Oxidative status – Age- and circadian variations? – A study in leukocytes/plasma

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Abstract

OBJECTIVES: In the present paper the circadian variation and age differences in human leukocyte reactive oxygen species (ROS) levels and plasma total antioxidant status were studied. Different ROS levels could influence disease during ageing and at different times of the day/night.

METHODS: Unstimulated and stimulated (12-O-tetradecanoyl-phorbol-13-acetate (TPA)) blood samples were analysed by flow cytometry using dihydroethidium (DHE) and dihydrorhodamine 123 (DHR) as probes. 60 healthy individuals were divided into five equal groups ((I) 1–2 days post partum, (II) 20 ± 2 years, (III) 40 ± 2 years, (IV) 60 ± 2 years and (V) 80 ± 2 years) and 6 healthy volunteers were followed with blood samples at 3 hour intervals for 24 hours.

RESULTS: We observed a significant peak of ROS levels in both monocytes and granulocytes at 6 pm and 3 am using DHE as probe. DHR showed in principle the same pattern. The monocytes (unstimulated and stimulated) and unstimulated granulocytes of the newborn group showed higher ROS values than all the other groups, whereas the groups of adults did not differ from each other. The plasma total antioxidant status was significantly higher in the newborn group and showed no circadian variation.

CONCLUSIONS: The present data show that there is a circadian variation of ROS production in leukocytes that might possibly influence the occurrence of cardiovascular incidents like myocardial infarction. No increase in ROS production was found in healthy elderly compared to younger individuals. The increased ROS levels and antioxidant status of newborns might influence neonate immunity and play a part in cell signalling and development.

ORIGINAL ARTICLE

Abbreviations:

ROS – reactive oxygen species
TPA – 12-O-tetradecanoyl-phorbol-13-acetate
DHE – dihydroethidium
DHR – dihydrorhodamine 123
PFA – paraformaldehyde
PBS – phosphate-buffered saline
MFI – mean fluorescence intensity
TAS – total antioxidant status

Introduction

Reactive oxygen species (ROS), e.g. superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$), are well-known products of metabolism in all aerobic cells. ROS constitute an essential part of the bactericidal system of phagocytes and function also as important mediators of exogenous influences such as radiation, UV light, cigarette smoking, fatty acids in foods, transition metals, ethanol, pollution, hyperoxia, vigorous exercise and ischemia [15, 25]. It has further been shown that ROS have many functions in modulating the activity of intracellular molecules and signalling pathways [15, 5, 21, 29]. Moreover, it is also well recognised that ROS in high concentrations have toxic effects and mediate oxidative damage to e.g. proteins, lipids and DNA and can thus cause cell death, mutations and other toxicities [15, 25, 5, 21, 29].

The potential onslaught of oxidants in different tissues/organs is normally counterbalanced by a wide variety of antioxidants [21, 29]. However, oxidative damage results from oxidative stress when an imbalance in the oxidant/antioxidant equilibrium develops, either via impairment in the generation of antioxidant principles or in association with increased production of ROS [29, 26].

Several studies have shown that ROS play a significant role in the pathophysiology of cardiovascular disease (e.g. atherosclerosis, cardiac ischemia and reperfusion damage) [5, 29] and cancer [21]. ROS have also been implicated to play a role in diabetes mellitus, cataract, Parkinson's disease, neurodegenerative-, liver-, renal- and lung diseases and male infertility [25].

The data supporting the free radical theory of ageing is growing. It is hypothesised that the accumulation of oxidative damage to mitochondria in differentiated cells is a major factor in ageing [28]. Several studies indeed show that oxidative damage to mitochondrial lipids, proteins and DNA is increasing with age [17, 22, 30] and that there is an inverse relationship between mitochondrial oxidant production and the longevity of mammalian species [12, 13].

