Escherichia coli engineered to produce eicosapentaenoic acid becomes resistant against oxidative damages

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Abbreviation: ACP, acyl carrier protein; CFU, colony-forming unit; DH5αEPA+, E. coli DH5α transformant producing EPA; DH5αEPA−, E. coli DH5α transformant producing no EPA; DH5α(EPA−), E. coli DH5α carrying no vector; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; H_2O_2+, in the presence of H_2O_2; H_2O_2–, in

the absence of H_2O_2; LB, Luria-Bertani; ORF, open reading frame; PUFA, polyunsaturated fatty acid; VBNC, viable but unculturable.

Abstract The colony-forming ability of Escherichia coli genetically engineered to produce eicosapentaenoic acid (EPA) grown in 3 mM hydrogen peroxide (H_2O_2) was similar to that of untreated cells. It was rapidly lost in the absence of EPA. H_2O_2-induced protein carbonylation was enhanced in cells lacking EPA. The fatty acid composition of the transformants was unaffected by H_2O_2 treatment, but the amount of fatty acids decreased in cultures of cells lacking EPA and increased in cultures of cells producing EPA, suggesting that cellular EPA is stable in the presence of H_2O_2 in vivo and may protect cells directly against oxidative damage. We discuss the possible role of EPA in partially blocking the penetration of H_2O_2 into cells through membranes containing EPA.

Key words: Eicosapentaenoic acid; Hydrogen peroxide; Oxidative stress; Protein carbonylation; Viable but nonculturable

1. Introduction

Bacteria synthesize eicosapentaenoic acid (EPA) and, presumably, docosahexaenoic acid (DHA) through a molecular oxygen-independent enzyme system that is similar to that for polyketide biosynthesis [1]. Five open reading frames (ORFs) involved in EPA biosynthesis were first reported in Shewanella sp. [2], and their homologues have been found in various types of EPA-producing bacteria: Shewanella sp. strain GA-22 [3], Photobacterium profundum strain SS9 [4], and a DHA-producing marine bacterium, Moritella marina MP-1 [5]. We recently reported on EPA-biosynthesizing Escherichia coli recombinant systems, by which various levels of EPA can be formed only at or below 25 °C by combining the five ORFs essential for EPA biosynthesis [6]. The system is a useful tool to investigate the biological functions of EPA.

Polyunsaturated fatty acids (PUFAs) such as EPA and DHA are cellular molecules with the greatest susceptibility to oxidative damage [7]. PUFAs in the bulk phase exposed to either oxygen or reactive oxygen species form lipid radicals or lipid peroxy radicals [7]. It has been assumed that PUFAs are more readily oxidized in biological systems [8], although PUFAs in an aqueous system are stable against peroxidation [9–11]. In liposomes made of phospholipids, the degree of unsaturation is inversely related to the stability of the constituent PUFAs [9]. Therefore, the oxidative stability of PUFAs in the bulk phase appears to differ dramatically from that in aqueous biological systems.

The anti-oxidative effects of PUFAs such as DHA have been reported in animal systems [12,13]. DHA effectively decreases the levels of cellular lipid peroxide [11–13]. Moreover, highly unsaturated fatty acids are more resistant to lipid peroxidation in human hepatoma cells supplemented with PUFAs, indicating that DHA incorporated into the cells is not peroxidized by exogenous hydrogen peroxide (H_2O_2) [14]. These findings contradict the classical concept that an

increase in DHA levels in biological systems has deleterious effects by enhancing lipid peroxidation [8]. At present, there is no evidence showing that this resistance of PUFAs to oxidative stress is directly involved in biological functions.

Here we used E. coli recombinants engineered to synthesize EPA to probe the possible protective effects in vivo of EPA against oxidative damage from H_2O_2.

2. Materials and methods

2.1 Bacterial cells and cultivation

The E. coli strain DH5α (Takara Shuzo, Kyoto, Japan) used as host was precultured by shaking at 180 rpm in Luria–Bertani (LB) medium at 20 °C and its recombinant strains were grown in LB medium in the presence of ampicillin and chloramphenicol (both at 50 μg/ml) at the same temperature. H_2O_2 (3 mM) was added at an optical density of the cultures at 600 nm of 1.0, and the cultures were grown under the same conditions with no added H_2O_2. To measure the number of colony-forming units (CFUs) every 15 min, 100 μl aliquots of 10^{-9}-times diluted cultures were spread onto LB agar plates and then cultured at 20 °C for two days.

