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European Journal of Pharmacology 426 (2001) 1–10

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Review

Pharmacological action of melatonin in shock, inflammation and ischemia/reperfusion injury

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Received 2 April 2001; received in revised form 27 June 2001; accepted 29 June 2001

Abstract

A vast amount of circumstantial evidence implicates oxygen-derived free radicals (especially, superoxide and hydroxyl radical) and high-energy oxidants (such as peroxynitrite) as mediators of inflammation, shock and ischemia/reperfusion injury. The aim of this review is to describe recent developments in the field of oxidative stress research. The first part of the review focuses on the roles of reactive oxygen species in shock, inflammation and ischemia/reperfusion injury. The second part of the review described the pharmacological action of melatonin in shock, ischemia/reperfusion, and inflammation. (1) The role of reactive oxygen species: Immunohistochemical and biochemical evidence demonstrate the production of reactive oxygen species in shock, inflammation and ischemia/reperfusion injury. Reactive oxygen species can initiate a wide range of toxic oxidative reactions. These include the initiation of lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inactivation of glyceraldehyde-3phosphate dehydrogenase, inhibition of membrane sodium/potassium ATP-ase activity, inactivation of membrane sodium channels, and other oxidative modifications of proteins. All these toxicities are likely to play a role in the pathophysiology of shock, inflammation and ischemia and reperfusion. (2) Treatment with melatonin has been shown to prevent in vivo the delayed vascular decompensation and the cellular energetic failure associated with shock, inflammation and ischemia/reperfusion injury. Reactive oxygen species (e.g., superoxide, peroxynitrite, hydroxyl radical and hydrogen peroxide) are all potential reactants capable of initiating DNA single-strand breakage, with subsequent activation of the nuclear enzyme poly (ADP-ribose) synthetase (PARS), leading to eventual severe energy depletion of the cells, and necrotic-type cell death. Recently, it has been demonstrated that melatonin inhibits the activation of poly (ADP-ribose) synthetase, and prevents the organ injury associated with shock, inflammation and ischemia and reperfusion.

Keywords: Free radical; Melatonin; Inflammation; Ischemia/reperfusion; Shock

1. Production and role of free radicals in shock, inflammation and ischemia/reperfusion injury

Oxygen-derived free radicals are continually generated within cells of aerobic organisms specifically because they utilize molecular oxygen (dioxygen or O_2) as the basis of their metabolism. Free radicals are molecules or portions thereof which possess one or more unpaired electrons in their outer orbital, a state which greatly increases their reactivity (DeGroot, 1994).

The best-known reactive species generated from O_2 include the superoxide anion radical (dioxide or O_2^-), the hydroxyl radical (OH) and the peroxynitrite anion ($ONOO^-$); these reactive oxygen metabolites are related as shown in Fig. 1. Because of their high reactivity, these radicals can be devastatingly toxic to other molecules and can cause cellular dysfunction and sometimes death of cells (DeGroot, 1994). This presents aerobic organism with a major paradox, where O_2 is required for life but its byproducts may eventually kill them.

O_2 is enzymatically reduced to H_2O_2 in the presence of a ubiquitously distributed enzyme, superoxide dismutase (SOD) (McCord and Fridovich, 1969). Superoxide dismutase, usually classified as an antioxidative enzyme that affords protection against free radical damage, in some cases can be associated with increased oxidative stress. Thus, the over-expression of superoxide dismutase such as in trisomy 21 (Down syndrome), may be responsible for many of the neurodegenerative changes and cataracts these

