Long-Chain Polyunsaturated Fatty Acids and Chemically Induced Diabetes Mellitus: Effect of ω-3 Fatty Acids

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In a previous study, we showed that prior oral feeding of oils rich in ω-3 eicosapentaenoic acid and docosahexaenoic acid and ω-6 γ-linolenic acid and arachidonic acid prevent the development of alloxan-induced diabetes mellitus in experimental animals. We also observed that 99% pure ω-6 fatty acids γ-linolenic acid and arachidonic acid protect against chemically induced diabetes mellitus. Here we report the results of our studies with ω-3 fatty acids. Alloxan-induced in vitro cytotoxicity and apoptosis in an insulin-secreting rat insulinoma cell line, RIN, was prevented by prior exposure of these cells to α-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid. Prior oral supplementation with α-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid prevented alloxan-induced diabetes mellitus. α-Linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid not only attenuated chemical-induced diabetes mellitus but also restored the anti-oxidant status to normal range in various tissues. These results suggested that ω-3 fatty acids can abrogate chemically induced diabetes in experimental animals and attenuate the oxidant stress that occurs in diabetes mellitus. *Nutrition* 2003; 19:213–228. ©Elsevier Science Inc. 2003

KEY WORDS: diabetes mellitus, alloxan, ω-3 fatty acids, eicosapentaenoic acid, antioxidants, glucose, nitric oxide, free radicals

INTRODUCTION

Tumor necrosis factor-α (TNF-α) and interleukin (IL)–1 produced by infiltrating macrophages, lymphocytes, and monocytes damage pancreatic β-cells[1,2] and produce type 1 diabetes mellitus (DM). Prostaglandin E_2, derived from arachidonic acid (AA; 20:4 ω-6), suppresses TNF-α and IL-1 production and is an immunosuppressor.[3] This suggests that inhibition of TNF α and IL-1 and enhancement of the production of prostaglandin E_2 may limit the process of insulitis, and this may inhibit the development of type 1 DM in experimental animals. This is supported by the observation that peroxisome proliferator-activated receptor-γ (PPAR-γ) activators conjugated linoleic acid (CLA) and troglitazone[4] prevented the development of DM in the diabetic Zucker fatty fa/fa rat[5,6] and suppressed the production of free radicals and TNF-α and IL-2.[7,8] However, there is some controversy about the action of CLA on IL-2 secretion.[9] We previously showed that oral supplementation of oils rich in polyunsaturated fatty acids (PUFAs) and PUFAs prevent the development of alloxan-induced DM in experimental animals.[10,11] These results are interesting because PUFAs serve as endogenous ligands of PPARs.[4] Oils rich in PUFAs contain a mixture of fatty acids. Hence, it is not clear which of the specific fatty acids that are present in these oils are responsible for the prevention of chemically induced DM. Therefore, we studied the effect of individual PUFAs on alloxan-induced cytotoxicity to insulin-producing cells in vitro and their ability to prevent alloxan-induced DM in experimental animals. We report the results of our studies with various ω-3 fatty acids: α-linolenic acid (ALA; 18:3 ω-3), eicosapentaenoic acid (EPA; 20:5 ω3), and docosahexaenoic acid (DHA; 22:6 ω-3).

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MATERIALS AND METHODS

In Vitro Studies

An insulin-secreting rat insulinoma cell line, RIN, was used in the present study.[10] These cells grow as a monolayer after attaching to the substratum and were cultured in minimum essential medium buffered to pH 7.2 and supplemented with 10% fetal calf serum, 50 U/mL of penicillin, and 50 μg/mL of gentamycin in a CO_2 incubator.[10] The cells were subcultured at regular intervals of 3 to 4 d when they became confluent. For the in vitro studies, the cells were harvested from the confluent cultures by treating them for 1 min with 0.125% trypsin and 1 mM of ethylene-diamine-tetraacetic acid, followed by inactivation of trypsin by serum. The harvested cells were transferred to 24-well culture plates and incubated in fresh medium for 18 h. At the end of this incubation period, the cells were used for various studies, as described below.

ALLOXAN AND VIABILITY OF RIN CELLS. For this study, RIN cells were seeded at a density of 1×10^5 cells/well in 0.5 mL of medium in 24-well tissue culture plates. Alloxan, dissolved in 50 mM of citrate buffer, pH 3.0, was added to the cells and incubated for 1 h to study its toxicity. At the end of this 1-h incubation, the medium was removed, fresh medium was added, and cells were incubated for an additional 18 h.[10] After this incubation period, cell viability was determined by the 3-(4,5-dimethylthiazil-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as described previously.[10,12,13]

EFFECT OF ω-6 AND ω-3 PUFAS ON ALLOXAN-INDUCED CYTOTOXICITY TO RIN CELLS. This study was done with 1×10^5 cells/well in 24-well culture plates. Three type of studies were performed to investigate the effect of fatty acids on alloxan-induced cytotoxicity,[10] as described below.

In the pretreatment protocol, the cells were first incubated with

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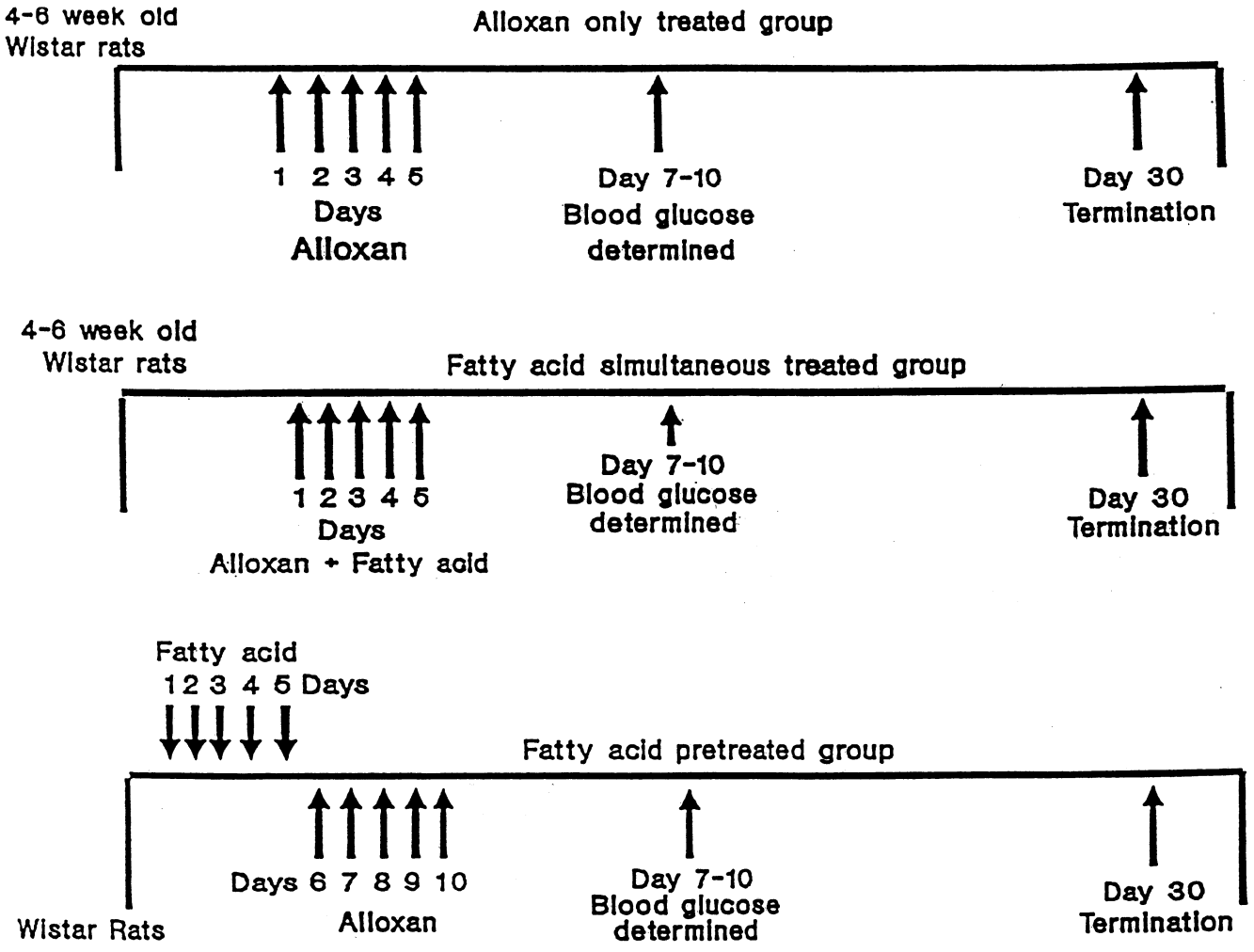

FIG. 1. Scheme showing the experimental protocols used to induce diabetes with alloxan and various treatments with fatty acids.

any one of the fatty acids for 5 h. At the end of the 5-h incubation, excess fatty acid was removed and replaced with fresh medium. To these cells, different amounts of alloxan were added and incubated for an additional 1 h. After this incubation, the medium was removed, replaced with fresh medium, and incubated for an additional 18 h. At the end of the 18-h incubation (total period of various incubations was 24 h), cell viability was determined by MTT assay.

In the simultaneous treatment protocol, the experiment was performed similar to that for pretreatment, except that the fatty acids and alloxan were added simultaneously and incubated for 1 h (before this addition, the cells were grown for 5 h in normal minimum essential medium). At the end of this period, the medium was removed, and fresh medium was added and incubated for 18 h. At the end of 18 h, cell viability was determined by MTT assay.

In the posttreatment schedule, the RIN cells were treated first with alloxan for 1 h, after which the medium was removed and replaced with fresh medium, and then the fatty acids were added to the cells and incubated for 18 h. At the end of the 18-h incubation, the cells were washed and incubated in fresh medium for an additional 5 h. Cell viability was assessed by MTT assay. In all these studies, appropriate controls were used.

Preliminary studies showed that optimum cytotoxicity of alloxan on RIN cells was seen after 1 h of incubation. Hence, in all subsequent studies, the cells were exposed to alloxan for 1 h. Previous studies showed that fatty acids are ingested by the cells within 30 min,[14] and maximum incorporation of fatty acids is seen at the end of 4 h.[15] In view of this, RIN cells were exposed to fatty acids for 5 h.

EFFECT OF ω-3 FATTY ACIDS ON ALLOXAN-INDUCED APOPTOSIS. Agarose gel electrophoresis was performed to determine whether ω-3 PUFAs alter alloxan-induced apoptosis of RIN cells in vitro. Cells, 1×10^6, were seeded in a six-well tissue culture plate. The cells were pretreated with various fatty acids (15 μg/mL) for 5 h. At the end of this incubation, the medium was replaced with fresh medium, and then the cells were treated with alloxan (6 mM) for 4 h. After the incubation, the cells were harvested and washed twice with ice-cold phosphate-buffered saline (pH 7.4). DNA from these cells was extracted by a phenol-chloroform method.[16] The amount of DNA in the sample was determined by checking the absorbance at 260 nm. An optical density of one was considered equivalent to 50 μg/mL of DNA. DNA samples were loaded at a concentration of 100 to 150 ng into the wells of the agarose gel. Electrophoresis was performed at a voltage of 50 V for 3 to 4 h. After the electrophoretic run, the gel was stained with 0.5 μg/mL of ethidium bromide and examined under an ultraviolet illuminator, and photographs were taken. This experiment was done with the fatty acids EPA and DHA.

