Vol. 207, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 6, 1995 Pages 105-110

OLEIC ACID BLOCKS EGF-INDUCED $[Ca^{2+}]_i$ RELEASE WITHOUT ALTERING CELLULAR METABOLISM IN FIBROBLAST EGFR T17

José L. Zugaza, Xesús A. Casabiell, Lázaro Bokser, and Felipe F. Casanueva‡

Molecular and Cellular Endocrinology Laboratory, Endocrine Section, Department of Medicine, Faculty of Medicine, Santiago de Compostela, Spain

Received November 29, 1994

SUMMARY: EGFR-T17 cells were pretreated with oleic acid and 5-10 minutes later stimulated with EGF, to study if early ionic signals are instrumental in inducing metabolic cellular response. Oleic acid blocks EGF-induced $[Ca^{2+}]_i$ rise and Ca^{2+} influx without altering 2-deoxyglucose and 2-aminobutiryc acid uptake nor acute, nor chronically. Oleic acid it is shown, in the first minutes favors the entrance of both molecules to modify the physico-chemical membrane state. On the other hand, oleic acid is unable to block protein synthesis. The results suggest that EGF-induced $Ins(1,4,5)P_3/Ca^{2+}$ pathway does not seem to be decisive in the control of cellular metabolic activity.

In recent years, the main steps of the transmembrane signalling by the EGF receptor have been elucidated. After binding to its receptor, EGF induces dimerization of the receptors, which is followed by activation of the receptor kinase domain leading to receptor autophosphorylation on tyrosine residues. In addition, a number of proteins are also tyrosine-phosphorylated, i.e. the GTPase-activating protein of *ras*, GAP-associated proteins (1), enzymes of the glycolitic pathway (2) and PLC-γ1 (3).

In addition to this signalling system, the activation of the PLC-γ1 by the EGF-receptor opens a second branch in the signalling pathway: after PIP_2 hydrolysis mediated by this enzyme, two intracellular second messengers are generated: inositol-1,4,5-trisphosphate and diacylglycerol (4-7). Diacylglycerol activates protein kinase C (PKC) which in turn phosphorylate several intracellular substrates (4, 8, 9). Simultaneously, $Ins(1,4,5)P_3$ leads to Ca^{2+} release from microsomal stores, inducing first a rise in cytosolic free Ca^{2+} concentrations $[Ca^{2+}]_i$, that is followed later by a Ca^{2+} influx across the plasma membrane by a mechanism not yet fully understood (5-7, 10). In the last few years, an extensive body of evidence has suggested that this IP_3-DAG pathway is involved in the stimulation of EGF-induced cell growth (4, 11-14).

‡To whom correspondence should be addressed at P.O. Box 563, 15780 Santiago de Compostela, Spain. Fax:(34)-81-572121.

Abbreviations used:
EGF: epidermal growth factor; IP_3: $Ins(1,4,5)P_3$; DAG: diacylglycerol; TK: tyrosin-kinase; AIB: 2-aminoisobutyric acid; NEFA: non-esterified free fatty acid; PLC-γ1: phospholipase C-gamma1; DMEM: Dulbecco's modified Eagle's medium; PBS: phosphate-buffer-saline.

0006-291X/95 $5.00

As EGF-induced cell activation finally leads to cell division, it is not surprising at all that basic metabolism processes are activated. In fact EGF administration to responsive cells induces glucose and aminoacid uptake in a matter of minutes (15-17). However, the overall regulation of such basic metabolic steps, as well as the role of cell activated early signal in this control are far from being understood at present.

We recently described that *cis*-unsaturated free fatty acids (*cis*-FFA) (but not saturated or *trans*-unsaturated FFA's) are able to selectively block the $[Ca^{2+}]_i$ signalling pathway in EGF-stimulated fibroblasts in a dosis-dependent manner (10-100μM), without altering the TK-dependent signal or the proliferative response (18, 19). In the present study we have taken advantage of this system in order to analyze the role of the Ca^{2+} signal in the induction of both 2-deoxyglucose and 2-aminoisobutyric acid (AIB) uptake, and protein synthesis in cultured mouse fibroblasts.

MATERIALS AND METHODS

Cell culture. EGFR-T17 is a NIH 3T3 derived mouse fibroblast cell line that has been transfected with the cDNA for the human EGF receptor (20). In order to measure the biological responses, cells were seeded in 100 mm or 24 well dishes and cultured to confluence for 2-3 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin.

