Changes in lipid peroxidation during pregnancy and after delivery in rats: effect of pinealectomy


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Pregnancy is a physiological state accompanied by a high energy demand of many bodily functions and an increased oxygen requirement. Because of the increased intake and utilization of oxygen, increased levels of oxidative stress would be expected. In the present study, the degree of lipid peroxidation was examined in different tissues from non-pregnant and pregnant rats after the delivery of their young. Melatonin and other indole metabolites are known to be direct free radical scavengers and indirect antioxidants. Thus the effect of pinealectomy at 1 month before pregnancy on the accumulation of lipid damage was investigated in non-pregnant and pregnant rats after the delivery of their young. Malonaldehyde and 4-hydroxyalkenal concentrations were measured in the lung, uterus, liver, brain, kidney, thymus and spleen from intact and pinealectomized pregnant rats soon after birth of their young and at 14 and 21 days after delivery. The same parameters were also evaluated in intact and pinealectomized non-pregnant rats. Shortly after delivery, lipid oxidative damage was increased in lung, uterus, brain, kidney and thymus of the mothers. No differences were detected in liver and spleen. Pinealectomy enhanced this effect in the uterus and lung. It is concluded that during pregnancy high levels of oxidative stress induce an increase in oxidative damage to lipids, which in some cases is inhibited by the antioxidative actions of pineal indoles.

Introduction
Pregnancy is a physiological state accompanied by a high metabolic demand and an increased requirement for tissue oxygen (Spätling et al., 1992). This increased oxygen requirement increases the rate of production of reactive oxygen species (ROS) (Halliwell and Gutteridge, 1990). It is well established that ROS damage cell membrane lipids and induce lipid peroxide formation (Tappel, 1973; Harman, 1982; Slater, 1984). In rats, lipid peroxidation remains low until mid-pregnancy and begins to rise after day 15 of pregnancy (Sugino et al., 1993). Likewise, the amount of peroxidized lipids, which are produced mainly in the placenta (Walsh, 1994), increases in the blood of pregnant women (Hubel, et al., 1989). Pregnancy also has an effect on maternal antioxidant enzyme activities. Other studies have demonstrated changes in the activity of superoxide dismutase and lipid peroxide production in the corpus luteum during pregnancy in rats (Mover-Lev and Ar, 1997). Furthermore, a measurable decrease in glutathione peroxidase in liver and placenta has been reported in pregnant rats (Mover-Lev and Ar, 1997). Collectively, these observations indicate that there is increased oxidative stress during pregnancy.

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One important pathological condition during pregnancy is pre-eclampsia. This human disorder is a leading cause of premature delivery and fetal growth retardation (Lim and Friedman, 1993). Pre-eclampsia is associated with increased lipid peroxidation in the maternal circulation and in the placenta (Morris et al., 1998; Wang and Walsh, 1998). Okatani et al. (1997) suggested the use of melatonin, because of its antioxidant properties, as a treatment for pre-eclampsia. This suggestion is supported by the observations of Chappell et al. (1999) who showed that supplementing women with a combination of vitamins C and E during pregnancy reduced the incidence of pre-eclampsia and decreased the ratio of plasminogen activator inhibitor 1 (PAI-1):PAI-2 in maternal blood during pregnancy. This index also indicates the potential benefit of antioxidants in reducing the signs of pre-eclampsia.

In the present study, the levels of lipid peroxidation were investigated in different tissues of pregnant rats after they delivered their young. Furthermore, owing to the important antioxidant properties of pineal indoles the effect of pinealectomy on the concentration of tissue lipid peroxidation products was studied in the mothers. It is well known that melatonin, as well as other pineal metabolites, are effective free radical scavengers and antioxidants both in vitro and in vivo (for reviews see Reiter, 1997, 1998).

