Mitochondrial oxidative stress is modulated by oleic acid via an epidermal growth factor receptor-dependent activation of glutathione peroxidase

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Mitochondria generate reactive oxygen species (ROS) under various pathophysiological conditions. In isolated mitochondria, fatty acids (FA) exhibit an uncoupling effect of the respiratory activity and modulate ROS generation. The effect of FA on intact cultured cells remains to be elucidated. The present study reports that FA (buffered by BSA) decrease the level of cellular ROS generated by the mitochondrial respiratory chain in cultured cells incubated with antimycin A. Both saturated and unsaturated FA are effective. This fatty acid-induced antioxidant effect does not result from a decrease in ROS production, but is subsequent to cellular glutathione peroxidase (GPx) activation and enhanced ROS degradation. This fatty acid-induced GPx activation is mediated through epidermal growth factor receptor (EGFR) signalling, since this response is (i) abrogated by the EGFR inhibitor AG1478 or by a defect in EGFR (in EGFR-deficient B82L fibroblasts), (ii) restored in B82LK+ cells expressing EGFR and (iii) mimicked by epidermal growth factor. These findings indicate that FA contribute to enhance cellular antioxidant defences against mitochondrial oxidative stress through EGFR-dependent GPx activation.

Key words: antimycin A, fatty acids, reactive oxygen species.

INTRODUCTION

Reactive oxygen species (ROS) are implicated in various pathophysiological processes. Almost all cells are capable of generating moderate and localized production of ROS that are involved in physiological cell signalling and regulation of essential cellular functions, such as gene expression, cell proliferation or death [1–4]. The level of ROS is modulated by a variety of antioxidant systems, such as small molecules (e.g. tocopherols, ascorbic acid, uric acid, polyphenolic compounds) and enzymes [e.g. superoxide dismutases (SODs), catalase, peroxidases] [1]. When ROS generation overwhelms antioxidant defences, the resulting oxidative stress may participate in pathological processes, such as cancer, neurodegenerative diseases, atherosclerosis, aging, autoimmune and inflammatory disorders [1,5–9].

Mitochondria are the major source of ROS, since it has been estimated that 1–2 % of consumed oxygen is converted into the superoxide anion (O_2^{-}), mainly generated at complexes I and III of the electron transport chain. O_2^{-} is converted into H_2O_2 by the mitochondrial SOD [10–12]. The generation of ROS in mitochondria can be stimulated by mitochondria inhibitors, such as the b–c1 complex inhibitor antimycin A (AM) [13].

Fatty acids (FA) are energy-rich substrates that may interfere at various sites with ROS formation. In mitochondria, FA catabolism (β-oxidation and acetyl-CoA degradation through the tricarboxylic cycle) supplies reducing equivalents to redox reactions at complexes I and II. The subsequent increase in the electron flow through the electron-transfer chain has been associated with an enhanced rate of H_2O_2 formation [14]. In contrast, FA are mild uncouplers that act through a protonophoric effect or through activation of uncoupling proteins (UCPs) and/or of the adenine nucleotide translocase [15–17], and may reduce mitochondrial H_2O_2 generation [18]. At higher concentrations, FA induce both respiration inhibition and enhanced H_2O_2 production [14]. These studies were conducted on isolated mitochondria, but the effect of FA on ROS generation in intact cells has been only poorly investigated. This led us to investigate whether FA could modulate mitochondrial oxidative stress in intact cells.

Our results suggested that moderate concentrations of FA may modulate ROS levels in cells treated with AM. This antioxidant effect results from glutathione peroxidase (GPx) activation and requires epidermal growth factor (EGF) receptor (EGFR) function.

MATERIALS AND METHODS

Chemicals

The ROS-sensitive fluorescent probe 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, diacetoxymethyl-ester (H2-DCFDA; DCF,2′,7′-dichlorodihydrofluorescein) was purchased from Molecular Probes (Eugene, OR, U.S.A.) and the EGFR inhibitor AG1478 was from Merck-Calbiochem (Fontenay, France). Recombinant EGF and anti-EGFR antibodies were from Santa Cruz/Peprotech/Tebu (Le Perray, France), and anti-phosphotyrosine antibody (4G10) was from UBI-Euromedex (Soufflenweysheim, France). FA, fatty-acid-free BSA (FAF-BSA), AM, horseradish peroxidase, decylubiquinone, NADH, NADPH, scopoletin and other reagents were purchased from Sigma-Aldrich. Fluorescent probes were dissolved in DMSO, and FA and mitochondrial inhibitors in ethanol.

Cell culture

Human ECV-304 cells (A.T.C.C., Rockville, MD, U.S.A.) were grown in RPMI 1640 medium containing 10 % (v/v) foetal calf serum. The murine B82L fibroblasts (EGFR-deficient) and B82LK+ (overexpressing EGFR), a gift from Dr M. Weber (Charlottesville, VA, U.S.A.), were grown under the conditions

Abbreviations used: AM, antimycin A; DCF, 2′,7′-dichlorodihydrofluorescein; EGF, epidermal growth factor; EGFR, EGF receptor; FA, fatty acids; FAF, fatty-acid-free; GPx, glutathione peroxidase; H2-DCFDA, 6-carboxy-DCF diacetate, diacetoxymethyl-ester; ROS, reactive oxygen species; SOD, superoxide dismutase; UCP, uncoupling protein.

