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ORIGINAL ARTICLE

Lipid Metabolism in Rats is Modified by Nitric Oxide Availability Through a Ca^{++}-Dependent Mechanism

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Abstract We studied lipid metabolism and the antioxidant defense system in plasma and liver of rats fed diets supplemented with L$^{\omega}$-nitro-L-arginine methyl ester (L-NAME), isosorbide dinitrate (DIS), L-arginine (Arg), or the associations of these drugs. Liver hydroperoxide and thiobarbituric-acid-reactive substance (TBARS) levels were decreased by Arg and increased by L-NAME or DIS treatments. Oxidized glutathione and conjugated dienes were increased by DIS. Nitrate + nitrite levels and serum calcium ([Ca^{++}]) were incremented by Arg or DIS and reduced by L-NAME. Superoxide dismutase and catalase activities decreased under Arg treatment, while L-NAME or DIS caused stimulation. Liver high-density lipoprotein (HDL) cholesterol was increased by DIS or NAME (alone or associated with Arg). Free fatty acids and neutral and polar lipids were increased by Arg, L-NAME, and DIS. However, predominating phospholipid synthesis increased the neutral/polar ratio. Decreased levels of nitric oxide (NO) (low [Ca^{++}]) was directly associated with increased fatty acid synthetase, decreased phospholipase A_2, carnitine-palmitoyl transferase, and fatty acid desaturase activities. Raised NO (high [Ca^{++}]) inversely correlated with increased phospholipase-A_2 and acyl-coenzyme A (CoA) synthetase and decreased fatty acid synthetase and β-oxidation rate. Arg or DIS produced changes that were partially reverted by association with L-NAME. Based on these observations, prolonged therapeutical approaches using drugs that modify NO availability should be carefully considered.

Keywords Oxidative stress · Calcium · Lipid metabolism · Rat liver · Nitric oxide

List of Abbreviations

NO	Nitric oxide
[Ca^{2+}]	Calcium concentration
[NOx]	Nitrite plus nitrate concentration
Arg	L-arginine
CAT	Catalase
DIS	Isosorbide dinitrate
FAS	Fatty acid synthetase
GSHPx	Glutathione peroxidase
GSHRd	Glutathione reductase
GSHTr	Glutathione transferase
LLC	Low-level chemiluminescence
L-NAME	Lω-nitro-L-arginine methyl ester
NL	Neutral lipids
PL	Polar lipids (phospholipids)
PL-A_2	Phospholipase A_2
ROOHs	Lipid hydroperoxides
SOD	Superoxide dismutase

Introduction

The central role of nitric oxide (NO) as a messenger molecule is well documented in various biochemical processes such as immune function [1, 2], inflammation [3–6], apoptosis and necrosis [7–10], neurotransmission

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[11, 12], mitochondrial respiration [13, 14], and endothelial function [10, 15–19], among others. It has also been reported that NO production is involved in the regulation of carbohydrate metabolism [20, 21] and inhibition of the Krebs cycle [22]. Lipid metabolism is strongly influenced by antioxidant status, although the mechanism involved remains unknown. Previous works demonstrated that deficiency of ascorbate [23, 24], α-tocopherol [25], and the oligoelements involved in the antioxidant system, such as selenium [26] or copper [27], were able to induce rapid and significant hyperlipidemia with a concomitant increase in liver very-low-density lipoprotein (VLDL) secretion [28]. A key study by Khedara et al [29] proved that serum concentration of nitrate negatively correlated with those of plasma triacylglycerides and total cholesterol. These authors concluded that lower NO levels in rats led to hyperlipidemia and that elevation in serum triacylglycerides might be due—at least in part—to reduced fatty acid (FA) oxidation [29, 30]. Taking into account that reduced NO production is one factor leading to hyperlipidemia and increased atherosclerotic risk [15–17, 29], we decided to further explore whether oxidative stress would affect lipid metabolism by either increasing or decreasing NO levels. Various enzymes of the antioxidant defense system and lipid metabolism, together with concentration of the major water- and lipid-soluble antioxidants, were also correlated with lipid composition in plasma and tissues of rats fed diets supplemented with L$^{\omega}$-nitro-L-arginine methyl ester (L-NAME), isosorbide dinitrate (DIS)—usually employed as an antihypertensive drug in humans—and L-arginine (Arg) alone or in combination with other drugs. Considering the central role of lipid metabolism and antioxidant defense system in the pathogenesis of atherosclerotic lesions [6, 9, 15–17, 19, 31, 32], we investigated the mechanism by which changes in NO production might affect biochemical pathways involved in FA and complex lipid metabolisms.

Materials and Methods

Chemicals

The following chemicals were purchased from Sigma Chem. Co, (Buenos Aires, Argentina): L-NAME, Arg, standards for high-performance liquid chromatography (HPLC) (retinol, oxidized and reduced glutathione, β-carotene, α- and β-tocopherols, FA methyl esters, glutathione, and ascorbate); snake venom (*Crotalus atrox* western diamondback rattlesnake); nicotinamide adenine dinucleotide $(NAD)^{+}$, hydrogenated NAD [NADH(H)], hydrogenated NAD phosphate [NADPH(H)], thiobarbituric acid; coenzyme A (CoA, lithium salt), *N*-ethylmaleimide, deferoxamine mesylate, organic and inorganic components for buffer preparations, triphenylphosphine; xylenol orange, tetraethoxypropane; sodium deoxycholate (grade II); delipidated serum albumin bovine serum albumin (BSA); fraction V from bovine); and butylated hydroxytoluene. DIS was purchased as Isordil from John Wyeth Lab. (Buenos Aires, Argentina). Sodium nitrite and nitrate were from Merck (Darmstadt, Germany). All solvents were HPLC grade and were provided by Carlo Erba, Milano, Italy. Silicagel G-60 plates for thin-layer chromatography (TLC) were provided by Fluka-Riedel-de Häen (Darmstadt, Germany). Lipid standards for TLC identification of neutral and polar lipids were from Serdary Research Laboratories (London, Ontario, Canada). Unlabeled FAs were provided by Nu-Chek Prep. (Elysian, MN, USA). Labeled [1-^{14}C]FA (palmitic, linoleic, α-linolenic, and eicosa-8,11,14-trienoic) (98–99% pure, 50–60 mCi/mmol) were obtained from Amersham Biosciences (Buckinghamshire, UK). All acids were stored in benzene under nitrogen atmosphere at –20 °C. Concentrations and purities were routinely checked by both gas liquid chromatography (GLC) and liquid-scintillation counting. Other chemicals used were reagent grade from local commercial sources.

Animal Treatment

Female Wistar rats from Comisión Nacional de Energía Atómica (Buenos Aires, Argentina) weighing 170 ± 10 g were bred and maintained on a control diet (Cargill type ''C'', Rosario, Argentina) throughout gestation and lactation. Dams were housed in plastic cages (one animal per cage) in a vivarium kept at 22 ± 1 °C with a 12-h light/dark cycle and relative humidity 60 ± 10%. After weaning, 36 male pups (weighing 46 ± 4 g/animal) were randomly divided into six groups of six animals fed ad libitum a balanced diet (group C) prepared in our laboratory as described in a previous paper [33]. Other groups were supplemented with Arg (40 g/kg)L-NAME (200 mg/kg), DIS (10 mg/kg), or a combination of Arg + L-NAME or DIS + L-NAME. During the ad libitum feeding period (35 days), body weight, water consumption, and food intake were determined every day. Blood samples (100–150 μl) were collected from the tail vein once a week to determine plasma calcium and nitrite + nitrate ([NOx]) levels. Animals were sacrificed on day 35 after feeding. To avoid individual differences among animals that might result from an ad libitum feeding, on day 34,

all rats were fasted for 24 h, re-fed with the corresponding diet for 2 h, and then killed by rapid decapitation without prior anesthesia 12 h after the refeeding period. All diet components used were purchased from Carlo Erba (Milano, Italy) or Mallinckrodt (New York, USA). Animal maintenance and handling were in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals [34].

Sample Collection and Subcellular Fraction Preparation

Liver, heart, kidneys, lungs, and brain from all experimental groups were rapidly excised and immediately placed in an ice-cold homogenizing medium [35]. The homogenates were processed individually at 1°C. Microsomal suspensions and cytosols (soluble fractions) were separated by differential centrifugation at 110,000 g, as described previously [35]. Microsomal pellets were resuspended in cold homogenizing solution up to a final protein concentration of 30 mg/ml. Mitochondrial suspensions from liver homogenates were obtained as described by Kler et al. [36]. Blood was also collected after killing the rats by decapitation. Samples were individually dispensed into heparinized tubes and fractionated by centrifugation at 500 g (10 min). Plasmas were immediately processed for calcium determination following the method described elsewhere [37, 38] using the procedure of Tomlinson and Dhalla [39]. Nitrite plus nitrate levels were measured according to Verdon et al. [40]. Lipid analysis was performed as described above. Erythrocyte ghosts were prepared by hypotonic lysis according to the procedure of Dodge et al. [41] modified by Berlin et al. [42].