2.2 E. coli recombinants

E. coli DH5α was engineered to produce EPA by transformation with ORFs derived from the EPA biosynthesis gene cluster (pEPA) from Shewanella sp. SCRC-2738 [1,2,6]. pEPA is a cosmid clone carrying a 38 kbp DNA fragment in pWE15 (Stratagene, La Jolla, CA, USA) that includes at least nine ORFs (ORFs 1–9), of which only five (ORFs 2, 5, 6, 7 and 8) are essential for EPA biosynthesis [1,6]. ORFs 2 and 8 tentatively encode phosphopantetheine transferase and enoyl reductase, respectively. ORF 5 and ORF 7 encodes a multifunctional protein including domains of

3-ketoacyl synthase, malonyl-CoA: acyl carrier protein (ACP) acyltransferase, six repeats of ACP, 3-ketoacyl-ACP reductase and that including domains of 3-ketoacyl synthase, chain length factor, and two 3-ketoacyl-ACP dehydratases, respectively [1].

pEPA was digested with XbaI enzyme to remove ORFs 1, 2, and 3. The resulting cosmid clone carried ORFs 4, 5, 6, 7, 8, and 9 (designated pEPAΔ1,2,3). ORF 4 and other unidentified ORFs of the clone are considered neutral in EPA biosynthesis. ORF 2 was cloned into pSTV28 (designated ORF 2/pSTV28; see ref. [6]). EPA in E. coli DH5α co-expressing pEPAΔ1,2,3 and ORF 2/pSTV28 comprised about 10% of the total fatty acids.

2.3 Analytical procedures

The E. coli cultures grown at 20 °C were treated with H_2O_2 as described above for the indicated times. Cells were harvested before and after the H_2O_2 treatment and then used for further analysis.

The catalase activity of the cell-free extracts of E. coli DH5α and its transformants was measured spectrophotometrically at 240 nm at room temperature, as described previously [15]. The standard reaction mixture for the assay contained 50 mM potassium phosphate buffer (pH 7.0), 59 mM H_2O_2, and 0.05 ml of crude extracts in a final volume of 1.0 ml. The reaction was run at 25 °C, and only the initial (60 s) linear rate was used to estimate the catalase activity. The molar absorption coefficient for H_2O_2 at 240 nm was 43.6 $M^{-1}cm^{-1}$. The enzyme activity unit (U) was defined as an amount of enzyme that decomposed 1 mmol of H_2O_2 per minute. Protein was assayed by the Bradford method [16].

The carbonyl content in oxidatively modified cellular proteins was determined as described [17]. Cells suspended in 50 mM HEPES buffer (pH 7.2) were disrupted by sonication. Cell-free extracts were prepared by centrifuging the sonicates at 10,000 × g for 15 min. Protein solutions of less than

5 mg/ml were subjected to determination of carbonyls, and the degree of carbonylation was expressed as nmol of protein carbonyls per milligram of protein.

Packed wet bacterial cells from 10 ml of cultures were subjected to methanolysis using 2 M methanolic HCl in the presence of 20 μg of heneicosanoic acid as an internal standard, as described previously [6]. The resulting fatty acid methyl esters (FAMEs) were analysed by gas–liquid chromatography and gas chromatography–mass spectrometry, as described previously [18]. The amount of fatty acid was expressed as micrograms of FAMEs per millilitre of culture.

3. Results

3.1 Effects of H_2O_2 on colony-forming ability

Figure 1A shows the number of CFUs of E. coli DH5α cells that produced or did not produce EPA (designated DH5EPAα+ and DH5αEPA−, respectively), grown at 20 °C in the presence (H_2O_2+) or absence (H_2O_2−) of 3 mM H_2O_2. The number of CFUs did not differ significantly between DH5αEPA+ and DH5αEPA− under H_2O_2− conditions. However, within 15 min of the addition of H_2O_2, the number of CFUs of strain DH5αEPA− decreased to 15% of the number at baseline, and then started to increase with no distinct time lag (Fig. 1A). However, strain DH5αEPA+ under H_2O_2+ conditions exhibited similar growth profiles as DH5αEPA− and DH5αEPA+ under H_2O_2− conditions, suggesting that EPA protects E. coli DH5α under oxidizing conditions.

3.2 Effects of H_2O_2 on catalase activity

The catalase activity of cell-free extracts prepared from two types of E. coli DH5α transformant cells grown at 20 °C was examined before and after the addition of H_2O_2 (Fig. 1B). At least for the first 45 min, catalase activity did not differ significantly between DH5αEPA+ and DH5αEPA− cells under H_2O_2+ or H_2O_2− conditions. The relatively unchanged activity suggests that catalase is not directly involved in the resistance of DH5αEPA+ to the oxidative damage caused by H_2O_2. E. coli DH5α carrying no vector [designated DH5α(EPA−)] had nearly the same catalase activity under H_2O_2+ or H_2O_2− conditions (Fig. 1C), indicating that the transformation of the strain had no effects on catalase activity.

