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Original Contribution

EFFECT OF L-ARGININE–NITRIC OXIDE SYSTEM ON CHEMICAL-INDUCED DIABETES MELLITUS

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Abstract—Several in vitro studies have suggested that nitric oxide may be the mediator of cytokine-induced beta-cell destruction. On the other hand, in vivo studies have given conflicting results: some studies suggesting that nitric oxide synthase inhibitors do not suppress streptozotocin-induced diabetes in mice, while others revealed that nitric oxide synthase inhibitors can reduce the incidence of insulin-dependent diabetes mellitus in rats. The results of the present study indicate that alloxan-induced diabetes in the male Wistar rats can be abrogated to a large extent by prior and simultaneous administration of the precursor of nitric oxide, L-arginine, where as N^G-monomethy-L-arginine (L-NMMA), a specific inhibitor of nitric oxide synthase, can completely block the beneficial action of L-arginine. Sodium nitroprusside, a nitric oxide donor, also showed significant inhibitory effect on the severity of diabetes induced by alloxan. Alloxan treatment reduced nitric oxide generation, whereas L-arginine and sodium nitroprusside, when given along with alloxan, enhanced nitric oxide production to control values. Induction of diabetes by alloxan in the experimental animals was associated with a marked elevation in plasma lactate, ketone body, and lipid peroxide levels with a simultaneous fall in plasma insulin and nitric oxide levels. Alloxan-induced diabetes also induced a fall in the levels of anti-oxidant enzymes such as superoxide dismutase, glutathione reductase, and total glutathione, and antioxidants: vitamin E and ceruloplasmin, and an increase in glutathione peroxidase and glutathione-*S*-transferase. All these biochemical abnormalities and antioxidant levels have improved to near normal levels in animals treated with insulin, L-arginine, and sodium nitroprusside. From the results of the present study, it is apparent that L-arginine and nitric oxide can prevent alloxan-induced beta-cell damage, and the development of diabetes, and restore the antioxidant status to near normal levels. © 1998 Elsevier Science Inc.

Keywords—Nitric oxide, L-Arginine, Insulin-dependent diabetes mellitus, Free radical, Antioxidants, Insulin

INTRODUCTION

Several in vitro studies showed that cytokines produced by the immune system cells infiltrating pancreatic islet cells can cause islet beta-cell destruction leading to the onset of insulin-dependent diabetes mellitus (IDDM) [1,2]. In a rat insulinomacellline (RINm5F), cytokine combination of interleukin-1 beta (IL-1 beta), tumor necrosis factor (TNF), and interferon-gamma (IFN-gamma)-induced DNA fragmentation, mitochondrial damage and death of RIN cells[1,2]. Glucose-induced insulin secretion is known to be inhibited by the cytokines IL-1 beta, IL-6, and TNF-alpha in cultured rat islets, by IL-1 beta, TNF, and IFN-gamma in mouse islets, and by combined treatment of IL-1 beta, TNF, and IFN-gamma in human islets [3]. Continued cytokine treatment in many cases leads to the destruction of islet cells. Subsequent studies as to the mechanism(s) involved in cytokine-induced beta-cell destruction led to the suggestion that nitric oxide (NO) could be the ultimate mediator of Islet beta cell damage [4–6]. It is known that TNF and other cytokines can enhance the production of NO, which is believed to be responsible for the destruction of beta cells because nitric oxide inhibitors can prevent the cytotoxic action of the cytokines.

In the mononuclear leukocytes infiltrating the pancreatic islets of autoimmune diabetes-prone nonobese diabetic (NOD) female mice, mRNA levels of inducible nitric oxide synthase (iNOS), IL-1 alpha, and IFN-gamma were found to be increased from 5 weeks of age

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to onset of diabetes (>13 weeks of age) [7]. Further, iNOS, IL-1 alpha and IFN-gamma mRNA levels were found to be higher in the mononuclear leukocytes isolated from the islets of diabetes-prone NOD female mice but not in the leukocytes of 12-week-old mice with low diabetes risk, NOD female mice injected with complete Freund's adjuvant at 4 weeks of age, NOD male mice or BALB\C female mice that do not develop diabetes [7]. These findings clearly suggest that IL-1 alpha and IFN-gamma may promote islet beta cell destruction atleast in part by upregulating iNOS expression or it is also likely that this enhanced iNOS expression may be a defensive and compensatory event in response to the inflammatory stress induced by cytokines.

Further support to the role of cytokines and NO in IDDM is obtained from the results of the studies performed by Kaneto et al. [8], who showed that nitric oxide induces apoptotic cell death of pancreatic beta cells in vitro. These in vitro studies are further strengthed by the work of Zunino et al., who showed that IL-1 promotes hyperglycaemia and insulitis in mice normally resistant to streptozotocin (STZ)-induced diabetes [9]. In addition, Sandborg et al. [10] showed that IL-1 receptor antagonist prevents low dose STZ induced diabetes in mice, suggesting a role for IL-1 in this in vivo model of IDDM.

It is also important to note that this presumed role of cytokines and NO in the induction of IDDM is not without controversy. For instance, it was reported that the adminitration of nitric oxide synthase inhibitor, N-nitro-L-arginine-methyl ester (NAME), does not suppress low dose streptozotocin-induced diabetes in mice [11]. On the other hand, Lindsay et al. [12] demonstrated that N-omega-nitro-l-arginine methyl ester, an inhibitor of NO synthase, reduces the incidence of IDDM in BB/E rats indicating that NO has a role in the destruction of pancreatic beta cells. Results reported by Lukic et al. [13] and Catanzaro et al. [14], which are supportive to each other, showed that NO synthase inhibition does indeed affects the induction of diabetes by streptozotocin in mice, and that streptozotocin-induced hyperglycemia can be decreased by NO inhibition, respectively. These results indicate that perhaps NO can modulate the cytotoxic action of STZ on beta cells.

