Vol. 145, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1987 Pages 15-24

POLYUNSATURATED FATTY ACIDS AUGMENT FREE RADICAL GENERATION IN TUMOR CELLS IN VITRO

U.N. DAS*, M.E. BEGIN, G. ELLS, Y.S. HUANG, AND D.F. HORROBIN

EFAMOL RESEARCH INSTITUTE, P O BOX 818,
KENTVILLE, N.S. CANADA B4N 4H8

Received March 2, 1987

S U M M A R Y : ***Polyunsaturated fatty acids (PUFAs) have been shown to inhibit both normal and tumor cell growth in vitro. As PUFAs are known to induce a respiratory burst and free radical generation in polymorphonuclear leukocytes and since free radicals are toxic to cells, we investigated the effect of PUFAs on a measure of free radical generation (nitroblue tetrazolium reduction) in normal human fibroblasts and breast cancer cells in vitro. Results suggested that linoleate (LA), gamma-linolenate (GLA), arachidonate (AA) and eicosapentaenoate (EPA) can enhance nitroblue tetrazolium reduction in tumor cells but not in normal cells. GLA, AA and EPA were 1 1/2 to 2 times more effective than LA in inducing free radical generation. This difference was not due to increased uptake of LA, AA and EPA by tumorcells. Infact, the uptake of LA was the same both in normal and tumor cells whereas that of AA and EPA occurred at approximately half the rate in the tumor cells compared to normal cells. This indicates that PUFA induced growth inhibition and cytotoxicity to tumor cells may, at least in part, be due to enhanced free radical generation.***

Polyunsaturated fatty acids (PUFAs) at adequate concentration have been shown to inhibit cell division of both normal and tumor cells in vitro (1,2,3). Tumor cells are known to have reduced activity of PUFA desaturase enzymes (4). Addition of PUFAs to a variety of tumor and normal cells in vitro can induce selective cytotoxicity (3,5) with tumor cells but not normal cells being killed. This selective tumoricidal action of PUFAs can be blocked by anti-oxidants such as vitamin E, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) indicating that free radicals and lipid peroxidation may have a role (3,6,7). This assumption is supported by the observation that radiation and some anti-cancer drugs augment free radical generation and lipid peroxidation in tumor cells, that toxicity due to adriamycin can be prevented by the anti-oxidant vitamin E (8,9) and free radicals are toxic to both

*PRESENT ADDRESS: DEPARTMENT OF CLINICAL PHARMACOLOGY AND MEDICAL RESEARCH, THE NIZAM'S INSTITUTE OF MEDICAL SCIENCES, PUNJAGUTTA, HYDERABAD-500 482, A.P., I N D I A.

0006-291X/87 $1.50

normal and tumor cells (10,11). Further, PUFAs are also known to augment superoxide production by human neutrophils (1,2). In order to verify whether PUFA induced cytotoxicity to tumor cells (3) is also mediated by enhanced production of free radicals, we studied the effect of LA, GLA, AA and EPA supplementation on nitroblue tetrazolium reduction in normal and tumor cells in vitro.

MATERIALS AND METHODS

Human breast carcinoma (AR-75-1) and established normal human fibroblasts (CCD-41 SK) were obtained from the American Type Culture Collection (ATCC). Stock cultures were grown in glass-bottles in bicarbonate buffered Dulbecco's modified minimal Eagles medium containing 10% heat-inactivated fetal calf serum, 50ug/ml gentamycin and 2.5 ug/ml fungizone.

Both the cell lines were seeded at $1X10^4$ cells/well in 24 well tissue culture plates. The cells were grown in 0.5 ml of bicarbonate-buffered Dulbecco's modified Eagle's medium (Flow laboratories) with or without added fatty acids at 37^0C in a 5% CO_2 humidified incubator as described earlier (3). One day after seeding, various fatty acid esters and/or control solutions were added depending on the experimental protocol. The esters were initially dissolved in 95% ethanol and the final concentration of ethanol is not more than 0.02% in all control and fatty acid supplemented cultures. All experiments were done at 20 ug/ml of fatty acid, as earlier studies showed that this is the optimum concentration to produce maximum cytotoxicity to tumor cells with least toxicity to normal cells. Ethyl esters of linoleic acid (LA, 18: 2 n-6), gammalinolenic acid (GLA, 18: 3 n-6), arachidonic acid (AA, 20: 4 n-6) were obtained from either Sigma Laboratories and methylester of eicosapentaenoic acid (EPA, 20:5 n-3) from Nu-chek laboratories. All other chemicals were obtained from Sigma Chemical Co., U.S.A.

