Detection of the Production of Reactive Oxygen Species by Neutrophils in Whole Blood: Modulation by Adamantanes and Triggering by Fe³⁺-ions

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Using indicators for the production of reactive oxygen species (ROS) such as the a) OH-radical type (α-keto-γ-methiolbutyric acid, KMB) or b) hypochlorous acid (1-amino-cyclopropyl-1-carboxylic acid, ACC) neutrophil activities can be both quantified and differentiated in whole blood via ethene production. Ethene is trapped in the head space of blood samples incubated in the presence of zymosan and the respective indicators, KMB or ACC. This procedure allows the detection of effects of aminoadamantanes (AAD) such as amantadine or memantine, compounds frequently used for the treatment of Morbus Parkinson and Morbus Alzheimer. In this report we describe the detection of OH-type oxidants produced by isolated activated neutrophils and whole blood. Immunomodulatory activities of AAD are deduced from the following observations: AAD-stimulated ethene formation from (KMB) as an indicator for production of OH-type reactive oxygen species by zymosan-stimulated neutrophils ("respiratory burst") is detectable with isolated neutrophils. In whole blood, however, this reaction is only measurable in the presence of Fe-EDTA-complex. Stimulating effects of AAD are observed within a concentration range between 10⁻⁸ and 10⁻⁴ M with a maximum at 1 μM. Ethene release from (ACC) as indicator for the myeloperoxidase reaction after degranulation is not stimulated by AAD but inhibited at concentrations higher than 100 μM. The presented results suggest that submicromolar concentrations of AAD only stimulate the respiratory burst and apparently not degranulation of zymosan-stimulated polymorphonuclear neutrophils (PMN).

Introduction

Aminoadamantanes (AAD) such as memantine (3,5-dimethyl-1-adamantamine, MEM) and amantadine (1-adamantamine, AA) are in use for the treatment of Morbus Alzheimer and Morbus Parkinson (Pantev et al., 1993; Schreiber and Kornhuber, 1994) and certain other neurodegenerative diseases (Brenner et al., 1989) and several reaction mechanisms have been discussed (Skuza et al., 1994; Deshpande et al., 1995; Schneider et al., 1984; Lupp et al., 1990; Klec, 1982; Netzer, 1989; Dimpfel, 1995; Osborne and Quack, 1992). AAD act as non-competitive NMDA-receptor antagonists thus influencing the glutaminergic system (Bormann, 1989). Activation of this system yields the production of ROS which in turn are discussed in context with neuronal cell death (Dawson and Dawson, 1996). In a recent communication we reported on the potential antioxidative activity of AAD (Fig. 1). We found that these AAD neither act as free radical scavengers nor as singlet oxygen quenchers but modulate oxygen activation by PMN after their preactivation by zymosan, by the chemotaetic tripeptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) or by the Ca²⁺-ionophor A23187 (Albrecht-Goepfert, 1996; Albrecht-Goepfert et al., 1998).

In another previous report we described a method for gaschromatographic differentiation of ROS produced either by the respiratory burst or by MPO-catalysis (v. Kruegener et al., 1995) based on the relatively specific reaction between KMB...
and OH-(Fenton)-type oxidants on the one hand and that of ACC with hypochlorite and related chloramines on the other hand. In both cases ethene is produced which can easily be quantified gaschromatographically. In this communication we describe experiments showing that ROS production by isolated neutrophils can be followed with KMB-headspace technique where AAD enhances ethene release. In whole blood, however, this reaction is only observable in the presence of added Fe$^{3+}$-ions.

Materials and Methods

Materials

All chemicals and enzymes were obtained either from Sigma or from Merck, Darmstadt. AAD were a generous gift from Dr. G. Quack, Merz & Co., Frankfurt.

PBS contained in 1 l: 9 g NaCl, 0.2 g KCl, 1.4 g NaH$_2$PO$_4$, 0.2 g KH$_2$PO$_4$, 0.1 g MgCl$_2$$\times$6H$_2$O, 0.07 g CaCl$_2$$\times$2H$_2$O. PBS was adjusted to pH 7.4.