Oxidative Stress Biomarkers

Thiobarbituric-acid-reactive substance (TBARS) were measured by the fluorometric method of Yagi [43]. Organo peroxides were determined with the FOX version II assay for lipid hydroperoxides (ROOHs) (FOX2) according to Nourooz-Zadeh et al. [44]. Contents of oxidized glutathione (GSSG) were determined following the HPLC method of Asensi et al. [45], while reduced glutathione (GSH) was measured following the glutathione-*S*-transferase assay described by Brigelius et al. [46]. Samples for GSSG analyses were obtained in the presence of *N*-ethylmaleimide and deproteinized using trichloroacetic acid (15% final concentration) [45]. α- and *β*-tocopherols, licopene, and *β*-carotene were determined by HPLC according to Butris and Diplock [47] as modified by Bagnati et al. [48]. Ascorbate was determined in deferoxamine mesylate-treated samples by the method of Benzie et al. [49]. Catalase activity (CAT) was measured according to Aebi [50]. Cytosolic (Cu,Zn) and mitochondrial (Mn) superoxide dismutase (SOD) activities were assayed as described by Flohé and Ötting [51]. Glutathione peroxidase (GSHPx), glutathione transferase (GSHTr), and glutathione reductase (GSHRd) were assayed according to Wheeler et al. [52], Habig et al. [53], and Carlberg et al. [54], respectively. Light emission by chemiluminescence from lipid peroxidation assays was measured following the procedure of Wright et al. [55] with some modification. Liver microsomal suspensions obtained from control Wistar rats (2 ml; 0.5 mg protein/ml) in 0.1 M TRIS–HCl buffer (pH 7.40) and 0.15 M KCl were incubated in glass vials at 25 °C with a solution containing 100 μM Fe^{2+} and 100 μM sodium ascorbate to initiate nonenzymatic lipid peroxidation. Low-level light emission was continuously monitored in the dark over a 130-min period and recorded as constant photocurrent method (cpm) using a liquid scintillation counter (1214 Rackbeta Scintillation Counter, Turku, Finland) in the out-of-coincidence mode. At zero time, membrane preparations were supplemented with 0.5 mg cytosolic proteins from the different experimental groups. Other vials lacking ascorbate, Fe^{2+}, or both were run as control assays in the same conditions. The sum of total chemiluminescence (areas under each curve) was used to calculate percent change in respect to the assay with no cytosol addition (reference curve).

Lipid Analysis and Enzyme Activities of Lipid Metabolism

Total lipids were extracted by the method of Folch et al. [56]. Phospholipid and neutral lipid fractions were separated from the Folch extracts by the microcolumn chromatography method described elsewhere [57] and/or TLC [58, 59]. GLC of fatty acid methyl esters (FAMEs) was performed as indicated in our previous paper [60], except that in this case, we used a capillary column mounted on a Hewlett Packard HP 6890 Series GC System Plus (Avondale, PA, USA) equipped with a terminal computer integrator. The FAMEs were identified by comparison of their relative retention times with authentic standards, and mass distribution was calculated electronically by quantification of the peak areas. Cholesterol content was enzymatically measured according to Allain et al. [61]. High-density lipoprotein (HDL) cholesterol was determined using a commercial kit (Wienner Lab. Rosario, Argentina). Total and neutral lipids were

estimated gravimetrically after evaporation of an aliquot of the corresponding lipid extract (Folch or silicic acid subfraction, respectively) up to constant weight [62]. Phospholipids were also measured as phosphorus content [63] after mineralization of an aliquot from the silicic acid partition. Phospholipase A_2 (PL-A_2) activity was determined with [^{14}C]phenylcyclohexene ([^{14}C]PC) (24.0 mCi/mmol, 99% pure) as substrate according to the method of Hirata et al. [64] with the modifications described in our previous paper [65]. To determine FA desaturase activities in microsomal suspensions, each FA used as substrate [[1-^{14}C] 16:0, [1-^{14}C] 18:2 (n-6), α-18:3 (n-3), or [1-^{14}C] 20:3 (n-6)] was diluted to a specific activity of 0.20–0.25 μCi/mol with the respective pure unlabeled FA. In order to compare results, enzymatic assays were conducted at saturated substrate concentrations. Analyses were done as described in previous papers [65, 66]. Acyl-CoA synthetase assays were performed on cytosol fractions obtained as supernatants of 110,000 g, according to the method of Tanaka et al. [67] modified as described in our previous paper [38]. FA synthetase activity was assayed according to Horning et al. [68]. Carnitine-palmitoyl transferase activity was measured following the method of Bieber and Fiol [69], while the rate of β-oxidation was determined as the production of acid-soluble metabolites derived from [1-^{14}C]palmitate in hepatic microsomal suspensions as described by Kler et al. [36]. Ketone bodies (acetoacetate plus 3-hydroxybutyrate) were measured according to Laun et al. [70] by an enzymatic assay using 3-hydroxybutyrate dehydrogenase. Free fatty acids (FFAs) were determined as described in the paper of Duncombe and Rising [71].

Other Methodologies

Calcium content was determined after sample mineralization by atomic absorption in a Shimatzu Atomic Absorption Spectrophotometer AA-630-12 (Shimatzu Corp., Kyoto, Japan) or calcium-sensitive electrode model 93-20 (Orion Res. Inc., Cambridge, MA, USA) as described elsewhere [37–39]. Protein content was determined by the micromethod of Bradford [72] with crystalline BSA as standard.

Graphic Software and Statistical Treatment of Data

All values represent the mean of six individual determinations (assayed in duplicate) ±1 standard error of the mean (SEM). Data were analyzed by either the Student's *t* test or by analysis of variance (ANOVA), with the aid of Systat (version 8.0 for Windows) from SPSS Science (Chicago, IL, USA). Results were also plotted and analyzed using Sigma Scientific Graphing Software (version 8.0) from Sigma Chem. Co. (St. Louis, MO, USA) and/or GB-STAT Professional Statistics Program (version 4.0) from Dynamic Microsystems Inc. (Silver Springs, NV, USA).

Results

Animal Model

We found that the addition of L-NAME, Arg, or a combination of these drugs to the standard diet produced no significant differences in final body weight or water consumption among the experimental groups (data not shown). From day 15 to the end of the experiment, DIS or DIS + L-NAME groups showed a 16% increase and a 19% decrease (mean values compared to control group, not statistically significant) in the water and diet consumption, respectively, either expressed as milliliter water/body weight, milliliter water/rat, or grams food/body weight, grams food/rat.

Oxidative Stress Biomarkers

Figure 1 shows the plasma [NOx] during the feeding period. Inclusion of DIS in the diet composition produced a rapid and sustained increase in [NOx] concentration detected 5 days after treatment. Values were maintained at this level up to the end of the experimental period. The effect of DIS was not significantly modified by association with L-NAME. Arg caused an increase in [NOx] levels 10 days after treatment, reaching a maximum (three-fold in respect to control values) on day 15. Then it tended to decline slightly. L-NAME decreased the [NOx] concentration from day 5 up to the end of the experimental period. However, when Arg was associated with L-NAME, [NOx] values were restored to control values after 10–15 days of the combined treatment. Levels of [NOx] in soluble (cytosolic) fractions from different tissues of control and treated rats are shown in Table 1. Arg-treated rats exhibited increased [NOx] concentrations in liver, heart, and lungs. Association with L-NAME completely abolished these increments. Supplementation with L-NAME alone caused a significant reduction of [NOx] levels in all tissues studied, but this effect did not impair the important increment produced by DIS treatment in all soluble fractions analyzed. As a measurement of the oxidant capacity of cytosol fractions obtained from the different experimental groups, we