It is also known that many diseases show diurnal variation in onset and outcome, e.g. the increased occurrence of cardiovascular incidents such as myocardial infarction, stroke and sudden cardiac death in the morning hours after awakening [18, 32]. Given the potential role of ROS in the pathogenesis of these disease states it has been speculated that circadian variations in ROS load may explain the accumulation of events in the late night/early morning hours. To support this assumption varying levels of oxidative "stress markers" like 8-hydroxydeoxyguanosine, malondialdehyde and 8-isoprostane have been demonstrated in human urine during day and night [11].

In this paper we describe two separate studies investigating the production and expression of ROS in human leukocytes; one study comparing different groups of individuals at different ages (span 0–80 years) and further one study investigating circadian variations in leukocyte ROS levels. Both studies recorded basal (unstimulated) intracellular ROS levels in both monocytes and granulocytes as well as levels after *in vitro* stimulation with the powerful agonist tetradecanoyl phorbol acetate (TPA).

Material & Methods

Study populations/blood sampling

Blood samples (anticoagulated with EDTA or heparin) were taken from healthy, non-smoking individuals. All participants were informed about the study and gave their written consent for participation. The regional ethics committee approved the study.

To study the influence of age on ROS production in leukocytes blood samples were taken from 60 subjects sub-divided into five groups; (I) 1–2 days post partum, (II) 20 ± 2 years, (III) 40 ± 2 years, (IV) 60 ± 2 years and (V) 80 ± 2 years. Each group comprised 12 individuals (6 men and 6 women). All samples were taken as fasting morning blood samples and kept on ice until further preparation within 60 min after collection.

For the study of circadian variation 6 healthy volunteering medical students (3 males and 3 females) between 21 and 25 years were followed for 24 hours with blood samples taken every 3 hours starting at 9:00 am. The subjects were offered general hospital meals; breakfast between 7 and 9 am, lunch between 11:30 am and 1 pm, dinner between 4 and 5 pm and a night snack between 7 and 8 pm. The subjects were sleeping at night, only interrupted by short wake-ups to take blood samples.

All subjects were told not to exert physical exercise before blood sampling, not to eat vegetables, fruits or berries in large quantities and not to take any vitamin preparations, dietary supplementations or medications of any kind the day before or the same day as blood samples were taken.

Reagents

Dihydroethidium (DHE, mainly reflecting superoxide anion), dihydrorhodamine 123 (DHR, mainly reflecting peroxynitrite and hypochlorous acid), 12-O-tetradecanoyl-phorbol-13-acetate (TPA), paraformaldehyde (PFA) and phosphate-buffered saline (PBS) tablets were all obtained from Sigma, St. Louis, MO, USA.

Preparation of blood leukocytes for flow cytometry

Ex vivo (**basal**) levels of ROS in leukocytes were measured by immediate postexsanguinational incubation of 50 µl EDTA blood with 5 µmol/L (final concentrations) of the ROS-sensitive probes DHE and DHR, respectively, for 15 min at 37 °C. After incubation a hypotonic lysis of erythrocytes was performed by adding 30 volumes of lysing solution (156 mmol/L NH_4Cl, 10 mmol/L $NaHCO_3$, 0.12 mmol/L EDTA) at room temperature in the dark for 15 minutes, followed by centrifugation at 300 g for 5 minutes at 4 °C. The supernatants were discarded and the leukocyte pellet was washed once in 2 ml PBS (pH 7.4). Finally, the leukocytes were resuspended in 0.5 ml 1% (w/v) PFA in PBS and stored in the dark and cold until flow cytometry was performed after 1–6 hours.

In vitro (**TPA-stimulated**) ROS production was examined by coincubating 50 µl aliquots of EDTA whole blood with TPA (final concentration 100 ng/ml) and DHE or DHR (final concentration 5 µmol/L) for 60 minutes at 37 °C (DHE) or for 90 minutes at 30 °C (DHR). At ended incubation the blood was lysed and washed as described above for basal samples and finally the leukocytes were resuspended in 0.5 ml 1% PFA in PBS.