Since Tan et al. (1993) described the ability of melatonin to neutralize the highly toxic hydroxyl radical, it has been
demonstrated that melatonin detoxifies several radicals and protects numerous organs against agents or conditions that cause free radical generation. Melatonin protects the liver against damage during ischemia-reperfusion (Sewerynek et al., 1996), prevents kainate-induced oxidative damage to brain tissue (Melchiorri et al., 1996) and inhibits 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP) neurotoxicity in mice (Acuña-Castroviejo et al., 1997). Melatonin also reportedly detoxifies the peroxyl radical which is generated during lipid peroxidation (Pieri et al., 1994, 1996) and the peroxy nitrite anion (Cuzzocreo et al., 1998) which is capable of oxidatively damaging membrane lipids. Melatonin is also known to reduce lipid peroxidation induced by a variety of toxins (Daniels et al., 1998; Princ et al., 1998; Siu et al., 1998) and preserves plasma membrane fluidity which is normally altered when the membrane contains oxidatively damaged polyunsaturated fatty acids (Garcia et al., 1997). Although melatonin is produced in other organs, the pineal gland is generally considered to be the main source of circulating melatonin concentrations (Reiter et al., 1995). In the present study, rats that had been surgically pinealectomized were used to determine whether circulating endogenous concentrations of melatonin are of significance in influencing oxidative stress during pregnancy.

Materials and Methods

Animals

A total of 48 adult female Sprague–Dawley rats (180–200 g), purchased from Harlan (Houston, TX), were used in this study. The animals were maintained under controlled temperature (22 ± 2°C) and constant photoperiodic conditions (14 h light:10 h dark; lights on from 07:00 h to 21:00 h). Food and water were available ad libitum. All the animals were killed by decapitation.

Experimental procedure

All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee. Half of the rats (24 animals) were anaesthetized with rodent cocktail (ketamine:xylazine) and the pineal gland was removed using the standard procedure described by Hoffman and Reiter (1965). After 3 weeks, when the animals had totally recovered, 18 intact and 18 pinealectomized rats were placed with males until they were pregnant, as determined by visual examination. The females were separated from the males and placed in individual cages before delivering their young. The exact time (h) of the delivery was determined by observing the animals frequently during the last 2 days of gestation (days 20 and 21). Six intact and six pinealectomized rats were killed by decapitation soon after delivering their young. Six control and six pinealectomized non-pregnant rats were killed by decapitation simultaneously with the rats that had delivered. The time of day that the animals were killed depended on the time the young were delivered, since the aim was to collect tissues from the mothers as soon as feasible after they delivered their young. All the rats that had delivered young were killed during the light period. The remaining animals (12 intact and 12 pinealectomized rats that had delivered young) were kept with their litters for an additional 14 or 21 days at which time they were killed during the light period (7:00–12:00 h).

Liperoxidation assay

After the rats were killed, tissues were collected, frozen on dry ice and stored at –80°C until assays were performed. Concentrations of malonaldehyde and 4-hydroxyalkenals were analysed using an LPO-586 kit purchased from Calbiochem (La Jolla, CA). For each assay, 100 mg tissue was homogenized in 1 ml of ice cold 50 mmol Tris–HCl buffer 1− (pH 7.4) with an Ultraturrax polytron giving a final concentration of 10% (w/v) homogenate. Samples were centrifuged at 3000 g for 10 min at 4°C and supernatants were collected. An aliquot (200 μl) of each sample was used to evaluate the concentration of malonaldehyde + 4-hydroxy alk enals as an index of lipid peroxidation. In the assay, the production of a stable chromophore after 40 min of incubation at 45°C was measured at a wavelength of 586 nm. The values are represented as nmol malonaldehyde + 4-hydroxyalkenals mg−1 protein. This procedure has been used widely for the measurement of products of lipid peroxidation (Chen et al., 1995; Melchiorri et al., 1996; Sewerynek et al., 1996; Escam es et al., 1997; Daniels et al., 1998).

Protein assay

Protein concentrations were determined using the method described by Bradford (1976) and analytical grade bovine albumin was used to establish a standard curve. Data were obtained from three different measurements.

Statistical analysis

All data are shown as means ± standard error. Statistical analysis was performed with one-way ANOVA followed by a Student Newman–Keuls test or with a t test between two groups when required.