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described previously [19]. Then the culture medium of subconfluent cells was replaced by fresh phenol-red-free RPMI 1640 medium (containing the indicated FAF-BSA and/or inhibitors) and used for experiments.

Determination of activities of complexes I and II/III

ECV-304 cells were permeabilized by digitonin as described by Chowdhury et al. [20]. Briefly, cells were incubated in 80 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 10 mM Tris/HCl (pH 7.2), 5 mM potassium phosphate containing 10 μM digitonin, and AM and/or oleic acid as indicated. The activity of complex I was evaluated by monitoring NADH oxidation (at 340 nm) in the presence of decylubiquinone as electron acceptor, according to the procedure described by Rigoulet et al. [21]. Briefly, the assay mixture (final volume, 1 ml) contained 0.3 mM decylubiquinone, 4 μM antimycin, 0.1 mM NADH in 10 mM Tris/maleate buffer (pH 7.2) and 50 μl of cell suspension (100 μg of cell protein). The assay for complexes II/III (succinate dehydrogenase/ubiquinol–cytochrome *c* reductase) contained 1 mM ferricyanide, 5 μM rotenone, 1 mM KCN and 50 μl of cell suspension (100 μg of cell protein) in 1 ml of permeabilization buffer. The activity was determined by monitoring ferricyanide reduction (absorbance at 436 nm) [21].

Evaluation of intracellular ROS

Intracellular ROS levels were estimated using the oxidant-sensitive H2-DCFDA probe, as described by Royall and Ischiropoulos [22]. Cells (ECV-304 or fibroblasts) were grown in 6-multiwell plates, then incubated in serum-free (phenol-red-free RPMI 1640) medium with H2-DCFDA (5 μM) for 30 min before adding AM and/or FA, as described above. Then cells were carefully washed in PBS, scraped off into 1 ml of water and the fluorescence was measured (excitation at 495 nm and emission at 520 nm).

Determination of cellular H_2O_2

H_2O_2 was determined on digitonin-permeabilized cells by horseradish-peroxidase-dependent quenching of scopoletin fluorescence, as described by Steinbrecher [23].

GPx activity

Control and stimulated cells were washed in PBS, scraped off the well and homogenized by sonication in PBS. GPx activity was determined as described previously [23]. Briefly, the enzyme reaction mixture contained 2 mM GSH, 0.15 mM NADPH, 0.05 units/l glutathione reductase in a buffer containing 20 mM Tris (pH 7.0), 0.5 mM EDTA and 25 μg of cell homogenate in a final volume of 1 ml. The reaction (at 37 °C) was initiated by the addition of cumene hydroperoxide (final concentration, 5 mM) and NADPH oxidation was monitored at 340 nm (extinction coefficient for calculating NADPH consumption was 6.22).

Western-blot analysis

After stimulation, cells were homogenized in a lysis buffer and Western-blot analysis was performed using anti-phosphotyrosine and anti-EGFR antibodies as described previously [24]. Protein concentrations were determined using the bicinchoninic acid method [25].

Statistical analysis

Statistical analysis was performed using analysis of variance (one-way ANOVA, Tukey test, SigmaStat software).

RESULTS

FA reduce the level of AM-induced mitochondrial ROS

In cultured ECV-304 cells, AM (10 μM) induced ROS generation, as assessed by the increase in DCF fluorescence (Figure 1A) and of peroxidase-catalysed scopoletin quenching (Figure 1B). ROS production was rapidly stimulated, reaching a maximum after 10–15 min. Determination of the activity of complexes I and III in cells pretreated with AM showed that complex III was inhibited, whereas complex I was fully active (their corresponding activities were 30 ± 5 and 115 ± 12% of the untreated control). These results suggest that supplementation of ECV-304 with AM inhibits complex III and triggers mitochondrial ROS generation in the intact cells, as in isolated mitochondria. This AM-induced increase in cellular ROS (monitored by DCF fluorescence and H_2O_2 production) was completely inhibited when cells were co-incubated with 50 μM oleic acid in the presence of 50 μM FAF-BSA (Figures 1A and 1B). This FA-induced antioxidant effect was dependent on the oleic acid concentration and oleic acid/FAF-BSA ratio (Figure 1C). The maximal FA-induced antioxidant effect was obtained with FAF-BSA ratio 1:1, but was lost when FAF-BSA ratio was less than 0.2 and greater than 2.