Flow cytometry

The labelled samples were analyzed in a Becton Dickinson FACSort™ flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an argon laser and Cell-Quest software (Becton Dickinson). The fluorescense intensity was daily calibrated with Flow-Set fluorescent microspheres (Coulter Corporation, Miami, FL, USA) according to the manufacturer's instructions. Ten thousand events were collected from each sample with light scatter gain set in the linear mode and the fluorescence gain set in the logarithmic mode. The leukocyte subpopulations, i.e. granulocytes, monocytes and lymphocytes, were identified by their light scatter characteristics, enclosed in electronic gates and separately analyzed for fluorescence intensity (Fig. 1). The results are expressed as mean fluorescence intensity (MFI). The intra-assay coefficients of variation were < 1,8% in unstimulated and < 4,7% in TPA-stimulated samples.

Total antioxidant status

Total antioxidant status (TAS) was measured in heparin plasma using a kit from Randox, Crumlin, UK, exactly following the manufacturers instructions. This assay is based on the peroxidase-mediated conversion of a chromogen to a blue-colored radical cation which can be detected at 600 nm. Antioxidants in the added plasma sample cause inhibition of this color production to a degree that is proportional to their concentration.

Statistics

Data are presented as mean values ± SEM. Age groups were compared using the non-parametric Mann-Whitney test. Data sets of circadian variation were analyzed *post hoc* using the Wilcoxon paired sample test. A p-value of < 0,05 was considered significant. All analyses were performed with SPSS 12.0 for Windows.

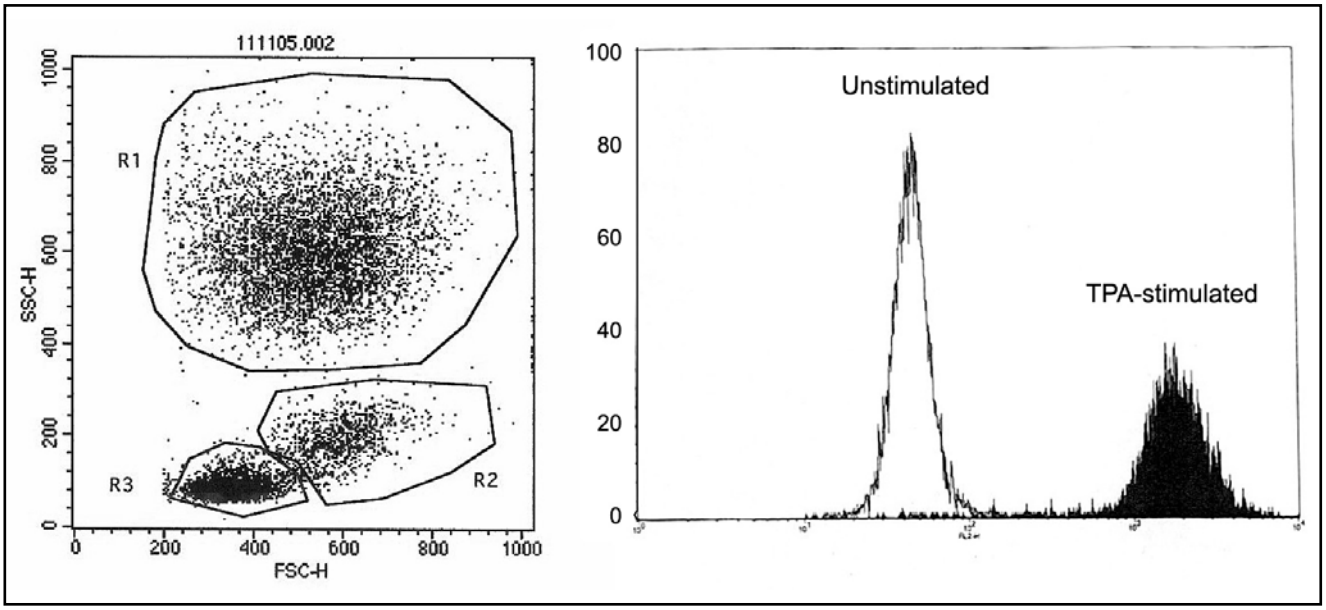

Fig 1: Representative examples of FACSort analyses: The left panel shows a forward/side scatter dot plot with gating of blood leukocytes, where the gates R1, R2 and R3 represent granulocytes, monocytes and lymphocytes, respectively. The histogram (right panel) illustrates the different DHE fluorescence pattern of unstimulated (open histogram) and TPA-stimulated (filled histogram) granulocytes. Horizontal axis: fluorescence intensity, vertical axis: cell number.

