Prooxidant action of crystalline serum albumin in lipid peroxidation during incubation of rat adipose tissue in vitro

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SUMMARY During in vitro incubation of rat adipose tissue in a medium containing crystalline serum albumin, a thiobarbituric acid-reacting chromogen (presumably malonaldehyde) appears in the medium, reflecting peroxidation of polyunsaturated fatty acids in the tissue.

Albumin which has been dialyzed, treated with EDTA and dialyzed, or subjected to gel filtration shows a markedly decreased ability to support malonaldehyde production. Prooxidant activity is found in the dialysate and in protein-free fractions appearing after albumin in the gel filtration.

The prooxidant that contaminates albumin has been identified as iron and citrate, the latter acting by forming a soluble complex with the iron.

KEY WORDS free fatty acids · peroxidation · adipose tissue · rat · incubation · ferrous ions · citrate · iron–citrate complex · albumin contaminants · prooxidant activity · malonaldehyde · gel filtration · dialysis

In a previous report we have shown that during the incubation of rat adipose tissue in a medium containing bovine serum albumin (BSA) a chromogen reactive with thiobarbituric acid, presumably malonaldehyde, is released into the medium (1). Malonaldehyde is a decomposition product of peroxidized polyunsaturated fatty acids (2). Peroxidation, which may occur as a nonenzymatic reaction catalyzed by certain metals such as Fe^{++}, characteristically develops in the tissues of vitamin E deficient animals (3, 4). In an earlier report (1) we demonstrated that the production of malonaldehyde by adipose tissue could be correlated with the release of free fatty acid, and that production was accentuated when lipolysis was stimulated by hormones. Supplementation of the chow diet with α-tocopherol reduced the production of malonaldehyde by incubated adipose tissue, without influencing lipolysis.

The present study was designed to explore some of the factors in the in vitro system which are responsible for peroxidation. Crystalline bovine serum albumin, which is employed by most investigators performing in vitro studies on rat adipose tissue, was found to be essential for maximal peroxidation. An explanation for this prooxidant role will be given.

METHODS AND MATERIALS

Male rats of the Wistar strain from our own colony weighing between 170 and 260 g were used. The animals were closely matched in weight in any given experiment. In early experiments the rats were fasted overnight, but when it was found that feeding did not influence malonaldehyde production, animals fed ad lib. were used (1). The diet was Purina rat chow. Epididymal fat pads were removed from rats anesthetized by the intraperitoneal injection of 5 mg of sodium pentobarbital per 100 g of body weight.

Incubations were carried out at 37° in a Krebs-Ringer bicarbonate medium (KRB), gassed with 95% O_2–5% CO_2, in stoppered 10-ml Erlenmeyer flasks in a Dubnoff metabolic shaker, oscillating 70–80 times per min. Adipose tissue from several animals was pooled and cut into small pieces for random distribution among flasks in some experiments, whereas in others entire fat pads were used,

* Deceased.

usually one serving as a control for the tissue of the opposite side.

Free fatty acids (FFA) in tissue and medium were determined according to Dole (5) and malonaldehyde was determined by a modification of the method of Wilbur et al. (6). In the initial studies the data were expressed as thiobarbituric acid optical density units per gram of tissue (1). Later, in keeping with the suggestion of Sinnhuber and Yu (7) 1,1,3,3-tetraethoxypropane, which is hydrolyzed by acid to malonaldehyde, was used as a standard. In this paper the data are expressed as mμmoles of malonaldehyde released per gram of tissue. For uniformity, all the original data have been converted to the same units using a standard calibration curve. Non-haem iron was determined by a modification of the method of Doeg and Ziegler (8) and citrate by the method of Ettinger et al. (9).

Samples of crystalline bovine serum albumin were purchased from Armour, Sigma and Pentex Laboratories. Human serum albumin was obtained from the Protein Foundation, Jamaica Plains, Mass.