Recently, we noted that oral administration of L-arginine, the precursor of NO, can enhance insulin secretion and reduce hyperglycemia, and that these beneficial actions are associated with increased formation of NO in patients with NIDDM [15]. These results suggested to us that in all probability increased formation of NO that may occur following L-arginine administration is beneficial to patients of diabetes mellitus, and perhaps it may protect the beta-cells of the pancreas. In view of these controversial results with regard to the role of NO in DM, we performed the studies reported here to resolve the role of the L-arginine-nitric oxide system in beta cell function and in IDDM.

MATERIALS AND METHODS

Male Wistar rats 4–5 weeks old were used in the present study.

Sources of chemicals

All the fine chemicals used in this study were obtained from Sigma Chemical Company, St. Louis, MO.

Induction of diabetes mellitus

Alloxan was used to induce damage to the pancreatic beta-cells and produce hyperglycemia and IDDM. The experimental and control animals were kept fasting for 12 h prior to the administration of alloxan. Freshly prepared alloxan in 50 mM citrate buffer, PH.3, was injected intraperitoneally to these animals at a dose of 75 mg/kg of body weight per day for 5 consequtive days. Both blood sugar and urine sugar was estimated once in 3 or 4 d, after the last dose of alloxan, in these animals by glucometer to confirm the development of diabetes mellitus. Usually these animals developed IDDM in about 2 to 3 weeks time after the first injection of alloxan. These animals showed weight loss (due to uncontrolled diabetes), severe hyperglycemia (fasting blood sugar >275 mg/dl), and a clear decline in the plasma levels of insulin. On an average, almost all the animals that received alloxan developed IDDM in our hands. To determine the role of the L-arginine–nitric oxide system in the development of IDDM, one group ($n = 10$) of animals received 24 h prior to alloxan treatment 50 mg of L-arginine, the precursor of NO, in 0.5 ml normal saline intraperitoneally. The next 5 d these animals received alloxan as usual. This group is called the prearginine group.

To another group of 10 animals, simultaneous intraperitoneal injections of 50 mg of L-arginine in 0.5 ml of normal saline and alloxan in 0.5 ml of 50 mM citrate buffer pH 3.0 was given for 5 consecutive days. This group is called as the simultaneous L-arginine and alloxan group.

To determine whether inhibition of NOS will have any effect on the development of IDDM, one group of 10 animals received intraperitoneally, freshly prepared L-NMMA (20 mg/kg) in 0.5 ml of normal saline and 50 mg of L-arginine simultaneously 24 h prior to alloxan treatment. In addition, another group of 10 animals received intraperitoneally L-NMMA (20 mg/kg), 50 mg of L-arginine and alloxan at the same time per day for 5

consecutive days. As a control, another group of 10 animals received L-NMMA and alloxan intraperitoneally at the same time for 5 consecutive days.

In a similar fashion, the NO donor, sodium nitroprusside, was also used in these studies. Similar to L-NMMA and L-arginine, sodium nitroprusside was administered to the respective groups of animals 24 h prior to alloxan treatment and simultaneously with alloxan for 5 consecutive days. For all the studies, appropriate controls were also used. Details of the experimental protocol used in the present study is given in Fig. 1.

Insulin treatment

For the control of IDDM, subcutaneous injections of intermediate acting zinc insulin (in the form of human monotard, Novo Nordisk, India) was administered at a dose of 4 units twice a day (at 0800 and 1600 h). As a result of this insulin injection, the fasting blood sugar that was around 275 mg/dl has dropped to less than 120 mg/dl in these animals. Hyperglycemia in this group of animals was thus controlled by insulin for a minimum period of 8 d and were killed on day 9, for further studies.