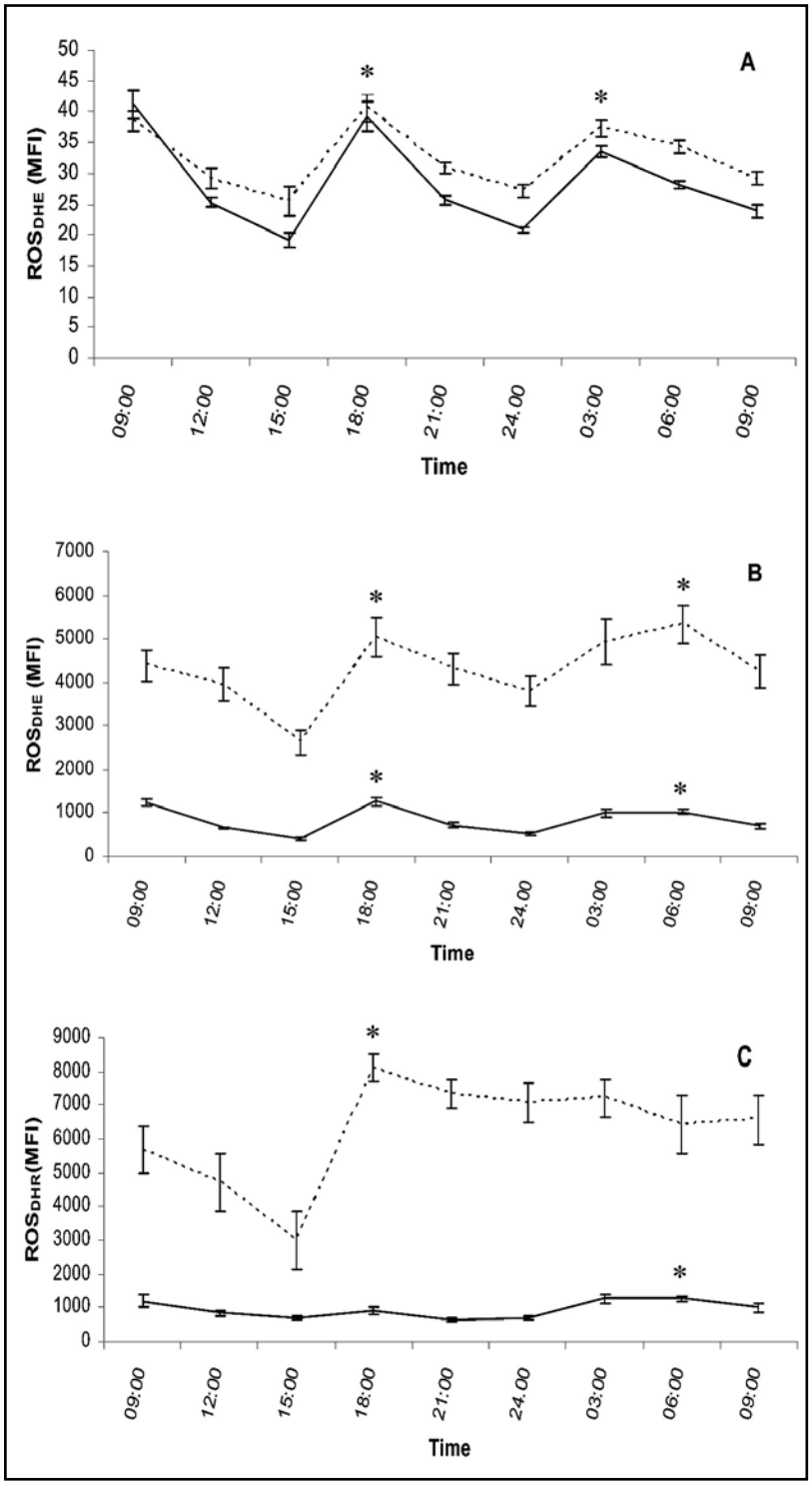

Fig 2: Circadian variation of ROS levels in granulocytes (dotted line) and monocytes (continuous line). DHE (**A** and **B**) and DHR (**C**) were used as probes. Values given are mean ± SEM (n = 6). (**A**) Unstimulated samples incubated for 15 min. (B and **C**) Samples stimulated with TPA (100 ng/ml) incubated for 60 min (**B**) and 90 min (**C**).
*= p≤0,05, differences vs. preceding minimum ROS levels.

Results

Circadian variation

When DHE was used as a probe, significant circadian variations in both granulocyte and monocyte intracellular ROS levels were recorded. Basal (nonstimulated) ROS levels reached its lowest values at 3 pm and 12 pm and showed significant peaks at 6 pm and 3 am in both monocytes and granulocytes (Fig. 2a). In granulocytes all individuals increased their ROS_{DHE} levels at least 19% (p = 0,027) during the afternoon peak and at least 31% (p=0,027) during the night/early morning peak. Corresponding values for monocytes were 71% (p = 0,027) and 48% (p = 0,027). It should also be noted that basal ROS levels were significantly higher at the start of the 24 h registration period compared to the end (both 9:00 am). TPA-stimulated cells increased their ROS_{DHE} levels (compared to nonstimulated cells incubated for 60 min) on average 66-fold and 9-fold (granulocytes and monocytes respectively) through the night and