Calcium measurements. Measurement of $[Ca^{2+}]_i$ in cell suspensions was performed using the fluorescent indicator fura 2 (21). Cell suspensions were incubated at 37 °C for 30 min with 3 μM fura 2/AM, diluted to a ratio 1/4 with KRH and maintained at room temperature until use. Cells were then placed in a cuvette positioned in a thermostatically controlled (37±1 °C) holder, and the fluorescence signal was measured under continuous stirring in a Perkin-Elmer LS-5B fluorimeter adjusted to λ 345 nm excitation and λ 490 nm emission.

2-Deoxyglucose uptake assay. Cells were cultured to 70% confluence with DMEM+10 % FCS in 24 well dishes. The medium was changed to serum free DMEM. After 36 hours of starvation the medium was substituted by DMEM/F12 (1:1). 2 hours later fresh medium supplemented with free fatty acid (50 μM) was added and ten minutes later cells were stimulated with EGF (10 nM). At the end of the stimulation period, the cells were washed with transport buffer I (10 mM Hepes, 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 2 mM Na_2HPO_4 pH 7.4) and then incubated for 5 min with transport buffer supplemented with 0.1% BSA. After the addition of 2-deoxyglucose (0.1 mM) and 2-deoxy-D-[1-^{3}H]glucose (0.2 μCi/well), transport was measured over a 5 min period (15). Uptake was stopped by washing the cells 3 times with ice-cold phosphate-buffer-saline (140 mM NaCl, 3 mM KCl, 6 mM Na_2HPO_4 pH 7.4). Cells were solubilized in NaOH and samples were neutralized with HCl before adding scintillation liquid and counting in a Beckman LS β-counter.

Amino acid uptake assay. Cells were grown to 70% confluence with DMEM+10 % FCS in 24 well dishes and were serum starved for 24 h in non supplemented DMEM. Free fatty acids (50 μM) and EGF (10 nM) were then added. At the end of the treatment period the cells were washed with transport buffer II (1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM KCl, 25 mM $NaHCO_3$, pH 7.4). At the indicated times, uptake was measured over 15 min in transport buffer containing 2-aminoisobutyric acid (0.1 mM), and 2-amino[1-^{14}C]isobutyric acid (0.2 μCi/well). After three washes with ice-cold PBS, samples were solubilized and counted in a β-counter.

Protein synthesis. Cells were grown in DMEM+10 % FCS for 48 h, and then starved for 24 h in Met-free DMEM (22). Oleic acid (50 μM) was added for 10 min, and finally were stimulated with EGF 10 nM. Labelling was performed by incubation with EXPRE^{35}S-^{35}S (50 μCi/ml, for 0, 6, 12, 24 hours). At the indicated time, monolayers were washed three times with ice-cold PBS, samples were solubilized and counted in a β-counter.

Materials. Culture media and sera were from GIBCO. Plastic culture dishes were purchased from Corning. Recombinant EGF was from Promega. Fura 2/AM was from

Calbiochem, 2-deoxy-D-[1-^{3}H]glucose, 2-amino[1-^{14}C]isobutyric acid, [9,10-^{3}H] oleic acid, EXPRE^{35}S^{35}S were from Amersham. Oleic and elaidic acids were from Sigma Chemical Co. (St. Louis Mo., USA).

RESULTS AND DISCUSSION

While the biochemical processes that activate both glucose and aminoacids uptake by growth factors like insulin have been comprehensively studied (15-17), the regulation of such events by EGF have been somewhat overlooked. In particular, the role of EGF-activated early intracellular signals like Ca^{2+} or IP_3 in the triggering of both glucose and aminoacid uptake, or protein synthesis is not know at present (9, 15). In agreement with previous reports (18), pretreatment of EGFR-T17 cells with 50 µM oleic acid for five minutes leads to a significant suppresion of the IP_3-generated $[Ca^{2+}]_i$ rise (Fig. 1). Such an elimination of this early intracellular step was highly selective because of i) EGF binding to its cognate receptor, ii) EGF receptor autophosphorylation and the internalization of the EGF-EGF receptor complex and iii) the fact that cell proliferation was not altered (18, 19).