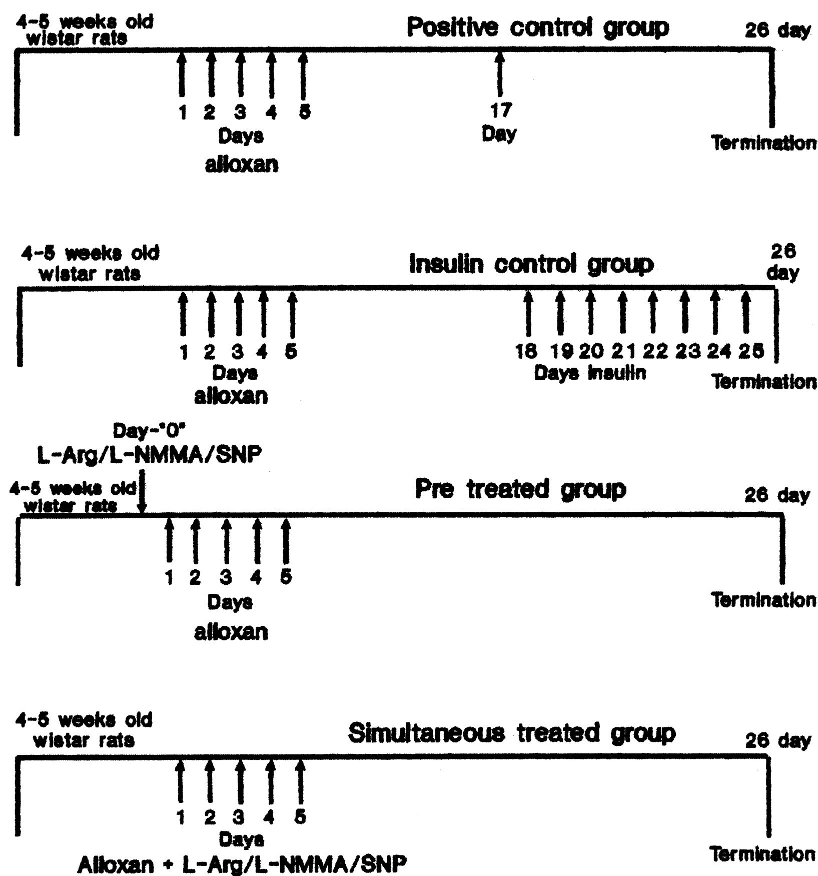

Fig. 1. Scheme of experimental protocol.

Table 1. Effect of Alloxan, L-Arginine, and L-NMMA Treatment on Plasma Glucose, Lactate, Aceto Acetate (Ketone Body) and Insulin Levels and on Body Weight and Survival in Wistar Rats

Treatment	Bl. Sugar (mg/dl)	Lactate (mg/dl)	Aceto Acetate (mg/dl)	Insulin (μU/ml)	Weight (g)	Surv. Rate (%)
Saline control (n = 10)	73.4 ± 5.6	42.5 ± 10.8	2.3 ± 0.48	45.4 ± 7.4	94.8 ± 4.2	100
L-Arginine (n = 10)	71.6 ± 6.8	43.6 ± 10.1	2.4 ± 0.51	42.2 ± 5.4	96.0 ± 3.5	100
L-NMMA (n = 10)	94.2 ± 9.8*	41.9 ± 8.3	2.6 ± 0.52	36.5 ± 5.3	102 ± 6.2	100
Alloxan (n = 10)	434.9 ± 86*	73.0 ± 10.5*	37.7 ± 26*	25.3 ± 11*	82.0 ± 3.1*	10
Insulin (n = 10)	110.7 ± 7.7*†	46.2 ± 6.8†	2.7 ± 0.67†	—	91.3 ± 8.7	100
Pre-L-Arg + allox (n = 10)	285.5 ± 68*†	61.3 ± 11.7*	20.6 ± 16*†	30.6 ± 10*	88.2 ± 2.5*	40
Simt. Arg + allox. (n = 10)	273.1 ± 83*†	58.6 ± 9.8*†	21.9 ± 15*†	31.8 ± 11*	89.4 ± 3.2†	60
Pre-Arg + L-NMMA & alloxan (n = 10)	384.3 ± 84*	69.1 ± 9.3*	32.5 ± 10*	21.3 ± 4.8*	85.0 ± 6.2	10
Simt. Arg + L-NMMA & alloxan (n = 10)	391.5 ± 70*	71.5 ± 7.5*	33.3 ± 8.7*	20.1 ± 3.7*	82.5 ± 4.8*	10
L-NMMA + alloxan (n = 10)	400.2 ± 36*	70.1 ± 10.5*	35.0 ± 15*	20.6 ± 5.0*	83.8 ± 4.4*	10

*$p \leq .05$ compared with control.
†$p \leq .05$ compared with alloxan-treated group.

Estimation of blood sugar

Plasma glucose levels were estimated by the glucose oxidase–peroxidase method using the commercially available kit obtained from Boehringer Mannheim, Germany [15].

Estimation of insulin levels

Plasma insulin levels were estimated by the Elisa method using the commercially available Elisa kits obtained from Boehringer Mannheim, Germany (Enzymuntest system, on ES 33 analyzer).

Estimation of acetoacetic acid in the urine

Multiple reagent strips of Miles India Ltd. were used for the estimation of urine ketone body, acetoacetic acid based on the principle that when acetoacetic acid reacts with nitroprusside the original buff pink (which indicates negative reaction) converts to purple.

Estimation of lactate

Plasma levels of lactate were measured by using a commercially available kit obtained from Boehringer Mannheim, Germany [16].

Estimation of nitric oxide as its metabolite nitrite

Nitric oxide decomposes rapidly in aerated solutions to form stable nitrite/nitrate products. In our study, nitrite concentrations were determined and used as an index of NO synthesis. Nitrite was quantified colorimetrically after its reaction with the Greiss reagent as described earlier [15,17].

Table 2. Effect of Sodium Nitropusside (SNP) on Alloxan-Induced IDDM and on Various Biochemical Parameters and Body Weight and Survival of Wistar Rats

