The antioxidant drug dipyridamole spares the vitamin E and thiols in red blood cells after oxidative stress

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Abstract

Objective: To test the antioxidant effect of therapeutic doses of dipyridamole on cellular membranes, human erythrocytes were chosen as an appropriate model to study oxidative stress induced by cumene hydroperoxide because of their high content in heme-Fe²⁺.

Methods: The oxidative stress was induced by incubation with 160 μmol l⁻¹ cumene hydroperoxide and expressed by three main factors: lipid peroxidation by means of kinetics of decrease in fluorescence emission of the probe incorporated in the cell membranes, vitamin E oxidation and intracellular thiol content. The concentrations of dipyridamole tested (2–20 μmol l⁻¹) did not exceed pharmacological doses.

Results: After 7 min of incubation at 25°C with the oxidant and 20 μmol l⁻¹ dipyridamole thiol content was 50.1% ± 2.6 compared with 31.5% ± 2.4 in the absence of the drug. After 12 min vitamin E content was 88.3% ± 2.3 compared with 64.7% ± 3.4 of untreated cells in the absence of dipyridamole. Dipyridamole added 5 min after the oxidation reaction suppressed the fluorescence decrease for a time proportional to the drug concentration.

Conclusions: Thus, at clinically realistic doses dipyridamole shows a concentration-dependent antioxidant effect. It protects membranes from oxidation and spares the antioxidant power of erythrocytes.

Keywords: Free radicals

1. Introduction

Dipyridamole is a pyrimidopyrimidine compound with antithrombotic and vasodilating actions [1,2], effective in the secondary prevention of cerebrovascular disease [3].

Although this drug has been used for many years, its mechanism of action is still unclear [4]. It has been suggested to inhibit platelet phosphodiesterase [5] to stimulate the release of prostacyclin [6–8], and to inhibit the carrier mediated transport of adenosine [9,10]. Recently, it has also been reported to have quite significant antioxidant activity in vitro by electron spin resonance (ESR) studies [11,12].

In particular, Iuliano et al. [11] have demonstrated that the antioxidant activity of dipyridamole is mainly due to the scavenging of peroxyl radicals. Pedulli et al. [12] suggested an alternative antioxidant mechanism of electron transfer, different from the classical chain breaking effect which operates in the case of phenol or amine antioxidant and involves the transfer of hydrogen atoms to peroxyl radicals.

The involvement of reactive oxygen species and lipid peroxidation in disease states such as atherosclerosis [13–15], ischemia-reperfusion injury [16,17], and sickle cell anemia [18–20] has focused much scientific interest on lipid peroxidation processes, outlining a possible role for antioxidant drugs in the care or prevention of these diseases.

It has been hypothesized that the antioxidant effect of dipyridamole may contribute to its therapeutic virtues [4,11]. However, the entity of the dipyridamole anti-radical
effect, its dose dependence in the drug concentration range achieved in the clinical arena, and its intimate mechanism of action remain largely unknown to date. To test the antioxidant effect of dipyridamole on cellular membranes red blood cells (RBCs) were chosen as an appropriate model to study the oxidative stress induced by hydroperoxides because of their high content of heme-Fe\(^{2+}\). Membrane soluble hydroperoxides such as cumene hydroperoxide (CHP) are frequently used in the study of oxidative processes as model compounds to initiate radical formation [21–23]. In our RBCs model CHP can be decomposed by metal ions giving rise to an alkoxyl radical that can initiate lipid peroxidation process accompanied by a parallel antioxidant consumption.

In this ex vivo study we evaluated the antioxidant capacity of pharmacological doses of dipyridamole in inhibiting lipid peroxidation and sparing endogenous vitamin E and cytosolic thiols in intact erythrocytes.

2. Materials and methods

2.1. Chemicals

Cumene hydroperoxide was obtained from Aldrich (St. Louis, MO), \( \alpha \)-tocopherol was obtained from Fluka (Switzerland); 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and adenosine deaminase were from Sigma (St. Louis, MO); 9,11,13,15-cis,trans,trans,cis-octadecatetraenoic acid (cis-parinaric acid, cis-PnA) was purchased from Molecular Probes (Eugene, OR). Dipyridamole was obtained from Boehringer Ingelheim Italia (Florence, Italy). All reagents and solvents used were of analytical grade.