In Vivo Studies

INDUCTION OF DM. Male Wistar rats, 4 to 5 wk old, weighing approximately 200 g, were used for this study. The animals

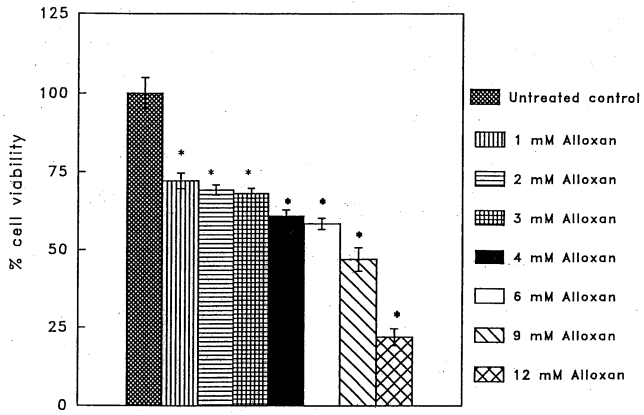

FIG. 2. Effect of alloxan on the viability of RIN cells. All values are expressed as mean ± standard error. *$P \leq 0.05$ versus control group.

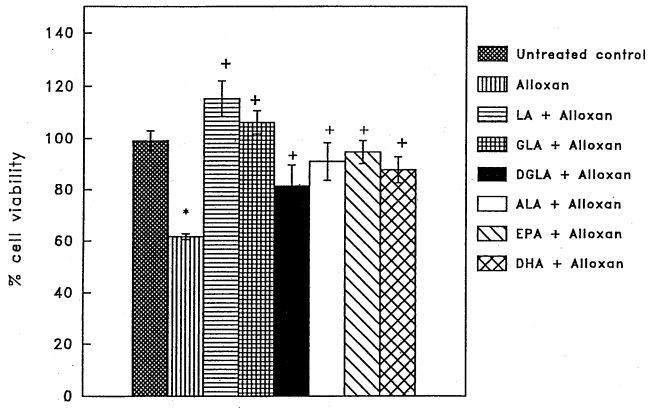

FIG. 4. Effect of pretreatment with different fatty acids (15 μg/mL) on alloxan (6 mM)–induced toxicity to RIN cells. All values are expressed as mean ± standard error. *$P \leq 0.05$ versus control group. +$P \leq 0.05$ versus alloxan-treated group. ALA, α-linolenic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; LA, linoleic acid.

were housed in a room maintained at 25°C with a 12-h dark and 12-h light cycle. The animals were given standard rat chow and water ad libitum. This study was approved by the ethics committee of the institute.

Alloxan was used to induce type 1 DM.[10,11,17] The animals were fasted 12 h before the administration of alloxan. Freshly prepared alloxan, 75 mg/kg of body weight, in 50 mM of citrate buffer, pH 3.0, was injected intraperitoneally everyday for 5 consecutive days. Blood and urine sugar values were estimated once every 3 to 4 d, after the last dose of alloxan, by glucometer to confirm the development of DM. These animals developed type 1 DM in approximately 2 to 3 wk after the first injection of alloxan. Only those animals showing substantial weight loss due to uncontrolled DM, severe hyperglycemia (fasting blood sugar > 275 mg/dL), and a clear decline in the plasma insulin levels were considered to have developed type 1 DM. Control animals received only citrate buffer.

TREATMENT WITH ω-3 PUFAS. All fine chemicals including the fatty acids were obtained from Sigma Chemical Company(St. Louis, MO, USA). The fatty acids used were at least 99% pure.

TREATMENT WITH FATTY ACIDS. To study the effect of the fatty acids on the incidence of DM in alloxan-treated animals, two types of treatments were performed: 1) pretreatment with fatty acids before the administration of alloxan and 2) simultaneous treatment with fatty acids and alloxan. For brevity, these groups are referred to as the pretreatment group and the simultaneous treatment group.

In the pretreatment group, the experimental animals received orally 100 μg of the fatty acid in 100 μL of 0.01% ethanol (which is about 500 μg/kg of body weight) for 5 consecutive days followed by intraperitoneal administration of alloxan (75 mg/kg) from day 6 for the next 5 consecutive days.

In the simultaneous treatment group, the animals received 100 μg of the fatty acid in 100 μL of 0.01% ethanol orally, and alloxan was given by intraperitoneal injections for 5 consecutive days. In all these studies, appropriate controls also were used. Additional details of the experimental protocols used in the present study are presented in Fig. 1. Each group consisted of at least 12 animals. Animals pretreated with the fatty acids formed the positive control group (previous studies showed no significant differences between the pretreatment and simultaneous fatty acid treated control animals). None of the animals received additional antioxidants.

COLLECTION OF BLOOD AND VARIOUS INTERNAL ORGANS. The animals were killed 30 d after the first dose of alloxan (Fig. 1). The weights of the animals were noted on the day of termination of the study.

Blood samples collected from the orbital sinus of the control and treated rats, as described previously,[10,11] were used for the

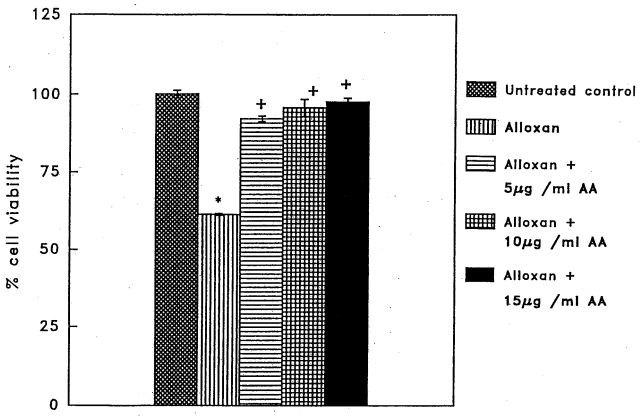

FIG. 3. Effect of pretreatment with AA on alloxan (6 mM)–induced toxicity to RIN cells. All values are expressed as mean ± standard error. *$P \leq 0.05$ versus control group. +$P \leq 0.05$ versus alloxan-treated group. AA, arachidonic acid.

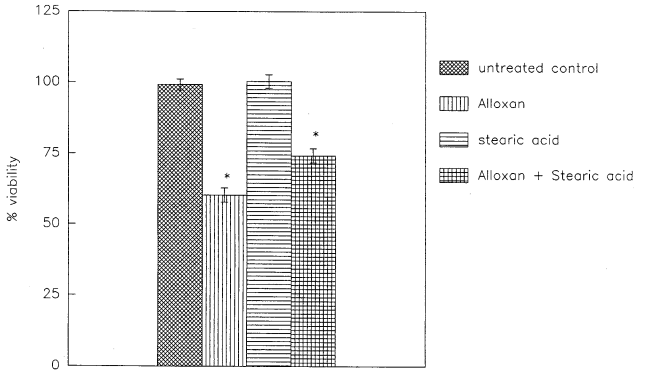

FIG. 5. Effect of stearic acid on alloxan-induced cytotoxicity in RIN cells. All values are expressed as mean ± standard error. *$P \leq 0.05$ versus control group.

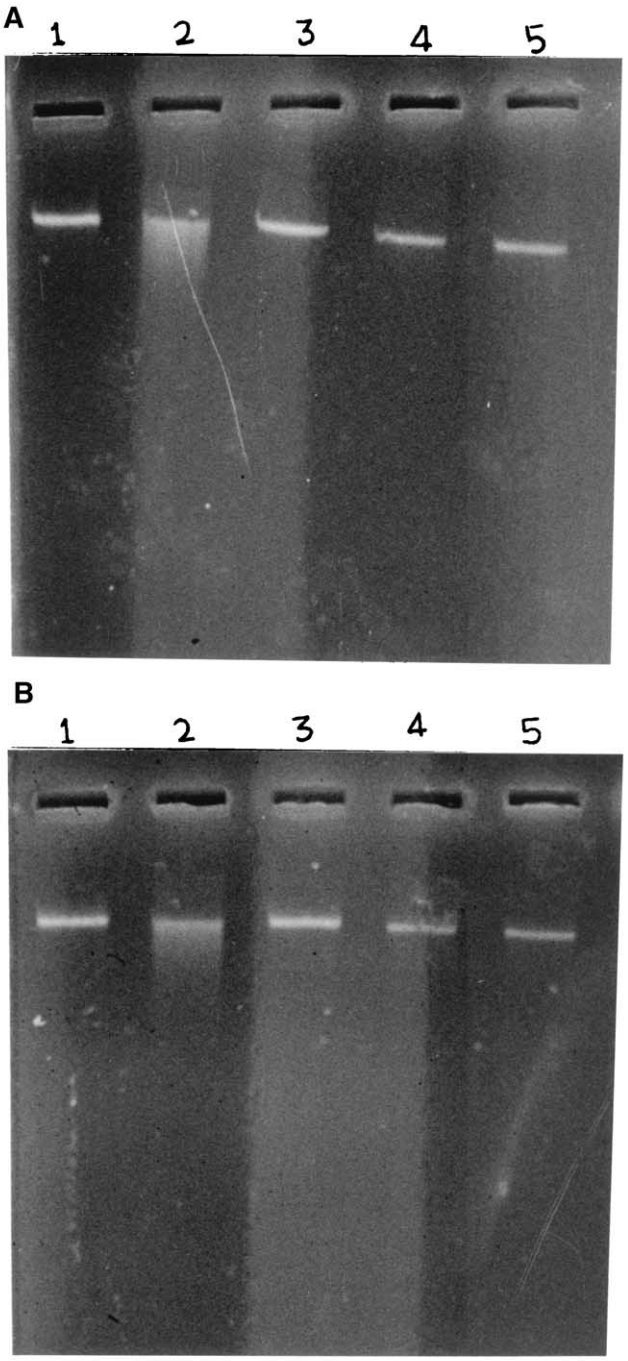

FIG. 6. Agarose gel electrophoresis of DNA isolated from RIN cells pretreated with polyunsaturated fatty acids and alloxan. (A) Treated with eicosapentaenoic acid. (B) Treated with docosahexaenoic acid. Lane 1: untreated control; lane 2: alloxan; lane 3: citrate; lane 4: fatty acid; lane 5: alloxan plus fatty acid.

estimation of glucose, lactate, insulin, nitric oxide (NO), lipid peroxides, urate, ceruloplasmin, and fatty acids. Red blood cells (RBCs) obtained from these samples were washed twice with phosphate-buffered saline, lysed, and used for the estimation of various antioxidants.

