Effects of Melatonin on Oxidative Stress in Streptozotocin-Induced Diabetic Rats

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Oxidative stress plays an important role in diabetes and other oxygen-related diseases. Melatonin, a pineal hormone thought to be a scavenger of oxygen radicals and a potentially advantageous therapeutic agent in diseases having oxidative stress, was administered (10 mg/kg ip, in gum tragacanth to prolong its absorption, once a day for 4 successive days) to normal and 30-day streptozotocin–induced diabetic Sprague-Dawley rats, after which markers of oxidative stress were assessed in the liver, kidney, intestine, and spleen. Alanine and aspartate aminotransferase activities in serum, which were increased after diabetes, were not increased further by melatonin administration, indicating that there was no melatonin-related liver toxicity. Most melatonin-induced effects were seen in the liver, and very few in extrahepatic tissues. In livers of diabetic rats, reduced concentration of nitrite and increased lipid peroxidation were both restored to normal levels following treatment with melatonin. Hepatic glutathione peroxidase activity was not changed in diabetics, but was decreased after melatonin administration in both normal and diabetic animals. Total glutathione concentrations were significantly decreased in livers of all diabetics and were not normalized by melatonin treatment. Hepatic superoxide dismutase activity was elevated following melatonin dosing in normal rats, but dropped below normal levels in diabetic rats and was not restored by melatonin treatment. Glutathione *S*-transferase activity was higher than normal in melatonin-dosed normal rat livers. These results suggest that after 4 days of administration, melatonin may enable various enzymes of the hepatic antioxidative defense system to better detoxify harmful oxygen radicals without producing overt toxicity in a disease such as diabetes.

Keywords Antioxidant, Catalase, Diabetes, Free Radical, Glutathione, Glutathione Peroxidase, Glutathione Reductase, Glutathione *S*-Transferase, Lipid Peroxidation, Melatonin, Oxidative Stress, Rat, Streptozotocin, Superoxide Dismutase, Thiobarbituric Acid

Melatonin, *N*-acetyl-5-methoxytryptamine, is a hormonal product of the pineal gland that plays many roles within the body, including control of reproductive functions, modulation of immune system activity, limitation of tumorigenesis, and effective inhibition of oxidative stress (Reiter 1993, 1997). One major function of melatonin is to scavenge radicals formed in oxygen metabolism (Reiter 1994; Hardeland et al. 1993), thereby potentially protecting against free radical–induced damage to DNA, proteins, and membranes (Reiter et al. 1995; Tan et al. 1993). A decrease in melatonin levels is believed to create an excess of oxidants in the body which may be linked to age-related diseases and possibly to the aging process itself (Reiter 1995, 1998; Poeggeler et al. 1993). Melatonin also has the potential to play an important role in naturally occurring free radical–related diseases, such as Parkinson's disease, multiple sclerosis, muscular dystrophy, emphysema, atherosclerosis, diabetes and others (Reiter 1994). Whereas some studies have examined the efficacy of exogenous melatonin as an antineoplastic agent (Anisimov, Popovich, and Zabezhinski 1997; Baldwin and Barrett 1998; Cini et al. 1998; Neri et al. 1998; Panzer 1997; Tan et al. 1993), other studies have focused on melatonin's immediate prophylactic effects as an antioxidant (Melchiorri et al. 1995; Montilla et al. 1998; Pierrefiche et al. 1993). However, whether melatonin also has potential in the treatment of chronic free radical–related diseases, such as diabetes, has not been delineated.

Diabetes is a prevalent disease in the United States, affecting 2% of the population. Streptozotocin (STZ)-induced diabetes is a well-established animal model of type I (insulin-dependent) diabetes (Kwon et al. 1994). Hepatic effects of diabetes include, among other things, increased oxidative stress. Exogenously administered melatonin may act to facilitate hepatic responses to oxidative stress indirectly through its effects on the defense system, or directly through its own demonstrated antioxidant properties.