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)01175-X

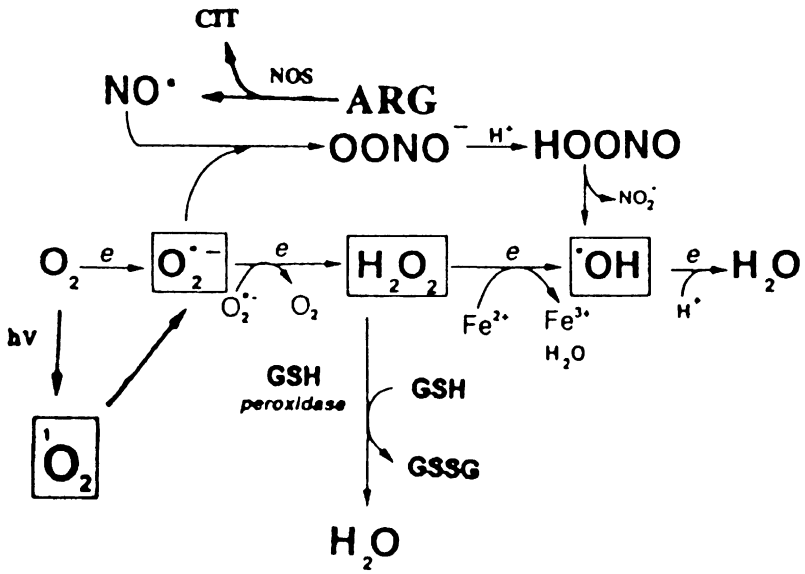

Fig. 1. Free radicals and reactive oxygen intermediates that are considered in this review. The three-electron (e) reduction of oxygen (O_2) generates the highly toxic hydroxyl radical ($^{\cdot}OH$); intermediates in this process include the superoxide anion radical ($O_2^{-\cdot}$) and the non-radical species hydrogen peroxide (H_2O_2). The $O_2^{-\cdot}$ is enzymatically converted to H_2O_2 by a widely distributed family of enzymes known as superoxide dismutase (SOD). Transition metals, e.g., Fe^{2+} generate the $^{\cdot}OH$ via the Fenton reaction. Singlet oxygen ($^1O_2$), an activated form of O_2, is also a toxic agent. H_2O_2 is metabolized to non-toxic products by GSH peroxidase; in the process reduced glutathione (GSH) is converted to oxidized glutathione (GSSG). Nitric oxide synthase (NOS) generates nitric oxide (NO) which can degrade into more reactive radicals. These interactions are explained in the text.

individuals experience at an early age (Kedziora and Bartosz, 1988).

H_2O_2 does not possess an unpaired electron and, therefore, is not a free radical per se. Thus, it is usually classified as a reactive oxygen intermediate or species. H_2O_2 can diffuse though membranes and it has a half-life much longer than that of $^{\cdot}O_2 \cdot H_2O_2$ has several fates intracellularly. It can be metabolized by one or two antioxidative enzymes, *i*. GSH-PX or catalase, and, in the worst case scenario, in the presence of the transition metals Fe^{2+} or Cu^{1+}, it is reduced to the $^{\cdot}OH$ via the Fenton reaction (Fig. 2) (Meneghini and Martins, 1993).

$^{\cdot}OH$ is fearsomely reactive and highly toxic. It indiscriminately reacts with any molecule it encounters. Among radicals, it could be classified as the radical's radical. Because of its large size and electroreactivity, it is not uncommon for $^{\cdot}OH$ to interact and produce damage to macromolecules such as DNA, proteins, carbohydrates, and lipids (Kehrer, 1993). Oxidative damage to macromolecules is especially noticeable because compared to the smaller molecules in cells, they are present in limited numbers. In the case of DNA, damage inflicted by the $^{\cdot}OH$ can lead to cancer (Dizdaroglu, 1993). The reactions of radicals with non-radicals (which most molecules in an organism are) lead to, by necessity, in the formation of a new radical; thus radicals beget radicals. In some cases, these newly formed radicals may also be rather toxic and, in fact, may initiate other damaging free-radical reactions. An example of this type of chain reaction is lipid peroxidation, where the lipid peroxyl radical ($ROO^{\cdot}$), once produced, abstracts a hydrogen atom from a neighboring polyunsaturated fatty acid (PUFA), thereby propagating the degeneration of lipids (Girotti, 1985).

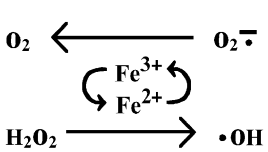

Fig. 2. The generation of the highly toxic hydroxyl radical ($^{\cdot}OH$) from the superoxide anion radical (O_2) and hydrogen peroxide (H_2O_2). This set of reactions was initially defined by Fenton and still bears his name.

A radical, however, can also interact with another radical to form a stable molecule. In this case, the unpaired electrons in each radical form a covalent bond. This is what happens when a $O_2^{-\cdot}$ encounters NO with the resultant formation of $ONOO^-$ (Beckman et al., 1990). The biological activity and decomposition of $ONOO^-$ is very much dependent on the cellular or chemical environment (presence of proteins, thiols, glucose, the ratio of nitric oxide (NO) and superoxide, carbon dioxide levels and other factors), and these factors influence its toxicity (Beckman et al., 1990; Rubbo et al., 1994; Villa et al., 1994).

$ONOO^-$ is cytotoxic via a number of independent mechanisms. Its cytotoxic effects include initiation of lipid peroxidation, inactivation of a variety of enzymes (most notably, mitochondrial respiratory enzymes and membrane pumps) (Villa et al., 1994) glutathione depletion (Phelps et al., 1995).