THYMIDINE INCORPORATION STUDIES

One day after seeding, 0.4 uCi of labelled thymidine (specific activity 20.0 Ci/mmol) was added to study DNA synthesis. Cells were washed atleast thrice with PBS (PH 7.4), detached by trypsinization and counted in a liquid scintillation counter on day 1,2 and 3 to assess DNA synthesis.

FATTY ACID UPTAKE STUDIES

One day after seeding, the cells were incubated with 0.1 uCi of labelled LA, AA and EPA (specific activity: LA: 52.6 m Ci/mmol, AA:54.5m Ci/mmol EPA: 55.4 m Ci/mmol) to study the uptake of these fatty acids by both normal and tumor cells. Labelled fatty acids were obtained from New England Nuclear Corporation, Boston, USA. The cells were washed thrice with PBS, detached by trypsinization and the total amount of fatty acid incorporated was counted in a liquid scintillation counter on day 1,2 and 3 for all the fatty acids tested.

NBT - REDUCTION

The superoxide anion can reduce nitroblue tetrazolium ion (NBT) to the insoluble blue formazan. It is generally accepted as a reliable and simple method of assaying superoxide anion and possibly other free radicals (13). The ability to reduce NBT was assayed by incubating the cells for 4 hours at 37^0C with 0.5 ml of 0.2% NBT dissolved in PBS (pH 7.4). Before incubating with NBT, the culture medium was removed and the cells were washed 2 or 3 times with PBS. To quantitate the amount of NBT reduction, after the reaction was over (i.e. after 4 hours of incubation with NBT solution), the cells were washed twice in PBS, detached with trypsin - EDTA treatment and lysed by the addition of 0.5 ml of distilled water. The lysate was centrifuged

at 1000 rpm for 10 minutes and the supernalant was read at 490 nm and control cells not treated with fatty acids but which underwent the same treatment with NBT was used as reference and instrument was adjusted to 0 reading with this control.

Earlier studies showed that incubation of cells with NBT alone is sufficient to produce NBT reduction and hence we avoided the need to add TPA (12-0-tetradecanoylphorbol-13-acetate) to stimulate superoxide generation as is the case with macrophages and neutrophils (12).

All experiments were done in quadruplicate and repeated at least twice. Our earlier studies showed that the cytotoxic action of PUFAs is evident from Day 4 of incubation (3). Hence in this study all experiments were done from day 1 to day 3, day 1 being one day after seeding since, we were interested in evaluating what reactions were occurring prior to overt cell damage.

RESULTS

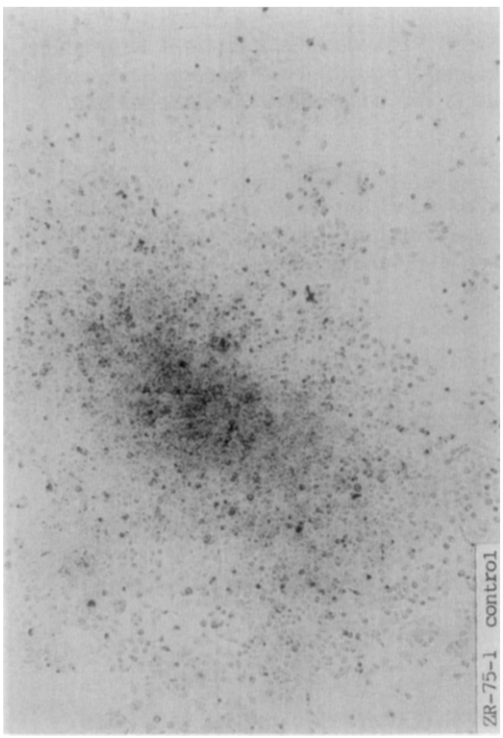
The results of thymidine incorporation are given in table 1. There was a gradual increase in thymidine incorporation from day 1 to day 2 but by day 3, 41-SK seemed to have ceased growing as there was no increase in thymidine uptake. There is no significant difference between 41 SK and ZR-75-1 cells suggesting that their growth rate is almost same during the first 3 days of culture.