To evaluate whether this inhibitory effect was dependent on FA structure, we compared the effect of various saturated (palmitic acid and its structural analogue bromopalmitic acid), monounsaturated (oleic acid) and polyunsaturated FA (arachidonic and eicosapentaenoic acids). Under the experimental conditions used (oleic acid/FAF-BSA ratio, 1:1), all these FA induced a similar inhibition of ROS (DCF fluorescence) regardless of the degree of unsaturation (Figure 1D). It is, therefore,

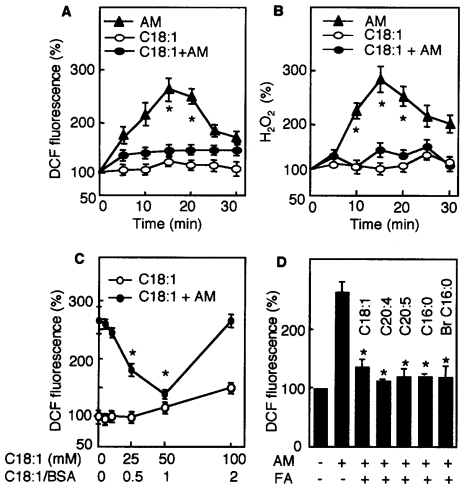

Figure 1 FA inhibit the AM-induced ROS increase in ECV-304 cells

Cells in serum-free medium containing 50 μM FAF-BSA, were loaded with H2-DCFDA (5 μM) for 30 min, then treated with AM (10 μM) and/or FA. Then the DCF fluorescence (intracellular ROS) or H_2O_2 was determined. Data are expressed as a percentage of the untreated control. (**A**) Time course of ROS generation. Cells were incubated for the indicated time with 10 μM AM and/or 50 μM oleic acid ($C_{18:1}$/FAF-BSA ratio, 1:1). (**B**) Digitonin-permeabilized cells were treated as in (**A**) and H_2O_2 was determined at the indicated times by fluorescence quenching of scopoletin in the presence of horseradish peroxidase. (**C**) Dose–response of oleic acid ($C_{18:1}$) on AM-induced ROS. Cells (in phenol-red-free RPMI medium containing 50 μM FAF-BSA) were incubated for 15 min with increasing concentrations of oleic acid (0–100 μM), with or without AM (10 μM). (**D**) Comparative effect of saturated (palmitic, $C_{16:0}$; bromopalmitic, $BrC_{16:0}$) and unsaturated (oleic, $C_{18:1}$; arachidonic, $C_{20:4}$; eicosapentaenoic, $C_{20:5}$) FA (50 μM for 15 min) on AM-induced increase in ROS. Data are expressed as a percentage of the untreated control. Results are means $\pm$ S.E.M. for at least four separate experiments (each in triplicate). $^*P < 0.01$ (treated versus untreated control).

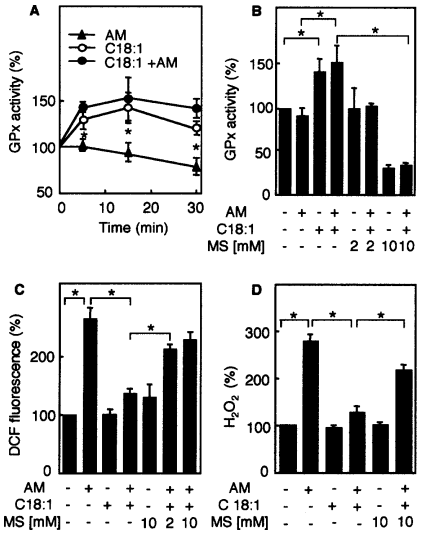

Figure 2 GPx is involved in the oleic acid-induced antioxidant effect

(**A**) Time course of GPx activity in cells treated with AM and/or FA (oleic acid, $C_{18:1}$), under the conditions described in Figure 1. Effect of the GPx inhibitor mercaptosuccinate (MS, 2 or 10 mM) on GPx activity (**B**), DCF fluorescence (**C**) and H_2O_2 (peroxidase-mediated scopoletin quenching) (**D**) in cells treated for 15 min with AM and/or FA. Data are expressed as a percentage of the unstimulated control (100%); means ± S.E.M. for three separate experiments (each in triplicate). $^*P < 0.01$ (treated versus untreated control).

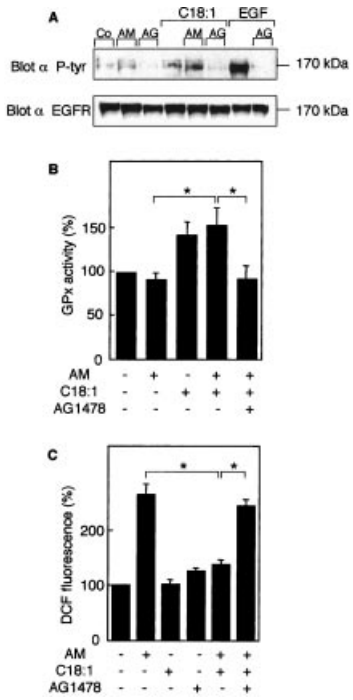

Figure 3 GPx activation and DCF fluorescence inhibition triggered by oleic acid are inhibited by the EGFR inhibitor AG1478