Important cardiovascular consequences of circulatory shock include reduced responsiveness of arteries and veins to exogenous or endogenous vasoconstrictor agents (vascular hyporeactivity), myocardial dysfunction, and disrupted intracellular energetic processes. These alterations have been previously suggested to be related to NO, due to the activation of the endothelial isoform of nitric oxide synthase (ecNOS, early stage) and expression of a distinct inducible isoform of nitric oxide synthase (iNOS, late stage) in the vascular smooth muscle cells, cardiac myocytes, and other cell types (Szabò and Thiemermann, 1994; Szabò, 1995). The outcomes of these studies prompted these conclusions, based mainly on results obtained with the use of NOS inhibitors, and did not or could not distinguish between the effects of NO vs. $ONOO^-$. Recent data demonstrate that authentic $ONOO^-$ is capable of mimicking many of the cardiovascular alterations associated with shock (endothelial dysfunction, vascular hyporeactivity, myocardial failure, and cellular energetic failure) (see above). In circulatory shock, pro-inflammatory cytokines invoke a pleiotropic cellular response, including the stimulation of oxygen-centered free radicals, such as $O_2^{-\cdot}$. The majority of NO produced by macrophages is converted to $ONOO^-$ (Ischiropoulos et al.,1992). The production of $ONOO^-$ (evidenced by increased nitrotyrosine immunoreactivity or increased oxidation of the fluorescent probe dihydrorhodamine 123 to rhodamine 123) has recently been demonstrated in endotoxic shock and in hemorrhagic shock (Wizemann et al., 1994; Szabo et al., 1995).

A large number of studies demonstrate the protective effect of superoxide dismutase (SOD) analogs in various models of endotoxic and hemorrhagic shock and splanchnic artery occlusion/reperfusion injury (McKechnie et al., 1986; Kapoor and Prasad, 1995; Rhee et al., 1991; Wang et al., 1990; Bitterman et al., 1988; Youn et al., 1991; McCord, 1993; Salvemini et al., 1999). Furthermore, there is a large amount of evidence to show that the production of reactive species such as $O_2^{-\cdot}$, H_2O_2 and $^{\cdot}OH$ occurs at the site of inflammation and contributes to tissue damage (Cuzzocrea et al., 1997, 1998a; Ohishi et al., 1989; Dawson et al.,1991; Peskar et al., 1991; Da Motta et al., 1994; Salvemini et al., 1996). Inhibitors of nitric oxide synthase activity reduce the severity of inflammation and support a role for NO in the pathophysiology associated with various model of inflammation (Cuzzocrea et al., 1998a, 1997; Salvemini et al., 1996; Tracey et al., 1995; Wei et al., 1995). In addition to NO, $ONOO^-$ is also generated during inflammation (Cuzzocrea et al., 1997, 1998a; Salvemini et al., 1996). The involvement of $ONOO^-$ in these conditions is strongly supported by direct measurements. For example, in arthritis, increases in plasma and synovial fluid nitrotyrosine levels have been reported (Kaur and Halliwell, 1994). In ileitis (Miller et al., 1995) and in endotoxin-induced intestinal inflammation (Chamulitat et al., 1996), there is immunocytochemical documentation (increased nitrotyrosine immunoreactivity in the inflamed tissues) of augmented $ONOO^-$ production.

The pathophysiological role of NO and $ONOO^-$ in the gastrointestinal damage elicited by endotoxin or chronic inflammation has been the subject of a variety of detailed investigations. The ability of authentic $ONOO^-$ to cause severe colonic inflammation has been documented (Rachmilewitz et al., 1993). The production of $ONOO^-$ in colitis may be even more pronounced because of the parallel down-regulation of superoxide synthase (Seo et al., 1995), which renders $O_2^{-\cdot}$ available for coupling with NO. Desferoxamine, a putative peroxynitrite scavenger (Denicola et al., 1995), or superoxide dismutase protects against the gastric damage elicited by NO donors, supporting the view that peroxynitrite (and not NO per se) is the cytotoxic species in these models (Lamarque and Whittle, 1995a,b).

The overproduction of reactive oxygen and nitrogen species during inflammation leads to a considerable oxidant stress as indicated by increased lipid peroxidation represented by high blood levels of malondialdehyde (MDA) and conjugated dienes as well as the depletion of the endogenous antioxidants vitamins C and E (Novelli et al., 1997; Cuzzocrea et al., 1999a).

Reactive species and $ONOO^-$ produce cellular injury and necrosis via several mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage (Cochrane, 1991; Szabó et al., 1996b). Oxygen byproducts produce strand breaks in DNA, which triggers energy-consuming DNA repair mechanisms and activates the nuclear enzyme poly (ADP-ribose) synthase resulting in the depletion of its substrate NAD in vitro and a reduction in the rate of glycolysis. Since NAD functions as a cofactor in glycolysis and the tricarboxylic acid cycle, NAD depletion leads to a rapid fall in intracellular ATP. This process has been termed *the PARS suicide hypothesis*. In a variety of in vitro studies in fibroblasts, endothelial cells, epithelial cells, smooth muscle cells and other cell types, it has been established that pharmacological inhibitors of poly (ADP-ribose) synthase, such as 3-aminobenzamide, protect against the cellular oxidant injury in response to oxyradicals, NO generator drugs and peroxynitrite (Cochrane, 1991; Szabó et al., 1996b). Recent investigations have concluded that inhibition of poly (ADP-ribose) synthetase exerts beneficial effects in endotoxic shock (Szabó et al., 1996b) and various forms of reperfusion injury (Zhang et al., 1994; Zingarelli et al., 1996; Thiemermann et al., 1997; Cuzzocrea et al., 1999b). There is recent evidence that the activation of poly (ADP-ribose) synthase may also play an important role in inflammation (Szabó et al., 1997, 1998; Cuzzocrea et al., 1998b,c).