day. Similar values for ROS_{DHR} were 156-fold and 3-fold, respectively. TPA-stimulated ROS levels also showed a significant peak at 6 pm (p=0,028) and a peak at 3–6 am (p=0,028) in both monocytes and granulocytes (Fig. 2b).

A circadian variation was obtained also with DHR as the ROS probe. Basal ROS levels showed a significant rise from 12 pm to 3 am in both monocytes (p = 0,046) and granulocytes (p= 0,027), the ROS levels fading gradually during the day until a minimum at 12 pm with no significant peaks at 6 pm (data not shown). In TPA-stimulated samples (Fig. 2c) monocytes showed a small ROS increase at 6 pm (not significant) and significantly increased levels at 3-6 am (p = 0,028) as also shown for DHE. In granulocytes a 2,7-fold increased ROS response was observed at 6 pm compared to 3 pm (p = 0,028), being retained at that high level during the whole night, gradually fading during the day reaching minimum levels at 3 pm.

The plasma total antioxidant status showed no variations and remained stable throughout the day and night (Fig. 3).

Age variation

We were not able to demonstrate significant variations between the four groups of adult people as regards either unstimulated (basal) or TPA-stimulated ROS levels (Fig. 4). However, the group of newborns differed

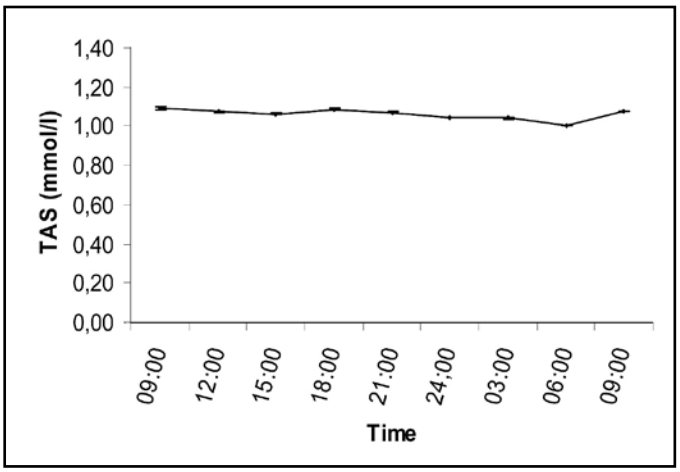

Fig 3: Plasma TAS levels (mean ± SEM) at different times of the day (n= 6).