Treatment	Bl. Sugar (mg/dl)	Lactate (mg/dl)	Aceto Acetate (mg/dl)	Insulin (μU/ml)	Weight (g)	Survi. Rate (%)
Control (n = 10)	76.62 ± 6.65	38.1 ± 4.5	2.50 ± 0.5	36.9 ± 6.1	98.7 ± 4.5	100
Alloxan (n = 10)	442.0 ± 37.6*	62.7 ± 8.7*	36.8 ± 9.2*	22.5 ± 6.0*	84.7 ± 5.7*	0
Insulin (n = 10)	107.1 ± 8.25*	41.0 ± 3.9†	2.7 ± 0.46	—	96.0 ± 6.4†	100
SNP 2 μg (n = 10)	75.50 ± 7.58	40.4 ± 10	2.4 ± 0.51	36.3 ± 5.6	97.2 ± 7.3	100
SNP 5 μg (n = 10)	73.83 ± 7.70	39.5 ± 5.7	2.6 ± 0.52	38.5 ± 6.5	98.6 ± 5.9	100
SNP 10 μg (n = 10)	77.00 ± 6.80	38.3 ± 5.0	2.4 ± 0.52	35.9 ± 6.6	99.2 ± 6.2	100
Pre-SNP 2 μg + alloxan (n = 10)	360.4 ± 36.2*	57.6 ± 8.0*	30.6 ± 6.2*	25.9 ± 8.5	87.6 ± 6.2*	37.5
Pre-SNP 5 μg + alloxan (n = 10)	336.5 ± 39.0*†	54.1 ± 6.4*†	21.2 ± 7.9*†	28.6 ± 6.0*†	90.2 ± 7.5*	62.5
Pre-SNP 10 μg + alloxan (n = 10)	343.8 ± 39.8*†	55.3 ± 5.5*	23.7 ± 8.3*†	27.9 ± 7.1*	88.6 ± 7.9*	50
Simult. SNP 2 μg + alloxan (n = 10)	310.7 ± 22.2*†	55.6 ± 6.0*	27.5 ± 8.3*†	24.9 ± 5.2*	91.2 ± 7.7	75
Simult. SNP 5 μg + alloxan (n = 10)	277.2 ± 36.6*†	50.4 ± 7.6*†	18.7 ± 5.1*†	30.1 ± 7.9	94.5 ± 6.8†	87.5
Simult. SNP 10 μg + alloxan (n = 10)	289.0 ± 39.8*†	51.5 ± 6.6*†	21.2 ± 6.9*†	26.7 ± 6.6*	92.2 ± 4.5*	75

*$p \leq .05$ compared with control.
†$p \leq .05$ compared with alloxan-treated group.

Table 3. Effect of Alloxan, L-Arginine, and L-NMMA on the Levels of Lipid Peroxides and Nitric Oxide in Wistar Rats

Treatment	Lipid Peroxides (nm)	Nitric Oxide (μM)
Saline control (n = 10)	1.63 ± 0.50	1.03 ± 0.25
L-Arginine (n = 10)	1.59 ± 0.10	1.40 ± 0.17
L-NMMA (n = 10)	1.65 ± 0.31	0.76 ± 0.09
Alloxan (n = 10)	2.41 ± 0.78*	0.51 ± 0.11*
Insulin (n = 10)	1.72 ± 0.44	1.14 ± 0.18†
Pre Arginine + alloxan (n = 10)	1.65 ± 0.26	1.12 ± 0.16†
Simult. Arginine + alloxan (n = 10)	1.74 ± 0.31	1.28 ± 0.31†
Pre Arg + L-NMMA + alloxan (n = 10)	2.21 ± 0.55*	0.61 ± 0.09*
Simult. Arg + L-NMMA + allox. (n = 10)	2.31 ± 0.57*	0.53 ± 0.07*
L-NMMA + alloxan (n = 10)	2.46 ± 0.49*	0.59 ± 0.09*

*$p \leq .05$ compared with control.
†$p \leq .05$ compared with alloxan-treated group.

Lipid peroxidation products

The total amount of lipid peroxidation products present in the plasma samples of the controls and experimental animals was estimated using the thiobarbituric acid (TBA) method [18], which measures the malondialdehyde (MDA) reaction products and hence is referred to as MDA-eq (MDA-equivalents) substances. 1,1,3,3-Tetraethoxy propane (TEP) was used as the standard.

Estimation of antioxidants and antioxidant enzymes

Estimation of plasma urate. This is done by using a commercially available kit obtained from Pinnacle marketing Pvt. Ltd, Hyderabad, India. This test is based on the principle that uric acid is oxidized by uricase to form allantoin and hydrogen peroxide. The hydrogen peroxide, when reacted with 3,5-dichloro-2-hydroxy benzene sulfonate and 4-amino antipyrine, produces a colored chromogen that can be measured at 520 nm.

Estimation of ceruloplasmin (E.C. No. 1.16.3.1.). Plasma levels of ceruloplasmin can be determined using an enzymatic method, which measures the oxidase activity of ceruloplasminin the presence of P-phenylene diamine dihydrochloride. Ceruloplasmin oxidizes P-phenylene diamine dihydrochloride in the presence of oxygen to form a purple product that can be measured at 530 nm.

Estimation of SOD, catalase, glutathione peroxidase, glutathione reductase, total glutathione, and vitamin E

The levels of various antioxidants such as superoxide dismutase (SOD,E.C. No. 1.15.1.1.), catalase (E.C. No. 1.11.1.6), glutathione peroxidase (E.C. No. 1.11.1.9), glutathione reductase (E.C.No. 1.6.4.2), glutathione-*S*-transferase (E.C. No. 2.5.1.18), and vitamin E in the RBC lysate of the experimental animals were determined as described earlier [19–21].

Statistical analysis

All the biochemical parameters mentioned above were measured in the blood samples obtained from the treated animals on day 26, i.e., at the time of termination of the study (see the Scheme of experimental protocol). All values obtained were expressed as mean ± SD. The statistical analysis of the results was done by Student's *t*-test.