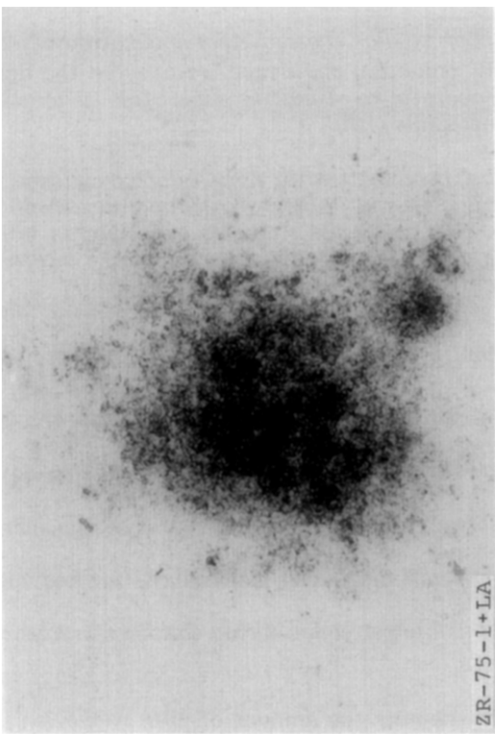
Fig.1 depicts the amount of NBT reduction in 41 SK and ZR-75-1 cells treated with different types of fatty acids at 20 ug/ml. It is evident that GLA, AA and EPA induced significant enhancement in NBT reduction by ZR-75-1 cells whereas they had a much smaller effect on normal human fibroblasts. This is substantiated by the NBT reduction observed at 490 nm (Table 2): GLA, AA and EPA treated ZR-75-1 cells are almost twice as potent as LA treated cells in inducing NBT reduction. This correlates well with our earlier results wherein it was observed that GLA, AA and EPA are more potent than LA in inducing selective cytotoxicity to tumor cells (3,7). The lack of cytotoxic action of PUFAs on normal cells may also be due to their inability to suf-

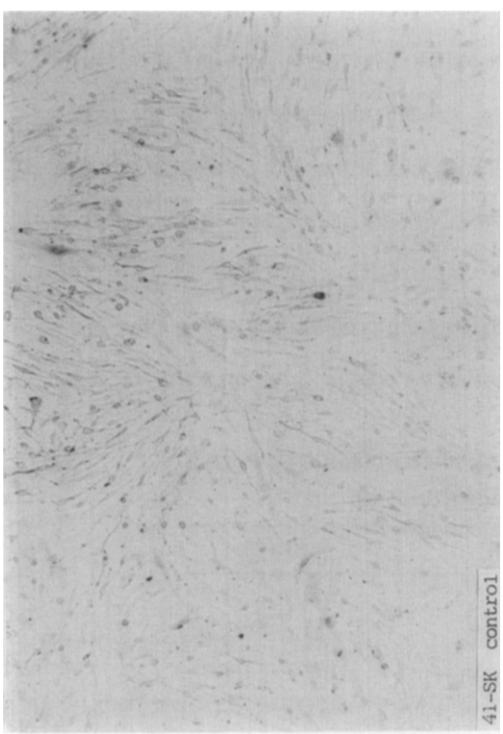
Table 1. Thymidine incorporation in human fibroblasts (CCD-41SK) and breast cancer cells (ZR-75-1)

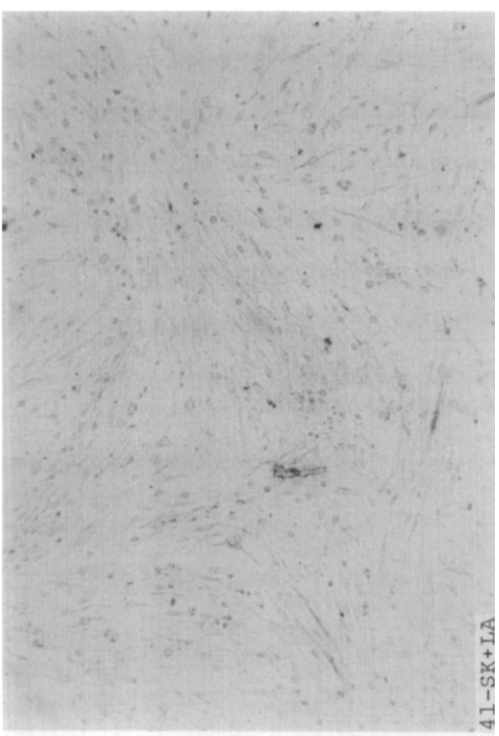
TIME	41 SK	ZR-75-1
3 hrs	424.1 ± 57.4	352.4 ± 32.4
24 hrs	601.3 ± 44.1	633.0 ± 78.6
48 hrs	1180.5 ± 212.4	1152.5 ± 136.7
72 hrs	895.2 ± 55.2	1016.2 ± 81.8