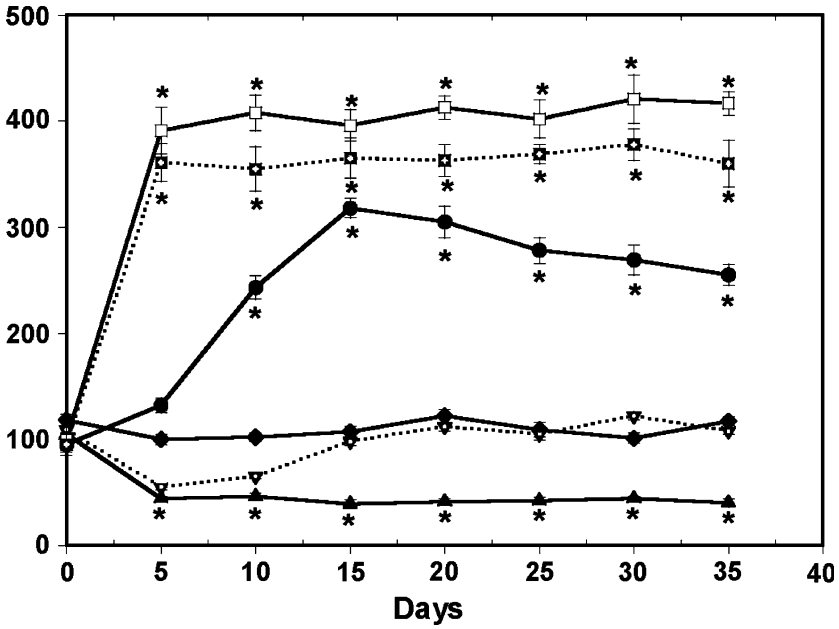

Fig. 1 Nitrate + nitrite [NOx] concentration was determined in plasma samples obtained from the rats' tail vein every 5 days during the entire feeding period. Determination was based on reduction of nitrate to nitrite followed by Griess reaction (see ''Materials and Methods''). Data are mean ± 1 standard error of the mean (SEM) (pmoles/mg protein) of duplicate or triplicate measurements from six independent animals. Control group (*filled diamond*); Lω-nitro-L-arginine methyl ester (L-NAME) (*filled triangle*); L-arginine (Arg) + L-NAME (*filled inverted triangle*); Arg (*filled circle*); isosorbide dinitrate (DIS) + L-NAME (*filled square*); DIS (*open square*). (*) Significantly different with respect to control data; $P < 0.01$

Table 1 Sum (Σ) of nitrite + nitrate [NOx] in soluble fractions from tissues of treated rats

Treatment	Liver	Heart	Brain	Kidney	Lung
C	0.25 ± 0.02	1.12 ± 0.03	3.06 ± 0.18	0.44 ± 0.02	0.88 ± 0.03
Arg	0.39 ± 0.01*	1.69 ± 0.04	2.78 ± 0.26	0.48 ± 0.03	1.52 ± 0.04*
L-NAME	0.12 ± 0.01*	0.75 ± 0.02*	2.36 ± 0.11*	0.25 ± 0.01*	0.66 ± 0.03*
Arg + L-NAME	0.22 ± 0.02	1.23 ± 0.03	3.15 ± 0.09	0.46 ± 0.05	1.16 ± 0.04
DIS	1.08 ± 0.03*	2.50 ± 0.11*	4.74 ± 0.12*	0.76 ± 0.03*	1.55 ± 0.02*
DIS + L-NAME	0.81 ± 0.04*	2.23 ± 0.08*	4.11 ± 0.07*	0.52 ± 0.05	1.36 ± 0.03*

The sum (Σ) of [NOx] was measured as described in ''Materials and Methods''. Results are expressed as pmoles/mg protein, and they are the mean of six independent determinations ±1 standard error of the mean (SEM)

C control, *Arg* L-arginine, L-*NAME* Lω-nitro-L-arginine methyl ester, *DIS* isosorbide dinitrate

*Significantly different with respect to the corresponding control value ($P < 0.01$)

tested the increase or decrease in low-level chemiluminescence (LLC) induced by Fe/ascorbate in vitro (Fig. 2). The curve indicated as ''P'' (without cytosol addition) showed the importance of endogenous components as antioxidants, as total CCL (area under the curve) increased 123% with respect to that obtained after the addition of control cytosol considered as the reference curve. Cytosols from DIS- or DIS + L-NAME-treated rats increased LLC by 250% and 259%, respectively, whereas cytosol from L-NAME-treated rats produced a 32% increase. Supplementation with Arg or Arg + L-NAME decreased LLC by –64% and –26%, respectively, with respect to the reference curve. ''M'' and ''R'' curves demonstrated that there was no spontaneous LLC—at least in a detectable extension—without induction by ascorbate or Fe/ascorbate.

Plasma from the Arg group showed increased values of TBARS, tocopherols, GSH, ascorbate, and carotene + glutathione, whereas retinol contents were decreased (Table 2) in comparison with the control group. L-NAME-treated rats showed increased levels of TBARS and ROOHs with respect to the control group. Ascorbate was also decreased in DIS- and DIS + L-NAME-treated rats and increased in Arg-supplemented animals. The combination of Arg +

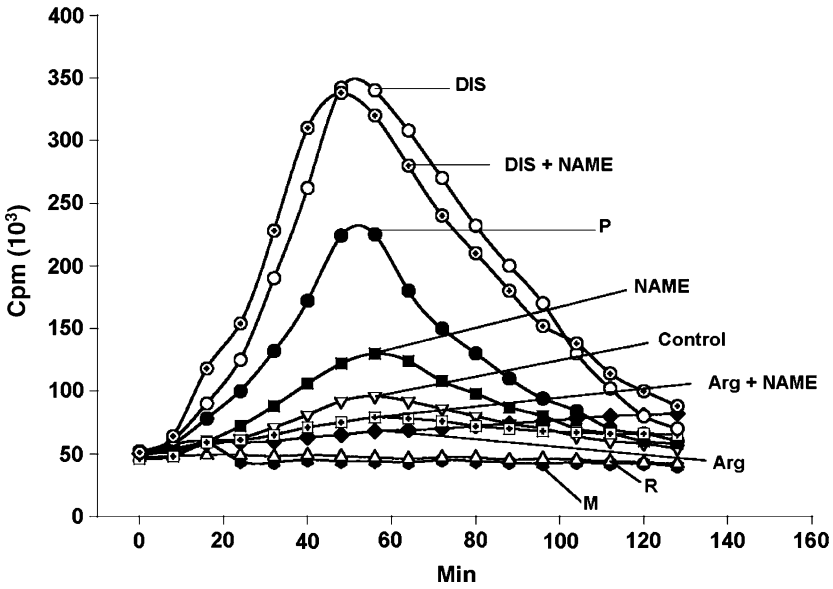

Fig. 2 Low-level chemiluminescence [constant photocurrent method (cpm) 10^3, mean of six determinations] induced by Fe/ascorbate in vitro at 25°C in control liver microsomes as a function of time. Control microsomes were incubated in a buffer solution (pH: 7.40) supplemented with cytosol fractions (0.5 mg protein/ml) obtained from liver of rats fed different experimental diets. Nonenzymatic peroxidation of lipids was induced by addition of Fe/ascorbate at zero time. *Curve "P"* represents data obtained in the presence of liver cytosol fraction from nonsupplemented diet. *Control* corresponds to no cytosol addition. *M* and *R* are curves obtained without addition of cytosol and lacking Fe or ascorbate, respectively. Cytosol from L-arginine (Arg)- (*filled diamond*); Arg + Lω-nitro-L-arginine methyl ester (L-NAME)- (*square with dot*); L-NAME- (*filled square*); P- (*filled circle*); DIS + L-NAME- (*circle with dot*); and DIS- (*open circle*) treated animals. Standard errors of the mean (SEM) were omitted for simplicity, and they were between 5% and 8% of each mean value

L-NAME restored all values to control levels with the exception of GSH. Ratios of GSH/GSSG for Arg, L-NAME, and Arg + L-NAME groups were not modified with respect to control group. DIS or DIS + L-NAME treatments produced similar alterations in all biomarkers assayed. ROOHs and TBARS were increased, lipid soluble antioxidants were dramatically reduced, and ascorbate was decreased by 27% ± 3% with respect to the control group. The ratios of GSH/GSSG in DIS and DIS + L-NAME groups were decreased by 51% and 84%, respectively, compared with the control group. In DIS-treated rats, conjugated dienes also showed a marked increase with respect to other groups.