Liver, pancreas, and kidney of control and treated animals were dissected out, perfused with normal sterile saline, and analyzed for various biochemical parameters. Identical portions of the pancreas from all animals were fixed in formalin for histopathologic examination.

ESTIMATION OF PLASMA GLUCOSE, INSULIN, AND LACTATE LEVELS. Plasma glucose levels were estimated by the glucose oxidase-peroxidase method with a commercially available kit (Boehringer-Mannheim, Mannheim, Germany). Plasma insulin levels were estimated by enzyme-linked immunosorbent assay with a commercially available kit (Enzymun, Boehringer-Mannheim) on an ESS 33 analyzer. Plasma levels of lactate were measured with a commercially available kit from Boehringer-Mannheim.

ESTIMATION OF PLASMA NO AS ITS STABLE METABOLITE NITRITE. NO decomposes rapidly in aerated solutions to form stable nitrite/nitrate products. In the present study, nitrite concentration was determined in plasma and used as an index of NO synthesis. Nitrite was estimated colorimetrically with the Griess reagent, as described previously.[17,18] The concentration of nitrite levels in plasma was determined with the use of a standard curve that was constructed with sodium nitrite as the standard.

ESTIMATION OF LIPID PEROXIDATION PRODUCTS. Malondialdehyde (MDA), the product of lipid peroxidation, in the biological samples reacts with thiobarbituric acid under acidic conditions at 95°C to form a pink complex with an absorbance maximum at 532 nm. The total amount of lipid peroxidation products in the plasma of the control and experimental animals was estimated with the thiobarbituric acid method, as described previously.[19,20] 1,1,3,3-Tetraethoxy-propane was used as the standard.

The concentrations of lipid peroxides and NO also were estimated in the liver and kidney samples of control and treated animals.

ESTIMATION OF ANTIOXIDANTS: PLASMA CERULOPLASMIN. Plasma levels of ceruloplasmin were measured with an enzymatic method that measures the oxidase activity of ceruloplasmin in the presence of *p*-phenylene diamine dihydrochloride.[21] Ceruloplasmin oxidizes *p*-phenylene diamine dihydrochloride in the presence of oxygen to form a purple product that can be measured at 530 nm.

PREPARATION OF RBC LYSATE. Two milliliters of blood collected was centrifuged, plasma and RBCs were separated as described previously,[10,11] and RBCs so obtained were lysed by the addition of distilled water for 2 h at 4°C (volume of lysate = 2 mL). Hemoglobin was removed by washing with chloroform: methanol (3:5, v/v), as described previously.[10,11] Hemoglobin-free lysate was used to estimate the activities of different antioxidants.

ESTIMATION OF HEMOGLOBIN. Hemoglobin was estimated by the cyanomethemoglobin method of Drabkin and Austin.[22] To 20 μL of RBC lysate, we added 5 mL of Drabkin's cyanide ferricyanide solution, which contains potassium ferricyanide. The contents were mixed well and allowed to stand at room temperature, and the hemoglobin–cyanomethemoglobin complex thus formed was compared with standard and reagent blanks. The absorbance was read at 540 nm.

ESTIMATION OF SUPEROXIDE DISMUTASE, CATALASE, GLUTATHIONE PEROXIDASE, AND GLUTATHIONE-S-TRANSFERASE IN RBCS. The activity of superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione-S-transferase in the RBC membranes were measured as described previously.[10,11,23,24] The activities of antioxidant enzymes catalase, SOD, glutathione peroxidase, and glutathione-S-transferase

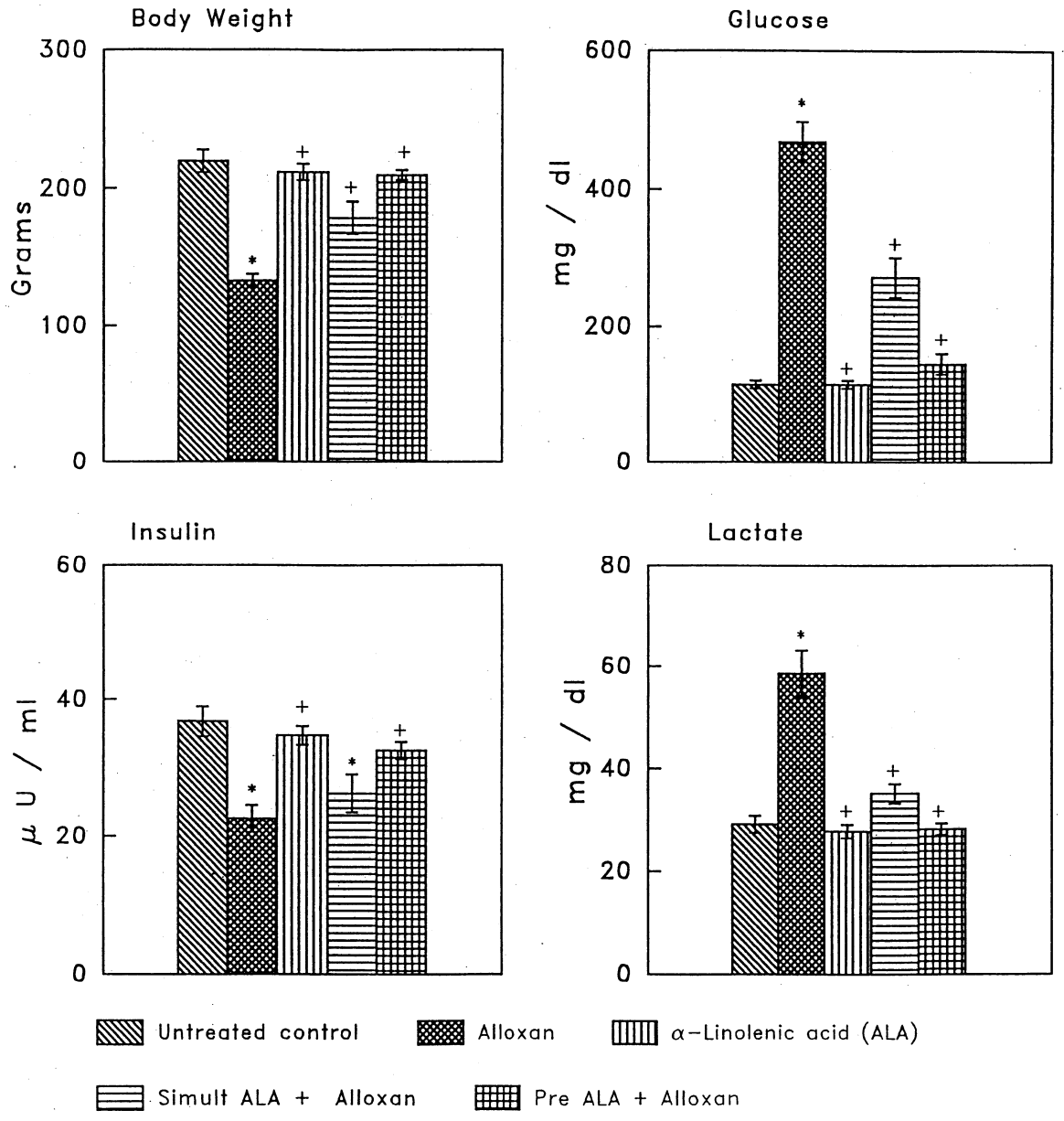

FIG. 7. Effect of alloxan and ALA on body weight, plasma glucose, insulin, and lactate in the plasma of experimental animals. All values are expressed as mean ± standard error ($n = 10$). *$P \leq 0.05$ versus control group. +$P \leq 0.05$ versus alloxan-treated group. ALA, α-linolenic acid.

also were estimated in the liver and kidney samples of the control and treated animals.

Statistical Analysis

All values obtained are expressed as mean ± standard error. The data were analyzed with one-way analysis of variance followed by Dunnett's *t* (two-sided) test and Bonferroni's test.

RESULTS

In Vitro Studies

ALLOXAN AND THE VIABILITY OF RIN CELLS. The toxicity of alloxan to RIN cells was assessed by studying the effect of various concentrations of alloxan (1 to 12 mM) on these cells. The results of this study are shown in Fig. 2. Alloxan at a concentration of 6 mM was able to kill about 42% of the cells compared with the control. All further studies were done with this concentration of alloxan.

EFFECT OF ω-3 FATTY ACIDS ON ALLOXAN-INDUCED CYTOTOXICITY TO RIN CELLS. To evaluate the effect of various PUFAs on alloxan-induced cytotoxicity to RIN cells, the cells were pretreated with individual fatty acids and then exposed to alloxan. These studies were performed initially with AA. Results of this study showed that pretreatment with AA protects RIN cells from alloxan-induced cytotoxicity at all three concentrations (5, 10, and 15 μg/mL) tested (Fig. 3). At these concentrations, AA was not toxic to RIN cells (data not shown). Hence, all fatty acids were used at a concentration of 15 μg/mL in all future studies, and RIN cells were pretreated with fatty acids before exposure to alloxan. It is evident from the results shown in Fig. 4 that ALA, EPA, and DHA also prevented alloxan-induced cytotoxicity to RIN cells, although they were less effective than linoleic acid (18:2 ω-6), γ-linolenic acid (18:3 ω-6), and AA. Stearic acid (18:0), a

TABLE I.

INCIDENCE OF DIABETES MELLITUS IN WISTAR RATS TREATED WITH ALLOXAN AND DIFFERENT POLYUNSATURATED FATTY ACIDS

Treatment	Alloxan only (n = 10)	Alloxan + simultaneous fatty acid treatment (n = 10)	Alloxan + pretreatment with fatty acid (n = 10)
SA*	10	—	10
OA†	10	—	10
LA‡	9	4	3
GLA‡	9	5	0
DGLA‡	10	5	2
AA‡	10	1	0
ALA§	9	7	1
EPA§	10	7	0
DHA§	10	3	0

* Saturated fatty acid.
† Monounsaturated fatty acid.
‡ ω-6 fatty acids.
§ ω-3 fatty acids.
AA, arachidonic acid; ALA; α-linolenic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; LA, linoleic acid; OA, oleic acid; SA, stearic acid

saturated fatty acid, was ineffective against alloxan-induced cytotoxicity to RIN cells (Fig. 5). We previously observed that the cytoprotective action shown by AA is not due to the formation of cyclooxygenase (COX) and/or lipoxygenase (LO) products of AA (reference), suggesting that AA by itself is effective. Hence, we did not perform further studies with COX and LO inhibitors and ω-3 fatty acids.

EFFECT OF EPA AND DHA ON ALLOXAN-INDUCED APOPTOSIS OF RIN CELLS. Alloxan induced apoptosis of RIN cells (Fig. 6). EPA and DHA prevented apoptosis of RIN cells induced by alloxan (Fig. 6), suggesting that these fatty acids can prevent alloxan-induced DNA fragmentation and apoptosis.