(**A**) EGFR activation (tyrosine autophosphorylation) induced by oleic acid and AM, and inhibition by AG1478. Cells were incubated for 15 min in a medium containing 50 μM FAF-BSA without (Co) or with (AM 10 μM), AG1478 (AG 5 μM), oleic acid ($C_{18:1}$; 50 μM) or EGF (1 nM) as indicated. Western blots were labelled either with anti-phosphotyrosine (P-tyr) or with anti-EGFR. Oleic acid-elicited GPx activation (**B**) and ROS decrease (evaluated by DCF fluorescence) (**C**) were inhibited by the EGFR inhibitor AG1478. Experimental conditions were as described in (**A**). Data are expressed as a percentage of the unstimulated control. Means ± S.E.M. for three separate experiments (each in triplicate). $^*P < 0.01$ (treated versus untreated control).

concluded that AM induces an increase in mitochondrial H_2O_2 generation in cultured ECV-304, which is inhibited by FA. This effect of FA is (i) independent of FA unsaturation, (ii) independent of mitochondrial metabolism of FA (since $BrC_{16:0}$ is not a substrate for the β-oxidation pathway) and (iii) not mediated by peroxidation derivatives of FA (since, under the conditions used, saturated FA do not undergo peroxidation). To investigate the mechanism by which FA prevent AM-induced oxidative stress (referred below as FA-induced antioxidant effect), we used oleic acid because this fatty acid is easily dispersed in the culture medium and is not very susceptible to peroxidation.

GPx is involved in the protective effect of oleic acid

Since FA are effective uncouplers of isolated mitochondria [17] and since uncouplers (including UCP) decrease both the mitochondrial membrane potential, $\Delta\psi_m$ [18,26], and ROS generated in state 4 mitochondria [17,27,28], we investigated whether the mechanism of the FA-induced antioxidant effect was due to mitochondrial uncoupling. Under our experimental conditions, oleic acid did not induce any decrease in $\Delta\psi_m$ on intact cells (results not shown), thus suggesting that FA do not induce any major uncoupling effect. Moreover, when isolated mitochondria [11,29] or cells [30] are treated with AM, uncouplers (such as carbonyl cyanide *m*-chlorophenylhydrazone) stimulate ROS generation. Altogether these results suggest that, under the conditions used (intact cells and FA buffered by FAF-BSA), oleic acid does not induce mitochondrial uncoupling, does not block the AM-induced ROS generation, but may act to enhance ROS degradation through up-regulation of cellular antioxidant defences.

The up-regulation of cellular antioxidant defences may (theoretically) result from gene induction or enzyme activation. A mechanism involving gene induction was unlikely because of the very rapid response triggered by FA (10–15 min) and the lack of effect of cycloheximide, used under conditions inhibiting protein biosynthesis (results not shown). This led us to investigate whether this effect of FA may result from activation of cellular antioxidant enzymes.

As the ROS released from AM-inhibited mitochondria have been identified as H_2O_2 [13,27], we first examined the activity of cellular catalase and GPx, two H_2O_2-degrading enzymes. Incubation of cells with oleic acid (used alone or in conjunction with AM) did not induce any change in catalase activity (results not shown), whereas it triggered a rapid and significant activation of GPx (Figure 2A). Moreover, the GPx inhibitor mercaptosuccinate inhibited both the oleic acid-induced GPx activation and the FA-induced antioxidant effect (Figures 2B–2D). These results strongly suggest that the FA-induced antioxidant effect could be mediated via GPx activation. As oleic acid did not activate GPx *in vitro* (results not shown), it was hypothesized that GPx activity may be regulated through cell signalling.

Oleic acid activates GPx through an EGFR-dependent mechanism

Since (i) EGF may regulate antioxidant enzymes [31], (ii) oleic acid is able to activate EGFR [19] and (iii) the effect of oleic acid was inhibited by broad-specificity inhibitors of tyrosine kinases (results not shown), we investigated whether the oleic acid-induced GPx activation was mediated through EGFR signalling.

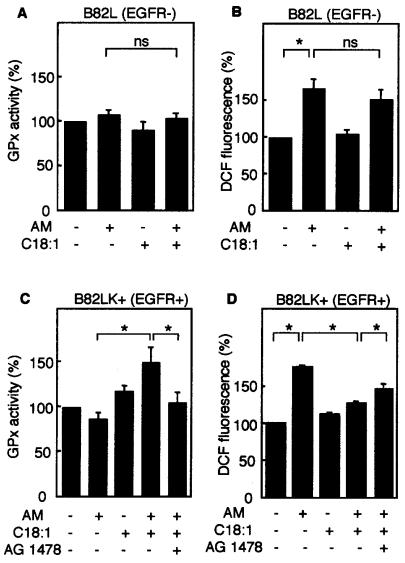

Figure 4 EGFR is required for GPx activation and oleic acid-induced antioxidant effect

Comparative effect of oleic acid on GPx activity (**A**, **C**) and ROS levels (**B**, **D**) in B82L (EGFR deficient) (**A**, **B**) and B82LK+ (overexpressing EGFR) (**C**, **D**) treated or untreated with AM and/or oleic acid ($C_{18:1}$) and/or AG1478 (in the case of B82LK+), under the experimental conditions as described in Figure 3. Data are expressed as a percentage of the untreated control; means ± S.E.M. for three separate experiments (each in triplicate). $^*P < 0.05$.