Mean ± S.D.


ZR-75-1 control


ZR-75-1+LA


41-SK control


41-SK+LA

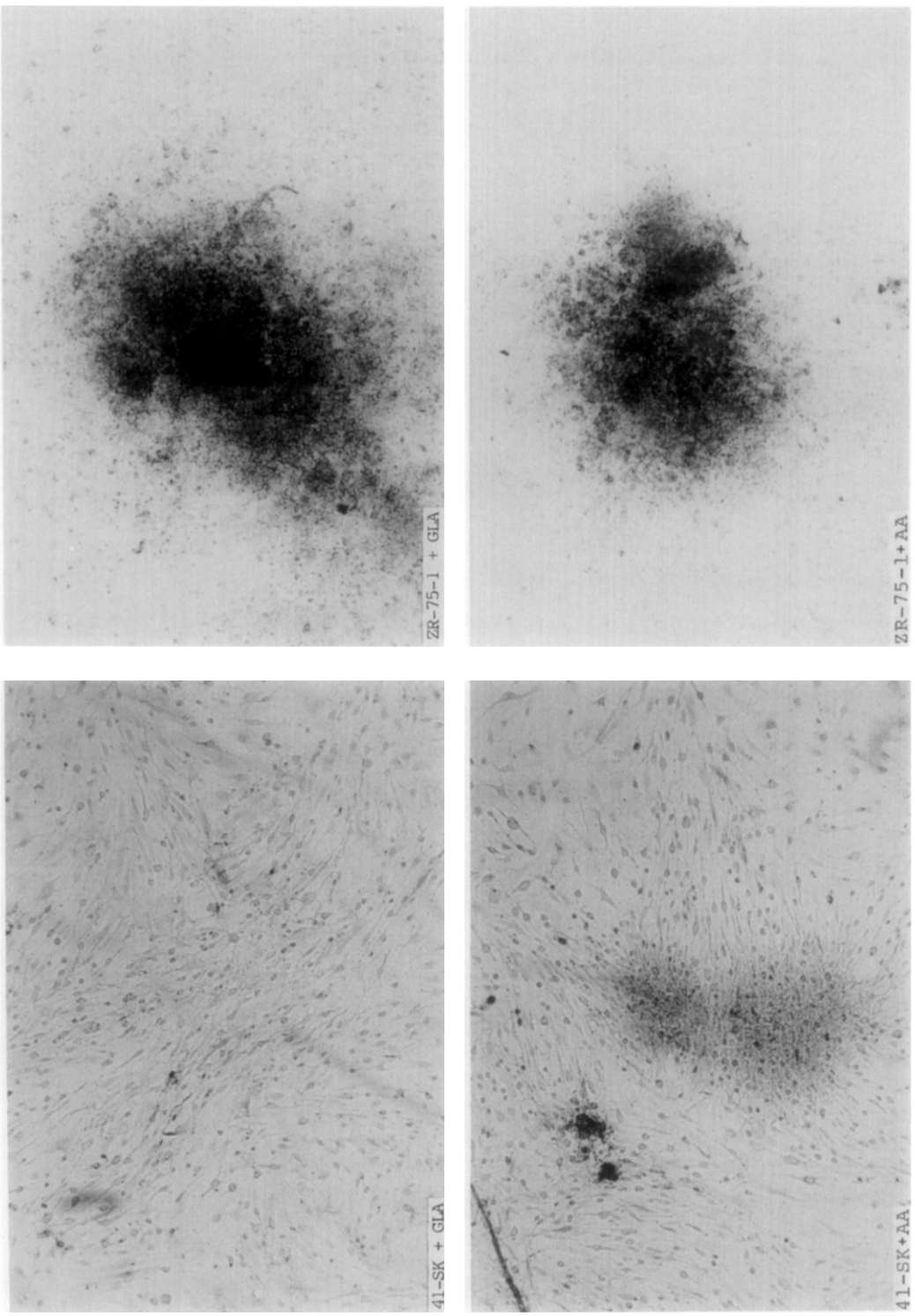
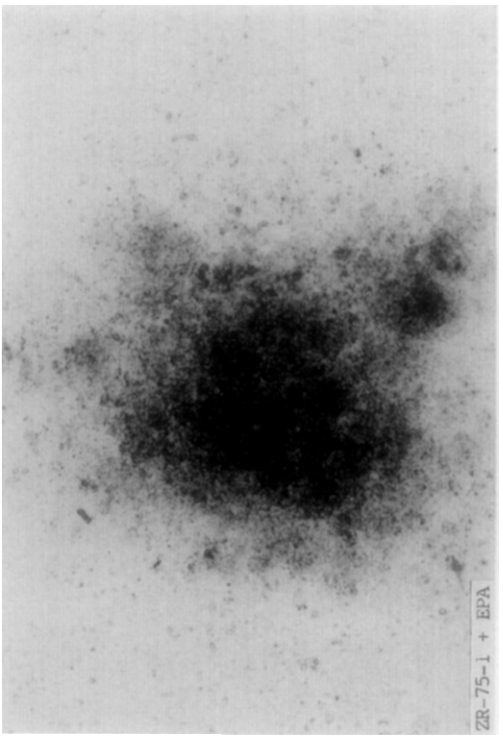