Table 3 shows the activities of some enzymes of the antioxidant defense system in plasma and erythrocytes from six experimental groups. Erythrocyte SOD and CAT activities were decreased by Arg treatment and increased by L-NAME supplementation. Glutathione-related enzymes were not significantly altered in either plasma or erythrocyte lysates from these experimental groups. Combined supplementation with Arg + L-NAME restored the activities of both enzymes to control values. DIS- or DIS + L-NAME-treated rats showed an important increase in SOD and GSHPx activities from plasma and erythrocytes, whereas CAT activity in erythrocytes was reduced by 41–45% with respect to the control group. Characteristic stress biomarkers in liver from treated rats are shown in Table 4. The pattern of alterations was similar to that observed in plasma (Table 2). Livers from the Arg group exhibited decreased contents of tocopherols and increased amounts of β-carotene + glutathione. Arg caused a reduction in ROOHs and TBARS, whereas L-NAME supplementation produced an increment in both biomarkers with an elevation in diene conjugate concentration. In the Arg group, tocopherols were significantly decreased. The addition of L-NAME to the Arg diet neutralized all these alterations. However, L-NAME was ineffective in restoring the modifications caused by DIS, such as the elevation in ROOHs, TBARS, and diene conjugate levels and the concomitant decreased in tocopherol and ascorbate contents. The ratio GSH/GSSG decreased in DIS-treated rats compared with control rats.

Table 5 shows enzyme activities of the antioxidant defense system in liver of treated rats. SOD activity was measured using selective inhibitors for Mn-, Cu, and Zn-dependent isoforms [sodium cyanide and sodium dodecyl sulfate (SDS)]. Arg decreased both SOD isoforms and, as a consequence, total SOD activity. CAT was also diminished compared with controls. Glutathione-related enzymes were not significantly

Table 2 Oxidative stress biomarkers in plasma from treated rats

Biomarkers	Treatments					
	C	Arg	L-NAME	Arg + L-NAME	DIS	DIS + L-NAME
Lipid hydroperoxidases (ROOHs)						
μM	4.6 ± 0.1	4.4 ± 0.3	5.5 ± 0.1*	4.7 ± 0.2	10.6 ± 2.1*	11.3 ± 1.9*
pmoles/mg protein	61.3 ± 4.4	58.4 ± 2.0	83.5 ± 2.8*	62.0 ± 1.1	137.7 ± 24.8*	148.7 ± 30.2*
pmoles/mg total lipids	1150 ± 41	927 ± 64	1522 ± 81*	1119 ± 55	1967 ± 123*	2288 ± 119*
Thiobarbituric-acid-reactive substance (TBARS)						
μM	1.3 ± 0.03	1.4 ± 0.1	2.9 ± 0.1*	1.4 ± 0.1	5.4 ± 0.3*	8.0 ± 0.3*
pmoles/mg protein	17.9 ± 1.0	28.6 ± 1.5*	22.4 ± 0.7*	18.2 ± 0.6	68.5 ± 1.9*	76.3 ± 2.0*
pmoles/mg total lipids	325 ± 16	348 ± 20*	644 ± 11*	334 ± 21	978 ± 61*	1089 ± 66*
($\alpha + \gamma$) tocopherols						
μM	16.1 ± 1.1	18.7 ± 0.6	13.9 ± 2.0	16.5 ± 1.2	9.8 ± 0.5*	9.1 ± 0.2*
pmoles/mg total lipids	4025 ± 133	4968 ± 84*	2106 ± 71*	3928 ± 107	1422 ± 61*	1404 ± 56*
Retinol						
μM	1.7 ± 0.1	1.8 ± 0.2	1.8 ± 0.3	1.7 ± 0.0	1.6 ± 0.2	1.5 ± 0.1
pmoles/mg total lipids	426 ± 24	285 ± 14*	273 ± 18*	407 ± 33	228 ± 21*	231 ± 12*
Oxidized glutathione (GSSG)						
nmoles/mg protein	65.5 ± 5.1	71.0 ± 4.2	54.3 ± 2.0*	68.6 ± 4.0	143.4 ± 3.0*	135.8 ± 2.7*
Reduced glutathione (GSH)						
nmoles/mg protein	833.0 ± 34.3	1041.6 ± 45.8*	841.5 ± 30.5	944.1 ± 42.1*	830.0 ± 55.0	839.4 ± 5.3
GSH/GSSG						
	12.7 ± 0.2	14.7 ± 0.2	15.5 ± 0.3	13.8 ± 0.4	5.8 ± 0.2*	6.2 ± 0.2*
Conjugated dienes						
ODU/mg total lipids	0.03 ± 0.0	0.03 ± 0.01	0.04 ± 0.01	0.02 ± 0.0	0.12 ± 0.01*	0.15 ± 0.01*
β-carotene + glutathione						
μM	1.5 ± 0.2	1.6 ± 0.4	1.3 ± 0.3	1.4 ± 0.3	0.8 ± 1*	0.7 ± 0.1*
pmoles/ mg total lipids	375 ± 18	413 ± 11*	217 ± 24*	334 ± 28	114 ± 9*	108 ± 12*
Ascorbate						
μM	35.1 ± 1.3	44.6 ± 0.8*	32.3 ± 1.0	33.7 ± 0.4	27.4 ± 0.5*	26.0 ± 0.7*
pmoles/mg protein	468.0 ± 12.4	594.7 ± 15.7*	431.0 ± 16.4	443.4 ± 11.3	355.8 ± 10.2*	342.1 ± 9.8*

Biomarkers were determined according to the methods described in ''Materials and Methods.'' Results were calculated in different ways and are expressed as the mean ± 1 standard error of the mean (SEM) of six independent determinations assayed in duplicate or triplicate

C control, *Arg* L-arginine, *L-NAME* Lω-nitro-L-arginine methyl ester, *DIS* isosorbide dinitrate

*Significantly different with respect to the corresponding control value ($P < 0.01$)

modified. L-NAME supplementation produced increased total SOD activity at the expense of the cytosolic isoform, with no changes in the other enzymes assayed. Modifications observed for Arg or L-NAME were abolished by combination of these two drugs. DIS and DIS + L-NAME groups showed significant increments in SOD isoforms, GSHPx, and GSHRd activities with concomitant reduction of CAT activity.

Lipid Metabolism Parameters

Enzymatically determined total cholesterol and HDL cholesterol in plasma and liver microsomal suspensions are shown in Table 6. Total cholesterol was increased by supplementation with Arg, L-NAME, DIS, or DIS + L-NAME. HDL cholesterol was reduced by DIS treatment either alone or in combination with L-NAME. As a result, the ratio HDL/total cholesterol was decreased in the Arg-, L-NAME-, DIS-, and DIS + L-NAME-treated groups. In liver microsomes only DIS supplementation produced a significant increase in cholesterol content that could not be reverted by simultaneous supplementation with L-NAME.

Supplements that modified NO levels also affected the absolute and relative amounts of neutral (NL) and polar (PL) lipids in liver microsomes and plasmas (Table 7). Liver NL and PL were increased by Arg, L-NAME, DIS, or DIS + L-NAME. The ratio NL/PL was decreased in the L-NAME-supplemented group. The same experimental groups exhibited elevated

Table 3 Enzyme activities in plasma and erythrocytes from treated rats

Enzymes	Treatments					
	C	Arg	L-NAME	Arg + L-NAME	DIS	DIS + L-NAME
Superoxide dismutase (SOD)						
Erythrocytes	1843.5 ± 96.1	1197.3 ± 71.8*	2396.1 ± 153.5*	1806.9 ± 122.5	2673.8 ± 139.5*	2581.5 ± 135.6*
Catalase						
Erythrocytes	143.9 ± 12.2	98.5 ± 6.3*	193.3 ± 19.1*	135.0 ± 19.8	79.6 ± 6.1*	84.5 ± 7.7*
Glutathione peroxidase (GSHPx)						
Plasma	5.0 ± 0.4	4.2 ± 0.3	5.1 ± 0.3	4.7 ± 0.4	8.6 ± 0.5*	9.2 ± 0.6*
Erythrocytes	62.5 ± 10.4	59.7 ± 18.5	71.8 ± 11.8	66.4 ± 10.1	136.9 ± 15.1*	144.0 ± 17.1*
Glutathione transferase (GSHTr)						
Plasma	2.7 ± 0.2	1.9 ± 0.3	2.5 ± 0.2	3.1 ± 0.4	3.0 ± 0.3	2.9 ± 0.2
Erythrocytes	15.3 ± 0.8	15.8 ± 0.7	14.3 ± 1.1	15.6 ± 0.9	16.2 ± 0.9	14.9 ± 0.8
Glutathione reductase (GSHRd)						
Plasma	2.1 ± 0.1	1.8 ± 0.2	2.2 ± 0.2	3.0 ± 0.3	2.7 ± 0.3	2.4 ± 0.2
Erythrocytes	8.8 ± 0.7	10.3 ± 0.6	9.1 ± 0.8	9.9 ± 0.6	10.1 ± 1.0	9.5 ± 0.9

Enzyme activities were determined as described in ''Materials and Methods.'' Results are expressed in units/mg protein (plasma) or units/g hemoglobin (erythrocytes) except for catalase, which is expressed in k instead of units. Each value represents the mean ± 1 standard error of the mean (SEM) of six independent determinations assayed in duplicate