Despite the fact that melatonin is available over the counter and is widely used in the United States, there is minimal information on the toxicology of melatonin (Lamberg 1996; Guardiola-Lemaitre 1997). Thought to be the wonder drug of the 1990s (Weaver 1998), no one has yet determined whether a high dose of melatonin would have beneficial or deleterious effects on the

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oxidative stress pathway. Therefore, this study was designed to determine if melatonin would alter the activities of several integral components of the oxidative stress pathway, including glutathione (GSH) and the enzymes superoxide dismutase (SOD), catalase, nitric acid synthetase, GSH peroxidase, GSH reductase, and GSH *S*-transferase, of the liver, kidney, spleen, and intestine in normal and chronic insulin-dependent diabetic rats.

MATERIALS AND METHODS

Animals and Materials

Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were allowed to acclimatize for 4 days prior to start of the experiment. They were given Purina Rat Chow (No. 5012, St. Louis, MO) and water ad libitum. Animal husbandry and experimentation were consistent with the *Public Health Service Guide for the Care and Use of Laboratory Animals*. All chemicals were purchased from Sigma Chemicals (St. Louis, MO).

Treatment

Because melatonin is not water soluble and is rapidly metabolized, 1% gum tragacanth was used as a vehicle for the suspension of melatonin and to prolong its absorption.

To determine the effect of melatonin upon diabetes, female Sprague-Dawley rats (225–250 g) were divided randomly into four groups of 6 to10 animals. Two groups remained normal, while two groups received STZ (50 mg/kg body weight, iv, in freshly prepared 10 mM sodium citrate, pH 4.5, in a volume of 1 ml/kg). One month later, all the groups received either melatonin (10 mg/kg body weight) or vehicle ip (4 ml/kg) once a day in the middle of the sleep cycle (11:00 AM) for 4 days. Then, 24 hours after the last injection, rats were anesthetized with 1.5% halothane by inhalation, blood was collected by cardiac puncture, and then livers, kidneys, spleens, and small intestines were excised, rinsed in ice-cold 1.15% potassium chloride and stored at − 80°C until analyzed.

Assays

Alanine and aspartate aminotransferases activities were assayed in serum using the kits available from Sigma (St. Louis, MO). Glucose concentration in serum was measured with Sigma Kit HK-20. An appropriate portion of each tissue was weighed and homogenized in ice-cold 0.1 M Tris buffer (pH 7.4) to prepare a 10% *w*/*v* homogenate. Homogenates were assayed for evidence of lipid peroxidation activity using the thiobarbituric acid (TBA) reaction (Ohkawa, Ohishi, and Yagi 1979). Supernatants (cytosol) from centrifuged homogenates (105,000*g*, 1 hour) were assayed for the activities of catalase (Luck 1963), SOD (Mirsa and Fridovich 1972), GSH peroxidase (Tappel 1978), GSH reductase (Carlberg and Mannervik 1975), and GSH *S*-transferase toward 1-chloro-2,4-dinitrobenzene (CDNB) (Habig, Pabst, and Jakoby 1974). All enzyme assays were performed in duplicate and were proportional to protein concentration. Concentrations of nitrite, the product of nitric oxide synthetase (Ding, Nathan, and Stuehr 1988), total GSH (Mokrasch and Teschke 1984), and protein (Lowry et al. 1951) were also determined.

Statistical Analysis

Means and standard error of the means were calculated for all data. Data were analyzed by analysis of variance (ANOVA) and Duncan's test, and significance was set at $p < .05$.

RESULTS

Activities in serum of alanine and aspartate aminotransferases were elevated in diabetic animals as compared to normal, and were decreased in diabetic rats after melatonin treatment (Table 1). Serum glucose concentrations were above 550 mg/dl in all diabetic rats, and were not changed by melatonin administration.