2.2. Red blood cells

Blood samples were obtained from healthy volunteers by venipuncture using ethylenediaminetetraacetic acid (EDTA) as anticoagulant. The study was approved by the Human Research Committee of the Institute of Clinical Physiology, National Research Council. Written consent was obtained from each subject. Hematocrit was determined and the cells were centrifuged at 400 g for 10 min at 4°C, washed three times in Ca\(^{2+}\) and Mg\(^{2+}\) free Hanks’ buffer and suspended in Hanks’ buffer. RBCs oxidative stress was induced as described by van den Berg et al. [24].

2.3. Measurement of oxidation kinetics of cis-PnA

The oxidative degradation of PnA was monitored in a continuous fluorescence assay on a spectrofluorimeter (excitation 313 nm; emission 455 nm). Full methodological details are given elsewhere [24,25]. The fluorescence signal was calibrated on 3 ml of a 1% RBC suspension in Hanks’ buffer. An ethanolic solution of \( \text{cis-PnA} \) was then added to the cell suspension up to an amount of 2.25 nmol, that corresponds to 3 mol.% of erythrocyte phospholipids [25].

The \( \text{cis-PnA} \) fluorescence signal was recorded for at least 3 min to reach its stability, and then 160 \( \mu \text{mol l}^{-1} \) cumene hydroperoxide was added and the subsequent decrease in fluorescence emission due to oxidation of PnA was monitored every 2 min over 50 min. Control tracings in the absence of cumene hydroperoxide were also recorded.

Measurements of oxidation kinetics of \( \text{cis-PnA} \) were carried out both in the absence and in the presence of dipyridamole, added 5 min after the oxidant at the concentrations reported, with results ranging from 0.8 to 16.5 \( \mu \text{mol l}^{-1} \).

2.4. Endogenous antioxidant evaluation

Red blood cells as a 1% suspension were incubated at 25°C with the oxidant, 160 \( \mu \text{mol l}^{-1} \) cumene hydroperoxide for 7 and 12 min to measure the acid soluble thiol and the \( \alpha \)-tocopherol content, respectively. Concentration and time of incubation of cumene hydroperoxide were chosen to induce a vitamin E consumption of about 30–35% of the value measured in cell suspensions from the same subject untreated with the oxidant.

Incubation with the oxidant was preceded by 5 min preincubation with dipyridamole. The dose-dependent effect of dipyridamole (2–20 \( \mu \text{mol l}^{-1} \)) was evaluated in RBCs of the same subject.

To exclude a possible adenosine mediated effect on dipyridamole activity some incubations were performed on RBCs pretreated with 290 mU ml\(^{-1} \) adenosine deaminase.

2.5. Acid soluble thiol extraction procedure

Intracellular thiols were determined by titration with DTNB according to a modified procedure by Beutler [26].

To the pellet obtained after centrifugation (1600 g, 10 min, 4°C) 6 ml of 1% RBC suspension 0.5 ml H\(_2\)O was added. Proteins were precipitated by the addition of 1 ml of fresh metaphosphoric acid solution (1.67 g metaphosphoric acid, 0.2 g EDTA–disodium salt and 30 g NaCl in 100 ml H\(_2\)O). The supernatant was separated from protein precipitate after 5 min by centrifugation and subsequently filtered on Puradisc 25AS (Whatman), 0.2 \( \mu \text{m} \) pore size. An aliquot of 0.5 ml of supernatant was combined with 0.5 ml of 300 mmol l\(^{-1} \) Na\(_2\)HPO\(_4\), and its blank was obtained by combining 0.5 ml of supernatant with 0.5 ml of H\(_2\)O. Then, 100 \( \mu \text{l} \) DTNB solution (20 mg DTNB in 100 ml 1% sodium citrate) was added to the blank and to the sample. Sample absorbance was read against the blank absorbance at 412 nm.
2.6. Vitamin E extraction procedure

To 7 ml of the RBCs incubation suspensions, 50 μl of 45 mmol l⁻¹ butylated hydroxytoluene were added, and immediately centrifuged in a table top centrifuge. Vitamin E was extracted from RBC pellets according to the procedure by Lang et al. [27]. Vitamin E concentrations were assessed by HPLC with fluorimetric detection (excitation 286 nm, emission 330 nm). The HPLC analysis was performed on a ODS C-18 column (Beckman) using methanol as eluent at 1 ml min⁻¹. Data from HPLC were collected and analyzed on a Hewlett-Packard computer using the System Gold program (Beckman).

