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N-acetylserotonin is a better extra- and intracellular antioxidant than melatonin

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Abstract Both melatonin and its precursor *N*-acetylserotonin have been reported to exert antioxidant properties both in vitro and in vivo. Since little is known about their antioxidant activity in lymphocytes, we investigated their effects on spontaneous and on oxidant-induced reactive oxygen species formation in human peripheral blood lymphocytes in comparison to the antioxidant trolox, a water-soluble analogue of α-tocopherol. Both melatonin and *N*-acetylserotonin exhibited antioxidant properties against t-butylated hydroperoxide- and diamide-induced reactive oxygen species formation in peripheral blood lymphocytes. *N*-acetylserotonin turned out to be about three times more effective than melatonin. In resting cells, the intracellular reactive oxygen species concentration was only decreased by *N*-acetylserotonin and trolox, melatonin had no effect. In t-butylated hydroperoxide-mediated cell death, *N*-acetylserotonin was as effective as trolox in protecting peripheral blood lymphocytes from cell death and required 10-fold lower concentrations than melatonin. Furthermore, in an aqueous cell-free solution, the capacity of *N*-acetylserotonin to scavenge peroxyl radicals was much higher than that of melatonin. These results clearly indicate *N*-acetylserotonin to be a much better antioxidant than melatonin.

Key words: Reactive oxygen species; Melatonin; *N*-acetylserotonin; Antioxidant activity; Lymphocyte

1. Introduction

Melatonin (MEL), the chief secretory product of the pineal gland, has been claimed to exhibit distinct antioxidant features in vitro as well as in vivo (for review see [1]). Since MEL was described to be a more effective scavenger of peroxyl and hydroxyl radicals than α-tocopherol and glutathione [2,3], it was postulated to be an essential element of the mammalian antioxidant defense system and to exert its physiological effects at least in part via influencing the cellular redox status [4]. Because of its low toxicity, MEL was considered to have therapeutic implications in deferring aging processes [5,6], where reactive oxygen species (ROS) are known to play an important pathogenetic role [7]. More recent studies, however, point to a rather limited direct antioxidant potency of MEL: to some extent it can scavenge hydroxyl [8] and peroxyl radicals [3], but not the less reactive superoxide anion [9]. A recent study has demonstrated that MEL acts as a retarder of the lipid peroxidation but not as a chain-breaking antioxidant [10]. This is consistent with results from lipid peroxidation assays, where the antioxidant capacity of MEL was much lower than that of α-tocopherol [11,12] and required markedly supraphysiological concentrations [13].

Indoleamines in general are known to influence biological oxidation processes [14] and *N*-acetylserotonin (NAS), the immediate precursor as well as a major product of in vivo back-transformation of MEL, has been reported to exert antioxidative properties [15,16,17]. To further differentiate the role and the physiological significance of MEL in the cellular antioxidant defense system, it was the aim of the present study to clarify the radical scavenging potency of MEL in comparison to NAS and the well-characterized antioxidant trolox (TX), a water-soluble analogue of α-tocopherol. Since little is known about antioxidant activities of MEL and NAS in lymphocytes, which are sensitive to oxidative stress [18,19], we investigated their effects on the spontaneous and oxidant-induced ROS formation, as well as on the ROS induced cell death in human peripheral blood lymphocytes (PBL).

2. Materials and methods

If not otherwise specified, substances were purchased from Sigma (St. Louis, MO, USA) and were of analytical grade or better. Dihydrorhodamine (DHR) was purchased from Molecular Probes (Eugene, OR, USA). 2,2′-azobis (amidinopropane hydrochloride) (AAPH) was obtained from Polysciences (Warrington, PA, USA).