RESULTS

Development of IDDM and survival of the animals

L-Arginine treatment. As already mentioned, all the animals that received alloxan developed IDDM as expected (Tables 1 and 2). In the alloxan-alone–treated group, the plasma blood glucose levels were high (about 430 mg/dl) and plasma insulin levels were low, and when were not treated with insulin for the control of diabetes they died by day 8

Table 4. Effect of Alloxan, L-Arginine, and L-NMMA Treatment on the Concentrations of Various Antioxidants in the Wistar Rats

Treatment	SOD (U/g Hb)	Catalase (KU/g Hb)	Vitamin E (μg/g Hb)	Ceruloplasmin (mg/dl)	Urate (mg/dl)
Control (n = 10)	2375 ± 250	24.2 ± 3.5	5.11 ± 0.84	26.46 ± 3.2	0.81 ± 0.11
L-Arginine (n = 10)	2355 ± 260	24.3 ± 2.5	4.78 ± 0.53	25.9 ± 1.29	0.78 ± 0.15
L-NMMA (n = 10)	2260 ± 297	26.5 ± 2.1	4.87 ± 0.72	24.96 ± 2.36	0.85 ± 0.11
Alloxan (n = 10)	1835 ± 285*	26.6 ± 2.3	3.66 ± 0.63*	19.5 ± 1.20*	0.91 ± 0.15
Insulin (n = 10)	2323 ± 195†	25.4 ± 1.7	4.74 ± 0.82†	24.75 ± 3.27†	0.86 ± 0.10
Pre-L-Arg + allox. (n = 10)	1845 ± 425*	27.0 ± 4.9	4.10 ± 1.0	21.4 ± 2.90	0.68 ± 0.12†
Simult. Arg + allox (n = 10)	2120 ± 345	24.8 ± 3.2	4.44 ± 0.74	28.48 ± 2.89	0.69 ± 0.18†
Pre-Arg + L-NMMA & alloxan (n = 10)	1755 ± 216*	25.7 ± 1.2	4.03 ± 0.65*	19.65 ± 1.34*	0.92 ± 0.15
Simlt. Arg + L-NMMA & alloxan (n = 10)	1695 ± 384*	26.1 ± 2.1	3.72 ± 0.44*	18.85 ± 1.48*	0.93 ± 0.09
L-NMMA + alloxan (n = 10)	1707 ± 205*	25.6 ± 1.1	3.78 ± 0.58*	20.66 ± 1.12*	0.90 ± 0.11

*$p \leq .05$ compared with control.
†$p \leq .05$ compared with alloxan-treated group.

Table 5. Effect of Alloxan, L-Arginine, and L-NMMA Treatment on the Concentrations of Glutathione Class Antioxidants in the Wistar Rats

Treatment	Glutatihone Peroxidase (μmol/NADPH/min/g Hb)	Glutathione reductase (U/g Hb)	Glutathione-*S*-transferase (μmol conj/min/g Hb)	Total Glutathione (μ mol/g Hb)
Control (n = 10)	19.76 ± 1.27	9.43 ± 0.93	2.46 ± 0.32	6.73 ± 1.39
L-Arginine (n = 10)	19.84 ± 3.04	9.17 ± 1.00	2.27 ± 0.34	6.56 ± 1.27
L-NMMA (n = 10)	22.98 ± 0.93	10.7 ± 0.96	2.99 ± 0.35	6.46 ± 0.98
Alloxan (n = 10)	30.01 ± 4.36*	5.47 ± 1.11*	3.61 ± 0.73*	4.78 ± 1.27*
Insulin (n = 10)	20.36 ± 2.70†	9.53 ± 1.07†	2.78 ± 0.34†	6.20 ± 1.36†
Pre Arginine + allox. (n = 10)	28.76 ± 6.10*	6.72 ± 0.80†	2.90 ± 0.81	5.00 ± 1.46
Simult. Arg + alloxan (n = 10)	24.95 ± 6.24	7.47 ± 0.97†	2.95 ± 0.43	5.56 ± 0.94
Pre-Arg + L-NMMA & alloxan (n = 10)	28.31 ± 1.22*	5.38 ± 0.97*	4.73 ± 0.50*†	5.14 ± 0.45*
Simult. Arg + L-NMMA & alloxan (n = 10)	29.16 ± 2.77*	5.14 ± 0.61*	5.05 ± 0.63*†	4.91 ± 0.70*
L-NMMA + alloxan (n = 10)	28.02 ± 1.59*	5.07 ± 0.82*	4.78 ± 0.45*†	4.81 ± 0.67

*$p \leq .05$ compared with control.
†$p \leq .05$ compared with alloxan-treated group.

due to diabetic keto-acidosis and lactic acidosis, as evidenced by severe hyperglycemia, increase in ketone bodies, and blood lactate levels (Tables 1 and 2). There was also a significant decrease in the weight of the animals in the alloxan treated group due to uncontrolled IDDM.

On the other hand, animals treated with insulin had near-normal plasma glucose levels, normal plasma lactate, and acetoacetic acid levels, and were normal in their body weight, and there were no deaths in this group (Tables 1 and 2).

Experimental animals that received L-arginine 24 h prior to and simultaneously along with alloxan also developed diabetes mellitus but was of much less severe degree, as evidenced by almost 50% lower blood sugar levels, significantly higher plasma insulin levels compared to the alloxan- alone–treated group, and lower plasma lactate and ketone body levels (Table 1). The animals in the prearginine and simultaneous arginine + alloxan treated groups showed significantly less loss of weight compared to the alloxan-alone–treated group. Even their survival rate (L-arginine + alloxan-treated groups) was better compared to the alloxan-treated group, even though they have not received insulin. These results suggest that L-arginine can prevent the development of diabetes and severity of diabetes to a significant degree, possibly by protecting the pancreatic islet cells from damage.