2.7. Statistical analysis

Data are expressed as mean±S.E. Statistical differences between variables were assessed by analysis of variance and a post hoc Bonferroni–Dunn test using the Statview 4.0 program (Abacus Concepts). A probability (P) value <0.05 was considered to be statistically significant.

3. Results

Lipid peroxidation of red blood cell membranes, induced by incubation in the presence of 160 μmol l⁻¹ cumene hydroperoxide, was monitored by the fluorescence emission of hydrophobic probe cis-parinaric acid pre- incorporated into membranes.

Fig. 1 shows the time course of the decay of cis-PnA fluorescence emission in the absence or in the presence of 4 μmol l⁻¹ and 12.5 μmol l⁻¹ dipyridamole.

Drug addition 5 min after exposure to the oxidant suppresses the probe fluorescence decay for a time proportional to the drug concentration (Fig. 1, inset).

The sensitivity towards oxidative stress was variable among subjects, both on vitamin E consumption (ranging from 28 to 55%, n=11) and thiol oxidation (ranging from 54 to 77%, n=10).

In the absence of the oxidant, 20 μmol l⁻¹ dipyridamole did not modify either vitamin E (326.4±10 pmol/10⁶ cells vs. 325.7±12 pmol/10⁶ untreated cells, n=4) or thiol content (2.07±0.20 mmol l⁻¹ of packed cells vs. 2.03±0.22 mmol l⁻¹ of untreated packed cells) in RBCs after at least 40 min of incubation.

To rule out a possible adenosine-mediated effect of dipyridamole some samples were incubated in the presence of 290 mU ml⁻¹ adenosine deaminase: the results were not modified by the presence of the enzyme.

Vitamin E content in RBCs, after 12 min incubation, was about 64.7%±3.4 (n=11) of untreated cells (351.6±20 pmol/10⁶ cells).

Increasing doses of dipyridamole, ranging from 2 to 20 μmol l⁻¹, preserve vitamin E in a dose-dependent fashion (Fig. 2). Vitamin E content was 70.6%±1.7, 77.2%±3.9, 84.0%±2.5 and 88.3%±2.3 of untreated cells in the presence of 2, 5, 10 and 20 μmol l⁻¹ dipyridamole, respectively.

Cytosolic thiol concentration after 7 min incubation in the presence of 160 μmol l⁻¹ cumene hydroperoxide was reduced to 31.5%±2.4 (n=10) of untreated cells (1.89±0.17 mmol l⁻¹ of packed cells). In the presence of...
initially yielding cumene alkoxyl radicals that can initiate clinical therapeutic applications: from 2
mediation of the absence of the metal ion [25]. Cumene-induced peroxi-
brates were challenged with acute chemical oxidative -PnA is not degraded in erythrocyte ghost membranes in pure in vitro assay of antioxidant activity. Cellular mem-
cis requires a metal ion cofactor (i.e. Fe\textsuperscript{2+}-heme) in fact was the ex vivo evaluation of red blood cells, rather than a
initiation of lipid peroxidation by cumene hydroperoxide in a clinically realistic framework. First, the model used
an oxidative stress induced by cumene hydroperoxide. The the present study perhaps help to put this antioxidant effect
®rmed by our experiments in red blood cells submitted to dipyridamole [11,12,29]. However, some peculiarities of
demonstrated in vitro by ESR studies [11,12], was con-
demonstrating the powerful antioxidant effects of


4. Discussion

The peroxyl radical scavenging activity of dipyridamole, demonstrated in vitro by ESR studies [11,12], was con-
irmed by our experiments in red blood cells submitted to an oxidative stress induced by cumene hydroperoxide. The initiation of lipid peroxidation by cumene hydroperoxide requires a metal ion cofactor (i.e. Fe\textsuperscript{2+}-heme) in fact cis-PnA is accompanied by hemoglobin oxida-
tion (met-hemoglobin and hemicrome formation) [24,25]. In this model the membrane soluble oxidant reacts gradual-
ly with both membrane-bound hemoglobin and cytosolic, initially yielding cumene alkoxyl radicals that can initiate lipid peroxidation, vitamin E consumption and thiol oxid-
ation. In the present work dipyridamole, at pharmacological doses, was demonstrated to behave as an active quencher of peroxy radicals due to its capability to extend the lag phase of cis-PnA peroxidation, and to reduce vitamin E consumption in red blood cell membranes.