2.1. Isolation of lymphocytes

Peripheral blood lymphocytes (PBL) from venous blood of healthy adult male donors were obtained by Ficoll-Hypaque gradient centrifugation. Isolated PBL were washed three times with PBS (10 mM sodium phosphate, 160 mM NaCl, pH = 7.4) and finally suspended in RPMI 1640 (PAA Laboratories, Exton, USA) plus 10% fetal calf serum (FCS, PAA Laboratories, Exton, USA) at a concentration of 1×10^6 cells/ml. Cells were then stimulated with the mitogen concanavalin A (Con A, 5 μg/ml) for 72 h, washed three times with PBS and cultured in RPMI 1640/10% FCS and 10 U/ml recombinant human interleukin-2 (IL-2, Boehringer Mannheim, Germany) at a concentration of 1×10^6 cells/ml. Medium and IL-2 were replaced every two days. For the experiments, activated PBL from day five to day 11 after stimulation were taken. The cell viability as determined with the trypan blue exclusion test was >95%.

2.2. Determination of ROS formation

Formation of ROS was monitored using the oxidation sensitive dye DHR, the uncharged and non-fluorescent reduction product of the cationic fluorescent dye rhodamine 123, which is suitable for ROS measurements in aqueous solutions [20]. In cellular systems, this dye passively diffuses across cell membranes and is oxidized within the cell to rhodamine, which is then located in the mitochondria. Thus, it is a

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Abbreviations: PBL, peripheral blood lymphocyte; ROS, reactive oxygen species; MEL, melatonin; NAS, *N*-acetylserotonin; TX, trolox; t-BHP, t-butylated hydroperoxide; PBS, phosphate buffered saline; DHR, dihydrorhodamine; FCS, fetal calf serum; PI, propidium iodide; DMSO, dimethyl-sulfoxide; AAPH, 2,2′-azobis(amidinopropane hydrochloride)

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useful tool for the detection of intracellular ROS formation as well [21].

2.2.1. Monitoring of the rhodamine fluorescence in an aqueous solution. 10 μM DHR (20 mM stock solution in dimethyl-sulfoxide (DMSO)) in PBS was incubated at 37°C. The ROS-induced rhodamine fluorescence was continuously monitored at 500 nm/560 nm (excitation/emission) after the addition of 1 mM AAPH as a radical-generating system using a Perkin-Elmer LS50-B spectrofluorimeter. The effect of MEL and NAS at the final concentration indicated (all solutions adjusted to a final concentration of 0.5% ethanol, required for the initial dispersion of MEL and NAS) on the peroxyl radical-induced oxidation was determined. Pure solvent (0.5% ethanol) was used as a negative control.

2.2.2. Monitoring of the rhodamine fluorescence in PBL. PBL (2×10^6 cells/ml) were incubated at 37°C in the presence of 2 μM DHR (20 mM stock solution in DMSO) for 10 min. After washing with PBS, cells were resuspended in RPMI (1×10^6 cells/ml) and left untreated or were incubated with 100 μM t-butylhydroperoxide (t-BHP), which forms peroxyl- and alkoxyl radicals in the presence of traces of transition metal ions [22] or 100 μM diamide, which oxidises sulfhydryl groups [23]. At the same time, the indicated concentrations of MEL, NAS and TX were added as a 500 mM stock solution in ethanol, The final ethanol concentration was 0,1%. Pure solvent (0.1% ethanol) was used as a negative control. After the indicated time periods, the rhodamine fluorescence was analyzed at 488 nm/525 nm (excitation/emission) using a Becton-Dickinson FACScan.

2.3. Determination of cell death

Cell death was monitored by means of the propidium iodide (PI) uptake [24]. After 4 h of treatment with t-BHP and various concentrations of MEL and NAS, PBL were washed twice with an ice-cold HEPES buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7,4), 10 μl of PI (50 μg/ml) was added to the cell pellet and incubated for 15 min at room temperature. The PI uptake, which occurs in dead cells, was analyzed using a Becton-Dickinson FACScan at an emission wavelength of 600 nm.

2.4. Statistical analysis

A data analysis was performed by a repeated measures ANOVA with a two factor repetition followed by a Tukey post-hoc test. The analysis was done with the SigmaStat 2.03 Software package.