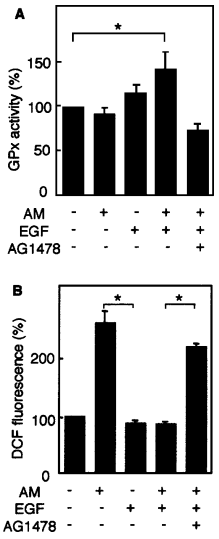

Figure 5 EGF-elicited EGFR activation induces GPx activation and decreases cellular ROS level

GPx activity (**A**) and cellular ROS (DCF fluorescence) (**B**) were determined in ECV-304 cells treated for 15 min with or without AM (10 μM), EGF (1 nM) and/or AG1478 (5 μM), under the conditions of Figure 3. Data are expressed as percentage of the untreated control; means ± S.E.M. for three separate experiments. $^*P < 0.01$.

As shown in Figure 3, oleic acid (in co-incubation with AM) rapidly elicited a moderate activation of EGFR (Figure 3A), an increase in the GPx activity (Figure 3B) and an increase in the inhibition of the AM-induced ROS (Figure 3C). All these effects of oleic acid were blocked by the EGFR inhibitor AG1478, thus supporting the hypothesis that the increase in the inhibition of the AM-induced ROS by oleic acid is mediated through an EGFR-dependent GPx activation (Figures 3A–3C).

To confirm the role of EGFR in the regulation of GPx, two sets of additional experiments (one using genetically engineered cells and the other using EGF as agonist) were performed. The efficacy of oleic acid was studied in B82L cells (EGFR-deficient) and B82LK+ cells (B82L cells transfected with human EGFR cDNA and overexpressing active EGFR) treated with oleic acid and/or AM. As shown in Figure 4, in B82L cells (EGFR deficient), oleic acid did not induce any GPx activation and did not prevent the increase in AM-induced ROS (Figures 4A and 4B), whereas, in B82LK+ cells (expressing EGFR), the effect of oleic acid (activation of GPx and inhibition of ROS) was recovered (Figures 4C and 4D).

Similarly, in ECV-304 cells treated with EGF (100 pM) and AM, we observed both the expected GPx activation and the subsequent increase in the inhibition of AM-induced ROS, which were eliminated by the EGFR inhibitor AG1478, thus confirming the crucial role of EGFR (Figures 5A and 5B). It was found that AG1478 tested alone had no effect on the basal level of ROS and GPx activity (results not shown).

Taken together these results strongly suggest that (i) a moderate activation of EGFR evokes GPx activation and (ii) the oleic acid-induced antioxidant effect countering the increase in AM-induced ROS is mediated by an EGFR-induced GPx activation.

DISCUSSION

To our knowledge, this is the first study indicating that FA are capable of counterbalancing mitochondrial ROS generation elicited by AM in intact cells, and that this antioxidant effect results from an EGFR-mediated GPx activation and subsequent ROS degradation. In mammalian cells, O_2^- generated by complexes I and III of the mitochondrial electron transport chain is rapidly converted into H_2O_2 by mitochondrial SOD [10,32]. Mitochondrial ROS generation is enhanced when the electron transport chain is inhibited by AM. AM inhibits the complex III by blocking the quinol cycle of the b–c1 complex at the quinone reducing centre, thereby inducing semiquinone accumulation and univalent reduction of O_2 to give O_2^- (and subsequently H_2O_2) [13].

In isolated mitochondria, FA (in the absence of AM) lower the production of H_2O_2 [18] and induce mitochondrial uncoupling, possibly mediated by mitochondrial UCPs and/or ATP/ADP antiporter [17]. This uncoupling effect of FA prevents the increase of the transmembrane electrochemical potential $\Delta\psi_m$ and the subsequent increase in mitochondrial ROS production [17]. In intact cells, however, the FA-induced antioxidant effect is probably independent of any uncoupling or protonophoric effect, because under our experimental conditions oleic acid induced no decrease but a small increase in mitochondrial membrane potential $\Delta\psi_m$ (determined by the uptake of rhodamine 123; results not shown). Moreover, FA and uncouplers reduce ROS production by state 4 mitochondria and, in contrast, they stimulate ROS generation when the electron-transfer chain is inhibited by AM, in isolated mitochondria [27,29] and in intact cells [30]. Altogether, these results suggest that the FA-induced antioxidant effect does not result from mitochondrial uncoupling and subsequent decrease in ROS production. These conclusions are in agreement with previous observations on intact hepatocytes [33] and perfused heart [34].