In ischemia/reperfusion injury superoxide, produced during the reperfusion phase, rapidly reacts with NO and forms $ONOO^-$. This has been demonstrated in the heart (Matheis et al., 1992; Schulz and Warnbolt, 1995; Naseem et al., 1995), liver (Ma et al., 1995), kidney (Yu et al.,

1994), intestine (Cuzzocrea et al., 1999b), brain (Fagni et al., 1994; Cazevicille et al., 1993; Gunasekar et al., 1995), and lung (Ischiropoulos et al., 1995; Kooy et al., 1995). Under these conditions, prevention of $ONOO^-$ generation by inhibition of NO biosynthesis markedly reduces reperfusion injury, as shown by reduced pulmonary lipid peroxidation (Schulz and Warnbolt, 1995) or improved myocardial mechanical performance (Schulz and Warnbolt, 1995).

A growing body of evidence supports a role for $ONOO^-$ and other reactive species in neuronal injury associated with ischemia/reperfusion injury in the central nervous system. The original proposition (Hammer et al., 1995) that $ONOO^-$ (and not NO or $O_2^{-\cdot}$, independently) is a major cytotoxic mediator in the neuronal injury during stroke and *N*-methyl-D-aspartate (NMDA) receptor activation, was based on theoretical considerations and previous evidence showing that reperfusion injury in the central nervous system is associated with activation of *N*-methyl-D-aspartate receptors, which then triggers the production of $O_2^{-\cdot}$ and NO. There is now indirect evidence to show that *N*-methyl-D-aspartate receptor activation is associated with a marked increase in a $^{\cdot}OH^-$-like activity in the brain (blocked by inhibition of NOS), which is presumably due to $ONOO^-$ generation (Dawson, 1995). The involvement of $O_2^{-\cdot}$ and the protective effect of $O_2^{-\cdot}$ neutralizing strategies (Salvemini et al., 1999; Fagni et al., 1994; Cazevicille et al., 1993; Gunasekar et al., 1995; Dawson, 1995; Beal et al., 1995; Dawson et al., 1993; Crow and Beckman, 1995) as well as the involvement of NO and the protective effect of NOS inhibition (Lafon-Cazal et al., 1993; Huang et al., 1994; Schulz et al., 1995) has been well established in various forms of central nervous system injury.

Similar to inflammation and shock, the mechanism of $ONOO^-$-induced cellular damage in the ischemia/reperfusion remains a subject for future investigations, but presumably involves multiple mechanisms. Both in vivo and in vitro evidence clearly suggests the involvement of poly (ADP-ribose) synthase in the neuronal damage associated with NO (or $ONOO^-$) production in response to *N*-methyl-D-aspartate receptor activation (Smith et al., 1994; Cosi et al., 1994; Wallis et al., 1993; Zhang et al., 1995; Eliassow et al., 1997).

2. Protection against free radicals: role of melatonin

While the effects of the pineal gland and melatonin were initially defined in terms of the endocrine physiology, particularly in relation to the neuroendocrine-reproductive axis (Reiter, 1980), in recent years it has become apparent that melatonin's actions transcended those of a hormonal modulator. By the early 1990s, melatonin had been shown to subtly influence the function of a variety of tissues and cells not generally considered in the endocrine category (Reiter, 1991). This led to a suspicion that melatonin had yet undefined actions at the cellular level. At about the same time, a publication appeared in a relatively obscure journal which claimed melatonin had both antioxidant and pro-oxidant activity in vitro, depending on its concentration in the medium (Ianas et al., 1991). If these actions of melatonin could be verified in subsequent more comprehensive studies, they could potentially explain the diverse effects of melatonin since free radical generation is ubiquitous in cells of aerobic organisms.

