs may be active per se or only through their oxidation derivatives (eg, 13(S)-hydroperoxyoctadecadienoic acid, and 13(S)-hydroperoxyoctadecadienoic acid, which are able to activate EGFR). OA is only slightly oxidizable and does not generate these oxidized lipids.

The EGFR phosphorolyzing activity by 50 μmol/L OA or arachidonic acid (AA) was grossly equivalent to 50 and 200 μmol/L EGF, respectively (Figure 1F). When OA was removed from the cell culture medium (cells were later incubated in delipidated medium), EGFR autophosphorylation persisted for 15 to 20 minutes after washout (data not shown). Similar to EGF, OA and AA were also able to induce EGFR dimerization, concomitant with autophosphorylation of the receptor (Figure 2A). As shown in Figure 2B, OA was effective in activating the EGFR pathway, as assessed by the
recruitment of SH2-containing proteins SHP-2, SHC, and GRB2.

**OA-Induced EGFR Activation Elicits MAPK Activation**

The OA-induced activation of the EGFR signaling pathway was associated with MAPK activation, as shown by tyrosine phosphorylation of p42/p44 MAPK (Figure 3A) and activation of MBP kinase activity of immunoprecipitated MAPK (Figure 3B). To investigate whether EGFR and MAPK activations were causally related or independent events, similar experiments were performed on genetically engineered B82L-derived cell lines expressing or not expressing EGFR (parental B82L cells were EGFR deficient and transduced B82LK1 cells overexpressed EGFR) (Figure 3C). As shown in Figure 3, D and E, OA (50 μmol/L, 15 minutes) induced no significant MAPK activation in cells lacking EGFR (parental B82L cells), whereas it elicited both EGFR autophosphorylation and MAPK activation in B82LK1 cells (expressing EGFR). Conversely, OA-induced MAPK activation was inhibited when EGFR autophosphorylation was inhibited with genistein (Figure 4). These events were not or were slightly influenced by the PKC inhibitor bisindolylmaleimide or phorbol-12-myristate-13-acetate–mediated down regulation of PKC (Figure 4). Taken together, these data suggest that the moderate OA-induced activation of the EGFR signaling pathway is effective for the inducement of MAPK activation (whereas classic PKC is apparently dispensable).

The next experiments were designed to understand the mechanism of the OA-induced EGFR. It was hypothesized that OA may (1) trigger an autocrine secretion of EGF or (2) interact (more or less directly) with and activate EGFR.

**EGFR Autophosphorylation and Activation by OA Is Independent of Any Autocrine Effect**

EGFR (parental B82L cells were EGFR deficient and transduced B82LK+ cells overexpressed EGFR) (31, 32) (Figure 3C). As shown in Figure 3, D and E, OA (50 μmol/L, 15 minutes) induced no significant MAPK activation in cells lacking EGFR (parental B82L cells), whereas it elicited both EGFR autophosphorylation and MAPK activation in B82LK+ cells (expressing EGFR). Conversely, OA-induced MAPK activation was inhibited when EGFR autophosphorylation was inhibited with genistein (Figure 4). These events were not or were slightly influenced by the PKC inhibitor bisindolylmaleimide or phorbol-12-myristate-13-acetate–mediated down regulation of PKC (Figure 4). Taken together, these data suggest that the moderate OA-induced activation of the EGFR signaling pathway is effective for the inducement of MAPK activation (whereas classic PKC is apparently dispensable).

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excluded because OA-induced activations of EGFR and MAPK were not inhibited by anti-EGF antibody (in contrast to that elicited by added exogenous EGF) and because the transfer of preconditioned medium (from cells pretreated with OA) triggered neither EGFR phosphorylation nor MAPK activation (Figure 5C). This conclusion (no requirement of autocrine EGF) was consistent with the very rapid response (2 minutes) and was confirmed by the lack of inhibition with cycloheximide and with phenylmethylsulfonyl fluoride (inhibitors of protein synthesis and of pro-EGF processing, respectively)\(^9\) (data not shown).