Fig. 1. NBT reduction in 41-SK and ZR-75-1 cells after treatment with various polyunsaturated fatty acids. 41-SK are normal human fibroblasts. ZR-75-1 are human breast tumor cells.



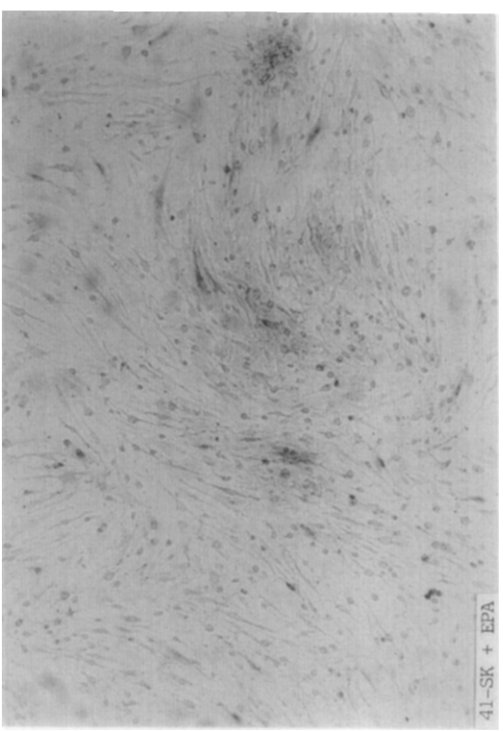


Fig. 1. *Continued.*

Table 2. NBT reduction by 41-SK and ZR-75 cells on day 2 & 3 of incubation with PUFAs (for procedure see material and methods). Expressed as O.D. at 490 mm

Day 2:

FATTY ACID	41 SK	ZR-75-1
Control	0.000 ± 0.000	0.000 ± 0.000
LA	0.013 ± 0.008	0.012 ± 0.007
GLA	0.010 ± 0.008	0.020 ± 0.014
AA	0.010 ± 0.006	0.015 ± 0.002
EPA	0.009 ± 0.005	0.012 ± 0.006

Day 3:

FATTY ACID	41 SK	ZR-75-1
Control	0.000 ± 0.000	0.000 ± 0.000
LA	0.008 ± 0.001	0.014 ± 0.002
GLA	0.006 ± 0.001	0.030 ± 0.004
AA	0.005 ± 0.003	0.023 ± 0.003
EPA	0.004 ± 0.005	0.023 ± 0.009

Mean ± S.D.

ficiently enhance free radical generation in them, as shown by the small change in NBT reduction seen in 41 SK cells in the present study.