Validation of melatonin's free radical scavenging ability was initially reported by Tan et al. (1993). In a cell free system in which $^{\cdot}OH$ were generated by the exposure of H_2O_2 to 254-nm ultraviolet light, melatonin was compared to reduced glutathione (GSH) and mannitol in terms of their ability to neutralize this highly toxic radical. In this system, the product of the reaction was quantified by high-performance liquid chromatography (HPLC) with electrochemical detection and verified by electron spin resonance spectroscopy and it was found that the relative efficacies of three molecules in scavenging $^{\cdot}OH$ were melatonin > GSH > mannitol (Reiter et al.,1999a). Several melatonin metabolites tested in the same system proved less effective than melatonin itself in scavenging the $^{\cdot}OH$. That melatonin functions as a direct free radical scavenger is supported by the observation that it also quenched $^{\cdot}OH$ generated via another means, i.e., the Fenton reaction (Reiter et al., 1999b). These findings suggest a role for melatonin in the antioxidative defence in all organisms that produce this indole (Reiter et al., 1999b; Poeggeler et al., 1993) particularly since melatonin crosses all morphophysiological barriers and enters all cells. Since the findings of Tan et al. (Reiter et al., 1999b), other investigators have also documented melatonin's ability to detoxify the $^{\cdot}OH$ (Susa et al., 1997; Bromme et al., 1999). Using both well-defined in vitro and in vivo methodology, these authors demonstrated melatonin's efficacy in neutralising the highly toxic $^{\cdot}OH$. From these studies, the rate constant for the interaction of melatonin with the $^{\cdot}OH$ was calculated to be in the range of $2.7–4.0 \times 10\ m^{-1}\ s^{-1}$. Furthermore, one of the reaction products of melatonin with $^{\cdot}OH$ was identified as cyclic 3-hydroxymelatonin (Tan et al., 1998). 3-Hydroxymelatonin is excreted in the urine and is a footprint molecule that represents melatonin's $^{\cdot}OH$ scavenging efficiency.

Shortly after the discovery by Tan et al. (Reiter et al., 1999b) that melatonin quenches $^{\cdot}OH$, Pieri et al. (1994) reported that melatonin was also capable of scavenging another toxic oxygen-related radical, the peroxyl radical ($LOO^{\cdot}$). $LOO^{\cdot}$ is produced during peroxidation of lipids and it is sufficiently reactive that it propagates the chain reaction, thereby leading to extensive destruction of fatty acids (Fig. 3) (Yu, 1994). Thus, molecules, which neutralize $LOO\cdot$, e.g., vitamin E (Lloyd, 1990), act as chain breaking antioxidants and play a critical role in preserving the functional integrity of membrane lipids and therefore cellular function. Pieri et al. (1994) compared melatonin's

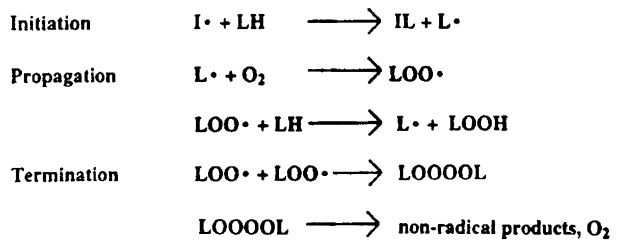

Fig. 3. Simplified summary of lipid peroxidation, a self-propagating process illustrating its three phases. Because the peroxidation process is a chain reaction that is re-initiated by the products, i.e. LOW, it generates cellular damage due to lipid peroxidation, which can be extensive. I′ = initiating radical; L′ = lipid radical; LOO′ = peroxyl radical; LH = lipid molecule.

ability to scavenge the LOO using what is referred to as the oxygen radical-absorbing capacity of the molecule with that of several other known scavengers. On the basis of these tests, they concluded that melatonin was roughly twice as effective as water-soluble vitamin E, i.e., Trolox, in neutralizing LOO. They came to a similar conclusion as a result of their further investigations (Pieri et al., 1995) and therefore suggested that melatonin is a better antioxidant against lipid peroxidation than this vitamin.

Subsequent studies by other workers have also examined melatonin's ability to quench the LOO. While Scaiano (1995) and Marshall et al. (1996) feel that melatonin has some LOO · -scavenging ability, neither group inferred that it was on a par with vitamin E in this regard. At this point melatonin's efficacy in scavenging the LOO remains questionable, although its effectiveness against lipid peroxidation both in vitro and in vivo is substantial (Reiter, 1996, 1995).

An activated form of $^{\cdot}O_2$, i.e., singlet oxygen ($^{\cdot}O_2$) is a toxic agent which inflicts damage to a variety of macromolecules (Agarwal et al., 1991). Although Poeggeler et al. (1993) first predicted that melatonin may quench $^{\cdot}O_2$, the first experimental evidence for this was provided by Cagnoli et al. (1995). They showed that melatonin protected primary cultures of cerebellar granule cells from the cytotoxicity of $^{\cdot}O_2$ molecules which were formed when the cells were exposed to both a photosensitive dye, rose bengal, and then to light. While this evidence is indirect and the mechanism by which melatonin limits $^{\cdot}O_{2-}$-induced cellular damage is unknown, it did appear that melatonin effectively neutralized $^{\cdot}O_2$ in this study. More direct evidence of melatonin's ability to quench $^{\cdot}O_2$ were recently demonstrated. Thus, Zang et al. (1998) found melatonin to be highly reactive with $^{\cdot}O_2$. The evidence for this conclusion is based on the observation that melatonin reduced the product of the interaction of $^{\cdot}O_2$ with a specific target molecule. The target molecule was quantified by electron spin resonance spectroscopy.