In Vivo Studies

EFFECT OF ω-3 FATTY ACIDS ON ALLOXAN-INDUCED DM. Incidence of DM. We next studied whether ω-3 fatty acids can prevent alloxan-induced DM. Almost all animals that received alloxan developed type 1 (insulin-dependent) DM approximately 17 d after alloxan treatment as demonstrated by high blood glucose (~350 to 400 mg/dL) and low plasma insulin levels compared with control levels[10,11,17] (Fig. 7). It is evident from the results shown in Table I that the sensitivity of animals to develop alloxan-induced DM depended on the type of fatty acid used and the time of treatment. In general, pretreatment with fatty acids showed better protective action against alloxan-induced DM than did simultaneous treatment. Simultaneous and pretreatment with ALA resulted in 70% and 10% incidence of DM, respectively. About 70% and 30% of the animals, which received simultaneously EPA and DHA, respectively, with alloxan developed DM. Pretreatment with EPA and DHA protected all animals from alloxan-induced DM, whereas pretreatment with ALA prevented 90% of the animals from developing DM. Of all the fatty acids tested, AA produced the best results. DHA was the next best fatty acid in its ability to prevent alloxan-induced DM (Table I). These results suggested that pretreatment with fatty acids is more effective in protecting animals from alloxan-induced DM. In view of this, all further studies were performed only with the pretreatment schedule.

Body weight. To determine the effect of alloxan-induced DM, the body weights of all animals were noted before the termination of the study. Significant decreases in body weight were noted in animals that developed alloxan-induced DM (Figs. 7 to 9). Animals that received ALA, EPA, and DHA with alloxan (pretreatment and simultaneous treatment) showed significantly less loss of body weight than did those that received alloxan only.

Plasma glucose. Significant increases in plasma glucose levels were noted in alloxan-treated animals (Figs. 7 to 9). In the alloxan-treated animals, a mean plasma glucose level greater than 400 mg% was observed. In pretreatment and simultaneous treatment (ALA, EPA, and DHA) groups also treated with alloxan, a significant decrease in plasma glucose levels compared with alloxan-alone group was noted (Figs. 7 to 9). In the pretreated ALA, EPA, and DHA groups, plasma glucose levels were normal as these animals did not develop DM after alloxan treatment (Figs. 7 to 9). Even in the simultaneous treated groups (where fatty acid was given orally and alloxan was administered intraperitoneally at the same time), even though they developed DM, its severity was much less (Figs. 7 to 9).

Plasma insulin levels. A significant decrease in the levels of insulin was observed in the alloxan-only group (positive control group) versus the control group suggesting that significant damage to the pancreatic β-cells occurred as expected. In the fatty acid supplemented groups, plasma insulin levels were restored to near normal. The extent of restoration of insulin levels to nearly normal levels depended on the ability of individual fatty acid to protect the animals from alloxan-induced DM. Thus, animals that received simultaneous ALA, EPA, and DHA with alloxan, wherein the fatty acids were not able to completely prevent the development of DM, plasma insulin were not restored to completely normal levels (Figs. 7 and 8), even though they tended to be slightly higher than those in the alloxan-alone animals. In contrast, in the pretreated animals (pretreatment with fatty acid followed by alloxan), plasma insulin levels were much higher than those in the simultaneous group. In the DHA-pretreated animals, plasma insulin levels were normal as these animals did not develop alloxan-induced DM (Fig. 9).

Plasma lactate. Plasma lactate levels were estimated in all groups as a biochemical marker of anaerobic metabolism that occurs in DM. Animals treated with alloxan showed a significant increase in plasma lactate levels compared with controls (Figs. 7 to 9). In the simultaneous groups treated with alloxan, plasma levels were elevated compared with those of the control group; however, in the groups pretreated with fatty acids, levels were restored to normal.

Plasma lipid peroxides and NO. Because uncontrolled DM is associated with increased free radical generation and lipid peroxidation, we measured plasma concentrations of lipid peroxides and NO in the plasma, liver, and kidney. A significant increase in plasma levels of lipid peroxides and a decrease in the concentrations of NO in the alloxan-treated group was noted in comparison with normal controls (Figs. 10 to 12). In the groups pretreated with EPA and DHA, the levels of lipid peroxides and NO were restored to normal. In the groups simultaneously treated with EPA and DHA, the induction of DM by alloxan was not completely prevented; hence, the levels of lipid peroxides were elevated and those of NO were lower. In the group treated simultaneously with ALA,

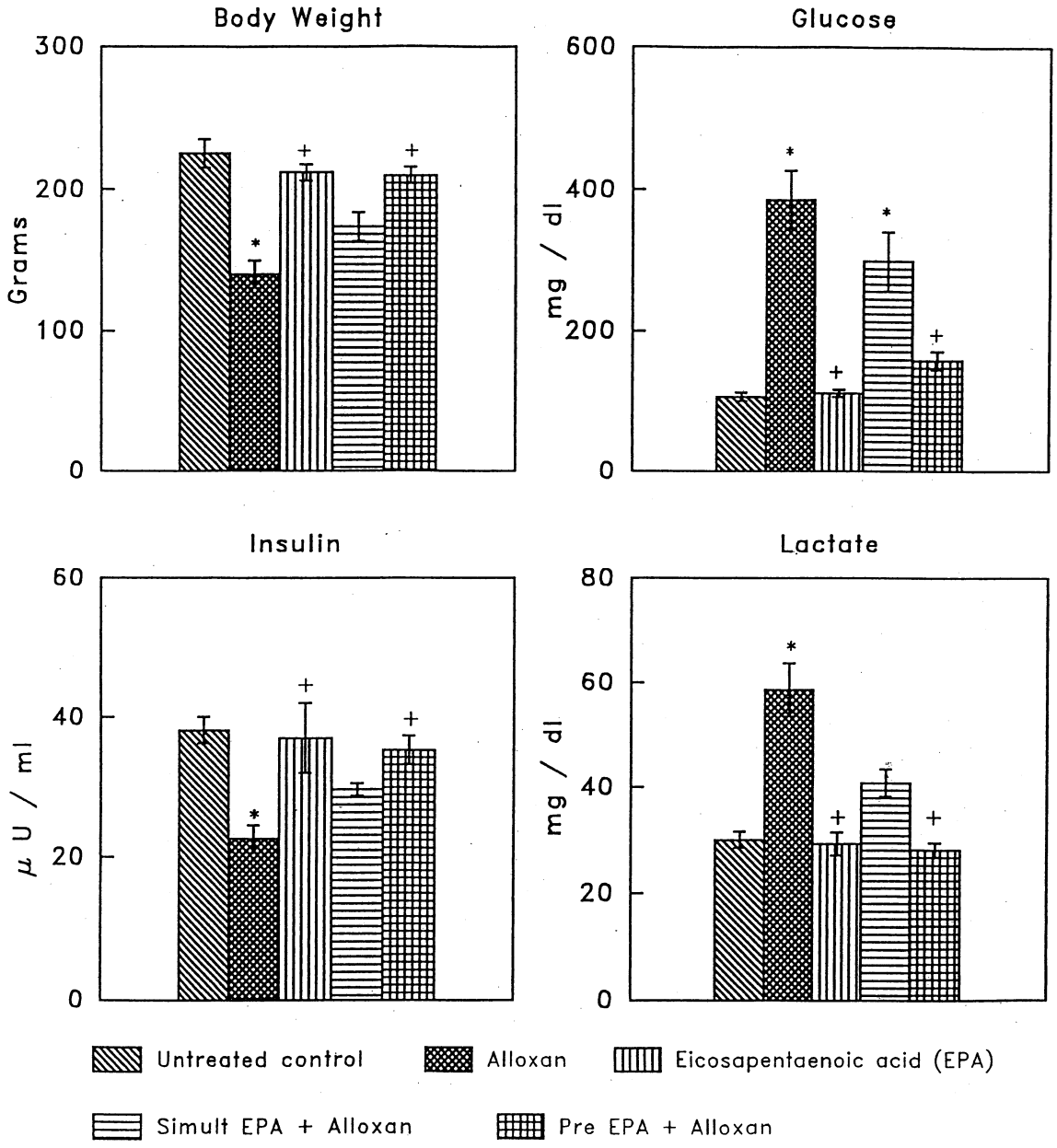

FIG. 8. Effect of alloxan and EPA on body weight, plasma glucose, insulin, and lactate in the plasma of experimental animals. All values are expressed as mean ± standard error ($n = 10$). *$P \leq 0.05$ versus control group. +$P \leq 0.05$ versus alloxan-treated group. EPA, eicosapentaenoic acid.

there was no significant change in the levels of lipid peroxides, even though NO concentrations were significantly lower compared with alloxan control group (Fig. 10). In the ALA-pretreated group, even though the levels of lipid peroxides were significantly lower, the concentrations of NO were similar to those seen in the simultaneously treated group.

To elicit the balance between pro-oxidants (lipid peroxides) and NO, the ratio between lipid peroxides and NO was measured in various tissues including plasma (Figs. 10 to 12). These results showed that the NO:MDA ratio was significantly lower in the plasma of alloxan-induced DM rats. This suggests a shift in the balance toward a pro-oxidant status. In the simultaneously treated ALA and EPA groups (and with alloxan), in which alloxan-induced DM was not completely prevented, the plasma NO:MDA ratio was lower. In all other groups of animals, plasma the NO:MDA ratio was maintained at normal levels (Figs. 10 to 12).

In the present study, the concentrations of lipid peroxides and NO (measured as its stable metabolite nitrite) also were measured in liver and kidney tissues. These results were similar to those seen in plasma; concentrations of lipid peroxides were increased in the liver and kidney homogenates of alloxan-treated animals compared with controls (data not shown). In the ALA plus alloxan and EPA plus alloxan simultaneously treated groups, the NO:MDA ratio in liver and kidney tissues was significantly lower, whereas in the DHA plus alloxan simultaneously treated group, it was restored to normal in liver tissue but not in kidney tissue. Conversely, in the ALA plus alloxan, EPA plus alloxan, and DHA plus alloxan pretreated groups, the NO:MDA ratio was restored to normal in liver tissue but not in kidney tissue (data not shown). This suggests that the pro- and antioxidant systems behave differently in different tissues in response to various ω-3 fatty acids.