In our assays on lipid peroxidation, dipyridamole was added to cell suspension 5 min after the initiation of oxidation. Since the drug takes time to be optimally incorporated into the membranes, it is reasonable to think that its protective action would be more effective with an earlier drug treatment. However, our aim was to test the capacity of dipyridamole to act as a real chain-breaking compound once the process of lipid peroxidation had begun.

Despite the inability of dipyridamole to penetrate the cytosol [28], in our experimental conditions the antioxidant effect of dipyridamole is spread also to cytosolic thiols.

The method we used to assess the thiol content is unable to distinguish the glutathione (GSH) from the other free thiols; however, it represents a fast and easy tool to observe the heavy effect of oxidative stress at cytosolic level. Free thiol oxidation can be the result of direct radical attack but can also occur indirectly through GSH — requiring repair processes such as the reduction of oxidised membrane protein thiol group, van den Berg et al. [24], using the same method, described (even if they did not show it) a rapid and massive consumption of GSH induced by both cumene hydroperoxide and hydrogen peroxide, with an initial drop in RBC GSH content within the first 5 min to a level that was dependent on oxidant concentra-
tion. Increasing oxidative stress caused a progressive inability to regenerate GSH, thus reducing the cytosolic thiol pool. Thus, the modest effect of dipyridamole on the cytosolic thiol content could be due in minimal part to the sensitivity of the method we used and to a larger extent on the capacity of cumene to overwhelm GSH-dependent antioxidant factors. The cytosolic antioxidant effect of the dipyridamole is the consequence of the reduction of radical species forming at the membrane site. Moreover, the moderate effect of the drug on free thiol content could also be due to the capacity of the oxidised GSH to move out the
cells.

Our study confirms and expands previous observations demonstrating the powerful antioxidant effects of dipyridamole [11,12,29]. However, some peculiarities of the present study perhaps help to put this antioxidant effect in a clinically realistic framework. First, the model used was the ex vivo evaluation of red blood cells, rather than a pure in vitro assay of antioxidant activity. Cellular mem-
branes were challenged with acute chemical oxidative stress, and, in this model, dipyridamole acted as a potent antioxidant shield. Second, the explored dose concentration range of dipyridamole was chosen in order to reproduce the whole spectrum which can be found with different clinical therapeutic applications: from 2 μmol l\textsuperscript{-1} achieved in plasma with 200 mg p.o. (the elective dosage for antiplatelet-neuroprotective applications) [10] up to 10-fold higher concentrations (achieved for cardiac surgery applications as an antiplatelet agent) [30]. Interestingly, an antioxidant effect was observed over the full explored range, but with a steep dose-dependence, suggesting that high intravenous dose regimens — such as those achieved in a cardiac surgery theater — are probably required to recruit the full antioxidant drug potential. Third, the study design allowed us to gain an insight into the underlying pathways of the antioxidant effect. Such pathways are multiple and complex, involving a vitamin E sparing effect, active quenching of peroxy radicals and — despite the inability of dipyridamole to cross the cell membrane — saving of cytosolic thiols. In addition, the antioxidant effect is due to dipyridamole per se and independent of adenosine accumulation.

Thereby, the antioxidant effect of dipyridamole is
important and detectable at doses achieved in clinical therapeutic applications of the drug. The anti-radical effect is achieved through multiple biochemical pathways, all of them independent of adenosine accumulation. The full recruitment of the antioxidant potential requires high dipyridamole concentration, clinically achievable with high intravenous doses. At this point, the antioxidant effect of dipyridamole cannot be considered a mere bench curiosity but might well account for some of the multiple effects observed in its clinical diagnostic and therapeutic applications. The stage is set for in vivo studies specifically addressing this issue.

References