TABLE 1A EFFECT OF DIALYSIS OF ALBUMIN ON FFA AND MALONALDEHYDE PRODUCTION BY ADIPOSE TISSUE

	Medium Malonaldehyde	Medium FFA
	mμmoles/g	*μmoles/g*
Undialyzed	49.5 ± 0.66	20.0 ± 1.1
Dialyzed	9.9 ± 0.90	27.3 ± 1.1

Each value is the mean ± SE of 4 observations.

An 8% albumin–KRB solution (20 ml) was dialyzed for 36 hr in 3 changes of bathing solution (1300 ml of KRB each). The dialyzed albumin–KRB (diluted with KRB to a 4% solution) was then compared with a freshly prepared 4% albumin–KRB solution. Pieces of adipose tissue weighing approximately 400 mg each were preincubated at 37° in 3 ml of KRB medium (without albumin) containing 10 μg of adrenaline per ml of medium. The gas phase was 95% O_2–5% CO_2. At the end of 30 min the tissues were removed, washed in 0.9% NaCl for 15 sec, and then transferred to 10-ml Erlenmeyer flasks containing 3 ml of the media to be tested and gassed with 95% O_2–5% CO_2. The flasks were stoppered and incubated for 3 hr in a Dubnoff shaker at 70–80 oscillations/min at 37°.

TABLE 1B

	Medium Malonaldehyde	Tissue FFA
	mμmoles/g	*μmoles/g*
KRB	6.8 ± 0.33	31.5 ± 1.8
Dialysate	34.5 ± 1.21	26.7 ± 2.3

Each value is the mean ± SE of 4 observations.

A 20% albumin–KRB solution (10 ml) was dialyzed in a bath of 140 ml KRB for 10 hr. The dialysate was then compared with fresh KRB as incubation medium. The adipose tissue was preincubated with adrenaline, as above, and then transferred to either KRB or dialysate for 3 hr. Tissue FFA was measured, as no albumin was present to transport FFA from tissue to medium.

TABLE 2 EFFECT OF FERROUS ION AND ASCORBIC ACID ON MALONALDEHYDE PRODUCTION BY ADIPOSE TISSUE INCUBATED IN KRB WITHOUT ALBUMIN

	Medium Malonaldehyde	Tissue FFA
	mμmoles/g	*μmoles/g*
KRB (22)	6.5 ± 0.77	26.1 ± 0.74
KRB + Fe^{++} (7)	10.6 ± 1.76	25.6 ± 0.97
KRB + ascorbic acid (7)	33.6 ± 5.51	26.0 ± 1.94
KRB + Fe^{++} + ascorbic acid (8)	59.6 ± 9.52	24.7 ± 1.30

* Mean ± SE. Number of observations in parentheses.

Incubation carried out as in Table 1. Additions of 0.22 μmole $FeSO_4$ and 1.2 μmoles ascorbic acid per ml of KRB were made as indicated.

RESULTS

When adipose tissue is preincubated with 10 μg of adrenaline per ml of medium for 30 min and then is further incubated for 3 hr in KRB containing 4% bovine serum albumin, malonaldehyde and FFA appear in the medium (Table 1A). When the albumin was dialyzed before use, its peroxidation-supporting activity was much reduced, despite a greater release of FFA. The dialysate (Table 1B) was shown to contain the factor(s) responsible for peroxidation. These data indicate that the albumin contained some factor or factors which promote lipid peroxidation.

In other tissue systems, ferrous ion and ascorbic acid or other reducing agents have been shown to be important factors in promoting peroxidation of unsaturated fatty acids through a cycle of coupled oxidations (4). Table 2 shows that when lipolysis by adipose tissue was stimulated by preincubation with adrenaline, and the tissue was then incubated in a KRB medium without albumin, the addition of 0.22 μmole of $FeSO_4$ per ml of medium had no significant influence on malonaldehyde production whereas 1.2 μmoles of ascorbic acid was highly effective. When both iron and ascorbic acid were added, amounts of malonaldehyde were produced that were comparable with those obtained when the medium was supplemented with albumin.