ANTIOXIDANT STATUS. Plasma ceruloplasmin levels. The pathobiology of DM is linked largely to the balance between oxidant and antioxidant status. Hence, activities of various antiox-

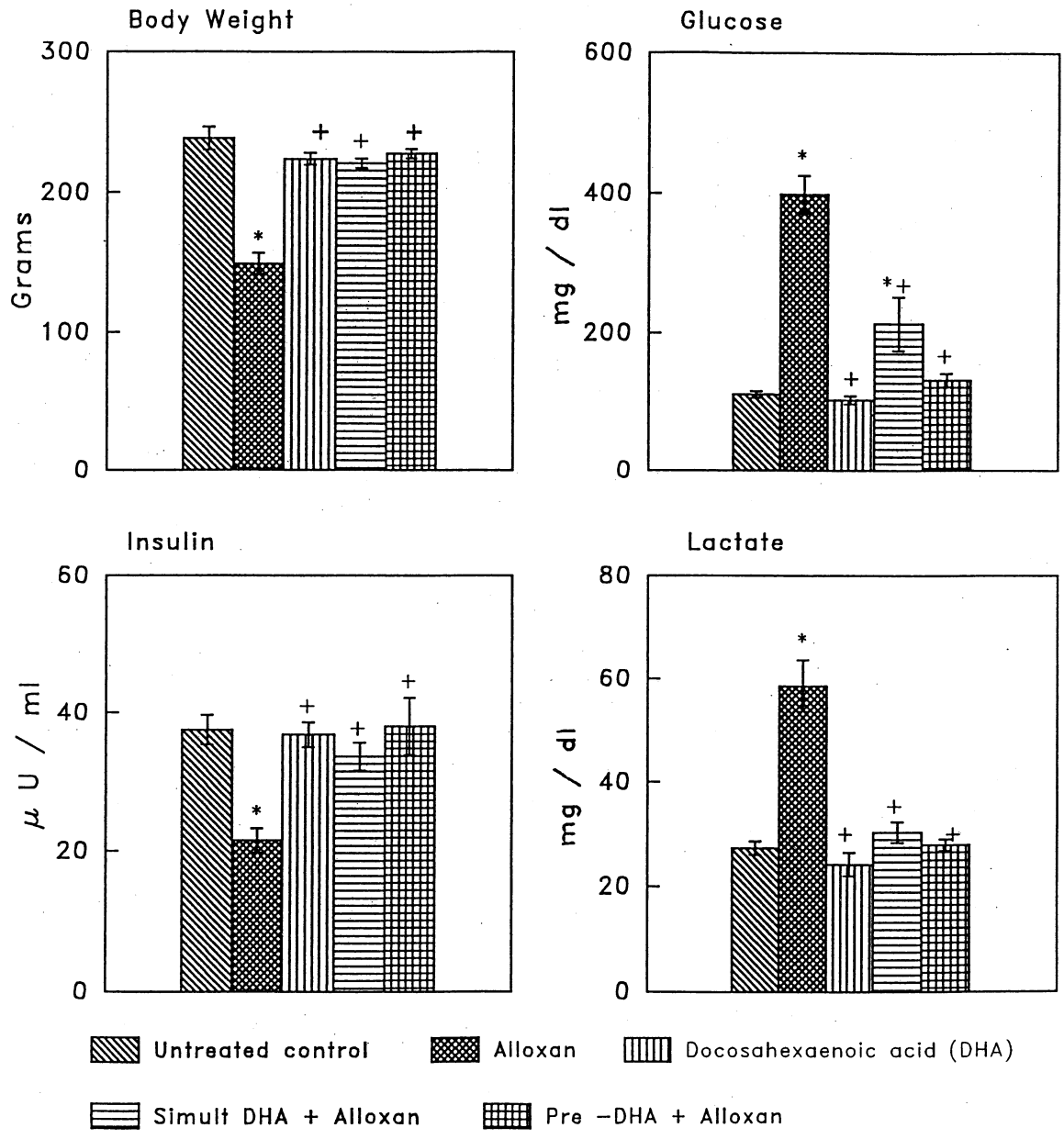

FIG. 9. Effect of alloxan and DHA on body weight, plasma glucose, insulin, and lactate in the plasma of experimental animals. All values are expressed as mean ± standard error ($n = 10$). *$P \leq 0.05$ versus control group. +$P \leq 0.05$ versus alloxan-treated group. DHA, docosahexaenoic acid.

idant enzymes, e.g., SOD, catalase, glutathione peroxidase, and glutathione-S-transferase, in the RBC membranes, liver and kidney homogenates, and plasma ceruloplasmin levels of the alloxan-induced DM and control animals were measured. Plasma ceruloplasmin levels were significantly higher in the alloxan treated group than in the untreated control (Figs. 10 to 12). This may be as a result of the oxidant stress induced by alloxan. The levels of ceruloplasmin were significantly lower in the simultaneous (plus alloxan) and pretreated ALA and DHA groups (Figs. 10 and 12). In contrast, ceruloplasmin concentrations were significantly higher in the untreated control than in the pretreated and simultaneous EPA-treated groups (Fig. 11).

Antioxidants in RBC lysate. It is evident from the results shown in Figures 13 to 15 that there was a significant decrease in the activity of SOD in the RBC lysate in alloxan-induced DM animals compared with control animals. In the ALA- and EPA-pretreated and DHA-treated (in pre- and simultaneous treatment) groups, the activity of SOD was restored to normal compared with alloxan-treated animals (Figs. 13 to 15). A significant increase in the activities of glutathione peroxidase and glutathione-S-transferase was observed in alloxan-induced DM animals, which can be attributed to oxidant stress induced by alloxan. The increased activities of glutathione peroxidase and glutathione-S-transferase were restored to near normal levels in the pretreated ALA group and the pretreated and simultaneous DHA groups (Figs. 13 and 15). In the simultaneous ALA-treated group, there was no significant change in the activities of either glutathione peroxidase or glutathione-S-transferase (Fig. 13). In the simultaneous EPA-treated group, the activities of glutathione peroxidase and glutathione-S-transferase did not change significantly compared with the alloxan-treated group, whereas in the pretreated EPA group, the activities of these enzymes were significantly lower in comparison with the alloxan-treated control group, although they were still higher than in the untreated control group (Fig. 14). It is interesting to note that ALA, EPA, and DHA control animals showed increased activity of SOD

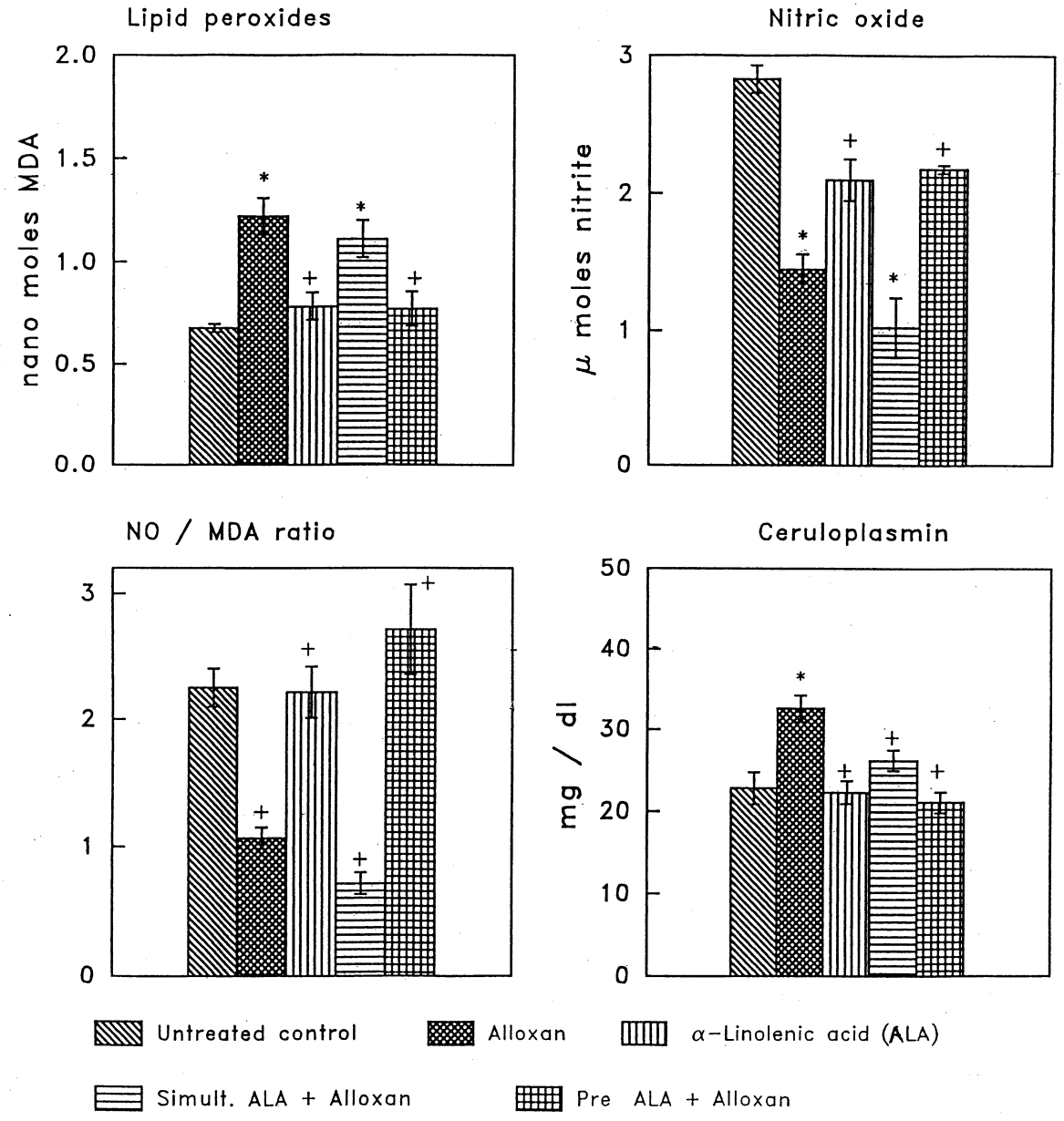

FIG. 10. Effect of alloxan and ALA on levels of lipid peroxides, NO, NO:MDA ratio, and ceruloplasmin in the plasma of experimental animals. All values are expressed as mean ± standard error ($n = 10$). *$P \leq 0.05$ versus control group. +$P \leq 0.05$ versus alloxan-treated group. ALA, α-linoleic acid; MDA, malondialdehyde; NO, nitric oxide.

in the RBC. This might explain, in part, why the activity of SOD was maintained at normal levels even after exposure to alloxan (which induces oxidant stress) in ALA-, EPA-, and DHA-treated (pre- and simultaneous treatments) animals. These results suggest that various ω-3 fatty acids have distinctly different effects on the activities of various antioxidant enzymes, at least in the RBCs. There were no significant changes in the activity of catalase in any group. A summary of the changes in the activities of various antioxidant enzymes in the RBC lysate is shown in Table II.

Histopathology of the Pancreas

Histopathologic examination of the pancreas of alloxan-treated animals showed decreased islet cell number, necrosis of pancreatic β-cells, reduction in the size of β-cells, and lymphocyte infiltration (data not shown). These changes were not observed in the pancreas of groups that were simultaneously or pretreated with AA (data not shown). Similar observations were made in the pancreas of animals in which alloxan-induced DM was prevented by other ω-3 fatty acids.