Among indicators that were altered in diabetic rat livers, two trends were observed. The first trend was the restoration of enzyme activity levels or cellular concentrations after melatonin treatment. Concentrations of nitrite and TBA reactants (Figure 1) were both altered in diabetic rat livers (decreased 52%, increased 190%, respectively) and brought back to or above normal levels with melatonin treatment. The second trend was the failure to normalize diabetic effects. For example, although SOD activity was significantly increased after melatonin treatment of normal rats (Figure 2), the 67% decline in activity seen in diabetics was reversed but not normalized after melatonin treatment. Melatonin treatment also did not restore GSH concentration (Figure 3) from the 82% decrease in diabetics. Enzymatic activities that did not follow either of these trends included hepatic GSH peroxidase (Figure 4), which was normal in diabetic rats, but was reduced after melatonin treatment in both normal (by 49%) and diabetic (by 25%) rats. Likewise, hepatic catalase activity (Figure 2) was increased by 40% in normal rats and by 43% in diabetic rats after melatonin treatment. GSH *S*-transferase activity toward CDNB (Figure 3) was increased in the livers of

TABLE 1
Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and glucose concentrations in normal and diabetic rat serum

	AST (U/ml)	ALT (U/ml)	Glucose (mg/dl)
Normal	72 ± 7	10 ± 2	108 ± 8
Normal + melatonin	38 ± 7*	11 ± 1	107 ± 15
Diabetic	115 ± 29*	32 ± 8*	787 ± 67*
Diabetic + melatonin	72 ± 9#	25 ± 9	804 ± 57*

Note: Results are means ± *SE* for 6 to 10 rats.
*Significantly different from normal controls at $p < .05$.
Diabetic + melatonin group is significantly different from untreated diabetic rats at $p < .05$.

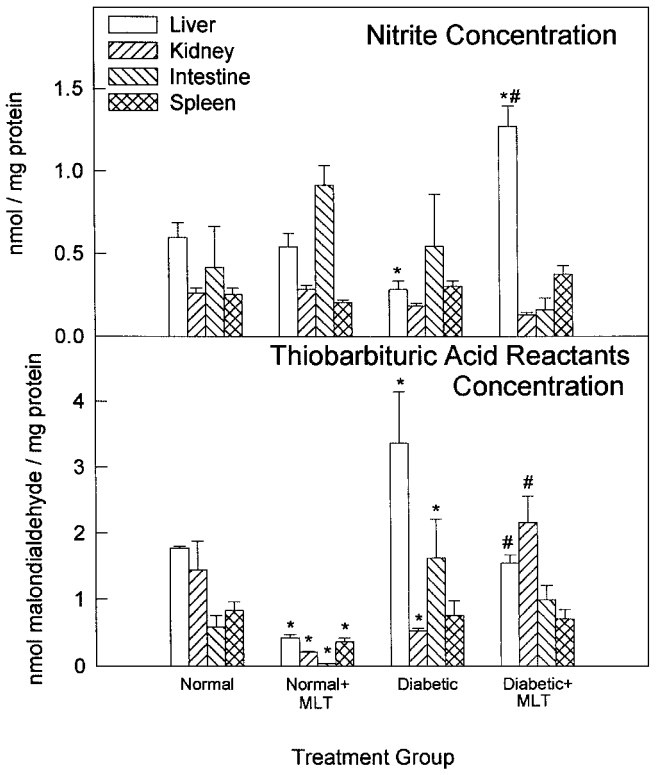

FIGURE 1

Effects of diabetes (30 days after STZ) and melatonin administration (10 mg/kg/day) for 4 days on nitric oxide synthetase, as reflected in the concentration of nitrite, and lipid peroxidation, as measured by concentration of thiobarbituric acid reactants. Each bar is the mean ± *SE* for 6–10 rats. *Significantly different from normal controls at $p < .05$. # Significantly different from diabetic controls at $p < .05$.