C control, *Arg* L-arginine, *L-NAME* Lω-nitro-L-arginine methyl ester, *DIS* isosorbide dinitrate

*Significantly different with respect to the corresponding control value ($P < 0.01$)

Table 4 Oxidative stress biomarkers in liver from treated rats

Biomarkers	Treatments					
	C	Arg	L-NAME	Arg + L-NAME	DIS	DIS + L-NAME
Lipid hydroperoxidases (ROOHs)						
pmoles/mg protein	155.6 ± 23.1	106.1 ± 12.0*	253.2 ± 17.4*	134.8 ± 19.2	340.7 ± 31.2*	329.7 ± 26.2*
pmoles/mg total lipids	389 ± 45	176.8 ± 21.7*	442 ± 23*	321 ± 50	486 ± 29*	508 ± 43*
Thiobarbituric-acid-reactive substance (TBARS)						
pmoles/mg protein	86.1 ± 5.3	53.0 ± 2.1*	177.6 ± 12.5*	70.5 ± 7.7	188.1 ± 18.3*	170.0 ± 23.4*
pmoles/mg total lipids	215 ± 11	88.3 ± 15.4*	296.5 ± 20.2*	170.0 ± 16.4	269.2 ± 24.4*	261.7 ± 34.6*
($\alpha + \gamma$) tocopherols						
pmoles/mg total lipids	1,505 ± 114	1349.1 ± 88.5*	920.6 ± 68.2*	1619.3 ± 106.7	741 ± 34*	809 ± 46*
Reduced glutathione (GSH)						
nmoles/mg protein	1,137 ± 72	1,095 ± 101	1,221 ± 135	1,088 ± 63	1,272 ± 66	1,304 ± 85
Oxidized glutathione (GSSG)						
nmoles/mg protein	53.0 ± 3.1	61.4 ± 4.3	40.5 ± 4.4*	60.0 ± 4.4	135.2 ± 4.0*	130.8 ± 5.1*
GSH/GSSG						
	21.5 ± 0.4	17.8 ± 0.3	30.1 ± 1.0*	18.1 ± 0.5	9.4 ± 0.2*	9.9 ± 0.3*
Conjugated dienes						
ODU/mg total lipids	0.06 ± 0.01	0.07 ± 0.01	0.110 ± 0.01*	0.06 ± 0.02	0.205 ± 0.03*	0.200 ± 0.02*
β-carotene + glutathione						
pmoles/mg total lipids	1,095 ± 154	1,281 ± 46*	700 ± 52*	1,023 ± 95	551 ± 67*	590 ± 54*
Ascorbate						
pmoles/mg protein	12.6 ± 0.9	12.8 ± 0.5	11.3 ± 0.6	12.4 ± 0.8	8.1 ± 0.1*	7.5 ± 0.2*

Biomarkers were determined according to the methods described in ''Materials and Methods.'' Results were calculated in different ways and are expressed as the mean ± 1 standard error of the mean (SEM) of six independent determinations assayed in duplicate or triplicate

C control, *Arg* L-arginine, *L-NAME* Lω-nitro-L-arginine methyl ester, *DIS* isosorbide dinitrate

*Significantly different with respect to the corresponding control value ($P < 0.01$)

Table 5 Enzymes activities in liver from treated rats

Enzymes	Treatments					
	C	Arg	L-NAME	Arg + L-NAME	DIS	DIS + L-NAME
SOD						
Total	26.3 ± 0.5	21.2 ± 0.5*	30.6 ± 0.7*	25.8 ± 0.7	35.4 ± 0.9*	37.2 ± 1.1*
Mitochondrial (Mn-SOD)	5.5 ± 0.2	4.2 ± 0.1*	5.7 ± 0.3	5.1 ± 0.2	7.2 ± 0.2*	6.9 ± 0.3*
Cytosolic (Cu ,Zn-SOD)	20.8 ± 0.4	17.0 ± 0.4*	24.9 ± 0.5*	20.7 ± 0.9	28.2 ± 0.8*	30.3 ± 0.5*
Catalase	0.9 ± 0.1	0.4 ± 0.1*	1.1 ± 0.2	0.9 ± 0.2	0.3 ± 0.05*	0.4 ± 0.1*
GSHPx	4.0 ± 0.2	3.8 ± 0.3	5.2 ± 0.3	4.1 ± 0.2	6.5 ± 0.3*	6.1 ± 0.2*
GSHTr	12.2 ± 0.3	13.1 ± 0.5	12.7 ± 0.6	10.1 ± 0.9	11.2 ± 0.6	10.7 ± 0.7
GSHRd	0.2 ± 0.03	0.2 ± 0.06	0.3 ± 0.03	0.2 ± 0.02	0.5 ± 0.10*	0.6 ± 0.20*

Enzyme activities were determined as described in ''Materials and Methods.'' Results are expressed in units per mg protein, except in the case of catalase, which are expressed in k/mg protein. Each value represents the mean ± 1 standard error of the mean (SEM) of six independent determinations assayed in duplicate

C control, *Arg* L-arginine, *L-NAME* Lω-nitro-L-arginine methyl ester, *DIS* isosorbide dinitrate, *SOD* superoxide dismutase, *GSHPx* glutathione peroxidase, *GSHTr* glutathione transferase, *GSHRd* glutathione reductase

*Significantly different with respect to the corresponding control value ($P < 0.01$)

Table 6 Cholesterol (CHO) content in plasma and liver microsomes from treated rats

Treatment	Plasma			Liver microsomes
	Total CHO	HDL-CHO	HDL/total	Total CHO
C	3.80 ± 0.10	2.40 ± 0.10	0.63 ± 0.01	0.14 ± 0.01
Arg	4.94 ± 0.20*	2.70 ± 0.15	0.55 ± 0.01*	0.16± 0.01
L-NAME	4.95 ± 0.04*	2.60 ± 0.10	0.52 ± 0.01*	0.13 ± 0.01
Arg + L-NAME	3.91 ± 0.06	2.80 ± 0.21	0.72 ± 0.03	0.14 ± 0.00
DIS	5.52 ± 0.03*	1.80 ± 0.10*	0.32 ± 0.01*	0.18 ± 0.02*
DIS + L-NAME	5.44 ± 0.02*	1.90 ± 0.12*	0.35 ± 0.01*	0.19 ± 0.02

Total cholesterol (CHO) and high-density lipoprotein (HDL)-CHO levels were determined enzymatically according to the method described in ''Materials and Methods.'' Results are expressed as nM concentration (plasma) or μmoles/mg protein (liver microsomal suspensions) and are the mean ± 1 standard error of the mean (SEM) of six independent determinations assayed in duplicate

C control, *Arg* L-arginine, *L-NAME* Lω-nitro-L-arginine methyl ester, *DIS* isosorbide dinitrate

*Significantly different from the corresponding control values ($P < 0.01$)

Table 7 Neutral and polar lipids of liver microsomes and plasmas from treated rats

Treatment	Liver microsomes			Plasma		
	Neutral lipids	Polar lipids	Neutral lipids/ polar lipids	Neutral lipids	Polar lipids	Neutral lipids/ polar lipids
C	0.07 ± 0.01	0.47 ± 0.03	0.15 ± 0.01	1.60 ± 0.01	3.01 ± 0.02	0.53 ± 0.02
Arg	0.09 ± 0.01*	0.66 ± 0.02*	0.13 ± 0.02	2.58 ± 0.02*	5.60 ± 0.04*	0.46 ± 0.02*
L-NAME	0.11 ± 0.01*	0.88 ± 0.02*	0.12 ± 0.01*	2.72 ± 0.03*	5.70 ± 0.05*	0.47 ± 0.01*
Arg + L-NAME	0.07 ± 0.00	0.50 ± 0.02	0.14 ± 0.02	1.62 ± 0.06	3.07 ± 0.03	0.53 ± 0.02
DIS	0.13 ± 0.02*	0.92 ± 0.02*	0.14 ± 0.02	2.91 ± 0.05*	6.85 ± 0.05*	0.42 ± 0.01*
DIS + L-NAME	0.12 ± 0.03*	0.89 ± 0.01*	0.13 ± 0.02	2.69 ± 0.08*	5.78 ± 0.04*	0.46 ± 0.02*

Liver microsomal suspensions and plasma from treated rats were extracted by the method of Folch et al. [56] and fractionated into NL and PL subclasses using microcolumn silicic acid partition as described in ''Materials and Methods.'' Results are expressed as nmoles/mg protein (microsomes) or nM concentration (plasma) and are the mean ± 1 standard error of the mean (SEM) of six independent analyses. Representative molecular weights were considered for calculations of neutral and polar lipid concentrations (800 and 880, respectively)

C control, *Arg* L-arginine, *L-NAME* Lω-nitro-L-arginine methyl ester, *DIS* isosorbide dinitrate

*Significantly with different respect to the corresponding control values ($P < 0.01$)

levels of NL and PL in plasma. The ratio NL/PL decreased significantly in Arg, L-NAME, DIS, and DIS + L-NAME groups.