In order to find out whether this difference in NBT reduction is due to differential uptake of fatty acids, we studied the uptake of labelled LA, AA and EPA in 41 SK and ZR-75-1 cells under the same conditions. The results given in Fig.2 show that the uptake of LA, AA and EPA was atleast twice as high in the fibroblasts compared to breast tumor cells at 24 and 48 hours. At 72 hours of incubation, the incorporation of LA and EPA was almost same both in normal and tumor cells, whereas that of AA is atleast 1 1/2 times low only in the tumor cells. We have also done the fatty acid uptake studies in another four cell lines: two normal and two tumor cell lines, and they are essentially same as that of the present study (data not shown). These results suggest that tumor cells incorporate less LA, AA and EPA compared to normal cells when incubated under in same conditions.

DISCUSSION

The present study demonstrates that even though tumor cells incorporate less LA, AA and EPA compared to normal cells, AA and EPA treated tumor cells have a

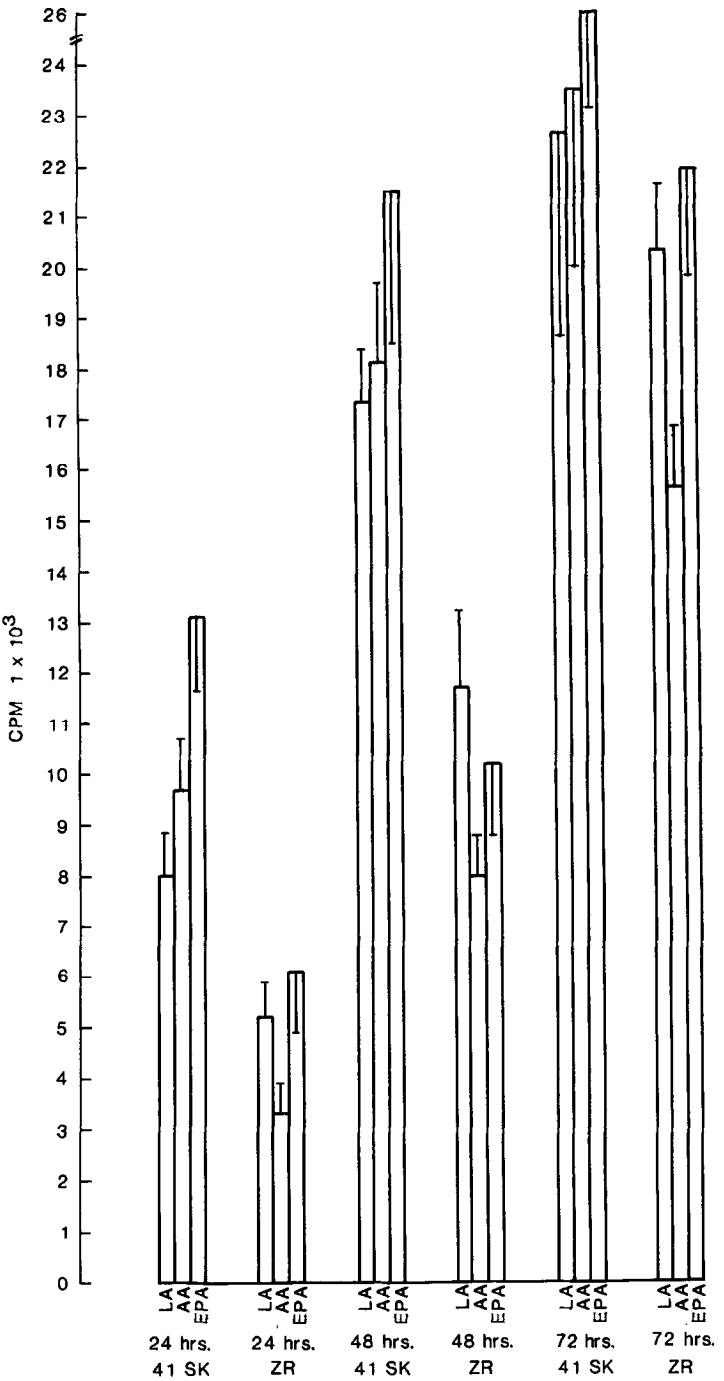


Fig.2. Incorporation of labelled LA, AA,and EPA by 41-SK and ZR-75-1 cells at 24, 48,and 72 hours.