3. Results

3.1. Formation of rhodamine in the presence of MEL and NAS in a cell-free system

In a cell-free system, we used 1 mM AAPH, which thermally decomposes to yield peroxyl radicals at a constant rate, as a ROS-generating system. Incubation of a solution of DHR with 1 mM AAPH led to a time-dependent increase of the rhodamine fluorescence (Fig. 1). Addition of MEL led to a reduced rate of fluorescence generation, indicating inefficient scavenging of peroxyl radicals, however, its respective capacity was much lower than that of NAS. Fig. 1 shows the relative efficiency of MEL, NAS and TX in inhibiting the peroxyl radical-induced oxidation of DHR. It is evident that NAS at 10 μM was an even more efficient radical scavenger than TX at the same concentration, while MEL was comparatively weak in retarding the oxidation. Ethanol alone did not affect the DHR oxidation by peroxyl radicals.

3.2. MEL and NAS effects on the intracellular DHR oxidation in human PBL

Resting PBL showed almost no change in their spontaneous intracellular ROS formation over a period of 4 h, as shown in Fig. 2. Nevertheless, the content of intracellular ROS in these cells can be reduced by the addition of TX and NAS. Interestingly, while NAS had an effect comparable to TX, MEL did not decrease the intracellular ROS content. After 4 h of incubation, even a slight enhancing effect of MEL on the ROS formation in resting PBL could be observed.

When PBL were incubated with the membrane-permeable oxidant t-BHP (100 μM), which forms peroxyl- and alkoxyl radicals [22], the intracellular rhodamine fluorescence showed a time-dependent increase with a maximum after 2 h of incubation (Fig. 3A). Addition of 1 mM MEL, NAS or TX, respectively, reduced the DHR oxidation, indicating an antioxidant effect of all substances. However, the capacity of MEL and NAS to scavenge t-BHP-induced ROS showed marked differences (Fig. 3B). While MEL significantly inhibited the ROS formation in a concentration range of 100 μM–1 mM with a maximal inhibition of $44.5\pm1.9\%$ compared to controls, NAS significantly decreased the DHR oxidation already at 30 μM with a maximal inhibition of $78.8\pm0.9\%$ at 1 mM, which is comparable to the antioxidant capacity of an equimolar concentration of TX (Fig. 3A).

Incubation with the sulfhydryl-oxidizing agent diamide (100 μM) also induced an increase of the intracellular DHR oxidation in PBL (Fig. 4) which was counteracted by MEL and NAS. The lowest concentrations of MEL and NAS tested, which showed an effect, were 300 μM and 100 μM, respectively. Maximal inhibition of the diamide-induced ROS for-

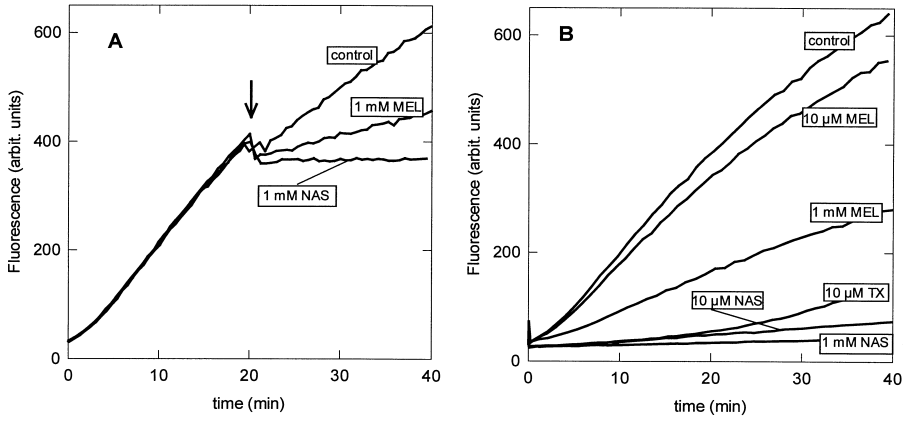

Fig. 1. Effects of MEL, NAS or TX on ROS formation in aqueous solution. 10 μM DHR in PBS was incubated at 37°C and the rhodamine fluorescence was monitored at 500 nm/560 nm (excitation/emission). Peroxyl radicals were generated by 1 mM AAPH and the effect of 10 μM TX, 10 μM and 1 mM MEL, 10 μM and 1 mM NAS on the DHR oxidation was monitored. All solutions contained 0.5% ethanol.