Results

Lipid peroxidation, represented as nmol malonaldehyde + 4-hydroxyalkenals mg−1 protein, was significantly increased in lungs of maternal rats killed soon after delivery of their young (Fig. 1). This response was enhanced when the animals were pinealectomized 1 month before pregnancy. However, pinealectomy in animals that never became pregnant had no effect on the amount of lipid peroxidation products in the lung.

In uterine tissue, pinealectomy alone significantly increased the amount of lipid peroxidation products (Fig. 2). Likewise,
Pregnancy also caused a significant increase in uterine malonaldehyde + 4-hydroxyalkenals when the mothers were killed shortly after delivery of their young. Pinealectomized rats that became pregnant showed a further increase in uterine malonaldehyde + 4-hydroxyalkenals.

In kidney (Fig. 3), brain (Fig. 4) and thymus (Fig. 5), pregnancy and delivery increased lipid peroxidation products in mothers, but these increases were not enhanced by pinealectomy. These differences were statistically significant in kidney and brain tissue. In the liver and spleen, neither delivery nor pinealectomy changed the amount of lipid peroxidation (data not shown).

The results obtained in the tissues of maternal rats that nursed their young for either 14 or 21 days after delivery are summarized (Table 1). In the lung and brain of these rats, earlier removal of the pineal gland enhanced the amount of the products of lipid peroxidation; a similar tendency was seen in the uterus and kidney but in these tissues the increases were not verified statistically. In kidney and lung tissues collected from mothers killed at 14 or 21 days after delivery, the amount of lipid peroxidation products was lower than in the same tissues taken from rats that had just delivered their young.

**Discussion**

This study shows that the physiological state of pregnancy and delivery causes an increase in the amount of lipid peroxidation products in lung, uterus, kidney, brain and thymus of rats. Pinealectomy also enhanced this damage in lung and uterine tissue. Furthermore, lipid peroxidation decreased in some tissues with increasing time after delivery; thus, the amount of lipid peroxidation products was lower in kidney and lung at 14 and 21 days after delivery compared with values measured in these tissues immediately after delivery of the pups.

**Table 1.** Changes in lipid peroxidation in intact (INT) and pinealectomized (PINX) rats at 14 and 21 days after delivery of their pups

<table>
<thead>
<tr>
<th></th>
<th>Lung</th>
<th>Uterus</th>
<th>Brain</th>
<th>Kidney</th>
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<tr>
<td></td>
<td>14 days</td>
<td>21 days</td>
<td>14 days</td>
<td>21 days</td>
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<tr>
<td>INT</td>
<td>1.84 ± 0.09</td>
<td>2.05 ± 0.11</td>
<td>1.65 ± 0.15</td>
<td>1.52 ± 0.06</td>
</tr>
<tr>
<td>PINX</td>
<td>2.13 ± 0.09*</td>
<td>2.22 ± 0.39</td>
<td>1.96 ± 0.24</td>
<td>1.65 ± 0.11</td>
</tr>
</tbody>
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Values are mean ± SEM.

n = 6 for each group.

*P < 0.05 versus INT group.
Lipid peroxides are formed when lipids interact with oxygen radicals. These oxidized lipid products are reactive and cause damage to cells and cell membranes (Mead et al., 1986; Hennig and Chow, 1988). Once initiated, lipid peroxidation can be amplified by a radical chain reaction of self-propagation (Halliwell and Gutteridge, 1990; Reiter, 1995). The human placenta produces lipid peroxides that are secreted mainly to the maternal side of the placenta (Walsh and Wang, 1993a) and markers of increased lipid peroxidation are observed during normal pregnancy (Ishihara, 1978; Wickens et al., 1981). Furthermore, the concentration of lipid peroxides in maternal blood decrease after the placenta has delivered (Wickens et al., 1981). Walsh and Wang (1993a) surmised that these compounds remain in the circulation for some time since some lipid peroxides, such as oxidized polyunsaturated fatty acids in low-density lipoproteins, have a half-life of 3 h (Gorog et al., 1989). In the present study, increased lipid peroxidation was observed within 3 h of delivery in a variety of rat tissues. Damaged lipid products produced in the placenta during pregnancy could act as the agents that initiate damage in other tissues.