Our results show that the FA-induced antioxidant effect results from GPx-mediated ROS degradation. GPx is known to be regulated by various physiological or pathological stimuli. For

instance, GPx level is down-regulated by selenium deficiency, oxidative stress or adduct formation through glycation or lipoxidation [35–37]. In contrast, GPx is up-regulated by laminar shear stress [38], ischaemia [39], hypertension [40], traumatic injury [41], endotoxin [42], physical training [43] and obesity [44]. The up-regulation of GPx gene expression is triggered (in part) by ROS [45] and mediated by nuclear factor-κB [46]. But, under our experimental conditions, FA-induced GPx activation was not mediated by gene induction but rather by signalling-dependent regulation of enzyme activity, since it did not require protein synthesis, and was blocked by tyrosine kinase inhibitors.

FA activate various signalling pathways, such as protein kinase C, mitogen-activated protein kinase, phosphoinositide 3-kinase [47–52] and EGFR [19,52]. Preliminary experiments investigating the effect of various inhibitors of cell signalling have shown that pretreatment of cells with tyrosine kinase inhibitors relieved the FA-induced antioxidant effect (results not shown). Our attention was focused on EGFR because FA are able to activate the EGFR-signalling pathway [19]. As the FA-induced GPx activation and subsequent ROS degradation were eliminated in cells treated with the EGFR inhibitor AG1478 and EGFR-deficient B82L cells, it was suggested that EGFR mediates this effect of FA. This was confirmed by the effect of low concentrations of EGF (that triggers GPx activation and ROS degradation) and by the use of genetically engineered cells (only cells expressing EGFR were responsive to FA). It may be noted that the FA-induced antioxidant effect reported in the present study was observed only at low-FA concentration (optimal molar ratio of FA/BSA was between 0.5 and 1). In contrast, we observed (results not shown) that higher concentrations of FA (FA/FFA-BSA greater than 2) induced an increase in intracellular ROS, in agreement with previous studies [53,54].

From a physiological point of view, the ability of EGFR to activate GPx and decrease cellular ROS may constitute an autoregulatory mechanism of the EGFR-signalling pathway, since EGFR stimulates H_2O_2 production and H_2O_2 induces and/or reinforces EGFR activation and signalling [53,55]. EGFR-induced GPx activation may constitute a feedback mechanism (decreasing H_2O_2 level and subsequent EGFR activation) of potential importance in cell-signal regulation and in cell defences against oxidative stress [56,57].