Two reports claim that melatonin also scavenges hypochlorus acid (HOCl) (Marshall et al., 1996; Chan and Tang, 1996). On the other hand, melatonin does not react directly with $^{\cdot}O_2$ (Hardeland et al., 1993), although indolyl cation radical is not thought to be as potent as melatonin, as well as several melatonin-related indoles, i.e., 5-methoxytryptophol, 5-methoxyindole acetic acid, and 5-methoxytryptamine (Poeggeler et al., 1995); this illustrates that they may have substantial electron-donating potential, at least under the conditions of these studies. Another analog of melatonin, 5-hydroxytryptamine (serotonin) was found to generate free radicals, i.e., be pro-oxidant in two separate studies (Reiter et al., 1999a,b Marshall et al., 1996) although in a recent report using another system to assess redox-active molecules, Daniels et al. (1996) found serotonin to possess antioxidant activity. The different outcome, reported in the work of Daniels et al. (1996), relative to those of earlier articles, presumably relates to the system that was used to measure antioxidant/pro-oxidant activity. That the hepatic metabolite of melatonin, 6-hydroxymelatonin sulfate, may also be a free radical scavenger was suggested by Pierrefiche et al. (1993) since it was shown to be capable of resisting lipid peroxidation in vitro. If this is the case, some of melatonin's antioxidant properties in vivo may occur after its metabolism to its chief hepatic degradation product, 6-hydroxymelatonin sulfate. It has been proposed that the product formed, i.e. the indolyl cation radical, when melatonin donates an electron, may secondarily scavenge the O_2^-. Melatonin has also no known direct interactions with H_2O_2, as described by Chan and Tang (1996) and by Poeggeler et al. (1994).

Besides its direct free radical-scavenging actions, melatonin may also protect cells from damage by acting cooperatively with other antioxidants. These potential interactions were examined by Sewerynek et al. (1995) in both a cell-free system and in the presence of brain homogenates. In these studies, the radical trapping reagent 2, 2′-azinobis-3-ethylbenz thiazoline-6-sulfonic acid (ABTS) was incubated with the Fenton reagents H_2O_2 and $FeSO_4$ to generate the ABTS cation radical ($ABTS^{\cdot+}$), which is measured at 420 nm of absorbance in a spectrophotometer. Melatonin, when given in combination with either of the chain-breaking antioxidants ascorbic acid, Trolox (vitamin E) or GSH, acted synergistically to suppress the formation of $ABTS^{\cdot+}$. It is well known that the chain-breaking antioxidants used in this study also act in a synergistic manner to control free radicals but this is the first work which report that melatonin also possesses this property.

Recently, Gilad et al. (1997) demonstrated that melatonin inhibits $ONOO^-$-induced oxidative processes. In this work, it was found that melatonin dose-dependently inhibits the oxidation of dihydrorhodamine 123 by $ONOO^-$, and its potency is comparable with the efficacious $ONOO^-$ scavengers glutathione and cysteine. Moreover, it is also shown that melatonin protects cultured cells against $ONOO^-$-induced injury. It is noteworthy that the protection provided by the highest concentration of melatonin tested (300 μM) was not completely effective in reversing the suppression of mitochondrial respiration induced by $ONOO^-$.

The mechanism of $ONOO^-$ scavenging by melatonin at the moment is not completely clear and requires further studies. On one hand, it is possible that melatonin reacts with ground state $ONOO^-$. It is also possible, however, that melatonin reacts with peroxynitrous acid (Cuzzocrea et al., 1999c; Zhang et al., 1999), or with its highly reactive cleavage products, either an intermediate with hydroxyl radical-like agent (homolytic cleavage) or with the nitronium ion (heterolytic cleavage). In this respect, it is noteworthy that melatonin also inhibits hydrogen peroxide-induced oxidative processes (Pieri et al., 1995; Scaiano, 1995).