Compared with untreated cells, addition of EGF to EGFR-T17 fibroblasts significantly increased the rate of 2-deoxyglucose uptake (five minutes pulse) (Figure 2A). This increase was observed 6 hours after EGF administration and was maximal at both 12 and 24 hours post-stimulus. Pretreatment with oleic acid did not alter the EGF-induced 2-deoxyglucose uptake. However, oleic acid *per se* was able to induce a significant increase in glucose uptake at 0 minutes (ten minutes after oleic acid addition).

These results powerfully suggest that the EGF-induced early intracellular signals like DAG, IP_3, $[Ca^{2+}]_i$ rise and cell hyperpolarization are not instrumental for 2-deoxyglucose uptake, and secondly, that oleic acid *per se* is able to actively enhance 2-deoxyglucose uptake. We have no clear explanation for this finding. Non-esterified fatty acids (NEFAs) are normal constituents of biological membranes (23), and are also common metabolites carried by the blood stream with levels oscillating widely during the day or in pathological states (24, 25). After their increase in plasma, NEFAs rapidly partition into biological membranes, thus influencing the physicochemical state of lipid domains (26). It has been shown that the effects of fatty acids on cellular membranes can be related to its spatial conformation (27). However, type A (like oleic acid) but not type B

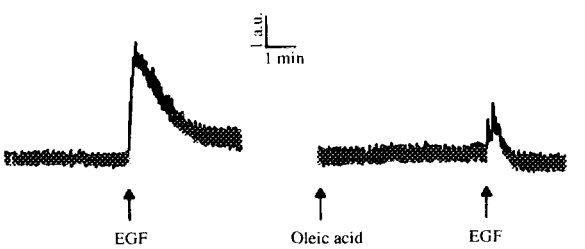

Fig. 1. **Effect of oleic acid on EGF-induced $[Ca^{2+}]_i$ rise in EGFR-T17 cells.**
Fibroblasts preincubated with fura 2/AM as described in Materials and methods. After recording the basal$[Ca^{2+}]_i$ for 5 min,50 µM of oleic acid or vehicle (ethanol) was added and five minutes later EGF 10 nM was added and calcium release monitored.

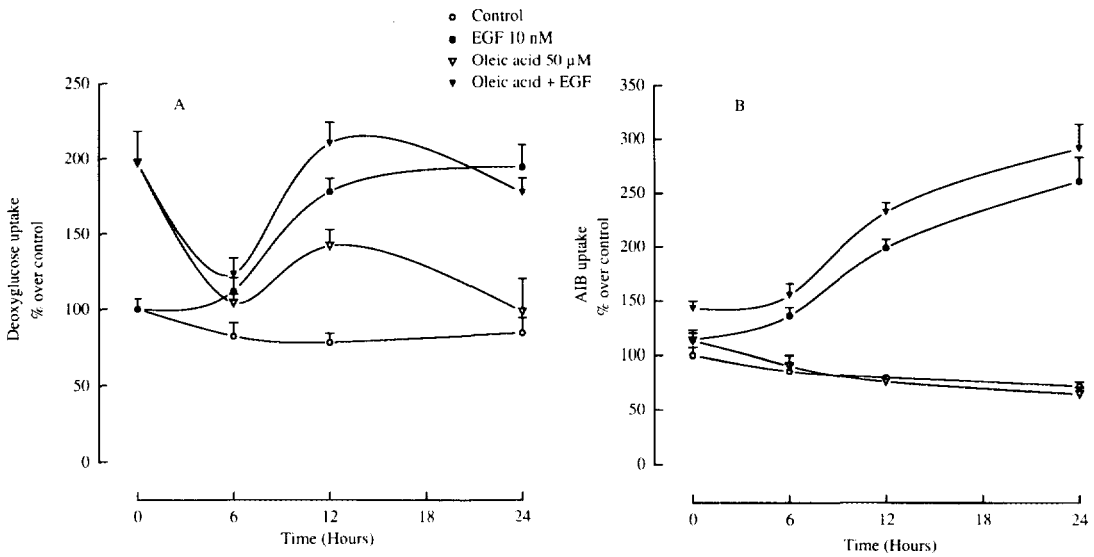

Fig. 2. **Effects of oleic acid on time-course of EGF-stimulated 2-deoxyglucose, and 2-aminoisobutyric acid uptake.**
EGFR T17 cells were preincubated with oleic acid 50 µM or vehicle and 10 min after was added EGF 10 nM. The treatment times were 0, 6, 12, 24 hours. At these times the 2-deoxiglucose uptake (A), and AIB uptake (B) were measured over 5, and 15 minutes,respectively. Uptake was stopped by washing three times with ice-cold PBS and solubilized with NaOH 0.1M, and samples were neutralized with HCl before adding scintillation liquid and counting in a β-counter. Results are expressed as a percentage of stimulation over basal uptake (mean± S.E. of three independent experiments).