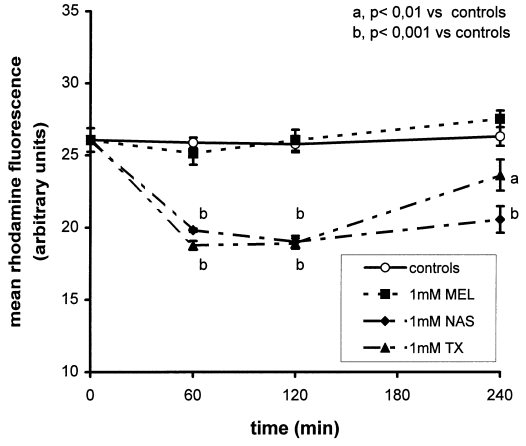

Fig. 2. Effects of MEL, NAS and TX on ROS formation in resting human PBL. PBL (2×10^6 cells/ml) were loaded with 2 μM DHR for 10 min at 37°C. After washing with PBS, cells were resuspended in RPMI (1×10^6 cells/ml) and incubated with the indicated concentrations of MEL, NAS, TX or solvent (0.1% ethanol in RPMI). After the indicated time periods, the rhodamine fluorescence was measured at 488 nm/525 nm (excitation/emission) using a Becton-Dickinson FACScan. Data represent mean ± S.E.M., $n=4$, each done in triplicate.

mation by MEL (1 mM) and NAS (1 mM) was 54.1 ± 7.7% and 84.0 ± 2.9%, respectively.

3.3. Influence of MEL and NAS on the ROS-induced cell death in human PBL

Since oxidants are known to induce lymphocyte cell death, possibly by an apoptotic pathway [25], we investigated the implications of the above described antioxidant features of MEL and NAS on the t-BHP-mediated cell death in PBL. As shown in Fig. 5, MEL as well as NAS were able to decrease the percentage of non-viable PBL after incubation with t-BHP. However, 100 μM NAS but MEL only in concentrations of more than 300 μM were effective in protecting against t-BHP-induced cell death and 100 μM NAS was as effective as 1 mM MEL, indicating that MEL is by far less efficient in protecting PBL against oxidative stress than NAS.

4. Discussion

Since MEL and NAS can easily cross cell membranes due to their amphiphilicity [26,27], we were interested to what extent these indoleamines influence intracellular redox processes. Our results clearly point to a considerably higher ROS-scavenging activity of NAS compared to that of MEL. In terms of protective effects against radical-induced cell death, the difference between NAS and MEL was even more pronounced. While 300 μM NAS decreased the t-BHP-induced cell death in PBL by 63%, the same concentration of MEL only showed a reduction by 18%. Furthermore, 100 μM NAS