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REFERENCES

1 Halliwell, B. and Gutteridge, J. M. C. (1989) Free Radicals in Biology and Medicine, Clarendon Press, Oxford
2 Thannickal, V. J. and Fanburg, B. L. (2000) Reactive oxygen species in cell signaling. Am. J. Physiol. Lung. Cell. Mol. Physiol. **279**, L1005–L1028
3 Hensley, K., Robinson, K. A., Gabbita, S. P., Salsman, S. and Floyd, R. A. (2000) Reactive oxygen species, cell signaling, and cell injury. Free Radicals. Biol. Med. **28**, 1456–1462
4 Leonarduzzi, G., Arkan, M. C., Basaga, H., Chiarpotto, E., Sevanian, A. and Poli, G. (2000) Lipid oxidation products in cell signaling. Free Radicals. Biol. Med. **28**, 1370–1378
5 Albers, D. S. and Beal, M. F. (2000) Mitochondrial dysfunction and oxidative stress in aging and neurodegenerative disease. J. Neural Transm. Suppl. **59**, 133–154
6 Wallace, D. C., Brown, M. D., Melov, S., Graham, B. and Lott, M. (1998) Mitochondrial biology, degenerative diseases and aging. Biofactors **7**, 187–190
7 Tabrizi, S. J., Workman, J., Hart, P. E., Mangiarini, L., Mahal, A., Bates, G., Cooper, J. M. and Schapira, A. H. (2000) Mitochondrial dysfunction and free radical damage in the Huntington R6/2 transgenic mouse. Ann. Neurol. **47**, 80–86
8 Taylor, D. E., Ghio, A. J. and Piantadosi, C. A. (1995) Reactive oxygen species produced by liver mitochondria of rats in sepsis. Arch. Biochem. Biophys. **316**, 70–76
9 Polla, B. S. and Cossarizza, A. (1996) Stress proteins in inflammation. EXS **77**, 375–391
10 Loschen, G., Azzi, A., Richter, C. and Flohe, L. (1974) Superoxide radicals as precursors of mitochondrial hydrogen peroxide. FEBS Lett. **42**, 68–72
11 Boveris, A., Oshino, N. and Chance, B. (1972) The cellular production of hydrogen peroxide. Biochem. J. **128**, 617–630
12 Chance, B., Sies, H. and Boveris, A. (1979) Hydroperoxide metabolism in mammalian organs. Physiol. Rev. **59**, 527–605
13 Turrens, J. F. (1997) Superoxide production by the mitochondrial respiratory chain. Biosci. Rep. **17**, 3–8
14 Cocco, T., Di Paola, M., Papa, S. and Lorusso, M. (1999) Arachidonic acid interaction with the mitochondrial electron transport chain promotes reactive oxygen species generation. Free Radicals. Biol. Med. **27**, 51–59
15 Wojtczak, L. and Schonfeld, P. (1993) Effect of fatty acids on energy coupling processes in mitochondria. Biochim. Biophys. Acta **1183**, 41–57
16 Hermesh, O., Kalderon, B. and Bar-Tana, J. (1998) Mitochondria uncoupling by a long chain fatty acyl analogue. J. Biol. Chem. **273**, 3937–3942
17 Skulachev, V. P. (1998) Uncoupling: new approaches to an old problem of bioenergetics. Biochim. Biophys. Acta **1363**, 100–124
18 Korshunov, S. S., Korkina, O. V., Ruuge, E. K., Skulachev, V. P. and Starkov, A. A. (1998) Fatty acids as natural uncouplers preventing generation of $O_2^{-\bullet}$ and H_2O_2 by mitochondria in the resting state. FEBS Lett. **435**, 215–218
19 Vacaresse, N., Lajoie-Mazenc, I., Auge, N., Suc, I., Frisach, M. F., Salvayre, R. and Negre-Salvayre, A. (1999) Activation of epithelial growth factor receptor pathway by unsaturated fatty acids. Circ. Res. **85**, 892–899
20 Chowdhury, S. K., Drahota, Z., Floryk, D., Calda, P. and Houstek, J. (2000) Activities of mitochondrial oxidative phosphorylation enzymes in cultured amniocytes. Clin. Chim. Acta **298**, 157–173
21 Rigoulet, M., Devin, A., Averet, N., Vandais, B. and Guerin, B. (1996) Mechanisms of inhibition and uncoupling of respiration in isolated rat liver mitochondria by the general anesthetic 2,6-diisopropylphenol. Eur. J. Biochem. **241**, 280–285
22 Royall, J. A. and Ischiropoulos, H. (1993) Evaluation of 2′,7′-dichlorofluorescein and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. Arch. Biochem. Biophys. **302**, 348–355
23 Steinbrecher, U. P. (1988) Role of superoxide in endothelial-cell modification of low-density lipoproteins. Biochim. Biophys. Acta **959**, 20–30
24 Suc, I., Meilhac, O., Lajoie-Mazenc, I., Vandaele, J., Jurgens, G., Salvayre, R. and Negre-Salvayre, A. (1998) Activation of EGF receptor by oxidized LDL. FASEB J. **12**, 665–671
25 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**, 76–85
26 Liu, S. S. (1997) Generating, partitioning, targeting and functioning of superoxide in mitochondria. Biosci. Rep. **17**, 259–272
27 Boveris, A. and Chance, B. (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem. J. **134**, 707–716
28 Negre-Salvayre, A., Hirtz, C., Carrera, G., Cazenave, R., Troly, M., Salvayre, R., Penicaud, L. and Casteilla, L. (1997) A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. FASEB J. **11**, 809–815
29 Cadenas, E. and Boveris, A. (1980) Enhancement of hydrogen peroxide formation by protophores and ionophores in antimycin-supplemented mitochondria. Biochem. J. **188**, 31–37
30 Mabile, L., Meilhac, O., Escargueil-Blanc, I., Troly, M., Pieraggi, M. T., Salvayre, R. and Negre-Salvayre, A. (1997) Mitochondrial function is involved in LDL oxidation mediated by human cultured endothelial cells. Arterioscler. Thromb. Vasc. Biol. **17**, 1575–1582
31 Price, L. T., Chen, Y. and Frank, L. (1993) Epidermal growth factor increases antioxidant enzyme and surfactant system development during hyperoxia and protects fetal rat lungs *in vitro* from hyperoxic toxicity. Pediatr. Res. **34**, 577–585
32 Boveris, A., Cadenas, E. and Stoppani, A. O. (1976) Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. Biochem. J. **156**, 435–444
33 Nobes, C. D., Hay Jr, W. W. and Brand, M. D. (1990) The mechanism of stimulation of respiration by fatty acids in isolated hepatocytes. J. Biol. Chem. **265**, 12910–12915
34 Hassinen, I., Ito, K., Nioka, S. and Chance, B. (1990) Mechanism of fatty acid effect on myocardial oxygen consumption. A phosphorus NMR study. Biochim. Biophys. Acta **1019**, 73–80
35 Aspberg, A. and Tottmar, O. (1994) Oxidative stress decreases antioxidant enzyme activities in reaggregation cultures of rat brain cells. Free Radicals. Biol. Med. **17**, 511–516
36 Mates, J. M., Perez-Gomez, C. and Nunez de Castro, I. (1999) Antioxidant enzymes and human diseases. Clin. Biochem. **32**, 595–603
37 Arthur, J. R. (2000) The glutathione peroxidases. Cell. Mol. Life Sci. **57**, 1825–1835