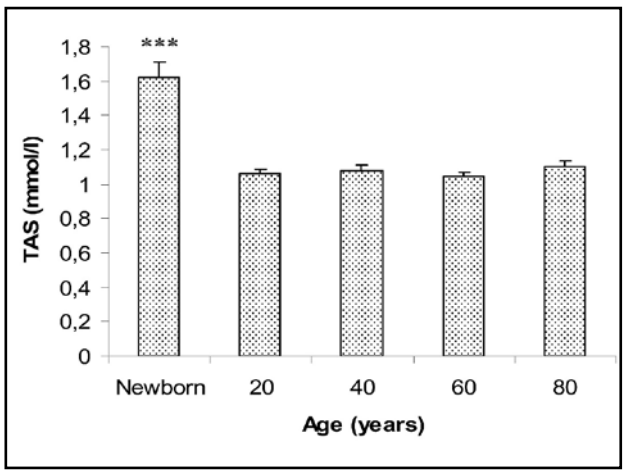

Fig 5: Plasma TAS levels (mean ± SEM) in the different age groups. (*** = p<0,001, difference vs. all other age groups)

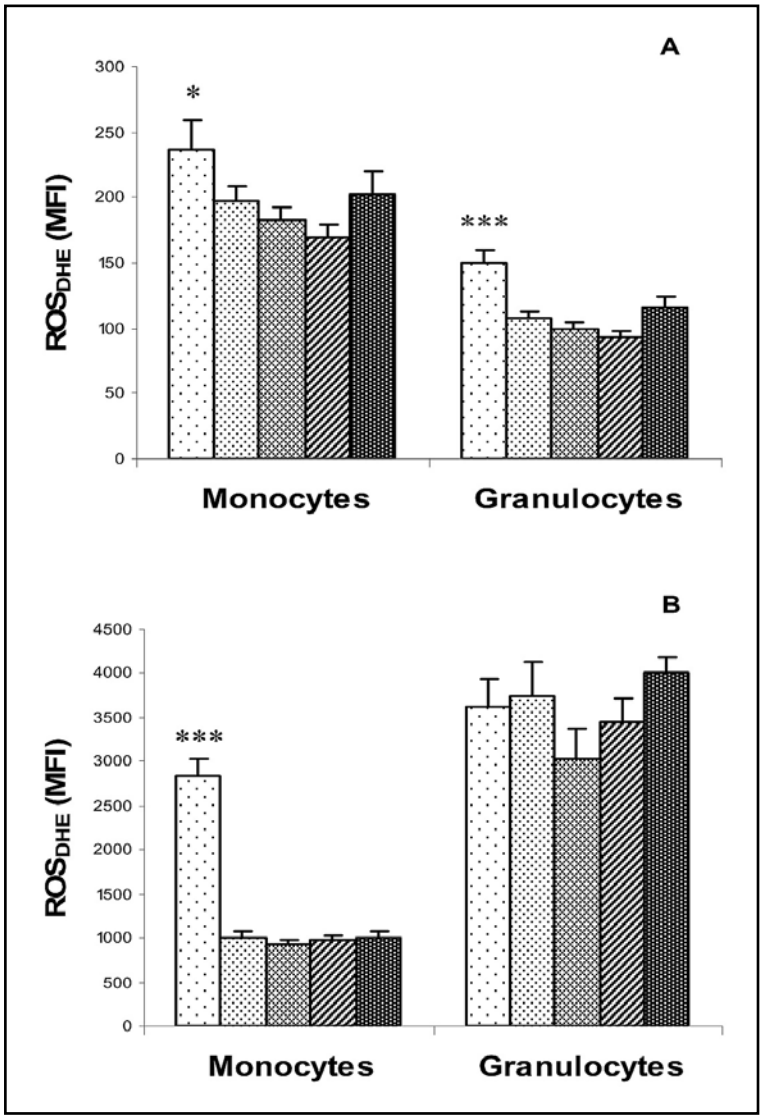

Fig 4 (left): ROS levels in monocytes and granulocytes of the different age groups.
From left to right;
newborn (n = 12),
20 ± 2 years (n = 12),
40 ± 2 years (n = 12),
60 ± 2 years (n = 12),
80 ± 2 years (n = 12).
DHE was used as ROS probe.
(**A**) Unstimulated samples incubated for 60 min.
(**B**) Samples stimulated with TPA (100 ng/ml) and incubated for 60 min. (* = p≤0,05, *** = p<0,001, difference vs. all other age groups)

from the other groups in several respects. Compared to other age groups there were slightly higher levels of ROS in unstimulated monocytes from newborns (p≤0,05) (Fig. 4a). In particular, TPA-stimulated monocytes from newborns demonstrated intracellular ROS levels which far exceeded those of monocytes from any other age group (p<0,001) (Fig. 4b). There were also significantly higher levels of ROS in the unstimulated granulocytes of newborn (p<0,001) (Fig. 4a), whereas the TPA-stimulated granulocytes (Fig. 4b) did not differ from the granulocytes of other age groups as regards ROS levels. These observations were the same irrespective

of whether DHE or DHR (data not shown) were used as a probe. The plasma total antioxidant capacity of newborns was also significantly higher ($p<0,001$) and differed from all other age groups (Fig. 5).

There were no significant differences between males and females within the groups and for the statistical analysis we chose to treat them as one group.

Discussion

In this study we demonstrated a circadian variation of ROS levels in leukocytes of young male and female individuals. This variation was most prominent when DHE was used as a probe. Both in unstimulated leukocytes (granulocytes and monocytes) and in leukocytes stimulated with the powerful ROS inducer TPA we demonstrated two peaks of ROS activity, one in the late afternoon and another in the night/early morning hours. However, in the case of DHR monitoring these two peaks were replaced by a more prolonged increased TPA response during night time. We also observed that the basal leukocyte and stimulated monocyte ROS_{DHE} levels registered at 9 am at the start of the observation period were significantly higher