These data are consistent with the hypothesis that the albumin caused peroxidation because it contained a metal catalyst and a reducing agent. For this reason the influence of the chelating agent ethylenediamine tetraacetate (EDTA) was investigated. Barber and Ottolenghi (10) have shown that EDTA prevents lipid peroxidation under a variety of circumstances, presumably by chelating ferrous ions. Table 3 shows that the addition of 1 μmole of EDTA per ml of medium significantly reduced malonaldehyde production in the presence both of untreated albumin and of the dialysate of albumin. The effectiveness of EDTA is apparently due to

TABLE 3 INFLUENCE OF EDTA ON MALONALDEHYDE PRODUCTION IN PRESENCE OF 4% BSA AND DIALYSATE OF BSA AGAINST KRB

	Malonaldehyde*	FFA*
	mμmoles/g	*μmoles/g*
BSA (8)	41.3 ± 5.41	20.6 ± 0.40†
BSA + EDTA (8)	13.3 ± 2.33	19.7 ± 0.83†
Dialysate (8)	46.5 ± 4.44	26.4 ± 0.63‡
Dialysate + EDTA (9)	17.3 ± 1.76	30.2 ± 0.88‡

Experimental design as in Tables 1A and 1B except for addition of 1 μmole EDTA/ml.

* Mean ± SE. Number of observations in parentheses.

† Medium.

‡ Tissue.

removal of a metal from the albumin and the dialysate rather than from the adipose tissue, for preincubation of adipose tissue for 1 hr with EDTA in a bicarbonate medium, free of Ca^{++} and Mg^{++}, does not prevent peroxidation when the adipose tissue is subsequently stimulated with adrenaline and then transferred to a medium containing untreated albumin (Table 4). When albumin is treated with EDTA and then dialyzed against KRB, its peroxidizing activity is reduced even more than by dialysis alone. Again activity can be restored with iron and ascorbic acid (data not shown).

The above studies demonstrate that a metal is involved in the production of lipid peroxides in this system. A sample of albumin was analyzed by Dr. B. L. Vallee, Harvard University, for trace metal content. A number of metals were found, but the iron content of 25 μg/g of protein was most relevant to the present study. This provides 20 mμmoles of iron per ml of an incubation medium containing 4% albumin. A comparable value was found in this laboratory later, using another analytical method (8).

With iron established as a reasonable candidate for the metallic prooxidant which contaminates albumin, the object of further studies was to identify the other essential dialyzable factor, represented in the reconstructed system by ascorbic acid. As expected, analysis of albumin for ascorbic acid showed it to be free of this compound. In view of the fact that serum albumin is obtained from citrated blood, the possibility was considered that the albumin samples were contaminated by citrate. Samples of Armour and Sigma brands of bovine albumin and of Protein Foundation human albumin were analyzed and found to contain 0.94, 0.60, and 1.37 μmoles of citrate respectively per ml of 4% solution. When adipose tissue, preincubated with adrenaline, is incubated in a KRB buffer with 4% dialyzed albumin for 2 hr there appear in the medium 3.5 ± 0.29 mμmoles of malonaldehyde per g in the absence of citrate and 13.1 ± 1.03 mμmoles/g in the presence of 0.57 μmole of citrate per ml. Dialyzed albumin contains no detectable citrate, but each milliliter of a 4% solution still contains 2.4 mμmoles of iron. From these data it seems a reasonable conclusion that iron solubilized as an iron–citrate complex is the prooxidant which contaminates albumin. The following studies were then done to determine whether the amounts of iron and citrate in the albumin would in fact account for the prooxidant activity of albumin.

Thirty milliliters of a 10% solution of Armour's crystalline bovine serum albumin in 0.5 M $NaHCO_3$ were placed on a 66 × 2.5 cm column of Sephadex G25. Samples were collected as 5 ml aliquots at a flow rate of 6–8 drops/min, using a Gilson Medical Electronics model V15 volumetric fractionator. The column was eluted with 0.05 M $NaHCO_3$. Fractions were analyzed for iron and citrate, and for albumin by measuring absorbance at 280 mμ with a Zeiss PMQ spectrophotometer. The prooxidant activity of each fraction was assayed by diluting 2.5 ml of $NaHCO_3$ eluate with 2.5 ml of the appropriate concentration of electrolytes to reconstitute the KRB buffer, and incubating one entire fat pad in 5 ml of a reconstituted fraction and the contralateral one in 5 ml of KRB. Controls for the fractions containing albumin were adjusted to contain the same concentration of albumin by adding the appropriate amount of untreated albumin. The results are shown in Fig. 1.