Because L-arginine forms the precursor to NO, and as uncontrolled diabetes is associated with increase in free radical generation and lipid peroxidation process, we measured the plasma levels of lipid peroxides and NO in these groups of animals. As shown in Table 3, the levels of NO were low and lipid peroxides were high in the alloxan-treated group compared to the control and alloxan + arginine groups. On the other hand, in the two L-arginine + alloxan-treated groups both nitric oxide and lipid peroxide levels were maintained at near-normal levels. These results suggest that L-arginine treatment can restore nitric oxide levels and lipid peroxidation process to normalcy. It can be seen from the results shown in Table 3 that in the L-arginine control group the nitric oxide concentrations tended to be at a higher level. This may be due to the increased formation of NO. These results also indicate that induction of diabetes can cause a fall in the synthesis of NO, as is seen in the present study in the alloxan-treated group that developed IDDM.

L-NMMA treatment

To confirm the results obtained with L-arginine treatment, we also performed studies with L-NMMA, a specific inhibitor of NO. It can be seen from the results given in Table 1 that L-NMMA treatment prevents the beneficial affect of L-arginine, because IDDM seen in

Table 6. Effect of Alloxan and Sodium Nitroprusside (SNP) Treatment on the Levels of Plasma Lipid Peroxides and Nitric Oxide in Wistar Rats

Treatment	Lipid Peroxides (nm)	Nitric Oxide (μM)
Control (n = 8)	1.56 ± 0.51	1.13 ± 0.19
Alloxan (n = 8)	2.27 ± 0.59*	0.54 ± 0.23*
Insulin (n = 8)	1.61 ± 0.51	1.06 ± 0.16†
SNP 2 μg (n = 8)	1.59 ± 0.21	1.27 ± 0.14
SNP 5 μg (n = 8)	1.55 ± 0.18	1.49 ± 0.15
SNP 10 μg (n = 10)	1.62 ± 0.16	1.32 ± 0.19
Pre-SNP 2 μg + alloxan (n = 10)	2.15 ± 0.48	0.79 ± 0.28†
Pre-SNP 5 μg + alloxan (n = 10)	2.02 ± 0.36	1.10 ± 0.28†
Pre-SNP 10 μg + alloxan (n = 10)	2.05 ± 0.36*	1.09 ± 0.20†
Simult. SNP 2 μg + alloxan (n = 10)	2.09 ± 0.44	1.15 ± 0.19†
Simult. SNP 5 μg + alloxan (n = 10)	1.83 ± 0.34	1.47 ± 0.19*†
Simult. SNP 10 μg + alloxan (n = 10)	1.89 ± 0.32	1.31 ± 0.23†

*$p \leq .05$ compared to control.
†$p \leq .05$ compared to alloxan-treated group.

Table 7. Effect of Alloxan and Sodium Nitroprusside (SNP) Treatment on the Concentrations of Various Antioxidants in the Wistar Rats

Treatment	SOD (U/g Hb)	Catalase (KU/g Hb)	Vitamin E (μg/g Hb)	Ceruloplasmin (mg/dl)	Urate (mg/dl)
Control (n = 8)	2670 ± 202	27.05 ± 1.99	4.92 ± 0.89	27.05 ± 2.67	0.82 ± 0.12
Alloxan (n = 8)	1996 ± 254*	26.92 ± 2.03	3.21 ± 0.92*	19.38 ± 1.41*	0.96 ± 0.16
Insulin (n = 8)	2573 ± 211†	27.87 ± 1.25	5.00 ± 0.65†	25.85 ± 1.89†	0.86 ± 0.11
SNP 2 μg (n = 8)	2655 ± 321	27.46 ± 1.83	5.03 ± 0.58	27.98 ± 2.31	0.85 ± 0.12
SNP 5 μg (n = 8)	2742 ± 131	25.73 ± 3.44	4.90 ± 0.87	26.90 ± 0.81	0.79 ± 0.07
SNP 10 μg (n = 8)	2646 ± 147	27.50 ± 1.56	5.02 ± 0.67	26.43 ± 1.03	0.84 ± 0.09
Pre-SNP 2 μg + alloxan (n = 8)	2141 ± 403*	26.27 ± 1.80	3.57 ± 0.92*	20.06 ± 1.69*	0.92 ± 0.13
Pre-SNP 5 μg + alloxan (n = 8)	2191 ± 261*	27.02 ± 1.60	3.95 ± 0.71*	21.41 ± 2.18*†	0.89 ± 0.12
Pre-SNP 10 μg + alloxan (n = 8)	2106 ± 233*	26.77 ± 1.93	3.85 ± 0.89†	21.13 ± 2.04*†	0.90 ± 0.13
Simult. SNP 2 μg + alloxan (n = 8)	2209 ± 221*†	26.70 ± 1.43	4.26 ± 0.86†	22.02 ± 2.58*†	0.89 ± 0.14
Simult. SNP 5 μg + alloxan (n = 8)	2448 ± 205*†	27.22 ± 1.43	4.63 ± 0.99†	23.30 ± 1.90*†	0.88 ± 0.12
Simult. SNP 10 μg + alloxan (n = 8)	2345 ± 156*†	26.65 ± 1.35	4.39 ± 0.86†	22.22 ± 2.53*†	0.93 ± 0.11