Table 8 shows modifications in FFA and ketone bodies in plasma from treated rats. Increased production of NO by Arg, DIS, or DIS + L-NAME supplementation increased FFA concentration. Ketone bodies were elevated in DIS and DIS + L-NAME groups.

Some key enzymes of long-chain FA metabolism were measured under different experimental conditions (Table 9). Particulate PL-A_2 activity was diminished by the addition of L-NAME to control diet and elevated by DIS or DIS + L-NAME supplementation. Transport of FA into mitochondria was decreased by Arg, L-NAME, DIS, or DIS + L-NAME treatments. The rate of FA oxidation was also decreased in the same experimental groups. L-NAME supplementation increased acyl-CoA synthetase activity and decreased all FA desaturase activities measured. Groups treated with either DIS or DIS + L-NAME showed decreased activity of the fatty acid synthase (FAS) complex. We also analyzed the composition of fatty acyl chains in NL and PL from plasma and liver subfractions under the different experimental conditions. Figure 3 shows a typical fatty acyl pattern expressed in terms of FA ratios corresponding to PL in liver microsomal fractions. Similar modifications were observed in total lipids from plasma and liver homogenates and in NL from liver (data not shown). Monoenoic/saturated FA ratio was decreased in all experimental groups except the Arg-supplemented one with respect to control animals (which was taken as 0% change). The ratio of linoleic/arachidonic FA was significantly increased only in the L-NAME group, whereas the docosahaexenoic/docosapentaenoic ratio was elevated in all experimental groups.

Table 8 Free fatty acids (FFA) and ketone bodies (KB) in plasma from treated rats

Treatments	FFA (nM)	KB (μM)
C	0.63 ± 0.01	223 ± 8
Arg	0.74 ± 0.01*	211 ± 10
L-NAME	0.79 ± 0.02*	230 ± 7
Arg + L-NAME	0.61 ± 0.02	214 ± 9
DIS	0.98 ± 0.02*	288 ± 12*
DIS + L-NAME	0.96 ± 0.03*	295 ± 15*

FFA and KB were determined as described in "Material and Methods." Results are expressed as nmoles of palmitate/L (FFA) or μM of 3-OH-butyrate/L (KB), and are the mean ± 1 standard error of the mean (SEM) of six independent determinations assayed in duplicate

C control, *Arg* L-arginine, *L-NAME* Lω-nitro-L-arginine methyl ester, *DIS* isosorbide dinitrate

*Significantly different with respect to the corresponding control value ($P < 0.01$)

Calcium Levels in Liver and Plasma from Treated Rats

Atomic absorption measurements of [Ca^{2+}] in liver postmitochondrial supernatants and microsomal suspensions are shown in Table 10. Arg supplementation increased the concentration of calcium, whereas L-NAME produced a significant reduction. Combination of these supplements had no effect. DIS or DIS + L-NAME groups exhibited increased contents of [Ca^{2+}]. Similar results were obtained in crude homogenates (data not shown). In liver microsomes, Arg, DIS, or DIS + L-NAME treatments elevated calcium levels, whereas L-NAME alone caused depletion. Total (free + linked to proteins) circulating [Ca^{2+}] was not modified by any treatment (Table 10).

Discussion

Calcium Concentration and Oxidative Stress Biomarkers

It is well known that NO can both promote and inhibit lipid peroxidation [31]. By itself, it acts as an efficient scavenger of lipid peroxyl radicals induced by a variety of oxidants, thus preventing the accumulation of oxidative chain propagators [73, 74]. However, in the presence of superoxide, NO forms peroxynitrite, which is a powerful oxidant capable of initiating lipid peroxidation and destroying water and lipid-soluble antioxidants and even deactivating antioxidant enzymes [74, 75]. We demonstrated that feeding rats with L-NAME decreased [NOx] levels in both plasma and homogenates from several tissues. Previous experimental data proved that more than 90% of [NOx] is derived from nitric oxide synthase (NOS) activity and that the contribution of the diet to [NOx] level is negligible [75]. Considering that the mean life of [NOx] in biological fluids is very short, the anion level indicates recent status in the activity of NOS isoforms [75]. We also observed how the level of [NOx] was increased after Arg supplementation in a more physiological way than that produced under the administration of pharmacological doses of DIS. This is very important, as supplementation with Arg reduces atherosclerotic risk, decreases platelet aggregation, and inhibits monocyte adhesion to endothelial cells [76–79], all of which are promising effects with therapeutic

Table 9 Enzyme activities of lipid metabolism in liver from treated rats

Enzyme specific activities	Treatments					
	C	Arg	L-NAME	Arg + L-NAME	DIS	DIS + L-NAME
Phospholipase A_2 (dpm/min mg protein)						
Soluble	403 ± 21	388 ± 22	452 ± 30	393 ± 19	431 ± 49	425 ± 47
Particulate	4,606 ± 77	5,215 ± 103	3,001 ± 64*	4,477 ± 105	6,815 ± 98*	5,907 ± 106*
Carnitine-palmitoyl transferase nmoles/min mg protein	3.26 ± 0.18	2.61 ± 0.11*	2.40 ± 0.08*	3.35 ± 0.21	1.66 ± 0.05*	1.78 ± 0.12*
Fatty acid β-oxidation rate nmoles acetate/min mg protein	4.12 ± 0.20	3.37 ± 0.10*	2.88 ± 0.06*	4.23 ± 0.16	1.95 ± 0.14*	1.91 ± 0.05*
Acyl-coenzyme A (CoA) synthetase nmoles/min mg protein	177.3 ± 7.8	168.5 ± 23.3	233.6 ± 14.0*	184.1 ± 15.2	161.7 ± 19.6	172.8 ± 11.5
Fatty acid synthetase ODU/ min mg protein	0.33 ± 0.02	0.30 ± 0.03	0.28 ± 0.04	0.36 ± 0.04	0.19 ± 0.02*	0.24 ± 0.03*
Fatty acid desaturases (nmoles/min mg protein)						
Δ9 (16:0 to > 16:1)	0.37 ± 0.02	0.33 ± 0.04	0.22 ± 0.03*	0.31 ± 0.06	0.34 ± 0.03	0.31 ± 0.03
Δ6 (18:2 n-6 to > 18:3 n-6)	0.72 ± 0.04	0.79 ± 0.03	0.36 ± 0.04*	0.77 ± 0.10	0.78 ± 0.10	0.75 ± 0.05
Δ6 (18:3 n-3 to > 18:4 n-3)	0.61 ± 0.03	0.65 ± 0.02	0.28 ± 0.03*	0.56 ± 0.05	0.60 ± 0.02	0.68 ± 0.05
Δ5 (20:3 n-6 to > 20:4 n-6)	1.02 ± 0.05	1.08 ± 0.07	0.69 ± 0.04*	0.98 ± 0.08	1.10 ± 0.09	1.06 ± 0.05

Enzyme activities were determined as described in "Material and Methods." Results are expressed in the units indicated and represent the mean ± 1 standard error of the mean (SEM) of six independent determinations assayed in duplicate

C control, *Arg* L-arginine, *L-NAME* Lω-nitro-L-arginine methyl ester, *DIS* isosorbide dinitrate

*Significantly different with respect to the corresponding control value ($P < 0.01$)