higher capacity to reduce NBT, suggesting enhanced superoxide production, although the limited speificity of the NBT reaction must not be forgotten. Since superoxide radicals and other free radicals can initiate lipid peroxidation and are toxic to tumor

cells (10, 14), this may explain why some PUFAs, especially GLA, AA and EPA have selective tumoricidal actions (3,4,6,7). In addition, tumor cells are known to contain reduced amounts of superoxide dismutase and glutathione peroxidase and their microsomal membrane contents of the electron carriers (NADPH-cytochrome C reductase, and cytochrome P-450) which generate oxygen intermediates and the NADPH - induced production of superoxide redicals are also low (15).

The increased NBT reduction observed in the tumor cells (as on supplementation with 20 ug/ml of GLA, AA and EPA compared to normal cells) in the present study may in part be attributed to their possibly low SOD and glutathione peroxidase activities. It is likely that both lipid peroxidation and generation of superoxide and other free radicals are low in the tumor cells due to substrate deficiency, namely their low content of PUFAs (especially those containing more than 2 double bonds) (15). On the other hand, the normal cells may be protected by free radical scavengers. This may explain in part why there is enhanced NBT reduction in tumor cells after supplementation with PUFAs.

Though, we have not investigated the exact machanism (s) by which this enhanced level of superoxide and possibly other free radicals is related to the tumoricidal action of PUFAs, it is likely that damage to DNA, induction of protein and nucleic acid cross-linking by lipid degradation products, or inhibition of mitrochondrial function leading to reduction in cellular ATP levels and loss of viability (16) may be involved. This possibility is supported by our observation that ATP can inhibit the PUFA - induced tumoricidal action (unpublished data) and that hematoporphyrin derivative - induced photosensitization, a procedure which also enhances free radical generation in the cells, in R 3230 AC mammary tumors causes a 60% reduction in cellular ATP levels (16).

REFERENCES

1. *Spector, A.A., Mathur, S.N., Kaduce, T.L. and Holman, R.T. (1981). Lipid Res. 19: 155.*
2. *Cornwell, D.G. and Morisaki, N.(1984). Free radicals in Biology, Academic Press, N.Y. p 95.*

3. Begin, M.E., Das, U.N., Ells, G. and Horrobin, D.F. (1985). Prostaglandins Leukotrienes Med. 19: 177-186.

4. Bailey, J.M. (1977). In Snyder F () ed ()., Lipid metabolism in mamals. Vol II Plenum Press, N.Y. P. 323.

5. Booyens, J., Engelbrecht, P., Le Ronx, S. Et at. (1984). Prostaglandins Leukotrienes Med. 15: 15-33.

6. Begin, M.E., Das, U.N. and Ells, G. Progr Lipid Res. in press.

7. Begin, M.E., Ells, G., Das, U.N. and Horrobin, D.F. JNCI, in press.

8. Lown, J.W. (1985) Adv. Free Radical Biol Med. 1: 125-264.

9. Guffy, M.M., Nort, J.A. and Burns, C.P. (1984). Cancer Res. 44: 1863-1866.

10. Rotilio, G., Boxi, A., Mavelli, I., Mordovi, B. and Strom, R.(1982). Proc. Fed Eur Biochem Soc, Symposium No. 62, P 1188-126.

11. Lown, J.W., Jushua, A.V. and chen, H.H. (1982). In Free Radicals, Lipid peroxidation and Cancer, (EDs) McBrien, D.C.H. and Slater, T.F. Academic Press, N.Y. P-305-328.

12. Badway,J.A., Curnutte,J.T., Karnovsky,M.L.(1981). J.Biol Chem 256: 12640-43.

13. Matsui, T., Nakao, Y., Kobayashi, N. (1985). Cancer Res. 45: 311-316.

14. Halliwell, B. and Gutteridge, J.M.C.(1984). Biochem. 219: 1-14.

15. Galeotti, T., Bautoli, G.M. and Bartoli, E.(1982). In Biological and clinical aspects of superoxide and superoxide dismutase, (eds) W.H. Bannister and J.V. Bannister Proc. Fed Eur Biochem Societies, Symposium No.62, P106-117.

16. Hilf,R., Murant,R.S., Narayanan,U and Gibson, S.L.(1986). Cancer Res.46:211-17.