3. Pharmacological intervention to reduce ROS generation in shock, inflammation and ischemia / reperfusion: role of melatonin

Interventions which reduce the generation or the effects of reactive oxygen species exert beneficial effects in a variety of models of inflammation and shock. These therapeutic interventions include a vitamin E-like antioxidant (Cuzzocrea et al., 1999a), a superoxide dismutase-mimetic (Wang et al., 1990; Cuzzocrea et al.,1999d) and a $ONOO^-$ decomposition catalyst (Salvemini et al., 1998). The therapeutic efficacy of superoxide dismutase itself in animals with systemic inflammation, haemorrhage or shock is controversial. The following reasons may explain the lack of effect of superoxide dismutase against the tissue injury associated with local or systemic inflammation: (1) Superoxide dismutase metabolised O_2^- to H_2O_2. Without efficient removal of the H_2O_2, however, H_2O_2 is converted to the highly toxic $^{\cdot}OH$ (Goode and Webster, 1993). Indeed, superoxide dismutase may function as a pro-oxidant by catalysing the conversion of H_2O_2 to $^{\cdot}OH$ (Yim et al., 1990) such as what is believed to be the case in Down syndrome. (2) Neither superoxide dismutase nor $O_2^{-\cdot}$ easily cross biological membranes. Thus, an increase in the amounts of extracellular superoxide dismutase does not attenuate the effects of $O_2^{-\cdot}$ generated by intracellular sources (Fridovich, 1995). In contrast to superoxide dismutase, spin trapping nitrones such as phenyl *N-tert*-butyl nitrone (PBN) consistently improve outcome in rat models of endotoxic (Mckechnie et al., 1986; Hamburger and McCay, 1989) and traumatic shock (Novelli, 1992; Novelli et al., 1986). The protective effects of melatonin have been demonstrated in various conditions of oxidant stress, including H_2O_2-induced lipid peroxidation (Sewerynek et al., 1995). It was also recently shown that melatonin acts as a modestly potent $ONOO^-$ scavenger, thereby protecting cultured cells from $ONOO^-$-induced injury (Gilad et al., 1997). Also, melatonin raises some interest as an anti-inflammatory agent.

The early phase of the inflammatory process is related to the production of histamine, leukotrienes, platelet-activating factor, and possibly cyclooxygenase products, while the delayed phase of the inflammatory response has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals and oxidants, such as H_2O_2, O_2^- and $^{\cdot}OH$, as well as to the release of other neutrophil-derived mediators (Ohishi et al., 1989; Dawson et al.,1991; Peskar et al., 1991; Da Motta et al.,1994;

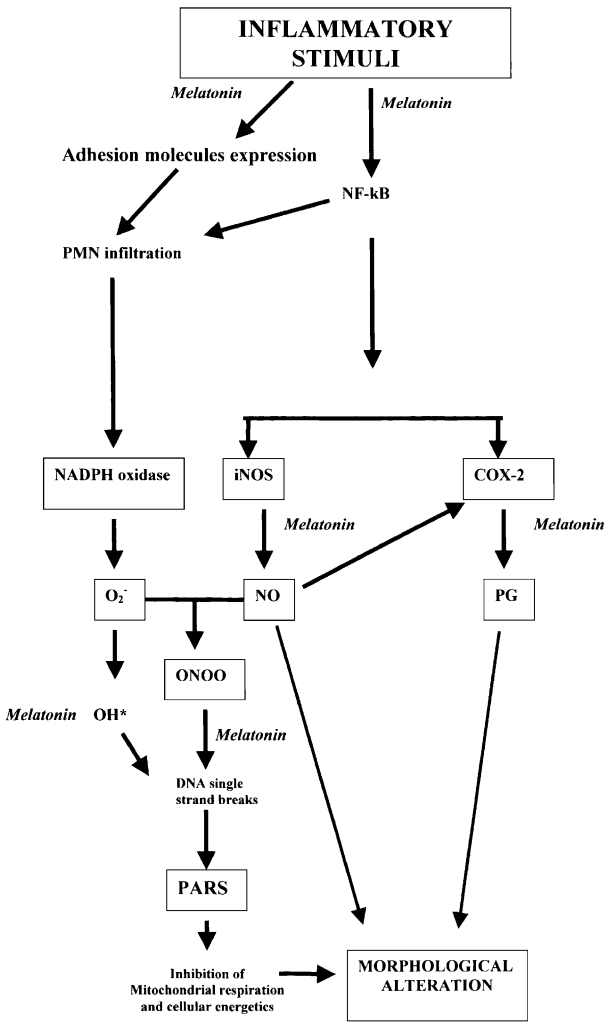

Fig. 4. Proposed scheme of some of the delayed inflammatory pathways involving nitric oxide (NO), hydroxyl radical (OH) and peroxynitrite ($ONOO^-$) in inflammation and potential sites of melatonin's anti-inflammatory actions. Inflammation triggers the expression of the inducible NO synthase (iNOS), at least in part, via the activation of nuclear factor KB (NF-κB). NO, in turn, combines with superoxide to yield $ONOO^-$. $OH^{\cdot}$ (produced from superoxide via the iron-catalyzed Haber–Weiss reaction) and $ONOO^-$ or peroxynitrous acid (ONOOH) induce cellular injury. Part of the injury is related to the development of DNA single-strand breakage, with subsequent activation of poly (ADP-ribose) synthase, leading to cellular dysfunction. NO can directly increase the catalytic activity of the inducible isoform of cyclooxygenase (COX-2), leading to enhanced production of pro-inflammatory prostaglandin metabolites. In this system, melatonin's anti-inflammatory effects may include (1) inhibition of the activation of NF-κB and prevention of the expression of iNOS; (2) direct inhibition of the catalytic activity of NOS; (3) $OH^{\cdot}$ scavenging; (4) $ONOO^-$ scavenging; (5) prevention of adhesion molecules expression; and (6) specific effects related to activation of melatonin receptors.