The major fractions containing albumin (tubes 27–35) were free of iron and citrate and exhibited lower prooxidant activity than controls containing untreated albumin. The pooled mean malonaldehyde release in fractions 27–35 was 5.4 ± 0.66 mμmoles/g (17 observations) compared with 16.1 ± 1.20 mμmoles/g (23 observations) for the controls. Addition of 0.25 μmole of $FeSO_4$ per ml to tubes 27–35 did not increase their activity, whereas 1.0 μmole of citrate and 0.05 μmole of $FeSO_4$ restored activity to 22.9 ± 1.82 mμmoles of malonaldehyde per g (19 observations) compared with 23.3 ± 2.30 mμmoles/g (10 observations) for the albumin controls.

TABLE 4 EFFECT OF TREATMENT OF ADIPOSE TISSUE WITH EDTA ON SUBSEQUENT PEROXIDATION IN PRESENCE OF BSA

	Malonaldehyde*	Medium FFA*
	mμmoles/g	*μmoles/g*
Control	49.3 ± 3.33	17.4 ± 0.27
Adipose tissue preincubated with EDTA	50.9 ± 3.66	13.4 ± 0.19

* Mean ± SE (4 observations).

Pieces of adipose tissue (400 mg) were preincubated in either KRB or KRB with 1 μmole EDTA per ml for 1 hr and then washed with two 25-ml changes of KRB for 30 min each. KRB was made without Ca^{++} and Mg^{++}. Thereafter, subsequent exposure to adrenaline and incubations with BSA were as in Table 1.

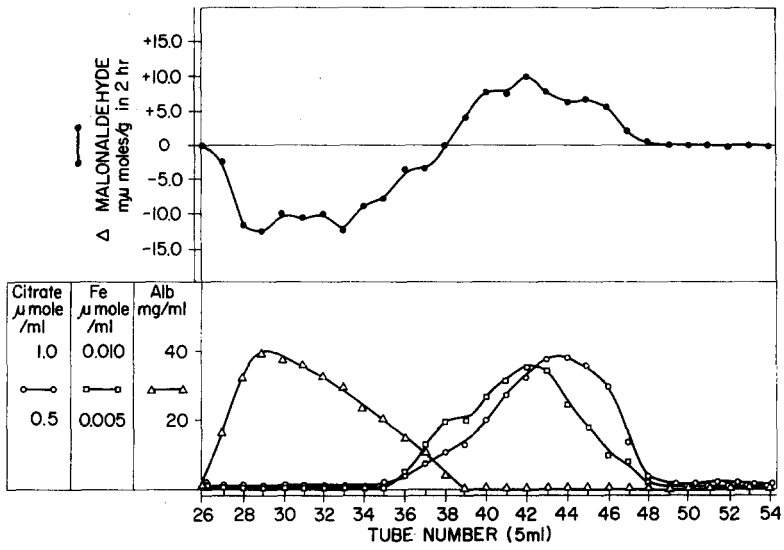

FIG. 1. Assay of lipid peroxidizing activity of fractions obtained by gel filtration of bovine serum albumin. Correlation of malonaldehyde production with albumin, iron, and citrate. Assay results are expressed as the difference between the amount of malonaldehyde produced in the fractions and in their corresponding controls. The concentrations of albumin, iron, and citrate in each table have been corrected to represent the actual amounts of these substances in 1 ml of the final incubation medium. Each point represents the mean of 3–5 determinations from five column fractionations.