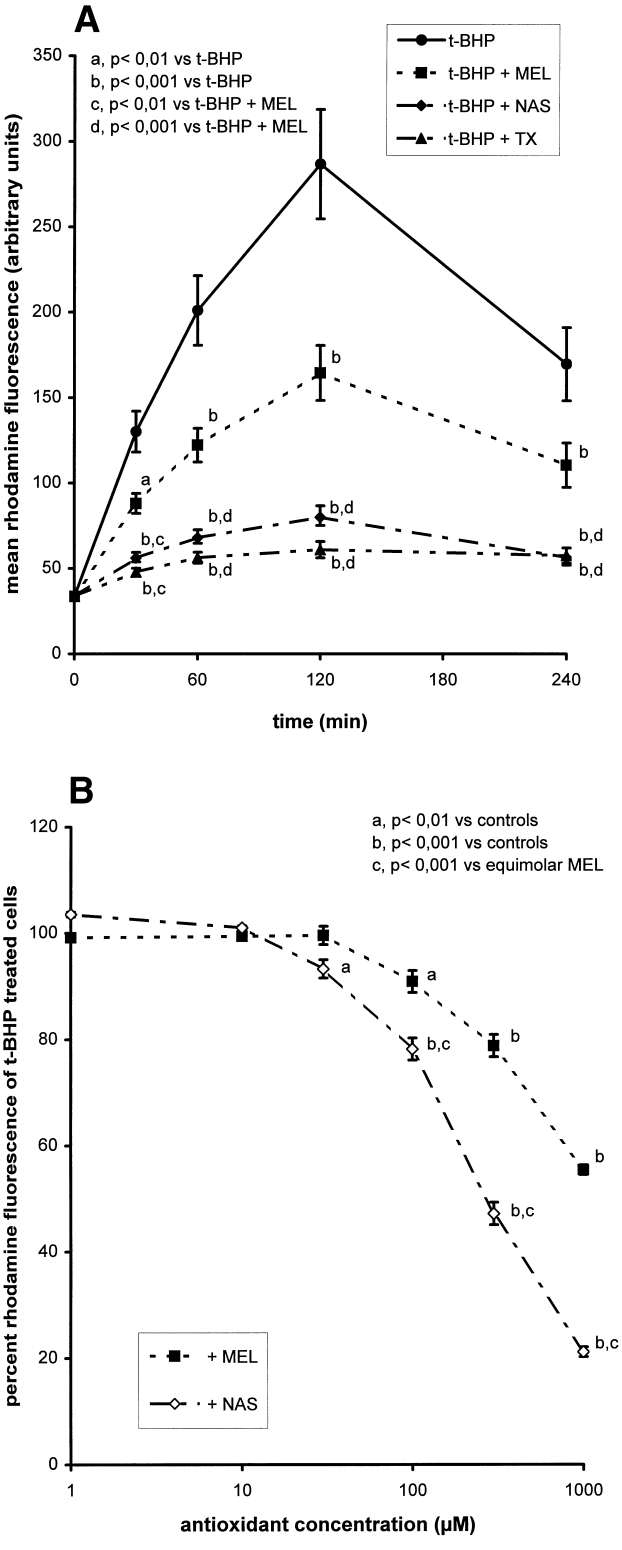

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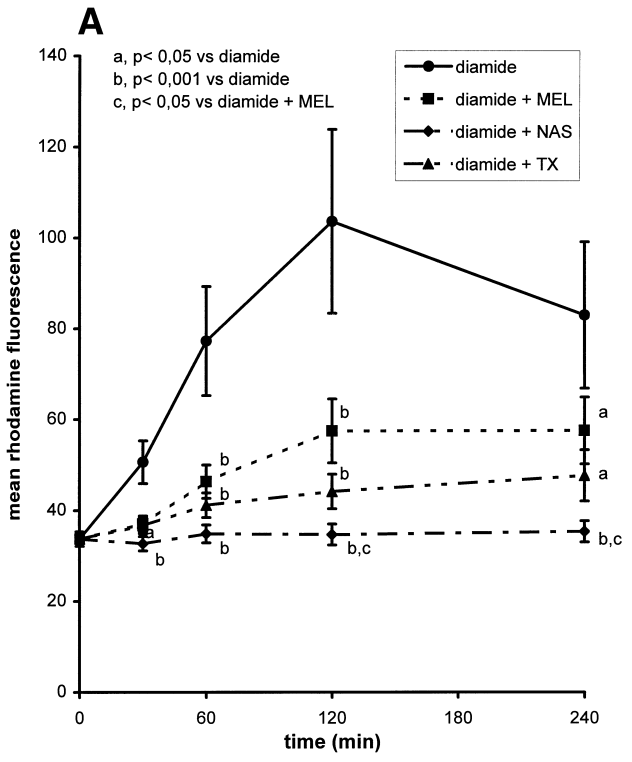
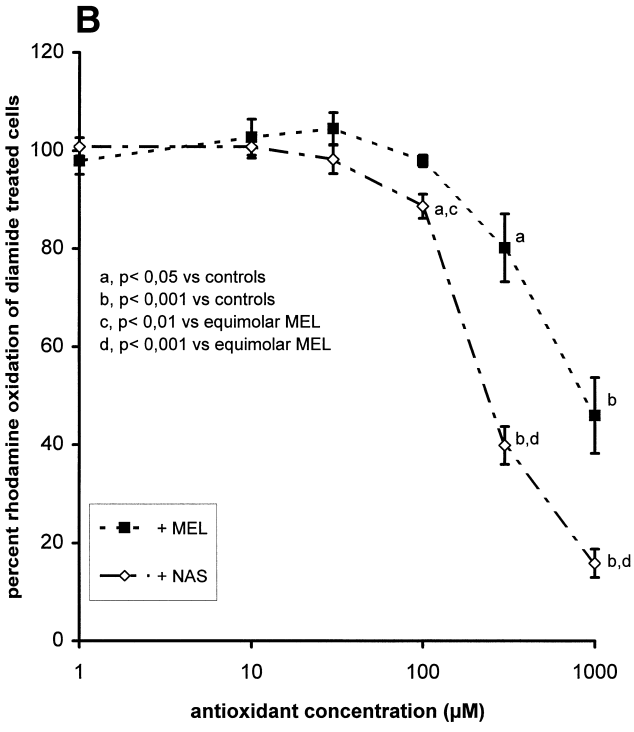
Fig. 3. Effects of MEL, NAS and TX on ROS formation in t-BHP-treated PBL. PBL (2×10^6 cells/ml) were loaded with 2 μM DHR for 10 min at 37°C. After washing with PBS, cells were resuspended in RPMI (1×10^6 cells/ml). (a) Cells were left untreated or were incubated with 100 μM t-BHP and 1 mM MEL, NAS, TX or solvent (0.1% ethanol in RPMI). After the indicated time periods, the rhodamine fluorescence was measured at 488 nm/525 nm (excitation/emission) using a Becton-Dickinson FACScan. Data represent mean ± S.E.M., $n=4$, each done in duplicate. (b) Cells were left untreated or were incubated with 100 μM t-BHP and the indicated concentrations of MEL, NAS or solvent (0.1% ethanol in RPMI). After 2 h of incubation, the rhodamine fluorescence was analyzed at 488 nm/525 nm (excitation/emission) using a Becton-Dickinson FACScan. Data represent mean ± S.E.M., $n=3$, each done in duplicate.