DISCUSSION

Alloxan is rapidly taken up by the β-cells, although it is not toxic by itself. It is the metabolites of alloxan that are toxic[25] to the islet cells of pancreas. Alloxan inhibits glucose-stimulated insulin release[26] and the generation of glucose-derived energy by inhibiting glycolytic flux and pyruvate oxidation.[27] Superoxide radicals generated during the redox cycling of alloxan results in the formation of H_2O_2. In the presence of Fe^{2+} ions, H_2O_2 reacts with superoxide to form OH· radicals. These OH· radicals are toxic to the pancreatic β-cells.[28] We previously showed that PUFA-rich oils prevent alloxan-induced damage to the pancreatic β-cells.[11] We

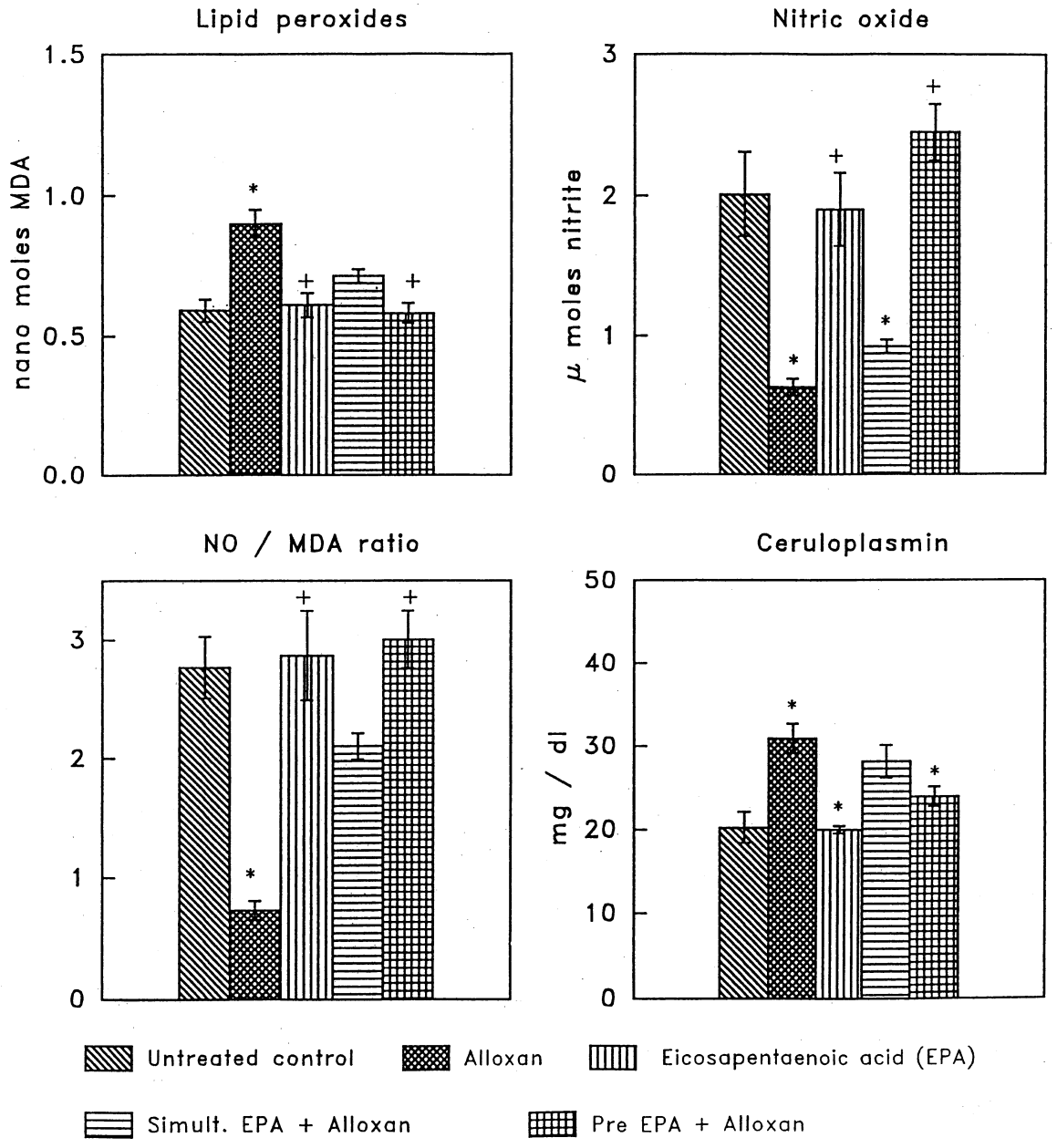

FIG. 11. Effect of alloxan and EPA on levels of lipid peroxides, NO, NO:MDA ratio, and ceruloplasmin in the plasma of experimental animals. All values are expressed as mean ± standard error ($n = 10$). *$P \leq 0.05$ versus control group. +$P \leq 0.05$ versus alloxan-treated group. EPA, eicosapentaenoic acid; MDA, malondialdehyde; NO, nitric oxide.

also observed that NO reduces the severity of alloxan-induced DM.[17] Agents or chemicals that quench free radicals protect against alloxan-induced damage to the pancreatic β-cells,[29,30] suggesting that alloxan-induced damage to the pancreatic β-cells is a process that depends on free radicals.

The present results support and extend our previous findings that PUFAs of ω-6 and ω-3 fatty acids can prevent alloxan-induced DM (type 1 DM) in experimental animals. But the previous studies were done with PUFA-rich oils,[11] so it was not clear which of the fatty acids in the oils was responsible for the protective action. The present results suggest that, of all the ω-3 fatty acids tested, EPA and DHA are effective in preventing alloxan-induced toxicity to β-cells in vitro and in vivo, especially when RIN cells and experimental animals were pretreated with these fatty acids. Because alloxan-induced cytotoxic action is a free radical–dependent process, these results raise the intriguing possibility that PUFAs can inhibit or quench the free radicals and, hence, prevent diabetes. The observation that ω-3 fatty acids, especially EPA and DHA, could revert the altered concentrations of lipid peroxides, NO, SOD, ceruloplasmin, glutathione peroxidase, glutathione-S-transferase, and catalase induced by alloxan to near normal levels in alloxan-treated animals supports this assumption. Under some well-defined conditions, these long-chain PUFAs (LCPUFAs) might have antioxidantlike actions or actually behave as antioxidants.

NO quenches the superoxide anion,[31,32] whereas SOD inactivates superoxide anion. Hence, the decreased concentrations of NO and SOD observed in alloxan-induced DM animals increase the half-life of the superoxide anion. This in turn may contribute to the cytotoxic action of alloxan. Further, catalase, SOD, both, or vitamin E prevented alloxan-induced cytotoxicity.[29,30,33,34]

In the present study, plasma lipid peroxides that increased significantly in alloxan-treated animals reverted to normal upon treatment with EPA and DHA. This suggests that the ability of

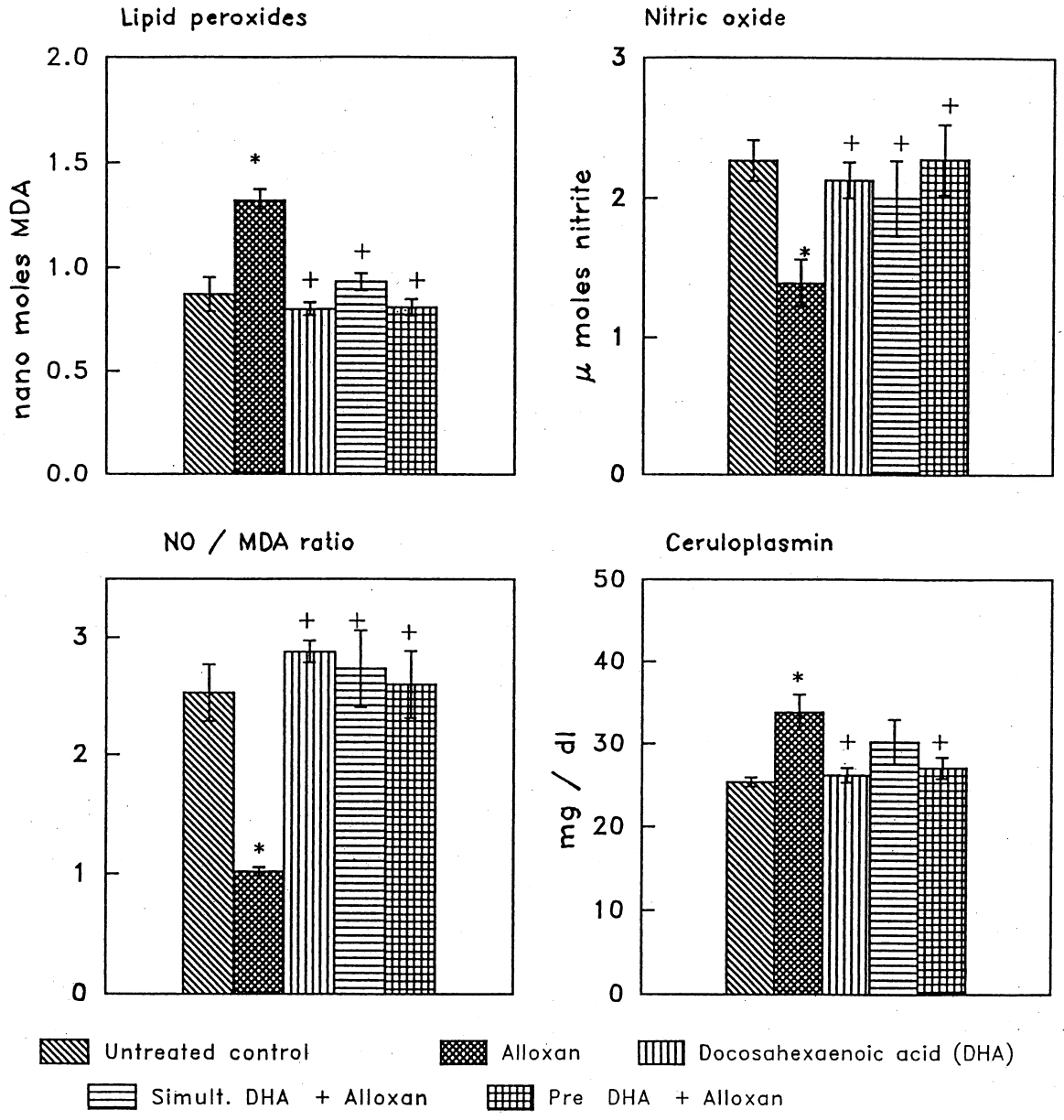

FIG. 12. Effect of alloxan and DHA on levels of lipid peroxides, NO, NO:MDA ratio, and ceruloplasmin in the plasma of experimental animals. All values are expressed as mean ± standard error ($n = 10$). *$P \leq 0.05$ versus control group. +$P \leq 0.05$ versus alloxan-treated group. DHA, docosahexaenoic acid; MDA, malondialdehyde; NO, nitric oxide.