than those at 9 am the second morning. This difference probably reflects the fact that the participants the first morning had to exert some physical activity to get to the laboratory in due time. In contrast, all participants had a quiescent start of the day the second morning, waking up in the hotel premises of the hospital where the blood samples also were taken. It is well established that physical exercise has an inducer effect on neutrophil ROS production [20], and it is probable that even light physical activity following a whole night of inactivity may add to or potentiate the morning peak of the diurnal ROS rhythm. Altogether, our findings demonstrate the fact that the specificity of the two fluorescent probes used (and others as well) is not known in detail, thus causing some difficulties in the interpretation of data. However, both probes used for ROS detection showed a seemingly consistent pattern of diurnal ROS variation.

We speculate that varying levels of ROS might contribute to higher risk of some disease incidents at certain times of the day/night. Cardiovascular events like myocardial infarction and sudden cardiac death are known to follow a circadian pattern with a peak of increased risk in the morning hours and a smaller peak in the afternoon [18, 32]. The high incidence of acute cardiovascular events in the morning after awakening and arising has been ascribed to the surge of heart rate and blood pressure due to sympathetic activation [18, 19] as well as other neural or humoral vasoactive factors and changes in hemostatic activity. However, myocardial infarction during night (sleep) has been thought to have a different pathogenesis and not be related to sympathetic activation or triggering events [18]. Based on our present findings it is possible that the increased leukocyte ROS in the night/early morning might contribute to cardiovascular events by representing an additional internal trigger of pathophysiological effects. The observation mentioned above that activity in the morning after arising may supervene on the diurnal ROS peak further strengthens this assumption. Oxidative stress is also believed to initiate a prothrombotic state [6, 14] and vasoconstriction [31] that adds to the increase in platelet aggregability on assuming the upright posture and the final trough of the fibrinolytic system [19].