Methods

500 ml blood was withdrawn from freshly sacrificed pigs, immediately mixed with 20 ml 2.5% EDTA and used within 5 h after storage at 5 °C. Neutrophils were isolated per dextran sedimentation and Histopaque 1119 density gradient centrifugation and washed twice with PBS (for more details see Hippeli et al., 1990). Viability of the isolated neutrophils was at least 98% as determined by trypan-blue exclusion. The following test systems were used:

a) Ethene release from KMB for the determination of superoxide-based ROS produced by the respiratory burst.

b) Ethene release from ACC for the determination of MPO-produced HOCI.

The following incubations with whole blood have been conducted in order to evaluate the different activities of PMN:

The KMB test system contained in 2 ml:
1 ml whole blood or $10^7$ isolated neutrophils; PBS-buffer (pH 7.4) ad 2 ml; 3 mM KMB; different concentrations of the AAD to be tested; 2.5 mg non-opsonized zymosan. To increase OH-radical formation 1 mM Fe$^{3+}$ has been added when indicated. The reaction was conducted for the times indicated in the individual figures at 37 °C in the dark.

The ACC test system contained in 2 ml:
1 ml whole blood; PBS-buffer (pH 7.4) ad 2 ml; 3 mM ACC; different concentrations of the AAD to be tested; 2.5 mg non-opsonized zymosan. The reaction was conducted for the times indicated in the individual figures at 37 °C in the dark.

Ethene release from KMB or ACC was determined gaschromatographically as described recently (v. Krüdener et al., 1995). All experiments were repeated twice with at least triplicates and standard deviations are expressed as $\sigma_{n-1}$.

Results

Modulation of neutrophil activities in whole blood by AAD

Respiratory burst and degranulation of isolated PMN is initiated by several compounds such as opsonized zymosan, FMLP, A 23187 or PMA. During this process of oxygen reduction, superoxide and hydrogen peroxide are produced, where OH-radical and hypochlorous acid are secondary products due to the catalytic activities of traces of iron ions and secreted myeloperoxidase, respectively. Effects of AAD on the production of reactive oxygen species by zymosan-prestimulated PMN was monitored by ethene formation from either ACC or KMB (v. Krüdener et al., 1995).

1) Ethene formation from KMB

Activated PMN ($10^7$ isolated PMN-cells in the presence of 2.5 mg opsonized zymosan and 1.5 mm KMB in 1 ml PBS buffer) oxidized KMB in a time-dependent manner linearly producing approximately 1600 pmol ethene within one hour. A small but significant acceleration of ethene release by
memantine is visible after 15 min of reaction (Fig. 2a). Testing increasing concentration of AAD in a range between $10^{-9}$ and $10^{-5}$ M on this 15-min effect, ethene release from KMB follows an optimum curve with maximal acceleration at a dosage of about $10^{-7} - 10^{-6}$ M AAD. (Fig. 2b).

If the same reaction is performed with 1 ml whole blood in 1 ml PBS buffer with 2.5 mg non-opsonized zymosan and 3 mM KMB, a biphasic rate of ethene production is observed lasting at least for 3 h. The presence of 1 μM MEM or DABCO have no influence on this reaction (Fig. 2c). Also, no significant increase in ethene release by MEM is observable after 15 min of reaction. If Fe$^{3+}$-ions are added to this incubation mixture, an enhancement of ethene formation at Fe$^{3+}$-concentrations higher than 500 μM is observed (Fig. 2d). Under these conditions (presence of 1 mM Fe$^{3+}$-ions) a stimulating effect of 1 μM MEM but not of DABCO is measurable between 10 and 45 min of incubation, where the strongest enhancement is visible after 15 min reaction time (data not shown).

If the effects of MEM and AA are compared with the DABCO-control after 15 min of reaction, a stimulation within the broad range between 10 nM and 1 μM (Fig. 3) and only inhibition at higher than 10 μM after 60 min reaction time (data not shown) are measurable.
II) Ethene formation from ACC

After appropriate stimulation by zymosan, isolated PMN produce HOCl which rapidly reacts with ACC forming ethene (Krüdener et al., 1995). If similar experiments as described for isolated PMN (Albrecht-Goepfert et al., 1998) are conducted in the presence of whole blood and 3 mM ACC instead of KMB, approximately 1700 pmol ethene are formed quasi-linearly within 30 min. After 30 min this activity continues for further 150 min with strongly reduced velocity. Neither MEM nor DABCO have any effect on this reaction (data not shown). In contrast to the KMB system addition of Fe$^{3+}$-ions has no effect on ethene release from ACC. Likewise, increasing concentrations of MEM or AA have no stimulatory effect around 1 only higher concentration than 100 μM inhibit the reaction both in the absence or in the presence of Fe$^{3+}$-ions as compared to the DABCO control (data not shown; c.f. Albrecht-Goepfert et al., 1998).