*$p \leq .05$ compared with control.
†$p \leq .05$ compared with alloxan-treated group.

these groups of animals was as severe as that observed in the alloxan control group. Further, prior and simultaneous L-NMMA treatment along with alloxan produced a significant increase in the plasma lactate, ketone body, and lipid peroxide levels and a fall in the plasma insulin and nitric oxide concentrations, which are almost comparable to the concentrations of these biochemical abnormalities seen in the alloxan-alone–treated control group. These results suggest that L-NMMA can completely block the beneficial actions of L-arginine in IDDM. It is also important to note that even in the L-NMMA control group, the plasma glucose levels were elevated to a significant degree, and the NO levels tended to be low in comparision to the control group. In addition, in all the three L-NMMA + alloxan-treated groups the body weight of the animals was found to be decreased due to IDDM, and mortality rates were similar to the alloxan-alone–treated group.

Antioxidant status in alloxan, L-arginine, and L-NMMA–treated animals

Because oxidant stress and antioxidants seem to play an important role in the pathobiology of diabetes mellitus and in the pathobiology of complications that occur in patients with diabetes mellitus, we studied the levels of various antioxidants in the present study. It is evident from these results (Tables 4 and 5) that experimental animals that develop IDDM due to alloxan treatment have low concentrations of SOD, glutathione reductase, total glutathione, vitamin E, and ceruloplasmin in comparison to the normal controls. At the same time, an increase in glutathione peroxidase and glutathione-*S*-transferase was observed in the diabetic animals (alloxan-alone–treated group).

On the other hand, insulin treatment has completely normalized the levels of all the antioxidants and antioxidant

Table 8. Effect of Alloxan and Sodium Nitroprusside (SNP) on the Concentrations of Glutathione Class Antioxidants in the Wistar Rats

Treatment	Glutathione Peroxidase (μmolNADPH/min/gmHb)	Glutathione Reductase (U/gmHb)	Glutathione-*S*-transferase (μmol conj/min/gmHb)	Total Glutathione (μmol/gmHb)
Control (n = 8)	23.01 ± 1.94	11.06 ± 1.26	2.83 ± 0.51	7.36 ± 0.67
Alloxan (n = 8)	31.07 ± 2.93*	6.23 ± 1.23*	4.27 ± 0.66*	5.26 ± 1.04*
Insulin (n = 8)	24.81 ± 1.81†	10.78 ± 1.29†	3.13 ± 0.70†	7.07 ± 0.79†
SNP 2 μg (n = 8)	22.40 ± 2.71	11.12 ± 1.16	2.79 ± 0.66	7.40 ± 1.23
SNP 5 μg (n = 8)	23.03 ± 1.65	10.59 ± 1.10	3.04 ± 0.50	7.73 ± 1.00
SNP 10 μg (n = 8)	23.43 ± 1.53	10.71 ± 1.09	2.92 ± 0.49	7.52 ± 1.13
Pre-SNP 2 μg + alloxan (n = 8)	29.64 ± 1.34*	7.11 ± 1.73*	3.94 ± 0.68*	5.61 ± 1.09*
Pre-SNP 5 μg + alloxan (n = 8)	27.71 ± 2.82*†	8.68 ± 1.51*†	3.79 ± 0.40*	6.00 ± 1.03*
Pre-SNP 10 μg + alloxan (n = 8)	28.09 ± 2.87*†	7.57 ± 1.15*†	3.98 ± 0.78*	5.73 ± 0.96*
Simult. SNP 2 μg + alloxan (n = 8)	27.65 ± 2.45*†	8.17 ± 1.31*†	3.67 ± 0.47*	6.27 ± 0.69*†
Simult. SNP 5 μg + alloxan (n = 8)	25.98 ± 2.05*†	9.90 ± 0.85†	3.17 ± 0.71†	6.80 ± 0.94†
Simult. SNP 10 μg + alloxan (n = 8)	26.48 ± 1.83*†	9.44 ± 1.39†	3.34 ± 0.58*†	6.60 ± 0.65†

*$p \leq .05$ compared with control.
†$p \leq .05$ compared with alloxan-treated group.

enzymes studied. This suggests that tight control of diabetes mellitus can restore the antioxidant status to normalcy.

In the pre-L-arginine + alloxan-treated group SOD, glutathione reductase, and uric acid levels remained low, whereas glutathione peroxidase levels were high in comparison to the control. In the L-arginine + alloxan simultaneously treated group, only glutathione reductase and uric acid levels were low.

In all the three L-NMMA + alloxan-treated groups, the concentrations of the antioxidant enzymes and antioxidants SOD, glutathione reductase, total glutathione, vitamin E, and ceruloplasmin levels were found to be low in comparison to the control similar to the alloxan-treated group. These results once again suggest that the beneficial actions of L-arginine seen in reversing the concentrations of antioxidants to normalcy is blocked by L-NMMA, the specific NO inhibitor, treatment. Thus, it can be suggested that some of the benefits of L-arginine treatment are presumably due to increased NO formation, and that this beneficial action is lost due to the inhibitory action of L-NMMA on nitric oxide synthesis in the L-NMMA + alloxan-treated groups (Tables 4 and 5).