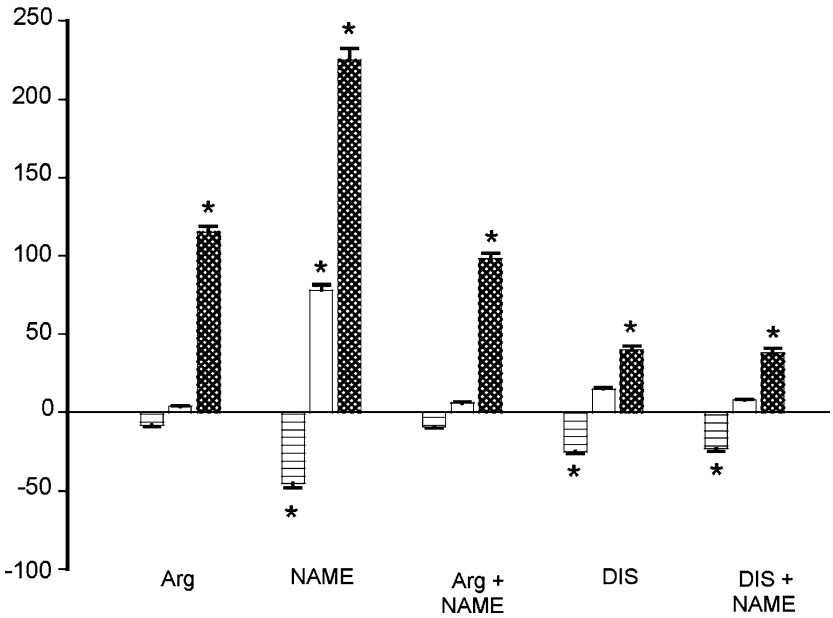

Fig. 3 Percent change [mean of percent ± 1 standard error of the mean (SEM) of six independent analyses assayed in duplicate] calculated from the content (namoles/mg protein) of selected fatty acids (FAs) of liver microsomal suspensions from rats fed different experimental diets. Within each grouped bars, the first one (horizontal pattern, *rectangle with vertical lines*) represents (16:1 + 18:1)/(16:0 + 18:0) FA, the second one (no pattern, *rectangle*) 18:2 (n-2)/20:4 (n-6), and the third one (crossing pattern, *rectangle with square*) 22:6 (n-3)/22:5 (n-6). (*) Significantly different with respect to control values, $P < 0.01$ or less

implications [80–83]. Considering the dual behavior of NO as a pro- and an antioxidant [31, 84], it is not surprising that inhibition of NO with L-NAME is currently considered a neuroprotective strategy without undesirable side effects [82, 83]. However, our study also demonstrated that alterations in NO levels may induce dangerous modifications in other components of the antioxidant defense system and in calcium concentration.

Although NO was first discovered as a mediator of vascular smooth relaxation, where it leads to a decrease in intracellular [Ca^{2+}] [85], other investigations carried out in several tissues demonstrated that treatments with NO and/or NO donors elicit increases in

Table 10 Calcium levels in liver and plasma from treated rats

Treatment	Liver		Plasma
	Postmitoch.	Microsomes	
C	1.48 ± 0.02	2.54 ± 0.03	2.81 ± 0.02
Arg	1.79 ± 0.01*	2.86 ± 0.03*	2.96 ± 0.02
L-NAME	1.04 ± 0.03*	1.41 ± 0.05*	2.70 ± 0.04
Arg + L-NAME	1.52 ± 0.06	2.61 ± 0.04	2.85 ± 0.05
DIS	1.96 ± 0.03*	3.17 ± 0.03*	2.94 ± 0.03
DIS + L-NAME	1.89 ± 0.04*	2.99 ± 0.02*	2.91 ± 0.05

Postmitochondrial (Post-Mitoch.) and microsomal suspensions from liver homogenates and plasma from treated rats were analyzed for calcium contents using atomic absorption spectrometry as described in ''Materials and Methods.'' Results are expressed as nmoles/mg protein (liver) or mM concentration (plasma) and are the mean ± 1 standard error of the mean (SEM) of six independent determinations assayed in duplicate

C control, *Arg* L-arginine, L-*NAME* Lω-nitro-L-arginine methyl ester, *DIS* isosorbide dinitrate

*Significantly different with respect to the corresponding control measurement ($P < 0.01$)

$[Ca^{2+}]$ [86]. This effect persists even in the absence of extracellular calcium and is dependent on ryanodine-sensitive calcium-release channels [86]. NO and peroxynitrite are responsible for $[Ca^{2+}]$ increase in the cytosolic compartment of various cell types [86–93]. Other reports suggested that peroxynitrite levels and, in general, NO availability determine calcium handling, especially from mitochondrial and endoplasmic reticulum stores [90, 91, 93]. Moreover, Berkels et al. [94] demonstrated a close correlation between NO and $[Ca^{2+}]$ by simultaneous detection using fluorometric probes. We found that there was a direct relationship between NO availability and $[Ca^{2+}]$ in liver homogenates, postmitochondrial supernatants, and microsomal suspensions. Perhaps this effect was produced through alterations in superoxide and/or peroxynitrite concentrations as suggested in previous reports [86, 88, 90, 93]. We observed unaltered total $[Ca^{2+}]$ in plasma. This may not be surprising and could be attributed to a compensatory mechanism that involves large calcium bone stores.

Modification in water- and lipid-soluble antioxidant levels may be an adaptive response to compensating peroxynitrite formation. It seems that there is an optimal concentration of NO that maintains antioxidant concentrations and GSH/GSSG ratio within the physiological range. Other authors suggested that there may be a relationship between NO availability and glutathione content [95, 96]. We also think that elevated NO may stimulate enzymes that synthesize glutathione, perhaps through a stimulatory effect of oxidative stress on γ-glutamyl-cysteinyl synthase activity as previously reported for the central nervous system [96]. Changes described for antioxidant contents were reflected in the ability of cytosol fractions for quenching the low-level chemiluminescence signal derived from Fe/ascorbate-induced lipid peroxidation. It is feasible to speculate that such an in vitro effect may correlate with similar actions displayed in vivo.

ROOHs and TBARS are usually used as indicators of lipid peroxidation. A slight increase in NO production exerted a protective effect in liver homogenates but not in plasma. Decreasing or significantly increasing NO generation led to an opposite effect that was more evident in liver homogenates and plasma. These changes agree with previously discussed pro- and antioxidant actions of NO [31, 72–75, 84].

SOD is one of the key enzymes for controlling excessive superoxide production in both cellular compartments (mitochondrial matrix and cytosol) through activity of their specific isoforms. Hydrogen peroxide generated by these isoenzymes is further reduced to water by GSH peroxidase and by catalase. Thus, the content of GSH and SOD are both considered the predominant antioxidant defense, at least in liver [52]. The relative importance of CAT as a scavenger of peroxide depends—in consequence—on the availability of GSH, and conversely, levels of GSH are affected by the specific activity of CAT. SOD and CAT are inducible by oxidative stress and incremented NO levels [52]. GSHRd is also incremented by elevation of the GSSG/GSH ratio. We found that SOD isoforms were elevated in both plasma erythrocytes and liver homogenates by high levels of NO. On the contrary, SOD activities decreased in liver homogenates and erythrocytes under a mild increment of NO. This phenomenon may be the consequence of a minor superoxide production due to the antioxidant effect of NO and, as discussed before, may involve therapeutic implications for NO donors [74–83]. Thus, when L-NAME suppressed NO production, the oxidative status derived from this event significantly increased SOD activity. Oscillations in the activities of SOD isoforms were also in accordance with changes in the GSH/GSSG ratio. There was an inverse correlation between this ratio and the SOD activity that was more evident in DIS-treated animals. Results obtained may be analyzed in different ways, and multiple comparisons can be performed. However, if we focus the impact evoked by dietary supplements on liver biomarkers, some key conclusions can be obtained. The ratios $[(GSHRd/GSHPx) \times 10^2]$, $[CAT/SOD) \times 10^2]$, and $[(GSHPx/SOD) \times 10^2]$ reflect the effort of the antioxidant defense system in maintaining the GSH/GSSG ratio between physiological values, neutralizing peroxide

overproduction, and reducing the formation of superoxide, respectively. Under excessive NO generation, the first ratio was increased significantly, as the stimulation of GSHRd observed in the DIS groups was insufficient to compensate for the level of SOD activity. Similarly, when NO levels were more physiological, CAT/SOD ratio was maintained at a mean value of 3.10. However, under excessive NO production, this ratio dropped to 0.90 because stimulation of CAT was unable to compensate for the SOD increase.