OA Triggers In Vitro EGFR Phosphorylation and EGFR Kinase Activation

Because immunopurified EGFR can be activated in vitro by EGF and by nonspecific "agonists," such as oxidized lipids and hydroxynonenal (4-HNE),\(^24\) we investigated whether OA was also able to stimulate in vitro autophosphorylation of immunopurified EGFR.
As shown in Figure 6, OA (50 μmol/L) incubated in vitro with EGFR (immunopurified from B82LK⁺ cells) induced both EGFR tyrosine phosphorylation (Figure 6A) and activation of EGFR intrinsic tyrosine kinase (Figure 6B).

These data suggest that OA may interact with EGFR, thereby activating it. This led us to investigate 2 possible mechanisms of the interaction between OA and EGFR: (1) through specific interaction at the binding site of EGF and (2) through nonspecific interaction involving the amphiphilic properties of OA.

**Study of Mechanism of EGFR Activation by OA: Analogy With Mild Surfactants**

As shown in Figure 7, [125I]EGF (30 pmol/L) binding was not altered by OA (50 μmol/L) (these EGF and OA concentration induced a grossly similar EGFR autophosphorylation). This suggests that OA does not interfere with the EGF-binding site of EGFR.

**Discussion**

AA and oxidized PUFAs induce EGFR activation, but the mechanism of action is largely unknown, except for
oxidized PUFAs, which inhibit protein tyrosine phosphatases (PTPases) and thereby increase tyrosine phosphorylation of cell proteins. In the present work, we investigated whether other major UFAs were able to activate EGFR and sought to identify their mechanism of action.

Because FAs are liberated and transported in the blood flow, they are in contact with vascular wall cells and may alter their physiology. This led us to use an endothelial cell line (ECV-304) that exhibits a stable phenotype, does not require exogenous growth factors, and expresses sufficient EGFR to perform Western blotting. Similar results were obtained with vascular smooth muscle cells and other cell types when EGFR was expressed (these data are not reported here to avoid redundant data).

The data reported here suggest that (1) EGFR is a primary target for UFAs, (2) UFAs activate the EGFR signaling pathway, (3) this UFA activity is correlated to their unsaturation degree and does not require FA oxidation, and (4) EGFR may act as a sensor of amphiphiles and of membrane fluidity changes. This sensitivity of EGFR to its microenvironment is not a general property of all membrane receptor tyrosine kinases, because OA triggered no significant activation of platelet-derived growth factor and insulin receptors (data not shown).

The OA-induced EGFR autophosphorylation is moderate but is effective in activation of the EGFR signaling pathway (ie, recruitment of SH2-containing substrates) and MAPK. In genetically engineered B82L cells, EGFR expression is necessary for OA-induced MAPK activation (no MAPK activation in EGFR-deficient cells B82L), but PKC activation is not required (because OA-induced MAPK activation is not inhibited by PKC inhibitors). This is consistent with the conclusions of Casabiell et al, but it cannot be excluded that the OA-induced PKC activation may be effective in other cell types.

To investigate the molecular mechanism of the OA-induced EGFR activation, several mechanistic hypotheses were considered: (1) autocrine secretion of EGF (or other mediators able to activate EGFR), (2) oxidative stress, lipid oxidation, or both, which in turn may induce EGFR activation, and (3) direct EGFR activation (with EGFR being a primary target).

An autocrine secretion of EGF was not involved in the OA-induced EGFR activation because OA-induced EGFR autophosphorylation is very rapid (2 minutes), is not blocked by cycloheximide (thus excluding de novo synthesis of EGF), is not inhibited by phenylmethylsulfonyl fluoride or leupeptin (two inhibitors of pro-EGF processing) (data not shown), is not blocked by anti-EGF antibody, and is not induced by the transfer of preconditioned medium.