NEFA's, are able to perturb the physico-chemical state of the plasma membrane leading to a more fluid state (28). It could be envisioned that in such a state the glucose transporters are somehow able to incorporate themselves into the plasma membrane from the cytoplasm. Saturated and *trans*-

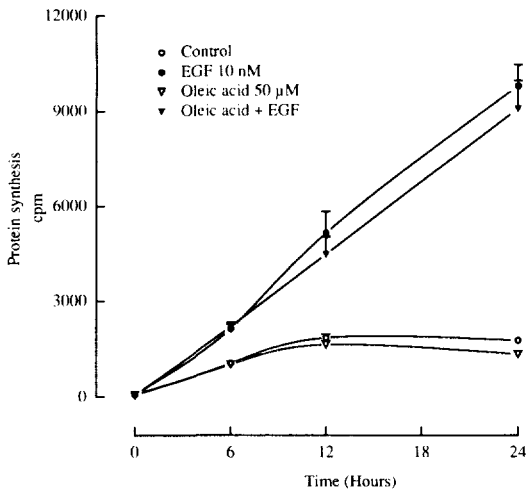

Fig. 3. **Effect of oleic acid on protein synthesis in EGFR-T17 cells.**
Cells were cultured in DMEM without methionine and serum free by 24 hours. After that cells were preincubated with oleic acid 50 µM or vehicle 10 min and finally stimulated with EGF 10 nM. Medium was supplemented with 50 µCi 35S-Met//ml. At the indicated times cells were washed three times with ice-cold PBS and solubilized with NaOH 0.1M and counting in a β-counter. Results are expressed as mean± S.E. of three independent experiments.

insaturated NEFAs are rather straight molecules that can pack themselves into the membrane without structural disruption of the lipid bilayer. *cis*-Unsaturated fatty acids, such as oleic acid, are angular molecules that cause a change in bilayer fluidity. It has been proposed that this change may directly help the activity of transport systems (28). In agreement with this interpretation, we have previously described that oleic acid, but not elaidic or stearic acids, can perturb some early transmembrane signals in EGF-activated cells, and in particular they block the intracellular Ca^{2+} peak that follows activation of the EGF receptor, probably due to an inhibition of the PLC γ (18).

AIB uptake is considered as a good index of aminoacid uptake by the cells (15-17). In fact, EGF addition to EGFR-T17 cells clearly enhanced the rate of AIB uptake, being evident at 6 hours and maximal at 24 hours (Fig. 2B). Pretreatment with oleic acid did not reduce EGF-stimulated AIB uptake. Although oleic acid *per se* did not modify AIB uptake versus control cells, it enhanced the EGF action at all time points.

Similarly to the glucose uptake results, we can conclude that: i) elimination of $[Ca^{2+}]_i$ rise by pretreating the EGF-stimulated cells with oleic acid does not alter the aminoacid uptake by the cells and ii) that oleic acid *per se* has a slight facilitatory action on such a process. Finally, EGF-stimulated protein synthesis was assessed as an overall index of cell metabolic activation. As Figure 3 shows, EGF clearly induced a time-dependent increase in protein synthesis. Pretreatment with oleic acid did not alter the EGF-induced in protein synthesis nor have an action on its own.

In the present work, elimination of the early ionic signals by pretreatment of the cells with oleic acid did not alter the EGF-induced stimulation of three relevant parameters of metabolic activation, i.e. glucose and aminoacid uptake and protein synthesis. These results are in agreement with previous work from our group showing that blockade of the $[Ca^{2+}]_i$ rise by oleic acid does not alter replication of EGF-stimulated cells. To our knowledge this is the first demostration that the early signals induced by EGF into the cell cytoplasm: $[Ca^{2+}]_i$, DAG, and IP_3 are not instrumental for the EGF-stimulated glucose and aminoacid uptake, and cell protein synthesis.