Salvemini et al., 1996). Cyclooxygenase (COX) is the rate-limiting enzyme in the biosynthesis of prostaglandins, thromboxane A2, and prostacyclin. In addition to the well-characterized constitutive form of cyclooxygenase (COX-1) (De Witt, 1991), an inducible isoform of cyclooxygenase (COX-2) is found in endothelial cells (Maier et al., 1990), fibroblasts (Raz et al., 1988), and macrophages (Fu et al., 1990; Masferrer et al., 1990) after treatment with proinflammatory agents including lipopolysaccharide (LPS) and interleukin 1β (IL-1β). Regulation of cyclooxygenase-2 shares similarities with the regulation of inducible nitric oxide synthase (iNOS) and is also under the regulation of nuclear factor kappa B (NF-κB) and mitogen-activated protein (MAP) kinase activation (Vane and Botting, 1995). Recent studies have demonstrated that melatonin inhibits NO production (Pozo et al.,1994), and reduces the expression of iNOS in the lung after carrageenan-induced pleurisy (Cuzzocrea et al., 1997). This reduction in iNOS is related to the known inhibitory effect of melatonin on the activation of the NF-κB (Mohan et al., 1995; Gilad et al., 1998; Lezovalch et al., 1998) since this transcription factor is involved in the process of iNOS expression (Nathan, 1999; Salzman et al., 1996). In addition, in vitro studies have demonstrated that melatonin reduces 6-keto-prostaglandin-Fα production in cultured J774 and RAW.264.7 macrophages activated by lipopolysaccharide (Lezovalch et al., 1998). Suppression of cyclooxygenase-2 expression by melatonin has been also demonstrated (Cuzzocrea et al., 1999c). One question that remains to be answered is the mechanism by which melatonin protects against the inflammatory injury.

There are a number of sites where melatonin may interfere with the inflammatory process (Fig. 4): (1) melatonin inhibits NO production and reduces the expression of iNOS in the lung after carrageenan-induced pleurisy (Cuzzocrea et al., 1997, 1998d); (2) melatonin influences the activation of the transcription factor NF-κB (Mohan et al., 1995; Gilad et al., 1998; Lezovalch et al., 1998); and (3) melatonin reduces the expression of iNOS at the transcriptional level (Pozo et al., 1994; Gilad et al., 1998; Bettahi et al., 1996; Maestroni, 1996). These findings are consistent with a proposed novel mechanism for melatonin's anti-inflammatory effect. Recently, prostaglandin (PG) levels in the exudate and cyclooxygenase-2 expression from carrageenan-treated rats were found to be completely inhibited by melatonin (Cuzzocrea et al., 1999c). This inhibitory response is likely to be related to a regulatory effect on gene expression as suggested by Gilad et al. (1998).

Finally, we have also found that melatonin attenuates the increase in poly (ADP-ribose) synthase activity associated with zymosan administration. Similarly, melatonin attenuated the formation of $ONOO^-$ by macrophages (ex vivo) obtained from rats that had been injected with zymosan. In these macrophages, melatonin also attenuated the fall in NAD associated with the enhanced formation of $ONOO^-$. Thus, we propose that the anti-inflammatory effects of melatonin may be partly induced through the activation of poly (ADP-ribose) synthase by indoleamine.

We conclude that the observed anti-inflammatory effects of melatonin may be dependent upon a combination of the following pharmacological properties of this agent: (1) Melatonin secondarily scavenges and inactivates $O_2^{-\cdot}$, which reduced the formation of $ONOO^-$. This, in turn, prevents the activation of poly (ADP-ribose) synthase and the associated tissue injury. (2) At the same time, melatonin lowers the synthesis of NO, thereby also reducing $ONOO^-$ formation. (3) In addition to $O_2^{-\cdot}$, melatonin also scavenges other radical oxygen species including $^{\cdot}OH$. (4) Melatonin additionally scavenges $ONOO^{-\cdot}$. (5) Finally, melatonin reduces the recruitment of polymorphonucleates into the inflammatory site. This effect of melatonin is very likely secondary to the reduction endothelial oxidant injury and, hence, a preservation of endothelial barrier function. These results support the view that the over-production of reactive oxygen or nitrogen species contributes to the acute inflammatory response and we propose that small molecules such as melatonin, which permeate biological membranes and function as intracellular radical scavengers, may be useful in the therapy of conditions associated with local or systemic inflammation.

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