Discussion

Morbus Parkinson and Morbus Alzheimer (Turner et al., 1996) are the most important and widespread neurodegenerative diseases, especially in elderly people (Schönberg et al., 1987). Since curing treatments are not available therapeutic possibilities are limited to symptom amelioration. Memantine (MEM, Akatinol®) may be a non-competitive NMDA-receptor antagonist yielding positive results in both diseases. Parkinson patients seem also to suffer from a selective abnormality in their immune response (Klüter et al., 1995). Certain brain areas ("neurofibrillary tangles") from Alzheimer patients (Good et al., 1996) seem to underly oxidative stress (Götz et al., 1995; Harris et al., 1995) indicated by the presence of nitrotyrosine as an indicator for the breakdown of peroxynitrite (PON), HONO. This reactive compound seems to be responsible for oxidative protein damage and is derived from the extremely rapid ($k = 6.7 \times 10^9$) interaction of superoxide with NO both stemming from activated neutrophils, macrophages or microglia. Peroxynitrite (PON) does not seem to be involved in KMB-fragmentation in whole blood since PON-driven ethene release from KMB is not influenced by iron ions (Hippeli et al., 1997). In the preceding paper we also described studies on possible antioxidative properties of different aminoadamantane derivatives. Since there exists a certain similarity between the chemical structures of aminoadamantanes and the singlet oxygen quencher, DABCO, this compound was used as "reference" in this report and in the present one. Concerning antioxidant or singlet quenching activites of these compounds it should be mentioned that DABCO has 2 reactive nitrogen atoms in the ring structure while MEM and AA have a saturated carbon atom (sp$^3$-hybrid) which is not reactive.

For the investigation of antioxidative properties of AAD as compared to DABCO, several radical-
generating systems as well as relevant indicators have been tested. In none of these systems radical scavenging properties have been found (Albrecht-Goepfert et al. 1998). From these results it can be concluded that AAD do not act as antioxidants in brain against ROS.

It could be shown, however, that AAD in micromolar concentrations function as immuno-modulators upregulating the respiratory burst in isolated prestimulated PMN. This effect could be shown with luminol-enhanced CL and with KMB as indicator for ROS other than HOCl. With ACC as a relatively specific indicator for HOCl production (v. Kruegener et al., 1995) or after stimulation with phorbolester (PMA), however, no AAD enhancement of PMN activation could be observed. A similar result has been reported by Berkow and colleagues (Berkow et al., 1987) showing that the diuretic Amlolid® had an influence on zymosan-, FMLP- or A 23187- prestimulated neutrophils but not on PMA-treated cells. They therefore talk about a “dissociation of neutrophil activating events”. Stimulation by low and inhibition by high concentrations of AAD of ROS production by zymosan- prestimulated PMN is in absolute agreement with the properties of theophylline which also stimulates ROS- production by PMN at ca. 1 μM and inhibits ROS production at higher concentrations (Banner and Page, 1996).

In this communication we extend our investigations on ethene release from KMB by neutrophils either isolated or in whole blood samples. AAD stimulated the respiratory burst of isolated neutrophils as detected with luminol-enhanced CL as well as measured by ethene production from KMB. Zymosan-treated whole blood samples showed this stimulatory effect of MEM or AA only significantly in the early phase of PMN-activation (i.e. after ca. 15 min) and in the presence of added Fe³⁺-ions. This indicates that “free” iron, which due to its toxicity is well regulated by iron transport proteins such as ferritin and thus limiting in blood, is catalyzing the production of OH⁻-type radicals which are driving the KMB fragmentation (Fig. 2c). Added iron ions thus “funnel” superoxide, primarily produced by the oxygen burst, into the OH⁻-radical path. The fact that only ethene can be measured and no ethane is due to the reactivity of KMB with Fenton-type oxidants releasing only ethene from KMB but no ethane, from ω3-C18:3 fatty acids in membranes of particulate blood components. In contrast, hypochlorite-dependent ethene formation from ACC as indicator for degranulation and thus MPO activity (production of HOCl) outside the PMN, was not further stimulated by MEM or AA, neither in the absence nor in the presence of iron ions (data not shown). Thus, no further enhancement of myeloperoxidase extrusion from white blood cells is initiated by AAD and apparently only the respiratory burst but not degranulation, MPO-activity respectively, is up-regulated by micromolar concentrations of AAD.