Oxidative stress (induced by UV-C irradiation or H$_2$O$_2$) and oxidized lipids or 4-HNE may activate EGFR either directly or indirectly through PTPase inhibition. The short-term OA-induced EGFR activation is probably not mediated through reactive oxygen species (ROS) generation or PTPase inhibition because (1) OA did not generate intracellular ROS, (2) antioxidants (probucol, tocopherol, trolox) did not inhibit the short-term OA-induced EGFR autophosphorylation, (3) OA-induced EGFR autophosphorylation occurs in vitro on immunopurified EGFR independent of any cellular generation of ROS, and (4) all of the in vitro assays on immunopurified EGFR contained Na$_2$VO$_3$, an inhibitor of PTPase (thus excluding a role for active PTPase in vitro). Moreover, OA-induced EGFR activation is probably not mediated via oxidation products of OA because OA is relatively resistant to autoxidation, and did not induce the formation of 4-HNE/protein adducts. However, it is not excluded that the relatively sustained phase of EGFR activation (30 to 45 minutes) may involve ROS generation, because EGFR activation induces H$_2$O$_2$ generation, which plays a role in EGFR pathway activation.

Chen et al recently reported that epoxyeicosatrienoic acid activates Src kinase (SrcK), which initiates a tyrosine kinase cascade (involving EGFR). Although OA is poorly oxidizable and cannot lead to epoxyeicosatrienoic acid formation, we investigated whether SrcK may be involved with the use of SrcK$^+$ and SrcK$^-$ (overexpressing a negative dominant SrcK$^-$) cell lines. OA induced EGFR autophosphorylation in both SrcK$^+$ and SrcK$^-$ cells, but the basal and OA-stimulated EGFR activations were higher in SrcK$^-$ (data not shown). This suggests that c-src is not strictly required for the OA-induced EGFR activation, which is in agreement with the in vitro OA-induced activation of immunopurified EGFR. Therefore, non oxidized OA may directly activate EGFR, via a mechanism different from that of epoxyeicosatrienoic acid, but it is not excluded that in vivo, EGFR activation induced by OA may be potentiated by c-src.

Finally, in vitro experiments suggest that OA interacts (probably directly) with an EGFR domain (different from the EGFR binding site), thereby activating it. Because of its amphiphilic properties, OA may interact with hydrophobic domains, such as with the transmembrane domain either directly (in vitro) or after insertion in membrane lipid bilayer, where it elicits changes in the membrane fluidity. This hypothetical mechanism was supported by EGFR activation induced by mild detergents (Tween-20 and Triton X-100), which is in agreement with the results of Igarashi et al. Amphiphiles may alter the membrane fluidity, thereby inducing conformational changes and activation of EGFR. This hypothesis is consistent with the data of Miloso et al, who report that point mutation in the EGFR transmembrane domain induce (probably through conformational change) a mild constitutive activation of EGFR. Finally, the reported data suggest that EGFR may act as a sensor for amphiphiles and membrane fluidity changes. In vivo, it cannot be excluded that OA may also modulate the activity of an EGFR ligand.

From a pathophysiological point of view, a local NEFA concentration effective in the activation of EGFR may be reached acutely during intravascular lipolysis of chyomicrons at the endothelial surface; during triglyceride lipolysis of adipocytes occurring during fasting, ketoacidosis, or other conditions associated with increased lipolysis; or during phospholipolysis by phospholipases during inflammation. Because the level of UFA (or the ratio of unsat-
urated to saturated FA) is largely dependent on diet composition, the reported data point out a new nutritional mechanism that regulates EGFR activity and subsequent cell functions. The EGFR pathway may have have interplay with the other NEFA-activated signaling pathways, which participate in the regulation of major intracellular events, such as cell proliferation, migration and adhesion, gene expression, glucose transport, and cellular metabolism. EGFR plays a role in wound healing and may be involved in repair processes, remodeling, and fibrosis of the vascular wall in response to injury in normal and atherosclerotic areas (where EGFR is highly expressed in proliferating cells). This may help to stabilize the plaque and may therefore in part account for the rather favorable effect of OA intake. The effects of PUFA are more complex because they are oxidizable and also lead to eicosanoid formation, which exhibits various potent effects on the vascular wall and the hemostasis equilibrium.

In conclusion, the reported data provide novel insight into the mechanism of cis-UFAs as mediators triggering cell signaling via EGFR, which apparently is a novel primary target of UFAs, acts as a sensor for amphiplic agents, and may participate in vascular wall biology regulation.

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