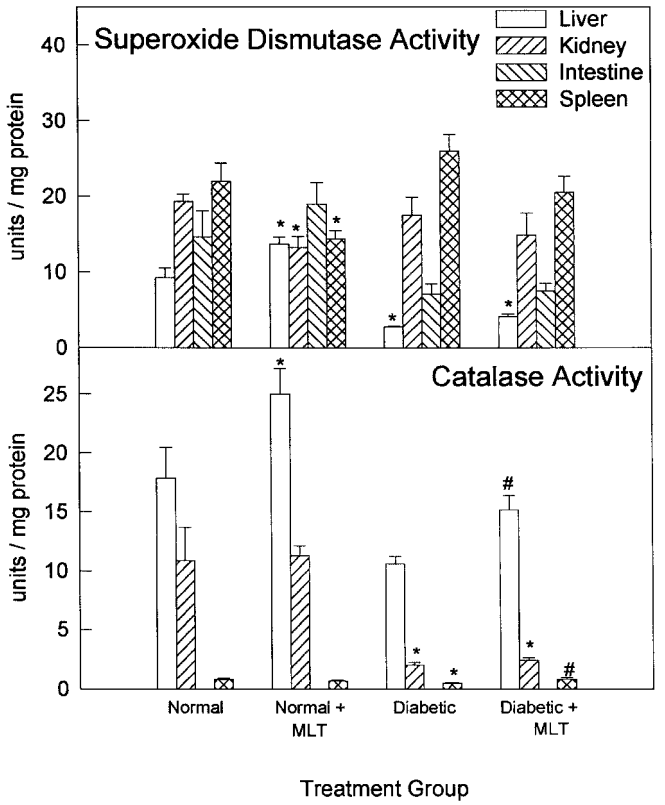

FIGURE 2

Effects of diabetes (30 days after STZ) and melatonin treatment (10 mg/kg/day) for 4 days on activity of superoxide dismutase and catalase. A unit of superoxide dismutase activity is defined as the quantity of superoxide dismutase required to produce 50% inhibition of the rate of reduction of cytochrome *c* under the conditions of the assay. A unit of catalase activity is defined as the amount of enzyme which liberates half the peroxide oxygen from a hydrogen peroxide solution in 100 sec at 25°C. Each bar is the mean ± *SE* for 6–10 rats. *Significantly different from normal controls at $p < .05$. # Significantly different from diabetic controls at $p < .05$.

normal rats after melatonin by 112%, but remained at normal levels in the livers of all diabetic rats, with or without melatonin treatment. Hepatic GSH reductase activity (Figure 4) was not significantly affected by diabetes or by melatonin treatment.

In contrast to hepatic results, effects of melatonin treatment in extrahepatic tissues were less consistent. Melatonin treatment resulted in an 85% decrease in lipid peroxidation (Figure 1) in normal kidneys, but the 63% decline in diabetic kidneys was reversed (increased by 307%) after melatonin treatment. Renal GSH *S*-transferase activity (Figure 3) was increased in diabetic rats and restored to normal levels after melatonin treatment. Decreased activity of renal SOD (Figure 2) was noted in normal rats after melatonin treatment. Decreased catalase activity (Figure 2) in kidneys of diabetic rats was not restored by melatonin. Intestinal TBA reactants (Figure 1) increased by 174% in diabetic rats; melatonin treatment decreased both normal levels (by 92%) and diabetic levels (by 40%) of TBA reactants. Intestinal GSH *S*-transferase (Figure 3) and GSH peroxidase (Figure 4) activities were increased after melatonin treatment in normal rats. Melatonin decreased TBA reactants (Figure 1) and SOD activity (Figure 2) in normal spleen, whereas decreased catalase activities (Figure 2) in diabetic rat spleen were not restored by melatonin. GSH *S*-transferase activity (Figure 3) increased by 85% in diabetic spleen, but was decreased after melatonin treatment in both normal (by 46%) and diabetic spleens (by 53%).