EPA and DHA to prevent the development of DM induced by alloxan is due to decreased production of free radicals and lipid peroxides. This is supported by the work of Sandhya and Ramesh[35] who showed that glucose prevents the toxic effects of streptozotocin by lowering the concentrations of lipid peroxides.

Increased production of free radicals and decreased concentrations of NO were reported in DM animals and patients with type 1 and 2 DM.[36–38] Hattori et al.[38] showed that SOD restores endothelium-dependent relaxation of the aorta of diabetic rats. The results of the present study support those observations.[38,39] The low levels of NO observed may be due to its inactivation by enhanced free radical generation (especially superoxide anion).[31,32] The observation that the levels of lipid peroxides were high in alloxan-induced diabetic rats (present study) supports this view.

NO is a free radical, but it also has a wide range of biological functions. NO is toxic to pancreatic islet cells. NO, produced by β-cells,[40] is an important effector molecule involved in macrophage-induced islet cell lysis.[41] NO-induced cell death is due to damage to DNA.[42,43] Exposure of islet cells to NO donors induces the activity of adenosine diphosphate ribose polymerase in cell nuclei[44] with concomitant depletion of intracellular nicotinamide adenosine dinucleotide (NAD^+). This causes insufficient energy generation in the cell, which ultimately leads to cell death.[45] This is supported by the observation that mice lacking the poly-adenosine diphosphate ribose polymerase (PARP) gene are resistant to the development of DM induced by streptozotocin.[46] In the absence of PARP, levels of NAD^+ remained normal even after exposure to streptozotocin and despite damage to DNA, and the mice did not develop diabetes and remained normoglycemic. This suggests that NAD^+ depletion leads to β-cell destruction; thus, its preservation in β-cells prevented the development of DM.

It is likely that prior administration of glucose would block alloxan-induced cytotoxicity to islet cells by preserving its NAD^+ content because glucose forms the energy source to these cells. The results of the present study showed that AA and other LCPU-

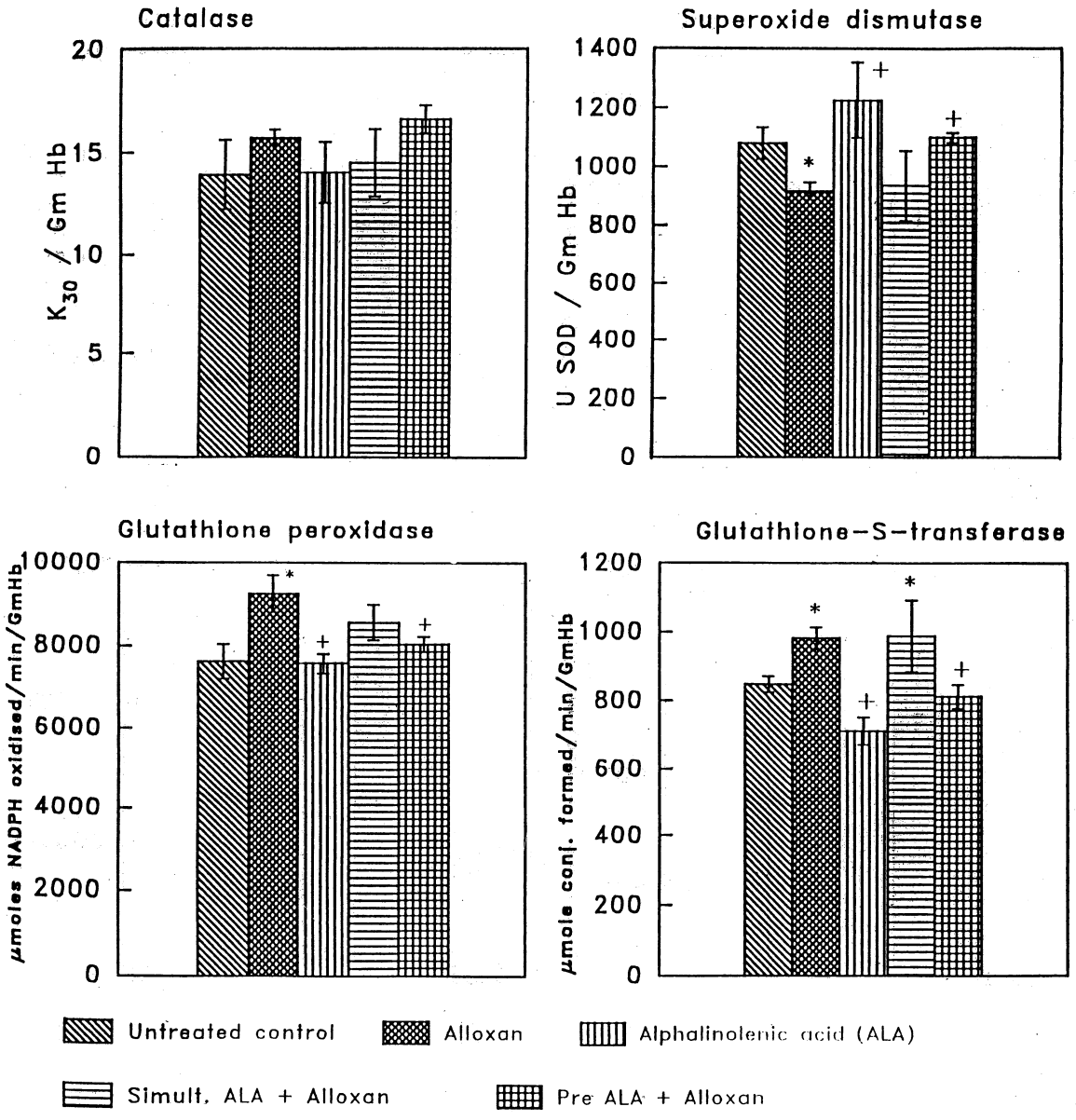

FIG. 13. Effect of alloxan and ALA on levels of different antioxidants in the red blood cell lysate of experimental animals. All values are expressed as mean ± standard error ($n = 10$). *$P \leq 0.05$ versus control group. +$P \leq 0.05$ versus alloxan-treated group. ALA, α-linolenic acid; NADPH, nicotinamide adenine dinucleotide phosphatase.

FAs prevent alloxan-induced destruction of β-cells in vitro and in vivo. We suggest that AA and other LCPUFAs can preserve NAD^+ content of β-cells by blocking the enzyme PARP. There is indirect evidence for this possibility. PARP activation occurs only when DNA is damaged. In the present study, EPA and DHA prevented apoptosis of RIN cells, suggesting that these fatty acids can prevent alloxan-induced DNA damage. In view of this, PARP activation is unlikely to occur in RIN cells, but there may be other mechanisms by which PARP activation would not occur. Possible direct action of these fatty acids to inhibit PARP activation also needs serious consideration. This suggestion needs to be explored in future studies. PUFAs themselves or their peroxidized products can bind to DNA,[47] and this may be yet another potential mechanism by which fatty acids can protect DNA from free radical attack.

Deficiency of antioxidants can result in tissue and organ damage due to free radicals. Antioxidant enzymes SOD, glutathione peroxidase, and catalase were low in the pancreatic tissue of normoglycemic diabetic-prone BB rats compared with the low-risk group.[48] In the present study, alloxan-induced DM animals showed low activities of SOD, glutathione peroxidase, and glutathione-S-peroxidase. Similar results were reported by other workers.[49,50] This decrease in the concentrations of various antioxidants can increase the formation of free radicals in DM. Supplementation of ω-3 fatty acids, especially EPA and DHA, increased significantly concentrations of various antioxidants in alloxan-induced DM rats (present study). This suggests that fatty acids can augment the tissue levels of antioxidants and thus protect them from chemically induced DM in rats. The fact that antioxidants such as butylated hydroxyanisole, vitamin E, and probucol can prevent alloxan-induced DM in rats supports this view.[29,30]

We previously reported that plasma levels of the saturated fatty acids stearic and palmitic (16:0) acids increase, whereas unsaturated fatty acids such as oleic acid (18:1 ω-9), linoleic acid, γ-linolenic acid, DGLA, and AA decrease in the plasma phospholipid fraction of alloxan-induced DM rats.[10,11,51] Plasma levels of

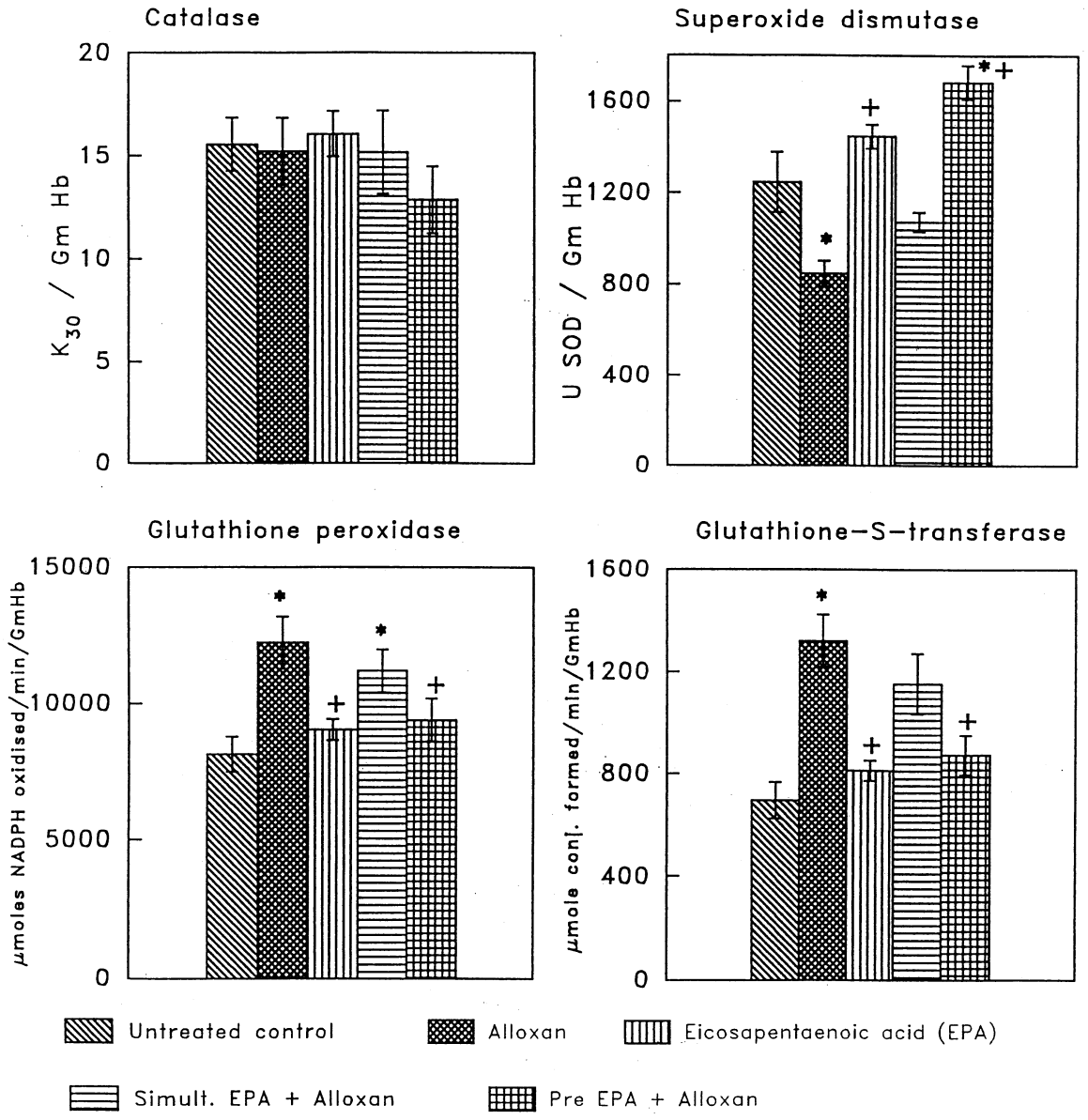

FIG. 14. Effect of alloxan and EPA on levels of different antioxidants in the red blood cell lysate of experimental animals. All values are expressed as mean ± standard error ($n = 10$). *$P \leq 0.05$ versus control group. +$P \leq 0.05$ versus alloxan-treated group. EPA, eicosapentaenoic acid; NADPH, nicotinamide adenine dinucleotide phosphatase.