Lipid Metabolism and NO Availability

Diets that modified NO availability produced a major impact on lipid metabolism at both systemic and liver levels. Our findings concerning the changes in cholesterol content were in agreement with those previously reported by Khedara et al. [29, 30]. These authors suggested a relationship between cholesterol and the concentration of plasma nitrates. In addition, we found that the increase in plasma total cholesterol produced by either increased or decreased NO production was accompanied by elevated cholesterol levels in liver microsomes under DIS supplementation (not reverted by L-NAME). Interestingly, HDL cholesterol decreased in DIS groups and, as a result, a significant reduction in HDL/total cholesterol ratio was observed in experimental groups in which NO was decreased or increased by dietary manipulation. It is important to note that this finding was also observed under Arg or L-NAME treatment; both are currently used in human therapeutic approaches [74–83]. Our results suggest that careful consideration should be taken in prolonged administration of Arg or L-NAME in order to consider their real impact on one factors leading to hyperlipidemia and increased atherosclerotic risk. Hyperlipidemia induced by dietary manipulation was not mediated by renal dysfunction, as previous papers demonstrated that kidney size, serum creatinine, and albumin were not modified by prolonged administration of Arg or L-NAME [29, 30]. This study further demonstrated an important decrease in the NL/PL ratio especially observed in plasma under both NO depletion and increment of NO production. In these two conditions, alterations of the NL/PL ratio were the consequence of significant increments of both types of glycerolipids, with a predominant increase in PL concentration. These results showed for the first time that NO availability can simultaneously modify neutral and polar glycerolipid content in plasma and liver microsomes. Thus, in addition to the effects exerted on cholesterol metabolism, defective or excessive formation of NO should be considered a key point in atherosclerotic patients receiving drugs that directly (or indirectly) modify NOS activity. The underlying mechanism that justifies these modifications is an issue that remains to be elucidated. Our study demonstrated that increased or decreased NO levels in plasma and tissues correlated with increased contents of plasma FFA and, in the case of NO overproduction, with a concomitant elevation of plasma ketone bodies. Misbalance in NO production established an oxidative stress condition that led to impairment of FA oxidation. In agreement with this, we found significant decreases in carnitine-palmitoyl transferase activity and acetate formation as an indicator of β-oxidation rate.

Interestingly, FA desaturase activities were inhibited in the L-NAME-treated rats in which availability of NO was depressed. This finding may be justified by our previous report concerning the general inhibitory effect exerted by deprivation of $[Ca^{2+}]$ on FA desaturase enzymes [37]. Only L-NAME-treated animals underwent a constant low level of NO and exhibited diminished $[Ca^{2+}]$ in liver homogenates and microsomal suspensions. In the same experimental group, acylation of FFAs was stimulated by calcium deprivation in accordance with our previous findings [38]. PL-A_2 is another key enzyme for glycerolipid metabolism. We found a direct correlation between NO levels, $[Ca^{2+}]$, and PL-A_2 activity. In the group in which $[Ca^{2+}]$ was decreased, PL-A_2 was inhibited, and conversely, increased NO levels and $[Ca^{2+}]$ stimulated PL-A_2 activity. This question is crucially important from the physiological point of view, as liberation of polyunsaturated fatty acids (PUFAs) (especially arachidonate) from PL stores plays a key role under different pathological conditions associated with modifications in local or systemic NO concentrations [97, 98]. We observed that the lipogenic enzyme FAS was inhibited when NO was generated in excess. Previous studies from other laboratories demonstrated that this enzyme was unaffected under discrete modifications of NO or administration of NO inhibitors [29]. However, DIS-treated animals showed a significant reduction of FAS activity, probably exerted via elevation of peroxynitrite and $[Ca^{2+}]$ [99], and/or the strong accumulation of FFAs observed in these animals [100]. In addition, Mohr et al. [20] demonstrated that excessive NO formation inhibits glyceraldehydes-3-phosphate dehydrogenase. This fact may lead to depletion of the glycolytic route that in conjunction with the inhibitory effect exerted by NO on the Krebs cycle [22, 101] and mitochondrial respiration [13, 14, 102, 103] produced a general failure in availability of precursors and energy for FA synthesis.

All these modifications in lipid metabolism were reflected on the fatty acyl pattern of liver and plasma lipids. Alterations observed are difficult to analyze, as they depend on at least three factors: precursor desaturation–elongation rate, antioxidant influence, and selective β-oxidation (catabolism) and/or peroxidation (oxidative damage). In brief, we think that monoenoic/saturated FA ratio was diminished in the L-NAME group as a consequence of the inhibition of Δ9 desaturase activity (via decrease in NO and [Ca^{2+}] availability) [37]. In DIS-treated rats, a less important decrease in this ratio may be attributed to a relative accumulation of saturated FA, perhaps due to a minor β-oxidation rate (the extension of which exceeded that of FAS inhibition). Decreased activities of Δ6 and Δ5 FA desaturases may account for the elevated linoleic/arachidonic FA ratio observed in L-NAME-treated animals. Biosynthesis of 22:6 (n-3) is carried out in mitochondria and depends critically on carnitine and α-tocopherol levels [104]. In fact, this ratio was employed as an indicator of α-tocopherol deficiency in mitochondria [105]. We found that there was a direct correlation between vitamin E levels and nmoles of 22:6 (n-3) per mg protein. However, the amount of 22:5 (n-3) also decreased at different proportions among the experimental groups, resulting in the ratios observed. Decrease of 22:5 (n-3) by peroxidation was intense in DIS-treated animals (data not shown). However, they exhibited minor values in the 22:6 (n-3)/22:5 (n-6) ratios. This may be the consequence of the extensive drop in the amount of docosahexaenoic acid, which correlates with the important deficiency of α-tocopherol in these experimental groups. Alterations in the ratio of PUFAs acylated to glycerolipids in general and to PL in particular should be considered of physiological relevance from at least two points of view: modifications in physicochemical properties of biomembranes (and indirectly most of their functions) as well as misbalance in the production of eicosenoids derived from the n-6 family (proinflammatory and proaggregating factors) and n-3 homologs (antiaggregating and anti-inflammatory) [106].

Conclusions

Figure 4 depicts a general view of our findings. Oxidative damage can occur due to either increased or decreased NO levels through alterations in the antioxidant defense system (antioxidant depletion and/or modifications of antioxidant enzymes). Key enzymes in the lipid metabolism were also modified in a calcium- and NO-dependent way. A failure in the main producing energy routes was the consequence of changes in NO and in the oxidative-stress-derived condition. Decreased β-oxidation of FA was accompanied by increased production of ketone bodies and FFA concentration that may be channeled, at least in part, to

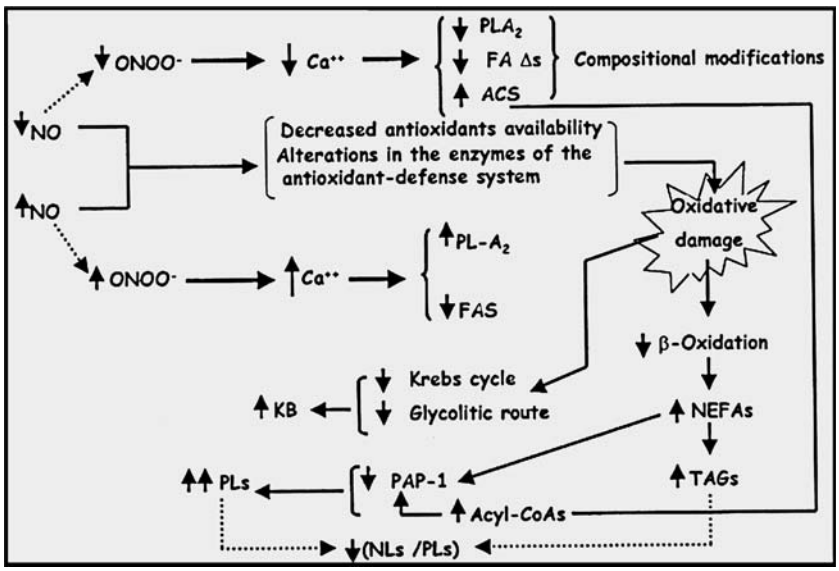

Fig. 4 General scheme showing the main changes evoked by oxidative stress due to increased or decreased nitric oxide (*NO*) levels. Alterations in the antioxidant defense system and in the activities of enzymes are also shown in relation to calcium concentration ([Ca^{++}]) and NO. *ACS* acyl-CoA-synthetase; *FA*Δ*s* fatty acid desaturases, *FA(s)* fatty acid(s), *FAS* fatty acid synthetase, *KB* Krebs cycle, *NEFAs* nonesterified fatty acids, *NLs* neutral lipids, *ONOO*$^-$ peroxynitrite, *PAP-1* phosphatidate-phosphohydrolase-1, *PL-A*$_2$ phospholipase A_2, *PLs* polar lipids (phospholipids), *TAGs* triacylglycerides

glycerolipid biosynthesis. Accumulation of FFA and increased acylation of CoA inhibited phosphatidate-phosphohydrolase-1 activity [107]. Such a condition resulted in more availability of phosphatidate to be transformed into glycerophospholipids. Increased PL biosynthesis overcomes that of NL and, as a result, LN/PL ratio decreased. This metabolic situation should be considered in detail when modifications of NO levels are implemented in an attempt to ameliorate the consequences of graft rejection, immune disorders, septic shock, and cardiovascular and neurological pathologies [78–85], among others [108].

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