DISCUSSION

It seems significant that antioxidant response is affected in many ways during diabetes (Saxena et al. 1993), as excessive levels of oxygen radicals cause cellular damage to nucleic acids, proteins, and lipids. Lipid peroxidation by oxygen radicals, known to be increased in diabetic rats (Mukherjee, Mukherjee, and Chatterjee 1994; Matkovics et al. 1982; Wolff 1993), causes a weakening in the cellular membrane which can lead to cell death. Therefore, the use of melatonin treatment in a chronic

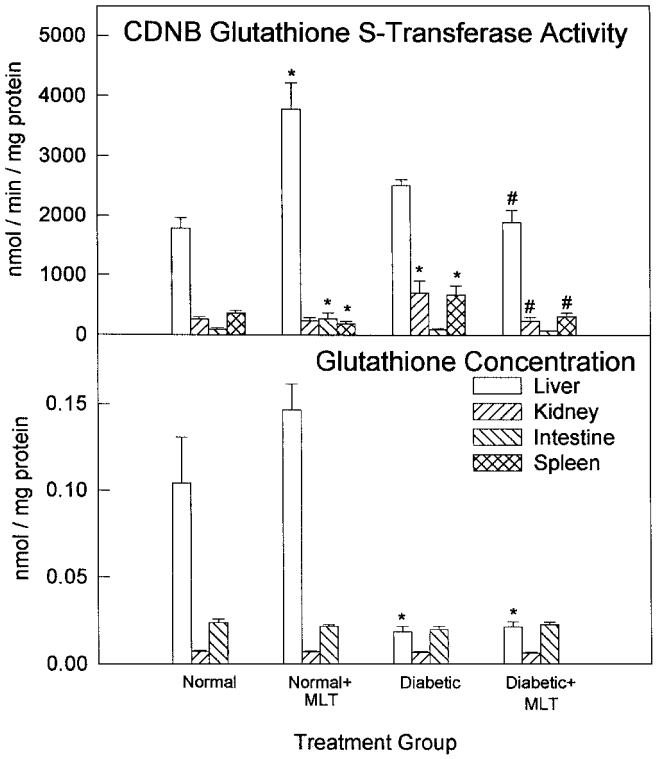

FIGURE 3

Effects of diabetes (30 days after STZ) and melatonin treatment (10 mg/kg/day) for 4 days on activity of glutathione *S*-transferase toward 1-chloro-2,4-dinitrobenzene and concentration of total glutathione. Each bar is the mean ± *SE* for 6–10 rats. *Significantly different from normal controls at $p < .05$. # Significantly different from diabetic controls at $p < .05$.

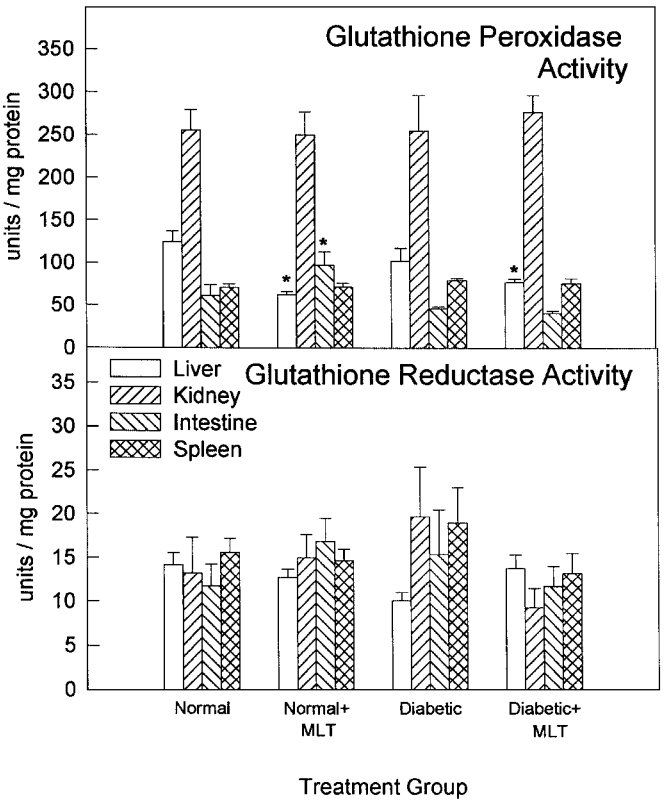

FIGURE 4

Effects of diabetes (30 days after STZ) and melatonin treatment (10 mg/kg/day) for 4 days on activity of glutathione peroxidase and glutathione reductase. A unit of glutathione peroxidase, like a unit of glutathione reductase, is defined as the amount of enzyme that transforms 1 μmol of NADPH to NADP in 1 min at 37°C. Each bar is the mean ± *SE* for 6–10 rats. *Significantly different from normal controls at $p < .05$.