E. coli can scavenge endogenous H_2O_2 by alkyl hydroperoxide reductase [19], although this enzyme is probably not involved in the elimination of exogenously added 3 mM H_2O_2 in our study, because it is saturated at a very low concentration (10^{-5} M) of H_2O_2 [19].

3.3 Effects of H_2O_2 on carbonyl content

The content of protein carbonyls of the strain DH5αEPA− was 28.7 nmol/mg protein at zero time. After cultivation for 15 min, the content of protein carbonyls of the same strain was 28.5 nmol/mg protein and 83.4 nmol/mg protein when grown under H_2O_2− and H_2O_2+ conditions, respectively (Table 1). In contrast, the content in strain DH5αEPA+ grown under the H_2O_2+ condition was 16.7 nmol/mg protein, which did not differ from that in cells grown under the H_2O_2− condition, but was significantly lower than that of DH5αEPA− grown under the H_2O_2+ conditions. These data indicate that the addition of H_2O_2 induced protein carbonylation in the DH5αEPA− strain only and that EPA partially or fully protects the carbonylation of proteins. Moreover, EPA

decreased the content of protein carbonyls of DH5αEPA+ even under H_2O_2− conditions (17.5 nmol/mg protein) compared with that of DH5αEPA− grown under H_2O_2− conditions (28.5 nmol/mg protein). This implies that oxidative stress causing the carbonylation of proteins occurs even in E. coli DH5α cells carrying a vector or growing in the presence of antibiotics, and that this stress is relieved by EPA. In addition, we found a slightly reduced content of protein carbonyls (23.8 nmol/mg protein) in E. coli DH5α cells carrying no vector [DH5α(EPA−)] when grown at 20 °C. The DH5α(EPA−) cells grown at 37 °C had a lower content of protein carbonyls (15.7 nmol/mg protein) than those grown at 20 °C, suggesting that growth at a suboptimal temperature enhances protein carbonylation through oxidative stress.

3.4 Effects of H_2O_2 on fatty acid amount and composition

Table 2 shows the amount and composition of fatty acids from E. coli DH5α transformants at zero time and after cultivation for 15 min under H_2O_2− and H_2O_2+ conditions. Treatment with H_2O_2 had little effect on fatty acid composition. These data suggest that EPA is not susceptible to attack by exogenous H_2O_2 in vivo. In contrast, the susceptibility of E. coli DH5α transformant cells to H_2O_2 differed. Compared with their respective baseline values, the amount of fatty acids extracted from cultures (1 ml) was reduced by ~8% in strain DH5αEPA− but increased by ~16% in DH5αEPA+ grown under H_2O_2+ conditions. The increased amount of EPA in DH5αEPA+ grown under H_2O_2+ conditions for 15 min was about the same as that in DH5αEPA+ grown under H_2O_2– conditions. Nearly the same amount of fatty acids was recovered from both strains under H_2O_2– conditions. EPA appears to protect E. coli DH5α cells from the decay and damage caused by 3 mM H_2O_2.

4. Discussion

Cellular damage by H_2O_2 is both direct and indirect. Some proteins can be oxidized directly by exogenous H_2O_2, which easily traverses the cell membrane. However, H_2O_2 that crosses the cell membrane probably reacts with iron or copper ions to form much more damaging species such as hydroxyl radicals (·OH) by the Fenton reaction [20]. These highly reactive radicals include lipid peroxy radicals [7] and account for much of the damage to proteins and probably to DNA in H_2O_2-treated cells. In the E. coli strain K12 exposed to 2 mM exogenous H_2O_2, various proteins such as elongation factor G, outer membrane protein A, and the β-subunit of F_0F_1-ATPase are carbonylated, leading to a transient growth arrest lasting 2–3 h [21]. This suggests that the decrease in CFUs of DH5αEPA− grown under 3 mM H_2O_2+ conditions (Fig. 1) was probably caused by enhanced carbonylation of proteins essential for growth. The de novo resynthesis of these damaged proteins might subsequently restore growth, as reported for H_2O_2-treated Staphylococcus aureus [22]. Oxidative damage to DH5αEPA– cells might have induced a dormant-like state [i.e., a viable but nonculturable (VBNC) state] [23–25], because the restoration of their growth had no distinct time lag (Fig. 1A). E. coli cells are known to enter the VBNC state in response to adverse environmental conditions such as low temperature and starvation causing oxidative stress [25]. Therefore the recovery of CFU of DH5αEPA– cells under H_2O_2+ conditions would be due mainly to the conversion of cells from the nonculturable to culturable state.