Both PMN and macrophages represent components of the immune system of blood. After massive local accumulation they may cause acute inflammations (Babior, 1984; Klebanoff, 1980; Noursharg, 1993) producing ROS via the respiratory burst and release oxidizing and degradative enzymes such as MPO, acid hydrolases and elastase (Janoff, 1985).

Activated PMN also seem to play a dominating role during inflammations after traumatic injury in the brain (Clark et al., 1994;) and after reperfusion of cerebral ischemia (Matsuo et al., 1994).

Immune reactions are also discussed as being involved in neuronal destructions in Morbus Parkinson (Kuhn and Müller, 1995) and during chronic brain ischemia in Morbus Alzheimer (Mast et al. 1995). Morbus Alzheimer is a chronic inflammatory process possibly initiated by activated microglia cells (Kalaria, 1993; McGeer and McGeer, 1995).

The effects of AAD on isolated PMNs or on PMNs in whole blood can be explained as follows: At first it is important to stress that AAD stimulate PMN respiratory burst (CL; KMB fragmentation) but apparently not degranulation (ACC fragmentation) at low concentrations (around 10⁻⁶ M) and inhibit both the burst and degranulation at higher concentrations (around millimolar). AAD like MEM seem to interact with membranes and accumulate intracellularly after multiple applications. This conclusion can be drawn on the basis of the observations that a) antiviral activity is apparently due to the interaction during viral “enveloping” inside animal cells (Havlichek, 1991) and b) MEM concentrations accumulate by 200 fold after multiple administration whereas the application of a single dose was reversible (Honegger et al. 1998). From these results it can be concluded that AAD do not act as antioxidants in brain against ROS.
et al., 1994). These observations allow the speculation that stimulation of preactivated peripheral blood cells by AAD is likely to occur in vivo since MEM-concentrations in the micromolar range are obtained in the peripheral blood stream. On the other hand inhibition of activated, tissue-bound cells such as microglia may be achieved by accumulation of MEM after multiple administration. Thus both down- and upregulation of ROS formation by preactivated immuneactive cells has to be taken into account.

Which interactions by AAD with pathways of signal transmission have to be envisaged? This question has been discussed in detail by Albrecht-Goepfert et al. (1998) and may be seen under the following assumptions:

Common features of AAD with 1) the metal chelate of inositol polyphosphate, 2) the adamantane derivative of benzoic acid p-hydroxymethylene-phosphonate and finally 3) tetrodotoxin, the poison of the “ball-fish” Spheroides porphyreus have to be discussed since

1) Inositolphosphates are involved in cellular signal transduction (Berridge and Irvine, 1984; Majerus, 1992) and the elementary structure of the metal chelate of inositol-tetraphosphate (Côté and Crain, 1993) is not dissimilar to the ADA skeleton (Albrecht-Goepfert et al., 1998):

2) the adamantane derivative of benzoic acid-p-hydroxymethylene phosphonate (Hallch and Sherman, 1980), is an effective inhibitor of inositol monophosphatase (Potter and Lampe, 1995) and its $k_i$ is similar to the values reported for upregulation of prestimulated PMN in our reports and may thus play a role in the treatment of neuropathological disorders (Manji et al., 1995; DelRio et al., 1996).

3) The fish poison tetrodotoxin is a Na$^+$-ion channel-blocker with neuroprotective properties (Taylor and Meldrum, 1995) since Na$^+$-ions stimulate Ca$^{2+}$- influx, ATP-depletion and thus cell death. It seems clear from the results presented that AAD act as immunemodulators in isolated PMN as well as in whole blood. It is also evident from several results that CL, independent of the used enhancer, lumicol or lucigenin, is an unspecific indicator. Therefore, in order to get more specific information on the type of activation and events expressed by PMN, gaschromatographic differentiation with the aid of KMB and ACC should be performed.

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