oxidative stress paradigm could yield insight into the role of radicals in oxidative stress–related diseases.

Melatonin, an in vivo antioxidant (Reiter et al. 1995; Melchiorri et al. 1995), prevents the initiation of diabetes by the diabetogen alloxan (Pierrefiche et al. 1993) and reduces lipoperoxidation, hyperglycemia, and protein glycosylation resulting from STZ treatment (Montilla et al. 1998). However, examination of antioxidant capabilities in an acute pretreatment paradigm does not adequately address the effect of melatonin in a chronic oxygen radical–related disease. Rats treated with STZ 30 days prior to investigation represent an established model of chronic insulin-dependent diabetes for determining whether melatonin can influence several enzymes that characteristically detoxify oxygen radicals.

Diabetes is known to alter hepatic glucose utilization and xenobiotic metabolism, as well as to produce cellular damage. An interesting effect of diabetes on the antioxidant defense system, as seen in this study and consistent with previous findings (Saxena et al. 1993; Matkovics et al. 1982), is the reduction in activity of key hepatic enzymes, including SOD, nitric oxide synthetase (reflected in the concentration of its product, nitrite), and catalase.

However, the interaction of enzymes, substrates, cofactors, and products leading to tissue damage is very complex, and it is not a simple matter to tease out causes and effects pertaining to oxidative stress. Nevertheless, two trends emerge in the antioxidant system of livers of diabetic rats after treatment with melatonin. The first trend is the recovery of enzyme activity or cellular concentration to normal or higher levels after melatonin therapy. The second observed trend is the failure to recover to normal levels after melatonin treatment. In this experiment, melatonin appears to have few consistent extrahepatic effects.

Melatonin treatment increases SOD activity in normal rat livers, but the decreased levels of hepatic SOD activity in diabetic rats are not restored after melatonin treatment (Figure 2). Depressed SOD activity in diabetic rats creates an enzymatic "dam"

(Winterbourn 1993), resulting in decreased conversion of superoxide anion to hydrogen peroxide. SOD thus becomes the rate limiting enzyme that generates toxic secondary effects by supplying superoxide radicals in diabetic rats. When not removed, superoxide radicals play an important catalytic role in the conversion of hydrogen peroxide to the more damaging hydroxyl radicals (Winterbourn 1993).

Nitric oxide synthetase provides a secondary method to neutralize superoxide radicals in the cell. Nitric oxide, produced by nitric oxide synthetase, reacts with the superoxide anion and is converted to nitrite (Josephy 1997). Concentrations of nitrite, an indicator of the effectiveness of nitric oxide synthetase, are depressed in the livers of diabetic rats but are restored after melatonin treatment to levels significantly higher than observed in either normal or diabetic rats (Figure 1). Restoration of this nitric oxide pathway by melatonin may help to eliminate excess superoxide radicals not detoxified by SOD.

Another important enzyme of the antioxidative defense system is catalase, which facilitates conversion of hydrogen peroxide to oxygen and water (Winterbourn 1993; Josephy 1997). Catalase activity levels, which appear slightly, but not significantly, reduced in diabetic rats, may reflect the reduced activity of SOD, which results in a lower concentration of hydrogen peroxide and thus a lower demand for catalase. Melatonin's scavenging of superoxide and hydroxyl radicals would allow hydrogen peroxide accumulation and thus restores the cellular need for catalase, an effect that is seen here, as both normal and diabetic catalase activities increase after melatonin treatment.