ACKNOWLEDGMENTS

Supported by grants from Fondo de Investigación Sanitaria, Spanish Ministry of Health, and grants from European Community, Copernicus Program, CIPA-CT92 3014. We thank Ms. M. Lage for excellent technical assistance.

REFERENCES

1. Ellis, C., Moran, M. F., McCormick, F. and Pawson, T. (1990) Nature 343, 377-381.
2. Reiss, N., Kanety, H. and Schlessinger, J. (1986) Biochem J. 239, 691-697.
3. Wahl, M. I., Daniel, T. O. and Carpenter, G. (1988) Science 241, 988-970.
4. Berridge, M. J. and Irvine, R. F. (1989) Nature 341, 197-205.
5. Pandiella, A., Malgaroli, A., Meldolesi, J. and Vicentini, L. M. (1987) Exp. Cell. Res. 170, 175-185.
6. Hepler, J. R., Nakahata, N., Lovenberg, T. W., DiGuiseppi, J., Herman, B., Earp, H. S. and Harden, T.K. (1987) J. Biol. Chem. 262, 2951-2956.
7. Pike, L. J. and Eakes, A.T. (1987) J. Biol Chem. 262, 2644-2651.
8. Pouyssegur, J. (1985) Trens Biochem. Sci. 9, 453-455.
9. Carpenter, G. (1987) Annu. Rev. Biochem. 56, 881-914.

10. Pandiella, A., Beguinot, L., Vicentini, L. M. and Meldolesi, J. (1989) Trends Pharmacol. Sci. 10, 411-414.
11. Jackson, T. R., Blair, A. C., Marshall, J., Goedert, M. and Hanley, M. R. (1988) Nature 335, 437-440.
12. Julius, D., Livelli, T. J., Jessel, T. M. and Axel, R. (1989) Science 244, 1057-1062.
13. Housey, G.M., Johnson, M.D., Hsiao, W. L. W., O'Brian, C. A., Murphy, J. P., Kirschmeier, P. and Weinstein, I. B. (1988) Cell 52, 343-354.
14. Fukami, K., Matsuoka, K., Nakanishi, O., Yamakawa, A., Kawai, S. and Takenawa, T. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 9057-9061.
15. Scimeca, J. C., Ballotti, R., Alengrin, F., Honegger, A. M., Ullrich, A., Schlessinger, J. and Van Obberghen, E. (1989). J. Biol. Chem. 264, 6831-6835.
16. Cohen, S. and Taylor, J. M. (1974) Epidermal growth factor: Chemical and biological characterization. In: Recent Progress in Hormone Research. R. O. Greep, ed. Academic Press, New York, vo. 30 pp.533-550.
17. Hollenberg, M. D. and Cuatrecasas, P. (1975) J. Biol. Chem. 250, 3845-3853.
18. Casabiell, X., Pandiella, A. and Casanueva, F. F. (1991) Biochem. J. 278, 679-687.
19. Casabiell, X., Zugaza, J. L., Pombo, C. M., Pandiella, A. and Casanueva, F.F. (1993) Exp. Cell. Res. 205, 365-373.
20. Velu, T. J., Beguinot, L., Vass, W. C., Willingham, M.C., Merlino, G.T., Pastan, I. and Lowy, D.R. (1987) Science 238, 1408-1410.
21. Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440-3450.
22. Bardocz, S. and Hesketh, J. E. (1989) Int. J. Biochem. 21, 1265-1268.
23. Gutknecht, J. (1988) J. Membr. Biol. 106, 83-93.
24. Hochachka, P. W. (1986) Science 231, 234-341.
25. Yoshida, S., Ikeda, M., Busto, R., Santiso, M., Martínez, E. and Ginsberg, M.D. (1986) J. Neurochem. 47, 744-757.
26. Renaud, G., Bouma, M.E., Foliot, A. and Infante, R. (1985) Arch. Int. Physiol. Biochim. 93, 313-319.
27. Karnovsky, M. J. (1979) Am. J. Pathol. 97, 212-221.
28. Melchior, D. L. and Czech, M.P. (1979) J. Biol. Chem. 254, 8744-8747.