was as effective as 1 mM MEL to protect PBL from ROS-induced cell death. These results indicate that MEL exerts only a little protective effect against oxidative stress in cells as compared to NAS.

Furthermore, in aqueous cell-free solutions, the capacity of MEL to scavenge peroxyl radicals was much lower than that of NAS. While 10 μM NAS was more effective than the same concentration of TX, MEL, even at millimolar concentrations, had only minor effects on the peroxyl radical-induced

Fig. 4. Effects of MEL, NAS and TX on ROS formation in diamide-treated PBL. PBL (2×10^6 cells/ml) were loaded with 2 μM DHR for 10 min at 37°C. After washing with PBS, cells were resuspended in RPMI (1×10^6 cells/ml). (a) Cells were left untreated or were incubated with 100 μM diamide, which oxidizes sulfhydryl groups, and 1 mM MEL, NAS, TX or solvent (0.1% ethanol in RPMI). After the indicated time periods, the rhodamine fluorescence was measured at 488 nm/525 nm (excitation/emission) using a Becton-Dickinson FACScan. Data represent mean ± S.E.M., $n=4$, each done in duplicate. (b) Cells were left untreated or were incubated with 100 μM diamide and the indicated concentrations of MEL, NAS or solvent (0.1% ethanol in RPMI). After 2 h of incubation, the rhodamine fluorescence was measured at 488 nm/525 nm (excitation/emission) using a Becton-Dickinson FACScan. Data represent mean ± S.E.M., $n=3$, each done in duplicate.
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DHR oxidation. This is in contrast to Pieri et al. [3], who reported the capacity of MEL to scavenge AAPH-derived peroxyl radicals to be twice that of TX. More recent studies, however, are in line with our findings. MEL showed only a weak protection of low density lipoproteins against oxidative damage as compared to α-tocopherol [11] and a lack of antioxidant activity of MEL against the peroxyl radical-induced lipid peroxidation in model membranes has been demonstrated recently [10]. According to Seegar et al. [15], who also investigated effects of MEL on the LDL oxidation, the antioxidative activity of MEL was negligible compared to other indoleamines, like serotonin, NAS and 6-hydroxymelanin. For serotonin, a higher efficiency to prevent lipid peroxidation than for MEL was reported [9]. A possible reason for this difference in their reactivity towards peroxyl radicals