Sodium nitroprusside (SNP) treatment

Because several in vitro studies have suggested that NO could be the ultimate mediator of cytokine-induced beta cell destruction (1–3), we studied the influence of sodium nitroprusside (SNP), a known NO donor, on alloxan-induced IDDM in vivo. It is evident from the results shown in Table 2 indicate that SNP treatment can partially abrogate the development of IDDM in the rats, especially when it is given simultaneously with alloxan. Although SNP pretreatment also decreased the severity of IDDM it was much less effective compared to the simultaneously treated group. From these results, it is evident that SNP is effective when it is given along with alloxan at a dose of 5 and 10 μg per animal. In addition to a decrease in the blood sugar levels, SNP-treated animals also showed lower levels of urinary acetoacetic acid, blood lactate, and lipid peroxides compared to the alloxan-treated group. The beneficial effect of SNP tretment against alloxan-induced damage to pancreatic beta-cells and the development of IDDM is supported by the observation that the plasma insulin levels are significantly higher in animals that received SNP both before and simultaneously with alloxan (Table 2). The beneficial effect of SNP treatment is further evident from the fact that SNP-treated animals did not show much weight loss and the survival is also better in these animals compared to the alloxan-alone–treated group.

Because SNP is a known NO donor, we estimated NO levels in these experimental animals. The results shown in Table 6 clearly show that animals that received SNP either before or simultaneously with alloxan have significantly higher levels of NO in comparison to the alloxan-treated group.

Antioxidant status in SNP-treated animals

Similar to the L-arginine treated groups, even in the animals that received SNP along with alloxan, a significant increase in the levels of SOD, glutathione reductase, total glutathione, vitamin E, and ceruloplasmin levels was noted (Tables 7 and 8), especially in animals that received 5 and 10 μg/animal of SNP (SNP + alloxan group). In addition, the levels of glutathione peroxidase and glutathione-*S*-transferase, which were increased in alloxan-treated animals, have reverted to almost normal levels following treatment with SNP (both in the pre- and simultaneously treated animals). These results indicate that SNP treatment can not only decrease the severity of IDDM induced by alloxan but can also restore the antioxidant status to near normalcy, which was associated with increase in the NO levels.

DISCUSSION

Insulin-dependent diabetes mellitus is an autoimmune disease characterized by infiltrating leukocytes into the islets of Langerhans of the pancreas and breakdown of glucose homeostasis as a result of destruction of insulin-producing pancreatic beta-cells. The leukocyte infiltrate, insulitis, composed of $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, macrophages, and dendritic cells play a major role in destroying the islet cells. $CD4^+$ T cells, and macrophages secrete gamma-interferon, tumor necrosis factor, and IL-1, which in turn induce high levels of NO and probably other free radicals such as superoxide anion, which may be the ultimate mediators of islet cell destruction. This assumption is supported by several in vitro results where in it was shown that NO can be directly toxic, whereas NO synthase inhibitors may protect the pancreatic beta cells [8,12]. But relatively few in vivo studies are available that documented the role of NO in IDDM. On the other hand, several in vivo studies revealed that NO and NO donors are useful in diabetes mellitus (reviewed in [15]). For example, it has been documented that endothelium-dependent vasodilation is impaired in humans with diabetes [16], and that it can be ameliorated by L-arginine, the precursor of NO [17]. Thus, these in vitro and in vivo results are contradictory to each other: in vitro studies suggesting that NO plays a role in the destruction of pancreatic beta-cells, whereas in vivo results indicated that NO is beneficial in DM. Hence, one has to be careful in extrapolating the in vitro results to an in vivo situation with regard to the role of NO in diabetes mellitus.

The results of the present investigation suggests that NO may, in fact, impart a beneficial action in chemical-induced IDDM. Studies performed with both L-arginine, the precursor of NO, and sodium nitroprusside, a donor of NO, indicate that when NO is present or administered simultaneously with the diabetogenic agent such as alloxan, the protective effect (of NO) on islet cells is considerable. Although pretreatment with L-arginine and SNP also showed some protective action against alloxan-induced beta-cell damage (as evidenced from less severe IDDM and histopathology of the pancreatic tissue showed that almost 70 to 75% 0f the beta cells are preserved compared to alloxan-treated animals, data not shown), the best results were obtained with simultaneous treatment.These results are further supported by the observation that L-NNMA, the NO synthase inhibitor, can block the beneficial action of L-arginine.

The fall in the concentrations of various antioxidants in alloxan-induced diabetic animals and their restoration to near normal levels by insulin treatment suggest that these changes may be secondary to the development of diabetes. Similarly, near normalization of the antioxidant status in SNP and L-arginine–treated animals also indicates that some of the metabolic abnormalities induced by alloxan and the diabetic status can be rectified by NO.

The protective and beneficial role of NO in alloxan-induced IDDM is further strengthened bythe observation that nitric oxide levels are low in alloxan-treated animals, and that they reverted to normal concentrations in L-arginine and SNP-treated groups. These changes in the NO levels was associated with a simultaneous change in the levels of the lipid peroxides in the opposite direction. These results clearly suggest that in IDDM the NO levels tend to be low, whereas lipid peroxides are high. The results of the present investigation also indicate that both L-arginine and SNP have the capacity to enhance NO levels in alloxan-induced diabetic animals and inhibit increase in the concentrations of lipid peroxides. These results can be interpreted to mean that free radicals, especially, superoxide anion, can be cytotoxic to islet cells in alloxan-treated animals, whereas NO has a cytoprotective function in preserving the beta-cell function. In view of these results, further studies are needed to exploit the beneficial action of NO in IDDM and possibly, in NIDDM.

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