We are not aware of earlier studies investigating the circadian variations of ROS production in intact human cells. The mechanisms of this variation are unknown. However, oxidative stress markers like 8-hydroxydeoxyguanosine, malondialdehyde and 8-isoprostane have been demonstrated in urine with a typical diurnal variation, all markers peaking in the afternoon at about 6–8 pm [11]. Theoretically, this peak of oxidative markers in urine might be a delayed reflection of oxidative damage taking place as a result of the late night/early morning peak of ROS in blood. The circadian variation of ROS levels could also be influenced by concentrations of antioxidant principles. For instance, melatonin levels increase during dark and peak at 3–4 am [7]. The antioxidant effect of melatonin might thus oppose the effect of ROS production during the night.

As reviewed briefly in the introduction oxidative damage also plays a major role in ageing. Some studies have shown increased oxidant production in rat hepatocytes [9, 27] and human granulocytes [16] as well as increased volatile organic breath markers of oxidative stress [23] with age. However, other studies have shown impairment of the oxidative burst with age [3, 24]. This might reflect a complex interaction between ROS production and ageing and that ageing can influence different transductional pathways in different ways [3]. Oxidative stress and damage play an important role in ageing, but on the other hand the significant decrease in the microbiocidal capacity of the polymorphonuclear cells with age is partly attributed to a decrease in ROS production [24].

In the present study we were not able to identify any increase or decrease of ROS production in leukocytes dependent on age, the only exception being newborns, *vide infra*. Likewise, the total antioxidant capacity of plasma did not differ between adults of all ages. This may reflect the fact that only healthy individuals were recruited to the study and that good health, despite old age, is characterized by a maintained oxidant/antioxidant balance. Derangement of the equilibrium between pro- and antioxidant principles is obviously a prerequisite for disease to progress and it is presumably a gradual wear-out of antioxidant mechanisms that eventually promotes ageing and also precipitates the variety of diseases associated with oxidative stress. The lack of difference in ROS production between the different age groups of adults could also indicate that the increasing oxidative stress in ageing is a result of

accumulation of oxidative damage over time, rather than increased oxidant production per se.

Few of the previous studies have included newborn babies as an age group. In the present study we found significantly increased TPA-stimulated ROS levels in newborn monocytes and increase of basal ROS levels in both granulocytes and monocytes of the newborn. This increased ROS generation capacity of newborns was, however, counterbalanced by a significantly increased total antioxidant capacity in plasma. This corresponds with previous studies that have shown an increased respiratory burst [8] and increased superoxide anion production (but decreased hydroxyl radical production) [1, 4] in neonate neutrophils compared with adult. A study of Phillips et al. [23] also showed increased oxidative stress in younger as well as in older humans. An increased leukocyte capacity for ROS production may have an impact on the function of the newborn immune system and its susceptibility to infections. It is also possible that this is a normal physiological response in childhood/youth as there is increasing evidence that ROS act as signal transducers during normal growth and development [23].

We also found significantly increased plasma total antioxidant capacity in the group of newborns compared to all other age groups. However, previous studies have shown that newborns, especially preterm babies, have a limited antioxidant protective capacity [10] and lower levels of the antioxidant vitamins E and A in infant cord blood compared to maternal blood [2]. We do not know the cause of this apparent discrepancy.

We conclude that there is a circadian variation of ROS expression in human leukocytes and that this might possibly influence the risk of cardiovascular disease incidents like myocardial infarction. We further demonstrated higher levels of ROS in leukocytes of newborns than in any other age group which might have implications for innate immunity and infection resistance. On the other hand, ROS in leukocytes and antioxidant capacity in plasma seemed to be at the same levels in all other age groups. This indicates that good health is associated with a balanced oxidant/antioxidant ratio independent of high age.

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