38 Takeshita, S., Inoue, N., Ueyama, T., Kawashima, S. and Yokoyama, M. (2000) Shear stress enhances glutathione peroxidase expression in endothelial cells. Biochem. Biophys. Res. Commun. **273**, 66–71
39 Chandrasekar, B., Colston, J. T. and Freeman, G. L. (1997) Induction of proinflammatory cytokine and antioxidant enzyme gene expression following brief myocardial ischaemia. Clin. Exp. Immunol. **108**, 346–351
40 Csonka, C., Pataki, T., Kovacs, P., Muller, S. L., Schroeter, M. L., Tosaki, A. and Blasig, I. E. (2000) Effects of oxidative stress on the expression of antioxidative defense enzymes in spontaneously hypertensive rat hearts. Free Radicals. Biol. Med. **29**, 612–619
41 Goss, J. R., Taffe, K. M., Kochanek, P. M. and DeKosky, S. T. (1997) The antioxidant enzymes glutathione peroxidase and catalase increase following traumatic brain injury in the rat. Exp. Neurol. **146**, 291–294
42 Spolarics, Z. (1996) Endotoxin stimulates gene expression of ROS-eliminating pathways in rat hepatic endothelial and Kupffer cells. Am. J. Physiol. **270**, G660–G666
43 Powers, S. K., Ji, L. L. and Leeuwenburgh, C. (1999) Exercise training-induced alterations in skeletal muscle antioxidant capacity: a brief review. Med. Sci. Sports Exerc. **31**, 987–997
44 Asayama, K., Nakane, T., Dobashi, K., Kodera, K., Hayashibe, H., Uchida, N. and Nakazawa, S. (2001) Effect of obesity and troglitazone on expression of two glutathione peroxidases: cellular and extracellular types in serum, kidney and adipose tissue. Free Radicals. Res. **34**, 337–347
45 Kondoh, M., Inoue, Y., Atagi, S., Futakawa, N., Higashimoto, M. and Sato, M. (2001) Specific induction of metallothionein synthesis by mitochondrial oxidative stress. Life Sci. **69**, 2137–2146
46 Zhou, L. Z., Johnson, A. P. and Rando, T. A. (2001) NF-κB and AP-1 mediate transcriptional responses to oxidative stress in skeletal muscle cells. Free Radicals. Biol. Med. **31**, 1405–1416
47 Sumida, C., Graber, R. and Nunez, E. (1993) Role of fatty acids in signal transduction: modulators and messengers. Prostaglandins Leukotrienes Essent. Fatty Acids **48**, 117–122
48 Bandyopadhyay, G. K., Hwang, S., Imagawa, W. and Nandi, S. (1993) Role of polyunsaturated fatty acids as signal transducers: amplification of signals from growth factor receptors by fatty acids in mammary epithelial cells. Prostaglandins Leukotr. Essent. Fatty Acids **48**, 71–78
49 Khan, W. A., Blobe, G. C. and Hannun, Y. A. (1995) Arachidonic acid and free fatty acids as second messengers and the role of protein kinase C. Cell. Signal. **7**, 171–184
50 Rao, G. N., Baas, A. S., Glasgow, W. C., Eling, T. E., Runge, M. S. and Alexander, R. W. (1994) Activation of mitogen-activated protein kinases by arachidonic acid and its metabolites in vascular smooth muscle cells. J. Biol. Chem. **269**, 32586–32591
51 Hardy, S., Langelier, Y. and Prentki, M. (2000) Oleate activates phosphatidylinositol 3-kinase and promotes proliferation and reduces apoptosis of MDA–MB-231 breast cancer cells, whereas palmitate has opposite effects. Cancer Res. **60**, 6353–6358
52 Dulin, N. O., Sorokin, A. and Douglas, J. G. (1998) Arachidonate-induced tyrosine phosphorylation of epidermal growth factor receptor and Shc-Grb2-Sos association. Hypertension **32**, 1089–1093
53 Bae, Y. S., Kang, S. W., Seo, M. S., Baines, I. C., Tekle, E., Chock, P. B. and Rhee, S. G. (1997) Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. J. Biol. Chem. **272**, 217–221
54 Inoguchi, T., Li, P., Umeda, F., Yu, H. Y., Kakimoto, M., Imamura, M., Aoki, T., Etoh, T., Hashimoto, T., Naruse, M. et al. (2000) High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells. Diabetes **49**, 1939–1945
55 Gamou, S. and Shimizu, N. (1995) Hydrogen peroxide preferentially enhances the tyrosine phosphorylation of epidermal growth factor receptor. FEBS Lett. **357**, 161–164
56 Brigelius-Flohe, R. (1999) Tissue-specific functions of individual glutathione peroxidases. Free Radicals. Biol. Med. **27**, 951–965
57 Ramakrishnan, N., Kalinich, J. F. and McClain, D. E. (1996) Ebselen inhibition of apoptosis by reduction of peroxides. Biochem. Pharmacol. **51**, 1443–1451

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