dihomo-γ-linolenic acid (20:3 ω-6), AA, ALA, and DHA decreased in patients with DM and diabetic nephropathy.[39,52] This suggests that the activities of δ-6-desaturase and δ-5-desaturase, which are necessary for desaturation of dietary linoleic acid and ALA to their respective metabolites are depressed in experimental DM and in humans with type 1 and 2 DM.[39,52–55] It is interesting to note that insulin can augment the activity of δ-6-desaturase activity.[56] This decrease in the levels of various PUFAs in DM can be attributed to a defect in the activities of desaturases, increased peroxidation by free radicals (because PUFAs may form substrates for peroxidation), or increased eicosanoid formation (i.e., increased use of substrate and PUFAs). If this is true, supplementation of PUFAs may be of benefit in DM. As evident from the present study, ω-3 fatty acids significantly improved the diabetic status of alloxan-induced DM Wistar rats: plasma glucose and lactate levels decreased and body weights decreased less compared with the alloxan-alone group. Further, an increase in NO, a decrease in the levels of lipid peroxides, and restoration of antioxidant status to near normal were observed in these animals. Fish-oil supplementation augments NO production in patients with type 2 DM[57] and increases the expression of hepatic antioxidant enzymes SOD, catalase, and glutathione peroxidase in lupus-prone mice and SOD activity in patients with systemic lupus erythematosus.[58,59] Because ω-3 fatty acids enhanced the activities of antioxidant enzymes in the present study, it is likely that PUFAs in general have the ability to increase the concentrations of various antioxidant enzymes.

IL-1, IL-6, TNF, and interferon-γ play major roles in pancreatic β-cell damage in type 1 DM by enhancing the formation of oxygen free radicals, lipid peroxides, and aldehyde production.[60–62] The ω-6 and ω-3 fatty acids inhibit IL-1, IL-2, and TNF production in human lymphocytes in vitro and in vivo, an action that does not depend on the formation of eicosanoids from PUFAs.[63–66] Hence, PUFAs likely protect the pancreatic β-cells by inhibiting cytokine production. However, in the present study, we did not measure various cytokines or C-reactive protein, so this proposal remains conjectural.

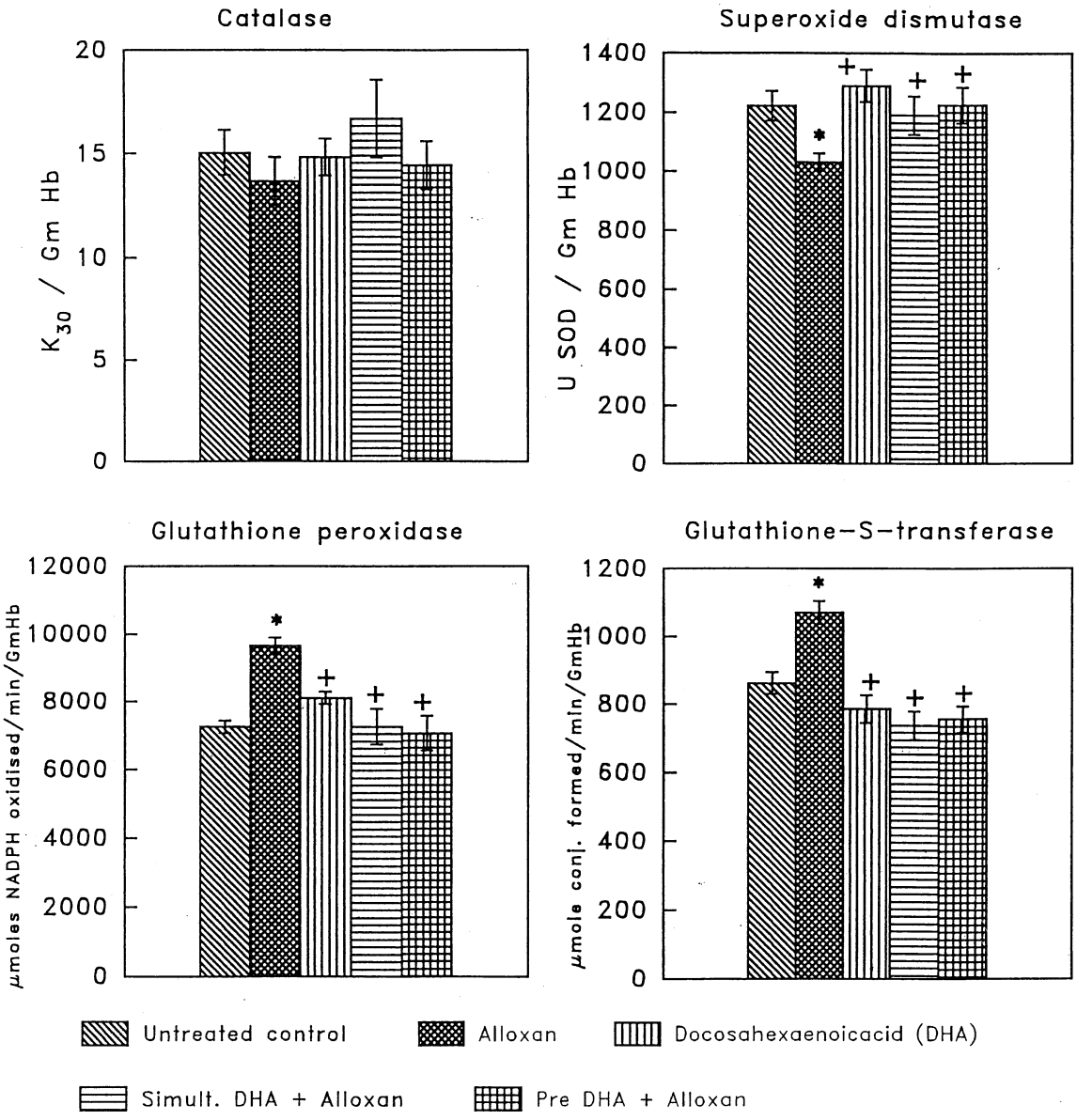

FIG. 15. Effect of alloxan and DHA on levels of different antioxidants in the red blood cell lysate of experimental animals. All values are expressed as mean ± standard error ($n = 10$). *$P \leq 0.05$ versus control group. +$P \leq 0.05$ versus alloxan-treated group. DHA, docosahexaenoic acid; NADPH, nicotinamide adenine dinucleotide phosphatase.

Activators of PPARs, troglitazone, and CLA also suppress free radical generation, and TNF and IL-1 production prevented DM in experimental animals.[4–6] PUFAs are endogenous ligands of PPARs and have actions similar to those of CLA and troglitazone, which suggests that they also possess the ability to prevent alloxan-induced DM in experimental animals. The results of the present study support this idea.

Previous studies investigating the effects of ω-6 and ω-3 fatty acids in DM concentrated on the possible beneficial actions of these fatty acids in the prevention or arrest of progression of diabetic complications,[67] but the results were not very conclusive. In contrast, we investigated the effects of pretreatment and simultaneous treatment with fatty acids and alloxan on the development of diabetes itself. The results of the present and previous studies[10,11] showed that some, if not all, PUFAs prevent chemically induced diabetes. This is supported by the observation that cod liver oil (a rich source of EPA and DHA) taken during pregnancy is associated with reduced risk for type 1 DM in the offspring.[68] Recent epidemiologic studies also reported that *trans*-fatty acids increase and PUFAs reduce the risk of type 2 DM in women.[69] *Trans*-fatty acids interfere with the metabolism of essential fatty acids and thus may reduce the availability of PUFAs to tissues (reviewed by Das[70] and Schrezenmeir and Jagla[71]). This may explain why *trans*-fatty acids prevent the beneficial action(s) of PUFAs. A negative correlation between the frequency and duration of breast feeding and type 1 DM also was reported.[72] Breast milk is rich in ω-6 and ω-3 PUFAs, so the beneficial action of breast feeding might be attributed to its high content of these fatty acids. Clearly more definitive studies are needed to establish this assumption. If PUFAs can prevent the development of type 1 DM, as in the present study in experimental animals, it would be interesting to study whether supplementation of these fatty acids can reduce the risk of type 1 and 2 DM in the high-risk population. It should be understood that type 1 DM is due to destruction of β-cells, whereas type 2 DM is due to peripheral insulin resistance. TNF-α seems to play a major role in both types: in type 1 DM, it

TABLE II.

SUMMARY OF THE ACTIONS OF ALLOXAN AND ω-3 FATTY ACIDS ON THE ACTIVITIES OF VARIOUS ANTIOXIDANT ENZYMES IN RED BLOOD CELL LYSATE*

Treatment	SOD	GP	GST	Catalase
Alloxan	↓	↑	↑	N
Pre-ALA + alloxan	N	N	N	N
Simult. ALA + alloxan	N	N	↑	N
Pre-EPA + alloxan	↑	N	N	N
Simult. EPA + alloxan	N	↑	N	N
Pre-DHA + alloxan	N	N	N	N
Simult. DHA + alloxan	N	N	N	N

* The activities of the antioxidant enzymes are shown as compared with control groups.
GP, glutathione peroxidase; GST, glutathione-S-transferase; N, normal; Pre, pretreatment with fatty acid and alloxan; Simult., simultaneous treatment with fatty acid and alloxan; SOD, superoxide dismutase

damages β-cells, whereas in type 2 DM increased amounts of TNF-α induce insulin resistance. EPA and DHA ω-3 fatty acids suppress TNF-α production and thus may be beneficial in type 1 and 2 DM. It may be emphasized that the present study was done in experimental animals, and the same results may or may not be extrapolated or obtained in humans.

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