It is unclear how DH5αEPA+ cells maintain their colony-forming ability (culturability) under H_2O_2+ conditions. It is possible that EPA or its metabolic derivatives accelerate the de novo synthesis of damaged proteins or the occurrence of reactive oxygen species scavenging systems, or both, to restore the reduced culturability of DH5αEPA+ grown under H_2O_2+ conditions. However, this is unlikely because we observed no arrest of growth in the DH5αEPA+ cells after exposure to H_2O_2. And only negligible levels of malondealdehyde, a peroxidative derivative of EPA [7], were

detected in DH5αEPA+ under H_2O_2+ conditions (data not shown). Any H_2O_2 molecules entering the cells would have been eliminated by catalase. It is more probable that some H_2O_2 is partially blocked from traversing the plasma membrane by EPA-containing phospholipids. According to a proton nuclear magnetic resonance study by Miyashita [26], DHA, another long-chain n-3 PUFA, has a much more rigid micellar structure in its aqueous phase than in chloroform. If this also applies to EPA, which has a similar structure to DHA, then EPA-containing phospholipids in the plasma membrane may act to shield against H_2O_2 entering the cell.

It is noteworthy that the increased production of EPA decreased the content of protein carbonyls even in H_2O_2-untreated cells. We found a lower content of protein carbonylation in the DH5αEPA+ strain than in the DH5αEPA− strain, and in the DH5αEPA+ strain than in the DH5α strain that carried no vector [DH5α(EPA−)], when grown at 20°C (Table 1). The catalase activity of strain DH5α(EPA−) grown at 20 °C under H_2O_2+ and H_2O_2− conditions was nearly identical to that of the DH5α transformant strains for the first 15 min (Figs 1B and 1C). In addition, the lowest level of protein carbonyls was detected in the DH5α(EPA−) strain grown at 37 °C (see Table 1). These results suggest that, in cells harbouring a vector, growth in the presence of antibiotics or at a low temperature might induce oxidative stress, leading to protein carbonylation. In other words, EPA in the plasma membrane may alleviate endogenously generated oxidative stress. This conclusion is consistent with the observations that culturability of starvation-stressed and low-temperature stressed E. coli O157 can be restored by using H_2O_2-degrading compounds [25], that some environmental stresses increase oxidative damage [27], and that long-term cultivation of E. coli at 20 °C enhances the expression of the genes for Mn-superoxide dismutase and catalase, and decreases the intracellular concentration of reduced glutathione [27].

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Table 1. Effects of H_2O_2 on protein carbonylation of E. coli DH5α strains with and without EPA.

Strain	Growth temp. (°C)	Amount of protein carbonyl (nmol/mg protein)		
		H_2O_2−		H_2O_2+
		0[a]	15[a]	15[a]
DH5αEPA−	20	28.7 ± 3.3	28.5 ± 3.5	83.4 ± 4.7
DH5αEPA+	20	17.0 ± 2.1	17.5 ± 1.9	16.7 ± 2.2
DH5α (EPA−)[b]	20	–[c]	23.8 ± 1.5	35.9 ± 3.2
DH5α (EPA−)[b]	37	–[c]	15.7 ± 0.6	19.4 ± 0.2

[a] Time after the addition of 3 mM H_2O_2 (min).

[b] E. coli DH5α carrying no vector.

[c] Not analysed.

Table 2. Effects of H_2O_2 on fatty acid compositions and fatty acid contents of E. coli DH5α strains with and without EPA.