Several studies have indicated that the antioxidative defence system is modified during pregnancy. Wisdom et al. (1991) showed that the activity of an important family of antioxidative enzymes, the superoxide dismutases (SOD), is reduced in the blood of pregnant women. In addition, Walsh and Wang (1993b) reported a deficiency in another antioxidative enzyme, glutathione peroxidase (GPx) during pregnancy. GPx is an important antioxidant enzyme present in virtually all tissues. The enzyme limits the generation of lipid peroxides and utilizes glutathione as its cofactor to convert lipid peroxides into relatively harmless hydroxylated fatty acids, water and glutathione disulfide (Krinsky, 1992; Levander, 1992). Given these findings, it might be expected that a deficiency in this enzyme would lead to increased lipid peroxidation during pregnancy. Gorog et al. (1989) proposed another mechanism to explain the increase in the amount of oxidative damage during pregnancy, based on the prolonged activation of leucocytes which generate superoxide anion...
radicals and hydrogen peroxide by oxidized fatty acids. Intravenous injection of oxidized fatty acids into rabbits resulted in an increase in the lipid peroxide content of plasma 6 h after the injection. The activated leucocytes could then generate additional lipid peroxides in the maternal circulation. As a consequence, reduced concentrations of the chief scavenger for the superoxide radical, that is SOD, during pregnancy may contribute to the enhanced damage observed.

In the present study, the highest concentrations of malonaldehyde + 4-hydroxyalkenals were measured in lung tissue. As the interface between the external environment and the blood, the lungs are exposed to a higher O2 pressure than any other organ. It is well demonstrated that during pregnancy ventilation increases significantly, enabling a higher O2 supply to this tissue (Spätling et al., 1992). In the present study, it was anticipated that oxidative damage accompanying the physiological state of pregnancy would be higher in lung tissue than in other organs and this was confirmed by the results.

Mover-Lev and Ar (1997) demonstrated no change in the activity of antioxidative enzymes in the brain of pregnant rats. If an increase in oxidative stress due to augmented oxygen utilization is not accompanied by an increase in the antioxidant defence system, higher levels of cellular oxidative damage would be expected in neural tissue, which has a higher rate of oxygen utilization than any other organ.

The increased lipid peroxidation in kidney and thymus tissue were unexpected since this has not been reported in other studies. The implication is that pregnancy and delivery induce a general state of increased oxidative stress.

The present study also showed that the high concentration of lipid peroxidation products, especially in the lung and kidney, decreased with increasing time after delivery. This is consistent with the results of Wickens et al. (1981) who showed that the concentration of lipid peroxides in maternal blood decreases significantly after the placenta is delivered.

A number of studies have focused on the role of oxidative stress in pathological conditions such as pre-eclampsia. Pre-eclampsia is a pregnancy-specific condition characterized by hypertension and proteinuria, both of which remit after delivery (Roberts et al., 1991). Some reports demonstrate that the increased oxidative stress in pre-eclampsia is accompanied by a higher production of lipid peroxides compared with that during normal pregnancy (Wisdom et al., 1991; Davidge et al., 1992; Walsh and Wang, 1993a; Shaarawy et al., 1998). It is also known that in pre-eclampsia maternal plasma concentrations of vitamin E (a well known inhibitor of lipid peroxidation) are decreased (Wang et al., 1991). This finding is consistent with the observations of Chappell et al. (1999) who gave pregnant women a combination of two vitamin antioxidants, vitamins C and E, and showed that the incidence of pre-eclampsia was reduced. It was also found that vitamin supplementation reduced the ratio of PAI-1:PAI-2 in the blood, further indicating that antioxidants may be of value in reducing the severity of pre-eclampsia. In light of these findings, antioxidant therapy is being considered for the treatment and prevention of pre-eclampsia (Gulmezoglu et al., 1997). Several studies have shown that melatonin has substantial antioxidant activity (Reiter, 1995, Reiter et al., 1995) and these observations were in part the stimulus for the present study.