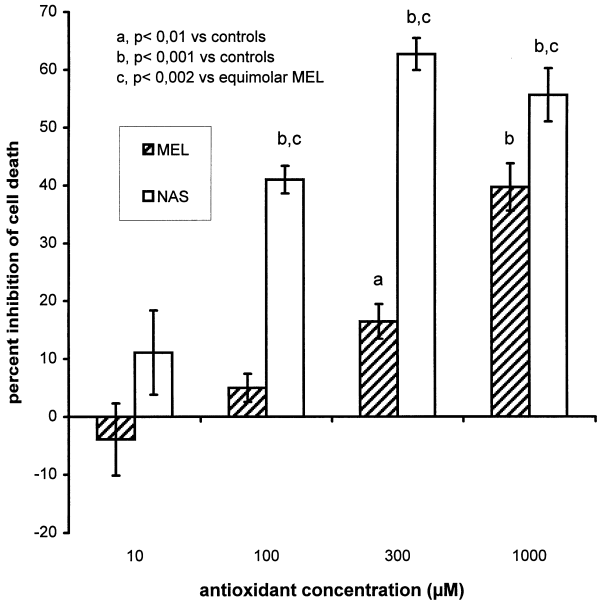

Fig. 5. The influence of MEL and NAS on the t-BHP-induced cell death in PBL. PBL (1×10^6 cells/ml) were left untreated or were incubated with 100 μM t-BHP and the indicated concentrations of MEL, NAS or solvent (0.1% ethanol in RPMI). After 4 h of treatment, PBL were washed twice with ice-cold HEPES buffer and incubated with PI (10 μg/ml) for 15 min at room temperature. The percentage of PI positive cells was determined using a Becton-Dickinson FACScan at an emission wavelength of 600 nm. Data represent mean ± S.E.M. of one experiment out of three giving identical results, $n=3$, each done in duplicates.

might be the phenolic OH-group in NAS and serotonin which is absent in MEL.

Although pinealectomy in rats, which abolishes circulating levels of MEL, has been reported to exaggerate the oxidative damage after treatment of rats with ROS-generating agents [28], the physiological significance of its antioxidant activity, which is observed only at micromolar concentrations, remains questionable, since the nocturnal peak serum concentration of MEL in humans as well as in other mammals is in the picomolar range [29]. Beside its endocrine release from the pineal gland, MEL was suggested to be produced and released by human PBL [30] as well as by cultured skin cells [31], indicating a paracrine or intracellular role as well. However, the concentrations of paracrine and intracellular MEL in lymphoid tissues or in the skin are not yet known.

In human serum, NAS is found in nanomolar concentrations [32], which are about 10–100-fold higher than MEL and NAS is produced and released by cultured skin cells [31] and by human PBL [30]. In skin cells, the amount of released NAS after serotonin addition was about 10 times higher than that of MEL. Furthermore, NAS is a major product of the in vivo back-transformation of MEL in various tissues [33], including human PBL [34]. Therefore, the concentration of NAS in various tissues is presumably much higher than that of MEL. This issue has to be clarified in further studies.

Our finding that MEL, in contrast to NAS and TX, is not able to decrease the intracellular ROS formation in resting PBL, but even led to a slight increase, points to a qualitative difference between MEL and NAS in influencing intracellular redox processes. This notion is corroborated by findings that MEL enhances the ROS formation in resting Jurkat cells, a human leukemic cell line, while NAS reduced it [35] and by Barsacchi et al. [36] reporting that vitamin E consumption in erythrocytes exposed to oxidative stress was reduced by NAS but enhanced by MEL.

Our results in a biochemical and a cellular experimental system demonstrate that NAS, the immediate precursor of MEL, is a better antioxidant than MEL itself. NAS exhibited antioxidative effects at 3–10-fold lower concentrations than MEL. Together with the fact that extracellular and intracellular NAS concentrations in vivo are considerably higher than those of MEL, these results clearly indicate NAS to be a physiologically more relevant antioxidant. Thus, an outstanding role of MEL as well as its physiological significance as anti-ageing principle due to its antioxidative features has to be questioned.

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