Iron and citrate first appeared in tube 36 and extended through tube 47. Prooxidant activity in the protein-free fractions corresponded to their content of iron and citrate. The mean production of malonaldehyde in tubes 39–46 was 8.0 ± 0.38 mμmoles/g (36 observations) compared to 0.5 ± 0.05 mμmole (31 observations) by the KRB controls. Addition of 0.25 μmole of $FeSO_4$ did not alter the activity (7.9 ± 0.48 mμmoles/g, 24 observations), whereas 2.0 μmoles of ascorbic acid increased it to 17.4 ± 2.36 mμmoles/g (8 observations).

The above data indicate a good correlation between the iron and citrate content of albumin and its prooxidant activity, but do not prove that no other undetected factors in the albumin may be involved. The data in Table 5 were obtained to determine whether the amounts of iron and citrate found in fractions 37–47 would support peroxidation when adipose tissue is incubated in a KRB medium without protein. Assays were carried out over 2 hr, using paired fat pads in each instance. In the KRB medium peroxidation was very low. Addition of either $FeSO_4$ (0.25 μmole/ml) or citrate (1.0 μmole/ml) resulted in comparable small, but significant, increases in malonaldehyde production. In the presence of citrate the amount of iron necessary to promote peroxidation was very small. Indeed, 0.5 μmole of citrate and 0.005 μmole of $FeSO_4$ were as effective as 1.0 μmole of citrate and 0.25 μmole of iron. The combination of 0.25 μmole of citrate and 0.0025 μmole of $FeSO_4$ was significantly more effective than 0.25 μmole of $FeSO_4$ alone. It is apparent that in the presence of citrate the smallest amount of iron detected in the protein-free fractions can account for their prooxidant activity. Experiments 12–14 were added to compare the effects of albumin before and after dialysis. Under the conditions of this experiment malonaldehyde production in the presence of 4% albumin containing 0.9 μmole of citrate and 0.014 μmole of Fe was quite comparable to that recorded in the protein-free medium with Fe and citrate additions. After dialysis (experiments 13, 14) the citrate was completely removed and iron reduced to 0.0024 μmole/ml. Citrate restored activity.

DISCUSSION

These studies demonstrate clearly that peroxidation of polyunsaturated fatty acids, as reflected in malonaldehyde production, occurs in adipose tissue during incubation in vitro and that the reaction depends on the availability of iron in a soluble form. Elsewhere we have shown that the amount of chromogen produced in the presence of thiobarbituric acid during incubation of rat adipose tissue is depressed by supplementing the chow diet with α-tocopherol (1). It is also depressed by incubating the tissues anaerobically or by incorporating an antioxidant, quercitin, into the media (unpublished results). Furthermore, agents such as Mn^{++}, MnO_4^-, Co^{++},

$Cr_2O_7^=$ and EDTA, which are known to inhibit peroxidation in other systems (10, 11), reduce the amount of thiobarbituric acid-reacting chromogen in adipose tissue. From these results it would seem reasonable to conclude that the chromogen formed during in vitro incubation of adipose tissue is a decomposition product of lipid peroxides, most probably malonaldehyde.

The role of crystalline serum albumin in promoting lipid peroxidation in adipose tissue was apparent from experiments with and without albumin. The complementary experiment in which adipose tissue was incubated with the albumin dialysate clearly demonstrated that dialyzable factors on the albumin were responsible for promoting lipid peroxidation. In this experiment, FFA accumulated in the tissues but did not enter the media, suggesting that peroxidation involves FFA within the tissues. Data have been presented elsewhere to support this conclusion (12).

Iron was suspected as one of the dialyzable factors responsible for promoting malonaldehyde production since previous studies have demonstrated the importance of iron in lipid peroxidation (3, 13, 14). Despite the fact that serum albumin contained iron, its role could not be corroborated until citrate was identified as the other factor. In the presence of citrate, Fe^{++} in a concentration as little as 10^{-6} M promotes peroxidation by adipose tissue incubated in a KRB medium at pH 7.4, whereas 100 times as much Fe^{++} is without effect in the absence of citrate. This finding explains why citrate promotes peroxidation when added to dialyzed albumin still containing traces of iron, but does not do so when added to albumin that has been treated with EDTA before dialysis.