Like catalase, GSH peroxidase catalyzes the conversion of hydrogen peroxide to water and may be rate-limiting as these two enzymes function interdependently (Gaetani et al. 1994). Hepatic GSH peroxidase activity levels are normal in diabetic rats, but decrease after melatonin treatments in both normal and diabetic rats (Figure 4). This effect of melatonin correlates inversely with increases in catalase activity. The activity levels of GSH reductase, the flavoenzyme that recycles GSH from its oxidized form to its reduced form (Carlberg and Mannervik 1975), and GSH peroxidase are also apparently codependent (Gaetani et al. 1994).

Another important complication of diabetes that can affect many cellular systems is the decreased concentration of GSH, which is not restored after melatonin treatment (Figure 3). GSH normally plays the role of an intracellular radical scavenger and is the substrate of many xenobiotic elimination reactions (Winterbourn 1993; Josephy 1997; Gregus and Klaassen 1996). This decrease in GSH concentration is not the result of the oxidation of GSH, because neither GSH peroxidase nor GSH reductase exhibit higher activity levels in livers of diabetic rats (Figure 4) and no observed differences in oxidized GSH were found (data not shown). Furthermore, the activity of GSH *S*-transferase, an enzyme involved in biotransformation and radical elimination reactions, is not stimulated in diabetics as in normal rats after melatonin dosage (Figure 3). This could indicate an effect not detected by these assays. Future research may reveal similar alterations in other cellular pathways due to the decrease in GSH concentrations.

Finally, the ultimate effect of oxidative stress in the liver of a diabetic animal is the increased level of lipid peroxidation, as measured by TBA reactants (Figure 1); our results are consistent with previous findings (Mukherjee, Mukherjee, and Chatterjee 1994). Highly destructive hydroxyl radicals attack cellular fatty acids, causing a weakening in many subcellular structures and the cell membrane. Unprevented, this damage can lead to cell death (Nigam, McBrien, and Slater 1988). Malondialdehyde is an end product of lipid peroxidation (Josephy 1997) that reacts with TBA, creating a spectrophotometrically measurable product (Ohkawa, Ohishi, and Yagi 1979). Although using the level of TBA reactants as a measure of lipid peroxidation has limitations because of the reactivity of malondialdehyde with other ketones and aldehydes (Janero 1990), we have compared our diabetic, melatonin-treated animals to both diabetic and normal animals, thus controlling for other possible TBA adducts in group comparisons. Because other TBA adducts have been shown to have absorption maxima at different wavelengths, TBA reactants remain a useful measure of the amount of malondialdehyde produced (Josephy 1997). After melatonin treatment of diabetic rats, the level of hepatic lipid peroxidation returns to normal. This correlates strongly with evidence for melatonin's primary role as a hydroxyl and free radical scavenger (Matuszak, Reszka, and Chignell 1997; Melchiorri et al. 1995; Pierrefiche et al. 1993; Reiter et al. 1995; Reiter 1995).

From these observations it is possible to conclude that melatonin's purported action of scavenging oxygen radicals alleviates some oxidative stress caused by diabetes, both directly and also indirectly, by allowing some of the enzymes of the antioxidant defense system to function more efficiently in neutralizing dangerous oxygen radicals without causing significant adverse effects. Ultimately, melatonin reduces lipid peroxidation, the most significant effect of the diabetes-induced depression of the antioxidative defense system. By scavenging dangerous hydroxyl radicals, melatonin can diminish lipid peroxidation and, potentially, some diabetes-induced cellular damage. The reduction of damage on the cellular level could possibly attenuate further tissue and organ damage induced by diabetes. Obviously, much future work is needed to fully understand the role of oxidant stress in diabetes and the potential utility of melatonin treatment in controlling complications of this debilitating disease. In addition, analysis of the effect of melatonin upon xenobiotic biotransformation and metabolism, in both diabetic and normal animals, as suggested by changes in GSH *S*-transferase activity, could shed light on additional actions of this important antioxidant.

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