Fatty acid[a]	Fatty acid composition (weight %)					
	DH5αEPA−			DH5αEPA+		
	H_2O_2−		H_2O_2+	H_2O_2−		H_2O_2+
	0[b]	15[b]	15[b]	0[b]	15[b]	15[b]
12:0	2.1 ± 0.2	2.0 ± 0.4	1.8 ± 0.3	1.9 ± 0.0	1.4 ± 0.3	1.4 ± 0.3
14:0	4.6 ± 0.2	5.0 ± 0.4	5.3 ± 0.4	4.5 ± 0.1	4.0 ± 0.1	4.4 ± 0.2
16:0	32.5 ± 0.5	33.6 ± 0.6	33.2 ± 0.1	33.4 ± 0.0	34.8 ± 0.2	34.9 ± 0.1
16:1	27.4 ± 0.1	27.9 ± 0.5	27.2 ± 0.4	19.2 ± 0.2	19.6 ± 0.3	19.4 ± 0.1
18:1	21.5 ± 0.0	20.5 ± 0.4	21.3 ± 0.2	18.8 ± 0.2	19.5 ± 0.1	19.6 ± 0.4
3-OH 14:0	10.4 ± 0.4	9.5 ± 0.4	9.6 ± 0.4	9.9 ± 0.0	10.0 ± 0.4	9.6 ± 0.3
20:5 (EPA)	n.d.[c]	n.d.[c]	n.d.[c]	9.4 ± 0.2	9.8 ± 0.4	9.9 ± 0.0
Others	1.5 ± 0.3	1.4 ± 0.1	1.7 ± 0.2	2.9 ± 0.2	0.8 ± 0.2	0.9 ± 0.1
Total FAMEs (μg/ml cell culture)	12.1 ± 0.8	14.4 ± 2.8	11.1 ± 1.5	12.5 ± 2.3	14.5 ± 1.5	14.5 ± 0.8
FAME recovery (%)	100	119.0	91.7	100	116.0	116.0

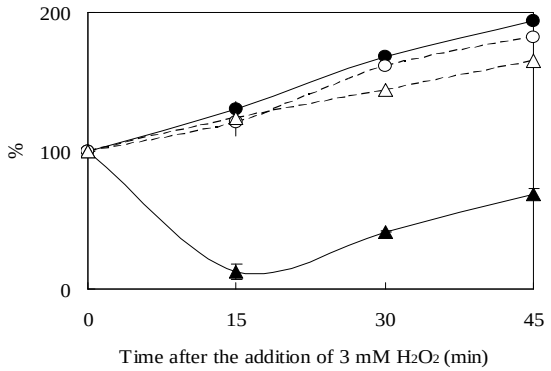
[a] Fatty acids are abbreviated as X:Y, where X and Y are the numbers of carbon atoms and double bonds, respectively. 3-OH 14:0 is 3-hydroxy 14:0.

[b] Time after the addition of 3 mM H_2O_2 (min).

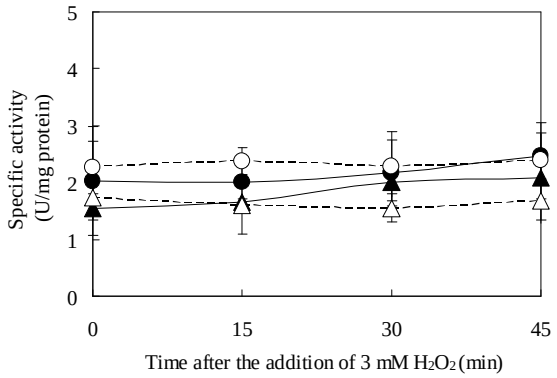
[c] Not detected.

Fig. 1.

A


200
%
100
0
0
15
30
45
Time after the addition of 3 mM H2O2 (min)

B


5
4
3
2
1
0
Specific activity
(U/mg protein)
0
15
30
45
Time after the addition of 3 mM H2O2 (min)

C

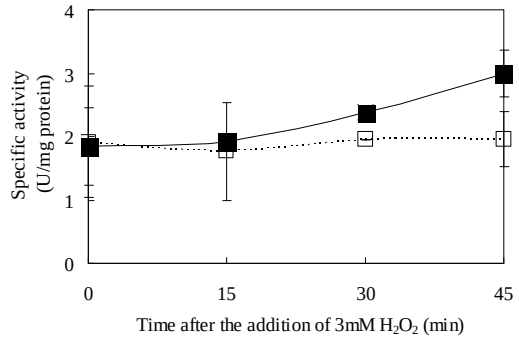

4
3
2
1
0
Specific activity
(U/mg protein)
0
15
30
45
Time after the addition of 3mM H2O2 (min)

Figure legend

Fig. 1. Effects of H_2O_2 treatment on the growth and catalase activity of E. coli DH5α strains producing or not producing EPA. Growth (A) and catalase activity (B) of E. coli DH5α transformant strains. Δ, strain DH5αEPA− grown under H_2O_2− conditions; ▲, DH5αEPA− grown under H_2O_2+ conditions; ○, DH5αEPA+ grown under H_2O_2− conditions; ●, DH5αEPA+ grown under H_2O_2+ conditions. (C) Catalase activity of E. coli DH5α cells carrying no vector [DH5α(EPA–)]. □, DH5α(EPA−) grown under H_2O_2− conditions; ■, DH5α(EPA−) grown under H_2O_2+ conditions. Cells were grown at 20 °C. The data are means ± standard errors for three independent experiments.