Confirmation of the importance of the citrate that contaminates serum albumin has recently come from observations with another brand of albumin (Pentex), which is not contaminated with citrate but does contain relatively large amounts of iron (0.041 μmole/ml of a 4% solution, compared to 0.012 μmole in Armour and 0.014 μmole in Sigma brand albumins). Incubation of adipose tissue with Pentex albumin does not stimulate malonaldehyde production, but if 1 μmole of citrate is added per ml the release of malonaldehyde during a 2 hr incubation is equal to that with Armour albumin (Pentex albumin, 2.4 ± 0.41 mμmoles/g; Pentex albumin + citrate, 15.9 ± 1.73 mμmoles/g; Armour albumin 16.1 ± 1.20 mμmoles/g). Significantly, passing the Pentex albumin in 0.05 M $NaHCO_3$ through a Sephadex G25 column removes only a small portion of the iron, which remains on the column until eluted with citrate.

The exact mode of action of citrate is not known but it does solubilize iron and perhaps makes it more available at the site of peroxidation. Previously, citrate has been shown to have an inhibitory effect on peroxidation and has even been used commercially to improve the stability of fats (15). This effect is contrary to that shown in our system, but the complexity of the peroxidation reaction undoubtedly permits varying results under different experimental conditions. It is relevant that Hochstein et al. (16) have recently reported a biphasic action of inorganic pyrophosphate on lipid peroxidation.

While the biological significance of the peroxidation reaction during incubation of adipose tissue still remains to be elucidated, the above data emphasize the importance of soluble iron in the incubation medium in catalyzing the reaction. The finding of significant amounts of iron and citrate in commercial albumin samples gives substance to the custom in many laboratories of dialyzing albumin before using it in metabolic experiments. Fortney and Lynn (17) and Hunter et al. (18) have also recently called attention to the artifacts resulting from iron contamination in studies on mitochondrial swelling. Finally, it should be emphasized again (1) that the peroxidation reaction in vitro is largely a reflection of the tocopherol inadequacy of chow diets in the rat, a point made previously by other investigators (19). Since the peroxidation of in vitro systems can be prevented or greatly reduced by supplementing the chow diet with tocopherol, it must be concluded that tocopherol levels which apparently are adequate for the intact animal are quite inadequate to protect isolated tissue, cellular or subcellular systems from traces of iron and other prooxidants. The implica-

TABLE 5 INFLUENCE OF IRON AND CITRATE ON MALONALDEHYDE PRODUCTION BY ADIPOSE TISSUE

Expt. No.	No. of Observations	Addition to Medium Citrate	Addition to Medium Fe^{++}	Malonaldehyde
		μmoles	*μmoles*	*mμmoles/g*
1	31	0	0	0.5 ± 0.05
2	36	0	0.25	3.9 ± 0.29
3	34	1.0	0	3.0 ± 0.34
4	10	1.0	0.25	13.1 ± 1.13
5	6	1.0	0.125	13.7 ± 1.50
6	6	1.0	0.050	13.6 ± 2.20
7	6	1.0	0.025	13.1 ± 0.64
8	6	1.0	0.0125	10.7 ± 0.84
9	6	1.0	0.005	10.9 ± 0.91
10	6	0.5	0.005	10.1 ± 1.04
11	6	0.25	0.0025	6.2 ± 0.46
12*	23	0.9	0.014	16.1 ± 1.20
13†	17	0	0.0024	3.5 ± 0.29
14†	17	0.6	0.0024	13.1 ± 1.03

Whole epididymal fat pads, ranging in weight from 500 to 900 mg, were preincubated in 5 ml of KRB with 10 μg adrenaline per ml for 30 min and then transferred to 5 ml of experimental KRB medium for 2 hr of incubation at 37° in a Dubnoff shaker in an atmosphere of 95% O_2–5% CO_2.

* 4% BSA in KRB; citrate and Fe content by analysis.

† 4% dialyzed BSA in KRB; Fe content by analysis, and citrate added as dictated.

tions of this for in vitro studies with tissues from rats fed chow diets are obvious.

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