No differences were observed between non-pregnant controls and pinealectomized rats with one exception, that is the uterus. This was not unexpected since under minimally oxidatively stressed conditions, a reduction in one antioxidant would not be expected to increase markedly the amount of free radical damage. A number of studies have shown that melatonin protects against lipid peroxidation induced by different insults (Chen et al., 1995; Escames et al., 1997; Garcia et al., 1997; Li et al., 1997; Daniels et al., 1998; Siu et al., 1998; Tesoriere et al., 1999). Although melatonin is a highly effective antioxidant in reducing lipid peroxidation when it is given as a pharmacological supplement, this is the first report showing that pinealectomy (and the consequential removal of a source of melatonin) increases the amount of lipid peroxidation in any organ. Moreover, the present study showed that pinealectomy enhances the increased levels of lipid peroxidation caused by pregnancy, at least in the lungs and uterus. In both tissues, the increased concentrations of malonaldehyde + 4-hydroxyalkenals due to pregnancy were statistically significant after loss of the pineal gland. Even at 14 days after delivery of the young, there were still significant differences in the amount of lipid peroxidation between control and pinealectomized rats in lung and brain tissues. These findings are consistent with the hypothesis that the low concentrations of melatonin may be responsible in part for maintaining high levels of oxidative lipid damage in these tissues. In the uterus and kidney, there also was a tendency for increased lipid peroxidation in pinealectomized rats but the differences were not statistically significant.

The mechanisms by which pinealectomy increased oxidative damage in pregnant rats are unknown. It is hypothesized that high ventilation frequency and the resultant increased lipid peroxide production in placenta during pregnancy causes high levels of oxidative stress. Melatonin, an important antioxidant that is reduced by pinealectomy, may be important in protecting cells from this molecular damage.

There are several mechanisms by which melatonin may reduce lipid peroxidation. Melatonin acts directly as an effective scavenger of both the hydroxyl radical (Tan et al., 1993, 1998; Stasica et al., 1998) and the peroxynitrite anion (Cuzzocrea et al., 1998), both of which are capable of initiating lipid peroxidation. Melatonin may also scavenge the peroxyl radical (Pieri et al., 1994, 1996) which propagates the chain reaction of lipid breakdown. In addition, melatonin reportedly increases the activity of the antioxidative enzyme glutathione peroxidase (Barlow-Walden et al., 1995), inhibits the pro-oxidative enzyme nitric oxide synthase (Bettahi et al., 1996; Guerrero et al., 1997) and increases mRNA for antioxidant enzyme SOD at least in some tissues (Antolin et al., 1996; Kotler et al., 1998). Thus, there are several mechanisms by which melatonin may have limited the generation of oxidatively damaged lipid in the present study.

Several studies have demonstrated a circadian rhythm in melatonin concentrations in the umbilical circulation of term fetuses and newborns (Acuña-Castroviejo et al., 1989; Muñoz-Hoyos et al., 1992). Melatonin is transferred from the maternal to the fetal circulation, generating a day–night difference in melatonin concentration in the circulation of the fetus (Muñoz-Hoyos et al., 1992). Okatani et al. (1997)
demonstrated that melatonin suppresses the vasospastic effect of H$_2$O$_2$ on the human umbilical artery and this suppressive effect is reduced by two antioxidants, mannitol and catalase. The suppressive action of melatonin on the vasospastic effects of peroxide may also have clinical implications in pre-eclampsia. It has been demonstrated that melatonin passes through the placenta more efficiently than antioxidants such as vitamin E and or S-adenosyl methionine (Schenker et al., 1998; Okatani et al., 1999). Hence, melatonin could be important as an antioxidant agent in the fetus as well. Administration of antioxidants, including melatonin, to mothers prevents oxidative stress associated with free radical damage in fetal and neonatal rats (Sastre et al., 1994; Wakatsuki et al., 1999). The role of melatonin, which is devoid of pro-oxidant effects (Marshall et al., 1996), in the prevention of maternal and fetal damage is still unknown and requires further study.

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